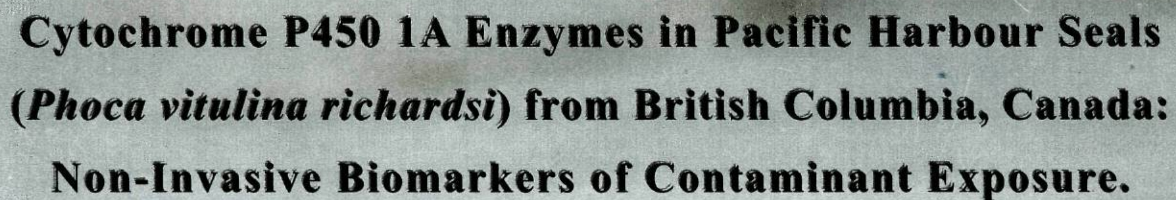

Cytochrome P450 1A Enzymes in Pacific Harbour Seals (*Phoca vitulina richardsi*) from British Columbia, Canada: Non-Invasive Biomarkers of Contaminant Exposure.

by

Marta Gonçalves Leite de Assunção

ICBAS – Institute of Biomedical Sciences Abel Salazar

University of Porto

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Marta Gonçalves Leite de Assunção

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Cover photo-Pacific harbour seal pup.

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Thesis Abstract

High levels of persistent contaminants like polyhalogenated aromatic hydrocarbons (PHAHs) are widespread in the marine environment. Many of these man-made chemicals are fat-soluble (*e.g.* PCBs, dioxins and furans) and tend to bioaccumulate in the food chain reaching worrying levels in top predators like marine mammals. These animals might therefore be particularly vulnerable to adverse health effects resulting from PHAH exposure. Biological responses measured as biomarkers in representative species can act as an early indicator of the exposure levels or toxicological risk that a natural population might be exposed to. Hepatic cytochrome P450 1A enzymes (CYP1A) have been associated with the biotransformation of PHAHs in a variety of wildlife species. The induction of these enzymes has been related to exposure to "dioxin-like" compounds in vertebrates and is therefore a well-established biomarker of contaminant exposure. The evaluation of biomarkers in marine mammals normally requires the use of liver, implying the use of dead animals. Therefore a non-invasive or minimally-invasive biomarker approach using skin biopsies is desirable. This approach has the potential to provide a tool for evaluating the risks associated with contaminant exposure in free-ranging marine mammals for which liver tissue cannot be obtained (*e.g.* cetaceans, endangered species). This study was conducted to investigate the use of CYP1A enzymes in Pacific harbour seal (*Phoca vitulina richardsi*) skin biopsies as a biomarker of exposure to "dioxin-like" contaminants. A comparative evaluation of the immunochemical expression of these enzymes in liver and skin and their relationship to contaminant load was aimed at. Considering that biopsies provide reduced amounts of tissue archived liver and skin from Pacific harbour seal pups were used to develop appropriate homogenization techniques. An optimized western blotting revealed measurable bands of CYP1A proteins in the obtained samples, using specific rat CYP1A antibodies. Additionally, small liver and skin/blubber biopsies were collected from twenty young free-ranging Pacific harbour seal pups captured from the Fraser River estuary (British Columbia, Canada) and housed temporarily in captivity. The biopsy sampling was conducted while animals were under a general anaesthetic. Despite the reduced amounts of tissue, liver and skin biopsies proved enough for the immunoquantification of CYP1A enzymes. The blubber was used for full congener analysis of PCBs, dioxins and furans. The CYP1A responsiveness in both tissues was further characterized in two additional *in vivo* studies by supplying orally or topically a non-toxic CYP1A inducer, ß-naphthoflavone (BNF). For the first time in pinnipeds, *in vivo* exposure to BNF was demonstrated to significantly induce hepatic and skin CYP1A proteins. The considerable skin CYP1A response to orally supplied BNF indicates that these enzymes in harbour seal skin might be responsive to "dioxin-like" contaminants that bioaccumulate in the food chain. Hepatic and skin CYP1A levels were significantly correlated in both untreated and BNF induced animals, supporting the use of skin as a surrogate for liver. Toxic Equivalents to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TEQs) measured in blubber were found not to correlate with CYP1A content in liver or skin of untreated animals. The measurement of skin CYP1A proteins as a biomarker of contaminant exposure in harbour seals and other marine mammals is promising although it requires further investigation.

Resumo da tese

Níveis elevados de hidrocarbonetos aromáticos polihalogenados (PHAHs) encontram-se dispersos no meio marinho. Muitos destes xenobióticos são lipofílicos (*e.g.* PCBs, dioxinas e furanos) e tendem a bioconcentrar-se na cadeia alimentar atingindo níveis preocupantes nos predadores do topo da cadeia trófica, como os mamíferos marinhos. Estes animais poderão estar particularmente vulneráveis aos efeitos adversos resultantes da exposição a PHAHs. A avaliação de biomarcadores (respostas biológicas) em espécies representativas, pode indicar níveis de exposição ou risco toxicológico a que uma população natural poderá estar sujeita. As enzimas da subfamília 1A pertencente aos citocromos P450 (CYP1A) têm sido associadas à biotransformação de PHAHs em várias espécies selvagens. Em vertebrados, a indução destas enzimas no fígado tem sido relacionada com a exposição a contaminantes com estrutura plana semelhante às dioxinas, portanto a indução de CYP1A é considerada um biomarcador de exposição bem estabelecido. A avaliação de biomarcadores em mamíferos marinhos requer normalmente o uso de amostras de fígado, o que implica a utilização de animais mortos. Uma aproximação não-invasiva ou minimamente-invasiva é desejável. Esta poderá fornecer uma ferramenta para a avaliação do risco associado com a exposição a contaminantes em populações selvagens de mamíferos marinhos das quais não é possível obter amostras de fígado (*e.g* cetáceos e espécies ameaçadas). O objectivo deste estudo foi investigar a utilidade das enzimas CYP1A em biopsias de pele da foca comum do Pacífico (*Phoca vitulina richardsi*) como um biomarcador de exposição a contaminantes com estrutura semelhante às dioxinas. Este estudo incluíu uma avaliação comparativa da expressão destas enzimas em biopsias de fígado e pele, assim como a sua relação com o nível dos contaminantes. Considerando a reduzida quantidade de tecido numa biopsia, fígado e pele arquivados de crias de foca comum do Pacífico foram utilizadas no desenvolvimento de técnicas apropriadas de homogenização. Utilizando um sistema de western blotting optimizado, bandas de proteinas CYP1A foram quantificadas nas amostras preparadas, usando anticorpos específicos para proteinas CYP1A de rato. Adicionalmente, pequenas biopsias de fígado e pele/blubber (camada adiposa por baixo da pele) foram recolhidas em vinte crias selvagens de foca comum do Pacífico, sob anestesia geral. Estes animais foram capturados no estuário do rio Fraser (Colúmbia Britânica, Canadá) e mantidos temporariamente em cativeiro. Apesar da reduzida quantidade de tecido, as biopsias mostraram-se suficientes para a imunoquantificação das enzimas CYP1A. A totalidade de congéneres de PCBs, dioxinas e furanos foram quantificados na camada adiposa. A avaliação da resposta das enzimas CYP1A foi complementada com dois estudos *in vivo* nos quais foi aplicado oralmente e topicamente um inductor não tóxico destas enzimas, o ß-naftoflavonóide (BNF). Pela primeira vez em pinípedes, foi demonstrado que a exposição *in vivo* ao BNF induz significativamente a expressão das enzimas CYP1A no fígado e na pele. Esta indução verificada nas amostras de pele de focas expostas oralmente ao BNF indica que as enzimas CYP1A da pele poderão ser induzidas por contaminantes com estrutura semelhante às dioxinas, que se acumulam na cadeia alimentar. Os níveis destas enzimas no fígado e na pele correlacionaram-se significativamente em focas não tratadas e induzidas com BNF, dando suporte ao uso de pele como tecido alternativo ao fígado. Os níveis de contaminantes na camada adiposa, expressos como equivalentes tóxicos à 2,3,7,8-tetraclorodibenzo-*p*-dioxina

(TEQs), não se correlacionaram com os níveis de enzimas CYP1A no fígado e na pele de animais não tratados. O uso dos níveis de CYP1A em biopsias de pele como biomarcador de exposição a contaminantes em foca comum, assim como em outros mamíferos marinhos, é promissor mas requer investigação adicional.

Table of contents

Title page........i
Thesis Abstract........iii
Resumo da tese........iv
Table of contents........vi
List of Tables........viii
List of Figures........x
Acknowledgements........xii
Dedication........xiii
Glossary........xv

Chapter 1

Introduction........1
Harbour seals........1
Environmental contaminants........1
Bioaccumulation, metabolism and toxicity........4
The xenobiotic-metabolizing enzymes of the CYP450 system........4
CYP450 forms in marine mammals........8
Toxic effects associated with environmental exposure........11
Toxic equivalency quotients (TEQs)........12
Monitoring the marine environment: the use of biomarkers........14
CYP1A enzymes as biomarkers of contaminant exposure........15
Immunochemical and catalytic evaluation of CYP1A enzymes........16
The non-destructive biomarker approach........18
Skin as an alternative tissue for biomarker evaluation in marine mammals........19
Aims of this study........20

Chapter 2–Immunoquantification of CYP1A enzymes in Pacific harbour seal liver and skin biopsies: tissue preparation and western blotting optimization.

Abstract....................22
Introduction....................23
Material and Methods....................25
Results and discussion....................35
Conclusions....................43

Chapter 3-Cytochrome P450 (CYP1A) levels in Pacific harbour seal skin biopsies as a biomarker of contaminant exposure.

Abstract....................44
Introduction....................45
Material and Methods....................49
Results....................58
Discussion....................72
Conclusions....................79

Appendix 1....................81
Appendix 2....................87

References....................99

List of Tables

Chapter 1

Table 1: Xenobiotic metabolizing CYP450 enzymes inducers and common substrates....8

Table 2: Toxic Equivalency Factors (TEFs) for "dioxin-like" PCBs in mammals according to Van den Berg *et al.* (1998)....13

Table 3: Toxic Equivalency Factors (TEFs) for dioxins and furans in mammals according to Van den Berg *et al.* (1998)....13

Chapter 2

Table 2: Primary and secondary antibodies and positive control used for immunoblotting....29

Chapter 3

Table 1: Primary and secondary antibodies and positive control used for immunoblotting....55

Table 2: Loading concentrations of liver and skin samples for each primary antibody....56

Appendix 1

Table 1: List of reagents and buffers prepared for total protein determination and western blotting....82

Table 3: Protein yield and CYP1A levels quantified per protein unit in samples prepared from seals PV0018 and PV00101 liver....85

Table 5: Seal PV0018 skin sample dilutions and average corrected CYP 1A levels quantified per protein unit....86

Appendix 2

Table 3: Physical parameters of the general study group of harbour seal pups captured at Swishswash island, BC....88

Table 4: BNF oral induction study design....89

Table 5: BNF topical induction study design....89

Table 6: Liver biopsies collected from harbour seal pups and total protein concentration after homogenization....90

Table 7: Skin biopsies collected from harbour seal pups and total protein concentration after homogenization....91

Table 8: CYP1A levels per protein unit (CYP1A/μg total protein) in liver samples from general study group and oral BNF induction study animals, determined by western blotting....92

Table 9: CYP1A levels per protein unit (CYP1A/μg total protein) in skin samples from general study group and oral BNF induction study animals, determined by western blotting....95

Table 10: CYP1A levels per protein unit (CYP1A/μg total protein) in skin samples from topical BNF induction study animals, determined by western blotting....97

Table 11: Total PCDDs, PCDFs, non-*ortho* and mono-*ortho* PCBs expressed as TEQs (total equivalency quotients in ng/kg lipid weight), according to Van den Berg *et al.* (1998) and total TEQs (sum of all) measured in the blubber of the twenty harbour seal pups....98

List of Figures

Chapter 1

Figure 1: General molecular structure and positioning numbering of PCBs, PCDDs and PCDFs....2

Figure 2: Proposed model for the AhR receptor-mediated changes in gene expression....6

Chapter 2

Figure 1: Correlation between the amount of liver from seals PV0018 (n=10) and PV00101 (n=10) homogenized and protein yield....35

Figure 2: Comparison of protein yield from harbour seal skin samples using different homogenizing time conditions (procedure A and B)....36

Figure 3: Immunoblot of seals PV00101 and PV0018 liver samples probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution....37

Figure 4: Correlation between the total protein loaded/well and the CYP1A levels detected for seals PV0018 (n=8) and PV00101 (n=6) liver samples....38

Figure 5: Correlation between the total protein loaded/well and the CYP1A2 levels measured for 3MC treated rat liver microsomes....39

Figure 6: Comparison of corrected liver CYP1A levels measured in two different gels. For seals PV00101 and PV0018, a liver sample (~15 μg total protein/well) and a 2-fold dilution of that sample were loaded in gel 1 and gel 2....40

Figure 7: Immunoblot of seal PV0018 skin sample probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution....41

Figure 8: Correlation between the total protein loaded/well and the CYP1A levels measured for seal PV0018 skin sample dilutions (n=4)....42

Chapter 3

Figure 1: Harbour seals (n=20) capture area in the mouth of the Fraser River estuary near Vancouver, British Columbia, Canada....49

Figure 2: Manual feeding of seal pups from the induced group....52

Figure 3: Liver biopsies collected from young harbour seals under a general anaesthetic....58

Figure 4: Skin/blubber biopsy....59

Figures 5 and 6: Biopsy sampling in hind flipper (5); sampling area 24 hours after collecting biopsy (6)....60

Figure 7: Correlation between the amount of tissue homogenized and the protein yield for all harbour seal liver and skin samples....60

Figure 8: Immunoblot of liver samples from control and BNF induced harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution.................61

Figure 9: Immunoblot of liver samples from untreated harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution...............................62

Figure 10: CYP1A protein bands densitometry in liver samples from BNF oral induction study..62

Figure 11: Immunoblot of skin samples from control and BNF orally induced harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution.........63

Figure 12: Immunoblot of skin samples from control and BNF topically induced harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution...63

Figure 13: Immunoblot of skin samples from untreated harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution...............................63

Figure 14: Immunoblot of liver and skin samples from BNF oral induction study seals, probed with monoclonal antibody against rat CYP1A1/2 (Mab 1-7-1) at 20μg IgG/ml. 64

Figure 15: Average CYP1A content on harbour seal liver (n=12) and skin (n=20) samples..65

Figure 16: Average content of CYP1A/ug total protein in liver (black bars) and skin (grey bars) samples from harbour seal pups...66

Figure 17: Liver and skin CYP1A levels in induced animals (n=3) and control animals (n=3) from the BNF oral administration study..66

Figure 18: CYP1A levels in skin samples from the induced area (n=6) compared to skin samples from the control area (n=6) in seals from the BNF topical application study...67

Figure 19: Correlation between CYP1A levels in liver and skin from untreated harbour seals from the general study group and BNF oral induction study group (n=15)..68

Figure 20: Correlation between CYP1A levels in liver and skin from induced and control harbour seals of the BNF oral induction study group (n=6)......................68

Figure 21: Percent average of total PCDD, PCDF and PCB (non-*ortho* and mono-*ortho*) congeners contributing to total TEQs (TEQ ng/kg lipid) in harbour seal pups (n=20)..68

Figure 22: Scattergram of liver (n=12) and skin (n=20) CYP1A levels and blubber total TEQs..69

Figure 23: Correlation between CYP1A levels in harbour seal skin samples (n=20) and non-*ortho* PCBs...70

Figure 24: Scattergram of CYP1A levels in liver (n=12) and skin samples (n=20) and body weight...70

Figure 25: Correlation between harbour seals body weight (n=20) and blubber total TEQs..71

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Dedication

To those who are always there for me.

"…nunca ninguém cometeu maior erro do que aquele que não fez nada, só porque podia fazer muito pouco..."

Edmund Burk

Glossary

PHAHs – Polyhalogenated aromatic hydrocarbons

PCDDs – Polychlorinated dibenzo-*para*-dioxins

PCDFs – Polychlorinated dibenzofurans

PCBs – Polychlorinated biphenyls

CYP450 – Cytochrome P450 enzymes family

AhR – Aryl hydrocarbon receptor

2,3,7,8-TCDD or TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

CYP1A – Cytochrome CYP450 1A enzymes subfamily

mRNA – Messenger RNA

ER – Endoplasmatic reticulum

ROS – Oxigen radicals

TEQs – Toxic equivalency quotients

TEFs – Toxic equivalency factors

MFO – Mixed function oxidase or monooxygenase system

PAH - Polycyclic aromatic hydrocarbons

BNF - ß-naphthoflavone

3MC - 3-methylcholanthrene

DDT - Dichloro-diphenyltricholoroethane

EROD – Ethoxyresorufin-*O*-deethylation

BaPH – Benzo(a)pyrene hydroxylation

AHH – Arylhydrocarbon hydroxylation

PAb – Polyclonal antibody

MAb – Monoclonal antibody

BPMO – Benzo(a)pyrene monooxygenase

PMS – Post mitochondrial supernatant

DMSO – Dimethyl sulfoxide

Chapter 1

Introduction

Harbour seals

The harbour seal (*Phoca vitulina;* Order-Pinnipedia; Family-Phocidae) is a relatively small mammal widely distributed around the northern hemisphere. Five geographically separated subspecies have been recognized, including the Pacific harbour seal (*Phoca vitulina richardsi*), which can be found on the eastern Pacific from Pribilof Islands to Baja California (Mexico). Over 100.000 harbour seals inhabit the coastal waters of British Columbia (Canada), with approximately one third of these in the Strait of Georgia between the Vancouver Island and the mainland (48.5°–51°N) (Olesiuk *et al.* 1990). Males can reach about 1.4-2 m and 70-140 kg and females 1.2-1.7 m and 50-110 kg. Adult seals feed on squid, molluscs and different fish, including rockfish (Family Scorpaenidae), herring (*Clupea arengus*), starry flounder (*Platichthys stellatus*), salmon (*Oncorhynchus spp.*), hake (*Merluccius productus*) and Pacific sandlance (*Ammodytes hexapterus*). Their diet is often subject to local prey availability and varies seasonally and regionally. These animals are non migratory and usually solitary, interacting for mating. The pupping season occurs in July-August (Cottrell *et al.* 2002; Temte *et al.* 1991) on sandy beaches or small rocky islands. The estimated nursing period is 24-42 days and the weaning mass 24 kg (Cottrell *et al.* 2002).

Due to their small size, wide distribution, large numbers and relative easy handling, this species has been well studied. As a top predator in the marine food chain, harbour seal becomes a species of great interest for toxicology studies (Ross 2000). The validation of methods using harbour seals biopsies allows the development of possible tools to be applied to other marine mammals.

Environmental contaminants

Persistent organic pollutants are widespread in the environment. Several of these pollutants, including the polyhalogenated aromatic hydrocarbons (PHAHs), have been

identified as contaminants in almost every environment compartment (Muir *et al.* 1999; Safe *et al.* 1985; Safe 1990). The PHAHs structure contains one or more aromatic rings substituted with a varying number of halogens, mainly chlorine and bromide. The most important PHAHs include the polychlorinated dibenzo-*para*-dioxins (PCDDs; n=75 congeners), polychlorinated dibenzofurans (PCDFs; n=135 congeners) and polychlorinated biphenyls (PCBs; n=209 congeners). PCDD and PCDF molecules have a similar, more or less rigid, planar configuration (Bosveld 1995) (Figure 1). In PCBs, a single carbon-carbon bond links the phenyl rings, allowing their free rotation (Safe *et al.* 1985) (Figure 1).

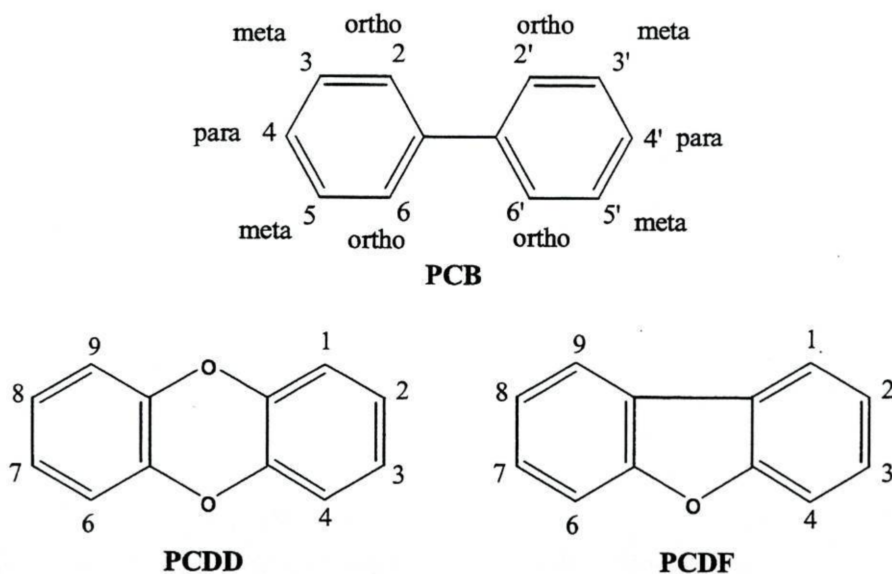


Figure 1: General molecular structure and positioning numbering of PCBs, PCDDs and PCDFs.

The coplanarity of these rings is influenced by the substitution pattern. The congeners that lack chlorine substitutions at all four *ortho* positions (non-*ortho* PCBs) or contain only one *ortho* chlorine atom (mono-*ortho* PCBs) can attain a planar molecular structure relatively easily (Bosveld 1995). As such, non-*ortho* PCBs and mono-*ortho* PCBs are, respectively, structural analogues and approximate structural analogues with PCDDs and PCDFs and can exhibit "dioxin-like" response in many species (similar type of toxicity) (Bosveld 1995). The multi-*ortho* PCBs have two or more *ortho* positions substituted with halogens atoms achieving, as a consequence, a

globular configuration (Bosveld 1995). PCBs, PCDDs and PCDFs are lipophilic compounds and the degree of lipophilicity is generally increased with increasing ring chlorination. Their resistance to breakdown by acids, bases, heat and hydrolysis largely contributed to their diverse industrial applications (Safe 1990) and to their environmental persistence.

The PCDDs and PCDFs have never been synthesised commercially, but are by-products of human activities still formed during the synthesis of a wide range of industrial chemicals, including herbicides, fungicides and PCBs (Safe 1990). In addition, PCDDs and PCDFs are also formed in combustion processes, such as municipal or hospital waste incinerators, car engines and chlorine bleaching processes associated with pulp and paper industry (Safe 1990).

PCBs were used for many industrial purposes from 1929 to the early 1970's. Their major application was as insulating and cooling materials in transformers and capacitors (Safe 1984). By the mid 1970's production of these persistent compounds was ceased in North America and Europe, but they are still used in closed systems (dielectric liquids in capacitors and transformers, heat-conducting fluids in heat exchangers and hydraulic fluids in mining equipment) (Safe 1984; Safe 1994). Since the majority of the 209 possible congeners is present in the commercial mixtures, biota may be exposed to a large number of different PCB congeners (Bosveld 1995).

Freshwater and land run-off, dumping activities and municipal and industrial waste represent the input of these contaminants into lakes and the marine environment (reviewed by Duinker and Boon 1985). In remote areas the atmosphere deposition is a significant source; halogenated organic compounds that entered the atmosphere through soils evaporation can move from temperate and tropical regions to the Artic within days to weeks (Macdonald 1996; Macdonald *et al.* 1997) and then enter into the aquatic environment. Sediments are part of the potential sink but may also act as a source for the overlaying water since benthic fauna circulation increases the transport of chemicals to the sediment surface facilitating their mobilization (Duinker and Boon 1985).

Bioaccumulation, metabolism and toxicity

In the marine environment, these fat-soluble chemicals can accumulate in fish and invertebrates mainly via surfaces in contact with the water (gills and skin), but also through diet. In marine mammals, food ingestion is the major route; these contaminants enter the food chain because they are associated with particulate matter (reviewed by Muir *et al.* 1999). Their concentration raises with increasing trophic level reaching high levels in the fatty tissues of fish-eating wildlife (Nakata *et al.* 1998; Ross and Troisi 2001).

In marine mammals, lipophilic contaminants are primarily stored in blubber being released when the fat reserves are used, for example, during breeding, lactation and moulting seasons (Nyman 2000). Usually, the contaminant load increases with age but reproducing females transfer part of their burden to their offspring mainly through lactation, but also by placental transfer (Addison and Brodie 1987; Addison and Stobo 1993). Furthermore, a selective transfer of certain PCBs compounds has been suggested (Addison and Brodie 1987; Wolkers *et al.* 2002). As a consequence, xenobiotics bioaccumulation patterns in marine mammals varies with age, sex, season and reproductive status (Ross and Troisi 2001).

In fish, birds and mammals, PHAH metabolism occurs predominantly in the liver These contaminants may undergo enzyme-mediated reactions, resulting in the formation of metabolites, which differ in physical and chemical properties (*e.g.* lipophilicity) from the parent compounds (Stegeman and Hahn 1994). The primary hepatic metabolic pathway is a two-phase process (Peakall 1992b). The initial phase (Phase I) including oxidative reactions is followed by a second phase were conjugating reactions occur (Phase II) (Lewis *et al.* 1998). More easily excretable compounds are formed, although reactive metabolites with potential toxicity/carcinogenicity can also be produced (Brouwer *et al.* 1995; Nyman 2000; Stegeman and Hahn 1994; Whyte *et al.* 2000).

The xenobiotic-metabolizing enzymes of the CYP450 system

The cytochrome P450 (CYP450) is a super family of heme-containing enzymes bound to the endoplasmatic reticulum (ER) membranes. Part of the mixed function oxidase

or monooxygenase system (MFO), can be found in most tissues in the body (reviewed by Peakall 1992b; Stegeman and Hahn 1994). They are organized in families and sub-families, depending on their genetic structure (for more detailed information see http://drnelson.utmem.edu/nelsonhomepage.html) and can be found in vertebrates, invertebrates, fungi, plants and some prokaryotes (reviewed by Nelson *et al.* 1996). The CYP450 enzymes mediate most of the Phase I oxidative metabolism of xenobiotics. In addition, these enzymes participate in the oxidative metabolism of endogenous compounds (prostaglandins, fatty acids and lipophilic vitamins), some of them with important regulatory functions (*e.g.* steroids, eicosanoids) (Stegeman and Livingstone 1998). Their ability to metabolize exogenous compounds, including man-made environmental pollutants, has attracted attention to the study of these enzymes.

