The effect of sewage effluent from De Beers marine diamond mining operations on the expression of cytochrome P450 (CYP1A) and vitellogenin (vtg)

A thesis submitted in fulfilment of the requirement for the degree of

MASTERS IN SCIENCE
(BIOCHEMISTRY)
of

RHODES UNIVERSITY

By

Louise De Almeida

November 2010

Abstract

Sewage effluents disposed into the marine environment from De Beers Marine Namibia diamond mining vessels have the potential to cause endocrine disruptive effects in marine organisms. Endocrine disruption refers to the alteration of the normal functioning of the endocrine system and various chemicals have the ability to mimic hormones, effecting endogenous hormone synthesis, transport, receptor interaction and intracellular signaling. The potential endocrine disruptive effects, caused by the release of different types of sewage effluents into the ocean, on fish species is a concern due to the commercial importance of fish species found in the mining area e.g. hake, sole, horse mackerel. Increased awareness of marine environmental degradation due to the presence of chemical contaminants has resulted in research being done on early warning systems, in the form of biomarkers.

Cytochrome P450 monooxygenase 1A (CYP1A) and vitellogenin (vtg) are important proteins found in fish liver and blood, that have been used as biomarkers for the detection of pollutants in fish. CYP1A is a subfamily of the P450 superfamily of enzymes and catalyzes the oxidation, hydrolysis and reduction of exogenous and endogenous compounds (phase I reactions) and thus has the capacity to regulate the metabolism of several organic contaminants. CYP1A expression is altered by exposure to planar xenobiotic compounds e.g. polyaromatic hydrocarbons. Vtg is an important precursor for egg yolk proteins and plays a role in the growth and development of an oocyte. Expression of this protein is altered upon exposure to estrogenic compounds.

The aim of this project was to isolate CYP1A from fish liver by differential centrifugation and optimize conditions for the CYP1A-mediated ethoxyresorufin-*O*-deethylase (EROD) assay and western blot analysis (to assess CYP1A expression). Another aim of this study was to evaluate the potential effects of biologically disruptive chemicals from sewage effluents, discharged into the marine environment, on the expression of CYP1A in two species of hake, *Merluccius capensis* and *M. paradoxus* (Cape hake). CYP1A in Cape hake is approximately a 60 kDa protein and the highest EROD activity was detected in the microsomal fraction after differential centrifugation.

Optimal EROD assay conditions were observed at pH 7.5, a temperature of 25 °C, 10 µl of sample and a reaction time of 30 seconds. Enzyme stability assays indicated a drastic decrease in enzyme activity after 30 seconds. The EROD assay was not NADPH dependent but was limited by NADPH supply, with an increase of 300% in EROD activity being observed with the addition of 0.1 M exogenous NADPH. The addition of dicumarol (40 µM), a phase II enzyme inhibitor, showed a 232% increase in EROD activity. This is because dicumarol inhibited enzymes with the capacity to metabolize the product (resorufin) of the EROD reaction. With regard to western blot analysis, the optimal primary (rabbit antifish CYP1A peptide) and secondary (anti-mouse/rabbit antibody-horseradish peroxidase conjugate (POD)) antibody dilutions were determined to be 1:1000 and 1:5000, respectively.

The comparison of CYP1A expression in Cape hake samples from De Beers Marine mining area and reference sites showed higher EROD activity (16.29 ± 0.91 pmol/min) in fish samples from the mining area in comparison to the reference site (10.42 ± 2.65 pmol/min). Western blot analysis was in agreement with the EROD assay results and a higher CYP1A expression was observed in fish from the mining sites. The increased CYP1A expression observed in fish from the mining area is not definitively an indication of a pollutant effect in the environment, as several environmental and biological factors (e.g. photoperiod and age) must also be considered before reaching this conclusion.

Another aim of this study was to purify vtg from Cape hake blood samples. Cape hake vtg was purified from fish plasma by selective precipitation with $MgCl_2$ and EDTA. Precipitated sample was subjected to anion exchange chromatography using fast protein liquid chromatography (FPLC). Vtg eluted as two broad peaks and had a molecular weight above 200 kDa. SDS-PAGE analysis also resolved smaller molecular weight proteins below 70 kDa, which were thought to be vitellogenin cleavage proteins, lipovitellin and phosphovitins. Western blot analysis was performed; however, it did not produce any conclusive results. The purification of vtg enables further studies in characterizing this protein and developing assay aimed at detecting estrogenic pollutants in the marine environment.

Table of contents

List of Figures

List of Tables

List of Abbreviations

AHR	Arylhydrocarbon receptor
APS	Ammonium persulphate
ARNT	AHR-nuclear translocator
BSA	Bovine serum albumin
CF/K	Fulton's condition factor
cm	Centimetre
CYP1A	Cytochrome P450 monooxygenase 1A
dH_2O	Distilled water
DOC-TCA	Deoxycholate trichloroacetic acid
DTT	DL-Dithiothreitol
EDC	Endocrine disrupting compound
EDTA	Ethylene diamine tetracetic acid disodium salt
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
EROD	7-Ethoxyresorufin-*O*-deethylase
FPLC	Fast protein liquid chromatography
g	Gram
HCl	Hydrochloric acid
kDa	Kilodaltons
LLTP	Large lipid transfer protein superfamily
M	Molar

µg	Microgram
µl	Microlitre
µM	Micromolar
mg	Milligram
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Millilitre
MLA	Marine license area
mM	Millimolar
MS	Microsomal sample
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre
OD	Optical density
P	Pellet
PAH	Polyaromatic hydrocarbons
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-*p*-dioxins
PCDF	Polychlorinated dibenzofurans
PEG	Polyethylene glycol
pmol	Picamolar
PMS	Post mitrochondrial sample
PMSF	Phenylmethanesulfonylflouride

POD	Horseradish Peroxidase Conjugate
S	Supernatant
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfatepolyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N',N'- tetramethylethylene- diamine
TN	Tris-sodium chloride
Tris	Tris (hydroxymethyl)- aminomethane
v/v	Volume/volume
vtg	Vitellogenin
w/v	Weight/volume

List of research outputs

A. POSTER PRESENTATION

De Almeida, L. Froneman, W. and Pletschke, B.I. (2010). The endocrine disrupting effects of sewage effluent from De Beers Marine Namibia diamond mining operation on the expression of CYP1A and vitellogenin in the hake, *Merluccuis capensis* and *M. paradoxus*. 22nd South African Society for Biochemistry and Molecular Biology (SASBMB) Congress. Ilanga Estate. Bloemfontein. 18-20 January 2010.

B. ANTICIPATED PUBLICATION IN A PEER-REVIEWED SCIENTIFIC JOURNAL

De Almeida, L. Froneman, W. and Pletschke, B.I. (2010). A review: Cytochrome P450 monooxygenase 1A (CYP1A) and vitellogenin (vtg) as biomarkers of endocrine disruption in fish. Article in preparation for submission.

Acknowledgements

I would like to take this opportunity to thank people without whom this study would not have been possible

My Supervisor Professor Brett Pletschke for constant support and invaluable advice during this project.

De Beers Marine Namibia (PTY) Ltd for funding this project.

My co-supervisor Professor William Froneman for supporting this project.

Lab 410 with special mention to Natasha Beukes and Susan Van Dyk for all the technical support.

To Balobi Trading, MCM Cape Town and the Ministry of fisheries in Namibia for the generous donation of fish samples.

To my family and friends for all the love and strength.

This thesis is dedicated to my late friend Alexandre Sladkov. You are dearly missed.

Chapter 1: Literature Review

1.1 De Beers Marine Namibia mining operations

De Beers Marine Namibia diamond mining company, is owned in equal share through a joint venture between De Beers and the government of Namibia (Namdeb)[1]. The mining area of interest for this study is the Atlantic 1 Mining License area (MLA) of Namdeb. Atlantic 1 MLA has been an important production source for Namdeb since 1991 and is located along the Namibian/ South African maritime border covering an area ~ 6000 km^2 (Japp, 2007) [2](Figure 1.1). Operations carried out on board the De Beers Marine Namibia vessels produce several types of wastes. The most hazardous wastes (including chemicals such as trichloroethylene, sulphuric acid and hydrofluoric acid) are removed from the vessels and transported to Cape Town for further treatment and disposal [3,4]. Sewage effluents are known to be one of the most important sources of contamination of coastal waters (Pinder *et al.,* 1999; Smith and Suthers, 1999). The products obtained from sewage treated in onboard treatment plants, wastewater generated from cleaning the vessels, oily-water and ferrosilicon containing water are examples of the different types of sewage effluents disposed into the marine environment from De Beers Marine Namibia diamond mining vessels.

[1] De Beers Marine (PTY) Ltd operating and financial review 2008
[2] CSIR report of risk assessment and data gap analysis workshop. Prepared for De Beers Marine Namibia (PTY) Ltd.
[3] De Beers Marine (PTY) Ltd environmental report 1997
[4] Namdeb Diamond Corporation (PTY) Ltd environmental programme I/II 1997

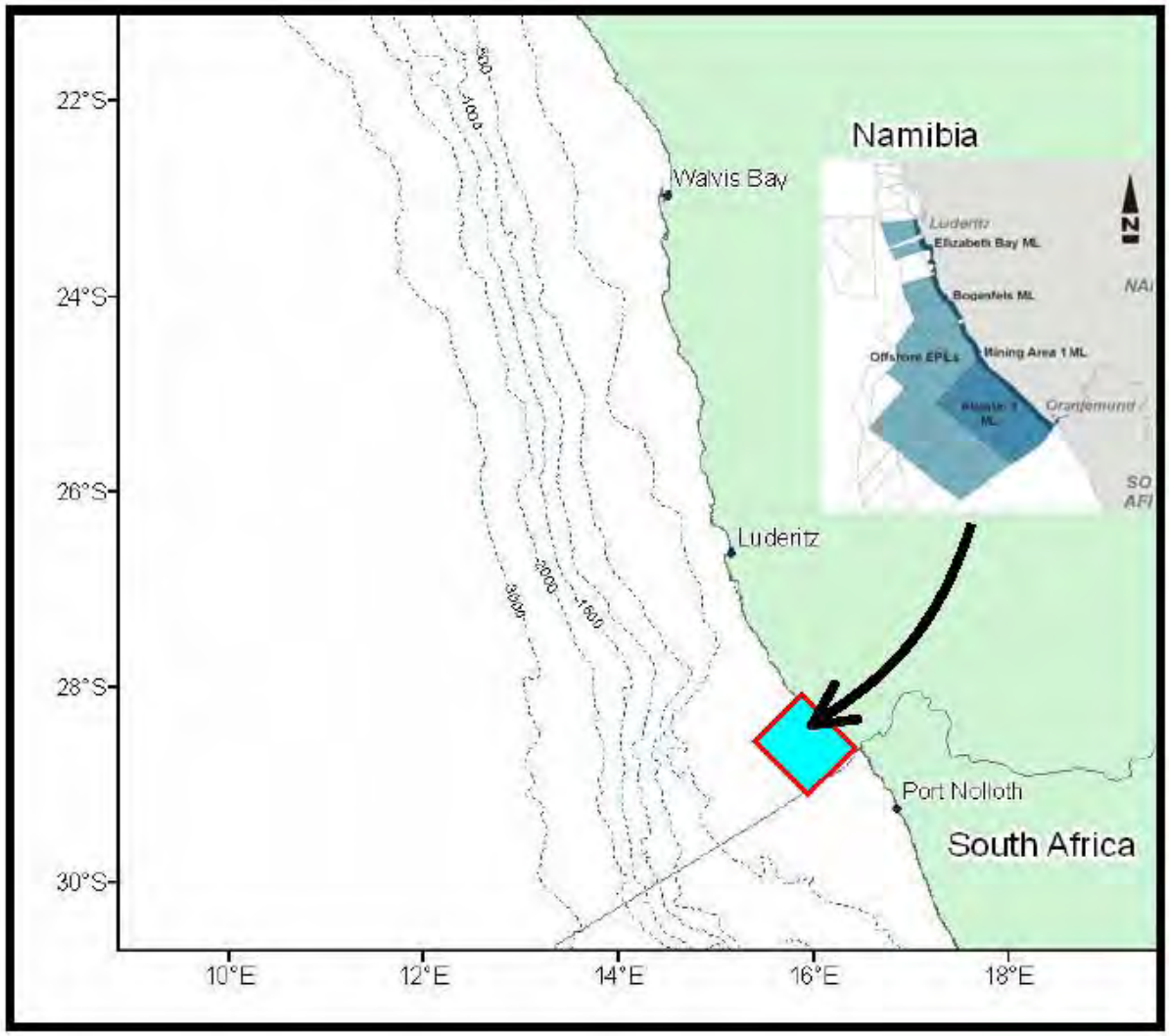


Figure 1.1: A map showing the approximate location of De Beers Marine Namibia Atlantic 1 mining license area (Japp, 2007).

1.2 Sewage effluent and endocrine disruption

Sewage effluents often contain a mixture of organic and inorganic chemicals, which may potentially disrupt the endocrine systems of marine organisms (chemicals present in the effluents become bioavailable to these organisms through aquatic respiration, osmoregulation, dermal contact with contaminated sediment and ingestion of contaminated food) (Lemos *et al.,* 2005; Mills and Chichester, 2005). Endocrine disruption refers to an

alteration in the normal functioning of the endocrine system. Endocrine disruption is caused by various chemicals that have the ability to mimic hormones (Larkin *et al.,* 2003). Endocrine disrupting chemicals (EDCs) is a term used to define a large group of compounds including polychlorinated biphenyls, polyaromatic hydrocarbons, dioxins, alkylphenols and esters (Barse *et al.,* 2007). These compounds confer the ability to 1) mimic the effects of hormones 2) alter the pattern of synthesis and metabolism of hormones and 3) modify hormone receptors (Cheek *et al.,* 2004; IEH Assessment, 1999; Pinder *et al.,* 1999). Endocrine disrupting compounds have an adverse effect on the organisms biological systems, including endogenous hormone synthesis, transport, receptor interaction and intracellular signaling within the organism (Cheek *et al.,* 2004; Fossi *et al.,* 2007). EDCs are mainly estrogenic in nature and thus interfere particularly with hormone pathways that control reproduction and sexual development (Paull *et al.,* 2008). Several studies have shown the affinity of EDCs for hormone receptors which regulate reproductive functions and may result in the induction of the female associated phosholipoproteins, e.g. vitellogenin in male fish species (Kirk *et al.,* 2003; Mills and Chichester, 2005; Porte *et al.,* 2006). Labadie and Budinski (2006) showed that the exposure of 17α-ethynylestradiol (EE2) obtained from wastewater treatment plant effluents contributed to estrogenecity in juvenile turbot (*Psetta maxima*). Noaksson *et al.* (2005) reported that the presence of EDCs in sewage treatment plant processing leachate resulted in the occurrence of reproductive disorders in female perch (*Percha fluviatilis*) and female brook trout (*Salvelinus fontinalis*).

1.3 Basic fish reproductive endocrinology

Steroid hormones are characterized as lipophilic molecules that are synthesized in steroidogenic tissues. Steroid hormones interact with specific target receptor sites to monitor and control several physiological functions within the organism (Yang *et al.,* 2008). The induction of vitellogenin is stimulated by steroid hormones produced by the organism's pituitary gland (Schulz *et al.,* 2001; Sole *et al.,* 2003). To gain a better understanding of the mechanism of endocrine disruption, it is important to understand basic fish reproductive endocrinology.

The basic biosynthetic pathway for steroid hormone production, shown in Figure 1.2, involves the hypothalamus, the pituitary gland and the gonads (the reproductive axis) (Kumakura *et al.,* 2004; Weltzien *et al.,* 2004). The synthesis and metabolism of steroid hormones is regulated by environmental cues. The decapeptide, known as gonadotrophin-releasing hormone (GnRH), located in the hypothalamus (in the brain) regulates the secretion of two types of gonadotrophin hormones (feedback mechanism) in the anterior pituitary gland, gonadotrophin I (GtH I) and gonadotrophin II (GtH II) (Blaquez *et al.,* 1998; Maitra *et al.,* 2007; Mateos *et al.,* 2002). GtH I and II are structurally analogous to follicle stimulating hormone (FSH) and luteinizing hormone (LH) and function in regulating gonadal steroidogenesis, gametogenesis and the release of sperm and ovulation (Blazquez *et al.,* 1998*;* Degani *et al.,* 1998). Although both hormones display similar steroidogenic activities, GTH I primarily functions to stimulate gametogenesis while GTH II is mainly involved in the final phase of reproductive maturation (Garcia-Hernandez *et al.,* 1997). The gonadotrophin hormones stimulate the production of steroid hormones in the gonads. The androgens or steroid hormones produced after GtH induction such as. 17β-estradiol and progesterones, bind to specific hormone receptors which results in the alteration of gene expression (Cuesta *et al.,* 2007). Estradiol targets the liver and stimulates the production of vitellogenin which is then subsequently incorporated into the oocyte. This is regulated by GtH I and occurs over several months until enough gametes are produced (Kime, 1999). The secretion of estradiol and 11-ketotestosterone is halted by progesterone which stimulates oocyte maturation (Greenwood *et al.,* 2001; Kime, 1999; Kortner and Arukwe, 2007; Lee *et al.,* 2004; Yaron, 1995).

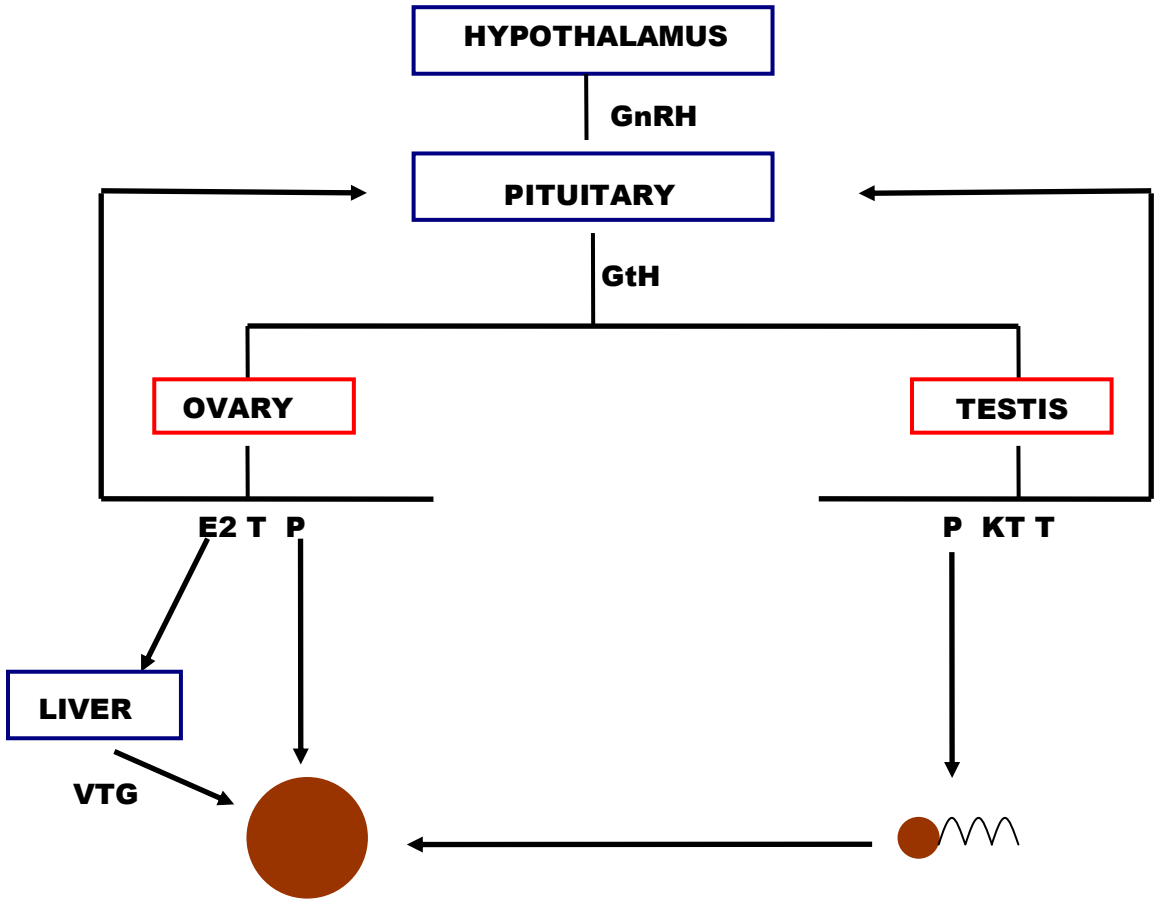


Figure 1.2: Basic biosynthetic pathway for steroid hormone production. GnRh- gonadotrophin releasing hormone; GtH- gonadotrophin I and II; E_2- estradiol; KT- 11-ketotestosterone; T-testosterone; P- progesterone (Kime, 1999)

1.4 Mechanism of endocrine disruption

The interactions between steroid hormones and their specific receptors represent several potential target sites for EDCs (You, 2004). Although the mechanism of endocrine disruption in fish is not completely understood, literature indicates the presence of five main intricate pathways (Arukwe, 2001). Endocrine disruptive chemicals may mimic hormone structures thus allowing EDC's to competitively bind to cell receptors. Some EDCs block the receptor binding site and inhibit the natural binding of hormones to the receptor. EDCs may interact both directly or indirectly with natural steroid hormones causing changes in subsequent

hormone signals (Scott and Sloman, 2004; Thomas, 2000). EDCs may also alter the natural pathway of hormone synthesis and metabolism, causing the imbalance or improper quantity of circulating hormones. EDCs are able to induce the formation of extra receptor sites in the cell amplifying the impact of the hormones on the cellular activity (Singleton and Khan, 2003). Larkin *et al.* (2003) confirmed the ability of estradiol (E_2) mimics to increase the number of estrogen receptors, inducing the synthesis of vitellogenin. Kirk *et al.* (2003) observed how nonylphenol affects steroid hydroxylation by cytrochrome P450 isoforms. Lavado *et al*. (2004) concur with this report and results obtained from their study indicated the ability of polyaromatic hydrocarbons (PAHs) to inhibit the activity of several enzymes (17β-hydroxysteroid dehydrogenase, P450 aromatase and cytochrome P17) involved in the endogenous metabolism of native hormones in flounders (*Platichthys flesus*).

1.5 Biomarkers for environmental pollution

An increase in the production and release of organic trace pollutants e.g. herbicides, metals, polychlorinated biphenyls, alkylphenols, insecticides and industrial effluent mixtures into the aquatic environment has caused an increased concern and awareness of the bioaccumulation, bioconcentration and biomagnification of these pollutants in marine organisms (Hall, 2002; Markert, 2007; Matozza *et al.,* 2008). Scientists are conducting extensive studies to assess the connection between contaminant exposure levels, tissue contamination and early adverse effects of these compounds (Oost *et al.,* 2003). In many populations, the toxic effects of pollutants only begin to manifest after a long period of time. As a result, research into early warning systems in the form of biomarkers for pollutants have increased more recently (Flammarion and Garric, 2002; Wells, 1999).

A biomarker is defined as a biological response (molecular, physiological or behavioural) which can be traced back to the exposure or the toxic effect of environmental pollutants, through the measurement of body fluids, cells or tissues (Oost *et al.,* 1994). Biomarkers can be divided into three different categories, biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility (Thilakaratne *et al.,* 2007). Due to the scope of this project,

focus was placed on defining biomarkers of effect. Biomarkers of effect are defined as biochemical or physiological changes within tissues or body fluids that are measurable e.g. butyrylcholineesterase (BChE) (Thilakaratne *et al.,* 2007). The response of biomarkers is often detected through bioassays e.g. cytochrome P450 (CYP1A) monoxygenase response is detected through the activity of ethoxyresorufin-*O*-deethylase (EROD) (Leino *et al.,* 2005).

Several biochemical biomarkers are used to indicate the biological effects of chemical contaminants in fish species, e.g. acetylcholinesterase (AChE), glutathione-S-transferase (GST), cytochrome P450 (CYP1A) monooxygenase activity (often measured as EROD activity), vitellogenin (Vtg) and catalase (CAT) (Hinck *et al.,* 2006; Kopecka and Pempkowiak, 2007; Lemos *et al.,* 2005). Although biomarkers provide vital insight into the effects of pollutants, they are highly influenced by abiotic and biotic factors such as water, temperature, reproduction and age which are very important considerations in the interpretation of data (Pain *et al.,* 2007). The variability of biomarkers can be reduced by 1) optimizing sampling techniques e.g. increased sample size and decreasing the range between the sizes of fish sampled 2) understanding of the physio-chemical parameters of the water and the reproduction cycle of the particular species and 3) data normalization (Sanchez *et al.,* 2008).

Cytochrome P450 (CYP1A) monooxygenase and vitellogenin (Vtg) are important proteins found in fish liver (Desantis *et al.,* 2005). CYP1A and vtg are commonly used as biomarkers in pollution detection, because their expression is altered upon exposure to contaminants. These proteins can provide vital biological information on the potential impact of pollutants on the fitness of organisms and the ecosystem (Rotchell *et al.,* 1999; Won *et al.,* 2005).

1.6 Cytochrome P450 1A (CYP1A)

1.6.1 CYP1A molecular properties and function

Xenobiotic metabolism is a two-phase process, with cytochrome P450s being considered the most important enzymes in Phase I metabolism. Phase I metabolism involves oxidative and reductive hydrolytic reactions (Gonzalez *et al.,* 2009; Siroka and Drastichova, 2004; Sole *et al.,* 2009). Cytochrome P4501A (CYP1A) is the subfamily of the P450 superfamily of enzymes. Several studies have indicated protein sizes ranging from 45-65 kDa (refer to Table 1.1) (Goksoyr and Forlin, 1992; Miota *et al.,* 2000). Although CYP1A is found predominantly in the liver, it has also been found in the kidneys, gill tissue and endoplasmic reticulum of fish (Assuncao *et al.,* 2007; Tom *et al.,* 2002). Oritz-Delgado and Sarasqueste (2004), as well as Sarasqueste and Segner (2000), describe cytrochome P450s as haemproteins, consisting of a single polypeptide chain (held together by hydrophobic forces, electrostatic and covalent bonds) with iron ligands bound to a porphyrin ring, a thiloate group in the peptide backbone and an oxygen binding site (Goksoyr and Forlin, 1992; Siroka and Drastichova, 2004).

Table 1.1 Different CYP1A sizes observed in liver microsomes of different fish species

Species	Size (kDa)	Reference
Balaenoptera acutorostrata	46.5	*Goksoyr, 1986*
Lithognathus mormyrus	55.0	*Tom et al., 2002*
Merluccius merluccius	55.0	*Mihailovic et al., 2006*
Oncorhynchus mykiss	55.0	*Aluru and Vijayan, 2006*
Oreochromis mossambicus	59.0	*Ueng et al., 1995*
Salma trutta	56.0	*Rodriguez-Cea et al., 2004*

Research into the function of cytrochrome P450s in fish has shown significant progress over recent years. The first successful purification and characterization of CYP1A was reported by Williams and Buhler (1983), using rainbow trout as a test species. Following this, CYP1A

has become the best studied enzyme within the members of the CYP gene family, due to its role in the catalytic breakdown of several xenobiotic compounds and its inducibililty by planar compounds e.g. 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD), dibenzofuran (PCDF), polychlorinated dibenzo-*p*-dioxin (PCDD) and biphenyl (PCB) (Takahashi *et al.,* 1995; Zabel *et al.,* 1995). To gain a better understanding of the interactions of CYP1A with different xenobiotics in the environment, it is important to have a basic understanding of how the cytochrome P450 system functions.

Cytochrome P450 catalyses the oxidation of the substrate through the cleavage of C-C/ C=N bonds and the addition of an oxygen atom to the substrate (Figure 1.3) thereby, increasing the polarity of the substrate (Siroka and Drastichova, 2004).

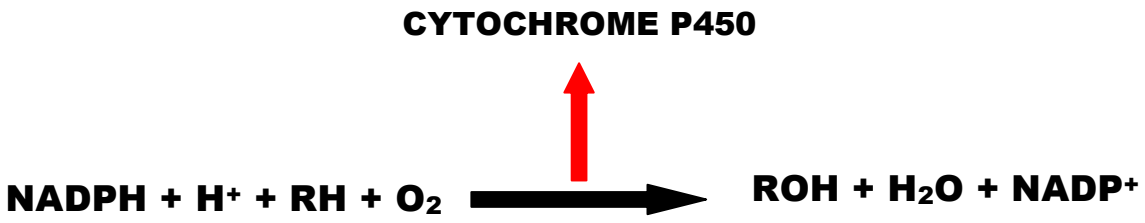


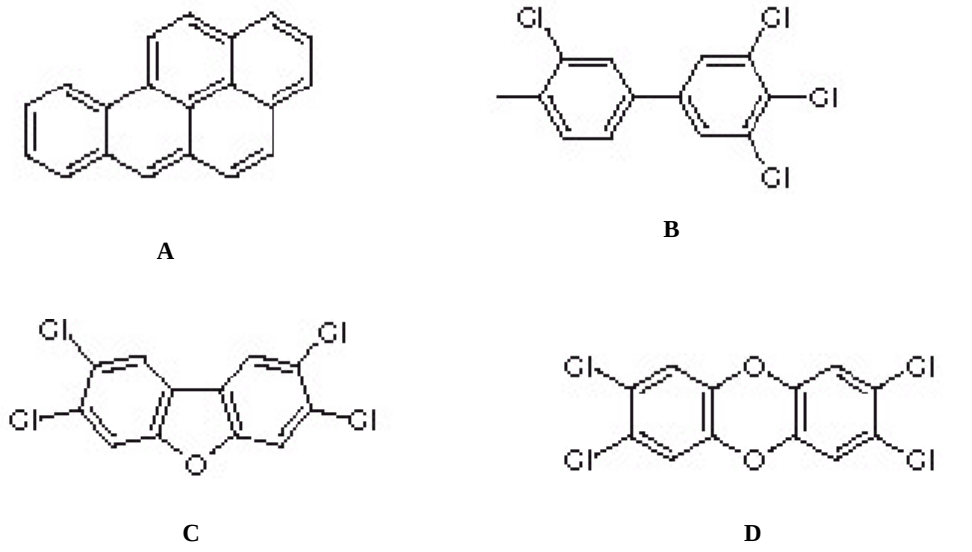
Figure 1.3: A brief outline of cytochrome P450 monooxygenase activity, RH: substrate which may consist of alkenes, alkanes, heterocyclic and/or aromatic rings (Siroka and Drastichova, 2004)

The reaction catalyzed by cytochrome P450 is more complex than indicated in Figure 1.3. The transfer of electrons for this NADPH/NADH dependent reaction is catalyzed by the enzymes NADPH reductase (microsomal), ferrodoxine and ferrodoxine reductase (in the mitochondria) and cytochrome P450 is not limited to utilizing molecular oxygen but may also obtain oxygen atoms from peroxides (Siroka and Drastichova, 2004). The cytochrome P450 family plays a role in many different oxidation reactions such as hydroxylation, H- and O- dealkylation, sulphoxidation and dehalogenation (Mansuy, 2007). Mansuy (1998) stated that this family of enzymes also participates in many other non-oxidation reactions; including isomerizations, dehydrations and dehydrogenations of different substrates.

Cytochrome P450s play a key role in the metabolism of cholesterol, the biosynthesis of bile acids, vitamin D_3 and steroid hormones in fish. The main function of CYP1A is the conversion of xenobiotic compounds through monooxygenation into more water-soluble products, which are detoxified further in Phase II metabolism (Livingstone, 1998; Masfaraud et al., 1990; Melancon and Lech, 1983; Miller *et al.,* 1988; Poelmans *et al.,* 2006; Sarasquete and Segner, 2000; Wootten *et al.,* 1995; Wootten *et al.,* 1996). Variations in the catalytic activities of cytochrome P450s are highly dependent on factors such as temperature, the hormonal cycle, food intake and sex (Jimenez *et al.,* 1987; Lindstrom-Seppa *et al.,* 1994; Marionnet *et al.,* 1997).

1.6.2 Fish CYP1A as a biomarker for environmental pollution

Polycyclic hydrocarbons (PAH), polychlorinated biphenyls (PCB), polychlorinated dibenzofurans (PCDF) and polychlorinated dibenzo-*p*-dioxins (PCDD) (Figure 1.4) are mutagenic and carcinogenic compounds that have been demonstrated to cause reproductive toxicity in many fish species (Steward, 1991; Viganno *et al.,* 1995). The inducibility of CYP1A by these compound groups are well established (Table 1.2). Thus the potential use of CYP1A as a biomarker for monitoring pollution and environmental management has been confirmed (Stegeman and Lech, 1991).