In mammals, the major xenobiotic-metabolizing forms belong to the CYP1, CYP2, CYP3 and CYP4 families (Lewis *et al.* 1998; Nelson *et al.* 1996). The highest concentrations and activity of these enzymes are found in the liver, although smaller concentrations have also been found in the extrahepatic tissues through which foreign compounds pass when entering the body (*e.g.* lung, skin and intestine) (Lewis *et al.* 1998; Peakall 1992b; Raunio *et al.* 1995). In addition to their regulation via chemicals and drugs, other physiological factors influence the expression of CYP450 enzymes. These include nutritional status, hormonal factors, inflammation or infection, disease, stress, gender and age (reviewed by Morgan *et al.* 1998).

The metabolism of certain PHAHs has been related to the CYP1A subfamily enzymes (Table 1), therefore this subfamily has become of particular interest to environmental toxicologists. The induction of CYP1A enzymes by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD or TCDD) and structurally related compounds like non-*ortho* and mono-*ortho* PCBs has commonly known to be mediated by the aryl hydrocarbon receptor (AhR) (Whyte *et al.* 2000). This cytosolic receptor functions as a ligand-activated transcription factor that controls the expression of several genes, including the induction of CYP1A subfamily (reviewed by Hahn 1998) (Figure 2). The binding of the planar ligand to the AhR receptor results in nuclear translocation of the complex, followed by release of a heat shock protein (Hsp90) and association to AhR nuclear translocator protein (ARNT) (Hahn 1998; Schmidt and Bradfield 1996). In the nucleus, this transformed AhR complex binds to specific dioxin responsive enhancer

(DRE) sequences (specific DNA regions) located in the promoter of CYP1A and other TCDD-responsive genes (Hahn 1998; Schmidt and Bradfield 1996). This will activate gene transcription (the AhR gene battery) to messenger RNAs (mRNA) and the subsequent translation into proteins involved in biotransformation reactions, including the CYP1A proteins (Hahn 1998; Hahn *et al.* 1998). These proteins are then inserted into the cytosolic ER membranes, where they metabolize TCDD and related compounds contributing to detoxification. However, metabolites with increased toxicity than their parent compounds can be produced.

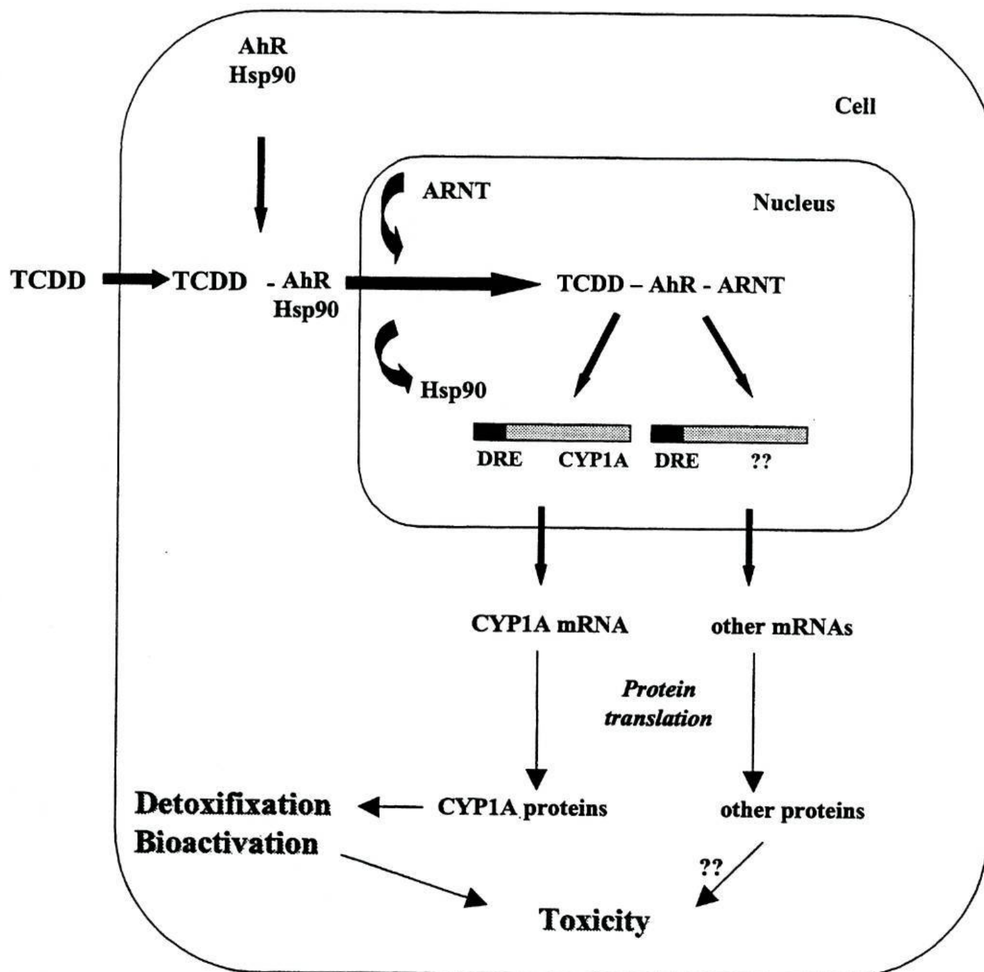


Figure 2: Proposed model for the AhR receptor-mediated changes in gene expression. Adopted from Stegeman and Hahn (1994) and Whyte *et al.* (2000).

The induction of the AhR gene battery has been linked to the production of oxygen radicals (ROS) (Nebert *et al.* 2000), lipid peroxidation and DNA oxidation damage when stimulated by high doses of planar PHAHs (Nyman 2000). According to Schlezinger *et al.* (1999) and Schlezinger and Stegeman (2001), in a vertebrate model a AhR receptor agonist (non-*ortho* PCB-3,3',4,4'-tetrachlorobiphenyl) might inactivate CYP1A catalytic cycle and stimulate the formation of ROS.

The AhR receptor might also participate in biological process distinct from the better characterized ligand-activated pathways (Schmidt and Bradfield 1996). In fact, an endogenous AhR ligand, yet unknown, has been proposed and evidence suggested that CYP1A might control the levels of this putative ligand (reviewed by Nebert *et al.* 2000). This endogenous metabolism might be involved in cell regulation, apoptosis and oxidative damage (Nebert *et al.* 2000). Therefore, knowing the nature of the endogenous ligand and its regulation would help to elucidate the toxic responses caused by exogenous AhR ligands (Whyte *et al.* 2000).

The AhR appears to be a phylogenetically ancient protein present in all living vertebrate groups (Hahn *et al.* 1998). In fact, AhR sequences have been identified in teleost, elasmobranch and agnathan fish (Hahn *et al.* 1998) that, in previous studies, had already shown good correlations between AhR presence and CYP1A inducibility (Hahn *et al.* 1992). In marine mammals, the CYP1A induction in environmentally exposed animals to planar PHAHs served as indirect evidence of the AhR function. This receptor has been characterized in the beluga whale (*Delphinapterus leucas*) (Hahn *et al.* 1992) and bottlenose dolphin (*Tursiops truncatus*) (Carvan *et al.* 1994) and latter partially sequenced in beluga and white-sided-dolphin (*Lagenorhyncus acutus*) (Jensen and Hahn 1996). More recently, cloning and sequencing of AhRs from beluga and harbour seal showed a closely related structure of these proteins (82% identical in overall amino acid sequence) among these species belonging to two distinct groups (pinnipeds and cetaceans) (Jensen and Hahn 2001; Kim and Hahn 2001). These AhR receptors also showed a higher binding affinity to TCDD, compared to human AhR (Jensen and Hahn 2001; Kim and Hahn 2001). Considering that marine mammals tend to bioaccumulate high concentrations of PHAHs, they might be more sensitive to PHAH effects than humans (Kim and Hahn 2001).

Table 1: Xenobiotic metabolizing CYP450 enzymes inducers and common substrates.

CYP450 subfamily	CYP proteins	Substrates	Common Inducers
CYP1A	1A1 / 1A2	7-ethoxyresorufin, PAH, planar PCBs, Acetanilide,estradiol, caffeine	PAH, BNF, 3MC, planar PCBs and PBBs, dioxins, furans
CYP2B	2B6	Pentoxyresorufin, barbiturates, steroids	PB, PCN, non-planar PCBs, DDT and other related pesticides
CYP3A	3A4 / 3A5	Steroids, antibiotics	PCN, PB, glucocorticoids
CYP4A	4A1	Lauric acid arachidonic acid	Clorofibrate, phthalates, PCBs

Abbreviations: PAH-polycyclic aromatic hydrocarbons; BNF-ß-naphthoflavone; 3MC-3-methylcholanthrene; PBBs-polybrominated biphenyls; PB-phenobarbital; PCN-pregnenolone-a-carbonitrile; DDT-dichloro-diphenyltricholoroethane.

Compiled from Stegeman and Hahn (1994) and Nyman (2000).

Xenobiotic metabolizing enzymes from CYP1 to CYP4 families appear to catalyze different enzyme activities with some overlap, as they seem to share substrates (Stegeman and Hahn 1994). In addition, orthologous CYP450 forms in different species may have different substrates, and interspecies differences in the presence, activity and inducibility of CYP450 forms have been reported (Gonzalez and Nerbert 1990, reviewed by Lewis *et al.* 1998). Therefore, the well documented properties of CYP450 enzymes in laboratory animals (rats, mouse and rabbits) and humans can only give an idea of the possible CYP450 forms and their properties in marine mammals (Lewis *et al.* 1998; Nyman 2000). In a toxicological perspective, all these facts indicate the importance of characterizing CYP450 forms involved in xenobiotic metabolism in marine mammal species.

CYP450 forms in marine mammals

After the comparative analysis of contaminants congener patterns between marine mammals and bivalve mollusc (assumed no biotransformation of PCBs) (Boon *et al.* 1989), or marine mammals and their prey (Boon *et al.* 1987; Boon *et al.* 1994; Boon *et al.* 1997; Bruhn *et al.* 1995; Storr-Hansen *et al.* 1995; Wolkers *et al.* 1998a) the

indirect presence of xenobiotic-metabolizing CYP450 enzymes in marine mammals was suggested. In fact, Boon *et al.* (1994) examined differences in the PCB patterns between phocid seals blood [harbour, ringed (*Phoca hispida*) and grey seals (*Halichoerus grypus*)] and their prey, in order to investigate the capacity of marine mammals to metabolize PCBs. The authors found that congeners without any vicinal hydrogen atoms (i.e. hydrogen atoms bound to adjacent carbon atoms of the phenyl ring) (class I) and with vicinal hydrogen atoms in *ortho* and *meta* positions, combined with 2 or more chlorine substituents at *ortho* positions (class II), were persistent and not metabolized by seals, as they showed equal PCBs pattern in prey and seal. Congeners similar to class II but bearing only one *ortho*-chlorine substituent (class III) were metabolized, suggesting that the ability to attain planar configuration is important for their metabolism (Boon *et al.* 1987; Boon *et al.* 1989). The metabolism of these categories of congeners by CYP1A subfamily was suggested (Boon *et al.* 1997). However, in congeners with vicinal hydrogen atoms at *meta* and *para* positions, either bearing 2 *ortho*-chlorine (class IV) or 3 or more *ortho*-chlorine substituents (class V), metabolism occurred, indicating that not attaining planar configuration does not prevent metabolism of these globular congeners (Boon *et al.* 1987). The enzymes belonging to CYP2B subfamily were suggested as responsible for this metabolism (Boon *et al.* 1997). Furthermore, when analysing PCB congeners in ringed seals, Wolkers *et al.* (1998a) found that congeners from classes II and III and classes IV and V showed a relation with CYP450 enzyme activities.

In pinniped species, protein levels and associated catalytic activities of putative CYP1A, CYP2B, CYP3A and CYP4A subfamilies have been reported in harbour seals, harp seals (*Phoca groenlandica*), hooded seals (*Cystophora cristata*), ringed seals, grey seals and southern sea lion (*Otaria flavescens*) (Addison and Brodie 1984; Addison *et al.* 1986; Fossi *et al.* 1997a; Fossi *et al.* 1997b; Goksøyr *et al.* 1992; Goksøyr 1995a; Nyman *et al.* 2000; Nyman *et al.* 2001; Tilley *et al.* 2001; Troisi and Mason 1997; Wolkers *et al.* 1998b; Wolkers *et al.* 1999). These enzymes have also been investigated in cetaceans. The CYP1A, 3A and 4A subfamilies, and associated catalytic activities, were reported in beluga whale, northern right whale (*Eubalaena glacialis*), southern right whale (*Eubalaena australis*), minke whale (*Balaenoptera acutorostrata*), fin whale (*Balaenoptera physalus*), striped dolphin (*Stenella

coeruleoalba), bottlenose dolphin and common dolphin (*Delphinus delphis*) (Fossi *et al.* 2000; Goksøyr *et al.* 1988; Goksøyr 1995a; Marsili *et al.* 1998; Moore *et al.* unpublished; White *et al.* 1994; Wilson *et al.* 2001). Considering the PCB congener patterns found in cetaceans compared to their diet (Boon *et al.* 1994), it has been suggested that CYP2B associated metabolism might be reduced in cetaceans compared to pinnipeds. Interestingly, putative CYP2B isoforms have been reported in beluga and minke whale and harbour porpoise (*Phocoena phocoena*) (Goksøyr 1995a; White *et al.* 1994; White *et al.* 2000). Thus, there is some evidence for the expression of CYP2B isoforms in cetaceans, but their metabolic capabilities remain to be determined.

Although xenobiotic metabolizing enzymes seem to be present in marine mammals, their metabolic ability is not well understood. Therefore, biotransformation of individual compounds has been studied *in vitro* assays using hepatic microsomes obtained from marine mammals. According to Boon *et al.* (1998), *in vitro* assays using microsomes of animals that died from natural causes can be a useful tool to understand Phase I metabolism. Van Hezik *et al.* (2001) reported the *in vitro* metabolism of chlorobornane congeners in seals microsomes. Whales appear to have low ability to metabolizing these compounds (Boon *et al.* 1998; Boon *et al.* 2001). In addition, beluga and pilot whale (*Globicephala melas*) microsomes were able to metabolize two non-*ortho* tetrachlorobiphenyl congeners (White *et al.* 2000). One of these congeners was not metabolized by sperm whale (*Physeter macrocephalus*) microsomes, which might indicate species-specific metabolizing abilities; nevertheless a non-*ortho* dichlorobiphenyl, a dioxin and furan congeners were significantly biotransformed *in vitro* (Boon *et al.* 2001).

Recently, CYP1A cDNA fragments were cloned from liver of five marine mammals and the respective amino acids partially sequenced (Teramitsu *et al.* 2000). One fragment classified as CYP1A1 was found for mink whale, dall's porpoise (*Phocoenoides dalli*), largha seal (*Phoca largha*) and ribbon seal (*Phoca fasciata*). For the steller sea lion (*Eumetopias jubatus*) two fragments, CYP1A1 and CYP1A2 were sequenced. The amino acid sequences for the five marine mammal species were found to be 73.9% to 94.3% identical to the CYP1A sequences of terrestrial mammals (Teramitsu *et al.* 2000). The identification of CYP1A genes in marine mammals is an

important step to assess the role of these enzymes in xenobiotic metabolism and possible contribution to toxicity.

Toxic effects associated with environmental exposure

The effects resulting from PCB, PCDD and PCDF exposure have been evaluated in laboratory animals and humans. These include dermal lesions, hepatotoxicity, immunosuppression, reproductive and developmental toxicities, genotoxicity, carcinogenisis, endocrine disruption and neurotoxicity (Brouwer *et al.* 1995; Safe 1984).

Free-ranging wildlife is exposed to complex chemical mixtures in which these contaminants might exert individual or interactive toxic effects, therefore their assessment can be extremely challenging. Furthermore, such confounding factors as age, sex and condition can affect the endpoint being measured (Ross 2000). However, there is growing concern since several studies related adverse effects in marine mammal populations to contaminant exposure. Persistent environmental contaminants have been implicated in premature births in California sea lions (*Zalophus californianus*) (Delong *et al.* 1973). Uterine closures, as well as skull asymmetry and lesions, have been observed in grey, harbour and ringed seals from the polluted Baltic Sea (Bergman *et al.* 1992; Helle *et al.* 1976; Mortensen *et al.* 1992; Zakharov and Yablokov 1990). The high contaminant burden in beluga whales from the St. Lawrence estuary was associated with tumours, immunosuppression and reproductive impairment (Béland *et al.* 1993; De Guise *et al.* 1995; Martineau *et al.* 1994). Furthermore, controlled captive studies have supported the possible effects caused by environmental pollutants. By exposing captive harbour seals through diet, consisting of fish from contaminated areas, experimental studies showed decreased reproductive success, decreased circulating levels of vitamin A and thyroid hormone, and impaired immune responses (Brouwer *et al.* 1989; De Swart *et al.* 1995; Reijnders 1986; Ross *et al.* 1995). More recently, environmental contaminants were associated with disrupted vitamin A dynamics in free-ranging harbour seal pups (Simms *et al.* 2000).

Although further studies are needed, contaminants are suspected as playing a role in a series of outbreaks of infectious diseases in free-ranging populations from polluted areas (Ross *et al.* 1996; Ross 2000; Ross 2002).

Toxic equivalency quotients (TEQs)

The risk assessment of PCBs, PCDDs and PCDFs can be performed using the 2,3,7,8-TCDD equivalents (TEQ) approach. The TEQ system allows expressing the toxicity of complex contaminant mixtures by using the toxic equivalency factor (TEF) for each congener. The 2,3,7,8-TCDD has been given a TEF of 1.0 because it binds most strongly to the AhR receptor and is the most toxic (Van den Berg *et al.* 1998). As a result of *in vivo* and *in vitro* studies, different "dioxin-like" PCB, PCDD and PCDF congeners have been assigned with a TEF reflecting their AhR binding potency relative to 2,3,7,8-TCDD (Tables 2 and 3) (reviewed by Safe 1990; Safe 1992; Van den Berg *et al.* 1998).

By multiplying the individual congener concentrations (*e.g.* ng/kg lipid weight) by the respective TEF values, the total amount of toxic equivalents (TEQs) present in the sample can then be calculated as a sum-parameter for all "dioxin-like" compounds (*e.g.* ng/kg lipid weight). One limitation of the TEQ system is that it assumes the same toxicity mechanism in all species, but according to Safe (1990) 2,3,7,8-TCDD and related compounds seem to cause their responses through a common receptor mediated mechanism of action (AhR). In addition, the AhR is suggested to be highly conserved throughout evolution (Hahn *et al.* 1998). The possible toxic response of the non-"dioxin-like" compounds or responses mediated by other toxicity mechanism are not included on this approach, neither are the possible interactive effects of these compounds in a contaminant mixture (Safe 1990; Safe 1992).

Despite these limitations, the TEQ system provides a useful means for simplifying the assessment of toxic risk in wildlife exposed to complex mixture of environmental contaminants.

Table 2: Toxic Equivalency Factors (TEFs) for "dioxin-like" PCBs in mammals according to Van den Berg *et al.* (1998).

PCB category	IUPAC number	Structure	TEFs
Non-*ortho*	77	3,3',4,4' – tetra CB	0,0001
	81	3,4,4',5 – tetra CB	0,0001
	126	3,3',4,4',5 – penta CB	0,1
	169	3,3',4,4',5,5' – hexa CB	0,01
Mono-*ortho*	105	2,3,3',4,4' – penta CB	0,0001
	114	2,3,4,4',5 – penta CB	0,0005
	118	2,3',4,4',5 – penta CB	0,0001
	123	2',3,4,4',5 – penta CB	0,0001
	156	2,3,3',4,4',5 – hexa CB	0,0005
	157	2,3,3',4,4',5' – hexa CB	0,0005
	167	2,3',4,4',5,5' – hexa CB	0,00001
	189	2,3,3',4,4',5,5' – hepta CB	0,0001

Table 3: Toxic Equivalency Factors (TEFs) for dioxins and furans in mammals according to Van den Berg *et al.* (1998).

Dioxins and furans	Structure	TEFs
PCDDs	2,3,7,8 – tetra CDD	1,0
	1,2,3,7,8 – penta CDD	1,0
	1,2,3,4,7,8 – hexa CDD	0,1
	1,2,3,6,7,8 – hexa CDD	0,1
	1,2,3,7,8,9 – hexa CDD	0,1
	1,2,3,4,6,7,8 – hepta CDD	0,01
	Octa CDD	0,0001
PCDFs	2,3,7,8 – tetra CDF	0,1
	2,3,4,7,8 – penta CDF	0,5
	1,2,3,7,8 – penta CDF	0,05
	1,2,3,4,7,8 – hexa CDF	0,1
	2,3,4,6,7,8 – hexa CDF	0,1
	1,2,3,6,7,8 – hexa CDF	0,1
	1,2,3,7,8,9 – hexa CDF	0,1
	1,2,3,4,6,7,8 – hepta CDF	0,01
	1,2,3,4,7,8,9 – hepta CDF	0,01
	Octa CDF	0,0001

Monitoring the marine environment: the use of biomarkers

The link between persistent contaminants found in the marine environment and detrimental health effects in free-ranging wildlife at the top of the food chain has increased the need for biomonitoring programs. Environmental biomonitoring studies have combined the measurement of contaminant levels with the use of biomarkers. A biomarker is a molecular and/or cellular alteration that occurs along the temporal and mechanistic pathways connecting ambient exposure to a toxicant and eventual disease (reviewed by DeCaprio 1997). The measurement of this response in an organism can be used as an early indicator of the toxicological risk to which a natural population is exposed. According to Depledge (1994), an ecotoxicological biomarker is defined as a biochemical, cellular, physiological or behavioural variation that can be measured in an organism tissue or body fluid, or also at the whole organism level, providing evidence of exposure to and/or effects of one or more chemical pollutant. *Exposure biomarkers* signal exposure of an organism, population or community to chemical pollutants, whereas *effect biomarkers* refer to responses that indicate adverse effects in an organism, population or community caused by pollutants (Depledge 1994).

A biomarker should fulfil several requirements before being considered as useful for biomonitoring purposes (Depledge 1994; Peakall 1992c):

-allow the early detection of exposure and/or effect;

-be easily assayed based in a well-established biochemical or physiological mechanism;

-be specific and able to be distinguished from natural sources of variability as ecological and physiological variables, species-specific differences and individual variability;

-respond to pollutants in a dose-response manner over a concentration of the pollutant that is environmentally meaningful .

Biomarkers used in environmental studies, which respond to toxic metals, PHAHs, polyaromatic hydrocarbons, carbamates and organophosphates, include parameters of DNA damage, enzyme induction, hormones, metal-binding proteins and immune response, among others (Peakall 1992c).

The measure of biomarkers in key marine mammal species can provide an indication on the chemicals that might threat top of the food chain consumers (Ross 2001). Laboratory models might not properly assess this issue, considering that contaminants reaching the top of the food chain might differ from the original mixtures introduced in the environment, as organism at the different trophic levels might preferentially metabolize or accumulate certain classes of contaminants (Ross 2001). Biomonitoring programs may also be relevant for human health since high levels of TCDD-like PHAHs have been found in fish consumers from industrialized areas and people inhabiting in remote areas that typically consume marine mammals (*e.g.* native Inuit in northern Canada) (Ross *et al.* 1996).

CYP1A enzymes as biomarkers of contaminant exposure

The synthesis of CYP1A subfamily enzymes in cell, after exposure to "dioxin-like" contaminants, as well as their participation in these chemicals metabolism, has led to several studies considering this CYP450 subfamily for environmental biomonitoring purposes. In fact, levels and activities of these enzymes (mainly in hepatic microsomes), in organisms occupying different positions on the marine food web have been evaluated and related to environmental exposure (Goksøyr and Husoy 1998; Peakall 1992b; Stegeman and Hahn 1994; Wilson *et al.* 2000; Yawetz *et al.* 1998). CYP1A induction through the AhR receptor has been used as a biomarker of "dioxin-like" contaminant exposure in different aquatic organisms (invertebrates, fish, birds and marine mammals) (Fossi *et al.* 1992; Fossi and Marsili 1997; Fossi *et al.* 1997a; Fossi *et al.* 1997b; Fossi *et al.* 2000; Goksøyr *et al.* 1991b; Goksøyr 1995a; Goksøyr 1995b; Marsili *et al.* 1998; Nyman 2000; Peakall 1992b; Stegeman and Hahn 1994; Troisi and Mason 1997; White *et al.* 1994; Whyte *et al.* 2000). Furthermore, induction studies using strong CYP1A inducers, like benzo(a)pyrene (PAH) and ß-naphthoflavone (BNF), have helped to assess its usefulness as an environmental biomarker in different fish species and also characterize their CYP1A enzymes (Celander *et al.* 1993; Goksøyr *et al.* 1991a; Haasch *et al.* 1993; Kloepper-Sams and Stegeman 1994; Wolkers *et al.* 1996).

Due to their often high trophic level, marine mammals are considered important species for monitoring the level and distribution of contaminants in the marine

ecosystem (Ross 2000). CYP1A expression and inducibility in marine mammals from different areas have therefore been assessed in several studies (Addison and Brodie 1984; Addison *et al.* 1986; Bandiera *et al.* 1995; Boon *et al.* 2001; Goksøyr 1995a; Wolkers *et al.* 1998b). CYP1A information has been related to contaminant burdens measured in blubber and/or food (Bandiera *et al.* 1997; Fossi *et al.* 2000; Letcher *et al.* 1996; Marsili *et al.* 1998; Moore *et al.* unpublished; White *et al.* 1994; Wolkers *et al.* 1998a; Wolkers *et al.* 1999). In addition, significant differences in the expression of CYP1A enzymes have been found between animals from polluted and less polluted areas (Fossi *et al.* 1997b; Nyman *et al.* 2000). Furthermore, the metabolism rate of "dioxin like" compounds tested *in vitro* was correlated with immunodetected hepatic CYP1A1 content in whale species (White *et al.* 2000).

Immunochemical and catalytic evaluation of CYP1A enzymes

Different methods have been used to characterize CYP450 forms. The most frequently approaches used for the CYP1A subfamily include the determination of mRNA or the protein content and its associated catalytic activity (Goksøyr 1995b).

For the CYP1A mediated activity, the most common assays include (Peakall 1992a; Whyte *et al.* 2000):

-ethoxyresorufin-*O*-deethylation (EROD),

-benzo(a)pyrene hydroxylation (BaPH),

-arylhydrocarbon hydroxylation (AHH).

After the development of antibodies raised against purified CYP1As, mainly from rodents and fish species, the CYP1A protein levels have been mostly determined by (Gelboin and Friedman 1985; Goksøyr *et al.* 1991b; Goksøyr 1995b; Husoy *et al.* 1996; Lin *et al.* 1998):

-western blotting,

-ELISA,

-immunohistochemistry.

The advantages of immunochemical techniques over catalytic measurements relay on the ability to analyse samples that lost their catalytic activity due to (a) improper

storage conditions, (b) to the presence of inhibiting compounds or (c) during preparation of enzyme fractions (Goksøyr *et al.* 1991b; Goksøyr and Husoy 1998). In addition, if more than one CYP450 form present is catalyzing the same reaction, measuring only the enzyme activity will not distinguish the forms that contribute to the tissues total enzyme activity (Gelboin and Friedman 1985). Since there are no available homologous antibody probes for marine mammal CYP450 proteins, immunochemical techniques still have to rely on the cross reactivity between heterologous antibodies prepared against purified CYP450 forms, from other mammals or fish, and the marine mammal CYP450 proteins itself.

The western blotting technique has been used to characterize marine mammal hepatic CYP1As (Boon *et al.* 2001; Goksøyr *et al.* 1988; Goksøyr 1995a; Nyman *et al.* 2000; White *et al.* 1994; White *et al.* 2000; Wolkers *et al.* 1998b; Wolkers *et al.* 1999) based on the cross reactivity obtained using polyclonal (PAb) and monoclonal (MAb) antibodies raised against rat and fish CYP1As. The specificities of the antibodies can range from highly specific CYP1A epitopes (antigenic determinants), recognizing only CYP1As from closely related species, or show a broader specificity, recognizing CYP1As from divergent groups (Stegeman and Hahn 1994). Monoclonal antibodies combine the ability of being produced in unlimited quantities with the high specificity for individual or classes of CYP450 (Gelboin and Friedman 1985; Goksøyr and Husoy 1998). According to Peakall (1992a), MAbs are a valuable tool in the identification and characterization of CYP450 enzymes as they can be used as highly specific probes to define CYP450 epitopes.

Recently, a specific polyclonal antibody was prepared against a partially sequenced CYP1A protein from harp seal; this antibody recognized two hepatic CYP1A forms in harp seal whereas a polyclonal antibody against trout CYP1A1 had previously recognized one hepatic CYP1A (Tilley *et al.* 2001). The heterologous antibodies that have been used suggest a close immunochemical relationship among CYP1A forms in vertebrates (Stegeman and Hahn 1994) and the deduced amino acid sequences of CYP1As fragments in some marine mammals were found to be 50% to 94.3% identical to vertebrate CYP1As (Teramitsu *et al.* 2000). Nevertheless, using antibodies developed against marine mammal CYP1A epitopes might allow a more valid

characterization of CYP450 forms present in this group, as well as, to better clarify when a subfamily has more than one form.

The non-destructive biomarker approach

The most commonly used biomarkers in biomonitoring programs require liver, but also, kidney, blood or brain tissues. Most biomarker studies in marine mammals have made use of liver samples (reviewed by Fossi and Marsili 1997). Typically, tissues are collected from animals captured as fisheries by-catch, in controlled hunts or those killed for sample collection, which raises ethical questions, especially if belonged to endangered populations. In other studies samples from stranded animals have been used, which might be unsuitable if the time between dead and sampling was long enough to influence the body contaminant burden (Boon *et al.* 1994), therefore cannot be considered representative animals. The development and validation of biomarker responses measured in biological samples that can be obtained on a non-invasively or minimally-invasive way are desirable (Fossi and Marsili 1997; Fossi *et al.* 1999; Goksøyr and Husoy 1998).

Measurement of organochlorine residues such as dioxins, furans, PCBs and chlorinated pesticides, as well as PAHs have been successful in blood and blubber samples collected from living specimens of northern elephant seals (*Mirounga angustirostris*) (Newman *et al.* 1994), sea lions (Jimenez *et al.* 1999), fin whales and striped dolphins (Marsili *et al.* 2001). In addition, blood, faeces, fur and skin/blubber biopsies have been described as biological materials to be used for biomarker evaluation. The biomarker responses measured in some of these tissues included DNA alterations, protein responses (including MFO), metabolic products (porphyrins), retinol and thyroid alterations and immunotoxicity parameters (Fossi and Marsili 1997; Fossi *et al.* 1999). Some of these have been proposed as non-destructive biomarkers of exposure to PHAHs, PAHs, toxic metals, organophosphates and carbamates in cetaceans, pinnipeds and sea birds (Fossi *et al.* 1997a; Fossi *et al.* 1997b; Fossi *et al.* 1999; Fossi *et al.* 2000; Marsili *et al.* 1998).

Another study refers to successful cell cultures (fibroblasts) obtained using skin biopsies from Mediterranean striped, bottlenose and common dolphins (Marsili *et al.* 2000). According to the authors, these *in vitro* systems can be useful for toxicological

studies in these species. More recently, the catalytic activity of CYP1A1 in rat peripheral blood lymphocytes was investigated and the author referred that the similarities observed in the regulation of lymphocytes CYP1A1 and hepatic CYP1A1 expression further suggest that blood can be used as surrogate for studying expression of CYP450 dependent xenobiotic metabolizing enzymes (Dey *et al.* 2001).

Besides the possibility of collecting biological tissue with minimal stress to the animals being sampled, a non-destructive biomarker approach also enables sequential analysis over a period of time of the same population, as well as, the evaluation of communities already at risk. If biomarkers developed using these alternative biological materials prove to be useful, more direct evidence of contaminant exposure, as well as related toxicity in marine mammals populations can be assessed.

Skin as an alternative tissue for biomarker evaluation in marine mammals

Skin is the major interface between the body and the environment, therefore also a major site of exposure to environmental agents. It is a complex tissue divided in two major components, epidermis and dermis, characterized by several types of cells. According to Bickers *et al.* (1986), skin possesses membrane-bound CYP450 dependent enzyme activity that responds to exposure to endogenous and exogenous compounds. While important knowledge has been gained from hepatic CYP450 studies, much less is known about this enzyme system in skin.

Using laboratory animals as models for the study of CYP450 enzymes in skin, it has been shown that these enzymes are present in higher concentration on epidermis, when compared to dermis and whole skin (Bickers *et al.* 1982). Analogous CYP450 forms have been found in both epidermis and liver microsomal fractions prepared from rats pretreated topically with 3-methylcholanthrene (3MC), although in smaller amounts in epidermis (Bickers *et al.* 1986). In addition, topical application of BNF and benz(a)anthracene (PAH) in rat epidermis and cultured human keratinocytes has increased AHH activity and CYP1A1 mRNA levels (Khan *et al.* 1992). In microsomes prepared from mouse skin, pretreated topically with benz(a)anthracene and pyridine (a solvent widely used in industry), significant increases in monooxygenase activities (including EROD and BaPH) were recorded (Agarwal *et al.* 1994, Ichikawa *et al.* 1989). In addition, major bands cross-reacted with antibodies

against CYP1A, 2B and 3A forms (western blotting) in these studies. Immunoblot analysis revealed induction of CYP1A, 2B, 2E and 3A forms in murine skin induced topically by dexamethasone, a known inducer of hepatic CYP450 forms used as topical agent in dermatological practice (Jugert *et al.* 1994). Significant increases in the related monooxygenase activities (including EROD) were also observed. These results suggested that rodent skin contains multiple and inducible CYP450 forms that play an important role in the metabolism of xenobiotics.

In a recent *in vivo* study, river otters (*Lontra canadensis*) were exposed to crude oil through diet and CYP1A1 was assessed by immunohistochemistry in skin biopsies (Ben-David *et al.* 2001). Skin endothelium CYP1A1 levels increased during exposure and decreased significantly during rehabilitation period. In different cetacean species biopsy-sampled in the Mediterranean Sea, mixed function oxidase activity [BPMO-benzo(a)pyrene monooxigenase] was investigated in skin (Fossi *et al.* 2000; Marsili *et al.* 1998). The authors reported a significant correlation between PCB or DDT levels and BPMO activity in male fin whales. In Atlantic bottlenose and common dolphin skin biopsies, the expression of CYP1A1 was primarily located in the vascular endothelial cells, with expression being higher in dermis, compared to epidermis (Wilson *et al.* 2001).

These results highlight the possible utility of skin as an alternative tissue for biomarker evaluation in free-ranging marine mammals; but also underscore the importance of validation steps.

Aims of this study

When considering the evaluation of biomarkers of contaminant exposure in free-ranging marine mammals, skin can be collected in a non-destructive and minimally-invasive manner. In addition, theoretical background provides evidence for the presence of CYP450 enzymes and its associated catalytic activity in mammalian skin, including marine mammals exposed to environmental contaminants.

In this study, we obtained liver and skin biopsy samples collected in a minimally-invasive way from healthy and free-ranging harbour seal pups. The immunochemical expression of CYP1A enzymes was evaluated in both tissues using western blotting.

This thesis is divided in two parts:

I. Immunoquantification of CYP1A enzymes in Pacific harbour seal liver and skin biopsies: tissue preparation and western blotting optimization.

II. Cytochrome P450 1A (CYP1A) levels in Pacific harbour seal skin as biomarker of contaminant exposure: a non-destructive approach.

The preparation and homogenization techniques for harbour seal liver and skin biopsies were developed (I). The western blotting was optimized for both tissues (I). Once the laboratorial procedures had been optimized, CYP1A expression in harbour seal liver and skin was measured (II). Furthermore, induction studies using BNF allowed a better characterization of these enzymes (II). This enabled a comparative evaluation of the biomarker analyzed in both body compartments (II). Finally, the CYP1A levels, in both liver and skin, were related to the contaminant concentrations measured, since contaminant analysis could be performed using the blubber attached to the skin biopsy (II).

Chapter 2

Immunoquantification of CYP1A enzymes in Pacific harbour seal liver and skin biopsies: tissue preparation and western blotting optimization.

Abstract

Considerable knowledge has been gained from the study of CYP450 in liver, but much less is known about this enzyme system in extrahepatic tissues. CYP450 concentrations are generally low in these tissues and substantial amounts of sample are often required despite purification techniques. When working with skin biopsies sampled from marine mammals, the small amounts obtained represent an additional challenge that must be addressed during the development of homogenization techniques. Western blotting has been widely used for the characterization of CYP450 xenobiotic-metabolizing forms like CYP1A enzymes, and is also considered suitable to the application to extrahepatic tissues. In this study, archived tissues from Pacific harbour seal pups were used. Homogenization protocols were adopted and developed for preparing liver and skin "post mitochondrial supernatant" fractions by differential centrifugation. CYP1A proteins were detected and quantified in both liver and skin samples by western blotting using a rat CYP1A specific antibody. A series of experiments were conducted to optimize western blotting measurements using harbour seal liver and skin samples. The system developed was reproducible; samples resolved in different gels/membranes had an average variability of 16.5 % between replicates. This is an appropriate technique for measuring CYP1A levels in liver and skin samples, and this may provide us with a sensitive biomarker approach to studying responses to contaminant exposure in harbour seals and other marine mammals.

Introduction

The majority of studies regarding cytochrome P450 enzymes (CYP450) refer to the hepatic system, as liver is the main drug metabolizing organ. In addition, liver characteristics make it suitable for investigating CYP450 system: high CYP450 concentrations, cellular homogeneity, easy subcellular fractionation and suitability for enzyme purification (Philpot 1991). Techniques have been developed to purify human, rat and fish hepatic CYP450s to apparent homogeneity (Goksøyr 1985; Guengerich *et al.* 1982; Guengerich and Martin 1998). However, CYP450 enzymes are not confined to liver and characterization of some isozymes has been successfully carried out in extrahepatic tissues in fish and rodents, including brain, kidney, lung, skin and bladder (Bickers *et al.* 1986; Philpot 1991; Stegeman and Hahn 1994). In contrast with liver, these tissues have low CYP450 levels (Warner and Gustafsson 1998). Warner and Gustafsson (1998) developed a technique to purify CYP450 enzymes from brain, breast and prostate, to evaluate detection by western blotting. Although this technique overcame the problem of lower CYP450s concentrations in extrahepatic tissues, it is time consuming and also requires substantial amounts of sample. As a consequence, this method might not be a solution when sample availability is limited, as when small biopsies of tissues are used.

When investigating CYP450 enzymes in liver, microsomal fractions (100 000 x g particulate fraction) obtained from tissue homogenates by differential centrifugation are preferred. Cytochrome P450 proteins are bound to the endoplasmic reticulum membranes (ER), and are therefore found in higher concentrations in microsomes prepared from crude liver homogenates. The contribution of ER in such fractions should be low, when preparing microsomal fractions from extrahepatic tissues (Philpot 1991). In addition, methods that work well for liver do not necessarily produce the same well-defined fractions or assure an efficient disruption of all cells in other tissues. As a consequence, the experimental development of optimal homogenization for different tissues is necessary (Warner and Gustafsson 1998).

Western blotting is an immunochemical technique that has been widely used to characterize the expression of xenobiotic metabolizing CYP450 forms. In this method,

proteins are identified by their mobilites during sodium dodecyl sulfate polyacrylamide gel electrophoresis, and also by their immunoreactivities. Several investigators have used antibodies against vertebrate CYP1As to probe for the presence of related hepatic CYP1A forms in fish (Goksøyr and Husoy 1998; Wilson *et al.* 2000), polar bears (Bandiera *et al.* 1997; Letcher *et al.* 1996), pinnipeds (Nyman *et al.* 2000; Wolkers *et al.* 1998; Wolkers *et al.* 1999) and cetaceans (Boon *et al.* 2001; Goksøyr 1995; White *et al.* 1994). To characterize the expression of CYP450 forms in extrahepatic tissues western blotting is considered an appropriate technique. As long as there are antibodies suitable for the detection of CYP450 forms, this system enables the investigation of extrahepatic tissues with concentrations of specific forms as low as 50 fmol/mg protein (Philpot 1991).

This study aimed to develop and validate methods for tissue homogenization and optimize western blotting to investigate CYP1A expression in harbour seal liver and skin by:

-Adapting existing protocols to homogenize small liver biopsies.

-Develop a protocol to homogenize skin biopsies.

-Determine if western blotting represents a technique adequate for the analysis of CYP1A expression in harbour seal liver and skin.

-Define optimal content of total protein in liver and skin samples for CYP1A quantification by western blotting.

Material and Methods

Harbour seal liver and skin biopsies

Archived liver and skin tissue from two Pacific harbour seal pups (*Phoca vitulina richardsi*; Order Pinnipidea, Family Phocidae) was used to develop and optimize the procedures. PV00101 was a recently orphaned pup that was captured at Creg Rocks, West of Sidney Island (BC, Canada) on July 2000. Liver and skin biopsies were collected immediately after euthanasia and preserved in liquid nitrogen (liquid N_2). PV0018 was a pup captured at Swishswash Island, Vancouver (BC, Canada), on August 2000, as part of a study group of twenty harbour seal pups. This seal was euthanised due to a life-threatening systemic infection associated with a pre-existing fracture of the lower mandible. Liver and skin biopsies were immediately collected and preserved in liquid N_2.

The animals were handled according to principles and guidelines of the Canadian Council on Animal Care and the oversight of the Animal Care Committee, Department of Fisheries and Oceans (DFO), Canada.

Sample preparation

This project was designed to use small liver and skin biopsies obtained from harbour seals. This fact alone was a limitation for preparing microsomal fractions, since the amount of tissue necessary could exceed the anticipated sample yield from biopsies. Fin whale skin biopsies (20 to 200 mg) were previously found to be too small to obtain microsomes (Marsili *et al.* 1998). Therefore a differential centrifugation of liver and skin homogenates at a medium speed (9 000 x g) was attempted. Considering that after centrifugation at this speed the ER small vesicles remain in the supernatant (Alberts *et al.* 2002) these "post mitochondrial supernatant" (PMS) or S9 fractions, should also be suitable for CYP1A quantification:

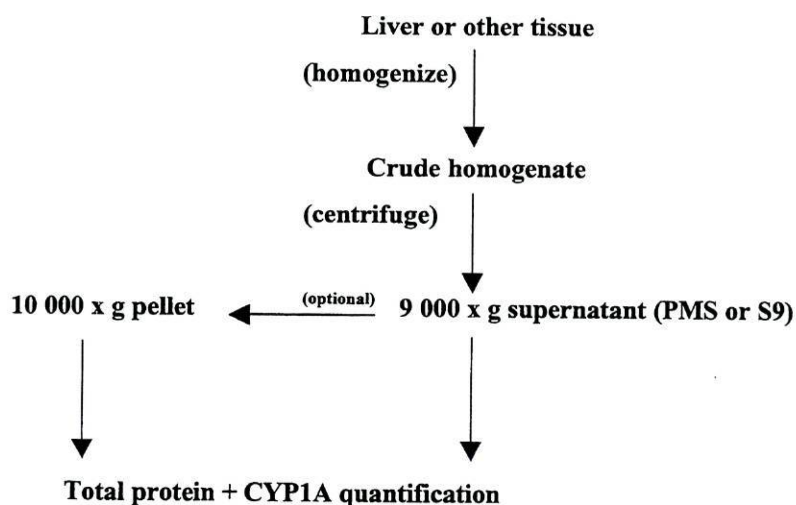


Diagram 1:Tissue preparation for determining total protein and CYP1A quantification.

Adapted from UNEP/FAO/IOC (1993).

Liver samples

Liver biopsies provide small amounts of tissue (30–100 mg), so the yield generated by the homogenization of different amounts of tissue was assessed. The protein yield from liver biopsies was also evaluated. Liver biopsies from both seals were individually homogenized according to protocol 1 (Appendix 1). Liver weighing approximately 5, 10, 15, 25 and 50 mg was prepared in duplicate. Using a manual pellet pestle (Kontes), liver was homogenized in ice cold homogenizing buffer [volume (µl)= 2 to 3 x sample weight (mg)] (Table 1-Appendix 1) using 1.5 ml Eppendorf tubes (Eppendorf®, Brinkman instruments, Ltd., Mississauga, ON, Canada). The tubes were kept on ice between bursts to minimize heat generation during the process. The homogenates were centrifuged at 9 000 x g for 20 minutes at 4°C (Biofuge 17 R, Baxter Scientific Products). The post-mitochondrial supernatant volume was recovered and measured (50 and 250 µl Hamilton glass syringes). Liver samples were immediately preserved at -80°C in labelled 0.6 ml Eppendorf tubes.

Skin samples

Skin sampling *in vivo* is usually limited to one biopsy (6 or 8 mm diameter; approximately 150–250 mg). Harbour seal skin is a light coloured tissue (approximately 5 mm deep) that has a tough "resilience". The border between

epidermis and dermis can be visualised. Although immunohistochemistry has revealed higher CYP1A levels in lower dermis compared to epidermis of cetacean skin (Wilson *et al.* 2001) no information is available regarding localization of CYP1A in pinnipeds skin. We therefore used entire skin biopsies during experiments.

To homogenize marine mammal skin, tissue grinders are preferred considering the abundant tough connective tissue. A homogenization protocol was developed using whole skin biopsies (6 mm diameter) obtained from seals PV0018 and PV00101. During homogenization, temperature was maintained (approximately 0°C) by keeping the tubes on crushed ice, and different time conditions were tested. The effect of homogenization time on the protein yield of skin samples was evaluated. Three skin biopsies were prepared per seal, for each time condition tested (procedures A and B). Individually, skin biopsies were weighed and carefully removed the hair with a razor blade. Skin was cut into small pieces. Skin pieces were frozen (liquid N_2) in a ceramic mortar, separated into two sets and homogenized one at a time, in ice cold homogenizing buffer [volume (μl)=4 x sample weight (mg)] using plastic tubes (1cm diameter). A polytron tissue homogeniser (Kinematica) equipped with stainless steel generator (7 mm diameter) was used. Homogenization conditions were the following:

Temperature conditions:

-Mortar kept on ice.

-Homogenization tube kept on ice while homogenizing.

-45 seconds break, between bursts, to minimize heat generation during the process.

-Remaining skin pieces snap frozen (liquid N_2) in mortar between bursts.

Time conditions:

Procedure A

- Burst duration (seconds) = 15.
- Number bursts/biopsy = 10.
- Remaining skin pieces snap frozen 3 times/biopsy in liquid N_2 between bursts.

Procedure B

- Burst duration (seconds) = 15.
- Number bursts/biopsy = 15.
- Remaining skin pieces snap frozen 4 times/biopsy in liquid N_2 between bursts.

The homogenates were centrifuged at 9 000 x g for 20 minutes at 4°C and the post-mitochondrial supernatant volume recovered and measured (500 µl Hamilton glass syringe). Skin samples were immediately preserved at -80°C in labelled 0.6 ml Eppendorf tubes.

Total protein determination

The total protein concentration (T protein in mg/ml) of liver and skin samples was determined (Lowry *et al.* 1951). Standard solutions were prepared using bovine serum albumin (BSA). To obtain a standard curve for determining the total protein concentration of liver samples 50, 20, 10, 5, 2, and 1 mg/ml BSA dilutions were prepared. For skin samples, BSA dilutions were 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg/ml BSA. Samples (5 µl) or standards (10 µl) were added to 0.5 ml of DMQ (Double Milli-Q water), and after inserting 5 ml of reagent F (Table 1-Appendix 1) the tubes were vortexed and left to stand for 10 minutes. Finally, 0.5 ml of reagent D (Table 1-Appendix 1) was added, the tubes vortexed and allowed to rest for 30 minutes. Samples and standards were analyzed in duplicate using a spectrophotometer (Spectronic 20; Thermo Spectronic Baush & Lomb analytical, Cambridge, England) at 695 nm against a reagent blank. The total protein concentration of liver and skin samples (mg/ml) was calculated using the equations determined from the polynomial fit obtained in the respective standard curves. The total protein yield (mg) of liver and skin samples was determined.

Western Blotting

Method settings were adopted from previous studies that used western blotting for CYP1A characterization and quantification in fish (Wilson *et al.* 2000) and marine mammals (Bandiera *et al.* 1997; Nyman *et al.* 2000). These studies provided a basis for the adapted protocols for electrophoresis and immunoblotting, including the antibodies and positive control used. The primary antibodies were specific for rodent CYP1A enzymes, such that the results were based on the cross-reactivity obtained with putative harbour seal CYP1As.

Antibodies and positive control

The antibodies and positive control used were the following:

Table 2: Primary and secondary antibodies and positive control used for immunoblotting.

	Product	Supplier
Primary antibody	Polyclonal rabbit antiserum against rat 1A2 (PAb)	Dr S. Bandiera, University of British Columbia (Vancouver, Canada)
Secondary antibody	Goat $F(ab')_2$ anti-rabbit IgG, Alkaline phosphatase (AP) conjugated	Biosource International (Camarillo, USA)
Positive control	3-methylcholanthrene (3MC) treated rat liver microsomes	Dr S. Bandiera, University of British Columbia (Vancouver, Canada)

The rabbit antiserum against rat 1A2 (polyclonal antibody-PAb) was kindly provided by Dr Stelvio Bandiera (UBC, Canada). This antibody recognizes both CYP1A1 and 1A2 forms, and has been successfully used to detect measurable CYP1A proteins in rodents, humans, polar bears and racoons (Bandiera, unpublished data). The secondary antibody was purchased from Biosource International, USA.

Sample preparation

Liver and skin samples were diluted in fresh sample diluting buffer (Table 1-Appendix 1), containing the detergent sodium dodecyl sulfate (SDS) to solubilize, denature and impart a strong negative charge to proteins. As a uniform amount of SDS binds per microgram of protein, a uniform charge density per unit mass is achieved providing the separation based on the mass of the polypeptide chain (Coligan *et al.* 1998). 2-mercaptoethanol was also added to reduce disulphide bonds and a blue dye (bromophenol blue) to track the proteins during electrophoresis. After vortexing, samples were denatured in a 95–100 °C water bath for 3 minutes. This procedure also prevented the degradation of the sample proteins by endogenous proteases (Coligan *et al.* 1998).

Electrophoresis

The CYP1A proteins were separated by electrophoresis according to methods described elsewhere (Laemmli 1970). The Mini-PROTEAN®II electrophoresis system

(Bio-Rad Laboratories, Inc., CA, USA) was used with vertical discontinuous polyacrylamide gels (0.75 mm thickness x 7 cm length x 8 cm width). The sandwich clamp assembly was attached to the casting stand and the 7.5% separating gel (Table 1-Appendix 1) was layered with hydrated butanol and allowed to polymerize for at least one hour. Once polymerized, the gel was washed with separating gel wash buffer (Table 1-Appendix 1). After placing a 15 or 10 well Teflon comb, the 3% stacking gel (Table 1-Appendix 1) polymerized for a minimum of 90 minutes before the comb was removed and the wells washed with stacking gel wash buffer (Table 1-Appendix 1). The wells were carefully dried with filter paper strips and samples loaded (volume=10 or 20 μl) using a 50 μl glass syringe (Hamilton). Pyronin Y solution (Table 1-Appendix 1) was loaded on the first and last wells to monitor the time required for the electrophoresis and the protein transfer efficiency. Two sandwich clamp assemblies were attached to the inner cooling core, forming the upper buffer chamber. This assembly was placed in the lower buffer chamber (tank) filled with electrode buffer (Table 1-Appendix 1). The upper buffer chamber was also filled with electrode buffer. The tank was placed on a tray with ice and the lid connected to a power supply. Gels were electrophoresed at constant current (15 mAmp/gel) for approximately 60 minutes. Proteins were separated according to their molecular size as they moved through the gel from the cathode (upper buffer chamber) towards the anode (lower chamber).

Immunoblotting

The CYP1A transfer and immunoblotting was performed according to methods described elsewhere (Coligan *et al.* 1998), using the Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad). Polyvinylidene difluoride (PVDF) membranes (8 cm length x 9 cm width; 0.45 μm pore) (Immobilon-P, Millipore) were pre-wet in 100% methanol followed by equilibration in DMQ water and transfer buffer (Table 1-Appendix 1). When the electrophoresis was completed, the stacking gel was discarded and the separating gel placed in a plastic mini-gel holder cassette, between a pair of porous fiber pads (8 cm length x 11 cm width) and filter paper (8 cm length x 11 cm width) soaked in transfer buffer. A PVDF membrane was carefully smoothed over the gel to ensure good contact while eliminating any air bubbles. By minimizing non-uniformity

protein transfer is improved. The holder cassette was inserted in the electrode module that was placed in the buffer tank. A bio-ice cooling unit, filled with frozen DMQ water, was also placed in the buffer tank to absorb the heat generated in the transfer. The lid was connected to the power supply and proteins electrotransferred at 100 volts (constant voltage) for 60 minutes in cold transfer buffer. A magnetic stirrer was used to circulate buffer solution. When the transfer was completed and after disassembling the holder cassette, PVDF membranes were stored at 4°C overnight in membrane blocking buffer (Table 1-Appendix 1) to block the non-specific binding sites on the membrane surface.