A **B**

C **D**

Figure 1.4: The general structure of inducers of CYP1A. A: Benzo[a]pyrene (PAH), B: 3,3', 4,4', 5-pentachlorobiphenyl (PCB), C: 2,3,7,8-tetrachlorodibenzofuran (PCDF), D: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (PCDD) (Whyte and Tillitt, 2000)

Table 1.2: A summary of studies of CYP1A induction on different fish species

Species	Common name	Compound/s	Reference	Induction*
Salvelinus namaycush	Lake Trout	2,3,7,8-tetrachlorodibenzo-p-dioxin	*Guiney et al., 1997*	+
Gadus morhua	Atlantic Cod	Polycyclic aromatic hydrocarbons	*Husoy et al., 1996*	+
Platichthys flesus	European Flounder			+
Oncorhynchus mykiss	Rainbow Trout	3,3',4,4'-tetrachlorobiphenyl Arclor 1254 3,4,3',4'-tetrachlorobiphenyl 7,12-dimethylbenz[a]anthracene	*Koponen et al., 1998* *Melancon & Lech, 1983* *Miranda et al., 1997*	+
Pimephales promelas	Freshwater Vendance	[^{14}C]-3,3',4,4'-tetrachlorobiphenyl	*Lindstrom-Seppa, 1994*	+
Fundulus heteroclitus	Killifish	β-napthoflavone 3-methylcholanthrene Benzo[a]pyrene	*Meyer et al., 2002* *Van veld et al.,* 1997	+
Scothalmus maximus	Turbot	benzo[a]pyrene γ-hexachlorocyclohexane	*Peters & Livingstone, 1995*	+
Mullus barbatus	Red Mullet	polycyclic aromatic hydrocarbon metabolites	*Porte et al., 2002*	+
Stentomus chrysops	Marine Fish Scup	3',3',4,4',5-pentochlorobiphenyl 3,3',4,4'-tetrachlorobiphenyl	*Schlezinger & Stegeman, 2001* *White et al., 1997*	+

*Induction of CYP1A observed: +

The induction of CYP1A by xenobiotic compounds, illustrated in Figure 1.5, is regulated at a transcriptional level (Miota *et al.,* 2000). The binding of the xenobiotic compound to the cytosolic arylhydrocarbon receptor activates the receptor, causing heat shock proteins and immunophilin-related proteins to dissociate (Kling *et al.,* 1999; Mortensen and Arukwe, 2007). This chain of events leads to the heterodimerization of the ligand-receptor complex with the arylhydrocarbon nuclear translocator, causing nuclear translocation of the complex (Hahn, 1998; Milhailovic *et al.,* 2006). The complex then binds specifically to the xenobiotic response element on DNA, which is found upstream from the CYP1A promoter, leading to the upregulation of transcription and increased CYP1A activity (Milhailovic *et al.,* 2006; Rice *et al.,* 1997).

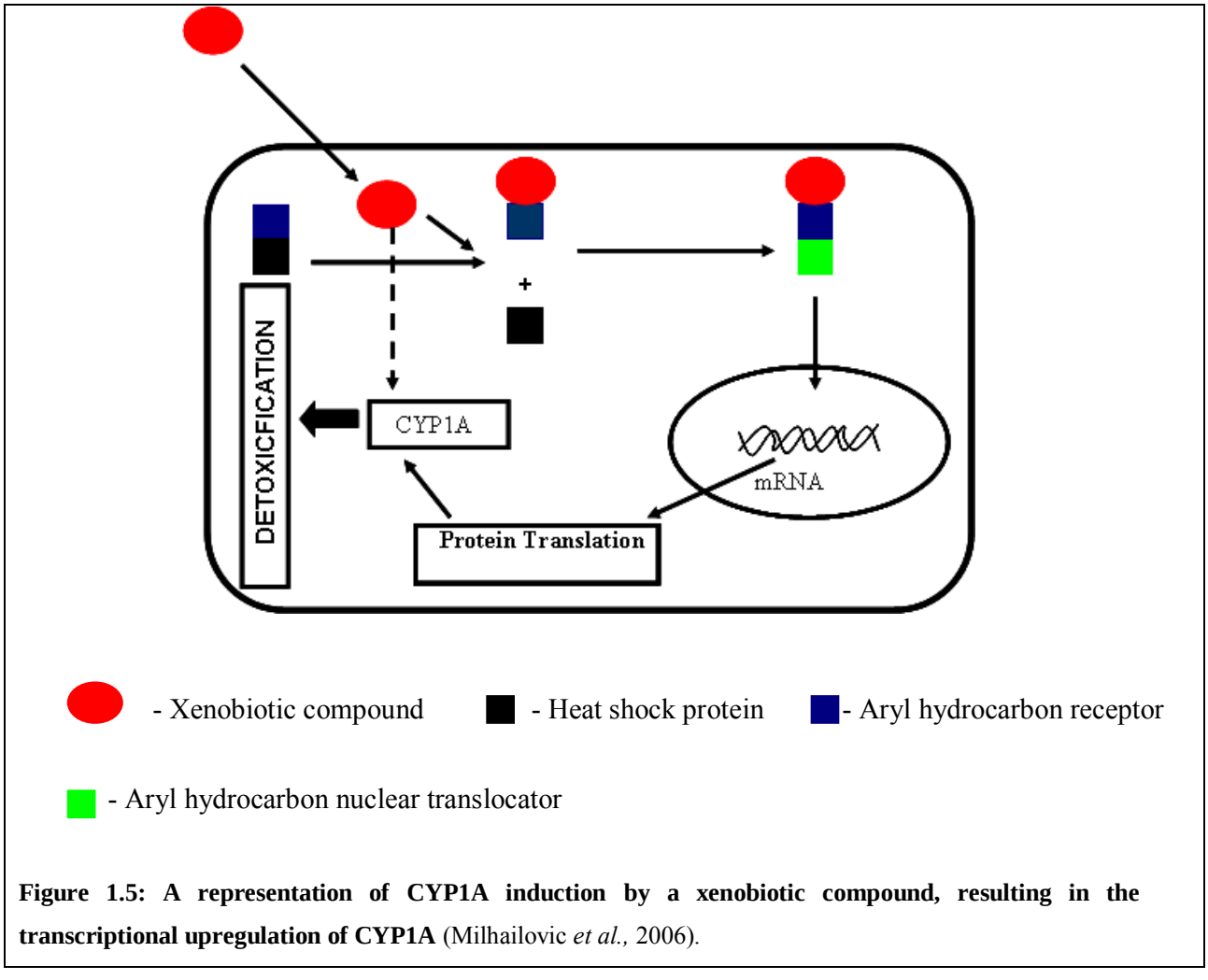


Figure 1.5: A representation of CYP1A induction by a xenobiotic compound, resulting in the transcriptional upregulation of CYP1A (Milhailovic *et al.,* 2006).

Although the study of CYP1A induction in fish provides useful insight into the status of the aquatic environment, precaution must be taken when using this tool to generate and discuss data, as recent studies have reported the ability of some compounds e.g. ethynylestradiol, to inhibit CYP1A activity (Hasselberg *et al.,* 2005). Findings by Machala *et al.* (1997) using Carp as a test species, concluded that some polycyclic and polyhalogenated aromatic hydrocarbons confer the ability to inhibit CYP1A activity. Huuskonen *et al.* (1995) demonstrated the inhibitory effect of paper mill effluent on the CYP1A mediated enzyme, 7-ethoxyresorufin-*O*-deethylase in Perch.

1.7 Vitellogenin (vtg)

1.7.1 Vitellogenesis in fish

The growth and development of an oocyte, shown in Figure 1.6, can be divided into four main stages: primary growth, followed by the formation of cortical alveoli, the vitellogenic phase and finally maturation (Arukwe and Goksoyr, 2003; Wood and Kraak, 2001). In the primary growth phase, oocytes act as storage vessels for RNA and proteins, which are required for future breeding seasons (Murua and Sabarido-Rey 2003; Picton *et al.,* 1998). The primary growth phase is rapidly followed by the cortical alveoli stage which is indicated by the appearance of yolk proteins, known as cortical alveoli vesicles, in the organelles (Murua and Sabarido-Rey 2003). During fertilization this stage is characterized by the increase in the number and size of cortical alveoli vesicles which release their contents into previtelline space within the egg membrane (Murua and Sabarido-Rey 2003). During the vitellogenic phase true yolk vesicles appear in the cytoplasm of oocytes, oocyte size continues to increase with the accumulation of yolk. This phase is halted once the oocytes have reached a fully developed size (Tyler *et al.,* 1997; Tyler *et al.,* 1990). Once the vitellogenic phase is complete oocytes undergo maturation, at this stage the first meiotic division takes place following the migration of the nucleas to the animal pole (Power *et al.,* 2007;). Towards the completion of the maturation stage the oocyte takes fluid up through its follicle (hydration phase) and the coalescence of yolk spheres and/or oil

droplets occurs (Murua and Sabarido-Rey, 2003; Power *et al.,* 2007) After ovulation a second meiotic division occurs and the oocytes become eggs (Murua-Sabarido *et al.,* 2003).

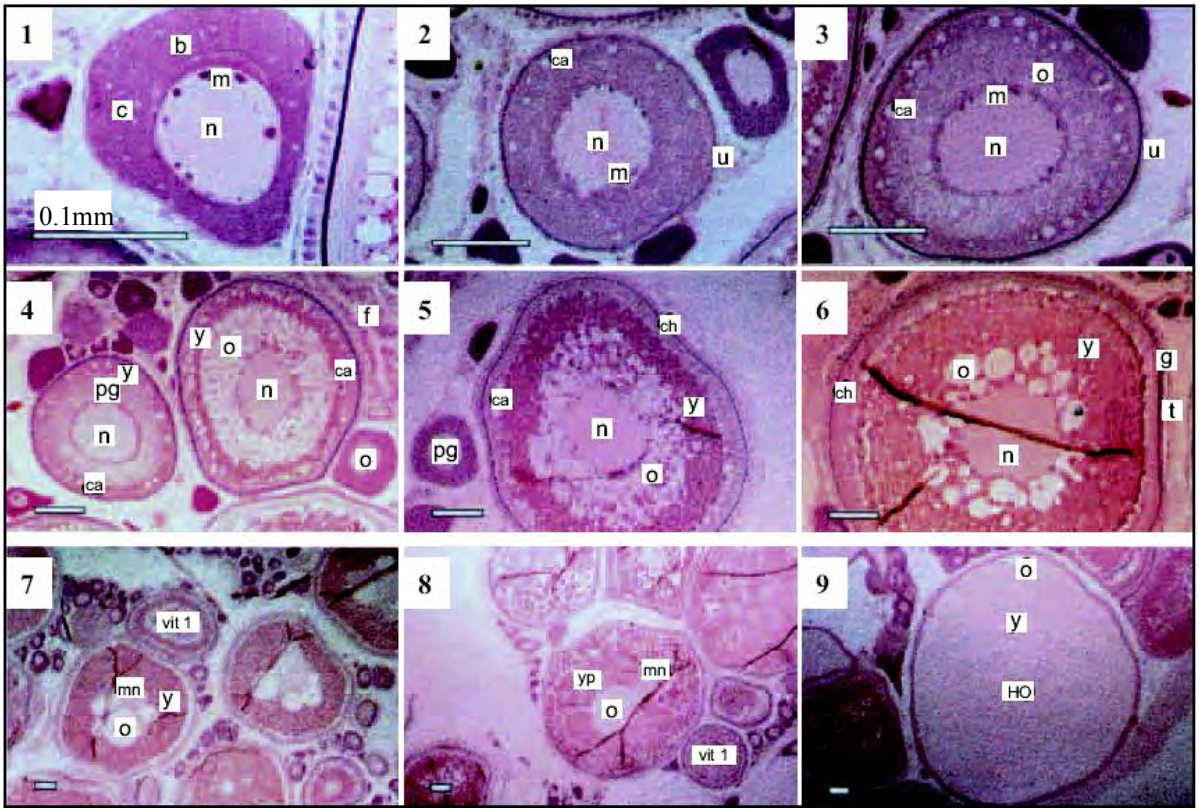


Figure 1.6: Oocyte development and maturation observed in *Merluccius merluccius.* 1: primary growth, 2+3: cortical alveoli stage, 4: early vitellogenic stage (right), 5+6: advanced vitellogenic stage, 7: early maturation stage, 8: final maturation, 9: hydrated oocyte. n: nucleas; c: cytoplasm; pg: primary growth; m: nucleolus; ca: cortical alveoli; t: follicle layer; u: oocyte envelope; y: yolk vesicles; o: oil droplets; mn: migratory nucleus; yp: yolk plates; ho: hydrated oocyte; b: balbiani bodies; ch: chorion; g: granulose; vit 1: vitellogenic oocyte; f: postovulary follicle (Murua and Sabarido-Rey, 2003)

Vitellogenin (vtg) is a high density dimeric lipoglycophosphoprotein (Matozza *et al.,* 2008) that is produced in sexually maturing female fish (Bulukin *et al.,* 2007). The genes that express this protein are also found in male species and can be induced by estrogenic

substances (Ferreira *et al.,* 2009; Jimenez *et al.,* 2007). In females the transcription of vtg-genes is coordinated by the GTH-induced estrogen (E2), 17β-estradiol, which binds to estrogen receptors (ER) in the liver inducing transcription (Bailey *et al.,* 2004; Crim and Idler 1978; Werawatgoompa *et al.,* 1997). Vtg is proteolytically cleaved into smaller proteins namely lipovitellin, phosvettes and phosphovitin (egg yolk proteins), which are then deposited as yolk granules (Arukwe and Goksoyr, 2003; Bailey *et al.,* 2004; Carnevali *et al.,* 1999; Davail *et al.,* 1998; Hiramatsu *et al.,* 2002; King *et al.,* 2008; Maitra *et al.,* 2007, Nath *et al.,* 2007; Penning *et al.,* 1977; Wallace and Begovac, 1985 and Wiley and Wallace, 1981). The main function of egg yolk is to provide the energy requirements and maintenance of the growing oocyte, but other functions of vtg include being a carrier for hormones and metal ions required to provide minerals to the developing embryo (Arukwe and Goksoyr, 2003; Won *et al.,* 2005). Recent reports by Liu *et al.* (2008) also indicate that vtg plays a role as a recognition molecule in the antibacterial defences of Carp (*Cyprinus carpio*).

To gain a deeper understanding on the process of vitellogenesis, studies have been carried out in an attempt to purify vtg from the plasma of different fish species. Nilsen *et al.* (2004) purified vtg from Carp and Medaka, using methods such as selective precipitation, anion exchange chromatography, gel permeation chromatography and SDS-PAGE analysis. Results obtained from this study indicate that Carp and Medaka vtg are approximately 150 and 130 kDa in size, respectively. Reports from Song *et al.* (2008) on Senegalese Sole purified using immunoaffinity methods show that the vtg of this particular species is 160 kDa in size. Manhor *et al.* (2005) concluded that catfish vtg purified using anion exchange resulted in a protein that was 200 kDa in size.

1.7.2 Fish vitellogenin as a biomarker for environmental pollution

The measurement of estrogen induced changes in fish vitellogenin, is a sensitive biomarker for the detection of estrogenic and anti-estrogenic like chemicals such as oestradiols, phthalates and alkyl-phenols, which have undesirable effects on the development and

reproduction process and sexual maturation in fish (Hansen *et al.,* 2001; Rodas-Oritz *et al.,* 2008; Zhang *et al.,* 2004).

The process of vitellogenin induction by xenoestrogens is not fully understood, but there has been evidence of transcriptional activation via competetitive receptor-ligand interaction (Kloas *et al.,* 2000; Nicolas, 1999; Nimrod and Benson, 1997; Soverchia *et al.,* 2005; Tenniswood *et al.,* 1983; Ternes *et al.,* 1999; Yadetie *et al.,* 1999). The use of vitellogenin as a biomarker for environmental pollution is well established (Table 1.3). Studies on vitellogenin induced effects on different fish species around the world confirm the importance of using vtg as a tool for the detection of aquatic pollutants in both laboratory and field studies (Gagnaire *et al.,* 2009).

Table 1.3: Studies on vitellogenin induced effects on different fish species, by xenoestrogens

Species	Common name	Compound	Reference
Carassius auratus	Gold fish	Bisphenol-A	*Ishibashi et al., 2001*
Danio rerio	Zebra fish	Metazachlor 17-α-ethinylestradiol	*Jurcikova et al., 2007* *Orn et al., 2006*
Lates calcarifer *Acanthopagrus butcheri*	Tropical Barrmundi Black Bream	17-β-estradiol	*King et al., 2008*
Platichythys flesus	European Flounder	Sewage effluent	*Lye et al., 1997*
Dicentrarchus labrax	Sea Bass	Estradiol	*Mananos et al., 1994*
Gadus morhua	Atlantic Cod	Alkylphenols	*Meier, 2007*
Salmo salar	Atlantic Salmon	Nonylphenol	*Meucci & Arukwe, 2005* *Yadetie et al., 1999*
Pimephales promelas	Fathead Minnows	17-β-estradiol	*Miles-Richardson et al, 1999*
Oryzias laptipes	Japanese Medaka	17-β-trenbolone	*Orn et al., 2006*
Paralichthys californicus	California Hailbut	Estrogen	*Palumbo et al., 2007*
Oreochromis niloticus	Nile Tilapia	Organochlorine pollutants	*Rodas-Oritz et al., 2008*
Micropogonias undulates	Atlantic Croaker	Arclor 1254	*Thomas, 1989*

1.8 Ecotoxicological bioassays for CYP1A and vitellogenin determination in fish

1.8.1 Bioassays

Bioassays are employed in ecotoxicological monitoring systems, as tools to investigate the potential toxicological effects different pollutants have on living organisms (Fent, 2001; Schlenk, 1999). Bioassays are often categorized according to the test matrices and test species that are being studied (Losso and Ghirardini, 2010). When conducting toxicological assays using test species, it is important to understand that the observed results for one species may not necessarily be valid for a different species. The variation in toxicological assays may be due to a number of factors including genetic variation and physiological differences, therefore, the development and validation of bioassays for different species is very important in ecotoxicological assessments (Van Der Oost *et al.,* 2003). To date several systems have been developed to determine the effects of EDCs on different fish species (Sanchez and Porcher, 2009; Shao *et al.,* 2005)

1.8.2 Vtg

Amongst several assays utilized for vtg detection, the most widely employed are direct assays. Studies carried out using these techniques have shown that these methods are successful in demonstrating the effects of estrogenic compounds. Vtg can be detected by enzyme linked immunosorbent assay (ELISA), western blot analysis (Jensen and Ankley, 2006; Okoumassoun *et al.,* 2002; Prakash *et al.,* 2007; Scott *et al.,* 2006; Sherry *et al.,* 1999; Tsukimura *et al.,* 2000; Watts *et al.,* 2003), radioimmunoassay and Vtg/Zrp mRNA determination through DNA hybridization (Bulukin *et al.,* 2007; Navas *et al.,* 2006; Rees *et al.,* 2003). Even though the direct assays are specific and sensitive they are limited by the cost, time and the expertise required to perform the assays (Kashiwada *et al.,* 2002; Liao *et al.,* 2006). Assays that have been developed and validated for vtg are shown in Table 1.4. Although there are various ways to detect vtg induction, the most popular method employed

by researchers is the ELISA method, due to its high degree of specificity and its ability to measure absolute (quantitative ELISA) or relative (semi-quantitative) values (Navas and Segner, 2006).

Table 1.4: Vtg bioassays developed and validated for different fish species

Species	Common name	Vtg bioassay	Reference
Pimephales promelas	Fathead Minnow	ELISA	*Parks et al., 1999*
Misgurnus angaillicaudatus	Chinese Loach	ELISA	*Shao et al., 2005*
Zoarces viviparous	Eelpout	ELISA	*Korsgaard & Pedersen, 1998*
Salvelinus alpines	Artic Char	ELISA	*Johnsen et al., 1999*
Cyprinus carpio *Perca fluviatilis*	Carp Perch	ELISA	*Hennies et al., 2003*
Danio rerio	Zebra fish	ELISA	*Fenske et al., 2001*
Pimephales promelas	Fathead Minnow	Dot Blot analysis	*Parks et al., 1999*
Cichlasoma dimerus	Cichlid	Immunocytochemistry	*Moncaut et al., 2003*
Misgurnus angaillicaudatus	Chinese Loach	Western blot analysis	*Shao et al., 2005*
Cyprinus carpio	Koi Carp	Quartz crystal microbalance assay	*Oshima et al., 2005*

1.8.3 CYP1A

The direct assays mentioned above (Section *1.8.2*) are all techniques that can also be utilized for CYP1A, but the most commonly employed and well established indirect strategy (optimized protocol) used to determine the induction of CYP1A is the measurement of CYP1A mediated EROD (7-ethoxyresorufin-*O*-deethylase) activity (Zapata-Perez *et al.,* 2005). The EROD assay is extremely sensitive and relatively inexpensive (Forlin *et al.,* 1995). The EROD assay demonstrates the effect of the uptake of toxic planar compounds in fish, whether the presence of these agents have been analytically detected or not (Abrahamson *et al.,* 2007; Beyer and Goksoyr *et al.,* 1993; Burgeot *et al.,* 1994; Eggens *et al.,* 1996; Galgani *et al.,* 1991, Hoeger *et al.,* 2004). EROD activity is measured in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) which initiates the CYP1A-mediated deethylation of the substrate 7-ethoxyresorufin (7-ER) for the production of resorufin (Figure 1.7), the product is measured using fluorimetry, spectrophotometry or high pressure liquid chromatography (HPLC) (Pikkarainen *et al.,* 2006; Stagg and Addison, 1994).

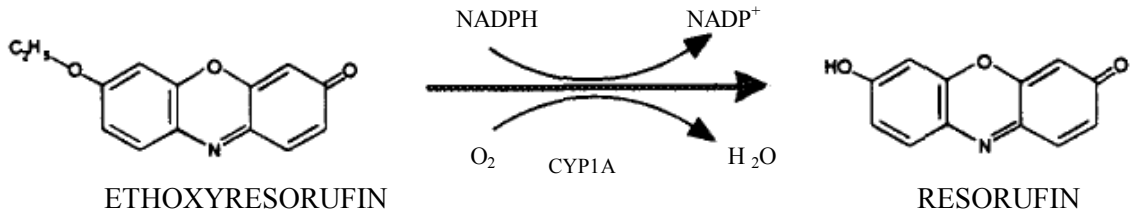


Figure 1.7: The 7-ethoxyresorufin-*O*-deethylase assay principle (Kennedy *et al.,* 1995)

The development and validation of the EROD assay for the detection of the pollutant effects in various fish species dates back to 1981, with Goddard *et al.* (1981) investigating CYP1A induction in freshwater viviparous fish (*Poecilliopsis lucida* and *Poecilliopsis monacha*) using the EROD assay. Further advancements into this technique lead to the adaptation of the

EROD assay into a microtitreplate technique (Grzebyk and Galgani, 1991). Kruner and Westerhagen (1999) employed the microtitre method developed by Grzebyk and Galgani (1991) in their research to optimize the assay conditions for the fish species *Limanda limanda* (dab). The long term studies that were conducted used the EROD assay as an early warning sign for pollution detection on the *Limanda* species in the German Bight (Kammann *et al.,* 2000). Other studies included a long term study conducted by Burgeot *et al.* (1994) who evaluated the biological effects of chemical pollutant exposure on the fish species *Callionymus lyra*, *Limanda limanda* and *Mullus barbatus* using the EROD microtitre plate assay. This assay has been developed and optimized for several tissues in different fish species (Table 1.5).

Table 1.5: Studies using the EROD assay as a bioassay for pollution detection in different fish species

Species	Common name	Tissue	Reference
Limanda limanda	Dab	Muscle	*Westerhagen et al., 1999*
Platichthys flesus	Flounder	Liver	
Oncorhynchus mykiss	Rainbow trout	Gill	*Carlsson et al., 1999*
Oncorhynchus mykiss	Rainbow trout	Gill epithelium	*Carlsson & Part, 2001*
Leuciscus cephatus	Chub	Liver	*Flammarion & Garric, 1999*
Gobio gobio	Gudgeon	Gonads	
Oncorhynchus mykiss	Rainbow trout	Pituitary cell lines	*Tom et al., 2001*
Liza saliens	Leaping mullet	Liver	*Arinc & Sen, 1999*
Solea vulgaris	Common sole	Liver	
Oncorhynchus mykiss	Rainbow trout	Liver cell lines	*Bols et al., 1999*
Oncorhynchus mykiss	Rainbow trout	Liver cell lines	*Babin et al., 2005*
Ctenopharyngodon idellus	Grass carp	Cultured hepatocytes	*Wan et al., 2004*
Oncorhynchus mykiss	Rainbow trout	Cultured hepatocytes	*Jos et al., 2007*
Scophthalmus maximus	Turbot	Bile	*Camus et al., 1998*
Rhombosoleu tapirina	Greenback flounder	Liver	*Mondon et al., 2001*

1.9 *Merluccius capensis* and *Merluccius paradoxus*

Merluccuis capensis and *Merluccuis paradoxus* (Cape hake) also referred to as shallow and deep water hake, respectively, belong to the family Gadidae and co-inhabit Namibian and South African waters (Von der Heyden *et al.,* 2007). Their geographical distribution is associated with the Benguela Current system (17° 30'S-29° 30'S) (Grote *et al.,* 2007).

M. capensis and *M. paradoxus* are morphologically similar and differences which separate them, for example; differences in the number of vertebrae and the pigmentation of the gill rakers are very minor, therefore, the two species are often considered as one stock (Figure 1.8) (Gordoa *et al.,* 2000; Mas-Riera, 1991). *M. capensis* occurs predominantly at a depth of 400 m and is the most abundant species off the coast of Namibia and the south coast of South Africa (Gordoa *et al.,* 2006). The distribution of *M. paradoxus* overlaps with the region inhabited by *M. capensis,* however, *M. paradoxus* is found at a depth of approximately 150-500 m. *M. paradoxus* is predominantly found along the west coast of South Africa (Figure 1.9) (Gordoa *et al.,* 2006).

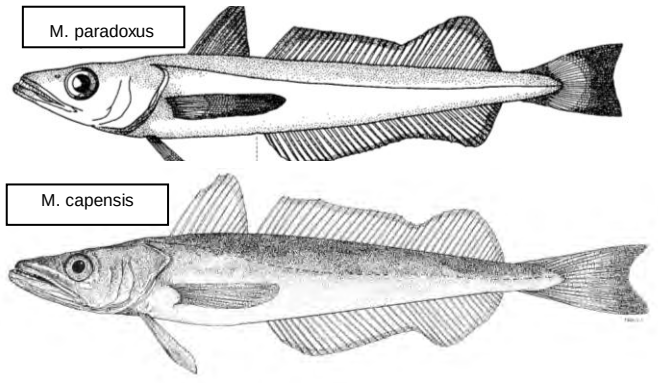


Figure 1.8: Diagnostic features of *M. capensis* and *M. paradoxus*. *M.capensis:* 54-58 vertebrae, colouration: dark brown with white belly; *M.paradoxus:* 48 vertebrae, colouration: lead grey with white belly. (adapted from Food and Agriculture Organisation of the United Nations, 2008)

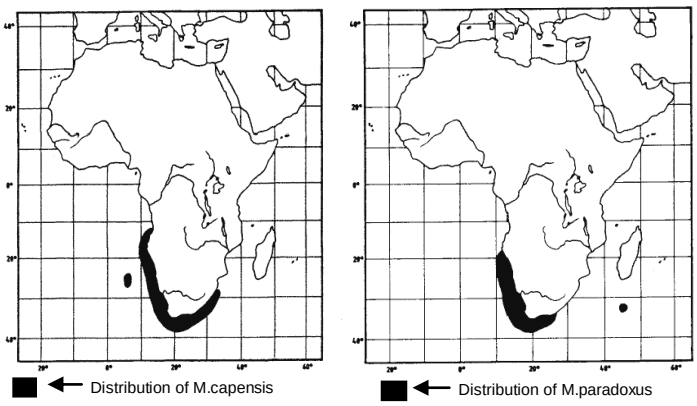


Figure 1.9: Maps indicating the distribution of *M.capensis* and *M. paradoxus* (adapted from Food and Agriculture Organisation of the United Nations, 2008)

This difference in the distribution and abundance of Cape hake is related to variations in temperature and oxygen (habitat preference)[5] (Von der Heyden *et al.,* 2007) *M. capensis/paradoxus* are migratory species, which migrate seasonally and exhibit vertical migration i.e. they are demersal during the day and nektonic at night (Gordoa and Macpherson, 1991). The peak spawning seasons occur during February, March and September, at which time the passive transport of pelagic eggs and larval stages occurs due to the Benguela and Agulhas Current (Rikhter and Golubiatnikova, 1997).

Both hake species spawn in midwater, with spawning occurring predominantly during early summer and a second spawning period occurring during late summer (August)[6]. *M. paradoxus* spawning occurs mainly along the edge of the Agulhas Bank, however, reports have indicated that spawning also takes place west of St. Helena Bay and off central Namibia [7]. *M. capensis* spawn at depths between 160-250 m off central Namibia, with spawning

[5] De Beers Marine Namibia (PTY) Ltd environmental impact assessment 2007

[6] Government Gazette of the Republic of Namibia 2001. Regulations relating to the exploitation of marine resources. Windhoek, Namibia No. 2591

[7] FAO Fisheries Report No. 695 2002

taking place in shallower waters (Powers *et al.,* 2004). The egg and larval populations of both species are concentrated inside the 200 m isobath, around the depth of the thermocline (Heyen *et al.,* 2007; Voges *et al.,* 2002). Juvenile *M. capensis* inhabit the entire Namibian coast but are more concentrated off Walvis Bay while *M. paradoxus* juveniles are most abundant off the Orange River and towards the extreme south to Cape Columbine (Grote *et al.,* 2007; Heyden *et al.,* 2007).

Cape hake are opportunistic predators which display a feeding pattern that is seasonally and spatially variable (Gordoa and Macpherson, 1991). Cape hake feed at the bottom and in midwater, with juvenile *M. capensis* and *M. paradoxus* feeding mainly on crustaceans, pelagic gobies and lanternfish. Adult diets are compromised of epipelagic fish, squid, mysids and euphasiids [3]; however, there has been evidence of cannibalism in larger fish (Shannon *et al.,* 2003). The variability in their diet makes these two species highly important predators in the Benguela system, with the annual consumption by Cape hake reaching approximately 6 million tons in South African and Namibian waters [4].

The role of hake in the aquatic food web, in terms of energy transfer from lower to higher trophic levels, means the study of pollution effects on these organisms is of great ecological relevance.

Chapter 2: Aims, objectives and hypothesis

2.1 Problem statement and motivation

The potential endocrine disruptive effects that may be caused by the release of different types of sewage effluent into the ocean on the Cape hake is of great concern, as Cape hake is the most valuable fish resource in Namibia. Economically, Cape hake represent one third of world fish stocks, with approximately 150 000 tons of stock being harvested yearly (Voges *et al.,* 2002). In addition, several marine top predators are dependent on hake as an energy source. Contaminant-induced impacts on fish populations could be associated with decreased food availability for these top predators, and in turn impact several other marine animals[8]. Studies on the effect of EDCs on the *Merluccius* species are also extremely limited (Mihailovic *et al.,* 2006).

Although several studies have established the impact of EDCs on different fish species (Arukwe *et al.,* 1998; Porte *et al.,* 2006; Rees *et al.,* 2003; Zapata-Perez *et al.,* 2005) this effect has not been demonstrated in practice by De Beers Marine Namibia. This highlights the importance of this project in terms of demonstrating possible biological impacts on the biophysical environment, and in future recommending an approach for managing discharges into the marine environment.

2.2 Hypotheses

2.2.1 Sewage effluents discharged into the aquatic environment from De Beers Marine (PTY) Namibia mining vessel operations contain compounds which effect the induction of CYP1A in Cape hake.

[8] Namdeb Diamond Corporation (PTY) Ltd Environmental programme I/II 1997

2.2.2 Vitellogenin can be successfully purified from Cape hake and detected via western blot analysis

2.3 Aims and Objectives

The induction response of the biotransformation enzyme, cytochrome P450 1A (CYP1A), in fish is often used as a biomarker for the detection of pollutants in marine environments (Goksoyr and Forlin, 1992). Biochemical methods such as western blot analysis and the CYP1A-mediated ethoxyresorufin deethylase (EROD) assay are commonly employed techniques used to assess possible contaminant effects. The aims of this project were: 1) to isolate CYP1A from fish liver samples and to optimize the conditions for the biochemical techniques mentioned above in the fish species *Merluccius capensis/ M. paradoxus* (Cape hake) 2) to evaluate potential endocrine disruptive effects in Cape hake samples, obtained from the De Beers Marine Namibia mining area on the induction of CYP1A 3) to purify a second biomarker also commonly used in the detection of estrogenic pollutants, vitellogenin (vtg), from blood samples obtained from the Cape hake samples, and 4) to optimize the western blot technique for vitellogenin detection in Cape hake.

Chapter 3: Preliminary fish studies

3.1 Introduction

Aquatic environments are recipients for a variety of natural and anthropogenic chemicals. Evidence for the presence of endocrine disrupting chemicals (EDCs) and their impact on aquatic organisms is well documented (Mills and Chichester, 2005). Fish models have played a significant role in toxicological studies used to assess the state of aquatic environments (Raisuddin and Lee, 2008; Winn, 2001). The use of fish models when investigating the effect of EDCs is advantageous, because: 1) these organisms are in constant contact and directly affected by a variety of chemicals which have potential endocrine disruptive capacities (Sebire *et al.,* 2008) 2) from an economic standpoint, several fish species are important in large commercial fishery and aquaculture industries (Mills and Chichester, 2005) 3) different fish species pose great ecological relevance and rapid feedback on the effects of contaminants can be obtained over the whole life cycle of an organism (Pritchard, 1993) and 4) using fish models has proven to be cost effective and less time consuming (Raisuddin and Lee, 2008; Winn, 2001).

Although the focus of this study was on *Merluccius capensis* and *Merluccius paradoxus* (Cape hake), preliminary studies were conducted on the fish species *Pomatomus saltatrix* (Blue fish) and *Mesopristes argenteus* (Silver Grunter) to optimize purification protocols for cytochrome P450 monooxygenase (CYP1A) and vitellogenin (vtg) (while awaiting the arrival of Cape hake samples). *P. saltatrix* is a pelagic fish species which inhabits warm, temperate and cool continental shelf waters (Silvano and Begossi, 2005). *M. argenteus* are benthopelagic perch like fish which inhabit inshore marine and brackish waters (Vari, 1978).

CYP1A was obtained from liver samples using differential centrifugation. Vtg purification involved the selective precipitation of plasma samples and anion-exchange chromatography.

3.2 Methods

3.2.1 Materials

Please refer to the chemical list in Appendix II for all materials and chemical suppliers used in this study.

3.2.2 Fish sampling, dissection and sample preparation

Frozen fish samples were obtained from St. Francis Bay area, Eastern Cape, South Africa (*P.saltatrix* n = 4, *M. argenteus* n= 1). Sample sizes were not uniform because these were the only fish samples available at the time of this study. Samples were thawed at approximately 4°C after which time, the total length (cm) and weight (g) of each fish was measured to determine Fulton's condition factors (CF). Livers (weight noted in g) were excised and blood volumes between 0.4-0.7 ml were drawn from the heart, using cold heparinized syringes (5U/ml) (Figure 3.1). Samples were stored at -20°C until further analysis.

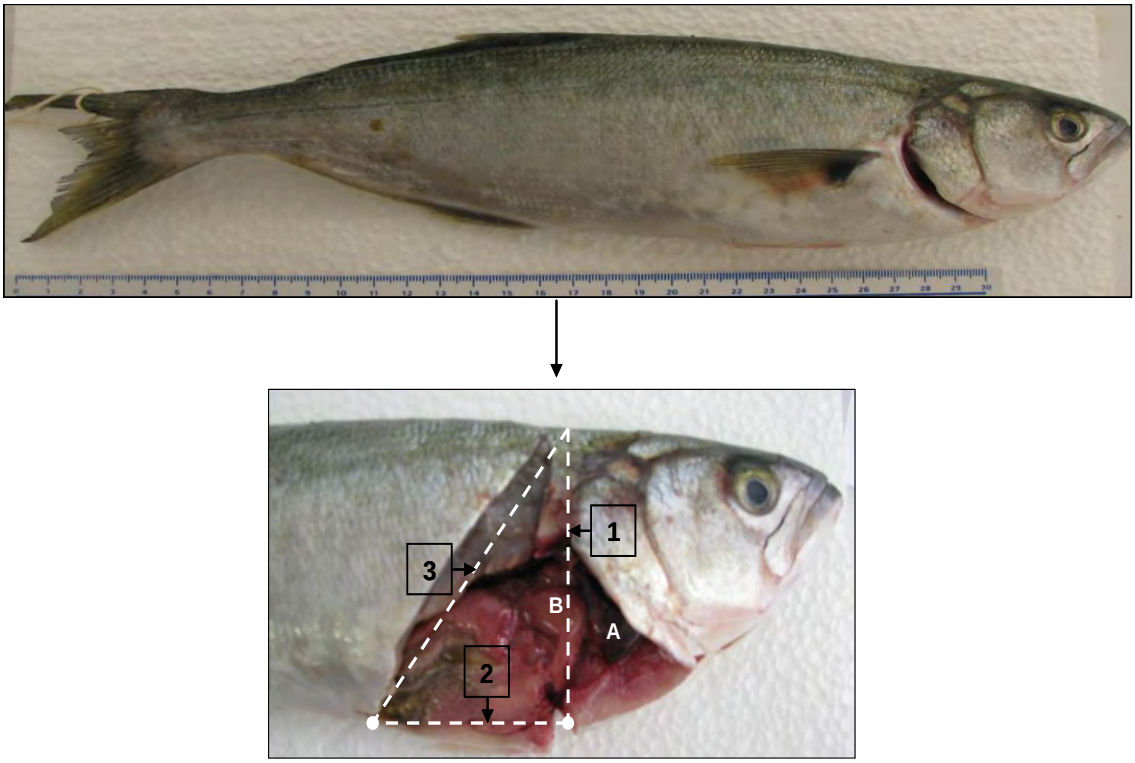


Figure 3.1: Demonstration of dissection protocol on *P. saltatrix* (2.6 cm) used to retrieve liver and blood samples. 1: incision one, 2: incision two, 3: incision three, A: heart, B: Liver. Image was retrieved using a Canon SX 100 camera, October 2008.

3.2.3 Partial purification of cytochrome P450 monooxygenase (CYP1A)

Liver preparations were carried out using a modified protocol of Nilsen *et al.* (1998). All preparations were carried out at 4°C. Liver samples (1.13-3.16 g) were homogenized in a Waring commercial blender in 1:4 (w/v) homogenization buffer (10 mM HEPES, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol) at pH 7.4. The post mitochondrial fraction (supernatant 1/S1) was obtained by centrifuging the homogenate at 12,000 × *g* for 20 minutes in a Beckman Coulter J2-21 Avanti® J-E centrifuge. Supernatant/(S1) was centrifuged again at 40,000 × *g* for 2 hours to obtain the microsomal fraction/pellet 2 (P2), which was resuspended in 1:1 (w/v) resuspension buffer (50 mM Tris, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol), pH 7.4. Fractions were retained and stored at -20°C

for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and protein determination was carried out using the Bradford assay (Bradford, 1976; Laemlli, 1970; Sole *et al.,* 2009).

3.2.4 Vitellogenin purification

Vitellogenin purification was carried out as described by Perez and Callard (1992). All preparations were carried out at 4°C. Blood samples were transferred from heparinized syringes (5U/ml) to microcentrifuge tubes and centrifuged at 5,000 rpm for 5 minutes in a Biofuge *Pico* Heraeus centrifuge. The resultant supernatant (plasma) was added to 1:4 (v/v) phosphate buffered saline (PBS) (1% PBS, 1 mM PMSF). Plasma samples were selectively precipitated with 0.02 M ethylene diamine-tetra acetic acid disodium salt (EDTA, pH 7.7) and 0.5 M magnesium sulfate($MgCl_2$, pH 7.7) and centrifuged at 2,500 × *g* for 5 minutes. The resultant pellet was resuspended in 50 mM Tris-HCl buffer containing 1 M NaCl, pH 7.5. The sample was subsequently centrifuged at 2500 × *g* for 30 minutes to remove any cellular debris present and the resultant supernatant was retained and added to 25 ml ice cold dH_2O containing 1 mM phenylmethanesulfonylflouride (PMSF). The mixture was then incubated for 24 hours at 4°C, after which time it was centrifuged at 2,500 × *g* for 15 minutes.The pellet was resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 M NaCl, and dialyzed against 50 mM Tris-HCl (pH 7.5) for 24 hours.

A 100 µl of dialyzed sample was subjected to anion exchange chromatography on a DEAE-cellulose column (Sigma-Aldrich, South Africa, D6418). The column (column dimensions: 5 ×3.4 cm) was equilibrated with 50 mM Tris-HCl, pH 7.5. The stepwise elution of vitellogenin was carried out using a NaCl gradient between 0 and 0.3 M. All samples were retained for protein determination and SDS-PAGE analysis.

3.2.5 SDS-PAGE analysis

SDS-PAGE was carried out according to the Bio-Rad, Mini-Protean® 3 cell instruction manual originally described by Laemlli (1970). This method was used to assess the purity of the sample after each purification step. Protein sample (10 µl) was added to 20 µl of SDS reducing buffer (0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 0.3% glycerol, 0.05% β-mercaptoethanol). Samples were incubated in a LABNET dry bath at 100°C for five minutes. Samples were then resolved in an acrylamide resolving gel and SDS-PAGE was run at 150 V in 1× SDS running buffer (3.0% (w/v) Tris, 14.4% (w/v) glycine, 1% (w/v) SDS, pH 8.3) for approximately 45 minutes using a Bio-Rad power pac™ basics. Protein bands were visualized using Coomassie Brilliant Blue (0.1% (w/v) Coomassie Brilliant Blue® R-250, 45% (v/v) methanol, 10% (v/v) acetic acid). When protein concentrations were lower than 0.1 mg/ml, gels were stained using Page Silver™ (Fermentas, South Africa).

3.2.6 Page silver staining

Silver staining was conducted according to Fermentas Page Silver™ Silver Staining Kit. All procedures were carried out at 23°C. Gels were incubated in gel fixing solution 1 (50% (v/v) ethanol, 10% (v/v) acetic acid) overnight to fix proteins to the gel matrix, prevent any protein diffusion and to remove any ions from the electrophoresis step that may interfere with the staining protocol. Each step was followed by a washing step, in which gels were agitated in dH_2O for 1 minute. Gels were then incubated in gel fixing solution 2 (30% (v/v) ethanol) for 60 minutes, removing the acetic acid from the first step and assisting in protein fixation to the gel matrix. Sensitizing solution (0.4% (v/v) sensitizing concentrate) was added and the gels were agitated for 1 minute. This step was used to enhance the sensitivity and contrast of the staining procedure. The sensitizing step was followed by staining the gels in staining solution (4% (v/v) staining reagent, 0.054% (v/v) formaldehyde) for 20 minutes. Gels were developed in developing solution (0.01% (v/v) sensitizing concentrate, 10% (v/v) developing reagent, 0.027% (v/v) formaldehyde)

until protein bands were sufficiently resolved (5-10 minutes) at which point development was halted by incubating gels in stop solution (8% (v/v) stop reagent) for approximately 5 minutes.

3.2.7 Bradford assay

Protein concentration in different samples was determined using a modified method of Bradford (1976). A lower (0-0.12 mg/ml) and higher (0-1.60 mg/ml) range standard curve was generated using bovine serum albumin as a reference protein (Appendix I), in order to quantify the protein concentration in samples. A 25 µl (high range)/ 10 µl (low range) sample aliquot was added to 225 µl (high range)/ 240 µl (low range) Bradford's reagent. Samples were incubated at 23°C for 5 minutes. Results were obtained at 595 nm using a Powerwave$_x$ spectrophotometer with KC junior software.

3.2.8 *Concentration of protein samples*

Samples with very low protein concentrations (below 1 µg/ml) were further concentrated to approximately 1-10 µg/ml using acetone or trichloroactetic acid precipitation to obtain better resolution on SDS-PAGE gels. These procedures were run according to Bollag *et al.* (1996). Samples were acetone precipitated by adding 1 ml ice cold acetone to 200 µl of protein sample. Samples were then vortexed using a Chiltern deluxe vortex mixer MT19 and incubated at -20°C for 10 minutes. Incubated mixtures were centrifuged at 13 000 rpm for 5 minutes in a Heraeus *pico*biofuge desk top centrifuge. The supernatants were discarded and remaining pellets were air dried until all the acetone had evaporated. Pellets were resuspended in 10 µl of the appropriate buffer (CYP1A: resuspension buffer, vtg: 50 mM Tris-HCl buffer, pH 7.5). TCA precipitation was carried out by adding 1 ml of protein sample to 0.15% deoxycholate-trichloroacetic acid (DOC-TCA), samples mixtures were vortexed and incubated at 23°C for 10 minutes. Trichloroacetic acid (100%) was then added to samples and the mixtures were vortexed again and spun at 10,000 × *g* for 5 minutes. The supernatant was removed and the pellet was resuspended in 50-100µl sodium hydroxide (0.1 M).

3.3 Results

3.3.1 Fulton's condition factor (CF)

The biological parameters of fish samples collected are indicated in Table 3.1. Fulton's condition factor is an index used to estimate the physiological condition of a fish (Table 3.2). It is based on the theory that fish with a certain weight: length ratio are in better physiological condition (Anene, 2005). This index is highly influenced by environmental factors and therefore gives useful insight into the status of the ecosystem which the fish inhabit (Ballon *et al.,* 2008). Condition factors were calculated using the following equation.

$K= W/L^3 * 100$

Where K is the condition factor, W is the weight (g) and L is the length (cm^3)[9]

Table 3.1: Biological data of fish samples, *P. saltatrix* and *M. argenteus*

Fish species	Sample number	Weight (g)	Liver weight (g)	Length (cm)	Blood volume (ml)
P. saltatrix	1	452.2	2.8	26.0	0.4
	2	456.0	3.2	25.5	0.3
	3	397.3	2.6	29.2	0.7
	4	389.1	1.1	27.0	0.7
M. argenteus	1	480.0	0.6	33.0	1.0

[9] Canada department of fisheries and oceans animal-user training template

Table 3.2: Condition factors (CF) for different fish samples

Fish Species	Sample number	CF (K)
P. saltatrix	1	2.5
	2	2.7
	3	1.6
	4	2.0
M. argenteus	1	1.3

3.3.2 Partial purification of cytochrome P450 (CYP1A) monooxygenase

After subcellular fractionation was conducted on liver homogenates, samples were analyzed using SDS-PAGE, shown in Figure 3.2, to determine whether CYP1A had been successfully isolated.

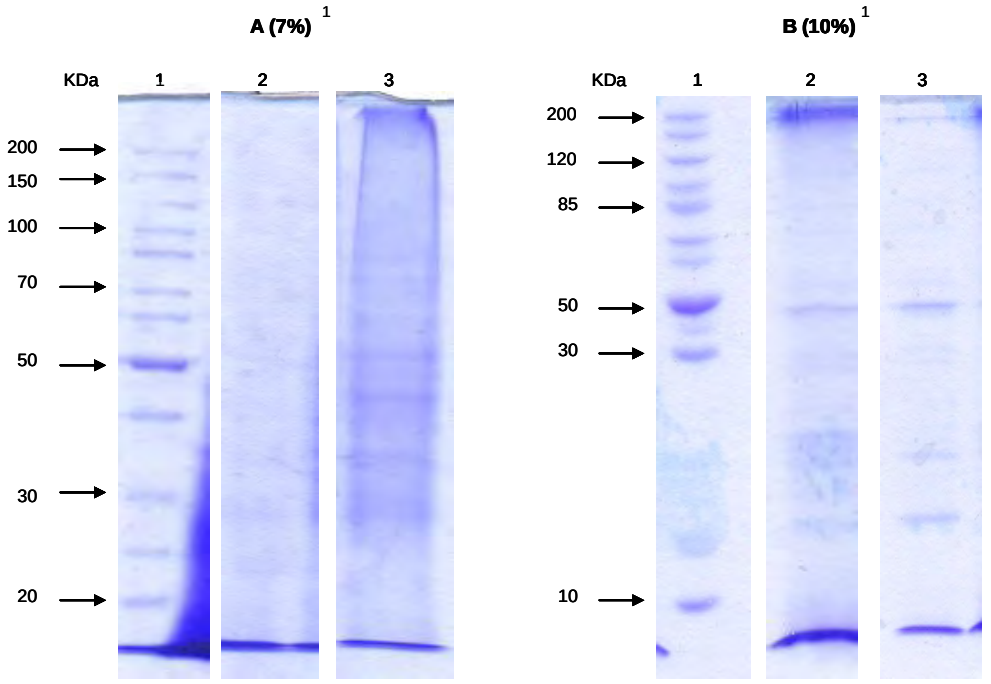


Figure 3.2: SDS-PAGE profiles of post-mitochondrial (PMS) (lane 2) and microsomal pellet fractions (lane 3) in (A) *P. saltatrix* and (B) *M. argenteus*. Peqgold molecular weight marker (Optima Scientific, South Africa) (lane 1). *Trichloroacetic acid precipitation was carried out on (A) to obtain a better resolution on the gel. [1] % Resolving gel.

SDS-PAGE analysis of PMS and MS fractions in *P. saltatrix* displayed four feint bands of approximately 50 kDa, 45 kDa, 35 kDa and 30 kDa in the MS fraction (Figure 3.2A). No apparent bands were observed in the PMS fraction. Results for *M. argenteus* demonstrated the presence of 3 bands of approximately 50, 40 and 15 kDa in both PMS and MS fractions (Figure 3.2B).

3.3.3 Vitellogenin purification

Lanes 2-8 in Figure 3.3A and B, show the SDS-PAGE profiles of different fractions collected at each purification step before anion exchange chromatography. Elution profiles for *M. argenteus* and *P. saltatrix* samples, after DEAE-cellulose chromatography, are presented in Figure 3.4.

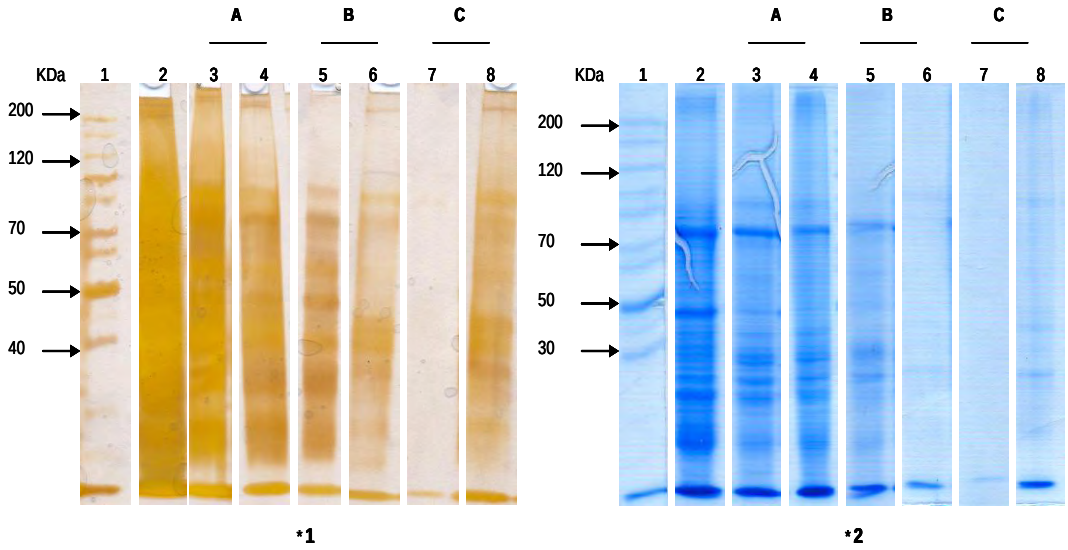


Figure 3.3: SDS-PAGE (7% resolving gel) profiles of vitellogenin purification steps (A) Centrifugation of blood samples to obtain plasma (B) Selective precipitation with $MgCl_2$/EDTA (C) O/N incubation in dH_2O containing 1mM PMSF, in *1: *P. saltatrix* (stained with PageSliver™ , Fermentas, South Africa) and *2: *M. argenteus* (stained with Comassie Brilliant Blue, Sigma-Aldrich, South Africa). Lane 1: PeqGold molecular weight marker; Lane 2: Crude; Lane 3: supernatant 1; Lane 4: pellet 1; Lane 5: supernatant 2; Lane 6: pellet 2; Lane 7: supernatant 3; Lane 8: pellet 3.

The SDS-PAGE profile obtained from the purification of vtg in *P. saltatrix* showed the presence of several protein bands in lane 4, 5, 6 and 8 with molecular weights > 200 kDa, and 85, 70, 50 kDa and below (Figure 3.3*1). Results for *M. argenteus* corresponded to those found in *P. saltatrix* with the exception of the protein band > 200 kDa (Figure 3.3*2).

The chromatographic profiles for *P. saltatrix* and *M. argenteus* showed the presence of several major peaks for *M. argenteus* and one major peak with several smaller peaks for *P. saltatrix*. Peak fractions were pooled, precipitated with acetone and subjected to 7% SDS-PAGE and stained with Page silver™ (data not shown). The protein in each pooled fraction, however, was too low and could not be visualized.

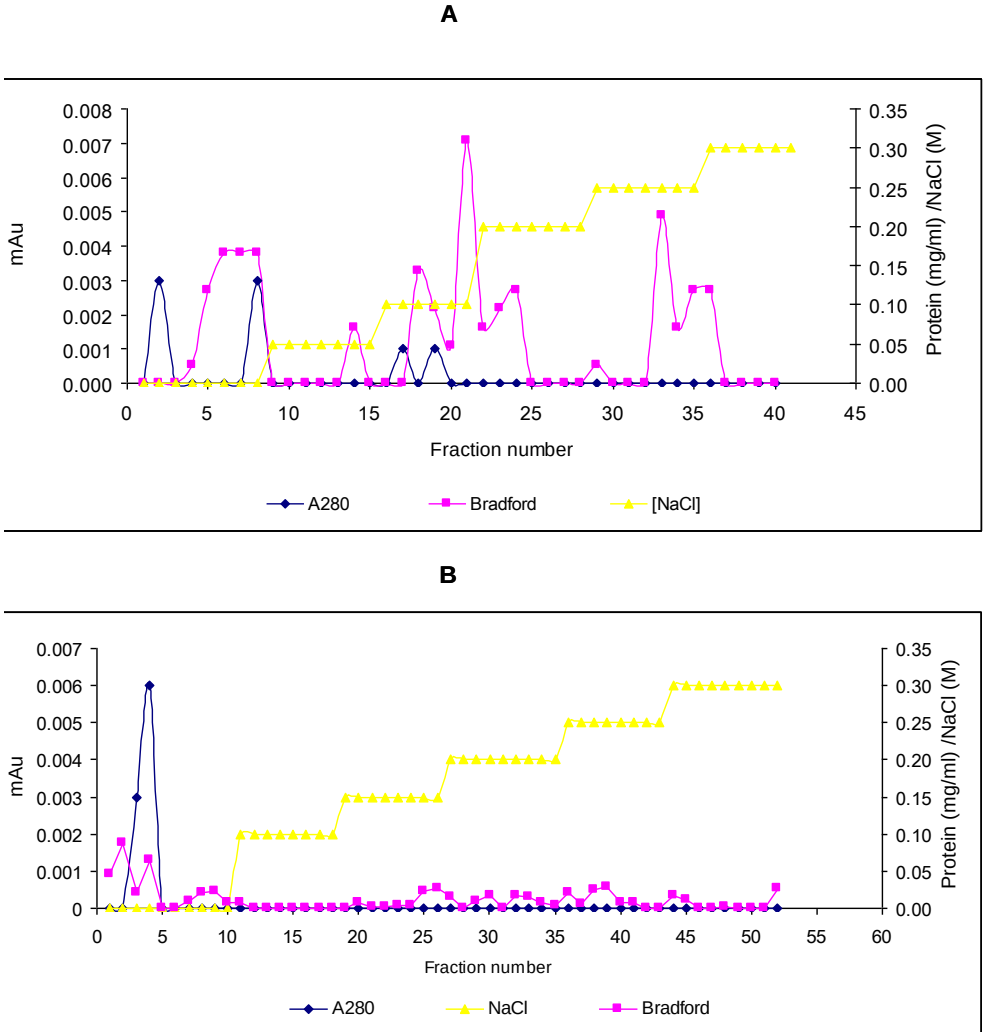


Figure 3.4: A chromatographic profile of vitellogenin purification in (A): *M. argenteus* and (B): *P. saltatrix*. The sample (100 µl) was subjected to anion exchange chromatography using a DEAE-cellulose column (5 × 3.4 cm) (Sigma-Aldrich, South Africa). Samples were eluted in Tris-HCl (50 mM, pH 7.5) with a stepwise NaCl gradient of 0-0.3 M at a flow rate of 1 ml/min.

3.4 Discussion

3.4.1 Fulton's condition factor (CF)

Fulton's condition factor (K) was employed to asses the general physiological condition of the fish species, *P. saltatrix* and *M. argenteus*. The Fulton's condition factor is an index which assumes that heavier fish (high K value) are in better physiological condition than lighter fish of the same length (low K value) (Jenkins, 2004). Studies on condition factors in *P. saltatrix* and *M. argenteus* are limited. According to condition factor studies conducted by Barnham and Baxter (1998) on the salmoid fish species, a K value between 1.4-1.6 is indicative of a fish in good physiological condition, whereas a K value of between 0.8-1.0 indicates that the fish are in poorer condition. Results presented in Table 3.2 indicate that all CF values lay within a narrow range with K values ranging from 1.4-1.6. It can, therefore, be assumed that all fish were in a good physiological condition, although this cannot be stated with complete certainty, as this is a general index which is susceptible to high degrees of variability (Robinson *et al.,* 2008).

3.4.2 Partial CYP1A purification

Studies previously carried out on fish species indicate the presence of CYP1A in the post mitochondrial supernatant (S1) and/or the microsomal fraction (P1) with observations indicating that CYP1A protein has a molecular weight ranging between 45-60 kDa (Goksoyr and Forlin, 1992). The results presented in Figure 3.2 are consistent with published literature. This purification protocol was selected for *M. capensis* and *M. paradoxus* (Cape hake) without any modifications. It is important to note that the focus of the preliminary studies was purely on the microsomal preparation of CYP1A from fish liver, although it can be speculated that CYP1A was successfully isolated. When working with Cape hake these results had to be confirmed by dot blot analysis (See Chapter 4, Section 4.3.3).

3.4.3 Vtg purification

SDS-PAGE analysis (Figure 3.3) from the initial vtg purification steps (before anion exchange chromatography) indicated the presence of two major bands at > 200 and 170 kDa, respectively, as well as several bands of lower molecular mass. The results presented here are consistent with several previous studies. Guzman *et al.* (2008) reported the size of vitellogenin in Senegalese sole (*Solea senegalensis*) to be approximately 172 kDa. Also, results from vtg purification studies undertaken on Chinese rare minnow indicated two major bands at above 200 kDa as well as 170 kDa (Song *et al.,* 2008). Although anion exchange chromatography resolved several elution peaks in both fish species (Figure 3.4), no SDS-PAGE profiles were obtained. Considering vtg is very sensitive to proteolytic cleavage (Song *et al.,* 2008) coupled with the low volume of blood samples to fish samples arriving frozen, the likelihood of vtg being lost during the anion exchange procedure was very high. This would explain why no protein bands were resolved during SDS-PAGE analysis. Reports by Arukwe *et al.* (1997) and Silversand *et al.* (1993) suggested the addition of proteolytic inhibitors such as aprotinin at the start of the purification process and the use of consistently low temperatures to prevent vtg degradation. When conducting the study on *Merluccius capensis/ M. paradoxus* (Cape hake), the DEAE-Cellulose column was substituted with a HiTrap Q HP column (strong anion exchange resin) and protein separation was carried out using Fast Protein Liquid Chromatography (FPLC), which reduced the purification time. Song *et al.* (2008) noted that vtg begins to degrade ~ 64 minutes into the purification process.

3.5 Conclusions

Results observed for the partial purification of CYP1A and purification of vtg allowed the following conclusions:

- The fish samples, *P. saltatrix* and *M. argenteus* were in good physiological condition according to Fulton's condition factor (K).

- The method employed for CYP1A isolation was successful and could be used to obtain CYP1A in Cape hake samples.

- Vtg was possibly lost due to proteolytic cleavage (no SDS-PAGE profiles were obtained) during the anion exchange step of purification. This protocol would be modified for vtg purification in Cape Hake.

Preliminary fish studies allowed insight into purification techniques employed for Cape hake. This study provided the foundation for subsequent studies presented in the following Chapters.

Chapter 4: Cytochrome P450 monooxygenase 1A

4.1 Introduction

The release of anthropogenic chemicals into aquatic environments has recently been reported to be the main cause of reduced reproductive success and increased mortality in several fish species (Ariese *et al.,* 1993; Sanchez *et al.,* 2008; Wozny *et al.,* 2008). Zabel *et al.* (1995) confirmed the link between embryo mortality and cytochrome P450 monooxygenase (CYP1A) induction as a direct effect of the congeners 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in Japanese medaka and Rainbow trout. Fish have the ability to accumulate chemicals, either by direct uptake via their gills (accumulation of the toxins occurs mainly in the gill pillar cells, the heart endothelium and general vasculature) and/or the ingestion of contaminated prey (toxins accumulate mainly in the gut mucosal epithelium) (Aubry *et al.,* 2005; Escartin and Porte, 1999; Pina *et al.,* 2007; Van Veld *et al.,* 1997). *Merluccius capensis* and *Merluccius paradoxus* were used in this study as indicator species of chemical contamination within the ecosystem they inhabit. The use of biomarkers poses an advantage to the use of analytical chemistry for the detection of pollutants in the aquatic environment, because a wide range of varying chemicals at different concentrations are introduced into this environment, making analytical detection very costly and highly difficult (Vindimian and Garric, 1989).

Cytochrome P450s are a superfamily of heme containing proteins which regulate metabolism of several endogenous and xenobiotic compounds (Hahn *et al.,* 1993). The function of this family of enzymes influences the susceptibility of these systems to the effects of toxic compounds (White *et al.,* 1997). Cytochrome P450s belonging to the subfamily 1A (CYP1A) are induced by polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). These compound groups have many different congeners with varying levels of toxicity (Aubry *et al.,* 2005; Hahn *et al.,* 1998; Zabel *et al.,* 1995).

Xenobiotic pollutants activate CYP1A genes via high affinity binding to the aryl hydrocarbon receptor (AHR) causing the activation of AHR-dependent signal transduction pathway. Once activated, the AHR complexes with AHR-nuclear translocator (ARNT) forming an active transcription factor, which then regulates the expression of CYP1A (Hahn *et al.,* 1998).

CYP1A is one of the most widely used biomarkers for the detection of chemical contamination in aquatic systems because it serves as a very sensitive biological response agent and shows a very short response time to chemical exposure (Meier *et al.,* 2001; Oost *et al.,* 2003). The induction of the CYP1A system is often measured by assessing the CYP1A-mediated 7-ethoxyresorufin-*O*-deethylase (EROD) activity, as this biomarker has proven to be cost effective, measurement of exposure in fish liver is easy and this tool has been found to be sensitive even in a complex mixture of compounds (Vindimian *et al.,* 1991).

The focus of this Chapter was the partial purification of CYP1A in the fish species *M. capensis* and *M. paradoxus* (Cape hake), the optimization of the EROD assay and western blot analysis for Cape hake. The optimization studies were followed by a comparison of CYP1A expression in Cape hake from the De Beers marine mining area and a reference site outside the mining area. *M. capensis* and *M. paradoxus* were considered as one stock.

4.2 Methods

4.2.1 Fish sampling, dissection and sample preparation

Frozen Cape hake samples were obtained off the coast of Luderitz, Namibia within the De Beers marine mining area (Figure 4.1, *M. capensis/M. paradoxus* n=11). Frozen Cape hake samples were also obtained from two reference sites, namely Marine coastal management (MCM) in Cape Town, South Africa and Balobi Trading, Mossel Bay,

Eastern Cape, South Africa (Figure 4.2) (*M. capensis/ M. paradoxus* n=11). Samples were thawed at approximately 4°C and the total length (cm) and weight (g) of each fish was measured to determine Fulton's condition factors (CF). Livers (weight noted in g) were excised and blood volumes between 0.1-0.5 ml were drawn from the heart, using cold heparinized syringes (5 U/ml). Samples were retained at -20 °C until further analysis.