The primary antibody was diluted (1:500) in antibody diluting buffer (Table 1-Appendix 1) and the membranes incubated for two hours in a 37°C water bath with mild shaking. The membranes were then washed (3 x 5-10 minutes) in wash buffer (Table 1-Appendix 1). The secondary antibody was diluted (1:3000) in antibody diluting buffer and membranes incubated for two hours in the conditions described above. The membranes were washed again (3 x 5-10 minutes) and air dried in a fume-hood (minimum 30 minutes).

After drying, the membrane bound antigens were visualized with 5-bromo-4-chloro-3-indolyl phosphate nitroblue tetrazolium (BCIP/NBT–Sigma-Aldrich), a chromogenic substrate. By submersing the membranes in BCIP/NBT, protein bands (alkaline phosphatase–precipitating substrates) appear within a few minutes. The reaction was stopped by submersing the membranes in DMQ water. Finally the membranes were dried between filter paper sheets and air dried.

Densitometry analysis

After scanning the membranes (PDI 420oe scanner, Bio-Rad), CYP1A protein bands densitometry was analysed by computer image analysis using Quantity One v4.2.2 software (The Discovery Series, Bio-Rad). The intensity (reflective density) and area (mm^2) of the pixels within the contour of each CYP1A band (contour quantity in reflective density x area-RD x mm^2) was determined. Since the primary antibody used was non species-specific, the results represent a *relative quantification* of CYP1A enzymes in harbour seal samples.

CYP1A immunoquantification in harbour seal liver and skin samples-optimization procedures

Since western blotting is a protein based assay, harbour seal CYP1A levels were corrected to protein unit to be comparable. A significant correlation between the sample total protein content and the CYP1A level obtained was necessary for the method to be reliable. Investigating this correlation also enabled to characterize sensitivity and identify an optimal total protein concentration range for the liver and skin samples to be loaded on the gels. Because the CYP1A content is higher in liver compared to extrahepatic tissues like skin, it was necessary to optimize the method for both tissues. Different experiments were conducted using harbour seal liver and skin samples prepared previously.

Protein concentration for reliable CYP1A measurements in liver samples

Liver samples from seals PV0018 and PV00101 were diluted equally (1:100) and loaded onto gels using a range of total protein concentrations between 1 and 33 µg/well (Table 3-Appendix 1). The two series of liver samples prepared per seal were loaded onto the same gel. Harbour seal hepatic CYP1A levels were quantified and the relationship between the amount of protein loaded per well and the CYP1A level measured was determined.

3-methylcholanthrene (3MC) induced rat liver as a positive control

As antibodies raised against rat CYP1A proteins have been used for western blotting, liver microsomes from rats treated with strong CYP1A inducers [*e.g.* 3MC and ß-naphthoflavone (BNF)] can be used as positive controls since both CYP1A1 and CYP1A2 enzymes are considerable induced. In this study, 3MC induced rat liver microsomes were used. Series dilutions (1 to 1:100) were prepared from a microsomal fraction (1µg total protein) and resolved in duplicates by western blotting. The intensity of the CYP1A2 band obtained in the different dilutions was plotted against the dilution factor. Based on these results a dilution factor that produced a strong band with intensity within the linear plot was selected.

When samples are resolved in different gels and CYP1A levels quantified in different membranes, a correction factor is needed to subtract the possible inter-gel variability and intensity differences resulting from membrane developing. The usefulness of 3MC rat liver microsomes in correcting inter-gel and membrane differences was tested using liver samples from seals PV0018 and PV00101. In gel 1, a sample from each seal was loaded in triplicates (~15 μg/well) and one well loaded with a sample of 3MC rat liver microsomes (1:10 dilution). A second gel (gel 2) was loaded in the identical manner. The same procedure was repeated using 2-fold dilutions (1:1) of these harbour seal liver samples. For each liver sample, the densitometry ratio of CYP1A band to the positive control CYP1A2 band was calculated. For each seal, the average CYP1A corrected levels obtained in the two gels were compared.

Determining intra- and inter-gel variability

To test intra- and inter-gel variability, a liver sample from each seal (PV0018 and PV00101) was loaded, at the same concentration of total protein, onto the same and into different gels. Liver samples (n=6) at 20 μg/well were loaded onto the same gel and into one well on 6 different gels. On each gel, a sample of 3MC rat liver microsomes (1:10 dilution) was loaded into one well. Harbour seal CYP1A bands were corrected as a ratio of the positive control CYP1A2 band densitometry and values obtained in the same and different gels averaged. The % of intra- and inter-gel variability was calculated as the coefficient of variation (CV) of the six readings:

CV= [(standard deviation*100)/average].

The % of intra- and inter-gel variability obtained for each set of harbour seal liver samples was calculated. For the western blotting system developed the % of intra- and inter-gel variability was determined by averaging the percentages obtained for seal PV0018 and PV00101.

Protein concentration for reliable CYP1A measurements in skin samples

One skin sample from each seal (PV0018 and PV00101), prepared previously using protocol 2 (Appendix 1), was diluted (2-fold series dilutions). Gels were loaded with these dilutions in increasing total protein concentration from 6.25 to 50 μg/well (Table

5-Appendix 1). Each diluted sample was loaded in triplicate onto different gels. On each gel, a sample of 3MC rat liver microsomes (1:10 dilution) was loaded into one well. For each sample, the densitometry ratio of the harbour seal CYP1A band to the positive control CYP1A2 band was calculated. The relationship between the amount of protein loaded per well and the corrected CYP1A level measured was determined.

Data and statistical analysis

Data was analysed using Sigma Plot©for Windows version 4.01 (SPSS, Inc.). Statistical analyses were performed using the STATISTICA©for Windows version 5.1 (StatSoft, Inc.). Data is presented as mean±standard error of the mean (SEM) of individual replicates. A two-tailed *t*-test was performed to determine total protein yield differences in homogenization of skin samples and evaluate densitometry differences when using corrected CYP1A levels (positive control). A p value of <0.05 was considered statistically significant. The relationship between the amount of tissue (liver and skin) homogenized and the protein yield, as well as the amount of protein loaded per well and the CYP1A levels measured (liver and skin samples) was evaluated by simple linear regression and its significance determined by testing for a significant departure of the slope from zero. A p value of <0.05 was considered statistically significant. The Pearson's product-moment correlation coefficient (r) was determined for each pair of variables. The coefficient of determination (R^2) was also determined as a percentage value of the common variation between the two variables. No data points were excluded as statistical outliers

Results and discussion

Liver samples

Duplicate sets of liver from seal PV0018, weighing between 5.1 to 50.8 mg, were homogenized yielding 0.10 to 3.27 mg of total protein. For seal PV00101, liver biopsies homogenized weighed 5.4 to 49.5 mg, yielding 0.24 to 3.22 mg of total protein (Table 3-Appendix 1).

A significant positive correlation was observed between the amount of liver homogenized and the protein yield obtained in liver samples from both seals (PV0018 r=0.9979; p<1.0x10^{-4} and PV00101 r=0.9932; p<1.0x10^{-4}) (Figure 1). These results indicate that, as expected, the protein yield obtained in liver samples reflected the amount of tissue homogenized. Tissue loss during the homogenization of such small amounts of liver did not cause variable protein yield in the samples prepared. The adopted homogenization protocol is therefore suitable for the homogenization of small liver biopsies.

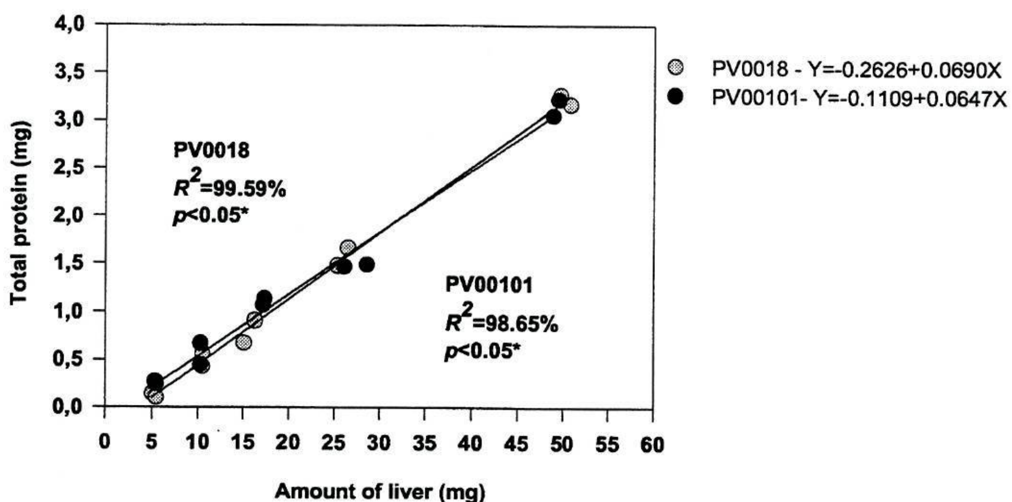


Figure 1: Correlation between the amount of liver from seals PV0018 (n=10) and PV00101 (n=10) homogenized and protein yield. Symbols refer to single readings.

Skin samples

The homogenization of skin biopsies (6 mm diameter) from both seals yield skin samples with approximately 1 mg of total protein. Longer homogenization did not result in skin samples with higher protein yield. For seal PV00101, the protein yield obtained from skin biopsies homogenized using procedure A (0.96 mg total protein) and procedure B (0.81 mg total protein) was not significantly different (t=1.0043, p=0.372). The same was observed when homogenizing PV0018 seal skin biopsies using procedures A (1.26 mg total protein) and B (0.92 mg total protein) (t=0.9133, p=0.413) (Figure 2). Protein yield obtained with procedure B was more variable for both seals; this was possibly due to the fact that after longer homogenization the homogenates were thicker and more difficult to recover, leading to variable final volumes of skin homogenates.

Time conditions in procedure A were therefore selected and a final protocol for preparation of samples from harbour seal skin biopsies developed (Protocol 2-Appendix 1).

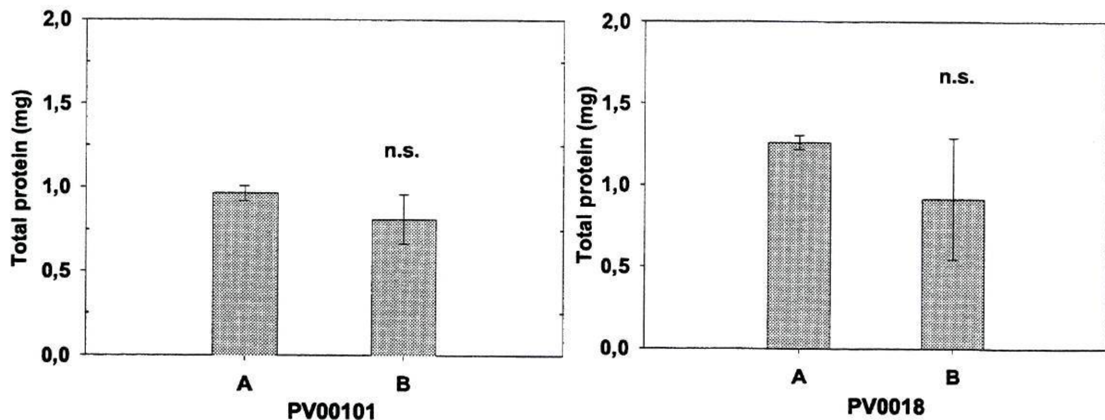


Figure 2: Comparison of protein yield from seals PV00101 and PV0018 skin samples using different homogenizing time conditions (procedure A and B). The bars refer to average of triplicates±SEM.
n.s.-non significant

Western blotting – Optimization

CYP1A in harbour seal liver samples

Protein bands showing similar mobility to CYP1A bands in the positive control (3-methylcholanthrene-induced rat microsomes) were detected in harbour seal liver samples prepared. Using the polyclonal antibody against rat CYP1A2, two protein bands could be detected in lane 3 containing microsomes from 3MC-treated rat liver (Figure 3). The first band corresponded to CYP1A1 (higher molecular weight; ~56 KDa) and the second more intensely stained band (lower molecular weight; ~52 KDa) to CYP1A2, as this antibody was more specific to rat CYP1A2. In seal liver samples, two proteins were recognized with mobility similar to rat CYP1A2, suggesting a similar molecular weight. These bands appeared as more distinct (separated) in liver sample from seal PV00101 compared to PV0018. To better confirm the identity of these putative CYP1A protein bands, a liver sample from an *in vivo* ß-naphthoflavone (BNF) induced harbour seal was included (Figure 3). The bands detected on harbour seal liver samples (lanes 1 and 2) appear to represent putative CYP1A proteins, since bands with the same mobility cross reacted strongly on the BNF induced liver sample (lane 4). Therefore, in the following immunoblots these two protein bands were quantified as CYP1A forms in harbour seal liver samples (see arrow in figure 3).

On the same blot, additional bands with faster mobility were detected in all lanes. These were attributed to non-specific binding considering that the primary antibody used was a relatively crude antibody preparation (not purified immunoglobulins).

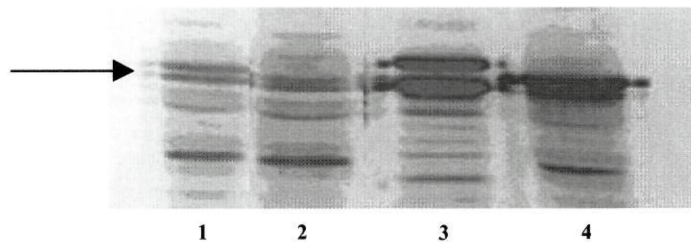


Figure 3: Immunoblot of seals PV00101 and PV0018 liver samples probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. Lane 1-PV00101 liver sample (25 µg total protein/well), lane 2-PV0018 liver sample (25 µg total protein/well), lane 3-3-methylcholanthrene (3MC) rat liver microsomes (1 µg total protein/well) and lane 4-ß-naphthoflavone (BNF) induced harbour seal liver sample (15 µg total protein/well).

Protein concentration for reliable CYP1A measurements in liver samples

No CYP1A bands were visible in wells loaded with 1-3 μg of total protein. However, starting at ~5 μg, an increase in the total protein loaded per well corresponded to increased CYP1A level measured. For all liver dilutions from both seals, a significant positive correlation between the total protein loaded per well and the CYP1A levels measured was observed; PV0018 (r=0.9258, R^2=85.71%, p=1.21x10^{-4}) and PV00101 (r=0.7638, R^2=58.34%, p=4.56 x10^{-2}). However, linearity was best in the range of 0 to ~15 μg of total protein (Figure 4). Within this protein range, when corrected the CYP1A levels to protein unit, the values obtained were similar (Table 3-Appendix 1). These results indicate that within the 0 to ~15 μg of total protein range, the CYP1A content measured in liver samples reflected the amount of total protein loaded per well. Problems with blots prevented the quantification of CYP1A levels in some of PV00101 samples, substantially reducing sample size. These findings suggest that, for the western blotting system in use, 10 to 20 μg of total protein per well should be loaded for untreated harbour seal liver samples.

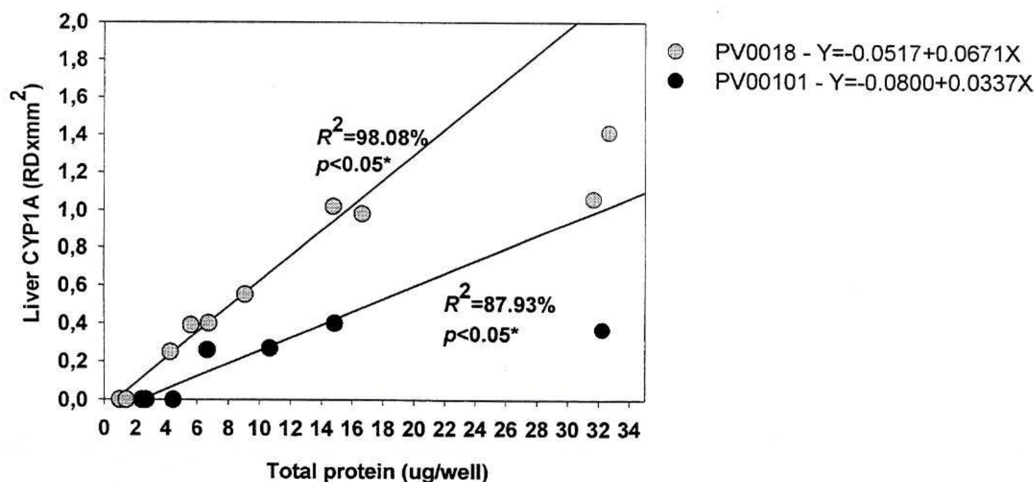


Figure 4: Correlation between the total protein loaded/well and the CYP1A levels detected for seals PV0018 (r=0.9903, p<1.0x10^{-4}; n=8) and PV00101 (r=0.9377, p=5.70x10^{-3}; n=6) liver samples. Symbols refer to single readings.

3-methylcholanthrene (3MC) induced rat liver as positive control

The rat CYP1A2 protein band intensity was correlated to all dilutions from 3MC rat treated liver microsomes tested (r=0.9519, p=9.49x10^{-4}), except the first one (1μg

total protein) that revealed an overdeveloped band and was excluded (blot not shown) (Figure 5). The dilution factor selected for the induced rat microsomes was 1:10. Several aliquots were prepared and stored at -80°C to be used as positive controls in the following gels prepared for resolving harbour seal samples.

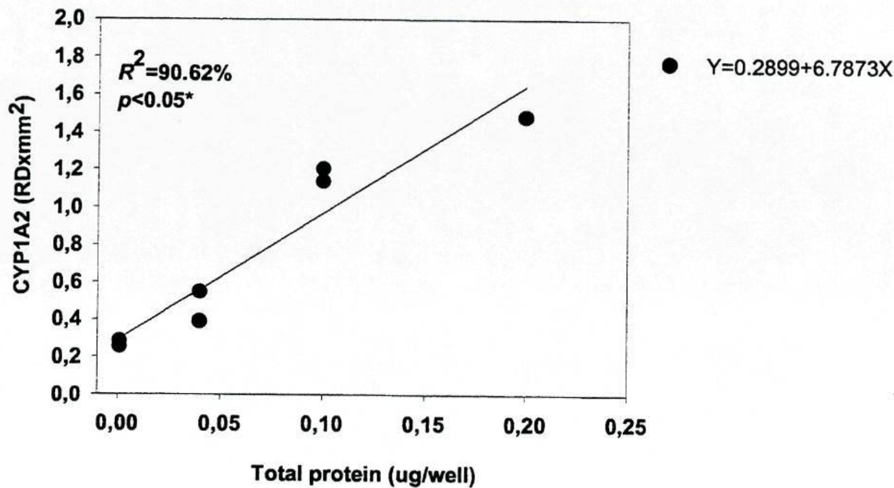


Figure 5: Correlation between the total protein loaded/well and the CYP1A2 levels measured for 3MC treated rat liver microsomes. Symbols refer to single readings.

When resolving the same liver samples from seal PV00101 in different gels, and correcting CYP1A levels to the positive control (harbour seal CYP1A RDxmm^2/3MC rat liver CYP1A2 RDxmm^2) no significant different levels of CYP1A were obtained (t=1.2760, p=0.292 and t=0.5406, p=0.618). The same was observed for PV0018 liver sample (t=-1.3797, p=0.240) loaded at higher concentration of total protein (Figure 6). These results indicate that if a 3MC induced rat sample is included in every gel as a positive control, its CYP1A2 band intensity can be used as an effective correction factor to subtract differences in CYP1A readings from harbour seal replicates resolved in different gels and blotted into different membranes.

For the 2-fold diluted sample of seal PV0018, the CYP1A levels measured in gel 1 and 2 were significantly different (t=3.5110, p=2.5x10^{-2}). As observed in figure 6, CYP1A levels detected in PV0018 liver samples were lower compared to PV00101, and considering that samples from both seals were loaded at 15 μg total protein per well, these differences might reflect different CYP1A induction degrees between the

two pups. The significantly different CYP1A levels of seal PV0018 (2-fold dilution) obtained in two different gels might indicate that for seal pups presumably less induced, loading samples in different gels with lower concentration of total protein (< 10 μg/well) might lead to non reliable CYP1A measurements. The amount of CYP1A present in those low concentrations of total protein might be too low for allowing a reliable CYP1A reading using western blotting. Therefore, loading concentrations for seal pups samples should preferentially be higher (15 to 20 μg total protein).

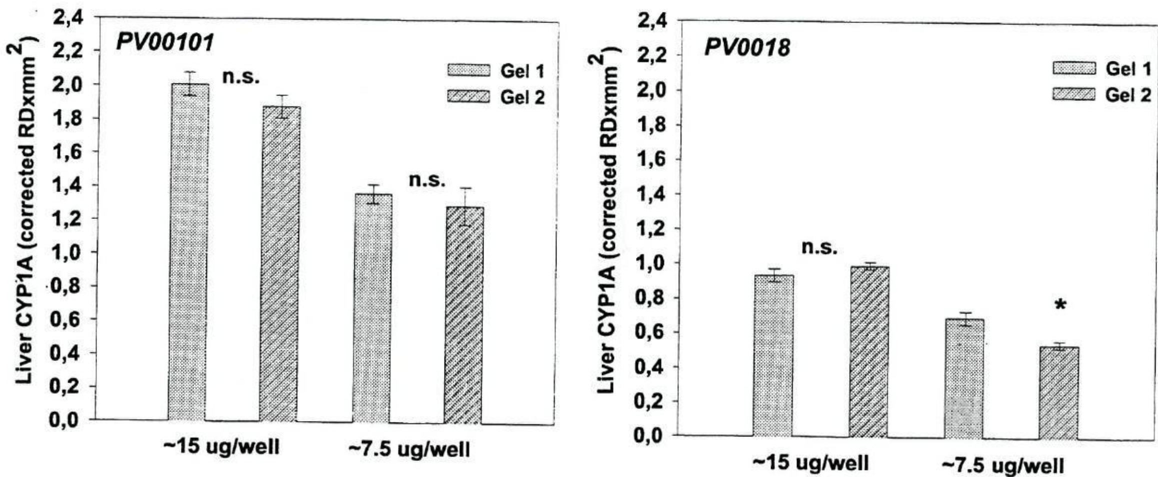


Figure 6: Comparison of corrected liver CYP1A levels measured in two different gels. For seals PV00101 and PV0018, a liver sample (~15 μg total protein/well) and a 2-fold dilution of that sample were loaded in gel 1 and gel 2. Bars refer to average of triplicates±SEM.
n.s.-non significant
*$p<0.05$

Determining intra- and inter-gel variability

In liver samples from both seals, higher variability was observed when the same sample was resolved in different gels and the CYP1A level was quantified in different membranes (Table 4). The average inter-gel variability determined for the system in use was 16.5 %, close to the limit usually accepted of 15%. Testing the same sample in the same gel and membrane revealed an average intra-gel variability of 10.8%. To account for the higher inter-gel variability, replicates of samples should be resolved in different gels/membranes.

Table 4: Intra- and inter-gel variability associated with western blotting method developed for quantification of harbour seal CYP1A. Liver samples from seal PV0018 (n=6) and PV00101 (n=6) were resolved in the same gel and in six different gels (loaded at 20 μg total protein/well).

Gel/membrane	Seal PV0018	Seal PV00101	Gel/membrane	Seal PV0018	Seal PV00101
N°	Liver CYP1A ($RD \times mm^2$)	Liver CYP1A ($RD \times mm^2$)	N°	Liver CYP1A ($RD \times mm^2$)	Liver CYP1A ($RD \times mm^2$)
1	0.88	1.60	1	0.88	1.60
1	1.12	1.58	2	1.02	1.58
1	1.26	1.30	3	1.10	1.11
1	1.14	1.27	4	0.96	1.53
1	1.08	1.32	5	0.73	1.81
1	1.15	1.40	6	0.77	1.89
Average	1.10	1.41	Average	0.91	1.59
Stdev	0.13	0.14	Stdev	0.14	0.27
% variability	11.36	10.26	% variability	15.82	17.20

CYP1A in harbour seal skin samples

In skin samples one band could be detected with similar mobility to the putative CYP1A bands in liver samples, using the polyclonal antibody against rat CYP1A2. Therefore, in the following immunoblots this protein band was assumed to represent CYP1A in harbour seal skin samples (see arrow in figure 7).

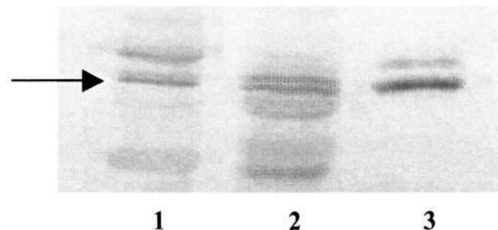


Figure 7: Immnublot of seal PV0018 skin sample probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. Lane 1-PV0018 skin sample (25 μg total protein/well), lane 2-harbour seal liver sample (15 μg total protein/well) and lane 3-3MC rat liver microsomes (1:10 dilution).

Protein concentration for reliable CYP1A measurements in skin samples

A significant positive correlation (r=0.9990, p=1.01x10^{-3}) was obtained between the total protein loaded per well and CYP1A levels measured throughout the protein range evaluated for PV0018 (Figure 8). When correcting the CYP1A levels to protein units, approximate CYP1A levels are obtained within the loading protein range of 12.5 to 50 μg (Table 5-Appendix 1). It was not possible to measure CYP1A levels in seal

PV00101 skin samples due to technical problems while preparing these samples for western blotting. Nevertheless, for the western blotting system in use, these findings suggest that untreated harbour seal skin samples should be loaded on gels within the range of 13 to 50 µg of total protein per well, with higher concentrations (*e.g.* 50 µg) possibly leading to more variable CYP1A readings.

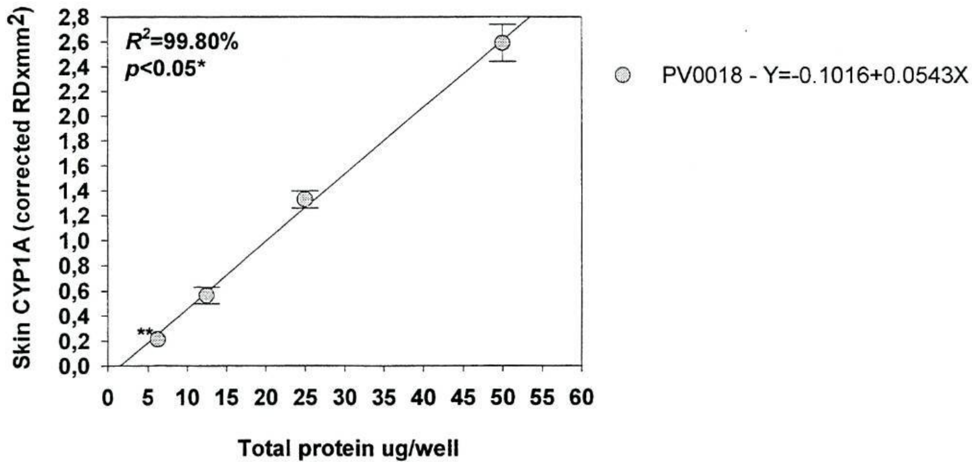


Figure 8: Correlation between the total protein loaded/well and the CYP1A levels measured for seal PV0018 skin sample dilutions (n=4). Symbols refer to average of triplicates±SEM except (**) indicating one CYP1A reading.