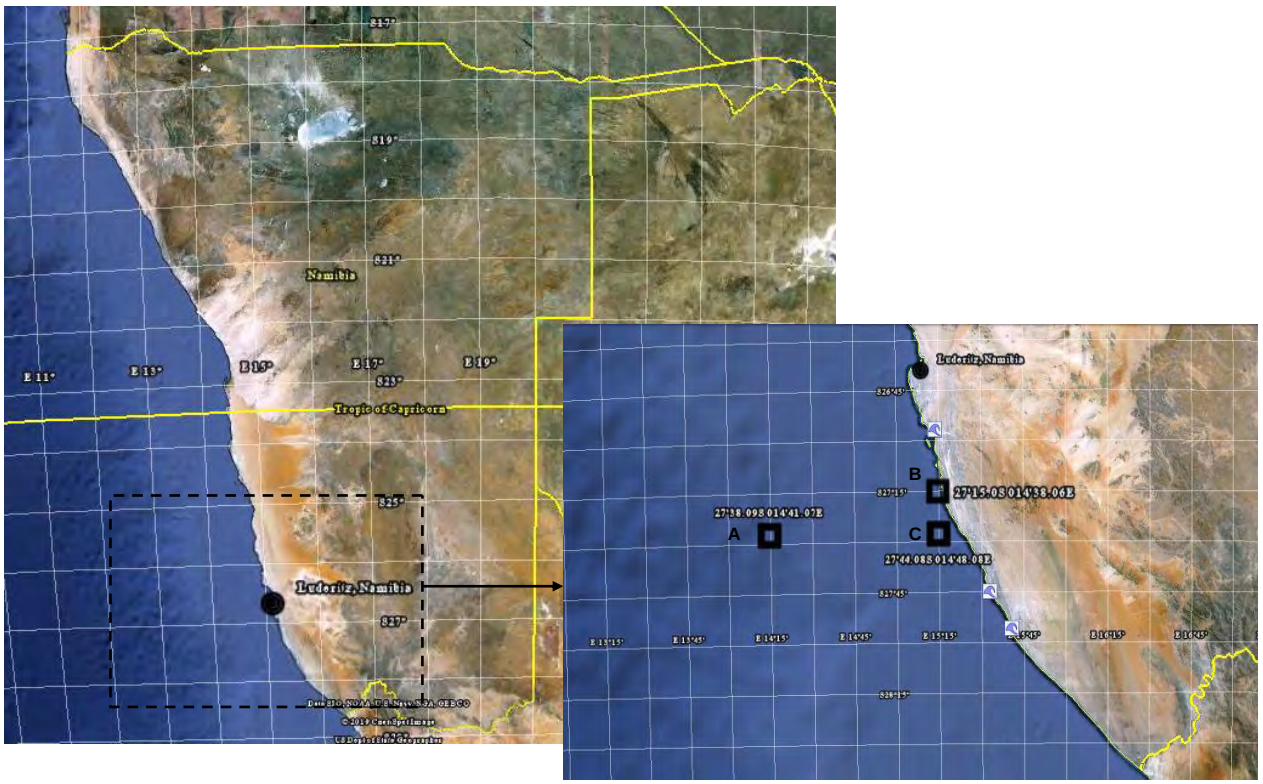


Figure 4.1: Map of *M. capensis/M. paradoxus* sampling area (A, B and C) off the coast of Luderitz, Namibia. The sampling area was within the De Beers Marine Namibia mining area (Images were obtained using Google Earth).

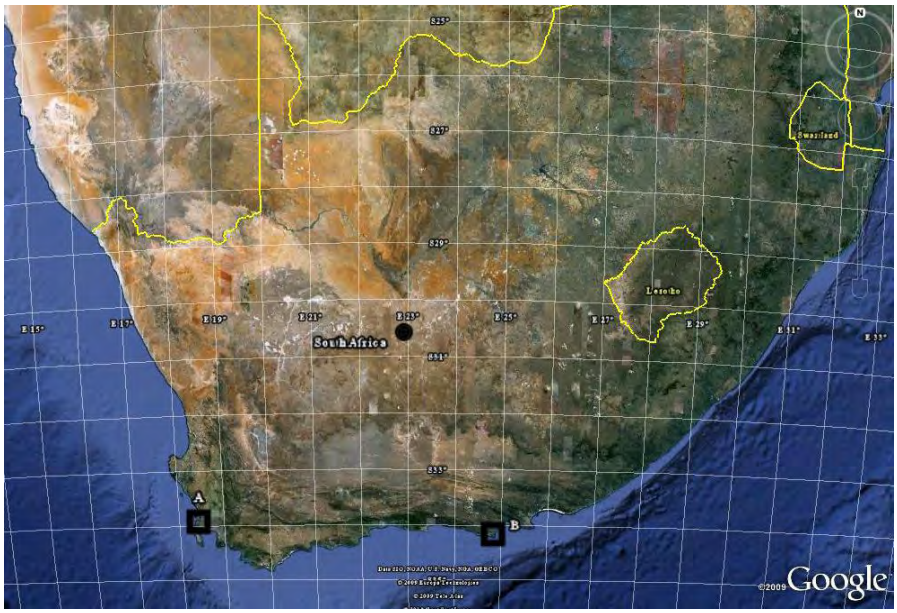


Figure 4.2: Map of *M. capensis/ M. paradoxus* reference sampling sites. A: MCM, Cape Town and Balobi Trading, Mossel Bay (Images were obtained using Google Earth).

4.2.2 Partial purification of cytochrome P450 monooxygenase (CYP1A)

The partial purification of CYP1A from Cape hake liver samples was carried out as described in Chapter 3, Section 3.2.3.

4.2.3 Discontinuous 7-ethoxyresorufin-O-deethylase activity (EROD) assay spectrophotometric and fluorescence study

EROD analysis was carried out using a modified method described by Pikkarainen *et al.* (2006), on both fractions in *M. capensis* and *M. paradoxus,* to determine which fraction contained the highest EROD activity. A comparative study using fluorimetry and spectrophotometry was investigated to determine which technique yielded the most accurate results.

Standard curves (Appendix II) were produced using resorufin, the product of the reaction as a reference compound, for both fluorimetry and spectrophotometric studies. Microsomal protein (10 µl) was added to a reaction mixture containing 7-ethoxyresorufin (0.097 mg/ml in methanol), Tris-NaCl (TN) buffer (0.05 M Tris, 0.1 M NaCl, pH 7.6) and 0.1 M nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was incubated in a Labnet drybath at 23°C for 15 minutes, after which time the reaction was stopped by the addition of 500 µl ice cold methanol (99.5%), bringing the total reaction volume to 1 ml. Samples were then centrifuged at 6,000 × *g* in a Heraeus Megafuge 1.0 R for 20 minutes at 4 °C to remove any cellular debris that may interfere with the results obtained. The resultant supernatant was analyzed spectrophotometrically at 572 nm (Klotz *et al.,* 1984; Shailaja *et al.,* 2006) in a Powerwave$_X$ spectrophotometer using KC Junior software. Results were also analyzed using a Hitachi Spectrofluorometer F2500, using excitation and emission wavelengths of 510 and 585 nm, respectively (Pikkarainen, 2006). All samples were conducted in triplicate with appropriate enzyme and substrate controls and with spectrophotometric and fluorimetric runs being performed in duplicate.

4.2.4 Dot blot analysis of microsomal preparations

Dot blot analysis was used to determine the presence of CYP1A in different liver fractions obtained from the partial purification procedure. A modified dot blot analysis was carried out as described by Desantis *et al.* (2005). Microsomal sample (2 µl) was spotted onto a nitrocellulose membrane and incubated at 23°C until sample spots had completely dried. Once dried, the membrane was blocked in 5 % bovine serum albumin (BSA), prepared in TBS-T buffer (50 mM Tris, 50 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5), for 1 hour at 23°C. Spots were then incubated with 1:5000 primary antibody (rabbit anti-fish CYP1A peptide) overnight at 4°C. After incubation the membrane was washed 3 times with TBS-T buffer and samples were incubated in 1:5000 secondary antibody, anti-mouse/rabbit-antibody-POD at 23°C for 1 hour. Another wash step (3 × TBS-T buffer and 1× TBS buffer) was employed before the chemiluminescent detection

reagent was incubated with the membrane for 1 minute at 23°C. Images were developed in a Uviprochemi gel documentation and analysis system using an optimized exposure time of 5 minutes. The negative control used was homogenization buffer (1 mM HEPES, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol).

4.2.5 EROD assay optimization

EROD assay conditions were optimized for Cape hake by assessing the following parameters: pH (5, 6, 6.5, 7, 7.5, 8, 8.5), reaction time (30 seconds- 40 minutes), temperature (23, 25, 30, 35, 40, 45 °C) and enzyme volume (1, 2, 5, 10, 15 µl). The nicotinamide adenine dinucleotide phosphate (NADPH) dependence of the EROD assay was also assessed by running parallel reactions in the absence and presence (0.1M) of the coenzyme. All experiments were conducted in triplicate with appropriate enzyme and substrate controls. Results were analyzed at 572 nm with the Powerwave$_x$ spectrophotometer using KC Junior software.

4.2.5.1 Phase II enzyme inhibition study

CYP1A was only partially purified from liver samples in Cape hake and phase II metabolic enzymes that may be present in solution are known to interfere with the EROD assay (Martel *et al.,* 1994).The addition of magnesium sulfate($MgSO_4$), isocitric acid and dicumarol has been reported to inhibit phase II metabolic enzymes (Martel *et al.,* 1994). A phase II enzyme inhibition study was conducted to determine whether any improvement in EROD activity could be observed. Varying concentrations of $MgSO_4$ (1, 2, 4, 6, 7 and 10 mM), isocratic acid (1, 2, 4, 6, 7 and 10 mM) and dicumarol (10, 20, 40, 60, 80 and 100 µM) were assessed. Reactions were conducted in triplicate with enzyme and substrate controls. Results were analyzed at 572 nm in a Powerwave$_x$ spectrophotometer using KC Junior software.

4.2.6 Western blot analysis

Western blot analysis was carried out according to instructions in the Roche, BM chemiluminescence western blotting kit (mouse/ rabbit). Primary antibody (rabbit anti-fish CYP1A peptide) and secondary antibody (anti-mouse/rabbit antibody-POD) dilutions were optimized for *M. capensis/ M. paradoxus*. Pellet 2 fractions (obtained using differential centrifugation) from liver samples were subjected to 8% SDS-PAGE, as described in Chapter 3 Section 3.2.5. After SDS-PAGE proteins were transferred onto nitrocellulose membrane in a Mini Trans-Blot cell at 100 V for 1 hour in ice cold transfer buffer (25 mM Tris, pH 8.3, containing 192 mM glycine, 20% (v/v) methanol). Following the transfer of proteins onto the nitrocellulose membrane, the membrane was stained in Ponceau S stain (0.5% (w/v) Ponceau S red, 1% (v/v) acetic acid) for 10 minutes to visualize the molecular weight marker and protein bands. The membrane was then destained with TBS and incubated in blocking solution (1% (v/v) block reagent prepared in TBS), for 1 hour at 23°C. The nitrocellulose membrane was subsequently incubated with primary antibody overnight at 4°C. After treating the membrane with the primary antibody it was washed with TBST buffer and blocking solution (0.5% blocking reagent prepared in TBS), followed by incubation with secondary antibody for 1 hour at 23°C. The membrane was washed with TBST buffer and incubated in chemiluminescence detection reagent (125 µl/cm^3) for 1 minute at 23°C. The western blot images were resolved using the Uviprochemi gel documentation and analysis system, at 5 minute exposure time.

4.2.7 Comparison of CYP1A expression in fish samples from De Beers marine mining area and the reference site (EROD assay and western blot analysis)

The average EROD activity was determined in liver fractions from 11 samples that were obtained from Luderitz, Namibia and the reference sites. The EROD assay was conducted under optimal EROD assay conditions with the protocol outlined in Section 4.1.3. Reactions were conducted in triplicate (duplicate run) with enzyme and substrate

controls. Results were analyzed with Powerwave$_X$ spectrophotometer using KC Junior software.

Comparative immunochemical analysis was conducted on 3 samples from each site using optimized primary and secondary antibody dilutions according to the protocol outlined in Section 4.1.6.

4.2.8 Statistical analysis

The potential differences in the condition factors of Cape hake samples from the different sites were tested employing "Student's" *t*-test. Statistical analysis was performed using the computer package, Microsoft Exel XP.

4.3 Results

4.3.1 Fulton's condition factor (CF)

The biological parameters of Cape hake samples used during this study are shown in Table 4.1. Table 4.2 indicates the condition factors (CF) of individual fish sampled from the different sites, the mean CF values of fish from Luderitz, Namibia and the reference sites are also summarized in Table 4.2.

Table 4.1: Biological data of Cape hake sampled from Luderitz, Namibia and reference sites

Sample number	Liver weight (g)	Body weight (g)	Length (cm)	Blood volume (ml)
R1*	1.09	410.50	38.40	-
R2	0.64	486.40	41.30	-
R3	2.30	422.40	39.20	-
R4	0.46	431.80	39.70	-
R5	0.50	453.50	41.0	-
R6	1.08	619.90	47.0	0.1
R7	1.10	531.90	43.60	-
R8	1.10	483.30	44.50	-
R9	1.17	510.50	41.70	-
R10	12.90	417.10	42.50	-
R11	5.70	675.90	44.00	0.1
H1*	1.70	211.80	31.00	-
H2	16.90	433.60	38.00	-
H3	9.40	400.70	37.00	-
H4	1.30	603.30	44.00	-
H5	5.30	85.40	22.00	-
H6	9.90	392.40	38.00	-
H7	11.40	511.90	41.00	-
H8	2.00	589.40	42.00	-
H9	16.60	167.5	30.00	-
H10	17.10	461.80	39.00	0.5
H11	14.30	977.00	47.00	0.1

*R: Cape hake samples obtained from the reference site *H: Cape hake samples obtained from the De Beers marine mining area. -: no blood sample retrieved

Table 4.2: Calculated condition factors (CF) for individual fish samples from different sites and mean CF values for each site.

Sample number	Condition factor (K)
R1	0.72
R2	0.69
R3	0.70
R4	0.69
R5	0.66
R6	0.60
R7	0.64
R8	0.55
R9	0.70
R10	0.54
R11	0.80
Average K:	**0.66 ±0.07**
H1	0.71
H2	0.79
H3	0.79
H4	0.71
H5	0.80
H6	0.72
H7	0.74
H8	0.80
H9	0.62
H10	0.78
H11	0.94
Average K:	**0.76 ±0.08**

Results of the student "*t*" test indicate that the CF values of hake collected from Namibian waters were significantly different from those calculated for the reference site ($t = 3.1$; $P < 0.05$).

4.3.2 Partial purification of CYP1A

Figure 4.3 shows the SDS-PAGE profiles of liver fractions obtained from *M. capensis/ M. paradoxus* at different stages of differential centrifugation.

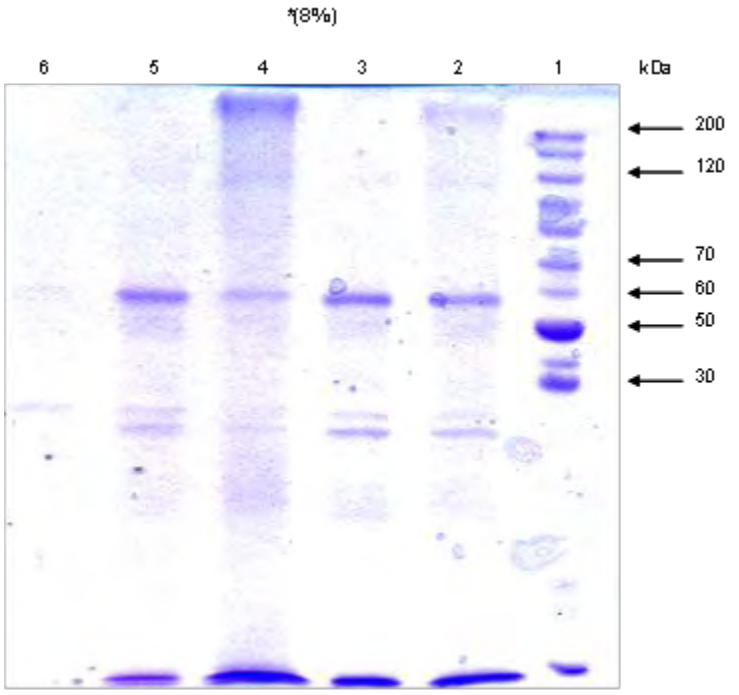


Figure 4.3: The SDS-PAGE profile of the partial purification of CYP1A in *M. capensis / M. paradoxus*. The gel was stained with coomassie brilliant blue R-250 O/N. Lane 1: Peqgold molecular weight marker; Lane 2: crude; Lane 3: supernatant 1; Lane 4: pellet 1; Lane 5: supernatant 2; Lane 6: pellet 2 **.** * % Resolving gel

SDS-PAGE analysis displayed 3 distinct bands, with a major band apparent at approximately 60 kDa and 2 bands ≤ 30 kDa in the crude fraction, supernatant 1, pellet 1 and supernatant 2. Two faint bands were observed in pellet 2- one at ~60 kDa and one band below 30 kDa. The profiles observed indicated the possible presence of CYP1A at approximately 60 kDa in all fractions.

4.3.3 Dot blot analysis

Results of anti-CYP1A dot blot analysis on different liver fractions from the partial purification of CYP1A are shown in Figure 4.4.

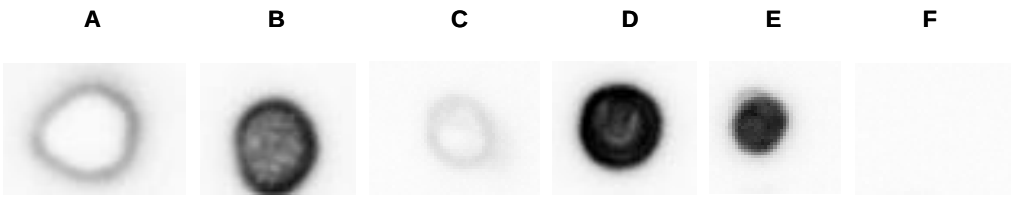


Figure 4.4: Dot blot analysis of CYP1A in different microsomal fractions in Cape hake. (A): Crude, (B): Supernatant 1; (C): Pellet 1; (D): Supernatant 2; (E): Pellet 2 (F) Negative control: Homogenization buffer. Protein samples (2 µl) from each fraction were spotted onto nitrocellulose membrane. Dot blots were developed in chemiluminescence detection reagent and resolved using a Uviprochemi gel documentation and analysis system. Pimary antibody (rabbit anti-fish CYP1A peptide): 1:5000, secondary antibody (mouse/rabbit antibody-POD): 1:5000. Note: no positive control was used as no commercial fish CYP1A for any fish species was available at the time of this study.

All fractions (A, B, C, D and E) showed positive immunoreactivity, confirming the presence of CYP1A. Fractions D (supernatant 2) and E (pellet 2) showed a higher response signal which could be attributed to a higher concentration of the CYP1A protein present in those fractions. No immunoreactivity was detected in the negative control (F).

4.3.4 EROD assay spectrophotometric and fluorescence study

The CYP1A mediated *O*-deethylation of 7-ethoxyresorufin for the production of resorufin was analyzed in all fractions obtained from the partial purification of CYP1A in Cape hake (Figure 4.5), to determine which fraction contained the highest EROD activity, using fluorimetry and spectrophotometric analysis.

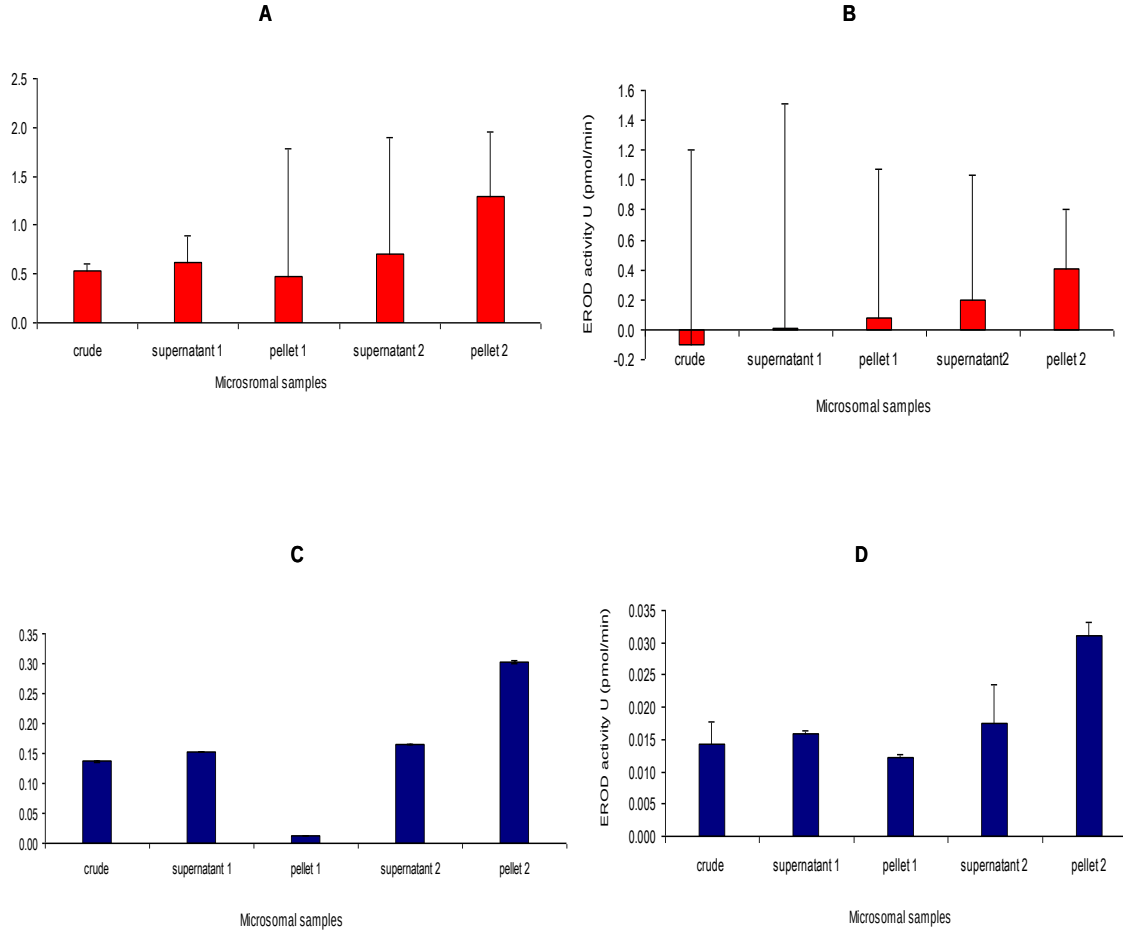


Figure 4.5: EROD activity (pmol/min) determinations for all fractions from the partial purification of CYP1A in *M. capensis/M. paradoxus* (Due to lack of samples, different purified samples were analyzed)**.** The study was conducted in duplicate runs using fluorimetry (A) and (B) (excitation wavelength: 510 nm; emission wavelength: 585 nm), as well as spectrophotometry (C) and (D) (wavelength: 572 nm). Datapoints represent mean values ±SD (n=3).

Results from both fluorimetric (Figures 4.5A and B) and spectrophotometric (Figures 4.5C and D) analysis are in agreement that the highest EROD activity was obtained in the pellet 2. Pellet 2 was thus selected for all any further analysis in this study. Fluorimetric results indicated high standard deviations in the duplicate runs in comparison to spectrophotometric analysis. Spectrophotometric analysis was, therefore, chosen as the standard EROD assay detection method for the remainder of this study.

4.3.5 EROD assay optimization

The 7-ethoxyresorufin-*O*- deethylase assay was optimized with respect to pH, temperature, reaction time and enzyme volume for Cape hake (Figure 4.6)

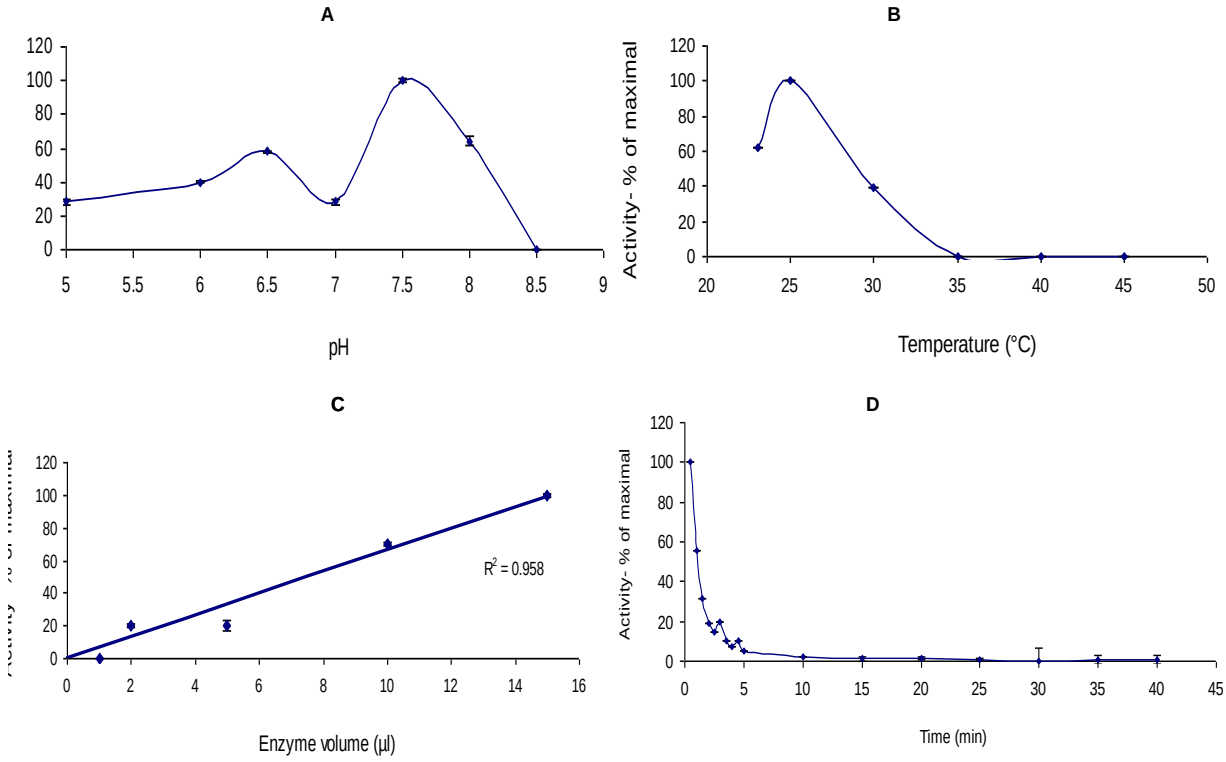


Figure 4.6: Assay optimization curves for *M. capensis/ M. paradoxus* showing influence of pH (A), temperature (B), enzyme amount (C) and assay time (D) on EROD activity in the pellet 2 fraction. Activities were determined spectrophotometrically at 572 nm and expressed as a % of maximal activity. Datapoints represent mean values ±SD (n=3)

Results in Figure 4.6 indicate the conditions required for optimal measurements of EROD activity. The EROD activity was observed to have a pH optimum of 7.5, even though

another peak was also observed at pH 6.5. The temperature optimum (Figure 4.6B) was observed at 25°C. When assessing the optimal time (Figure 4.6D) for the EROD assay,

activity decreased drastically after 1 minute (33.26 pmol/min; 55.73% activity) with the highest activity (60.00 pmol/min; 100% activity) being observed 30 seconds into the reaction. No EROD activity was observed after approximately 15 minutes. Increased enzyme amounts displayed a linear increasing relationship to EROD activity ($r = 0.962$). Based on these four studies the reaction conditions chosen for the EROD assay were as follows: pH 7.5, 25°C, sample amount 10 µl and reaction time 30 seconds.

CYP1A stability over time was evaluated and a typical result was obtained (Figure 4.7). Enzyme stability analysis indicated that CYP1A exhibits a very low degree of stability (96% decrease in EROD activity after 1 minute) with the complete absence of EROD activity after approximately 10 minutes (Figure 4.7).

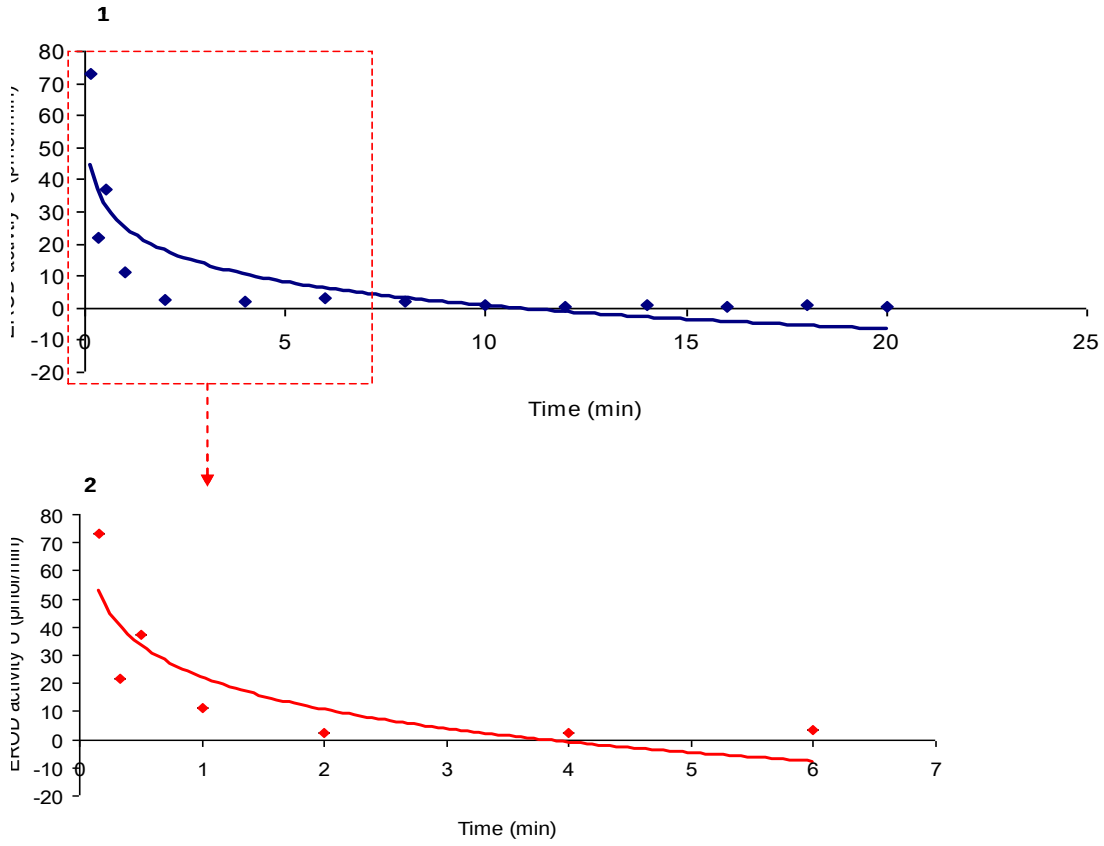


Figure 4.7: Typical regression curves showing the effect of time on CYP1A stability. EROD activity was measured over a period of 20 minutes (1) and 6 minutes (2) and results were obtained spectrophotometrically at 572 nm. Datapoints represent mean values ±SD (n=3)

The NADPH-dependence of the assay was evaluated for the EROD assay, and is presented in Figure 4.8.

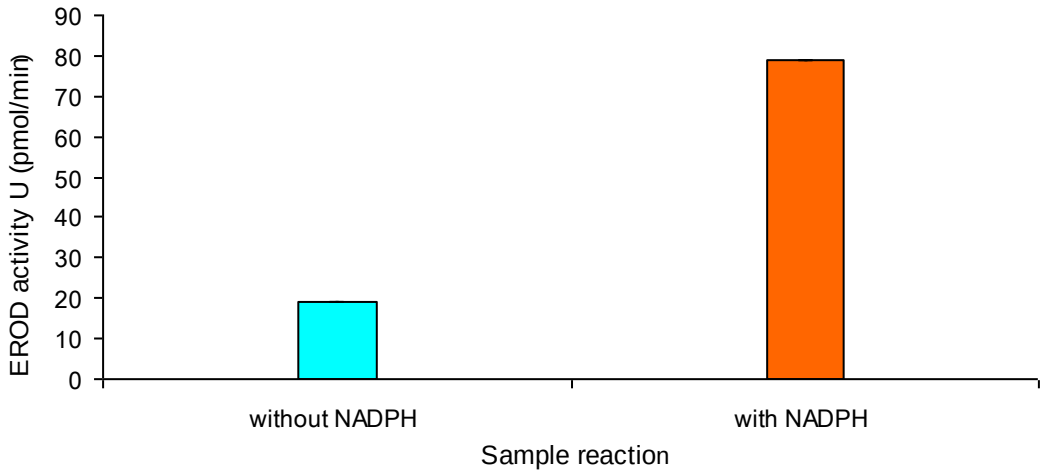


Figure 4.8: The NADPH-dependence of the EROD activity in *M. capensis /M. paradoxus* . Results were obtained spectrophotometrically at 572 nm. Datapoints represent mean values ±SD (n=3). Standard deviation bars are indicated but cannot be observed because standard deviations were ≤ 0.013.

Results show that the CYP1A mediated deethlyation of 7-ethoxyresorufin is able to take place in the absence of NADPH, therefore, indicating that the reaction is not NADPH-dependent (Figure 4.8). The addition of NADPH to the reaction, however, markedly increased EROD activity (up to 300%). NADPH (0.1 M) was added to EROD reaction mixtures for the remainder of this study.

4.3.5.1 Phase II enzyme inhibition study

The effect of magnesium sulphate, isocitric acid and dicumarol on phase II enzyme inhibition are shown in Figure 4.9.

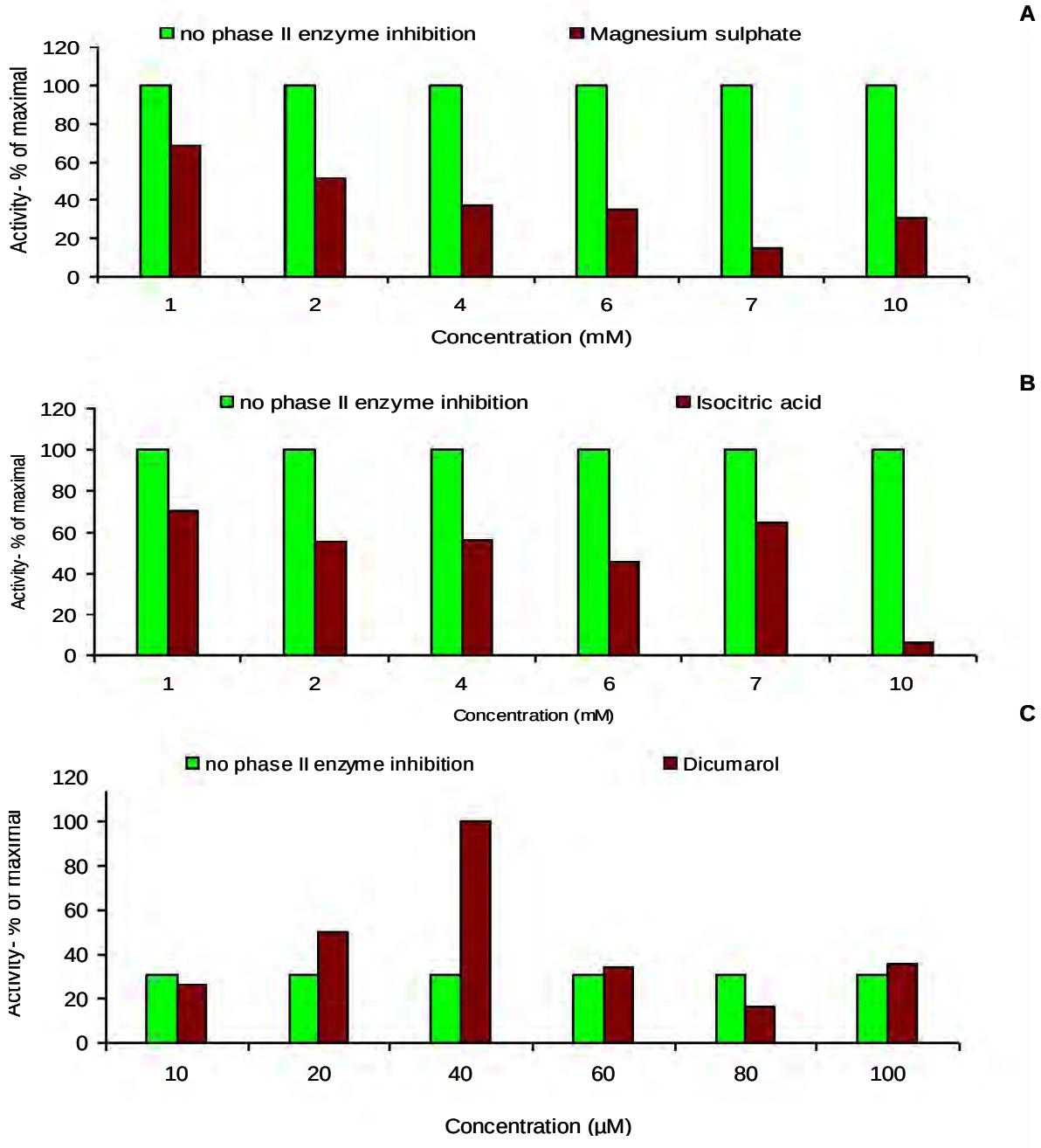


Figure 4.9: The phase II enzymes inhibition study in *M. capensis/ M. paradoxus* using magnesium sulfate(A) Isocitric acid (B) and (C) Dicumarol as inhibitors. Results were obtained spectrophotometrically at 572 nm and expressed as a % of maximal activity. Datapoints represent mean values ±SD (n=3). Standard deviation bars are indicated but cannot be observed in cases were standard deviations were ≤ 0.081.

The addition of magnesium sulfate($MgSO_4$) (Figure 4.9A) to the EROD reaction generated an overall inhibitory effect on the EROD assay. The results observed for isocitric acid correspond to those observed for $MgSO_4$ (Figure 4.9B). Results observed for dicumarol indicate an increase in EROD activity at dicumarol concentrations of 20, 40, 60, and 100 µM, with the highest activity being noted at a concentration of 40 µM (132.84 pmol/min; 100% activity). Dicumarol (40 µM) was, therefore, added to EROD reaction mixtures for the remainder of the study.

4.3.6 Western blot optimization

Results showing the optimized primary and secondary antibody dilutions for western blot analysis in Cape hake pellet 2 fractions are presented in Figure 4.10. Immunoblot analysis of pellet 2 fractions at varying primary (Figure 4.10A) and secondary (Figure 4.10B) antibody dilutions revealed faint bands at approximately 60 kDa and 30 kDa at primary antibody dilutions of 1:500 and 1:1000. Secondary antibody analysis revealed a band at approximately 60 kDa at a dilution of 1:5000. A great deal of smearing was observed which decreased the resolution of the western blot analysis. A primary antibody dilution of 1:1000 was selected for further studies because less background interference was observed in comparison to the 1:500 dilution. The optimal secondary antibody dilution selected was 1:5000.

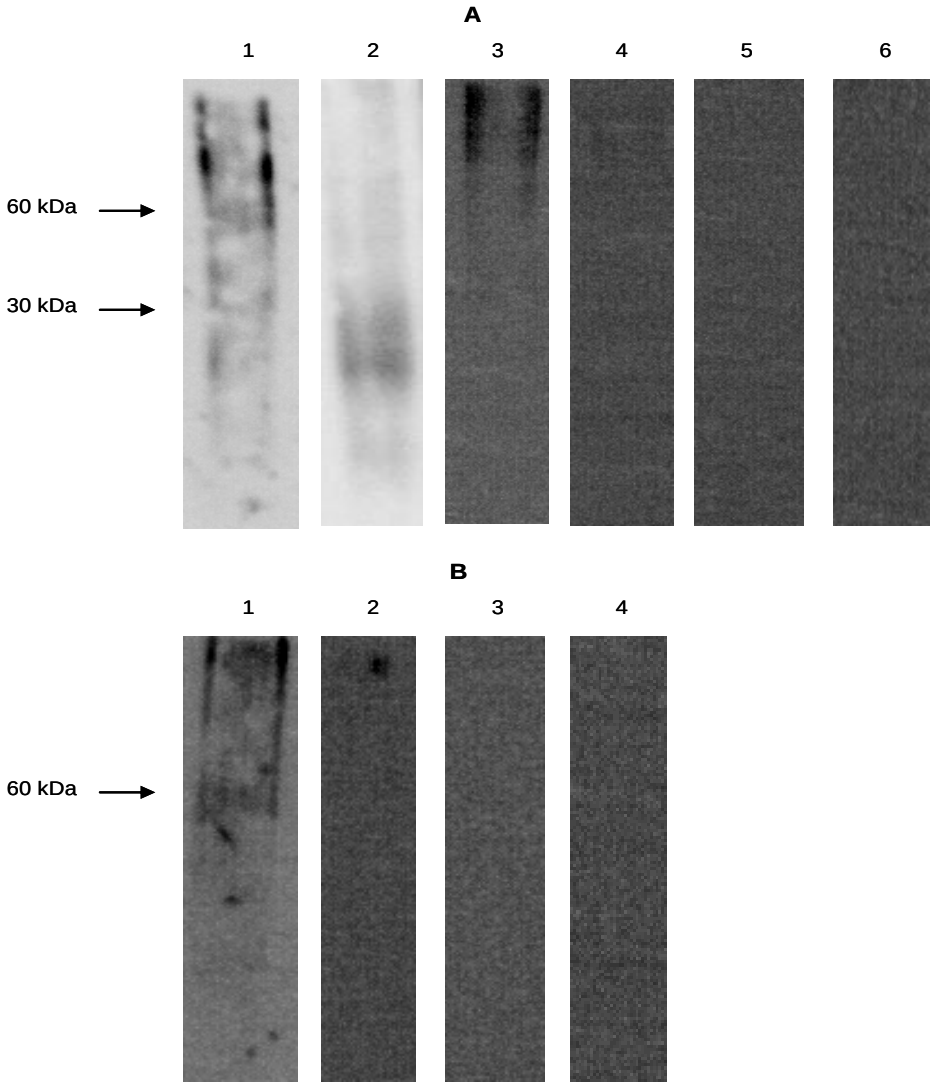


Figure 4.10: Western blots of pellet 2 liver fractions from *M. capensis/ M. paradoxus* at varying primary (A) and secondary (B) antibody concentrations. Primary antibody: rabbit anti-fish CYP1A peptide, Secondary antibody: anti-mouse IgG-POD/ anti-rabbit IgG-POD. A: primary antibody concentrations investigated were 1:500 (A1), 1: 1000 (A2), 1: 5000 (A3) , 1:10000 (A4) and 1:50 000 (A5); secondary antibody dilution (1:5000). Secondary antibody concentrations investigated were 1: 5000 (B1), 1:10000 (B2) and 1:50 000 (B3); primary antibody dilution (1:1000). (A6) and (B4): negative control: homogenization buffer. Ten µl of sample were loaded for SDS-PAGE.

4.3.7 Comparison of CYP1A expression in fish samples from De Beers marine mining area and the reference site (EROD and western blot analysis)

The mean EROD activity encountered in the liver pellet 2 fractions of the fish samples (n =11 for each region) from Luderitz, Namibia and the reference sites are presented in Figure 4.11A. Results for the western blot analysis in the pellet 2 fractions of *M. capensis/ M. paradoxus* from different sites are shown in Figure 4.11B.

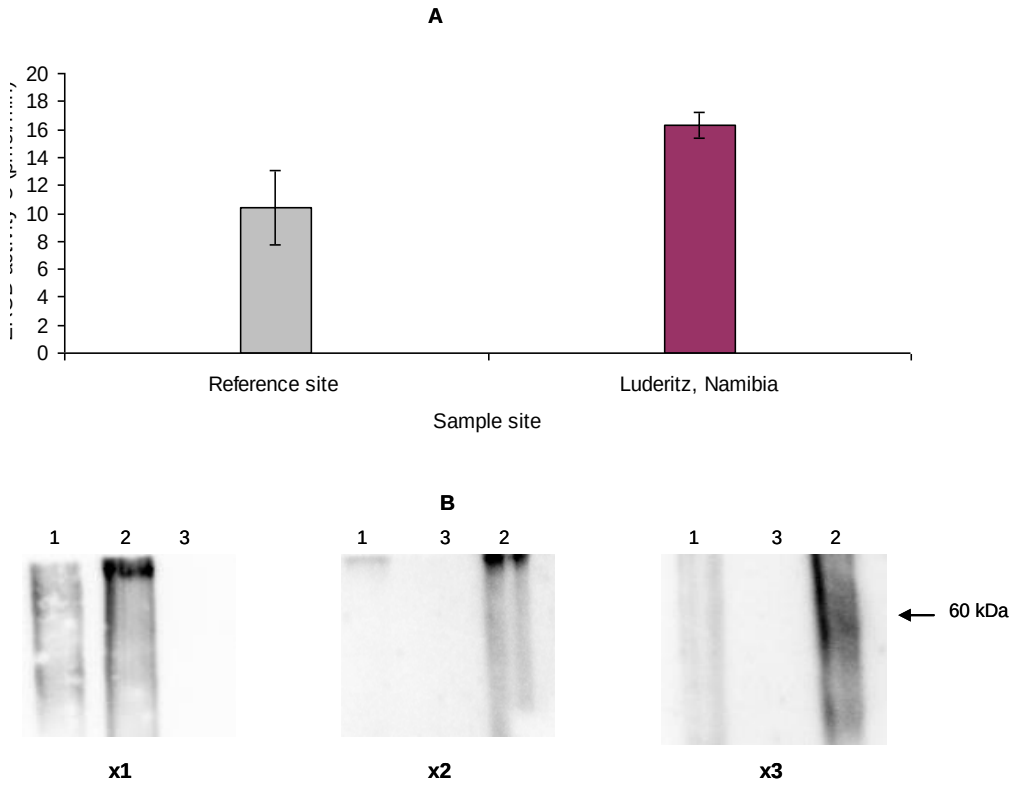


Figure 4.11: Average EROD activity (pmol/min) (A) in the pellet 2 liver fractions of *M. capensis/ M. paradoxus*. 11 samples were analyzed from each site. EROD activity was measured spectrophotometrically at 572 nm. Datapoints represent the mean values ± SD (n=6). **(B) Western blot analysis of pellet 2 fractions in *M. capensis/ M. paradoxus* from De Beers marine mining site (2) and the reference site (1). (3): negative control; homogenization buffer.** Three samples from each site were analyzed. Optimum primary (1:1000) and secondary (1:5000) antibody dilutions were used. x1: (sample number R1 and H1), x2: (sample number R7 and H7), x3: (sample number R8 and H8). Samples (10 µl) were loaded for SDS-PAGE.

The average EROD activity of the Cape hake from the two sites indicated a higher EROD activity in samples retrieved from Luderitz, Namibia (16.29 ± 0.91 pmol/min) in comparison to the mean EROD activity observed in samples from the reference sites (10.40 ± 2.65 pmol/min) (Figure 4.11A). Although the resolution obtained from western blot analysis (Figure 4.11B) was poor due to smearing, western blot analysis can be used to semi-quantitatively assess the concentration of proteins. When looking at results in Figure 4.11B one can infer that

the concentrations of CYP1A in samples from the Luderitz mining area were higher than that observed for samples obtained from the reference site.

4.4 Discussion

4.4.1 Partial purification of CYP1A, dot blot analysis and EROD assay

SDS-PAGE analysis of fractions taken from the partial purification of CYP1A (Figure 4.3) for Cape hake indicated the presence of a major protein band at approximately 60 kDa and minor band(s) below 30 kDa in all fractions (crude, supernatant 1, pellet 1, supernatant 2 and pellet 2). Although studies on CYP1A in *M. capensis/ M. paradoxus* are limited, reports by Goksoyr and Forlin (1992) have stated that fish CYP1A proteins have a molecular weight ranging between 45-60 kDa, depending on the species from which CYP1A is isolated. Andersson *et al.* (1993) indicated that the size of CYP1A isolated in the male rainbow trout pituitary gland was 58 kDa and Beyer and Goksoyr (1993) confirmed the same result in Gulf fish species. Investigations by Mihailovic *et al.* (2006) on the hake, *Merluccius merluccius,* stated that the molecular weight of CYP1A was 55 kDa. Most studies present the CYP1A protein as a monomer (Beyer and Goksoyr, 1993; Goksoyr *et al.,* 1992); therefore, it was assumed that the lower molecular proteins observed in Figure 4.3 were the resultant degradation products formed during the isolation procedure.

Dot blot analysis of the fractions collected from the partial purification procedure, indicated immunoreactivity for CYP1A in all fractions (Figure 4.4). CYP1A detection was expected in the post-mitochondrial supernatant (supernatant 1) and/or the microsomal pellet (pellet 2). High signal response was observed in supernatant 2 and pellet 2 which lead to the assumption that these two fractions contained the highest concentrations of CYP1A (Figures 4.4D and E). This was confirmed by conducting the ethoxyresorufin-*O*-deethylase (EROD) assay on each fraction. This assay was used to select the appropriate fraction for further analysis. Fluorimetric and spectrophotometric

EROD analysis results for the different fractions (Figures 4.5A, B, C and D) indicated the overall highest EROD activity to be in the pellet 2 (microsomal) fraction, therefore, this fraction was selected for all further analyses.

Both spectrophotometric and fluorimetric analysis was conducted in duplicate runs to test which method achieved more accurate results (Figure 4.5). This was determined by assessing the variation in triplicate samples and the variation of results between runs (reproducibility). Fluorimetry results (Figure 4.5A and B) demonstrated greater variation within triplicate samples and less consistency in EROD activities between runs (Figures 4.5C and D). Investigations into these two detection methods have been compared by Klotz *et al.* (1984), although observations in that study disagree with results obtained and presented here (fluorimetry faired slightly better than spectrophotometric analysis, in respect to sensitivity and accuracy). The study does, however, state that the visible assay is a reliable method and poses other advantages over fluorimetric detection; these include the visible assay being less laborious and the use of small assay volumes. Spectrophotometric analysis (572 nm) was, therefore, selected as the EROD assay detection method for the duration of this study.

4.4.2. EROD assay optimization

EROD assay conditions were optimized with respect to pH, temperature, enzyme volume and time. The pH and temperature optimum has been well documented in literature for different fish species (Figures 4.6A, B, C and D). Pikkarainen (2006) stated that most fish species have temperature optima between 20 and 25°C and pH optima around 7.8. Work conducted on a variety of fish species have reported similar findings to those presented here. Kruner and Westerhagen (1999) conducted studies on Dab from the North Sea and showed the pH optimum of this assay to be between pH 7.5 and 7.6.

Results observed for the time study (Figure 4.6D) and enzyme stability (Figure 4.7) demonstrated substantial decreases in EROD activity after 30 seconds into the assay

reaction (Figures 4.6D and 4.7). Two possibilities can account for these observations. All Cape hake samples arrived frozen, which may have lead to the loss of enzyme integrity. Considering CYP1A is a membrane bound protein and any alteration in the membrane structure due to freezing may have severely hampered the catalytic activity (Goksoyr *et al.,* 1991). It is worth noting that Monod and Vindimian (1991) reported a 50% reduction in CYP1A activity when analyzing frozen liver samples obtained from rainbow trout. *M. capensis/ M. paradoxus* inhabit water temperatures ranging from 4-12°C (Gordoa *et al.,* 1995) and although enzymatic activity was observed to be optimal for enzyme activity at 25 °C, higher temperatures may have compromised the stability of the protein.

The NADPH-dependence of the EROD assay (Figure 4.8) indicated that the absence of exogenous NADPH did not prevent the reaction from taking place. This is likely the result of biological systems within the fish liver microsomes that have the capacity to generate endogenous NADPH (Behrens *et al.,* 1997). The addition of NADPH, did however, increase EROD activity by 300%, indicating that the EROD assay is limited by the amount of NADPH that is present. NADPH (0.1 M) was, therefore, supplemented to all EROD reaction mixtures for the duration of this study. Although the EROD assay in this particular case was limited by exogenous NADPH supply, other studies using intact fish hepatocytes have demonstrated that the addition of this coenzyme to the overall EROD reaction is not necessary as the resulting increase in EROD activity was not significant (Behrens *et al.,* 1997; Hahn *et al.,* 1996). There was also evidence of *ex vivo* studies using fish liver post-mitochondrial supernatant fractions, which have suggested that additional co-enzymes (i.e. NADPH) are not required for the EROD assay (Jonsson *et al.,* 2002).

The phase II enzyme inhibition study was conducted to assess the degree of underestimation of EROD activity during this study. Considering that only partially pure CYP1A samples were analyzed, there was a large possibility that phase II enzymes such as DT-diaphorase and other cytosolic oxidoreductases were present within the mixture. These enzymes may have interfered with results obtained, as these enzymes have

the capacity to further metabolize the product of the reaction, namely, resorufin (Jonsson *et al.,* 2002). The compounds magnesium sulphate, isocitric acid and dicumarol have been reported to inhibit these enzymes (Martel *et al.,* 1994). The addition of magnesium sulfateand isocitric acid had an overall inhibitory effect on the EROD assay, and were thus not supplemented into the reaction mixtures during this study (Figures 4.9A and 4.9B). The addition 40 µM dicumarol to the EROD reaction (Figure 4.9C) showed a significant increase in EROD activity from 40 pmol/min (in the absence of dicumarol) to 133 pmol/min (in the presence of dicumarol), which represented a 232% increase in overall activity. The effects of dicumarol on the EROD assay have been well established in literature with similar findings being reported. Jonsson *et al.* (2002) showed that the addition of 10^{-5} M of dicumarol increased resorufin concentrations by 33%. Investigations by Das *et al.* (2004) were in agreement with these findings, as they confirmed the reappearance of resorufin in an incubation system (by the addition of 10 µM dicumarol) that contained depleted levels of this product. Dicumarol was, therefore, included when conducting EROD assays.

4.4.3 Western blot optimization

The anti-CYP1A immunochemical analysis of pellet 2 from Cape hake, showed the optimal primary and secondary antibody dilutions to be 1:1000 and 1:5000, respectively (Figures 4.10A and B). A great deal of smearing resulted in the poor visualization of bands, but a band at approximately 60 kDa and around 30 kDa were observed (Figures 4.10A(1) and B(1)). The smearing may have been the result of proteosomal degradation, as noted by Lee *et al.* (2008) whose studies into the degradation of cytochrome P450 2B proteins resulted in smears during western blot analysis. Degradation may have also been due to storage of samples at -20°C for a prolonged period of time, as samples were analyzed approximately 3 months after collection.

4.4.4 Comparison of CYP1A expression in Cape hake samples from Luderitz, Namibia and reference sites

Comparative observations using EROD activity and western blot analysis to assess CYP1A expression in Cape hake samples from the De Beers marine mining area and reference sites (Figure 4.11 A and B), showed a higher EROD activity (16.29 ± 0.91 pmol/min) in fish samples from the De Beers mining area in comparison to the reference site (10.42 ± 2.65 pmol/min), although western blot analysis (Figure 4.11 B) shows smearing and poor band visibility, the different signal intensities observed reflect differences in CYP1A concentration, and this data concurs with what was found with the EROD activity assays, with CYP1A expression being higher in hake samples from the De Beers marine mining area.

This study was designed to assess the possible presence of contaminant compounds in the De Beers marine mining area, but with the data obtained in Figure 4.11 it cannot be conclusively stated that a higher EROD activity is a definitive sign of a pollutant effect. Several factors must be considered when using biomarkers as indicators of pollution. The "Student's" *t-test* analysis of condition factors for the Cape hake sampled from different sites indicated $p<0.01$ which meant that samples from the different sites should be considered different in their physical characteristics. Therefore, differences in the catalytic and immunochemical data could be due to inappropriate sample design.

The cytochrome P450 system is highly influenced by both physio-chemical (e.g. photoperiod and salinity) as well as biological factors including sex, age and reproduction. These facts contribute to large variations in the activities of these enzymes (Goksoyr and Forlin, 1992). Although it may be argued that background variations in the activity of CYP1A would be less apparent than changes in CYP1A activities caused by toxic exogenous compounds, CYP1A is hardly detectable in fish unexposed to pollutants (Rodriguez-Cea *et al.,* 2004). If the mean value obtained for EROD activities (16.29 pmol/min) from fish samples obtained from the De Beers marine mining site is

considered, one cannot conclusively say whether this is result of anthropogenic or natural factors, because as stated by Pikkarainen (2006), EROD activities in fish from unpolluted areas range from 2.6 ± 0.4 to 22.6 ± 4.8 pmol/min depending on the area, and the value obtained for this study lies within that range.

Several different chemicals are known to either inhibit or activate CYP1A expression. CYP1A systems are not induced by phenolbarbitol-type contaminants, which presents another limitation to this study, as the results cannot be conclusively assessed without taking these factors into consideration (Williams *et al.,* 1998). Hake are considered important as indicator species, due to e.g. their economic importance, large bathymetric range and high enzymatic activities (Sole *et al.,* 2009). However, Cape hake are also a migratory species, and therefore using these fish as indicator species poses some limitations. When analyzing this data it is difficult to conclude that the results are indeed representative of the surrounding area being studied, i.e. Luderitz Namibia and the De Beers marine mining area.

This study has, however, showed the potential feasibility of using CYP1A as a biomarker and emphasizes the need to take several important factors into account before analyzing data. Further studies into *M. capensis/M. paradoxus* CYP1A are also required before these fish species are be considered appropriate indicator species.

4.5 Conclusions

- CYP1A isolated in liver samples from Cape hake *is* a 60 kDa protein.

- The resultant pellet 2 samples from the partial purification of CYP1A liver fractions from Cape hake displayed the highest EROD activity.

- Spectrophotometric analysis proved more accurate in comparison to fluorimetric analysis as a suitable detection assay for EROD

- EROD reaction conditions were optimized to the following parameters: pH 7.5, a temperature of 25 °C, 10 µl enzyme fraction and a reaction time of 30 seconds.

- The EROD assay was not dependent on the addition of exogenous NADPH, but was limited in the absence of NADPH.

- Dicumarol (40 µM) addition to the EROD reaction increased EROD activity by inhibiting the activity of phase II enzymes.

- Optimal primary and secondary antibody dilutions for western blot analyses were 1:1000 and 1:5000, respectively.

- CYP1A expression was higher in samples retrieved from the De Beers marine mining site in comparison to reference sites but this could not be conclusively attributed to the presence of contaminants within the mining area.

Method development studies for Cape hake CYP1A were followed by purification studies for the second biomarker, vtg. This is presented in Chapter 5

Chapter 5: Vitellogenin

5.1 Introduction

The general state or health of aquatic ecosystems has traditionally been determined using analytical chemical monitoring (Depledge *et al.,* 2009). This approach has several drawbacks including the detection range, half life of pollutant compounds, chemical speciation, cost and complexity of analysis (Goodsell *et al.,* 2009; Hagger *et al.,* 2009). These limitations led to investigations into the use of biological endpoints i.e. biomarkers of effect, which assess measurable biochemical or physiological changes in tissue or body fluids of an organism (Hagger *et al.,* 2009; Thilakaratne *et al.,* 2007). Biomarkers offer insight into the potential impacts of contaminants on the health of an organism, and provide vital information into the bioavailability and uptake of toxic compounds (Douhri and Sayah, 2009; Faria *et al.,* 2009). Biomarkers have also proven to be essential tools for the inexpensive, sensitive and rapid detection and measurement of effects of contaminants on the aquatic environment (Durou *et al.,* 2007; Humphrey *et al.,* 2007).

As mentioned previously, vitellogenin (vtg) is an egg yolk protein which is synthesized in the liver and translocated to the ovaries, where vtg serves as an energy source for developing embryos during oocyte development (Celius and Walther, 1998). Vtg also plays an important role in the formation of the extracellular eggshell (vitelline envelope) (Celius and Walther, 1998). Vtg has been commonly used as a screening tool to detect the estrogenic effects of compounds on aquatic organisms (Ebrahimi, 2005; Yamauchi *et al.,* 2008; Zaccaroni *et al.,* 2009). Estrogenic compounds e.g. 4-tert-nonylphenol and bisphenol A are responsible for the induction of this protein (Yamauchi *et al.,* 2008).

As discussed previously, the normal expression of vtg occurs in mature female fish under the control of estrogen and mediated by estrogen receptors (ERs) (Sun *et al.,* 2009). Although male or juvenile fish species do not express this protein they carry the vtg gene

and have ERs, which are inducible in the presence of estrogenic compounds (Maitra *et al.,* 2007). The measurement of vtg is, therefore, a useful biomarker when screening for potential endocrine disrupting pollutants in fish (Vijayavel and Balasubramanian, 2008; Watts *et al.,* 2003).

Vitellogenin is a species-specific phospholipo-glycoprotein and its structure varies among different fish (Nicolas, 1999). Vtg is most commonly isolated as a dimeric protein from the serum of vertebrates (Moussavi *et al.,* 2009). The purification of vtg from different fish species is fundamentally important as it leads to a better understanding of the physiological significance of this protein in reproduction which is critical before assay development (for EDC detection) is considered (Maitra *et al.,* 2007). Several vtg isolation techniques have been developed for different fish species and include ion exchange and size exclusion chromatography (Hennies *et al.,* 2003).

The aim of this study was to purify vitellogenin from Cape hake by selective precipitation and anion exchange chromatography. Purified vtg was used to develop and optimize western blot analysis.

5.2 Methods

5.2.1 Vitellogenin purification

Vitellogenin purification was conducted according to Guzman *et al.* (2008). Blood samples were centrifuged at 3,000 × *g* in a Heraeus Megafuge 1.0R for 15 minutes at 4°C to retrieve the plasma. Proteolysis was prevented by the addition of (2.5% v/v) aprotinin. Plasma samples were then centrifuged at 12,000 × *g* for 5 minutes at 4 °C and the resultant supernatant was selectively precipitated with 20 mM ethylene diamine tetraacetic acid disodium salt (EDTA, pH 7.7), 500 mM magnesium sulfate($MgCl_2$, pH 7.7) and distilled water. The mixture was then centrifuged at 5,000 × *g* for 30 minutes

and the resultant pellet was resuspended in 1 M sodium chloride (NaCl). A FPLC system was used to perform anion exchange chromatography using a pre-packed HiTrap Q HP column (5×5 ml) (Amersham Biosciences, Sweden), equilibrated with 20 mM Tris-HCl (pH 8.0). The resuspended sample (100 µl) was subjected to anion exchange chromatography and eluted with a linear NaCl gradient (0-0.5 M); followed by 1 M NaCl to remove any remaining adsorbed proteins. Fractions (0.5 ml) were collected at a flow rate of 1 ml/min and optical density (OD) measurements were conducted at 280 nm. Fractions suspected to contain purified vitellogenin were dialyzed overnight at 4°C, against a 1:1 ratio of dialysis buffer (20 mM Tris-HCl, pH 8) and glycerol. Dialyzed fractions were concentrated using polyethyleneglycol 20,000 (PEG) and analyzed by SDS-PAGE (refer to Chapter 3, Section 3.2.5 for full protocol). Protein concentration was determined using Bradford's assay (Chapter 3, Section 3.2.7)

5.2.2 Dot blot analysis

Dot blot analysis was used to determine the presence of vtg in different FPLC fractions obtained during the purification procedure. This analysis was conducted according to a modified protocol outlined by Desantis *et al.* (2005). The full details of this protocol are presented in Chapter 4, Section 4.2.4. No anti-hake antibodies were available commercially at the time of this study. A polyclonal primary antibody that represented the sequence of vtg conserved across several fish species was selected, namely; rabbit anti-salmon vitellogenin (1:5000). This polyclonal antibody detects epitopes in other gadiforms e.g. cod which increased the likelihood of epitope detection in Cape hake, which are also gadiforms. The secondary antibody (1:5000) used was anti-mouse/rabbit antibody-POD. Atlantic salmon vitellogenin served as the positive control and the negative control was 20 mM Tris-HCl (pH 8.0). Dot blot images were developed using the Uviprochem gel documentation and analysis system, using an optimized exposure time of 20 minutes.

5.2.3 Western blot optimization

Western blot analysis was carried out according to instructions in the Roche, BM chemiluminesence western blotting kit (mouse/rabbit) (catalogue no. 11520709001). (Please refer to Chapter 4, Section 4.2.6 for the detailed protocol). Primary antibody (rabbit anti-salmon vitellogenin) and secondary antibody (anti-mouse/rabbit antibody-POD) were optimized for Cape hake samples. Western blot images were developed using the Uviprochem gel documentation and analysis system, using an exposure time of 20 minutes.

5.2.4 Concentration of protein samples

Acetone and trichloroacetic acid precipitation were used for the concentration of protein samples. This protocol is outlined in Chapter 3, Section 3.2.8.