Conclusions

In summary, the tissue homogenization protocols described in this chapter allowed preparing suitable samples from small amounts of liver and skin obtained from harbour seals. Therefore they can be applied in similar studies using biopsy sampling. Additional studies should be carried out in order to improve skin homogenization techniques by testing more suitable, less noisy and time consuming grinding equipment.

CYP1A proteins were detected in samples generated from small biopsies of liver and skin. The antibodies raised against rat CYP1A forms are suitable for the detection of corresponding enzymes in harbour seal liver and skin. The use of a 3MC treated rat liver sample as a positive control in all gels allows correcting variation associated with inter-gel and membrane differences. The variability of sample replicates resolved in different gel/membranes was less than 16.5%. This suggests that the assay is reproducible, although 2-3 replicates are recommended. Western blotting represents an adequate technique for measuring CYP1A levels in samples prepared from liver and skin biopsies from young harbour seal pups.

Chapter 3

Cytochrome P450 1A (CYP1A) levels in Pacific harbour seal skin biopsies as a biomarker of contaminant exposure.

Abstract

As top predators in the food web, fish-eating marine mammals bioaccumulate high levels of fat-soluble persistent contaminants (*e.g.* PCBs, dioxins and furans) and may be vulnerable to adverse health effects, including reproductive impairment and immunotoxicity. Induction of hepatic CYP1A enzymes has been associated with exposure to "dioxin-like" compounds in vertebrates and is therefore touted as a biomarker of contaminant exposure. A biomarker approach using skin biopsies has the potential to provide a tool for evaluating the risks associated with contaminant exposure in free-ranging marine mammals for which liver samples cannot be obtained (*e.g.* cetaceans, endangered species). In this study, twenty free-ranging Pacific harbour seal pups (*Phoca vitulina richardsi*) were live-captured from the Fraser River Estuary (British Columbia, Canada) and housed temporarily in captivity. Small liver (approximately 30-150 mg) and skin/blubber (approximately 150-250 mg) biopsies were collected while seals were under a general anaesthetic. CYP1A enzyme levels were detected and quantified by western blotting using rat CYP1A specific antibodies, in samples obtained from liver and skin biopsies. To further evaluate CYP1A responsiveness, two additional induction studies were carried out using β-naphthoflavone (BNF), a non-toxic CYP1A inducer. Seals (n=6) were exposed to BNF orally (50 mg BNF/kg body weight), topically (0.12 mg BNF/cm^2 skin) or not at all. A significant CYP1A induction in skin and liver was observed in the orally exposed group, but not in the topically induced group, suggesting that the skin CYP1A system, like the hepatic one, responds to *in vivo* exposure through diet (internal processes). Both untreated and induced CYP1A levels in skin were significantly correlated to those in liver, supporting the use of skin as a surrogate for liver. CYP1A content in liver or skin of untreated animals did not correlate with Toxic Equivalents to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD or TCDD) (TEQs) measured in blubber. Further investigation is necessary to evaluate the utility of skin CYP1A system measurements as a biomarker of exposure to contaminants in harbour seals and other marine mammals.

Introduction

Persistent chemicals, such as the polyhalogenated aromatic hydrocarbons (PHAHs) are widely distributed in aquatic environments and subsequently accumulate in aquatic food webs. Considering their resistance to biological degradation, these lipophilic compounds tend to bioaccumulate in the blubber of marine mammals, often top predators in the food chain. Adverse health effects including reproductive impairment and immunotoxicity have been linked to the elevated contaminant concentrations in these animals (Ross *et al.* 1996; Ross 2000). These contaminants are also suspected of playing a role in facilitating the emergence of infectious diseases in free-ranging marine mammals (Ross 2001; Ross 2002).

PHAHs including polychlorinated biphenyls (PCBs), dioxins (polychlorinated dibenzo-*para*-dioxins-PCDDs), and furans (polychlorinated dibenzofurans-PCDFs) have been found in free-ranging harbour seal tissues (Addison *et al.* 2000; Bernhoft and Skaare 1994). In addition, the disruption of vitamin A dynamics, a dietary nutrient which is important to the development and health of mammals, has been linked to these contaminants (Ross *et al.* 1996; Simms and Ross 2001).

Environmental toxicology consists of different approaches to investigate the effects of contaminants on biological endpoints in suitable organisms, with evaluation of molecular or cellular alterations to these chemical stressors. Such responses, often called "biomarkers", can provide a signal of exposure and/or effect of xenobiotics in an individual (Addison 1996).

The cytochrome P450 (CYP450) enzymes consist of a large superfamily of enzymes responsible for the oxidative metabolism (mixed function oxidase or monooxygenase system-MFO) of several endogenous (*e.g.* steroids, fatty acids and vitamins) as well as exogenous compounds (*e.g.* xenobiotic chemicals). In mammals, the enzymes belonging to CYP1A subfamily, CYP1A1 and CYP1A2, represent the major xenobiotic-metabolizing forms (Nelson *et al.* 1996). Several studies have linked the degree of CYP1A induction in wild fish and mammals to the degree of contamination by "dioxin-like" PCBs, dioxins and furans (Stegeman and Hahn 1994). "Dioxin-like" PCBs include the non-*ortho* and mono-*ortho* congeners that are structurally related to

attain a planar configuration. The "dioxin-like" compounds trigger the synthesis of CYP1A enzymes by activating the cellular aryl hydrocarbon receptor (AhR) (Hahn 1998). Hepatic CYP1A therefore represents one of the most widely studied biomarkers of exposure to "dioxin-like" contaminants, in the aquatic organisms including marine mammals.

Induction studies have helped to characterize CYP1A enzymes in different fish species and laboratory rodents (Goksøyr *et al.* 1991a; Rodrigues and Prough 1991; Wolkers *et al.* 1996). The polyaromatic hydrocarbon carcinogen 3-methylcholanthrene (3MC), and flavones like ß-naphthoflavone (BNF) are strong CYP1A inducers and have been used as induction agents (Khan *et al.* 1992; Vyas *et al.* 1983), preferentially inducing CYP1A1 protein in humans, rats, rabbits and mice (Nebert and Gonzalez 1987). While 3MC represents a more potent CYP1A1 inducer (Rodrigues and Prough 1991), BNF is non-carcinogenic and less toxic, and therefore represents a preferable induction agent in non-terminal studies.

Hepatic CYP1A-like proteins and their associated catalytic activity (mainly ethoxyresorufin-*O*-deethylase-EROD) have been previously reported in phocid seals including harbour seals (*Phoca viltulina*), harp seals (*Phoca groenlandica*), hooded seals (*Cystophora cristata*), ringed seals (*Phoca hispida*) and grey seals (*Halichoerus grypus*) (Addison and Brodie 1984; Addison *et al.* 1986; Goksøyr *et al.* 1992; Goksøyr 1995a; Nyman *et al.* 2000; Wolkers *et al.* 1998b; Wolkers *et al.* 1999). Although different methods have been used to evaluate CYP1A proteins, immunochemical techniques are often considered preferable over catalytic measurements, since samples that have lost their catalytic activity retain measurable CYP1A proteins. In addition, immnunochemical techniques permit the characterization of specific CYP1A isoforms whereas catalytic endpoints likely integrate the activity of multiple enzymes (Gelboin and Friedman 1985; Goksøyr and Husoy 1998).

Until recently, the evaluation of contaminant-associated risks in marine mammals had to rely on tissues obtained from stranded, by-catch captured or sacrificed animals. The use of non-destructive sampling techniques and the analysis of alternative tissues for biomarkers of exposure in marine mammals from which liver samples cannot be obtained (*e.g.* cetaceans), is desirable for ethical, legal and logistical reasons. The

harbour seal is a top predator in the marine food web, and also represents a well-studied and widely distributed species. For these reasons, the harbour seal is becoming an important "sentinel" of marine ecosystem contamination. Since the harbour seal is exposed to a complex "real world" mixture of contaminants, their study complements laboratory-based studies of other mammals like rodents (Ross 2000). The subspecies used in this study, the Pacific harbour seal (*Phoca vitulina richardsi*), can be found in large numbers (over 100.000 individuals) on the Canadian British Columbia coast line (Olesiuk *et al.* 1990). In addition, methods have been developed which allow *in vivo* collection of microsamples of liver, blood, blubber and skin in a minimally-invasive manner.

Although CYP450 enzymes are most concentrated in liver, they are also present in extrahepatic tissues such as skin (Bickers *et al.* 1986; Raunio *et al.* 1995). Several induction studies using laboratory animals have demonstrated the CYP1A responsiveness to known CYP450 inducers, including 3MC and BNF (Agarwal *et al.* 1994; Bickers *et al.* 1986; Ichikawa *et al.* 1989; Jugert *et al.* 1994; Khan *et al.* 1992). In cetaceans sampled in the Mediterranean Sea, and southern sea lions of the coast of Argentina (Mar del Plata), levels of benzo(a)pyrene monooxygenase activity (MFO) have been measured in skin biopsies (Fossi *et al.* 1997b; Fossi *et al.* 2000; Marsili *et al.* 1998). A correlation was observed between these levels and PCBs and dichloro-diphenyltrichloroethane (DDT) concentrations in male fin whales (*Balaenoptera physalus*) (Fossi *et al.* 2000; Marsili *et al.* 1998). CYP1A appears not to be uniformly expressed in skin of Atlantic Ocean common (*Delphinus delphis*) and bottlenose dolphins (*Tursiops truncatus*), being more abundant in the lower dermis (Wilson *et al.* 2001). The expression of CYP1A1 in skin endothelium of river otters (*Lontra Canadensis*) was elevated following chronic exposure to oil (Ben-David *et al.* 2001). The evaluation of CYP1A protein expression in marine mammal skin biopsies might be of particular value, since this tissue can be relatively easily obtained with minimal stress to the animals being sampled.

This study aimed to investigate the measurement of CYP1A enzymes in harbour seal skin biopsies, as a biomarker of exposure to "dioxin-like" contaminants by:

-Using healthy recently weaned harbour seal pups in order to control for factors such as age, reproductive status and condition, so that individuals within the study group would be comparable.

-Obtaining skin and liver biopsies using innovating minimally-invasive procedures.

-Evaluating CYP1A expression in liver and skin from seal pups using western blotting.

-Testing the responsiveness of CYP1A enzymes in harbour seal liver and skin to a strong CYP1A inducer (BNF).

-Investigating the correlation between liver and skin CYP1A levels from untreated and induced harbour seal pups.

-Investigating the correlation between CYP1A levels from both tissues and the Toxic Equivalents to 2,3,7,8-TCDD (TEQs) measured in seals blubber.

Material and Methods

Seal capture and care

Twenty free-ranging Pacific harbour seal pups (*Phoca vitulina richardsi*) (females n=5; males n=15) were live-captured on Swishswash Island, Vancouver (British Columbia, Canada) in August 2000 (Figure 1). Seals were captured using a rapid deployment of a seine net from small vessels as described elsewhere (Jeffries *et al.* 1993).

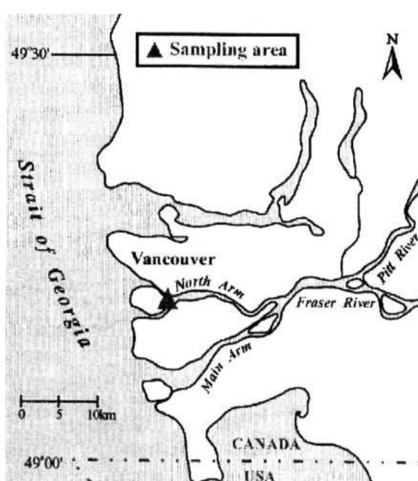


Figure 1: Harbour seals (n=20) capture area in the mouth of the Fraser River estuary near Vancouver, British Columbia, Canada.

In this area, mudflats exposed during low tide are used by harbour seals as haul-out sites. Briefly, the site was approached using two outboard-powered boats and the animals captured using a 100-170 m long net with 20-30 cm stretch mesh #36 nylon netting. The net had paired floats every 1m (float line) and 454 g of lead every 2 m (lead line). The leading boat approached the resting seals, tossed a large float attached to one end of the capture net and proceeded to encircle the area in front of the haul-out site. A second boat crew, which followed immediately, recovered the float and pulled the net ashore. After encircling the seals, the first boat crew pulled the opposite end of the net ashore. Seals were caught in the net as they returned to the water, and were immediately retrieved from the net. Pups that were considered too small and adult seals were immediately released back into the water. The selected animals were

placed into individual canine kennels and kept cool and wet during transport to the captive facilities. The seals were housed for six weeks at a Department of Fisheries and Oceans (DFO) field research station. Four outdoor circular cement pools (22 500 L) with 3 m diameter haul-out sites and constant flow-through fresh water (maintained at 12°C) were used and cleaned on a regular basis. Each seal was identified with a numbered interdigit tag on the right hind flipper and body weight, length, axillary girth, sex and general body condition were determined, as described elsewhere (Cottrell *et al.* 2002). All seal pups appeared recently weaned and age was estimated assuming 11.2 kg as mean birth weight and a daily growth rate of 350 g, as described for this subspecies (Cottrell *et al.* 2002). Seals were fed herring (*Clupea arengus*) approved for human consumption twice a day, starting with hand feeding in order to accustom them to whole fish. An animal care technician from the Marine Mammal Center (Sausalito, CA, USA) was responsible for the feeding and care of the seals during the captivity period.

A group of eight seals was released back into the area where they had been captured on August 31st. The remaining seals were used for two additional studies and released at the same location on September 18th.

All the animals were handled under veterinary supervision in accordance to principles and guidelines of the Canadian Council on Animal Care and the oversight of the Animal Care Committee, DFO, Canada.

Tissue sampling

Liver and skin/blubber biopsies were collected from seals by wildlife veterinarians in a dry indoors room. All sampling procedures were done under controlled and aseptic conditions.

Seals were fasted for at least 12 hours and placed under general anaesthetic (Isofluorine gas) for these procedures. Tissue samples, once collected, were washed in cold Phosphate Buffered Saline Solution (1X), placed in labelled cryovials and snap frozen in liquid nitrogen (liquid N_2). Animals were allowed to recover overnight in dry pools and were returned to their pools and fed the next day.

Liver biopsies

On the ventral side of the seal body an approximate 5 cm x 5 cm area was shaved and subsequently cleaned with Betadine™ (Purdue Frederick, Pickering, ON, Canada), Hibitane disinfectant (Ayerst laboratories, Montreal, QC, Canada) and 95% isopropyl alcohol (Rhone Merieux, Victoriaville, QC, Canada). Liver was obtained using a J-118 14G x 10 cm soft-tissue biopsy needle (PGI EZ Core, Products Group International, Inc., USA) through a 1 cm incision (number 11 scalpel blade) as described elsewhere (Ross *et al.*, unpublished). This procedure was repeated approximately four times for each animal, depending on the amount of tissue collected.

Skin/blubber biopsies

On the left lateral side of the body (anterior to the pelvis) of each animal, a 4 cm x 4 cm area was cleansed with Betadine, Hibitane and 95% isopropyl alcohol and a local anaesthetic applied by interdermal injection (Lidocaine™, Langford, Guelph, ON, Canada). Using an 8 mm biopsy punch (Acuderm, Ft. Lauderdale, FL, USA) two skin/blubber biopsies were obtained. The area was cleaned with Betadine and a topical anaesthetic was subsequently sprayed (Xylocaine™, Astra Pharma, Mississaugua, ON, Canada). The two tissues were separated immediately with scissors and stored in separate vials in liquid N_2. Only one of the skin biopsies was used for the present study; the other biopsy was aimed for a parallel study (Mos and Ross 2002).

ß-naphthoflavone (BNF) induction studies

Three weeks later and after a full recovery, two groups of seals (n=6) were selected for two additional induction studies. Animals with similar body weights were selected. BNF induction dosages applied in the oral and topical studies were based on *in vivo* induction studies using rodent models (Khan *et al.* 1992; Vyas *et al.* 1983). It was hypothesized that a partial CYP1A induction could be achieved in harbour seal.

Oral induction

Two treatment groups of three male seal pups were selected. The induction agent was supplied orally with the food. Individuals in the induced group (average 19.77±3.18 kg body weight) were given gelatine capsules containing 200 mg of BNF to achieve a daily dose of 50 mg/kg body weight and the control group (average 23.80±1.31 kg body weight) a fixed amount of empty capsules. The capsules were introduced into the herring through the operculum. The induced group was directly hand fed to ensure delivery of each dose (Figure 2) and the control animals were allowed to free feed.

Figure 2: Manual feeding of seal pups from the induced group.

Fish ingestion by the seals was supervised to ensure that the whole fish was eaten. This procedure was repeated on each of three days. On the fourth day a liver biopsy and a skin biopsy (left hind flipper) were obtained from each animal, using a general anaesthetic and the previously described procedures. Both tissue samples were washed in cold Phosphate Buffered Saline Solution (1X), placed in labelled cryovials and stored in liquid N_2 until further analysis.

Topical induction

In a second group of six seal pups (females n=2 and males n=4; total average body weight=23.32±1.82 kg), a 2 cm x 2 cm area was shaved at one site on the interdigital membrane of both hind flippers. The left flipper was used as the induction site and 0.36 mg of BNF dissolved in 30 µl of dimethyl sulfoxide (DMSO) were applied (Eppendorf 100 µl micropipette) to the shaved area to achieve a single induction dose of 0.12 mg BNF/cm^2 skin, assuming an application area of 3 cm^2. The right flipper was used as control area, and 30 µl of DMSO was applied (Eppendorf 100 µl micropipette). After the topical application, the skin was allowed to dry and the treated area measured and encircled with a permanent pen. The animals were placed in dry pools indoors for 24 hours. A skin biopsy was then taken at 24 hours post–induction from the centre of the marked area, in both hind flippers of each animal representing control and induced sites. After being washed on cold Phosphate Buffered Saline Solution (1X), samples were placed in labelled cryovials and stored in liquid N_2 until further analysis.

Liver and skin samples preparation

To prevent thawing during handling in the laboratory, liver and skin biopsies were processed entirely on frozen aluminium weigh boats. Each weigh boat was placed on a frozen rectangular metal tray, sitting on a mixture of dry ice and isopropanol in a fume hood. Liver and skin samples were prepared as post mitochondrial supernatants (PMS) of tissue homogenates for total protein determination and CYP1A density analysis (western blotting).

Liver samples

Liver biopsies (approximately 30 to 40 mg) were weighed individually and homogenized in 80 to 100 µl of ice cold homogenizing buffer (Table 1-Appendix 1), according to the established protocol 1 (Appendix 1). The supernatants were recovered and their volume measured with a 250 µl glass syringe (Hamilton). Liver samples were preserved immediately at -80°C in labelled 0.6 ml Eppendorf tubes.

Skin samples

Skin biopsies were removed from cryovials and a piece cut using a razor blade (approximately 10% of the biopsy) and immediately preserved in liquid N_2 for other study purposes. Each biopsy was weighed and cut into small pieces after the hair was carefully removed with a razor blade. Skin pieces were divided into two sets, placed in a ceramic mortar on ice and homogenized according to the protocol developed (Protocol 2-Appendix 1). Briefly, skin pieces were placed in plastic tubes (1cm diameter) containing 360 to 730 µl of ice cold homogenizing buffer (Table 1-Appendix 1). A polytron tissue homogenizer (Kinematica) equipped with stainless steel generator (7 mm diameter) was used. For each biopsy, homogenization consisted of ten bursts (15 seconds) with stops (45 seconds) in between, to minimize heat generation during the process. Additionally, skin pieces in the mortar were snap frozen three times with liquid N_2 to enhance the homogenization procedure. The homogenates were centrifuged at 9 000 x g for 20 minutes at 4°C. A 500 µl glass syringe (Hamilton) was used to recover and measure the supernatants volume. Skin samples were immediately preserved at -80°C in labelled 0.6 ml Eppendorf tubes.

Total protein determination

The total protein concentration (T protein in mg/ml) of the samples was measured (Lowry *et al.* 1951), following the procedures described on chapter 2. The total protein yield (mg) of liver and skin samples was determined.

CYP 1A quantification (western blotting)

Western blotting technique was used to detect and quantify CYP1A enzymes in harbour seal liver and skin samples. The procedure followed the optimization settings defined on chapter 2.

Antibodies and positive control

The antibodies and positive control used were the following:

Table 1: Primary and secondary antibodies and positive control used for immunoblotting.

	Product	Supplier
Primary antibody	Polyclonal rabbit antiserum against rat CYP1A2 (PAb)	Dr S. Bandiera, University of British Columbia (Vancouver, Canada)
Primary antibody	Monoclonal mouse against rat CYP1A1/2 (MAb 1-7-1)	Dr H. Gelboin, National Cancer Institute (Maryland, USA)
Secondary antibody	Goat $F(ab')_2$ anti-rabbit IgG, Alkaline phosphatase (AP) conjugated	Biosource International (Camarillo, USA)
Secondary antibody	Goat $F(ab')_2$ anti-mouse IgG, Alkaline phosphatase (AP) conjugated	Biosource International (Camarillo, USA)
Positive control	3-methylcholanthrene (3MC) treated rat liver microsomes	Dr S. Bandiera, University of British Columbia (Vancouver, Canada)

Dr S. Bandiera (Vancouver, Canada) and Dr H. Gelboin (Maryland, USA) kindly provided the primary antibodies. The polyclonal rabbit antiserum against rat 1A2 antibody recognizes both CYP1A1 and 1A2 forms, and has been successfully used to detect measurable CYP1A proteins in rodents, humans, polar bears and racoons (Bandiera, unpublished data). The monoclonal mouse anti-rat 1A1/2 also recognizes both CYP1A forms and produced detectable CYP1A bands in liver samples from ringed and grey seals (Nyman *et al.* 2000). In addition, this antibody also showed immunoreactivity in rat epidermis pretreated with 3MC (Khan *et al.* 1989). Secondary antibodies were purchased from Biosource International, USA.

Sample preparation

Harbour seal liver and skin samples and the positive control (3MC treated rat liver microsomes) were prepared as described in chapter 2. The optimal total protein concentrations to be loaded into each well for liver and skin samples from BNF induced animals were determined in preliminary experiments using both primary antibodies. Samples were loaded onto the gels with the following concentrations of total protein:

Table 2: Loading concentrations of liver and skin samples for each primary antibody.

Primary antibody	Samples	Total protein (μg)/well
Polyclonal rabbit anti-rat 1A2	Harbour seal liver	20
	Harbour seal skin	25
	Oral BNF harbour seal liver	1.5
	Oral BNF harbour seal skin	10
	Topical BNF harbour seal skin	20
	3MC rat liver	1:10 dilution
MAb 1-7-1	Harbour seal liver	20
	Harbour seal skin	25
	Oral BNF harbour seal liver	10
	Oral BNF harbour seal skin	25
	3MC rat liver	1:5 dilution

Electrophoresis, immunoblotting and densitometry analysis

These procedures were performed as described in chapter 2. Liver and skin samples were analysed in duplicates, loaded into different gels. For each sample, the densitometry (contour quantity in reflective density x area-RD x mm^2) ratio of the CYP1A band to the positive control CYP1A2 band was calculated. Duplicate readings from different gels/membranes were accepted if the percentage of variability was within the limit defined for the system (16.5%); otherwise the experiment was repeated. Data refers to the average of replicates corrected densitometry values per microgram of total protein loaded per well.

Contaminant analysis

Harbour seal blubber samples were analyzed by the Regional Contaminant Laboratory at the Institute of Ocean Sciences, DFO (BC, Canada), for full congener-specific PCBs, PCDDs and PCDFs, using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) according to methods described elsewhere (Ross *et al.* 2000). Toxic Equivalency Quotients (TEQs) were calculated using the most recent Toxic Equivalency Factors for mammals (Van den Berg *et al.* 1998). TEQs were determined for non-*ortho* and mono-*ortho* PCBs congeners, PCDDs and PCDFs, as well as total TEQs, in ng per kg of lipid weight.

Data and statistical analysis

Data was analysed using Sigma plot©for Windows version 4.01 (SPSS, Inc.). The CYP1A levels and the different contaminant classes were tested for normality using the Shapiro-Wilks test. Normal distribution was assumed if $p>0.05$, otherwise data was log-transformed prior to statistical treatment. Statistical analyses were performed using STATISTICA©for Windows version 5.1 (StatSoft, Inc.). Data is presented as mean±standard error of the mean (SEM) of individual replicates and/or group of seals. A two-tailed *t*-test was performed to evaluate differences in hepatic CYP1A band densitometry from BNF induced and control animals, in CYP1A content between liver and skin samples, and in CYP1A levels between control and induced animals in BNF oral and topical induction studies. A p value of <0.05 was considered statistically significant. The relationship between the amount of tissue homogenized (liver and skin) and the protein yield, the CYP1A content in liver and skin samples, as well as the CYP1A content and different contaminant concentrations (TEQs) was evaluated by simple linear regression and its significance determined by testing for a significant departure of the slope from zero. A p value of <0.05 was considered statistically significant. The Pearson's product-moment correlation coefficient (r) was determined for each pair of variables. The coefficient of determination (R^2) was also determined as a percentage value of the common variation between the two variables. No data points were excluded as statistical outliers.

Results

General study group

The twenty seal pups captured included five females and fifteen males. The captured seals average body weigh was 23.98±0.82 kg, which allowed estimating an average age of 38.2±2.6 days (Table 3-Appendix 2). Most of the animals were approaching weaning or were weaned, as this occurs between 26 to 40 days in harbour seal with mean weaning mass estimated at 23.6 kg (Cottrell *et al.* 2002).

All pups were in good condition except seal #18, which was euthanised with the agreement of all veterinarians, due to a life-threatening systemic infection associated with a pre-existing fracture of the lower mandible.

BNF induction study groups

The physical parameters of the harbour seal pups used for the oral and topical induction studies, as well as the study designs used are listed in Tables 4 and 5 (Appendix 2).

Liver biopsies

On the first sampling days (19th and 20th August), an attempt was made to collect liver biopsies from all twenty animals. However, tissue was only obtained from seventeen seals (average weight=51.5±7.7 mg) since the biopsy needle failed to penetrate the liver capsule in some cases (Figure 3).