5.3 Results

5.3.1 Vitellogenin purification

After selective precipitation of *M. capensis/ M. paradoxus* plasma using $MgCl_2$ and EDTA, the resulting samples were subjected to anion exchange chromatography (Figure 5.1). Five minor peaks and two major peaks eluted were observed in the elution profile (Figure 5.1). Fractions potentially containing the purified vitellogenin were pooled (fraction A: tubes 1-6, fraction B: tubes 16-29, fraction C: tubes 30-32), dialyzed and concentrated using PEG 20,000.

SDS-PAGE analysis of pooled PEG concentrated fractions from anion exchange chromatography are presented in Figure 5.2.

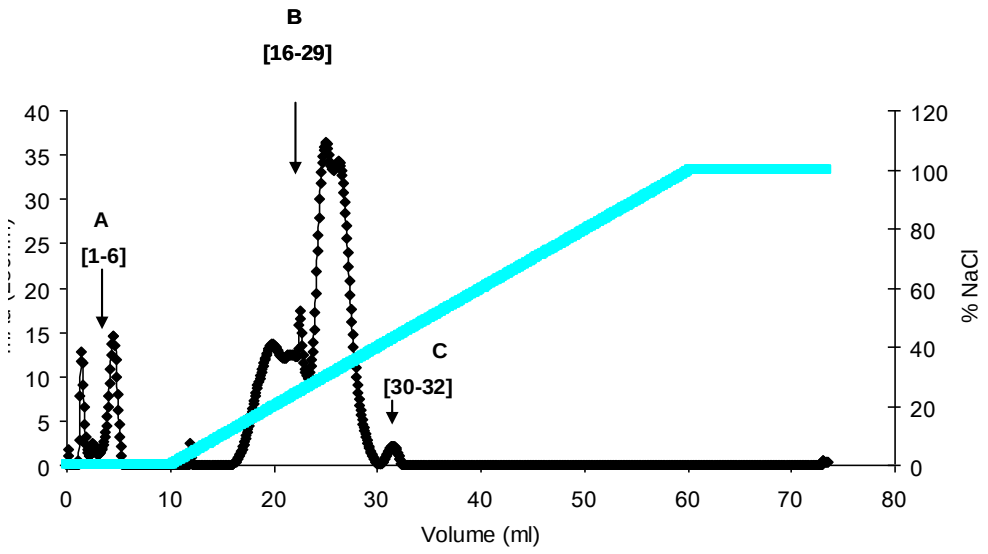


Figure 5.1: Chromatographic profile of vitellogenin purification in *M. capenis/ M. paradoxus*, from EDTA/$MgCl_2$ percipitated plasma sample. FPLC was used to perform anion exchange chromatography using a 5×5 ml Hi Trap Q HP column. Samples were eluted in Tris-HCl (20 mM, pH 8) buffer with a linear NaCl gradient of 0-0.5 M. Flow rate: 1ml/min. Peaks eluted between A: fraction 1-6, B: fraction 16-29 and C: fraction 30-32.

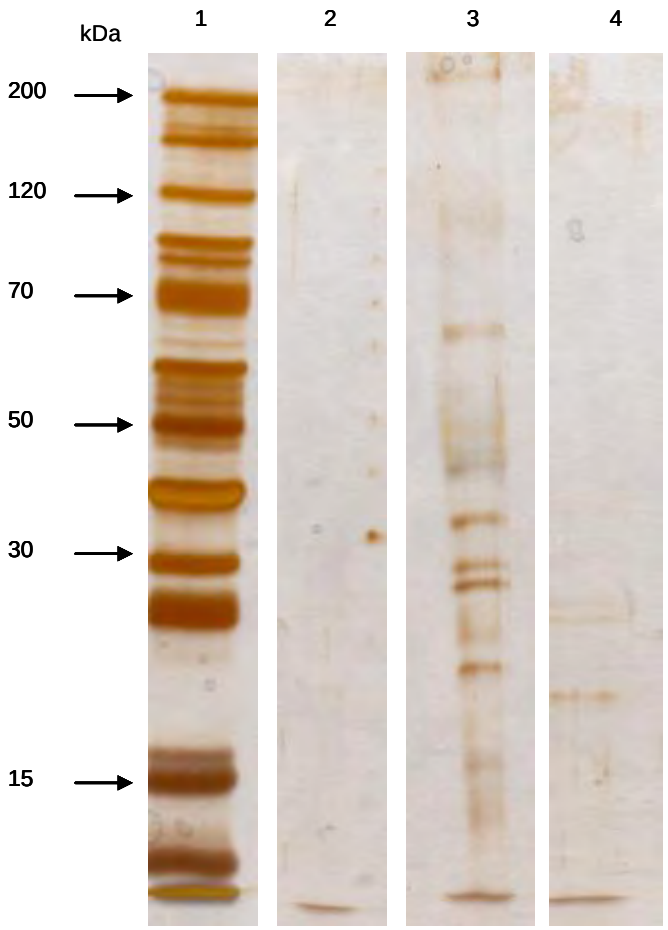


Figure 5.2: SDS-PAGE profile of purified vtg from Cape hake. Lane 1: peqGold molecular weight marker, Lane 2: pooled fraction 1-6, Lane 3: pooled fraction: 16-29, Lane 4: pooled fraction 30-32. Samples were subjected to SDS-PAGE (7% resolving gel) and stained with Page Silver™ (Fermentas).

Results showed the presence of several bands in fraction B (16-29) (lane 3) and a band above 200 kDa (Figure 5.2). Smaller molecular weight protein bands were also observed at below 70 kDa. No bands were apparent in fraction A (1-6), A_{280} analysis is susceptible to baseline background interference, therefore, the peak observed in fraction 1-6 could be attributed to this interference. Three faint bands appeared below 30 kDa in fraction C (30-32).

5.3.2 Dot blot analysis

Dot blot analysis was employed to confirm the presence of vtg in the pooled fractions from the anion exchange chromatography process, using the polyclonal rabbit anti-salmon vitellogenin as primary antibody (Figure 5.3).

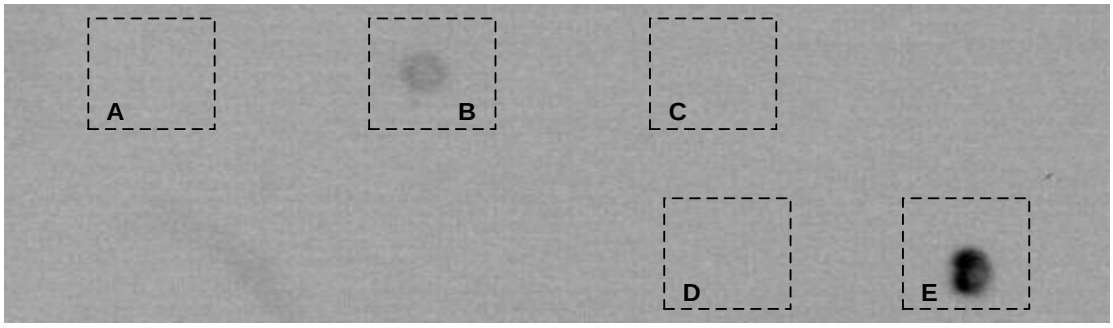


Figure 5.3: Dot blot analysis of pooled fractions from anion exchange chromatography. A: pooled fraction 1-6, B: pooled fraction 16-29, C: pooled fraction 30-32, D: negative control (assay buffer), E: positive control (6 µg of Atlantic salmon vitellogenin). Primary antibody (rabbit anti-salmon vtg): 1:5000, Secondary antibody (anti-mouse/rabbit antibody-POD); 1:5000. Dot blots were developed in chemiluminescence detection reagent and resolved using a Uviprochemi gel documentation and analysis system.

Dot blot analysis (Figure 5.3) indicated that the polyclonal antibody selected had the capacity to detect *M. capensis/ M. paradoxus* vtg epitopes and confirmed the presence of vtg in fraction B (positive immunoreactivity). No immunoreactivity was observed in fractions A and C. When comparing signal intensities between the positive control (E) which contained 6 µg of Atlantic salmon vtg and the Cape hake vtg it can be assumed that the lower signal intensity observed in the vtg purified from Cape hake was due to lower specificity of the antibody for hake vtg, because the Bradford's protein results indicated that fraction B contained 0.015 mg/ml (15 µg/ml) protein (data not shown).

5.3.3 Western blot optimization

Figure 5.4 shows the results obtained for primary antibody optimization for Cape hake vtg. No immunoreactivity was detected in PEG 20,000 concentrated vtg purified from Cape hake when primary antibody dilutions for this protein was optimized (Figure 5.4 A). Although samples were further concentrated using acetone (Figure 5.4B, Q2) and TCA (Figure 5.4B, Q3) precipitation, this did not produce any immunoreactive bands and only the positive control was detected throughout this experiment (Figure 5.4 A, P1 and B, Q1). No secondary antibody optimization was conducted as no positive results were obtained for the target antigen (Figure 5.4A, P3 and B, Q2 and Q3).

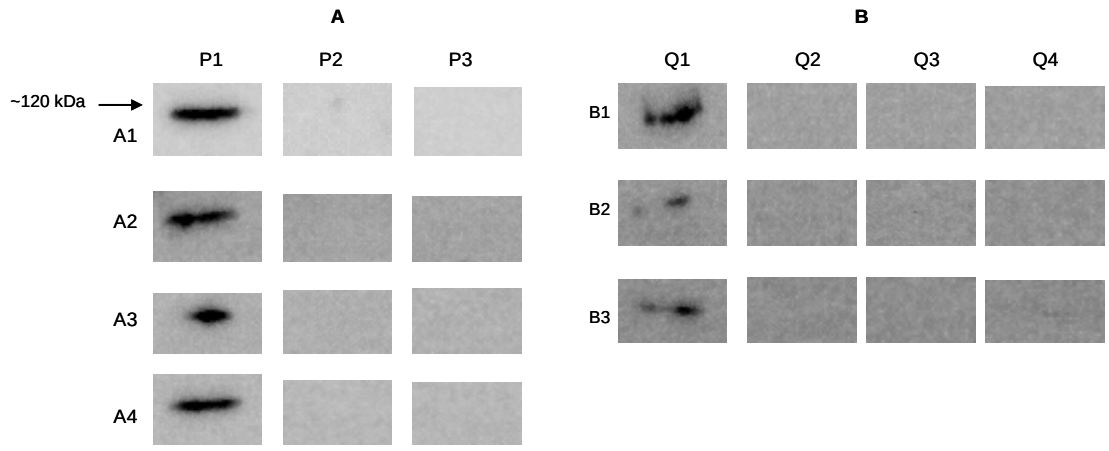


Figure 5.4: Western blot Primary antibody optimization on purified *M. capensis/M. paradoxus* vitellogenin. Purified PEG 20,000 concentrated vtg (A) was analyzed at varying primary antibody dilutions: A1: 1:1000, A2: 1:2000, A3: 1:3000, A4: 1:5000. P1: positive control (Atlantic salmon vtg), P2: negative control (assay buffer) and P3: PEG concentrated *M. capensis/ M. paradoxus* vtg. Acetone and TCA precipitated vtg (B) was also analyzed at varying primary antibody dilutions: B1: 1:500, B2: 1:1000, B3: 1:2000. Q1: positive control (Atlantic salmon vtg), Q2: acetone precipitated Cape hake vtg, Q3: TCA precipitated Cape hake vtg and Q4: negative control (assay buffer). Primary antibody: rabbit anti-salmon vitellogenin, secondary antibody: anti-mouse/rabbit antibody-POD.

5.4 Discussion

5.4.1 Vitellogenin purification

M. capensis/M. paradoxus vitellogenin was successfully purified using the method described by Guzman *et al.* (2008). The chromatographic profile (Figure 5.1) obtained here was similar to chromatographic profiles observed in studies on other fish species (Copeland and Thomas, 1988; Guzman *et al.,* 2008; Song *et al.,* 2008). Although the elution profile indicated the presence of several peaks (Figure 5.1), vtg was eluted in peak fraction B (16-29) which was subsequently confirmed by dot blot analysis (Figure 5.3). Currently no antibody is being specifically produced against Cape hake vitellogenin, but the polyclonal antibody selected for this study (rabbit anti-salmon vitellogenin) showed successful cross-reactivity with Cape hake vitellogenin (Figure 5.3).

SDS-PAGE analyses indicated the presence of several protein bands in fraction B (Figure 5.2). Vitellogenin appeared as a major band at above 200 kDa. Maitra *et al.* (2007) state that teleost vitellogenin is a high molecular protein that circulates as dimer, with molecular weights ranging between 300 kDa and 600 kDa. Wiley and Wallace (2008) have also reported that vitellogenin has a molecular weight at approximately 200 kDa. Manhor *et al.* (2005) isolated vitellogenin with a molecular weight of 200 kDa from breathing catfish and vitellogenin in greenback flounder which appeared as three major bands at 155, 104 and 79 kDa (Watts *et al.,* 2003). SDS-PAGE analysis of fraction B also revealed the presence of lower molecular weight bands below 70 kDa. Vitellogenin belongs to a large lipid transfer protein superfamily (LLTP) and amino acid sequences in this family are divided into several domains which are located in a linear fashion (Lubzens *et al.,* 2010). These domains include a heavy chain of lipovitellin, a phosvitin domain, a light chain of lipovitellin and a β-component. These domains correspond to different yolk proteins namely, lipovitellins, phosphovitins and phosvettes (Lubzens *et al.,* 2010). Vitellogenin serves as a precursor for these yolk proteins (Wallace and Begovac, 1985) and therefore the lower molecular weight bands observed in Figure 5.2

may be a result of the proteolytic cleavage of vitellogenin by lysosomal enzymes such as Cathepsin D (Lubzens *et al.,* 2010; Werawatgoompa *et al.,* 1997). This finding is not unexpected as studies conducted on *Xenopus* species (teleost phosophovitins have similar molecular weights to those found in amphibians) indicated the presence of lipovitellins with molecular weights ranging from 30-34 kDa, phosphovitins with molecular weights between 33-34 kDa as well as smaller phospholipids with lower molecular weights of 13-19 kDa (Wallace and Begovac, 1985; Wiley and Wallace, 2008). Vlaming *et al.* (1980) also reported the presence of a lower molecular weight protein during vtg purification in goldfish and found this to be lipovitellin and phosphovitellin.

5.4.2 Western blot analysis

Western blot optimization results observed indicated no immunoreactive bands for purified Cape hake vitellogenin (Figure 5.4). The primary and secondary antibody dilution optimization for Cape hake, using the polyclonal antibody, rabbit anti-salmon vitellogenin was, therefore, unsuccessful. This cannot be attributed to the technique employed because the positive control (Atlantic salmon vitellogenin) appeared as an immunoreactive band at approximately 120 kDa throughout this study. It is known that protein loss occurs at each step during a purification procedure. Considering that only 100 µl of selectively precipitated sample was subjected to anion exchange chromatography (Figure 5.1) protein loss (due to low protein concentrations) during western blot analysis is likely. The susceptibility of vitellogenin degradation is dependent on the ambient water temperatures that fish species occur in (Guzman *et al.,* 2008). Cape hake are cold water species, which means vitellogenin in this species is more unstable and susceptible to degradation during the purification procedure than warm water species e.g. Senegalese sole vitellogenin which was stable with no signs of degradation, when purification procedures were conducted at room temperature (~20°C) (Guzman *et al.,* 2008).

Although western blot analysis was unsuccessful, vitellogenin was successfully isolated from Cape hake and the purification of vitellogenin from Cape hake served as the first

step in further characterizing this protein and developing a biomarker assay for detecting the effect of estrogenic compounds.

5.5 Conclusions

- Vitellogenin was successfully isolated from *M. capensis/ M. paradoxus* as a protein with the molecular weight of 200 kDa, with the presence of proteolytically cleaved proteins, assumed to be lipovitellins and phosphovitellins.

- The polyclonal antibody rabbit anti-salmon vitellogenin can be utilized to immunochemically detect Cape hake vitellogenin.

- Western blot optimization of Cape hake was unsuccessful, possibly due to loss of protein because of low initial blood sample volumes obtained, low protein concentration in the final purified sample, combined with the susceptibility of this protein to proteolytic degradation.

Chapter 6: General discussion and future recommendations

6.1 General Discussion and Conclusions

The first objective of this study was the partial purification of cytochrome P450 monooxygenase 1A (CYP1A) from the liver of *Merluccius capensis/M. paradoxus* (Cape hake) (the two fish species were not differentiated and were considered as one stock). A differential centrifugation protocol was initially tested on two different fish species (*Pomatomus salatatrix* and *Mesopristes argenteus*) and the results obtained indicated a high probability that this technique would also be successful in Cape hake. The partial purification of CYP1A from liver fractions obtained from Cape hake was, therefore, performed using differential centrifugation.

The CYP1A mediated ethoxyresorufin-*O*-deethylase (EROD) assay and western blot analysis are common techniques employed when using CYP1A as a biomarker for the detection of chemical contamination (Abrahamson *et al.,* 2007). The next objective of this study was to develop the optimum conditions for these two assays. The following parameters were optimized for the EROD assay: pH, temperature, reaction time and amount of enzyme. A spectrophotometric and fluorimetric study was also conducted to determine which detection system was best suited to the EROD assay. The nicotinamide adenine dinucleotide phosphate (NADPH) dependence of the EROD assay and the effect of phase II enzyme inhibitory compounds (dicumarol, isocitric acid and magnesium sulphate) on EROD activity were also investigated. Western blot analysis was optimized by determining optimal primary and secondary antibody dilutions.

The next objective of this study was to assess the possible presence of contaminants in the marine waters of the De Beers mining area. This was accomplished by comparing the EROD activities of fish sampled from the marine mining area to fish sampled from a

reference site. Western blot analysis was also used to assess contaminant exposure. All assays were conducted under the optimized conditions.

Another aim of this study was to purify a second biomarker, vitellogenin (vtg), from Cape hake blood and optimize western blot analysis of this protein in terms of optimal primary and secondary antibody dilutions. Preliminary studies on the fish species *P. saltatrix* and *M. argenteus* resulted in the degradation of vtg during the purification procedure. This allowed insight into possible modifications required in the purification protocol when purifying vtg from Cape hake. The purification of Cape hake vitellogenin was accomplished. Western blot optimization for Cape hake vtg was unsuccessful, however.

6.1.1 Partial purification of CYP1A from Cape hake liver samples

The isolation of CYP1A from *P. saltatrix* and *M. argenteus* using differential centrifugation (preliminary studies) resulted in protein bands of ~50 kDa being resolved in the post-mitrochondrial and microsomal fraction. This method was used without modification to isolate CYP1A from Cape hake liver. The partial purification of CYP1A in *M. capensis/ M. paradoxus* resulted in the isolation of a CYP1A protein of approximately 60 kDa with lower molecular weight degradation products being observed. SDS-PAGE profiles obtained were similar in all fractions obtained from the purification procedure and the presence of CYP1A in all fractions was confirmed with dot blot analysis, these results were attributed to the poor resolution of the differential centrifugation technique, therefore, the EROD assay was used to select for the fraction with the highest EROD activity. The highest EROD activity was obtained in the microsomal fraction (pellet 2), which was the fraction chosen for all further analysis.

6.1.2 EROD assay and western blot optimization

Spectrophotometric analysis (at 572 nm) was selected as the preferred detection method for the EROD assay when compared to fluorimetric analysis, because it yielded lower standard deviations and higher reproducibility. EROD assays were conducted under the following conditions; pH 7.5, temperature of 25°C, and 10 µl of enzyme. The optimal reaction time was observed at 30 seconds since enzyme stability rapidly declined after 30 seconds. The integrity of CYP1A may have been compromised due to the fish samples arriving frozen which would alter membrane structure. Cape hake inhabit cold waters (temperatures of between 4 and 12°C) which would affect the stability of this protein when conducting EROD assays at 25 °C, the highest EROD activity was, however, observed at this temperature. The EROD assay is not dependent on exogenous NADPH as fish microsomes generate endogenous NADPH. This reaction was, however, limited by NADPH supply. The addition of exogenous NADPH resulted in a 300% increase in EROD activity. NADPH (0.1 M) was added to all EROD reactions for the remainder of the study. The addition of isocitric acid and magnesium sulfateto the EROD reaction resulted in the overall inhibition of EROD activity. The addition of dicumarol (40 µM) increased overall EROD activity by 232% because it inhibited phase II enzymes e.g. DT-diaphrose present in the partially pure CYP1A sample, which have the capacity to metabolize the product of the reaction, resorufin and therefore interfere with the EROD assay. Dicumarol (40 µM) was, therefore, added to all subsequent EROD reactions.

The optimal primary (rabbit anti-fish CYP1A peptide) and secondary (anti-mouse/rabbit antibody-POD) dilutions were 1:1000 and 1:500, respectively, for western blot analysis. Smearing in western blot was observed due to proteosomal degradation which resulted in poor band visibility, but signal intensities could still be used to semi-quantitavely assess the concentration of CYP1A.

6.1.3 Comparison of CYP1A expression in fish samples from De Beers marine mining area and the reference site

EROD activities observed for fish samples in the De Beers marine mining area showed higher mean activity (16.29 ± 0.91 pmol/min) to that in fish from the reference site (10.42 ± 2.65 pmol/min). This result was in agreement with data obtained with western blot analysis which indicated higher CYP1A expression in samples from the marine mining area.

As a consequence, data obtained from this study cannot conclusively state that the increased CYP1A expression observed in the mining area was due to the presence of pollutant compounds. CYP1A expression is influenced by several physical environmental and biological factors which result in large variations in the catalytic activity of this protein. Results of the t-test indicate that the CF values for the fish from the mining area and reference site were significantly different from one another ($P<0.05$), therefore, results could possibly be attributed to unsuitable sampling. Furthermore different compounds are known to inhibit or activate CYP1A expression and finally, Cape hake are migratory species, thus results observed cannot be linked specifically to the test area.

6.1.4 Vitellogenin purification from Cape hake blood and western blot optimization

Modifications to the purification protocol used to purify vtg from *P. salatrix* and *M. argenteus* allowed the successful purification of vtg from Cape hake blood samples. After anion exchange chromatography, the vtg eluate was found to contain a high molecular weight protein with a molecular weight above 200 kDa. Vitellogenin cleavage products of lower molecular weight were also present in the SDS-PAGE profile and these were assumed to be the yolk proteins lipovitellin and phosphovitin.

Results obtained from western blot optimization (no Cape hake immunoreactive bands appeared) could not be attributed to the experimental technique employed as the positive

control (Atlantic salmon vitellogenin) was detected in each instance during the study. Considering the limited amount of blood volume retrieved from frozen fish samples and the susceptibility of Cape hake vtg to degradation, because Cape hake inhabit cold waters (4-12°C) and therefore the vtg in this fish species would be less stable at temperatures higher than those they inhabit, vtg loss due to degradation is more likely explanation for the results obtained.

6.1.5 Summary

In summary, the hypothesis that sewage effluents discharged into the aquatic environment from De Beers Marine (PTY) Namibia mining vessels could contain compounds that affect the induction of CYP1A in Cape hake was not supported by the results observed. The data obtained could not yield conclusive results, as several additional factors have to be taken into consideration. This study did, however, demonstrate the potential of using CYP1A as a biomarker for studies on the health condition of aquatic systems. Furthermore, assay conditions for Cape hake were optimized which would serve as an important tool for future studies using these fish species. This study has also highlighted the need for several important factors to be taken into account before analyzing data and the importance of further studies into Cape hake CYP1A before these fish species can be considered suitable indicator species.

The second hypothesis suggesting that vitellogenin could be successfully purified from Cape hake was validated. This investigation represents an important step in characterizing Cape hake vtg and developing assays for the detection of estrogenic pollutants.

Overall further development of the biomarker approach to pollution detection in aquatic environments and the standardization of biomarker assays will allow further development to ecotoxicological modeling, which will be invaluable when evaluating the state of health of a marine environment.

6.2 Future recommendations

This study successfully isolated CYP1A from liver samples obtained from Cape hake, and the chemical contamination detection tools, namely the EROD assay and western blot analysis, were optimized for this particular fish species. The comparative data obtained to determine whether any contaminant compounds were present in the marine mining area were inconclusive which suggests that further studies need to be conducted. The use of fresh, unfrozen fish samples is advised; as it has been reported by Monod and Vindimian (1991) that the conservation and storage procedures utilized drastically effect protein integrity.

Sample design can be optimized by increasing the fish sample size, but this is not as important as retaining sample homogeneity (individual variability must be minimized in terms of e.g. size, age and reproductive status) (Beliaeff and Burgeot, 1997; Goksoyr and Forlin, 1992). Variations in results can also be limited by selecting reference sites and potentially contaminated sites which have similar hydrology and geochemistry (Handy *et al.,* 2003).

The environmental and biological interactions (e.g. temperature, salinity, size and age) that cause fluctuations and variations in biomarkers should also be assessed before attributing any variations in results to the presence of a pollutant. This is accomplished by determining the seasonal, spatial and temporal variability in a biomarker (Goksoyr and Forlin, 1992; Tom and Auslander, 2005). These variations have been well documented for CYP1A and vtg in other fish species (Hinck *et al.,* 2006; Kopecka and Pempkowiak, 2007; Maitra *et al.,* 2007; Moussavi *et al.,* 2009; Sanchez *et al.,* 2008; Sole *et al.,* 2009). Assessing the temporal and spatial variations in CYP1A would also be highly useful when using a migratory species like Cape hake as an indicator species.

Although the EROD assay is a valid technique for assessing environmental degradation, the use of one or two biomarkers is limited, i.e. CYP1A is inducible by a specific range of chemical compounds. Future research should move towards a multiple biomarker approach using enzymes (e.g. the second phase metabolism or oxidative stress indicators) combined with chemical analysis of water samples. This would allow for a more complete analysis (Goksoyr and Forlin, 1992; Handy *et al.,* 2003; Siroka and Drastichova, 2004; Tamara *et al.,* 2004).

Although vtg was successfully purified from Cape hake, further characterization of this protein is required in terms of structure and function in Cape hake, before assays for the detection of estrogenic pollutants are developed. It is important to further characterize vtg in Cape hake because it is a species specific protein and therefore the vtg structure may be significantly varied in different fish species (Nicolas, 1999).

.

Chapter 7: References

Abrahamson A. Brandt I. Brunstrom B. Sundt R.C. Jorgensen E.H. 2007. Monitoring contaminants from oil production at sea by measuring gill EROD activity in Atlantic cod (*Gadus morhua*). *Environmental Pollution 1-7*

Aluru N. and Vijayan M. 2006. Resveratol affects CYP1A expression in rainbow trout hepatocytes. *Aquatic Toxicology* 77: 291-297

Andersson T. Forlin L. Olsen S. Fostier A. Breton B. 1993. Pituitary as a target organ for toxic effects of P4501A1 inducing chemicals. *Molecular and Cellular Endocrinology* 91: 99-105

Anene A. 2005. Condition factor of four cichlid species of a man-made lake in Imo state, southeastern Nigeria. *Turkish journal of Fisheries and Aquatic Sciences* 5: 43-47

Ariese F. Kok S.J. Verkaik M. Gooijer C. Velhorst N.H. Hofstraat W. 1993. Sychronous fuorescence spectrometry of fish bile: a rapid screening method for the biomonitoring of PAH exposure. *Aquatic Toxicology* 26: 273-286

Arinc E. and Sen A. 1999. Hepatic cytochrome P4501A and 7-ethoxyresorufin-*O*-deethylase induction in mullet and common sole as an indicator of toxic organic pollution in Izmir Bay, Turkey. *Marine Environmental Research* 48: 147-160

Arukwe A. 2001. Cellular and molecular response to endocrine-modulators and the impact on fish reproduction. *Marine Pollution Bulletin* 42: 643-655

Arukwe A. and Goksoyr A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population and evolutionary implications of endocrine disruption. *Comparative Hepatology* 2: 1-21

Arukwe A. Knudsen F.R. Goksoyr A. 1997. Fish zona radiate (eggshell) protein: a sensitive biomarker for environmental estrogens. *Environmental Health Perspectives* 105: 419-421

Assuncao M.G.L. Miller K.A. Dangerfield N.J. Bandiera S.M. Ross P.S. 2007. Cytochrome P450 1A expression and organochlorine contaminants in harbour seals (*Phoca vitulina*): evaluating a biopsy approach. *Comparative Biochemistry and Physiology C* 145: 256-264

Aubry E. Rime H. Monod G. 2005. Beta-napthoflavone inhibits the induction of hepatic estrogen dependent proteins by 17 alpha-ethynylestradiol in mosquitofish (*Gambusia holbrooki*). *Biomarkers* 10: 439-455

Babin M. Casado S. Chana A. Herradon B. Segner H. Tarazona J.V. Navas J.M. 2005. Cytochrome P4501A induction caused by the imidazole derivative prochloraz in a Rainbow trout cell line. *Toxicology in Vitro* 19: 899-902

Bailey K.M. Merati N. Helser M. Hiramatsu N. Hara A. 2002. Developmental changes in egg yolk proteins of walleye pollock, *Theragra chalcogamma,* and a comparative study of immunoreactivity of other north pacific teleosts and invertebrate eggs. *Bulletin of Fish Science* 53: 95-105

Ballon M. Wosnitza-Mendo C. Guevara-Carrasco R. Bertrand A. 2008. The impact of overfishing and El Nino on the condition factor and reproductive success of Peruvian hake, *Merluccius gayi peruanus*. *Progress in Oceanography* 79: 300-307

Barnham C. and Baxter A. Condition factor K, for salmonoid. 1998. *Department of Primary Industries.State of Victoria*

Barse A.V. Chakrabati T. Ghosh T.K. Pal A.K. Jadhao S.B. 2007. Endocrine disruption and metabolic changes following exposure of *Cyprinus carpio* to diethyl phthalate. *Pesticide Biochemistry and Physiology* 88: 36-42

Behrens A. Schirmer K. Bols N.C. Segner H. 1997. Microassay of rapid measurement of 7-ethoxyresorufin-*O*-deethylase activity in intact fish hepatocytes. *Marine Environmental Research* 46: 369-373

Beliaeff B. and Burgeot T. 1997. Sampling design optimization for EROD measurements in fish. *Marine Ecology Progress Series* 153: 239-246

Beyer J. and Goksoyr A. 1993. Cytochrome P450 observation in gulf fish. *Marine Pollution Bulletin* 27: 293-296

Blazquez M. Bosma P.T. Fraser E.J. Van Look K.J.W. Trudeau V.L. 1998. Fish models for the neuroendocrine regulation of reproduction and growth. *Comparative Biochemistry and Physiology C* 119: 345-364

Bollag D.M. Rozycki M.D. Edelstein S.J. 1996. *Protein Methods*. 2nd ed. New York: Wiley-Liss Inc

Bols N.C. Schirmer K. Joyce E.M. Dixon D.G. Greenberg B.M. Whyte J.J. 1999. Ability of polycyclic aromatic hydrocarbons to induce 7-ethoxyresorufin-*O*-deethylase activity in trout liver cell line. *Ecotoxicology and Environmental Safety* 44: 118-128

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254

Bulukin E. Meucci V. Minunni M. Pretti C. Intorre L. Soldani G. Mascini M. 2007. An optical immunosensor for rapid vitellogenin detection in plasma from carp (*Cyprinus carpio*). *Talanta* 72: 785-790

Burgeot T. Bocquene G. Truquet P. Le Dean L. Galgani F. 1994. Induction of EROD activity in Red mullet (*Mullus barbatus*) along the French Mediterranean coasts. *The Science of the Total Environment* 142: 213-220

Camus L. Aas E. Borseth J.F. 1998. Ethoxyresorufin-*O*-deethylase activity and fixed wavelength fluorescence detection of PAHs metabolites in bile in turbot (*Scophthalmus maximus L.*) exposed to a dispersed topped crude oil in a continuous flow system. *Marine Environmental Research* 46: 29-32

Carlsson C. and Part P. 2001. 7-ethoxyresorufin-*O*-deethylase induction in rainbow trout gill epithelium cultures on permeable supports: asymmetrical distribution of substrate metabolites. *Aquatic Toxicology* 54: 29-38

Carlsson C. Part P. Brunstrom B. 1999. 7-ethoxyresorufin-*O*-deethylase induction in cultured gill epithelial cells from rainbow trout. *Aquatic Toxicology* 47: 117-128

Carnevali O. Carletta R. Cambi A. Vita A. Bromage N. 1999. Yolk formation and degradation during oocyte maturation in seabream *Sparus aurata* : involvement of two lysosomal proteinases. *Biology of Reproduction* 60: 140-146

Celius T. and Walther B.T. 1998. Oogenesis in Atlantic salmon (*Salmo salar L.*) occurs by zonagenesis preceding vitellogenesis in *vivo* and in *vitro*. *Journal of Endocrinology* 158: 259-266

Cheek A.O. King V.W. Burse J.R. Borton D.L. Sullivan C.V. 2004. Bluegill (*Lepomis macrochirus*) vitellogenin: purification and enzyme-linked immunosorbent assay for detection of endocrine disruption by papermill effluent. *Comparative Biochemistry and Physiology C* 137: 249-260

Copeland P.A. and Thomas P. 1988. The measurement of plasma vitellogenin levels in a marine teleost, the spotted seatrout (*Cynoscion nebulosus*) by homologous radioimmunoassay. *Comparative Biochemistry and Physiology B* 91: 17-23

Cuesta A. Vargas-Chacoff L. Garcia-Lopez A. Arjona F.J. Martinez-Roddroguez G. Meseguer J. Mancera J.M. Esteban M.A. 2007. Effect of sex-steroid hormones, testerone and estradiol, on humoral immune parameters of gilthead seabream. *Fish and Shellfish Immunology* 23: 693-700

Crim L.W. and Idler D.R. 1978. Plasma gonadotrophin, estradiol and vitellogenin and gonad phosvitin levels in relation to the seasonal reproduction cycles of female brown trout. *Ann. Biol. Anim. Biochem. Biophys* 18: 1001-1005

Das M. Rastogi S. Khanna S.K. 2004. Mechanism to study 1:1 stoichiometry of NADPH and alkoxyphenoxazones metabolism spectrophotometrically in subcellular biological preparations. *Biochimica et Biophysica Acta* 1675: 1-11

Davail B. Pakdel F. Bujo H. Perazzolo L.M. Waclawek M. Schneider W.J. Le Menn F. 1998. Evolution of oogenesis: the receptor for vitellogenin from rainbow trout. *Journal of lipid Research* 39: 1931-1937

Degani G. Boker R. Jackson K. 1998. Growth hormone, sexual maturity and steroids in male carp. *Comparative Biochemistry and Physiology C* 120: 433-440

Desantis S. Corriero A. Cirillo F. Deflorio M. Brill R. Griffiths M. Lopata A.L. de la Serna J.M. Bridges C.R. Kime D.E. and De Metrio G. 2005. Immunohistochemical localization of CYP1A, vitellogenin and Zona radiata proteins in the liver of swordfish (*Xiphias gladius L.)* taken from the Mediterranean Sea, South Atlantic, South Western Indian and Central North Pacific Oceans. *Aquatic Toxicology* 71: 1-12

Douhri H. and Sayah F. 2009. The use of enzymatic biomarkers in two marine invertebrates *Nereis diversicolor* and *Patella vulgate* for the biomonitoring of Tangiers bay (Morocco). *Ecotoxicology and Environmental Safety* 72: 394-399

Durou C. Poirier L. Amiard J.C. Budzinski H. Gnassia-Barelli M. Lemenach K. Peluhet L. Mouneyrac C. Romeo M. Amiard-Triquet C. 2007. Biomonitoring in a clean and a multi-contaminated estuary based on biomarkers and chemical analyses in the endobenthic worm *Nereis diversicolor*. *Environmental Pollution* 148: 445-458

Ebrahimi M. 2005. Vitellogenin assay by enzyme-linked immunosorbent assay as a biomarker of endocrine disruptor chemicals pollution. *Indian Journal of Occupational and Environmental Medicine* 9: 66-70

Eggens M.L. Opperhuizen A. Boon J.P. 1996. Temporal variation of CYP1A indices, PCB and 1-OH pyrene concentration in flounder, *Platichthys flesus,* from the Dutch Wadden sea. *Chemosphere* 8: 1579-1596

Escartin E. and Porte C. 1999. Assessment of PAH pollution in coastal areas from the NW Mediterranean through the analysis of fish bile. *Marine Pollution Bulletin* 38: 1200-1206

Faria M. Carrasco L. Diez S. Riva M.C. Bayona J.M. Barata C. 2009. Multi-biomarker responses in the freshwater mussel *Dreissena polymorpha* exposed to polychlorobiphenyls and metals. *Comparative Biochemistry and Physiology C* 149: 281-288

Fenske M. Van Aerle R. Brack S. Tyler C.R. Segner H. 2001. Development and validation of homologous zebrafish (*Danio rerio* Hamilton-Buchanan) vitellogenin enzyme-linked immunosorbent assay (ELISA) and its application for studies on estrogenic chemicals. *Comparative Biochemistry and Physiology C* 129: 217-232

Fent K. 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicology in vitro* 15: 477-488.

Ferreira F. Santos M.M. Castro L.F.C. Reis-Henriques M.A. Lima D. Vieira M.N. Monteiro. 2009. Vitellogenin gene expression in the intertidal blenny *Lipophrys pholis*: a new sentinel species for estrogenic chemical pollution monitoring in the european atlantic coast?. *Comparative Biochemistry and Physiology C* 149: 58-64

Flammarion P. and Garric J. 1999. A statistical approach for classifying the extent of EROD induction of fish sampled in clean and contaminated waters. *Water Research* 33: 2683-2689

Flammarion P. and Garric P.N. 2002. The measurement of cholinesterase activities as a biomarker in chub (*Leuciscus cephalus*): the fish length should not be ignored. *Environmental Pollution* 120: 325-330

Food and Agriculture Organisation of the United Nations (2008) *Fao fisheries and aquaculture-species fact sheets.* Fisheries and Aquaculture Department

Forlin L. Lemaire P. Livingstone D.R. 1995. Comparative studies of hepatic xenobiotic metabolizing and antioxidant enzymes in different fish species. *Marine Environmental Research* 39: 201-204

Fossi M.C. Casini S. Marsili L. 2007. Potential toxicological hazard due to endocrine-disrupting chemicals on Mediterranean top predators: State of art, gender differences and methodological tools. *Environmental Research* 104: 174-182

Gagnaire B. Gagne F. Andre C. Blaise C. Abbaci K. Budzinski H. Devier M.H. Garric J. 2009. Development of biomarkers of stress related to endocrine disruption in gastropods : Alkali-labile phosphates, protein bound lipids and vitellogenin-like proteins. *Aquatic Toxicology* doi: 10.1016/j.aquatox.2009.01.012

Galgani F. Bocquene G. Lucon M. Grzebyk D. Letrouit F. Claisse D. 1991. EROD measurements in fish from the northwest part of France. *Marine Pollution Bulletin* 22: 494-500

Goddard K.A. Schultz R.J. Stegeman J.J. 1981. Uptake, toxicity and distribution of Benz[$_a$]pyrene and monooxygenase induction in the topminnows *Poeciliopsis monacha* and *Poeciliopsis lucida. Drug metabolism and Disposition* 15: 449-455

Goksoyr A. 1986. Initial characterization of the hepatic microsomal cytochrome P450 system of the piked whale (Minke) *Balaenoptera acutorostrata. Marine Environmental Research* 19: 185-203.

Goksoyr A. and Forlin L. 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic Toxicology* 22: 287-312.

Goksoyr A. Larsen H.E. Blom S. Forlin L. 1992.Detection of cytochrome P450 1A1 in North Sea dab liver and kidney. *Marine Ecology Progress Series* 91: 83-88

Goksoyr A. Larsen H.E. Husøy A.M. 1991. Application of a cytochrome P450 1A1 ELISA in environmental monitoring and toxicological testing in fish. *Comparative Biochemistry and Physiology C* 100: 157-160

Gonzalez J.F. Reimschuessel R. Shaikh B. Kane A.S. 2009. Kinetics of hepatic phase I and II biotransformation reactions in eight finfish species. *Marine Environmental Research* 67: 183-188

Goodsell P.J. Underwood A.J. Chapman M.G. 2009. Review: Evidence necessary for taxa to be reliable indicators of environmental conditions or impacts. *Marine Pollution Bulletin* 58: 323-331

Gordoa A. Lesch H. Rodergas S. 2006. Bycatch: complementary information for understanding fish behaviour. Namibian Cape hake (*M. capensis* and *M. paradoxus*) as a case study. *ICES Journal of Marine Science* 63: 1513-1519

Gordoa A. and Macpherson E. 1991. Diurnal variation in the feeding activity and catch rate of cape hake (*Merluccius capensis* and *M. paradoxus*) off Namibia. *Fisheries Research* 12: 299-305

Gordoa A. Macpherson E. Olivar M.P. 1995. Hake: fisheries, ecology and markets. Chapter 3: Biology and fisheries of Namibian Hakes (*M. paradoxus* and *M. capensis*). Editors: Alheit J. and Pitcher T.J.

Gordoa A. Maso M. Voges L. 2000. Monthly variability in the catchability of Namibian hake and its relationship with environmental seasonality. *Fisheries Science* 48: 185-195

Greenwood L.N. Scott A.P. Etienne L. Vermeirssen M. Constantinos C.M. Pavlidis M. 2001. Plasma steroids in mature common dentex (*Dentex dentex*) stimulated with a gonadotropin-releasing hormone agonist. *General and Comparative Endocrinology* 123: 1-12

Grote B. Ekau W. Hagen W. Huggett J.A. Verheye H.M. 2007. Early life-history of cape hake in the benguela upwelling region. *Fisheries Research* 86: 179-187

Grzebyk D. and Galgani F. 1991. Measurement of the effect of organic pollution on marine organisms: rapid determination of EROD induction using plate readers. *Aquatic Living Resource* 4: 53-59

Guiney P.D. Smolowitz R.M. Peterson R.E. Stegeman J.J. 1997. Correlation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induction of cytochrome P4501A in vascular endothelium with toxicity in early life stages of lake trout. *Toxicology and Applied Pharmacology* 143: 256-273.

Guzman J.M. Norberg B. Ramos J. Mylonas C.C. Mananos E.L. 2008. Vitellogenin steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). *General and Comparative Endocrinology* 156: 285-297

Hagger J.A. Galloway T.S. Langston W.J. Jones M.B. 2009. Application of biomarkers to assess the condition of European marine sites. *Environmental Pollution* doi: 10.1016/j.envpol.2009.02.038

Hahn M.E. Lamb T.M. Schultz M.E. Smolowitz R.M. Stegeman J.J. 1993. Cytochrome P4501A induction and inhibition by 3,3'4,4'-tetrachlorobiphenyl in an Ah receptor containing fish hepatoma cell line (PLHC-1). *Aquatic Toxicology* 26: 185-208

Hahn M.E. Woodin B.R. Stegeman J.J. Tillitt D.E. 1998. Aryl hydrocarbon receptor function in early vertebrates: inducibility of cytochrome P450 1A in agnathan and elasmobranch fish. *Comparative Biochemistry and Physiology C* 120: 67-75.

Hahn M.E. Woodward B.L. Stegeman J.J. Kennedy W. 1996. Rapid assessment of induced cytochrome P4501A protein and catalytic activity in fish hepatoma cells grown in multiwall plates: response to TCDD, TCDF, and two planar PCBs. *Environmental toxicology and Chemistry* 15: 582-591

Hall J.E. 2002. Bioconcentration, bioaccumulation, and biomagnification in Puget Sound biota: assessing the ecological risk of chemical contaminants in Puget Sound. *Unpublished Thesis*. University of Washington Tacoma

Handy R.D. Galloway T.S. Depledge M.H. 2003. A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotoxicology* 12: 331-343

Hansen T. Karlsen O. Taranger G.L. Hemre G.I. Holm J.C. Kjesbu O.S. 2001. Growth, gonadal development and spawning time of Atlantic cod (*Gadus morhua*) reared under different photoperiods. *Aquaculture* 203: 51-67

Hasselberg L. Grosvik B.E. Goksoyr A. Celander M.C. 2005. Interactions between xenoestrogens and ketoconazole on hepatic CYP1A and CYP3A, in juvenile atlantic cod (*Gadus morhua*). *Comparative Hepatology* 4: 1-15.

Hennies M. Wiesmann M. Allner B. Sauerwein H. 2003. Vitellogenin in carp (*Cyprinus carpio*) and perch (*Perca fluviatilis*): purification, characterization, and development of an ELISA for the detection of estrogenic effects. *The Science of the Total Environment* 309: 93-103

Hinck J.E. Schmitt C.J. Blazer V.S. Denslow N.D. Bartish T.M. Anderson P.J. Coyle J.J. Dethloff G.M. Tillitt D.E. 2006. Environmental contaminants and biomarker responses in fish from the Columbia River and its tributaries: spatial and temporal trends. *Science of the Total Environment* 366: 549-578

Hiramatsu N. Hara A. Hiramatsu K. Fukada H. Weber G.M. Denslow N.D. Sullivan C.V. 2002. Vitellogenin-derived yolk proteins of whit perch, *Morone americana*: purification, characterization, and vitellogenin-receptor binding. *Biology of Reproduction* 67: 655-667

Hoeger B. Heuvel M.R. Hitzfeld B.C. Dietrich D.R. 2004. Effects of treated sewage effluent on immune function in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* 70: 345-355

Humphrey C.A. King S.C. Klumpp D.W. 2007. A multibiomarker approach in barramundi (*Lates calcarifer*) to measure exposure to contaminants in estuaries of tropical North Queensland. *Marine Pollution Bulletin* 54: 1569-1581

Husoy A.M. Myers M.S. Goksoyr A. 1996. Cellular localization of cytochrome P450 (CYP1A) induction and histology in atlantic cod (*Gadus morhua L.*) and european flounder (*Platichthys flesus)* after environmental exposure to contaminants by caging in Sorfjorden, Norway. *Aquatic Toxicology* 36: 53-74

Huuskonen S. and Lindstrom-Seppa P. 1995. Hepatic cytochrome P4501A and other biotransformation activities in perch (*Perca fluviatilis*): the effects of unbleached pulp mill effluents. *Aquatic Toxicology* 31: 27-41

IEH (1999) *IEH Assessment on the ecological significance of endocrine disruption: Effects on reproductive function and consequences for natural populations* (Assessment A4), Leicester, UK, MRC Institute for Environment and Health

Ishibashi H. Tachibana K. Tsuchimoto M. Soyano K. Ishibashi Y. Nagae M. Kohra S. Takao Y. Tominaga N. Arizono K. 2001. In vivo testing system for determining the estrogenic activity of endocrine disrupting chemicals (EDCs) in goldfish (*Carassius auratus*). *Journal of Health Science* 47: 213-218

Japp D.W. 2007. Potential impacts on offshore fisheries of mining by De Beers Marine Namibia in Atlantic 1. De Beers Marine Namibia Expert Report Fisheries Atlantic 1

Jenkins J.A. 2004. Fish bioindicators of ecosystem condition at the Calcasieu estuary, Louisiana. USA: National Wetlands Research Center, USGS

Jensen K.M. and Ankley G.T. 2006. Evaluation of a commercial kit for measuring vitellogenin in the fathead minnow (*Pimephales promelas*). *Ecotoxicology and Environmental Safety* 64: 101-105

Jimenez B. Mori G. Concejero M.A. Merino R. Casini S. Fossi M.C. 2007. Vitellogenin and zona proteins as biomarkers of endocrine disruption in peregrine falcon (*Falco peregrinus*). *Chemosphere* 67: 375-378

Jimenez B.D. Cirmo C.P. McCarthy J.F. 1987 Effects of feeding and temperature on uptake elimination and metabolism of benzo (a) pyrene in the bluegill sunfish (*Lepomis macrochirus*). *Aquatic Toxicology* 10: 41-57

Johnsen H.K. Tveiten H. Willassen N.P. Arnesen A.M. 1999. Artic charr (*Salvelinus alpinus*) vitellogenin: development and validation of an enzyme-linked immunosorbent assay. *Comparative Biochemistry and Physiology B* 124: 355-362

Jönsson E.M. Brandt I. Brunström B. 2002. Gill filament-based EROD assay for monitoring waterborne dioxin-like pollutants in fish. *Environmental Science & Technology* 36: 3340-3344

Jos A. Segner H. Herradon B. Repetto G. Navas J.M. 2007. Induction of EROD activity by 1-phenylimidazole and β-naphthoflavone in rainbow trout cultured hepatocytes: A comparative study. *Toxicology in Vitro* 21: 1307-1310

Jurcikova J. Mikula P. Dobsikova R. Nemethova D. Svobodova Z. 2007. Effects of metazachlor on vitellogenin induction in zebrafish (*Danio rerio*). *Act. Vet. Brno.* 76: 61-66

Kammann U. Lang T. Vobach M. Wosniok W. 2000. Ethoxyresorufin-*O*-deethylase (EROD) activity in dab (*Limanda limanda*) as biomarker for marine monitoring. *Environmental Science & Pollution Research* 12: 140-145

Kashiwada S. Ishikawa H. Miyamoto N. Ohnishi Y. Magara Y. 2002. Fish test for endocrine-disruption and estimation of water quality of Japanese rivers. *Water Research* 36: 2161-2166

Kime D.E. 1999. A strategy for assessing the effects of xenobiotics on fish reproduction. *The Science of the Total Environment* 225: 3-11

King S.C. Hassell K. Nugegoda D. Kristiansen S.I. 2008. The assessment of vitellogenin as a biomarker of exposure to estrogenic compounds in two australian perciformes. *Marine Environmental Research* 66: 116-118

Kirk C.J. Bottomley L. Minican N. Carpenter H. Shaw S. Kohli N. Winter M. Taylor W.E. Waring R.H. Michelangeli F. Harris R.M. 2003. Environmental endocrine disrupters dysregulate estrogen metabolism and Ca^{2+} homeostasis in fish and mammals via receptor-independent mechanisms. *Comparative Biochemistry and Physiology A* 135: 1-8

Kling C.M. Bowers J.L Kulakosky P.C. Kamboj K.K. Swanson H.I. 1999. The aryl hydrocarbon receptor (ARNT) heterodimer interacts with naturally occurring estrogen response elements. *Molecular and Cellular Endocrinology* 157: 105-119

Klotz A.V. Stegeman J.J. Walsh C. 1984. An alternative 7-ethoxyresorufin-*O*-deethylase activity assay: a continuous visible spectrophotomeric method for measurement of cytochrome P450 monooxygenase activity. *Analytical Biochemistry* 140: 138-145

Kopecka J. and Pempkowiak J. Temporal and spatial variations of selected biomarker activities in Flounder *(Platichthys flesus*) collected in the Baltic proper.2007. *Ecotoxicology and Environmental Safety* doi: 10.1016/j.ecoenv.2007.05.017

Koponen K. Kukkonen J.V.K. Lindstrom-Seppa P. 1998. Chemical accumulation and biotransformation enzyme activities of rainbow trout embryos in waterborne exposure to PCB-77. *Marine Environmental Research* 46: 475-478

Korsgaard B. and Pederson K.L. 1998. Vitellogenin in *Zoarces viviparus*: purification, quantification by ELISA and induction by estradiol-17β and 4-nonylphenol. *Comparative Biochemistry and Physiology C* 120: 150-166

Kortner T.M and Arukwe A. 2007. The xenoestrogen, 4-nonylphenol, impaired steroidogenesis in previtellogenic oocyte culture of Atlantic cod (*Gadus morhua*) by targeting the StAR protein and P450*scc* expressions. *General and Comparative Endocrinology* 150: 419-429

Kruner G. and Westerhagen H.V. 1999. Sources of measurement error in assays of EROD activity of fish for biological effects monitoring. *Helgol Marine Research* 53: 250-256

Kumakura N. Okuzawa K. Gen K. Yamaguchi S. Lim BS. Kagawa H. 2004. Effects of gonadotrophin-releasing hormone on pituitary-ovarian axis of one-year old pre-pubertal red seabream. *General and Comparative Endocrinology* 138: 105-112

Labadie P. and Budinski H. 2006. Alteration of steroid hormone profile in juvenile turbot (*Psetta maxima*) as a consequence of short-term exposure to 17α-ethynylestradiol. *Chemosphere* 64: 1274-1286

Laemlli U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685

Larkin P. Knoebl I. Denslow N.D. 2003. Differential gene expression analysis in fish exposed to endocrine disrupting compounds. *Comparative Biochemistry and Physiology* 136: 149-161

Lavado R. Thibaut R. Raldua D. Martin R. Porte C. 2004. First evidence of endocrine disruption in feral carp from the Ebro river. *Toxicology and Applied Pharmacology* 196: 247-257

Lee C.M. Kim B.Y. Li l. Morgan E.T. 2008. Nitric oxide-dependent proteasomal degradation of cytochrome P450 2B proteins. *Journal of Biological Chemistry* 283: 889-898

Lee YH. Du JL. Shih YS. Jeng SR. Sun LT. Chang CF. 2004. In vivo and in vitro sex steroids stimulate seabream gonadotropin-releasing hormone content and release in the protandrous black porgy, *Acanthopagrus schegeli. General and Comparative Endocrinology* 139: 12-19

Leino R.L. Jensen K.M. Ankley G.T. 2005. gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Pharmacology* 19: 85-98

Lemos N.G. Dias A.L. Silva-Souza A.T. Mantovani M.S. 2005. Evaluation of environmental waters using the comet assay. *Environmental Toxicology and Pharmacology* 19: 197-201

Liao T. Jin S. Yang FX. Hui Y. Xu Y. 2006. An enzyme-linked immunosorbent assay for rare minnow (*Gobiocypris rarus*) vitellogenin and comparison of vitellogenin responses in rare minnow and zebrafish (*Danio rerio*). *Science of the Total Environment* 364: 284-294

Lindstrom-Seppa P. 1985. Seasonal variation of the xenobiotic metabolizing enzyme activities in the liver of male and female vendace. *Aquatic Toxicology* 6: 323-331.

Lindstrom-Seppa P. Korytko P.J. Hahn M.E. Stegeman J.J. 1994. Uptake of waterborne 3, 3', 4, 4'- tetrachlorobiphenyl and organ and cell-specific induction of cytochrome P4501A in adult and larval fathead minnow *Pimephales promelas*. *Aquatic Toxicology* 28: 147-167.

Liu Q.H. Zhang S.C. Li Z.J. Gao C.R. 2008. Characterization of a pattern recognition molecule vitellogenin from carp (*Cyprinus carpio*). Doi: 10.1016/j.imbio.2008.10.003

Livingstone D.R. 1998. Review: The fate of organic xenobiotic in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comparative Biochemistry and Physiology A* 120: 43-49.

Losso C. and Ghirardini A.V. 2010. Review article: overview of ecotoxicological studies performed in the Venice Lagoon (Italy). *Environmental International* 36: 92-121

Lubzens E. Young G. Bobe J. Cerda J. 2010. Oogenesis in teleosts: how fish eggs are formed. *General and Comparative Endocrinology* 165: 367-389

Lye C.M. Frid C.L.J. Gill M.E. McCormick D. 1997. Abnormalities in the reproductive health of flounder *Platichthys flesus* exposed to effluent from sewage treatment works. *Marine Pollution Bulletin* 34: 34-41

Machala M. Nezveda K. Petrivalsky M. Jarasova A.B. Piacka V. Svobodova Z. 1997. Monooxygenase activities in carp as biochemical markers of pollution by polycyclic and polyhalogentaed aromatic hydrocarbons: choice of substrates and effects of temperature, gender and capture stress. *Aquatic Toxicology* 37: 113-123

Maitra S. Sahu R. Trehan N. Garg S.K. Nath P. 2007. Circannual variation in plasma vitellogenin and gonadotrophin II levels in relation to annual ovarian cycle in female mrigal, *Cirrhinus mrigala*. *Aquaculture* 265: 370-384

Mananos E. Zanuy S. Le Menn F. Carrillo M. Nunez J. 1994. Sea Bass (*Dicentrarchus labrax L.*) vitellogenin. I-Induction, purification and partial characterization. *Comparative Biochemistry and Physiology B* 107: 205-216

Manhor D. Rao G.D. Sreenivasulu G. Senthilkumaran B. Gupta A.D. 2005. Purification of vitellogenin from the air breathing catfish, *Clarias gariepinus*. *Fish Physiology and Biochemistry* 31: 235-239

Mansuy D. 1998. Review: the great diversity of reactions catalysed by cytochromes P450. *Comparative Biochemistry and Physiology C* 121: 5-14

Mansuy D. 2007. A brief history of the contribution of metalloporphyrin models to cytochrome P450 chemistry and oxidation catalysis. *C.R. Chimie* 10: 392-413

Marionnet D. Taysse L. Chambras C. Deschaux P. 1997. 3-methylcholanthrene-induced EROD activity and cytochrome P450 in immune organs of carp (*Cyprinus carpio*). *Comparative Biochemistry and Physiology C* 118: 165-170

Markert B. 2007. Definitions and principles for bioindication and biomonitoring of trace metals in the environment. *Journal of Trace Elements in Medicine and Biology* 21: 77-82

Martel P.H. Kovacs T.G. O'Connor B.I. Voss R.H. 1994. A laboratory exposure procedure for screening pulp and paper mill effluents for the potential of causing increased mixed function oxidase activity in fish. *Environmental Pollution* 89: 229-240

Masfaraud J.F. Monod G. Devaux A. 1990. Use of the fish cytochrome P450 dependent 7-ethylresorufin-*O*-deethylase activity as a biochemical indicator of water pollution. Study of the liver and the kidney of male and female Nase *(Chondrostoma nasus)* from the river Rhone. *The Science of the Total Environment* 97/98: 729-738.

Mas-Riera J. 1991. Changes during growth in the retinal structure of three hake species, *Merluccius spp.* (Telestei: *Gadiformes*), in relation to their depth distribution and feeding. *Journal of Experimental Marine Biology and Ecology* 152: 91-104

Mateos J. Mananos E. Carrillo M. Zanuy S. 2002. Regulation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) gene expression by gonadotrophin-releasing hormone (GnRH) and sexual steroids in the Mediterranean Sea bass. *Comparative Biochemistry and Physiology* 132: 75-86

Matozzo V. Gagne F. Marin M.G. Ricciardi F. Blaise C. 2008. Review: vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates. *Environmental International* 34: 531-545

Meier S. Andersen T.E. Hasselberg L. Kjesbu O.S. Klungsoyr J. Svardal A. 2007. Hormonal effects of C_4-C_7 alkylphenols on cod (*Gadus morhua*). *Institute of Marine Research*, Norway 1-56

Melancon M.J. and Lech J.J. 1983. Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquatic Toxicology* 4: 51-61.

Meucci V. and Arukwe A. 2005. Detection of vitellogenin and *zona radiata* protein expressions in surface mucus of immature juvenile atlantic salmon (*Salmo salar*) exposed to waterborne nonylphenol. *Aquatic Toxicology* 73: 1-10

Meyer J.N. Nacci D.E. Di Giulio R.T. 2002. Cytochrome P4501A (CYP1A) in killfish (*Fundulus heteroclitus*): heritability of altered expression and relationship to survival in contaminated sediments. *Toxicological Science* 68: 69-81.

Miles-Richardson S.R. Kramer V.J. Fitzgerald S.C. Render J.A. Yamini B. Barbee S.J. Giesy J.P. 1999. Effects of waterborne exposure of 17 β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquatic Toxicology* 47: 129-145

Milhailovic M. Arambasic J. Bogojevic D. Dinic S. Grdovic N. Grigorov I. Ivanovic-Matic S. Labus-Blagojevic S. Martinovic V. Petrovic M. Uskokovic A. Vidakovic M. Poznanovic G. 2006. Expression of CYP1A in the hepatopancreas of *Merluccius merluccius*, *Trigla lucerna* and *Liza ramada* (pisces) in the wider vicinity of Bar harbor Montenegro. *Archives of Biological Science* 58: 165-170

Miller M.R. Hinton D.E. Blair J.J. Stegeman J.J. 1988. Immunohistochemical localization of cytochrome P450E in liver, gill and heart of Scup (*Stenotomus chrysops*) and Rainbow Trout (*Salmo gairdneri*). *Marine Environmental Research* 24: 37-39

Mills L.J. and Chichester C. 2005. Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations?. *Science of the Total Environment* 343: 1-34

Miota F. Siegfried B. Scharf M.E. Lydy M.J. 2000. Atrazine induction of cytochrome P450 in *Chironomus tentans* larvae. *Chemosphere* 40: 285-291

Miranda C.L. Henderson M.C. Williams D.E. Buhler D.R. 1997. *In vitro* metabolism of 7.12-dimethylbenz[a]anthracene by rainbow trout liver microsomes and trout P450 isoforms. *Toxicology and Applied Pharmacology* 142: 123-132

Moncaut N. Lo Nostro F. Maggese M.C. 2003. Vitellogenin detection in surface mucus of the South American cichlid fish *Cichlasoma dimerus* (Heckel, 1840) induced by estradiol-17β, effects on liver and gonads. *Aquatic Toxicology* 63: 127-137

Mondon J.A. Duda S. Nowak B.F. 2001. Histological, growth and 7-ethoxyresorufin-*O*-deethylase (EROD) activity responses of greenback flounder *Rhombosolea tapirina* to contaminated marine sediment and diet. *Aquatic Toxicology* 54: 231-247

Monod G. and Vindimian E. 1991. Effect of storage conditions and subcellular fractionation of fish liver on cytochrome P450-dependent enzymatic activities used for monitoring of water pollution. *Water Research* 25: 173-177

Mortensen A.S. and Arukwe A. 2007. Effects of 17-α-ethynyestradiol on hormonal responses and xenobiotic biotransformation system of atlantic salmon (*Salmo salar*). *Aquatic Toxicology* 85: 113-123

Moussavi M. Nelson E.R. Habibi H.R. 2009. Seasonal regulation of vitellogenin by growth hormone in goldfish liver. *General and Comparative Endocrinology* doi: 10.1016/j.ygcen.2008.12.009

Murua H. and Sabarido-Rey F. 2003. Female reproductive strategies of marine fish species of the North Atlantic. *Journal of Northwestern Atlantic Fisheries Science* 33: 23-31

Nath P. Kabita S.K. Bhattacharya D. 2007. Vitellogenesis with special emphasis on Indian fishes. *Fish Physiology and Biochemistry* 33: 359-366

Navas J.M. and Segner H. 2006. Vitellogenin synthesis in primary cultures of fish liver cells as endpoint for in vitro screening of the (anti) estrogenic activity of chemical substances. *Aquatic Toxicology* 80:1-22

Nicolas J.M. 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. *Aquatic Toxicology* 45: 77-90

Nilsen B.M. Berg K. Eidem J.K. Kristiansen S.I. Brion F. Porcher J.M. Goksoyr A. 2004. Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine screening. *Analytical and Bioanalytical Chemistry* 378: 621-633

Nilsen B.M. Berg K. Goksoyr A. 1998. Methods in molecular biology: cytochrome P450 protocols. 1st ed. Totowa NJ: Humana Press

Nimrod A.C. and Benson W.H. 1997. Xenobiotic interaction with and alteration of channel catfish estrogen receptor. *Toxicology and Applied Pharmacology* 147: 381-390

Noaksson E. Linderoth M. Tjarnlund U. Balk L. 2005. Toxicological effects and reproductive impairments in female perch (*Perca fluvitilis*) exposed to leachate from Swedish refuse dumps. *Aquatic Toxicology* 75: 162-177

Okoumassoun L.E. Averill-Bates D. Gagne F. Marion M. Denizeau F. 2002. Assessing the estrogenic potential of organochlorine pesticides in primary cultures of male rainbow trout (*Oncorhynchus mykiss*) hepatocytes using vitellogenin as a biomarker. *Toxicology* 178: 193-207

Oost R. Beyer J. Nico P. Vermeulen E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* 13: 57-149

Oost R. Van Gastel L. Worst D. Hanraads M. Satumalay K. Van Schooten J. Heida H. Vermeulen N.P.E. 1994. Biochemical markers in feral roach (*Rutilus rutilus*) in relation to the bioaccumulation of organic contaminants. *Chemosphere* 29: 801-817

Oritz-Delgado J.B. and Sarasquete C. 2004. Toxicity, histopathological alterations and immunohistochemical CYP1A induction in the early life stages of the seabream, *Sparus aurata*, following waterborne exposure to B (a) P and TCDD. *Journal of Molecular Histology* 35: 29-45

Orn S. Yamani S. Norrgren L. 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and japenese medaka after exposure to 17α-ethinylestradiol and 17β-trebolone. *Archives of Environmental Contamination and Toxicology* 51: 237-243

Oshima K. Nakajima H. Takahashi S. Kera Y. Shimomura M. Miyauchi S. 2005. Quartz crystalmicrobalance assay for determination of plasma vitellogenin. *Sensors and Actuators B* 105: 473-478

Pain S. Devin S. Parant M. 2007. Biomarker *versus* environmental factors: Seasonal variations and modeling multixenobiotic defense (MXD) transport activity in transplanted zebra mussels. *Science of the Total Environment* 373: 103-112

Palumbo A.J. Linares-Casenave J.L. Jewell W. Doroshov S.I. Tjeerdema R.S. 2007. Induction and partial characterization of California halibut (*Paralichthys californicus*) vitellogenin. *Comparative Biochemistry and Physiology A* 146: 200-207

Parks L.G. Cheek A.O. Denslow N.D. Heppell S.A. McLachlan J.A. LeBlanc G.A. Sullivan C.V. 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. *Comparative Biochemistry and Physiology C* 123: 113-125

Paull G.C. Van Look K.J.W. Santos E.M. Filby A.L. Gray D.M. Nash J.P. Tyler C.R. 2008. Variability in measures of reproductive success in laboratory-kept colonies of zebrafish and implications for studies addressing population-level effects of environmental chemicals. *Aquatic Toxicology* 87: 1-12

Penning T.M. Merry A.H. Munday K.A. Akhtar M. 1977. Studies on the biosynthesis, assembly and secretion of vitellogenin, an oestrogen-induced multicomponent protein. *Journal of Biochemistry* 162: 157-170

Perez L.E. and Callard I.P. 1992. Identification of vitellogenin in little skate (*Raja erinacea*). *Comparative Biochemistry and Physiology B* 103: 699-705

Peters L.D. and Livingstone D.R. 1995. Studies on cytochrome P4501A in early and adult life stages of sea turbot (*Scophthslmus maximus L.*). *Marine Environmental Research* 39: 5-9

Picton H. Briggs D. Gosden R. 1998. The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology* 145: 27-37

Pikkarainen AL. 2006. Ethoxy-*O*-deethylase (EROD) activity and bile metabolites as contamination indicators in Baltic Sea perch: determination by HPLC. *Chemosphere* 65: 1888-1897

Pina B. Casado M. Quiros L. 2007. Analysis of gene expression as a new tool in ecotoxicology and environmental monitoring. *Trends in Analytical Chemistry* 26: 1145-1154

Pinder L.C.V. Pottinger T.G. Billinghurst Z. and Depledge M.H. (1999). *Endocrine function in aquatic invertebrates and evidence for disruption by environmental pollutants.* Report No. E67, Plymouth Environmental Research Centre, Brussels.