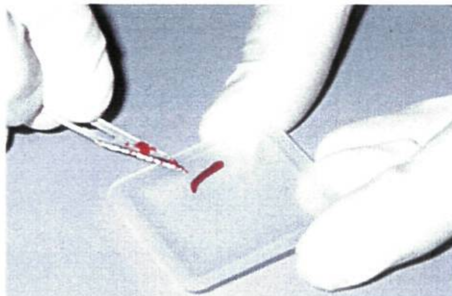


Figure 3: Liver biopsies collected from young harbour seals under a general anaesthetic.

Twelve liver biopsies were used for this study since the remaining tissue was used for other endpoints. The liver biopsies homogenized weighed on average 35.1±2.1 mg and the average total protein concentration obtained in liver samples was 2.02±0.14 mg (Table 6–Appendix 2).

After the BNF oral induction study (8th September), liver biopsies were obtained from all six seals. Considering the larger amount of liver obtained by biopsy at this point (average weight=148.2±26.0 mg) only a fraction of the tissue collected was homogenized (average weight=40.8±0.6 mg). The average total protein concentration obtained in liver samples was 3.06±0.14 mg (Table 6–Appendix 2).

Skin biopsies

One skin biopsy was obtained from the lateral left side of the body of each seal (average weight=~134 mg; 8mm diameter; 2 cm in depth including blubber) (Figure 4). The average total protein concentration obtained in skin samples was 2.17±0.13 mg (Table 7–Appendix 2).

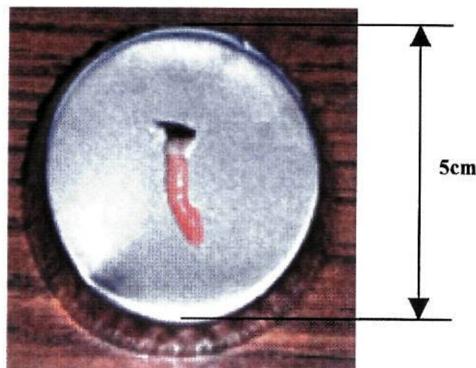


Figure 4: Skin/blubber biopsy.

Skin biopsies were collected from the hind flippers as previously discussed for the BNF induction studies (Figure 5 and 6).

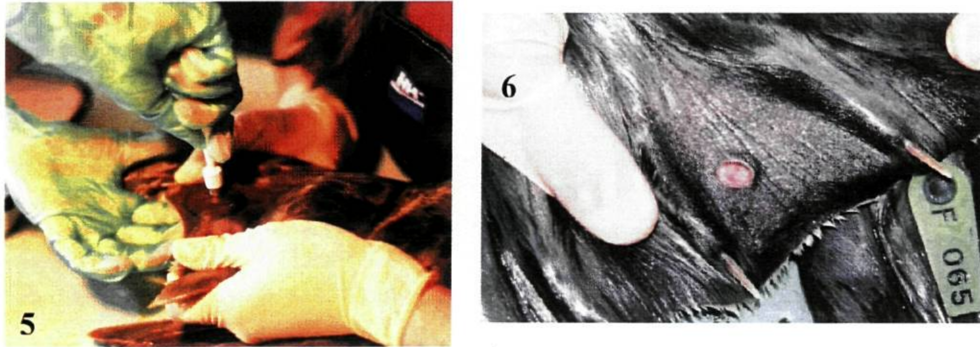


Figure 5 and 6: Biopsy sampling in hind flipper (5); sampling area 24 hours after collecting biopsy (6).

The average weight of skin biopsies collected from hind flippers was less than skin biopsies obtained previously from the lateral body (average weight = ~60 mg). The total protein concentration was on average 1.06±0.09 mg (Table 7–Appendix 2).

The protein yield of liver and skin samples prepared correlated with the amount of tissue homogenized, has shown in figure 7. This suggests that the tissue homogenization protocols used for small harbour seal liver and skin biopsies are robust, and that protein levels are proportionate to the mass of tissue.

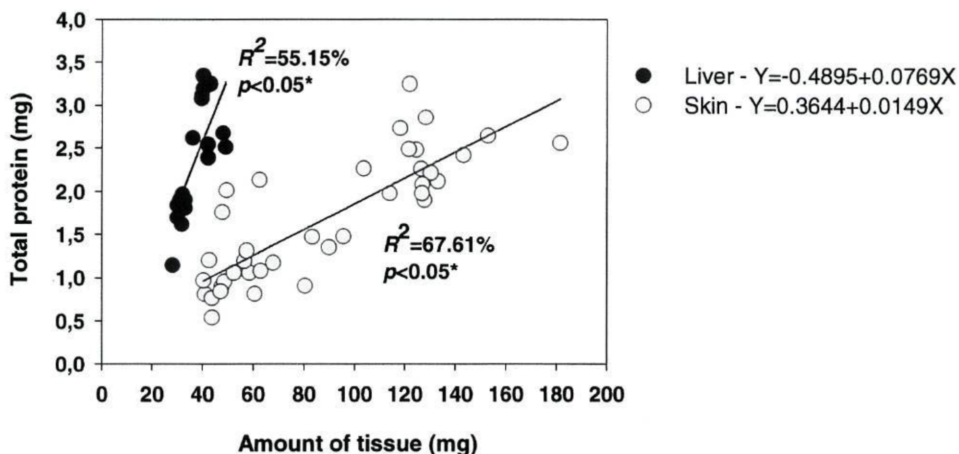


Figure 7: Correlation between the amount of tissue homogenized and the protein yield for all harbour seal liver (r=0.7426, p=4.15x10^{-4}; n=18) and skin (r=0.8223, p<1.0x10^{-4}; n=38) samples. Symbols refer to single readings.

Western blotting quantification of CYP1A enzymes in harbour seal liver and skin fractions

CYP1A proteins were separated and quantified by western blotting in all liver and skin samples. Given the protein yield obtained in these samples, the amount of tissue necessary to evaluate CYP1A levels in both liver and skin represented a small percentage of the biopsy tissue homogenized.

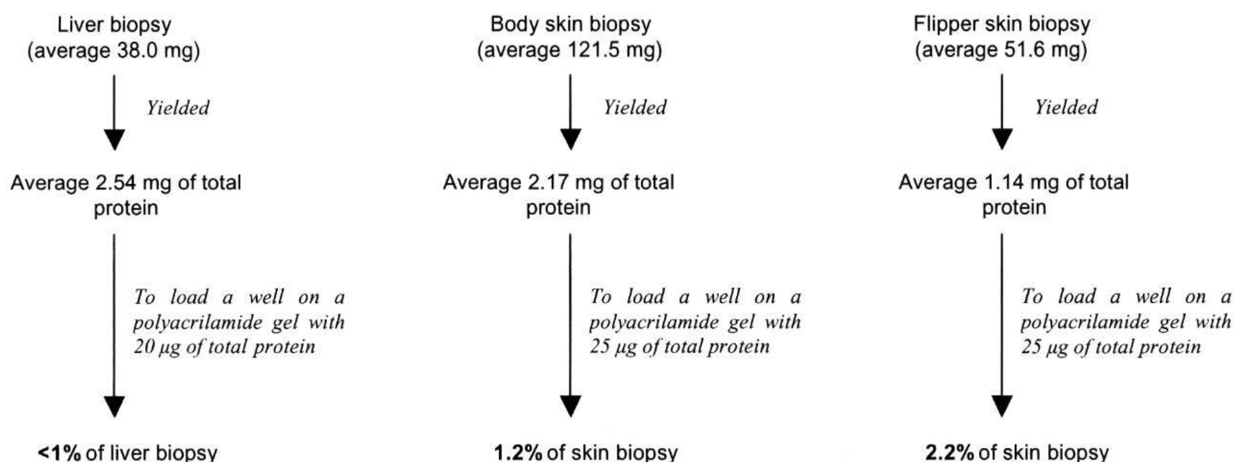


Diagram 1: Percentage of harbour seal liver and skin biopsies necessary for CYP1A immunoquantification.

* The diagram refers to all liver and skin biopsies collected from seals of the general study group, BNF oral induction and BNF topical induction study groups.

The amount of tissue in liver or skin biopsy used in this study is therefore sufficient for CYP1A detection by western blotting.

Liver samples

In liver samples from the BNF oral induction study animals (Figure 8) and general study group (Figure 9), the polyclonal anti-rat CYP1A2 recognized two protein bands (band 1 and band 2) immunochemically related to rat CYP1A1 and CYP1A2 forms.

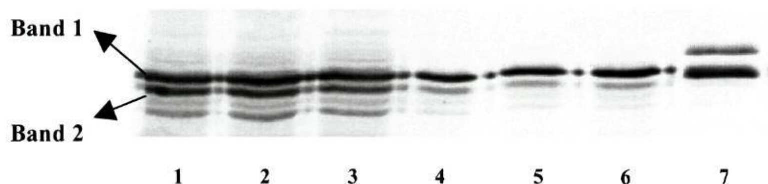


Figure 8: Immunoblot of liver samples from control (lane 1, 2 and 3 at 20 µg total protein/well) and BNF induced (lane 4, 5 and 6 at 1.5 µg total protein/well) harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. 3MC induced rat microsomes (lane 7 at 1:10 dilution) were included as positive control.

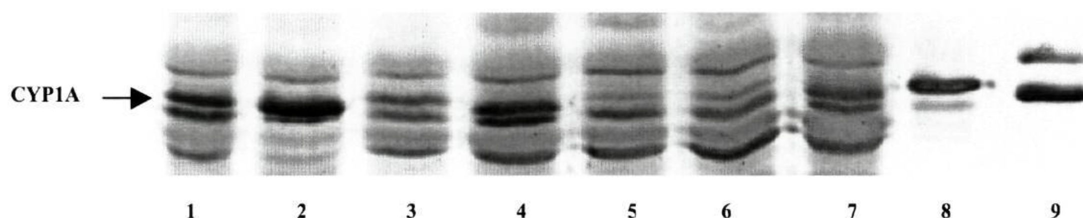


Figure 9: Immunoblot of liver samples from untreated harbour seals (lane 1, 2, 3, 4, 5, 6 and 7 at 20 μg total protein/well), probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. A liver sample from a BNF orally induced seal (lane 8 at 1.5 μg total protein/well) was included as reference and 3MC induced rat microsomes (lane 9 at 1:10 dilution) as positive control.

In the BNF oral induction study, a significant increase in hepatic CYP1A levels was observed for both CYP1A protein bands in induced seals (band 1, t=-13.0365, p=2.00x10^{-4}) (band 2, t=-33.3494, p=4.82x10^{-6}), suggesting that two CYP1A forms may be expressed in harbour seal liver (Figure 8 and 10). Additionally, in the band with higher molecular weight (band 1) a 10 fold increase was observed between control and induced animals, while a 4.5 fold increase was observed in the second band (band 2), which might suggest that band 1 refers to a putative CYP1A1 protein.

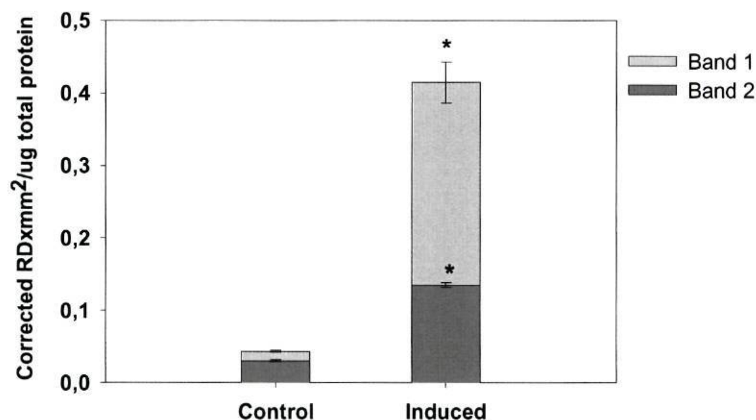


Figure 10: CYP1A protein bands densitometry in liver samples from BNF oral induction study. Control group-seals# 1, 7 and 16; induced group-seals# 12, 14 and 15. Bars refer to average densitometry levels of replicates per treatment group ±SEM.
* p<0.05

Skin samples

One protein band with similar mobility to the band 1 in liver samples cross-reacted in skin samples from seals given BNF orally (Figure 11) and topically (Figure 12).

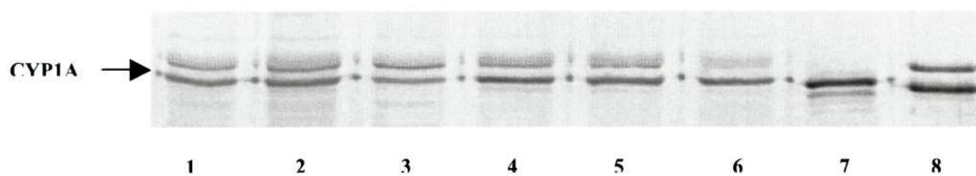


Figure 11: Immunoblot of skin samples from control (lane 1, 2 and 3 at 25 μg total protein/well) and BNF orally induced (lane 4, 5 and 6 at 10 μg total protein/well) harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. A liver sample from a BNF orally induced seal (lane 7 at 1.5 μg total protein/well) was included as reference and 3MC induced rat microsomes (lane 8 at 1:10 dilution) as positive control.

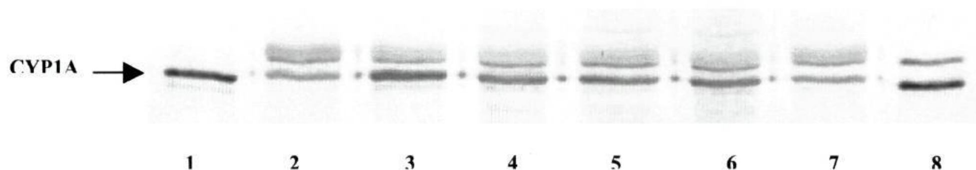


Figure 12: Immunoblot of skin samples from control (lane 2, 4 and 6 at 25 μg total protein/well) and BNF topically induced (lane 3, 5 and 7 at 20 μg total protein/well) harbour seals, probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. A liver sample from a BNF orally induced seal (lane 1 at 1.5 μg total protein/well) was included as reference and 3MC induced rat microsomes (lane 8 at 1:10 dilution) as positive control.

This same band was detected in skin samples from the general study group of seals and a thinner second band (faster mobility) could also be observed in some lanes (Figure 13). As this band is faint and not present in all skin samples, only the first band was considered. The nature of this second band was not determined due to time limitations.

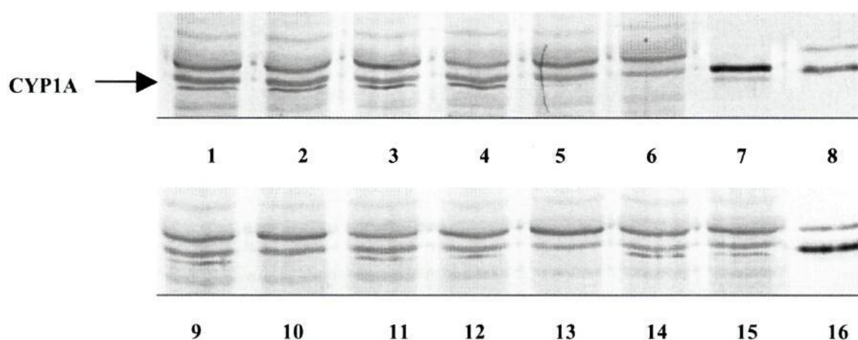
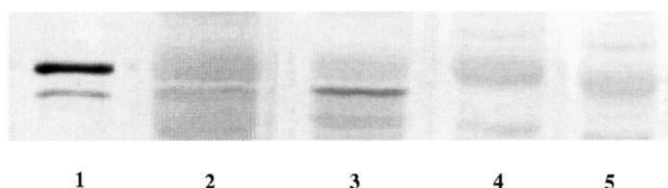


Figure 13: Immunoblot of skin samples from untreated harbour seals (lane 1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 13, 14 and 15 at 25 μg total protein/well), probed with polyclonal antibody against rat CYP1A2 at 1:500 dilution. A liver sample from a BNF orally induced seal (lane 7 at 1.5 μg total protein/well) was included as reference and 3MC induced rat microsomes (lane 8 and 16 at 1:10 dilution) as positive control.

To confirm the results obtained with the polyclonal antibody, a monoclonal antibody (MAb 1-7-1) that recognizes both CYP1A proteins in rat was used on liver and skin samples from both control and BNF orally induced animals. Only a single band was detected in a liver sample from BNF induced seal; this band is coincident in migration with band 1 (putative CYP1A1) previously detected. The second protein band (band 2) was not detected, perhaps because this antibody recognizes rat CYP1A1 protein over CYP1A2, as illustrated by the different intensity of these two bands obtained in 3MC induced rat microsomes (Figure 14–lane 1).

The same blot showed more faint cross reactivity with skin samples from BNF induced animals and skin and liver samples from control animals, where no distinct bands were detected.



1 2 3 4 5

Figure 14: Immunoblot of liver and skin samples from BNF oral induction study seals, probed with monoclonal antibody against rat CYP1A1/2 (Mab 1-7-1) at 20 μg IgG/ml. Lane 1-3MC induced rat liver microsomes (1:5 dilution), lane 2-control liver sample (20 μg total protein/well), lane 3-BNF induced liver sample (10 μg total protein/well), lane 4—control skin sample (25 μg total protein/well), lane 5-BNF induced skin sample (25 μg total protein/well).

For the general study group, immunoquantification of the two protein bands in liver samples averaged a CYP1A content ($5.68 \times 10^{-2} \pm 1.50 \times 10^{-2}$ CYP1A/μg total protein) which was 3 times higher than the single CYP1A band quantified in skin samples ($1.93 \times 10^{-2} \pm 3.50 \times 10^{-3}$ CYP1A/μg total protein) (Figure 15). This difference was significant (t=10.8049, p=0,00).

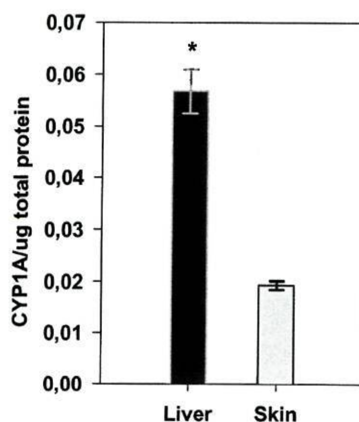


Figure 15: Average CYP1A content on harbour seal liver (n=12) and skin (n=20) samples. Bars refer to average CYP1A levels per tissue ±SEM.
* $p<0.05$

For comparative purposes, the same CYP1A protein band was quantified in both tissues analyzed. Considering that in harbour seal skin samples only the putatitve CYP1A1 form (band 1) was consistently detected, only the corresponding band in liver samples was considered. Since the nature of this protein was not further confirmed with a specific antibody against rat CYP1A1, it is referred as CYP1A in the following results.

The CYP1A levels detected by western blotting in the liver and skin samples of the general study group are summarised in tables 8 and 9; both data sets were determined to be normal (Shapiro-Wilks test; $p>0.05$-Tables 8 and 9-Appendix 2). Table 8 (Appendix 2) also includes the CYP1A levels for the liver samples of the BNF oral induction study group. The CYP1A levels detected by western blotting for the skin samples of the BNF oral induction study group and the induced and control groups of BNF topical induction study are reported on table 9 and 10 (Appendix 2).

General study group

The CYP1A protein band quantified in liver and skin samples showed variable intensity among individual seal pups (Figure 16). For the group of twelve pups for which both tissues were obtained, the average CYP1A level in liver ($1.98\times10^{-2}\pm8.50\times10^{-3}$CYP1A/µg total protein) was only slightly higher than skin ($1.79\times10^{-2}\pm2.80\times10^{-3}$CYP1A/µg total protein).

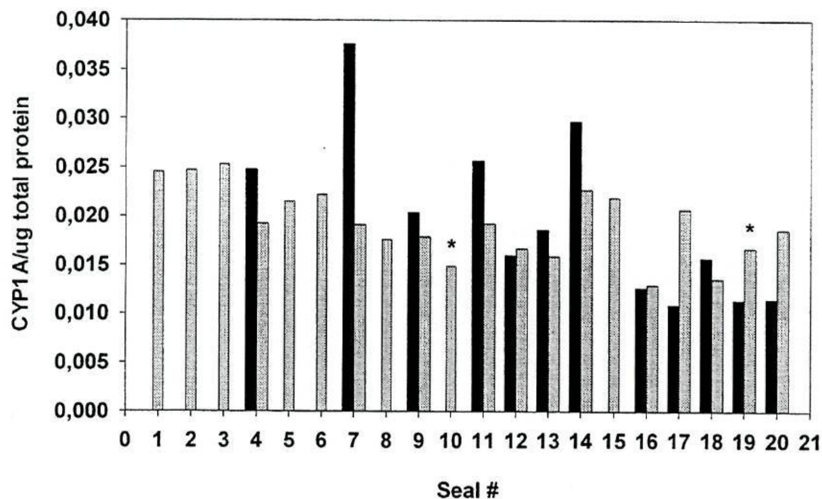


Figure 16: Average content of CYP1A/ug total protein in liver (black bars) and skin (grey bars) samples from harbour seal pups. Symbols refer to average of individual replicates except (*) indicating one CYP1A reading.

BNF oral induction group

Oral *in vivo* induction of harbour seals with BNF resulted in significant increases in CYP1A levels in liver and skin samples (Figure 17). The CYP1A level was significantly higher in liver samples from induced animals (t=-13.0365, p=2.00x10^{-4}) compared to control animals, representing a 10-fold increase in CYP1A protein concentration. A 3-fold increase in CYP1A protein concentration was observed in skin samples from induced animals (t=-5.5235, p=5.25x10^{-3}).

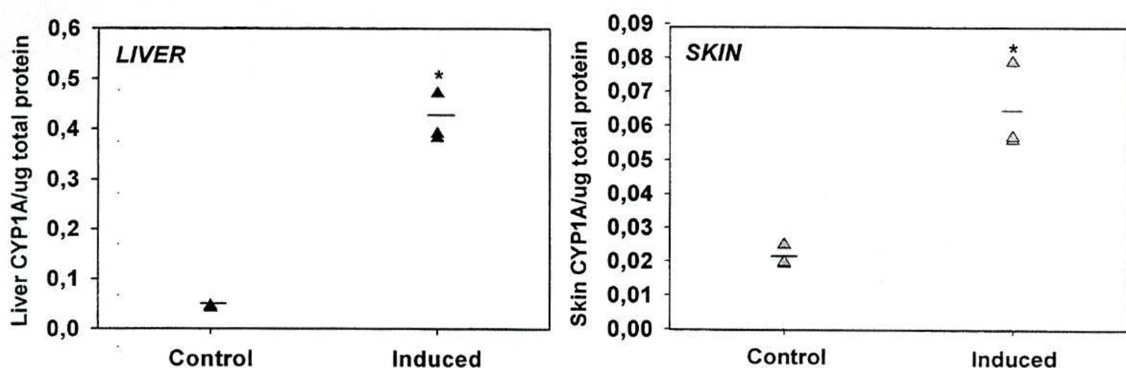


Figure 17: Liver and skin CYP1A levels in induced animals (n=3) and control animals (n=3) from the BNF oral administration study. Symbols refer to average CYP1A of individual replicates and lines to average CYP1A of treatment group.
* $p<0.05$

BNF topical induction group

The degree of induction was more variable when the induction agent was applied topically. The fixed volume applied to the shaved area on the left and right hind flipper of each animal spread variably (Table 5-Appendix 2). On average CYP1A levels in samples from induced flipper skin were 1.33 times higher but this difference was not significant (t=-1.1558, p=0.275). There was a small increase in skin CYP1A levels in induced flipper site, except in seal 20 were levels were reduced. Within the topical treatment group, CYP1A levels were also variable (Figure 18).

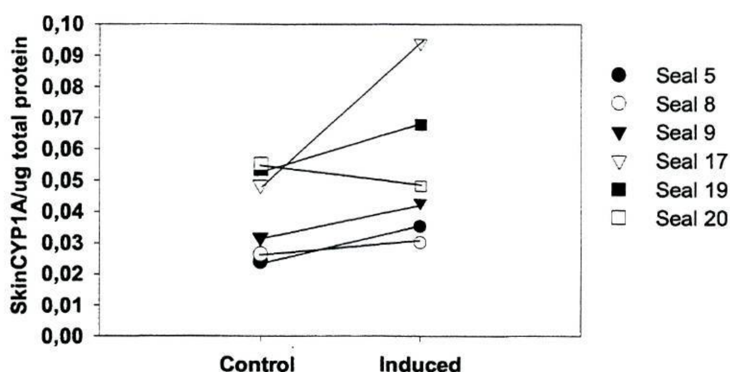
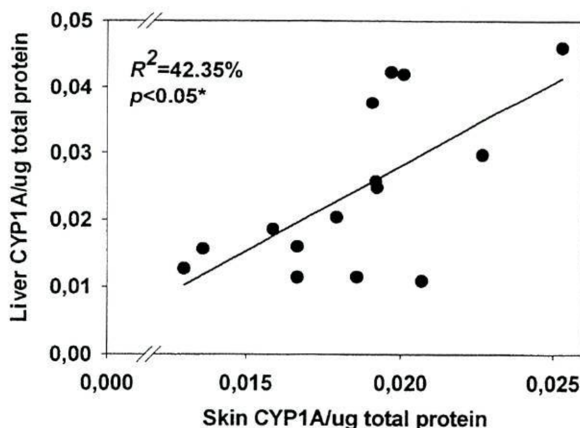


Figure 18: CYP1A levels in skin samples from the induced area (n=6) compared to skin samples from the control area (n=6) in seals from the BNF topical application study. Symbols refer to average CYP1A of individual replicates.

Correlating harbour seal CYP1A in liver and skin

A significant correlation between the CYP1A levels in liver and skin samples obtained from non-induced animals was established (r=0.6508, $p=8.60x10^{-3}$) (Figure 19), indicating that skin CYP1A levels tend to predict hepatic CYP1A content. Although the sample size was limited in the BNF oral induction study (n=6), it was interesting to observe that the CYP1A levels expressed in skin and liver also correlated among induced animals (r=0.9800, $p=5.94x10^{-4}$) (Figure 20).



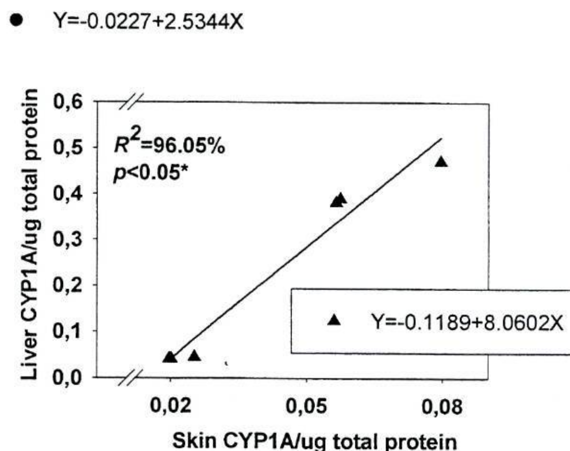


Figure 19: Correlation between CYP1A levels in liver and skin from untreated harbour seals from the general study group and BNF oral induction study group (n=15). Symbols refer to average CYP1A of individual replicates.

Figure 20: Correlation between CYP1A levels in liver and skin from induced and control harbour seals of the BNF oral induction study group (n=6). Symbols refer to average CYP1A of individual replicates.

Contaminant analysis

Blubber PCDD, PCDF and "dioxin-like" PCB residue levels (lipid weight basis) were expressed as toxic equivalency quotients (TEQs) (Table 11-Appendix 2).

The rank of TEQ contribution was mono-*ortho* PCBs > non-*ortho* PCBs > PCDDs > PCDFs (Figure 21). Mono-*ortho* PCBs, which are relative strong CYP1A inducers, accounted for almost half of the residues measured (47%) and strong CYP1A inducers, the non-*ortho* PCBs, accounted for 31% of the total TEQ. PCBs contributed therefore to 78% to the total TEQ. Dioxins (20%) and furans (2%) contributed the least.

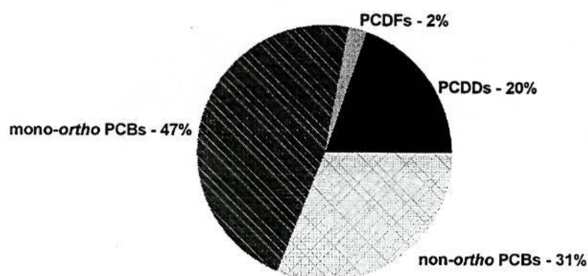


Figure 21: Percent average of total PCDD, PCDF and PCB (non-*ortho* and mono-*ortho*) congeners contributing to total TEQs (TEQ ng/kg lipid) in harbour seal pups (n=20).

In addition to assessing the relationship between the contaminants concentrations in all 20 seals from which skin samples were obtained, these four contaminant classes, as well as total TEQs were also grouped for the 12 seals from which liver samples were obtained.

All data sets were determined to be normal except for non- and mono-*ortho* PCBs and total TEQs for all the seals (n=20) and mono-*ortho* PCBs, for the smaller group (n=12) (Shapiro-Wilks test; $p>0.05$-Table 11-Appendix 2). For consistency, all data sets were therefore log-transformed.

The CYP1A levels in liver and skin were not significantly correlated to the planar contaminants burden present in seals blubber. Weak correlations were found between liver ($r=0.2610$, $R^2=6.81\%$, $p=0.413$) and skin ($r=0.2989$, $R^2=8.94\%$, $p=0.200$) CYP1A levels and total TEQs (Figure 22).

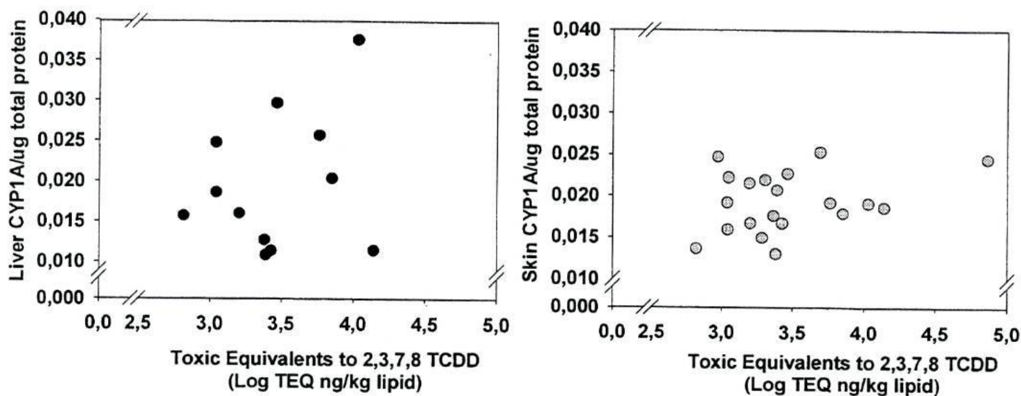


Figure 22: Scattergram of liver (n=12) and skin (n=20) CYP1A levels and blubber total TEQs. Symbols refer to individuals from the general study group.

When analysing the four contaminant classes separately similar trends were obtained. The correlations between hepatic CYP1A levels and non-*ortho* PCBs ($r=0.3362$, $R^2=11.30\%$, $p=0.285$), mono-*ortho* PCBs ($r=0.3053$, $R^2=9.32\%$, $p=0.335$), dioxins ($r=0.0048$, $R^2=0\%$, $p=0.988$) and furans ($r=-0.4849$, $R^2=23.51\%$, $p=0.110$) were non significant.

Skin CYP1A levels did not correlate significantly with mono-*ortho* PCBs (r=0.1367, R^2=1.87%, p=0.566), dioxins (r=0.1760, R^2=3.10%, p=0.458) or furans (r=-0.1369, R^2=1.87%, p=0.565), although a weak but significant correlation between non-*ortho* PCBs (r=0.4471, p=4.81x10^{-2}) and skin CYP1A was observed (Figure 23). A "heavily contaminated" outlier might have driven this correlation as its removal leads to a non-significant relationship (r=-0.2872, R^2=8.25%, p=0.233, n=19).

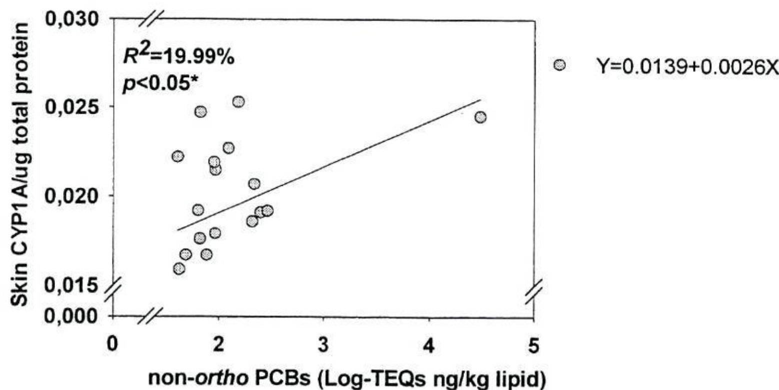


Figure 23: Correlation between CYP1A levels in harbour seal skin samples (n=20) and non-*ortho* PCBs. Symbols refer to individuals from the general study group.

No correlations were found between liver (r=-0.1767, R^2=3.12%, p=0.583) or skin (r=0.0093, R^2=0%, p=0.969) CYP1A content and body weight, a crude surrogate for age and to a lesser extent condition (Figure 24).

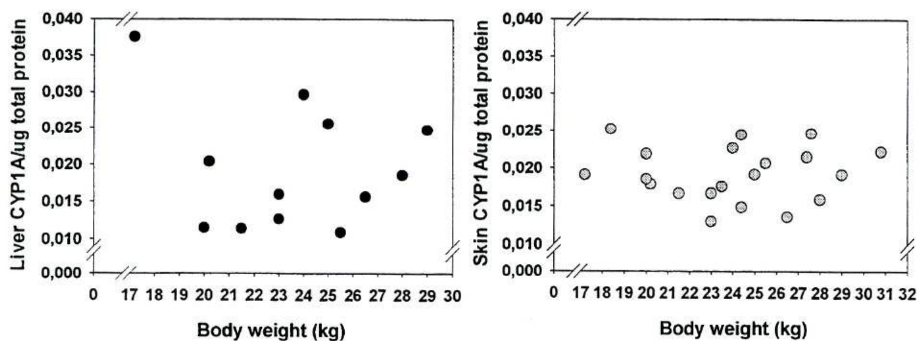


Figure 24: Scattergram of CYP1A levels in liver (n=12) and skin samples (n=20) and body weight. Symbols refer to individuals from the general study group.

When relating the pups body weight with total TEQs, a significant inverse correlation was found (r=-0.5495, p=1.21x10^{-2}) (Figure 25).

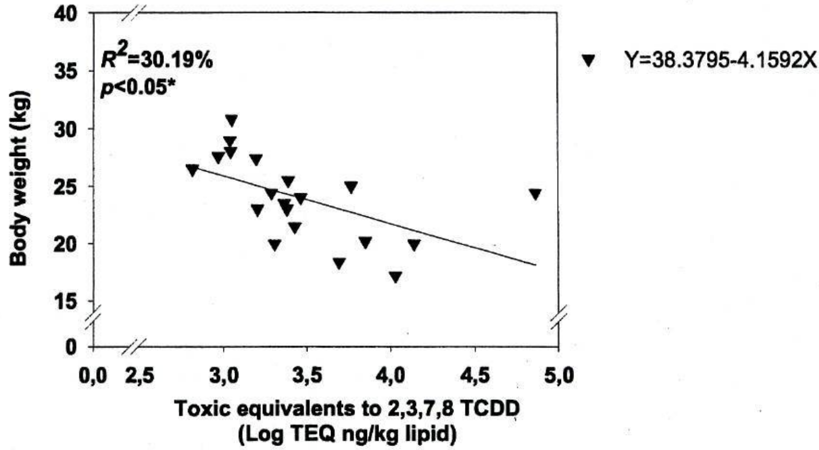


Figure 25: Correlation between harbour seals body weight (n=20) and blubber total TEQs. Symbols refer to individuals from the general study group.

Discussion

When investigating marine mammals, the use of biological samples that can be obtained in a non-invasive or minimally-invasive way is desirable. In this study, liver and skin biopsies were collected from free-ranging harbour seal pups using innovating minimally-invasive techniques. Despite the reduced amount of tissue obtained in liver and skin biopsies, the homogenization procedures developed provided sufficient samples to be resolved by western blotting. Previous studies demonstrated that skin biopsies provided suitable samples for the evaluation of benzo(a)pyrene monooxigenase (BPMO) activity in striped dolphins (*Stenella coeruleoalba*), bootlenose dolphin (*Tursiops truncatus*), common dolphin (*Delphinus delphis*), fin whales (*Balaenoptera physalus*) and southern sea lion (*Otaria flavescens*), and CYP1A proteins by immunohistochemistry in northern and southern right whales (*Eubalaena glacialis* and *Eubalaena australis*) (Fossi *et al.* 1997b; Fossi *et al.* 2000; Moore *et al.* unpublished).

The immunodetection of CYP1A forms in marine mammals relies on the cross reactivity obtained with antibodies raised against fish and rodents CYP1A enzymes. Due to the conserved nature of these enzymes among vertebrates (Stegeman and Hahn 1994), the results obtained for marine mammals using non species-specific antibodies have been considered reliable. In the present study, two protein bands, which appeared to be immunochemically related to CYP1A rat forms, were detected in harbour seal liver samples. The electrophoretic mobility of both proteins was within the expected range for rat CYP1A forms (approximately 52 and 56 KDa) (Imai 1978; White *et al.* 1994). The two forms were induced in seals by oral administration of BNF, a strong CYP1A inducer, suggesting that in harbour seals like in terrestrial mammals (Nelson *et al.* 1996) two hepatic CYP1A forms are present. The protein band with the higher molecular weight (band 1) appeared to be more responsive to BNF than the second band (band 2). Based on observations of preferential BNF induction of CYP1A1 compared to CYP1A2 in humans and rodents (Nebert and Gonzalez 1987), this result suggests that band 1 might be CYP1A1 and band 2 CYP1A2 (Rodrigues and Prough 1991). However, the specific

identity of both forms could not be confirmed. The monoclonal antibody against rat CYP1A1/2 (MAb 1-7-1) recognized only the putative CYP1A1 band in one liver sample from a BNF orally induced harbour seal. This antibody cross-reacted stronger with rat CYP1A1 compared to CYP1A2, and therefore the cross-reaction with the hepatic CYP1A2 in harbour seal might have not been detectable. Alternatively, this putative CYP1A2 protein might lack the epitope recognized by this antibody. The Mab (1-7-1) had previously recognized hepatic CYP1A forms in ringed and grey seals from the polluted Baltic Sea (Nyman *et al.* 2000). The present study results therefore suggest that a similar hepatic CYP1A-like form exists in the three species of pinnipeds.

The results of this study are in agreement with previous ones where at least one hepatic CYP1A form was characterized in pups and adult phocid seals. A single hepatic CYP1A protein band (~54 KDa) cross-reacted strongly on adult harbour seal (Dutch waters), using a specific antibody for rat CYP1A1 protein (Goksøyr 1995a). In sub-adult harp seal and adult ringed seals (Svalbard) liver samples, a single protein band cross reacted with an anti-rat CYP1A antibody; this band had a molecular weight similar to the BNF induced rat CYP1A bands (Wolkers *et al.* 1998a; Wolkers *et al.* 1999). However, one and two hepatic CYP1A forms have been reported for the same species when using antibodies developed against CYP1A proteins from different vertebrates. In fact, when using a non species-specific antibody, if the epitope (antigenic determinant) used to produce it is absent from the CYP1A protein of the species under investigation, this protein will not be detected. Pups and adult harp and hooded seals, sampled on West Ice, revealed one hepatic CYP1A band (54 KDa) using an antibody against rat CYP1A1 (Goksøyr 1995a), however, an antibody against cod CYP1A1 had previously recognized two protein bands with (~54 and 52 KDa) in liver samples from adult seals, of the same two species and geographical area, suggesting that the two CYP1A forms were present (Goksøyr *et al.* 1992). Recently, a polyclonal antibody prepared against CYP1A from harp seal clarified the issue, by revealing two distinct protein bands (~54 KDa) in harp seal liver microsomes (Tilley *et al.* 2001). The partial characterization of CYP1A genes, already carried out for some marine mammals (Teramitsu *et al.* 2000), combined with the development of group or species-

specific antibodies, might provide the answers regarding which CYP1A forms are expressed in the different species of phocid seals, including the harbour seal.

In harbour seal skin samples, a BNF inducible CYP1A protein corresponding to the putative hepatic CYP1A1 form was measured. Considering the thinner second band present in the skin samples from some untreated animals, the possibility of a second CYP1A form in skin cannot be excluded. This second band was only expressed in some pups but was absent in BNF induced animals, suggesting no responsiveness to this strong CYP1A inducer. In *in vivo* induction studies using rodents (Rodrigues and Prough 1991) have found that, contrary to liver, CYP1A2 protein was not well expressed in extrahepatic tissues after treatment with common CYP1A inducers (polycyclic aromatic hydrocarbons). This suggests different induction patterns of CYP1A forms between tissues. Time limitations prevented investigating the identity of a possible second CYP1A protein (non BNF responsive) in harbour seal skin. The monoclonal antibody (MAb 1-7-1) failed to reveal any CYP1A form in skin samples from BNF induced seals. Previously, this antibody showed immunoreactivity in epidermal microsomes from neonatal rats topically induced with 3MC (Khan *et al.* 1989). Although in this study MAb 1-7-1 recognized a CYP1A protein in liver samples from an induced animal, the degree of CYP1A induction in skin might not have been high enough to be detectable by this antibody.

In free-ranging harbour seal pups, immunoreactive skin CYP1A levels were significantly lower than hepatic levels. This is in agreement with a previous observation on a stranded Southern sea lion, where the levels of BPMO activity measured in liver were five fold higher compared to skin activity (Fossi *et al.* 1997b), although this study did not include CYP450 protein immunoquantification. The same study revealed the presence of similar CYP450 isoforms, between 45 and 66 KDa, in both liver and skin after SDS-PAGE electrophoresis, suggesting that both tissues may express similar forms (Fossi *et al.* 1997b). However, these results should be considered with caution as they refer to one animal.

The present study revealed that both harbour seal skin and liver tissues appear to share one BNF inducible CYP1A protein. Although sample size was small (n=6), liver and skin levels of this CYP1A protein increased significantly after oral

exposure to BNF, and correlated between the two tissues. These results suggest a similar pharmacokinetic distribution of BNF in both tissues and consequent CYP1A synthesis. However, the CYP1A induction was higher in liver compared to skin, which might be expected considering that liver is the main organ for BNF metabolism (Stegeman and Hahn 1994; Vyas *et al.* 1983). The results of the oral BNF study further indicate that CYP1A induction in harbour seal skin can occur from exposure through diet, as previously observed in skin endothelium of river otters fed crude oil (Ben-David *et al.* 2001). This result is relevant considering that marine mammals uptake lipophilic contaminants that bioaccumulate through the food chain.

The harbour seal skin CYP1A system appears to also respond to topical BNF application. Although not significant, average CYP1A levels increased slightly after topical application. Within control and induced group, CYP1A levels were quite variable. This might be due to experimental difficulties while performing the *in vivo* application. Although the animals were physically restrained, when applying the liquid to the flipper some momentarily motion caused it to spread variably in the shaved area. This likely resulted in differing degrees of induction among the animals. Alternatively, BNF has proven to be readily metabolized in marine fish and mammals (Stegeman and Hahn 1994; Vyas *et al.* 1983). Considering that in our study the same animal provided one control and one induced sample, a quick absorption and circulation of this compound may have prevented detecting differences between control and induced samples. However, it is unlikely that the systemic distribution of a reduced dosage of topically-applied BNF would cause a measurable CYP1A induction in both flippers within the 24 hours. Previously, (Khan *et al.* 1992) provided evidence that CYP1A1 mRNA levels, as well as aryl hydrocarbon hydroxilase (AHH) activity increased in adult rat and human cutaneous tissue after a single topical application of BNF. In addition, CYP1A1 mRNA levels also increased in epidermis when the same induction was performed in 4-day-old rats (Khan *et al.* 1992). The response of skin CYP1A proteins to different CYP1A inducers has also been observed in rat epidermis (Bickers *et al.* 1986), mice skin (Agarwal *et al.* 1994; Ichikawa *et al.* 1989) and murine skin (Jugert *et al.* 1994). The results obtained in the BNF induction studies performed

using harbour seal pup skin, indicate that this pinniped possesses a responsive cutaneous CYP1A system at an early age.

Although both skin and liver CYP1A systems have proven to be inducible in very young rodents, as well as adults (Pegram *et al.* 1995; Khan *et al.* 1992) no relationship between these proteins expression in the two tissues has previously been observed. In the present study, a significant correlation of CYP1A levels was obtained between liver and skin from untreated pups. The results suggest that CYP1A expression in skin might in part reflect expression in liver although other factors may regulate the expression of these enzymes in skin of young pups that are still developing. Furthermore, factors controlling the expression of mammalian CYP1A proteins in skin are poorly described (Khan *et al.* 1992) compared to the hepatic system. The significant correlation between harbour seal liver and skin CYP1A levels seems to be also present in BNF induced animals, suggesting a similar response of CYP1A system to induction in both tissues. This further supports the use of skin as an alternative biological material for the evaluation of contaminant exposure in marine mammals.

Marine mammals may transfer organochlorine residues such as PCBs and chlorinated pesticides from mother to pup mainly through lactation, as females mobilize large amounts of blubber reserves to produce a lipid-rich milk (approximately 35%-50% fat content) (Addison and Brodie 1987; Wolkers *et al.* 2002). As these contaminants tend to be lipid associated pups are exposed to substantial amounts by ingesting the mothers milk (Addison and Brodie 1987). In the present study, toxic equivalency quotients (TEQs) were calculated for AhR binding (i.e. "dioxin-like" or planar) PCBs, dioxins and furans to characterize the possible CYP1A induction of the contaminants ingested by the study animals. Mono-*ortho* PCBs, followed by non-*ortho* congeners, contributed the most to total TEQs, presumably reflecting PCB inputs associated with industry, as previously observed in harbour seals pups from Puget Sound (Washington) and British Columbia (Simms *et al.* 2000). PCBs were also found to represent an important fraction of the total TEQs in adult harbour seals samples from an earlier study (1991-1992) in the Strait of Georgia (Addison *et al.* 2000). In this study, the contribution of dioxins and furans to total TEQs was higher compared to Puget

Sound pups, but in agreement with the values obtained for British Columbia harbour seals (Addison *et al.* 2000; Simms *et al.* 2000). These site differences have been related to the historic input of PCDDs and PCDFs from pulp mills in coastal waters from BC (Addison *et al.* 2000; Ross and Troisi 2001), resulting in higher concentrations of dioxins and furans in blubber of seals inhabiting areas influenced by pulp mill effluents.

In the current study, a significant relationship between any of the potential CYP1A inducers (TEQs) and CYP1A expression in liver or skin from harbour seal pups was not detected. These results suggest that the immunoquantified CYP1A expression in liver and skin was not reflecting the pups "dioxin-like" contaminant burden, although greater sample size might have further clarified these relationships. One possible reason would be that these enzymes were induced by other contaminants, not included on the TEQs approach. Alternatively, in harp seal pups low hepatic ethoxyresorufin-*O*-deethylase (EROD) activities that did not correlate with PCBs in pups blubber and the mothers milk were thought to be due to an underdeveloped CYP1A associated catalytic activity (Wolkers *et al.* 2002). Although the enzyme activity of CYP1A was not determined in this study, it is possible that CYP1A protein expression, like their catalytic activity, might also be influenced by other factors in developing harbour seal pups. As a further possibility, in sub-adult harp seals sampled in the summer, an absent correlation between CYP450 activities, including EROD, and PCB burdens suggested a diet-related cause; the high carotene content of crustaceans, the main summer diet of harp seals, might have resulted in a CYP450 induction (Wolkers *et al.* 1999). Carotenes are known to be strong hepatic CYP1A inducers in rats, increasing EROD activity and expression of CYP1A1 and 1A2 (western blotting), even at low dosages (Gradelet *et al.* 1996). In the current study, faeces collected from some seals in the same and next day after the captures, showed that some of the pups were already preying on shrimps, so a possible influence of prey carotenes on CYP1A induction might not be excluded.

In this study, a negative correlation was observed between the body weight of the pups and their total TEQs burden. This is not likely due to metabolism and excretion of "dioxin–like" contaminants as no correlation was established between total TEQs and hepatic or skin CYP1A levels. Furthermore, Addison and Stobo

(1993) suggested that in the first four months of life, no metabolic degradation or excretion might influence organochlorine deposition in grey seals and other phocid seals species. Generally, organochlorine levels concentrate in blubber after weaning, as fat reserves are being used, and then as pups became self-sufficient through predation, blubber contaminants decline because are "diluted" by the fat deposition in blubber (Addison and Stobo 1993). The trend observed in this study might suggest that a growth-associated "dilution" was taking place in these seals. As a consequence, older pups might have lower contaminant concentrations in blubber compared to younger pups.

The use of skin biopsies as biological material for the evaluation of biomarkers of contaminant exposure deserves further investigation. Considering the results obtained in the present study using reduced amounts of liver and skin from harbour seals, skin biopsies might play a potential role as a surrogate for liver. Furthermore, skin can be easily obtained causing minimal disturbance and one skin biopsy assures enough sample to perform western blotting.

Conclusions

In summary, it was demonstrated that biopsy samples of liver and skin can be obtained from harbour seals causing minimal disturbance and without interfering with the animals' welfare.

The following study showed that CYP1A enzymes can be immunoquantified in microsamples of harbour seal liver. Skin biopsies proved as well to provide enough tissue for the immunoquantification of CYP1A enzymes. When measuring these enzymes, non-destructive sampling techniques might be therefore an option for marine mammals sampling.

This study revealed that harbour seal hepatic CYP1A system includes two putative CYP1A forms and skin CYP1A includes one form. The question regarding a possible non-BNF inducible second CYP1A protein in skin was unresolved. In further studies regarding this issue, the use of a larger gel system is advised to improve the separation of these proteins, as their similar molecular weight might prevent a good separation when samples are resolved on a minigel system. Furthermore, the use of a recently developed seal specific antibody might provide valuable information on the CYP1A forms present in both liver and skin from harbour seals.

It was demonstrated for the first time in pinnipeds, that *in vivo* exposure of harbour seals to BNF results in a significant increase of hepatic and skin CYP1A proteins. In this species both liver and skin CYP1A systems are therefore responsive to strong CYP1A inducers. Although some increase was also measured in skin CYP1A levels after BNF topical application, the fact that a significant induction occurred in skin after exposure to BNF through diet (oral induction study) indicates that skin CYP1A might be responsive to contaminants that are common CYP1A inducers and bioaccumulate in the food chain due to their lipophilic nature.

In the present study it was also verified that harbour seal hepatic CYP1A levels correlated significantly with CYP1A levels measured in skin of untreated and BNF induced animals. The fact that skin CYP1A partially predicted hepatic CYP1A content further supports the use of skin CYP1A measurements to infer the levels of

these enzymes in liver. However, the immunochemical expression of hepatic and skin CYP1A proteins appeared not to be induced by "dioxin-like" contaminants in blubber of untreated harbour seal pups (general study group), as no significant correlations were established between these enzymes and the contaminant levels. As a consequence, to strengthen and further validate the use of skin biopsies the responsiveness of skin CYP1A system to contaminant levels in harbour seals should be further characterized. The use of juveniles and adults subjected to different degrees of environmental exposure, combined with animals from contaminated and pristine areas, might be useful to further evaluate this relation. In addition, other contaminant classes should be also evaluated as possible CYP1A inducers.

In conclusion, this study addressed the need for a non-destructive approach, within biomarkers of "dioxin-like" contaminants exposure for marine mammals. This was achieved by providing evidence on the immunoquantification of CYP1A proteins in liver, and for the first time in skin biopsies obtained in a minimally-invasive way from a representative species of marine mammals. This work is a valuable contribution for the development of biopsy sampling methods coupled with simple analytical techniques, to evaluate well-established biomarkers of contaminants exposure. This approach is desirable for the early detection of environmental contaminant exposure in free ranging marine mammal populations, including those from which liver samples cannot be obtained.

Appendix 1

Table 1: List of reagents and buffers prepared for total protein determination and western blotting.

Reagents and buffers	Content
Homogenizing buffer	50 mM Tris(hydroxymethyl)aminoethane hydrochloride (Tris-HCL), (pH 7.4 at 4°C); 150 mM potassium chloride; 2mM ethylenediaminetetraacetic acid (EDTA)
Reagent F	0.01% cupric sulfate pentahydrate; 0.02% sodium tartrate tetrahydrate; 2% sodium carbonate anhydrous in 0.1 M sodium hydroxide
Reagent D	Folin-Phenol reagent (1:1 dilution with DMQ)
Sample diluting buffer (2 X)	0.0625 M Tris-HCl (pH 6.8 at room temperature); 10% glycerol; 0.001% bromophenol blue; 1% sodium dodecyl sulphate (SDS); 2-mercaptoethanol (at 19:1dilution)
Separating gel	0.375 M Tris-HCl (pH 8.8 at room temperature); 7.5% acrylamide:N,N'methylenebisacrylamide (BIS); 0.042% ammonium persulphate; 0.01% SDS; 0.03% N,N,N',N'-tetramethylethylenediamine (TEMED)
Separating gel wash buffer	0.675 M Tris-HCl (pH 8.8 at room temperature); 0.182% SDS
Stacking gel	0.125 M Tris-HCl (pH 6.8 at room temperature); 3% acrylamide: BIS; 0.08% ammonium persulphate; 0.1% SDS; 0.05% TEMED
Stacking gel wash buffer	0.227 M Tris-HCl (pH 6.8 at room temperature); 0.182% SDS
Pyronin Y	0.165 mM Pyronin Y; 2 % glycerol; 0.1% SDS (1:9 dilution with sample diluting buffer)
Electrode buffer	50 mM Tris(hydroxymethyl)aminomethane (Trizma); 6.9 mM SDS; 384 mM glycine
Transfer buffer	24.7 mM Trizma; 128 mM glycine; 0.35 mM SDS; 20% methanol
Membrane blocking buffer	5% Carnation non fat milk powder; 50 mM sodium chloride; 10 mM Tris-HCl (pH 7.4 at room temperature); 1mM EDTA
Antibody diluting buffer	300 mM sodium chloride; 5 mM potassium chloride; 16mM sodium phosphate; 3 mM potassium phosphate; 0.4 mM EDTA (pH 7.4); 20 gl^{-1} bovine serum albumin; 0.05% polyoxythylenesorbitan monolaurate (Tween 20); 5% goat serum
Wash buffer	0.05% Tween 20; 0.26 M sodium chloride; 4.75 mM potassium chloride; 15 mM sodium phosphate; 0.3 mM potassium phosphate; 0.04 mM EDTA, pH 7.4

Note: All solutions prepared with double Milli-Q water (DMQ).

Protocol 1:

PMS fractions from harbour seal liver biopsies adapted from Thomas *et al.* (1983).

Tissue processing:

-Wash liver biopsy in cold 1xPBS (Phosphate–Buffered Saline) and determine weight.

Tissue homogenization:

-Use 1.5 ml Eppedorf tubes and add ice cold homogenizing buffer [volume (μl) = 2 to 3 x liver biopsy weight (mg)].

-Homogenize using a manual pellet pestle.

Temperature conditions:

-Keep tube on ice while homogenizing.

-Stop between bursts for 10 seconds to minimize heat generation during the process.

Time conditions:

-Burst duration (seconds) = 10.

-Number bursts/biopsy = 5.

PMS fractions:

-Centrifuge homogenate at 9 000 x g for 20 minutes at 4°C (cool down centrifuge previously).

-Recover and measure supernatant volume and save or discard pellet.

-Keep supernatant at -80°C.

Protocol 2:

PMS fractions from harbour seal skin biopsies.

Tissue processing:

-Wash the skin/blubber biopsy in cold 1xPBS (Phosphate–Buffered Saline).

-Determine biopsy total weight.

-Using a razor blade, remove and weight blubber and weight skin after removing hair.

-Keep hair and blubber in liquid N_2 or –80 C° freezer.

-Cut the skin in small pieces (as small as possible).

-Separate skin pieces in two sets, freeze with liquid N_2 and homogenize one group at a time.

Tissue homogenization:

-Add ice cold homogenizing buffer [volume (µl) = 4 x skin sample weight (mg)] to a plastic tube (1 cm diameter).

-Homogenize using a polytron tissue homogenizer equipped with stainless steel generator (7mm diameter).

Temperature conditions:

-Keep mortar on ice.

-Keep tube on ice while homogenizing.

-Stop between bursts for 45 seconds to minimize heat generation during the process.

-Snap freeze (liquid N_2) the remaining skin pieces in mortar between bursts.

Time conditions:

-Burst duration (seconds) = 15.

-Number bursts/biopsy = 10.

-Snap freeze skin pieces 3 times/biopsy.

PMS fractions:

-Centrifuge homogenate at 9 000 x g for 20 minutes at 4°C (cool down centrifuge previously).

-Recover and measure supernatant volume and save or discard pellet.

-Keep supernatant at -80°C.

Table 3: Protein yield and CYP1A levels quantified per protein unit in samples prepared from seals PV0018 and PV00101 liver (5 to 50 mg).

Seal #	Amount liver (mg)	Total protein liver sample (mg)	Gel	Total protein in gel (µg/well)	Contour Quantity (Cqty) (RDxmm^2)	Cqty/µg total protein
PV0018	5.5	0.10	1	1.02	nd	-
PV0018	10.5	0.56	1	5.62	0.390	0.0694
PV0018	16.3	0.91	1	9.09	0.546	0.0601
PV0018	25.3	1.48	1	14.77	1.015	0.0687
PV0018	49.7	3.27	1	32.65	1.412	0.0432
PV0018	5.1	0.14	1	1.43	nd	-
PV0018	10.5	0.43	1	4.30	0.247	0.0574
PV0018	15.1	0.68	1	6.76	0.403	0.0596
PV0018	26.5	1.67	1	16.66	0.983	0.0590
PV0018	50.8	3.17	1	31.66	1.059	0.0334
PV00101	5.5	0.24	2	2.43	nd	-
PV00101	10.3	0.45	2	4.48	nd	-
PV00101	17.2	1.07	2	10.70	0.272	0.0254
PV00101	28.6	1.49	2	14.88	0.401	0.0269
PV00101	49.5	3.22	2	32.24	0.370	0.0115
PV00101	5.4	0.27	2	2.72	nd	-
PV00101	10.3	0.67	2	6.69	0.259	0.0387
PV00101	17.3	1.13	2	11.31	nm	-
PV00101	26.1	1.47	2	14.67	nm	-
PV00101	48.9	3.05	2	30.52	nm	-

nd –not detected.
nm–not measured.

Table 5: Seal PV0018 skin sample dilutions (total protein of 50 to 6.25 μg) and average corrected CYP 1A levels quantified per protein unit.

Seal #	Gel	Total protein in gel	Contour Quantity (Cqty)	Positive control	Corrected Cqty	Average	Corrected Cqty/μg total protein
		(μg/well)	(RDxmm^2)	(RDxmm^2)	(RDxmm^2)		
PV0018	1	50	0.730	0.278	2.626	2.584	0.0517
PV0018	2	50	1.022	0.363	2.815		
PV0018	3	50	0.670	0.290	2.310		
PV0018	1	25	0.372	0.278	1.338	1.327	0.0531
PV0018	2	25	0.524	0.363	1.444		
PV0018	3	25	0.348	0.290	1.200		
PV0018	1	12.5	0.189	0.278	0.680	0.564	0.0451
PV0018	2	12.5	0.164	0.363	0.452		
PV0018	3	12.5	0.162	0.290	0.559		
PV0018	1	6.25	nd	0.278	-	0.207	0.0331
PV0018	2	6.25	0.075	0.363	0.207		
PV0018	3	6.25	nd	0.290	-		

nd–not detected.

Appendix 2

Table 3: Physical parameters of the general study group of harbour seal pups captured at Swishswash island, BC.

General study group						
Seal #	**Tag #**	**Sex**	**Weight**	**Length**	**Girth**	**Age**
			19/08/00 (kg)	**(cm)**	**(cm)**	**(days-approx.)**
1	F061	M	24.45	95	76.0	37.4
2	F062	M	27.63	91	82.5	46.6
3	F063	F	18.35	90	70.0	20.3
4	F064	M	28.99	97	84.0	48.3
5	F065	M	27.45	101	88.0	58.9
6	F066	F	30.81	94	89.0	58.9
7	F067	M	17.17	84	67.0	16.9
8	F068	M	23.54	95	75.0	34.6
9	F069	M	20.17	89	67.5	23.1
10	F070	M	24.45	91	76.0	38.0
11	F071	F	25.00	92	83.0	42.0
12	F072	M	23.00	93	75.0	34.9
13	F073	M	28.00	100	82.0	49.1
14	F074	M	24.00	94	76.0	36.3
15	F075	M	20.00	91	69.0	27.7
16	F076	M	23.00	88	79.0	36.3
17	F077	M	25.50	92	80.0	43.4
18*	F078	M	26.50	95	82.0	47.7
19	F079	F	21.50	91	75.0	33.4
20	F081	F	20.00	91	74.0	29.1
AVERAGE:	-	-	23.98	93	77.5	38.2
SEM:	-	-	0.82	0.9	1.4	2.6

SEM-Standard error of the mean.
* Euthanised seal.

Table 4: BNF oral induction study design: BNF was supplied orally with the food at a daily dose of 50 mg BNF/kg body weight in 200 mg gelatine capsules to the induction group. Control animals were given empty capsules.

BNF oral induction study								
Seal #	**Tag #**	**Treatment**	**Sex**	**Weight**	**BNF Daily dosage**	**Day 1**	**Day 2**	**Day 3**
				5/09/00 (kg)	**(mg BNF/ n° capsules)**	**(n° capsules)**	**(n° capsules)**	**(n° capsules)**
12	F072	Induced	M	21.82	1091.00/5.46	5	5	6
14	F074	Induced	M	21.36	1068.00/5.34	5	5	6
15	F075	Induced	M	16.14	807.00/4.04	4	4	5
1	F061	Control	M	26.36	-	5*	5*	5*
7	F067	Control	M	22.27	-	5*	5*	5*
16	F076	Control	M	22.73	-	5*	5*	5*
AVERAGE:	-	-	-	21.78	988.67/4.94	-	-	-
SEM	-	-	-	1.34	91.08/0.46	-	-	-

SEM-Standard error of the mean.
* Empty capsules

Table 5: BNF topical induction study design: BNF was applied topically in a single dose of 0.36 mg of BNF dissolved in DMSO (dosage of 0.12 mg BNF/cm^2 skin assuming an application area of 3 cm^2) in the left hind flipper of all seals. DMSO was applied on the right hind flipper of each animal as control area.

BNF topical induction study							
				Left hind flipper - *Induced*		**Right hind flipper - *Control***	
Seal #	**Tag #**	**Sex**	**Weight**	**Volume/Treated area**		**Volume/Treated area**	
			12/09/00	**(µl)**	**(cm^2)**	**(µl)**	**(cm^2)**
5	F065	M	28.18	30	3.93	30	1.57
8	F068	M	29.55	30	3.53	30	1.57
9	F069	M	19.55	30	1.96	30	2.94
17	F077	M	21.82	30	4.12	30	2.94
19	F079	F	21.82	30	2.36	30	4.12
20	F081	F	19.10	30	2.94	30	3.53
AVERAGE:	-	-	23.34	-	3.13	-	2.77
SEM:	-	-	1.82	-	0.29	-	0.35

SEM-Standard error of the mean.

Table 6: Liver biopsies collected from harbour seal pups and total protein concentration after homogenization.

	Liver biopsies - 19 and 20/08/2000			Liver biopsies - 8/09/2000		
	General study group			Oral BNF induction study		
Seal #	Liver (mg)	Liver homogenized (mg)	Total protein (mg)	Liver (mg)	Liver homogenized (mg)	Total protein (mg)
1	-	-	-	154.5	40.1	3.34
2	17.1	*	*			
3	-	-	-			
4	33.0	33.0	1.81			
5	16.3	*	*			
6	17.6	*	*			
7	57.7	28.0	1.15	185.1	39.5	3.08
8	-	-	-			
9	36.0	36.0	2.62			
10	21.5	*	*			
11	52.8	30.0	1.70			
12	80.9	30.0	1.84	98.6	42.1	2.39
13	48.0	48.0	2.67			
14	104.1	33.0	1.90	88.7	40.3	3.19
15	15.2	*	*	107.8	39.6	3.11
16	71.4	42.0	2.54	254.5	43.0	3.25
17	82.9	32.0	1.97			
18	>1000	31.7	1.62			
19	66.9	49.0	2.51			
20	102.5	31.0	1.90			
AVERAGE:	51.5**	35.1	2.02	148.2	40.8	3.06
SEM	7.7	2.1	0.14	26.0	0.6	0.14

* Liver samples used for other studies. ** Excluding seal # 18.
SEM-standard error of the mean.

Table 7: Skin biopsies collected from harbour seal pups and total protein concentration after homogenization.

	Skin biopsies-19 and 20/08/2000		Skin biopsies-8/09/2000		Skin biopsies-13/09/2000		Skin biopsies-13/09/2000	
	General study group		Oral BNF induction study		Topical BNF induction study-*Induced*		Topical BNF induction study-*Control*	
Seal #	**Skin homogenized (mg)***	**Total protein (mg)**	**Skin homogenized (mg)***	**Total protein (mg)**	**Skin homogenized (mg)***	**Total protein (mg)**	**Skin homogenized (mg)***	**Total protein (mg)**
1	126.4	2.26	40.9	0.81				
2	124.4	2.48						
3	89.8	1.35						
4	83.2	1.47						
5	95.6	1.48			42.6	1.20	52.3	1.06
6	103.6	2.26						
7	133.0	2.11	47.5	0.90				
8	80.4	0.90			48.5	0.95	56.4	1.20
9	130.3	2.21			43.7	0.54	57.3	1.31
10	126.9	2.08						
11	114.0	1.98						
12	143.1	2.42	60.5	0.81				
13	121.8	3.24						
14	127.8	1.90	58.4	1.06				
15	118.1	2.73	43.6	0.76				
16	121.5	2.48	40.3	0.97				
17	128.2	2.86			62.4	2.13	49.4	2.01
18	181.5	2.56						
19	152.8	2.64			47.1	0.84	62.9	1.08
20	126.8	1.98			47.7	1.76	67.8	1.17
AVERAGE:	121.5	2.17	48.5	0.89	48.7	1.24	57.7	1.31
SEM	5.3	0.13	3.6	0.05	2.9	0.24	2.8	0.15

*Biopsy weight after removing blubber and small skin piece (see material and methods); SEM–Standard error of the mean.

Table 8: CYP1A levels per protein unit (CYP1A/µg total protein) in liver samples from general study group and oral BNF induction study animals, determined by western blotting.

	LIVER SAMPLES													
	General study group							Oral BNF induction study						
Seal #	Total protein/ well (µg)	Contour Quantity (cqty) ($RDxmm^2$)	Corrected Cqty ($RDxmm^2$)	Average	Stdev	Variability %	Average CYP1A/µg total protein	Total protein/ well (µg)	Contour Quantity (cqty) ($RDxmm^2$)	Corrected Cqty ($RDxmm^2$)	Average	Stdev	Variability %	Average CYP1A/µg total protein
1								20	1.419	0.764	0.846	0.116	13.7	0.0423
1								20	1.728	0.928				
4	20	0.544	0.510	0.496	0.0205	4.1	0.0248							
4	20	0.618	0.481											
7	20	0.781	0.733	0.733	-	-	0.0376	20	1.807	0.973	0.921	0.0742	8.1	0.0460
7	20	nm						20	1.617	0.868				
7	20	0.427	0.666	0.666	-	-								
7	20	nm												
7	20	0.625	0.854	0.854	-	-								
7	20	nm												
9	20	0.466	0.437	0.408	0.0410	10.1	0.0204							
9	20	0.488	0.379											
11	20	0.641	0.601	0.466	0.192	41.2	0.0257							
11	20	0.425	0.330											
11	20	0.391	0.610	0.610	-	-								
11	20	nm												
11	20	0.364	0.497	0.497	-	-								
11	20	nm												
11	20	0.300	0.528	0.528	-	-								
11	20	nm												

Seal #	Total protein/	Contour	Corrected	Average	Stdev	Variability	Average	Total protein/	Contour	Corrected	Average	Stdev	Variability	Average
	well	Quantity (cqty)	Cqty				CYP1A/µg	well	Quantity (cqty)	Cqty				CYP1A/µg
	(µg)	(RDxmm^2)	(RDxmm^2)			%	total protein	(µg)	(RDxmm^2)	(RDxmm^2)			%	total protein
12	20	0.242	0.227	0.227	-	-	0.0160	1.5	1.115	0.600	0.573	0.0382	6.7	0.382
12	20	nm						1.5	1.016	0.546				
12	20	0.298	0.465	0.465	-	-								
12	20	nm												
12	20	0.195	0.266	0.266	-	-								
12	20	nm												
13	20	0.327	0.307	0.307	-	-	0.0186							
13	20	nm												
13	20	0.291	0.454	0.454	-	-								
13	20	nm												
13	20	0.219	0.299	0.299	-	-								
13	20	nm												
13	20	0.242	0.426	0.426	-	-								
13	20	nm												
14	20	0.626	0.587	0.463	0.175	37.9	0.0297	1.5	1.146	0.617	0.585	0.0453	7.7	0.390
14	20	0.436	0.339					1.5	1.029	0.553				
14	20	0.455	0.710	0.710	-	-								
14	20	nm												
14	20	0.561	0.766	0.766	-	-								
14	20	nm												
14	20	0.321	0.565	0.565	-	-								
14	20	nm												
15								1.5	1.336	0.719	0.706	0.0184	2.6	0.471
15								1.5	1.291	0.693				
16	20	0.278	0.242	0.254	0.0170	6.7	0.0127	20	1.409	0.759	0.841	0.115	13.7	0.0420

Seal #	Total protein/ well (µg)	Contour Quantity (cqty) (RDxmm^2)	Corrected Cqty (RDxmm^2)	Average	Stdev	Variability %	Average CYP1A/µg total protein	Total protein/ well (µg)	Contour Quantity (cqty) (RDxmm^2)	Corrected Cqty (RDxmm^2)	Average	Stdev	Variability %	Average CYP1A/µg total protein
16	20	0.436	0.266					20	1.717	0.922				
17	20	0.263	0.229	0.217	0.0170	7.8	0.0109							
17	20	0.335	0.205											
18	20	0.333	0.290	0.290	-	-	0.0160							
18	20	nm												
18	20	0.313	0.273	0.273	-	-								
18	20	nm												
18	20	0.437	0.381	0.381	-	-								
18	20	nm												
19	20	0.260	0.227	0.228	0.00071	0.3	0.0114							
19	20	0.373	0.228											
20	20	0.254	0.221	0.230	0.0127	5.5	0.0115							
20	20	0.392	0.239											
W							0.9054							

Note: Samples which duplicate readings exceeded 16.5 % of variability or provided only one reading were resolved again. For each sample, the final average CYP1A content includes all readings.

nm–not measured; Stdev-Standard deviation ; % Variability= (Stdev*100)/average); W–Shapiro-Wilks test.

Table 9: CYP1A levels per protein unit (CYP1A/µg total protein) in skin samples from general study group and oral BNF induction study animals, determined by western blotting.

SKIN SAMPLES	General study group							Oral BNF induction study group						
Seal #	Total protein/ well (µg)	Contour Quantity (cqty) (RDxmm^2)	Corrected Cqty (RDxmm^2)	Average	Stdev	Variability %	Average CYP1A/µg Total protein	Total protein/ well (µg)	Contour Quantity (cqty) (RDxmm^2)	Corrected cqty (RDxmm^2)	Average	Stdev	Variability %	Average CYP1A/µg Total protein
1	25	0.693	0.620	0.613	0.00990	1.6	0.0245	25	0.619	0.462	0.494	0.0445	9.0	0.0197
1	25	0.755	0.606					25	0.811	0.525				
2	25	0.679	0.607	0.617	0.0134	2.2	0.0247							
2	25	0.779	0.626											
3	25	0.677	0.606	0.632	0.0368	5.8	0.0253							
3	25	0.819	0.658											
4	25	0.626	0.450	0.481	0.0438	9.1	0.0192							
4	25	0.815	0.512											
5	25	0.532	0.476	0.537	0.0863	16.1	0.0215							
5	25	0.744	0.598											
6	25	0.600	0.537	0.554	0.0240	4.3	0.0222							
6	25	0.711	0.571											
7	25	0.746	0.537	0.477	0.0849	17.8	0.0191	25	0.854	0.637	0.632	0.00707	1.1	0.0253
7	25	0.665	0.417					25	0.969	0.627				
8	25	0.466	0.417	0.439	0.0311	7.1	0.0176							
8	25	0.574	0.461											
9	25	0.638	0.459	0.449	0.0148	3.3	0.0179							
9	25	0.698	0.438											
10	25	0.489	0.373	0.373	-	-	0.0149							
10	25	nm												
11	25	0.738	0.531	0.480	0.0721	15.0	0.0192							
11	25	0.683	0.429											

Seal #	Total protein/ well (µg)	Contour Quantity (cqty) ($RDxmm^2$)	Corrected Cqty ($RDxmm^2$)	Average	Stdev	Variability %	Average CYP1A/µg Total protein	Total protein/ well (µg)	Contour Quantity (cqty) ($RDxmm^2$)	Corrected cqty ($RDxmm^2$)	Average	Stdev	Variability %	Average CYP1A/µg Total protein
12	25	0.640	0.460	0.418	0.0594	14.2	0.0167	10	0.688	0.513	0.566	0.0742	13.1	0.0566
12	25	0.599	0.376					10	0.956	0.618				
13	25	0.630	0.453	0.398	0.0778	19.5	0.0159							
13	25	0.546	0.343											
14	25	0.818	0.588	0.569	0.0276	4.9	0.0227	10	0.740	0.552	0.575	0.0325	5.7	0.0575
14	25	0.874	0.549					10	0.924	0.598				
15	25	0.558	0.499	0.547	0.0672	12.3	0.0219	10	0.569	0.424	0.490	0.0933	19.0	0.0797
15	25	0.739	0.594					10	0.860	0.556				
15								10	1.075	1.042	1.103	0.0863	7.8	
15								10	1.800	1.164				
16	25	0.374	0.362	0.325	0.0523	16.1	0.0130	25	0.345	0.257	0.327	0.0983	30.1	0.0201
16	25	0.446	0.288					25	0.612	0.396				
16								25	0.765	0.741	0.677	0.0912	13.5	
16								25	0.946	0.612				
17	25	0.556	0.539	0.518	0.0297	5.7	0.0207							
17	25	0.769	0.497											
18	25	0.379	0.367	0.341	0.0375	11.0	0.0136							
18	25	0.486	0.314											
19	25	0.430	0.417	0.417	-	-	0.0167							
19	25	nm												
20	25	0.521	0.505	0.465	0.0566	12.2	0.0186							
20	25	0.657	0.425											
W							0.9700							

Note – Samples which duplicate readings exceeded 16.5 % of variability or provided only one reading were not resolved again due to time limitations.

Note – Samples which duplicate readings exceed 16.5 % of variability or provided only one reading were resolved again. For each sample, the final average CYP1A content includes all readings.

nm–not measured; Stdev-Standard deviation ; % Variability= (Stdev*100)/average); W–Shapiro-Wilks test.

Table 10: CYP1A levels per protein unit (CYP1A/μg total protein) in skin samples from topical BNF induction study animals, determined by western blotting.

SKIN SAMPLES	Topical BNF induction study - *Induced*							Topical BNF induction study - *Control*						
Seal #	Total protein/ well (μg)	Contour Quantity (cqty) ($RDxmm^2$)	Corrected Cqty ($RDxmm^2$)	Average	Stdev	Variability %	Average CYP1A/μg Total protein	Total protein/ well (μg)	Contour Quantity (cqty) ($RDxmm^2$)	Corrected cqty ($RDxmm^2$)	Average	Stdev	Variability %	Average CYP1A/μg Total protein
5	20	0.723	0.697	0.706	0.0120	1.7	0.0353	25	0.606	0.584	0.602	0.0247	4.1	0.0241
5	20	0.774	0.714					25	0.671	0.619				
8	20	0.583	0.562	0.604	0.0587	9.7	0.0302	25	0.698	0.673	0.660	0.0191	2.9	0.0264
8	20	0.699	0.645					25	0.700	0.646				
9	20	0.916	0.883	0.860	0.0325	3.8	0.0430	25	0.876	0.845	0.798	0.0672	8.4	0.0319
9	20	0.907	0.837					25	0.813	0.750				
17	20	1.172	1.200	1.200	-	-	0.0939	25	0.751	0.769	0.769	-	-	0.0486
17	20	nm						25	nm					
17	20	2.869	2.064	2.217	0.216	9.7		25	1.710	1.230	1.440	0.296	20.6	
17	20	3.108	2.369					25	2.164	1.649				
19	20	0.893	0.914	0.914	-	-	0.0678	25	0.831	0.851	0.851	-	-	0.0536
19	20	nm						25	nm					
19	20	2.091	1.504	1.576	0.101	6.4		25	2.085	1.500	1.584	0.118	7.5	
19	20	2.161	1.647					25	2.187	1.667				
20	20	0.594	0.608	0.608	-	-	0.0482	25	0.780	0.798	0.798	-	-	0.0553
20	20	nm						25	nm					
20	20	1.603	1.153	1.143	0.0141	1.2		25	2.273	1.635	1.675	0.0566	3.4	
20	20	1.486	1.133					25	2.250	1.715				

Note - Samples with only one reading were resolved again. The final average CYP1A content includes all readings.

nm–not measured; Stdev-Standard deviation ; % Variability= (Stdev*100)/average).

Table 11: Total PCDDs, PCDFs, non-*ortho* and mono-*ortho* PCBs expressed as TEQs (total equivalency quotients in ng/kg lipid weight), according to Van den Berg *et al.* (1998) and total TEQs (sum of all) measured in the blubber of the twenty harbour seal pups.

BLUBBER SAMPLES					
Seal #	^{98}TEQ PCDD	^{98}TEQ PCDF	Total non-*ortho* PCB ^{98}TEQ	Total mono-*ortho* PCB ^{98}TEQ	Total ^{98}TEQ
1	14.586	0.801	88.455	25.600	129.443
2	4.672	0.423	6.176	8.230	19.502
3	6.816	1.086	8.864	23.303	40.069
4	5.655	0.456	6.055	8.724	20.890
5	5.571	0.539	7.147	11.224	24.481
6	4.040	0.740	4.984	11.329	21.093
7	8.091	0.758	11.000	36.336	56.185
8	9.313	1.415	6.165	12.099	28.991
9	11.946	0.908	7.139	26.971	46.963
10	6.166	0.867	5.710	14.082	26.826
11	4.586	0.378	11.748	26.319	43.031
12	6.484	0.837	5.395	11.932	24.648
13	5.108	0.707	5.073	10.063	20.952
14	7.658	0.988	8.084	15.254	31.984
15	7.214	0.739	7.059	12.344	27.356
16	7.003	0.987	5.826	15.638	29.455
17	6.308	1.225	10.361	11.838	29.732
18	2.495	0.576	5.608	8.019	16.697
19	10.598	1.232	6.582	12.446	30.859
20	9.152	1.330	10.170	42.253	62.906
AVERAGE:	7.173	0.850	11.380	17.200	36.603
W^a	0.9437	0.9652	0.3309*	0.8106*	0.6597*
W^b	0.9865	0.9658	0.8770	0.8377*	0.9128

W^a–Shapiro Wilks test for n=20; W^b–Shapiro Wilks test for n=12.
*$p<0.05$

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