Poelmans S. Verslycke T. Monteyne E. Noppe H. Verheyden K. Janssen C.R. Brabander. 2006. Testosterone metabolism in *Neomysis integer* following exposure to benzo (a) pyrene. *Comparative Biochemistry and Physiology* 144: 405-412

Porte C. Escartin E. De La Parra M.G. Biosca X. Albaiges J. 2002. Assessment of coastal pollution by combined determination of chemical and biochemical markers *Mullus barbatus. Marine Ecology Progress Series* 235: 205-216

Porte C. Janer G. Lorusso L.C. oritz-Zarragoitia M. Cajaraville M.P. Fossi M.C. Canesi L.2006. Endocrine disruptors in marine organisms: approaches and perspectives. *Comparative Biochemistry and Physiology C* 143: 303-315

Power D.M. Llewellyn L. Faustino M. Nowell M.A. Bjornsson B.T.H Einarsdottin I.E. Canario A.V.M Sweeney G.E. 2001. Review: Thyroid hormones in growth and development of fish. *Comparative Biochemistry and Physiology C* 130: 447-459

Powers J. Japp D. Tingley G. Hough A. 2004. *South African hake trawl fishery.* United Kingdom: Moody International Certification

Prakash O. Goswami S.V. Sehgal N. 2007. Establishment of ELISA for murrel vitellogenin and choriogenin, as biomarkers of potential endocrine disruption. *Comparative Biochemistry and Physiology C* 146: 540-551

Raisuddin S. and Lee J.S. 2008. Fish models in impact assessment of carcinogenic potential of environmental chemical pollutants: a appraisal of hermaphroditic mangrove killfish *Kryptolebias marmoratus*. *Interdisciplinary Studies on Environmental Chemistry- Biological Responses to Chemical Pollutants* 1: 7-15

Rees C.B. McCormick S.D. Heuvel J.P.V. Li. W. 2003. Quantitative PCR analysis of CYP1A induction in Atlantic salmon (*Salmo salar*). *Aquatic Toxicology* 62: 67-78

Rice C.D. Schlenk D. Ainsworth J. Goksoyr A. 1998. Cross-reactivity monoclonal antibodies against peptide 277-294 of rainbow trout CYP1A1 with hepatic CYP1A among fish. *Marine Environmental Research* 46: 87-91

Rikhter V.A. and Golubiatnikova P. 1997. Comparison of biology and abundance of Silver hake (*Merluccius bilinearis*) from Scotian Shelf and Cape hake (*Merluccius capensis*) from Namibia area. *NAFO Science Council Studies* 30: 57-63

Robinson M.L. Gomez-Raya L. Rauw W.M. Peacock M.M. 2008. Fultons body condition factor *K* correlates with survival time in a thermal challenge experiment in juvenile Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*). *Journal of Thermal Biology* 33: 363-368

Rodas-Oritz J.P. Ceja-Moreno V. Chan-Cocom M.E. Gold-Bouchot G. 2008. Vitellogenin induction and increased plasma 17β-estradiol concentrations in male nile tilapia, *Oreochromis niloticus*, exposed to organochlorine pollutants and polycyclic aromatics hydrocarbons. *Bulletin of Environmental Contamination and Toxicology* 81: 543-547

Rodriguez-Cea A. Del Rosario M. De La Campa F. Sanz-Medel A. 2004. Brown trout as a sentinel organism for organic pollution in the field using catalytic and immunochemical assays of cytochrome P-450 1A. *Journal of Environmental Monitoring* 6: 368-373

Rotchell J.M. Bird D.J. and Newton L.C. 1999. Seasonal variation in ethoxyresorufin O-deethylase (EROD) activity in European eels *Anguilla anguilla* and flounders *Pleuronectes flesus* from the Severn Estuary and Bristol Channel. *Marine Ecology Progress Series* 190: 263-270

Sanchez W. and Porcher J.M. 2009. Fish biomarkers for environmental monitoring within the water framework directive of the European Union. *Trends in Analytical Chemistry* 28: 150-158

Sanchez W. Goin C. Brion F. Olsson P.E. Goksoyr A. Porcher J.M. 2008. A new ELISA fro the three-spined stickleback (*Gasterosteus aculeatus L.*) spiggin, using antibodies against synthetic peptide. *Comparative Biochemistry and Physiology C* 147: 129-137

Sanchez W. Palluel O. Lagadic L. Ait-Aissa S. Porcher J.M. 2008. Biochemical effects of nonylphenol polyethoxylate adjuvant, diquat herbicide and their mixture on the three-spined stickleback. *Marine Environmental Research* 62: 29-33

Sarasquete C. and Segner H. 2000. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. *The Science of the Total Environment* 247: 313-332

Schlenk D. 1999. Necessity of defining biomarkers for use in ecological risk assessments. *Marine Pollution Bulletin* 39: 48-53

Schlezinger J.J. and Stegeman J.J. 2001. Induction and suppression of cytochrome P4501A by 3, 3', 4, 4', 5-pentachlorobiphenyl and its relationship to oxidative stress in the marine fish scup (*Stenotomus chrysops*). *Aquatic Toxicology* 52: 101-115

Schulz R.W. Vischer H.F. Cavaco J.E.B. Santos E.M. Tyler C.R. Goos H.J.Th. Bogerd J. 2001. Gonadotrophins their receptors, and the regulation of testicular functions in fish. *Comparative Biochemistry and Physiology B* 129: 407-417

Scott A.P. Katsiadaki I. Witthames P.R. Hylland K. Davies I.M. McIntosh A.D. Thain J. 2006. Vitellogenin in the blood plasma of male cod (*Gadus morhua*): a sign of oestrogenic endocrine disruption in the open sea. *Marine Environmental Research* 61: 149-170

Scott G.R. and Sloman K.A. 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology* 68: 369-392

Sebire M. Allen Y. Bersuder P. Katsiadaki I. 2008. The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. *Aquatic Toxicology* 90: 37-47

Shailaja M.S. Rajamanickam R. Wahidulla S. 2006. Increased formation of carcinogenic PAH metabolites in fish promoted by nitrile. *Environmental Pollution* 143: 174-177

Shannon L.J. Moloney C.L. Jarre A. Field J.G. 2003. Trophic flows in the southern Benguela during the 1980s and 1990s. *Journal of Marine systems* 39: 83-116

Shao J. Shi G. Song M. Jiang G. 2005. Development and validation of an enzyme-linked immunosorbent assay for vitellogenin in Chinese loach (*Misgurnus angaillicaudatus*). *Environmental International* 31: 763-770

Sherry J. Gamble A. Fielden M. Hodson P. burnison B. Solomon K. 1999. An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. *The Science of the Total Environment* 225: 13-31

Silvano R.A.M. and Begossi A. 2005. Local knowledge on a cosmopolitan fish ethnoecology of *Pomatomus saltatrix* (Pomatomidae) in Brazil and Australia. *Fisheries Research* 71: 43-59

Singleton D.W. and Khan S.A. 2003. Xenoestrogen exposure and mechanisms of endocrine disruption. *Frontiers in Biosciences* 8: 110-118

Siroka Z. and Drastichova J. 2004. Biochemical markers of aquatic environment contamination-cytochrome P450 in fish-a review. *Acta Veterinaria BRNO* 73: 123-132

Smith A.K. and Suthers I.M. 1999. Effects of sewage effluent discharge on the abundance, condition and mortality of hulafish, *Trachinops taeniatus* (Plesiopidae). *Environmental Pollution* 106: 97-106

Sole M. Raldua D. Piferrer F. Barcelo D. Porte C. 2003. Feminization of wild carp, *Cyprinus carpio*, in a polluted environment: plasma steroid hormones, gonadal morphology and xenobiotic metabolizing system. *Comparative Biochemistry and Physiology C* 136: 145-156

Sole M. Rodriguez S. Papiol V. Maynou F. Cartes J.E. 2009. Xenobiotic metabolism markers in marine fish with different trophic strategies and their relationship to ecological variables. *Comparative Biochemistry and Physiology C* 149: 83-89

Song M. Lv X. Wang H. Jiang G. 2008. Fast purification of trace vitellogenin from chinese rare minnow using protein A-immobilized antibody. *Analytical and Bioanalytical Chemistry* 390: 2151-2157

Soverchia L. Ruggen B. Palmero F. Mosconi G. Cardinaletti G. Scortichini G. Gatti G. Polzonetti-Magni A.M. 2005. Modulation of vitellogenin synthesis through estrogen receptor beta-1 in goldfish (*Carassius auratus*) juveniles exposed to 17β-estradiol and nonylphenol. *Toxicology and Applied Pharmacology* 209: 236-243

Stagg R.M. and Addison R.F. 1994. An inter-laboratory comparison of measurements of ethoxy-*o*-deethylase activity in Dab (*limanda limanda)* liver. *Marine Environment Research* 40: 93-108

Stegeman J.J. and Lech J.J. 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environmental Health Perspectives* 90: 101-109

Steward A.R. Kandaswami C. Chidambaram S. Ziper C. Rutkowski J.P. Sikka H.C. 1991. Disposition and metabolic fate of benzo[a]pyrene in the common carp. *Aquatic Toxicology* 20: 205-218

Sun L. Zha J. Wang Z. 2009. Interactions between estrogenic chemicals in binary mixtures investigated using vitellogenin induction and factorial analysis. *Chemosphere* doi: 10.1016/j.chemosphere.2008.11.083

Takahashi N. Miranda C.L. Henderson M.C. Buhler D.R. Williams D.E. Bailey G.S. 1995. Inhibition of *in vitro* aflatoxin B_1-DNA binding in rainbow trout by CYP1A inhibitors: α-napthoflavone, β-napthoflavone and trout CYP1A peptide antibody. *Comparative Biochemistry and Physiology C* 110: 273-280

Tamara S. Galloway R.J. Brown M.A. Browne A.D. Malcom D.L. Jones B. Depledge M.H. 2004. A multibiomarker approach to environmental assessment. *Environmental Science & Technology* 38: 1723-1731

Tenniswood M.P.R. Searle P.F. Wolffe A.P. Tata J.R. 1983. Rapid estrogen metabolism and vitellogenin gene expression in *Xenopus* hepatocyte cultures. *Molecular and Cellular Endocrinology* 30: 329-345

Ternes T.A. Stumpf M. Mueller J. Haberer K. Wilken R.D. Servos M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants- I. investigations in Germany, Cananda and Brazil. *The Science of the Total Environment* 225: 81-90

Thilakaratne I.D.S.I.P. McLaughlin J.D. Marcogliese D.J. 2007. Effects of pollution and parasites on biomarkers of fish health in spottail shiners *Notropis hudsonius* (Clinton). *Journal of Fish Biology* 71: 519-538

Thomas P. 1989. Effects of aroclor 1254 and cadmium on reproductive endocrine function and ovarian growth in atlantic croaker. *Marine Environmental Research* 28: 499-503

Thomas P. 2000. Chemical interference with genomic and nongenomic actions of steroids in fishes: role of receptor binding. *Marine Environmental Research* 50: 127-134

Tom D.J. Lee L.E.J. Lew J. Bols N.C. 2001. Induction of 7-ethoxyresorufin-*O*-deethylase activity by planar chlorinated hydrocarbons and polycyclic aromatic hydrocarbons in cell lines from rainbow trout pituitary. *Comparative Biochemistry and Physiology A* 128: 185-198

Tom M. and Auslander M. 2005. Transcript and protein environmental biomarkers in fish- a review. *Chemosphere* 59: 155-162

Tom M. Myers C.R. Waterman M.R. 2002. Evaluating molar CYP1A level in fish hepatic microsomes by competitive ELISA using recombinant membrane-free CYP1A standard protein. *Aquatic Toxicology* 59: 101-114

Tsukimura B. Bender J.S. Linder C.J. 2000. Development of anti-vitellin ELISA for the assessment of reproduction in the ridgeback shrimp. *Comparative Biochemistry and Physiology A* 127: 215-224

Tyler C.R. Pottinger T.G. Coward K. Prat F. Beresford N. Maddin S. 1997. Salmonid follicle stimulating hormone (GTHI) mediates vitellogenic development of oocytes in the rainbow trout, *Oncorhyncus mykiss. Biology of Reproduction* 57: 1238-1244

Tyler C.R. Sumpter J.P. Witthames P.R. 1990. The dynamics of oocyte growth during vitellogenesis in the rainbow trout (*Oncorhyncus mykiss*). *Biology of Reproduction* 43: 202-209

Ueng Y.F. Liu T.Y. Ueng T.H. 1995. Induction of cytochrome P450 1A1 and monooxygenase activity in Tilapia by sediment extract. *Bulletin of Environmental Contamination and Toxicology* 54: 60-67

Van Der Oost R. Beyer J. Vermeulen N.P.E. 2003. A review: fish bioaccumulation and biomarkers in environmental risk assessment. *Environmental Toxicology and Pharmacology* 13: 57-149

Van Veld P.A. Vogelbein W.K. Cochran M.K. Goksoyr A. Stegeman J.J. 1997. Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. *Toxicology and Applied Pharmacology* 142: 348-359

Vari R.P. 1978. The Terapon perches (Percoidei, Terapondae), a cladistic analysis and taxanomic revision. *Bulletin of American Museum of National History* 159: 175-340

Viganno L. Arillo A. De Flora S. Lazorchak J. 1995. Evaluation of microsomal and cytosolic biomarkers in a seven-day larval trout sediment toxicity test. *Aquatic Toxicology* 31: 189-202

Vijayavel K. and Balasubramanian M.P. 2008. Reproductive dysfunction induced by naphthalene in an estuarine crab *Scylla serrata* with reference to vitellogenesis. *Ecotoxicology and Environmental Safety* 69: 89-94

Vindimian E. Namour P. Migeon B. Garric J. 1991. In situ pollution induced cytochrome P450 activity of freshwater fish: barbell (*Barbus barbus*), chub (*Leuciscus cephalus*) and nase (*Chondrostoma nasus*). *Aquatic Toxicology* 21: 255-266

Vindimian E. and Garric J. 1989. Freshwater fish cytochrome P450-dependent enzymatic activities: a chemical pollution indicator. *Ecotoxicology and Environmental Safety* 18: 277-285

Voges E. Gordoa A. Bartholomae C.H. Field J.G. 2002. Estimating the probability of different levels of recruitment for Cape hakes *Merluccius capensis* off Namibia using environmental indices. *Fisheries Research* 58: 333-340

Von der Heyden S. Lipinski M.R. Matthee C.A. 2007. Mitrochondrial DNA analyses of the Cape hakes reveal an expanding panmictic population for *Merluccius capensis* and population structuring for mature fish in *Merluccius paradoxus. Molecular Phylogenetics and Evolution* 42: 517-527

Wallace R.A. and Begovac P.C. 1985. Phosvitins in *Fundulus* oocytes and eggs, preliminary chromatographic and electrophoretic analyses together with biological considerations. *The Journal of Biological Chemistry* 260: 11268-11274

Wan X. Ma T. Wu W. Wang Z. 2004. EROD activities in a primary cell culture of grass carp (*Ctenopharyngodon idellus*) hepatocytes exposed to polychlorinated aromatic hydrocarbons. *Ecotoxicology and Environmental Safety* 58: 84-89

Watts M. Pankhurst N.W. Pryce A. Sun B. 2003. Vitellogenin isolation, purification and antigenic cross-reactivity in three teleost species. *Comparative Biochemistry and Physiology B* 134: 467-476

Wells P.G. 1999. Biomonitoring the health of coastal marine ecosystems- the roles and challenges of microscale toxicity tests. *Marine Pollution Bulletin* 39: 39-47

Weltzien F.A. Andersson E. Anderson O. Schalchian-Tabrizi K. Norberg B. 2004. The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (*Pleuronectiformes*). *Comparative Biochemistry and Physiology A* 137: 447-477

Werawatgoompa S. Piyatiratitivorakul S. Nitithamyong C. Aranyakanonda P. Moree N. Vajanamarhutue C. Ruangvejivorachai P. Menasveta P. 1997. Plasma vitellogenin and growing oocytes of grouper (*Cephalopholia pachycentron*). *Journal of Marine Biotechnology* 5: 137-141

Westerhagen H.V. Kruner G. Broeg K. 1999. Ethoxyresorufin-*O*-deethylase (EROD) activity in the liver of dab (*Limanda limanda L.*) and flounder (*Platichthys flesus L.*) from the German Bight. EROD expression and tissue contamination. *Hegol Marine Research* 53: 244-249

White R.D. Shea D. Solow A.R. Stegeman J.J. 1997. Induction and post-transcriptional suppression of hepatic cytochrome P450 1A1 by 3,3',4,4'-tetrachlorobiphenyl. *Biochemical Pharmacology* 53: 1029-1040

Wiley H.S. and Wallace R.A. 1981. The structure of vitellogenin. *The Journal of Biological Chemistry* 256: 8626-8634

Wiley H.S. and Wallace R.A. 2008. The structure of vitellogenin, multiple vitellogenins in *Xenopus laevis* give rise to multiple forms of the yolk proteins. *The Journal of Biological Chemistry* 256: 8626-8634

Williams D.E. and Buhler D.R. 1983. Comparative properties of purified cytochrome P-448 from β-napthoflavone treated rats and rainbow trout. *Comparative Biochemistry and Physiology C* 75: 25-32

Williams D.E. Lech J.J. Buhler D.R. 1998. Xenobiotics and xenoestrogens in fish: modulation of cytochrome P450 and carcinogenesis. *Mutation Research* 399: 179-192

Winn R.N. 2001. Transgenic fish models in environmental toxicology. *Institute for Laboratory Animal Research Journal* 42: 322-329

Won S.J. Novillo A. Custodia N. Rie M.T. Fitzgerald K. Osada M. and Callard A.I.P. 2005. The freshwater mussel (*Ellipito complanata)* as a sentinel species: Vitellogenin and steroid receptors. *Integrated & Comparative Biology*. 45: 72-80

Wood W. and Kraak G. 2001. Apoptosis and ovarian function: novel perspectives from the teleosts. *Biology of Reproduction* 64: 264-271

Wootton A.N. Goldfarb P.S. Lemaire P. O'Hara S.C.M. Livingstone D.R. 1996. Characterisation of the presence and seasonal variation of CYP1A-like enzyme in digestive gland of the common mussel *Mytilus edulis*. *Marine Environmental Research* 42: 297-301

Wootton A.N. Herring C. Spry J.A. Wiseman A. Livingstone D.R. Goldfarb P.S. 1995. Evidence for the existence of cytochrome P450 gene families (CYP1A, 3A, 4A, 11A) and modulation of gene expression (CYP1A) in the mussel *Mytilus* spp. *Marine Environmental Research* 39: 21-26

Wozny M. Brzuzan P. Luczynski M.K. Gora M. Bidzinska J. Jurkiewicz P. 2008. Effects of cyclopenta[c]phenanthrene and its derivatives on zona radiate protein, ERα, and CYP1A mRNA expression in liver of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Chemico-Biological Interactions* 174: 60-68

Yadetie F. Arukwe A. Goksoyr A. Male R. 1999. Induction of hepatic estrogen receptor in juvenile atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. *The Science of the Total Environment* 233: 201-210

Yamauchi R. Ishibashi H. Hirano M. Mori T. Kim J.W. Arizono K. 2008. Effects of synthetic polycyclic musks on estrogen receptor, vitellogenin, pregnane X receptor, and cytochrome P450 3A gene expression in the livers of male medaka (*Oryzias latipes*). *Aquatic Toxicology* 90: 261-268

Yang L. Lin L. Weng S. Feng Z. Luan T. 2008 Sexually disrupting effects of nonylphenol and diethylstilbestrol on male Silver carp (*Carassius auratus*) in aquatic microcosms. *Ecotoxicology and Environmental Safety* doi: 10.1016/j.ecoenv.2008.01.007

Yaron Z. 1995. Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture* 129: 49-73

You L. 2004. Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. *Chemico-Biological Interactions* 147: 233-246

Zabel E.W. Cook P.M. Peterson R.E. 1995. Toxic equivalency factors of polychlorinated dibenzo-*p*-dioxin, debenzofuran and biphenyl congeners based on early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). 31: 315-328

Zaccaroni A. Gamberoni M. Mandrioli L. Sirri R. Mordenti O. Scaravelli D. Sarli G. Parmeggiani A. 2009. Thyroid hormones as a potential early biomarker of exposure to 4-nonylphenol in adult male shubunkins (*Carassius auratus*). *Science of the Total Environment* 407: 3301-3306

Zapata-Perez O. Del-Rio M. Dominguez R.C. Ceja V. Gold-Bouchot G. 2005. Preliminary studies of biochemical changes (ethoxycoumarin-*O*-deethylase activities and vitellogenin induction) in two species of shrimp (*Farfantepenaeus duorarum* and *Litopenaeus setiferus*) from Gulf of Mexico. *Ecotoxicology and Environmental Safety* 61: 98-104

Zhang F. Bartels M.J. Brodeur J.C. Woodburn K.B. 2004. Quantitative measurement of fathead minnow vitellogenin by liquid chromatography combined with tandem mass spectrometry using a signature peptide of vitellogenin. *Environmental Toxicology and Chemistry* 23: 1408-1411

Appendix I: Standard curves for the determination of protein concentration and EROD activity

I.I *Protein standard curve*

A lower (0-0.12 mg/ml) and higher range (0-1.60 mg/ml) protein standard curve was generated according to a modified Braford protein assay, first described by Bradford (1976) (Figure I.I and I.II). Bovine serum albumin (BSA) was used as a reference protein and results were determined at 595nm using a Powerwave$_x$ spectrophotometer with KC junior software.

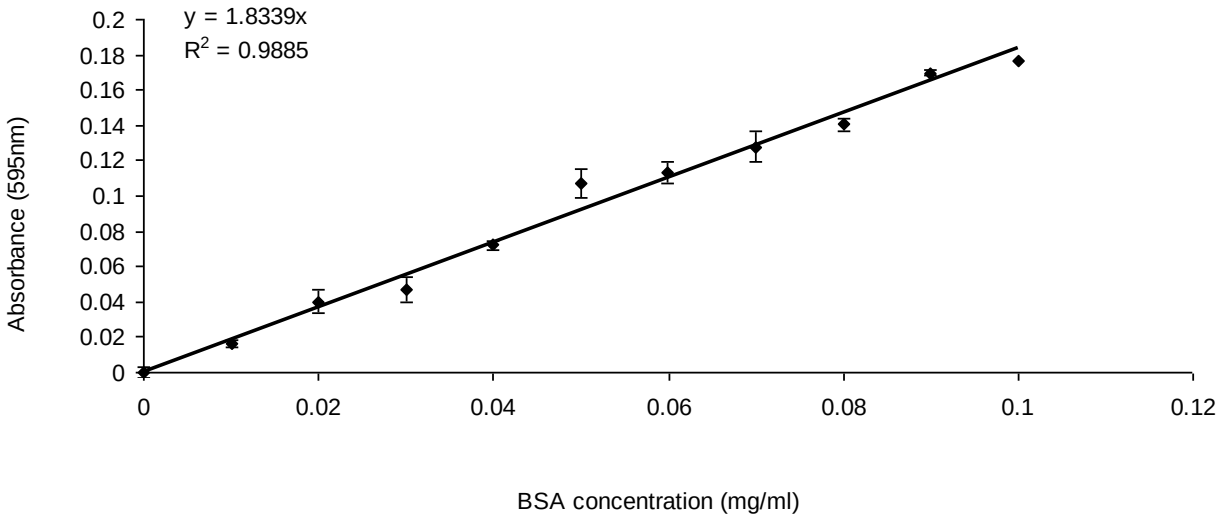


Figure I.I: A lower range protein standard curve using varying concentrations of the reference protein BSA. The standard curve was generated in the computer package Microsoft Excel (SD± 3)

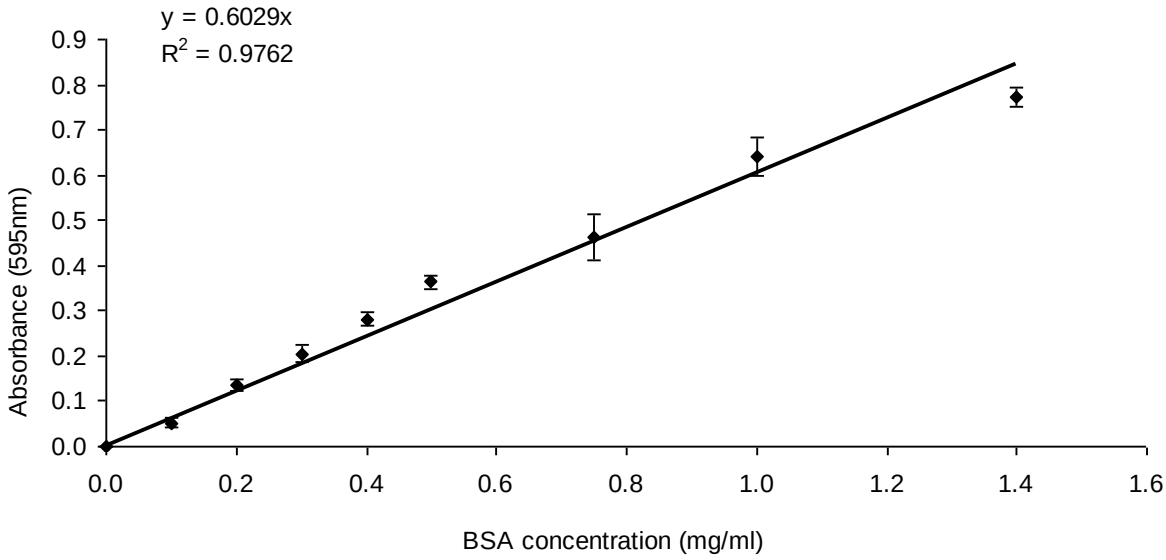


Figure I.II: A higher range protein standard curve using varying concentrations of the reference protein BSA. The standard curve was generated in the computer package Microsoft Excel (SD± 3)

I.II *EROD assay standard curve*

EROD assay standard curves were generated according to the method described by Pikkarainen *et al.* (2006). Resorufin (the product of the enzyme reaction) was used a the reference compound.

Lower (0- 120 pmol) and higher (0- 250 pmol) range standard curves (Figure I.III and I.IV) for fluorimetric studies were analyzed using a Hitachi spectrofluorimeter F2500 with excitation and emission wavelengths of 510 and 585 nm respectively. The standard curve produced for spectrophotometric studies was analyzed at 512nm in a Powerwave$_x$ spectrophotometer using KC junior software (Figure I.V)

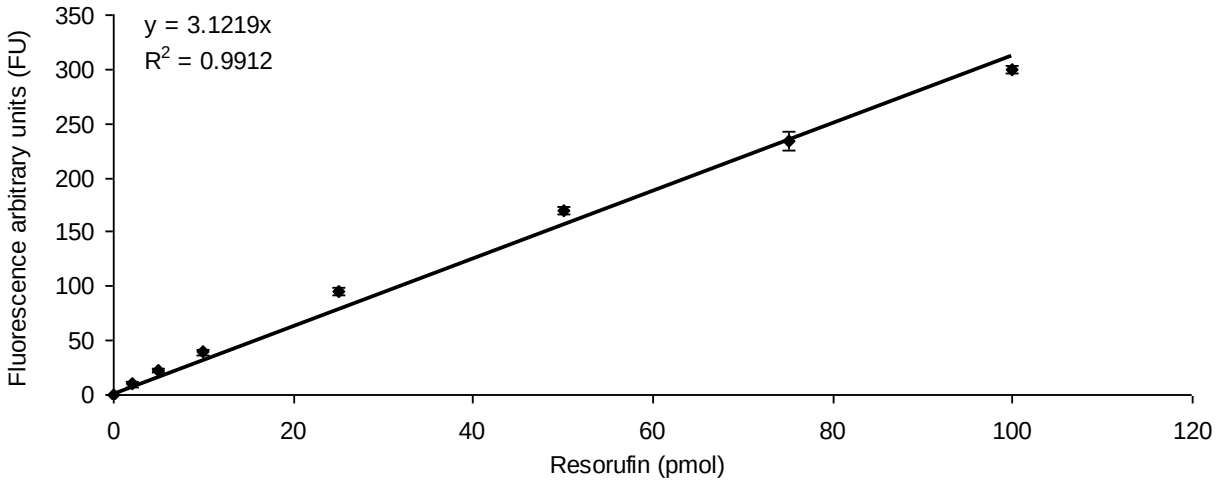


Figure I.III: The lower range EROD assay standard curve produced for fluorimetric studies at varying resorufin (pmol) concentrations. The standard curve was analyzed at emission and excitation wavelengths of 510 and 585nm and generated in the computer package Microsoft Excel (SD± 3).

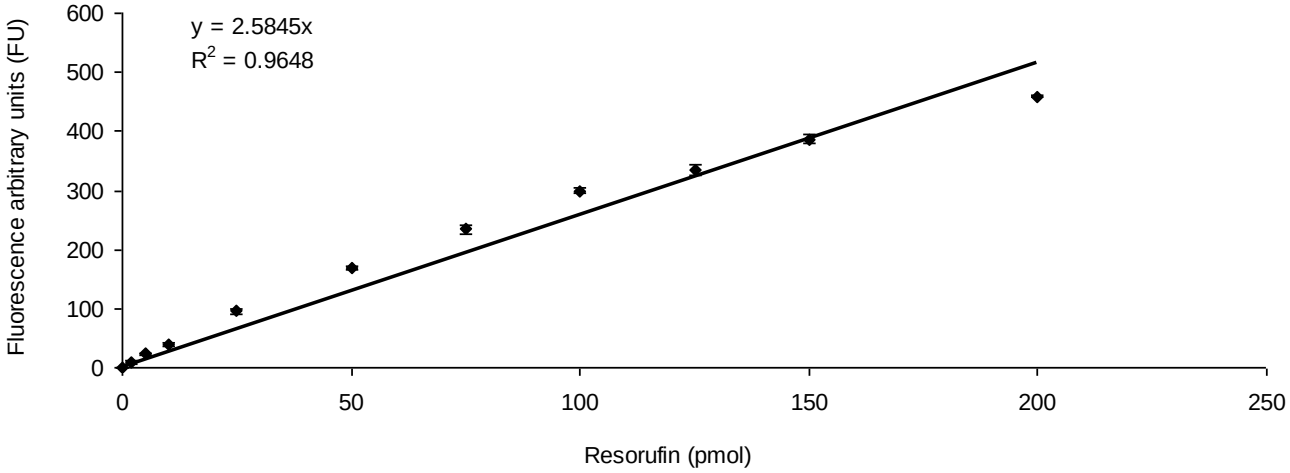


Figure I.IV: The higher range EROD assay standard curve produced for fluorimetric studies at varying resorufin (pmol) concentrations. The standard curve was analyzed at emission and excitation wavelengths of 510 and 585nm and generated in the computer package Microsoft Excel (SD± 3).

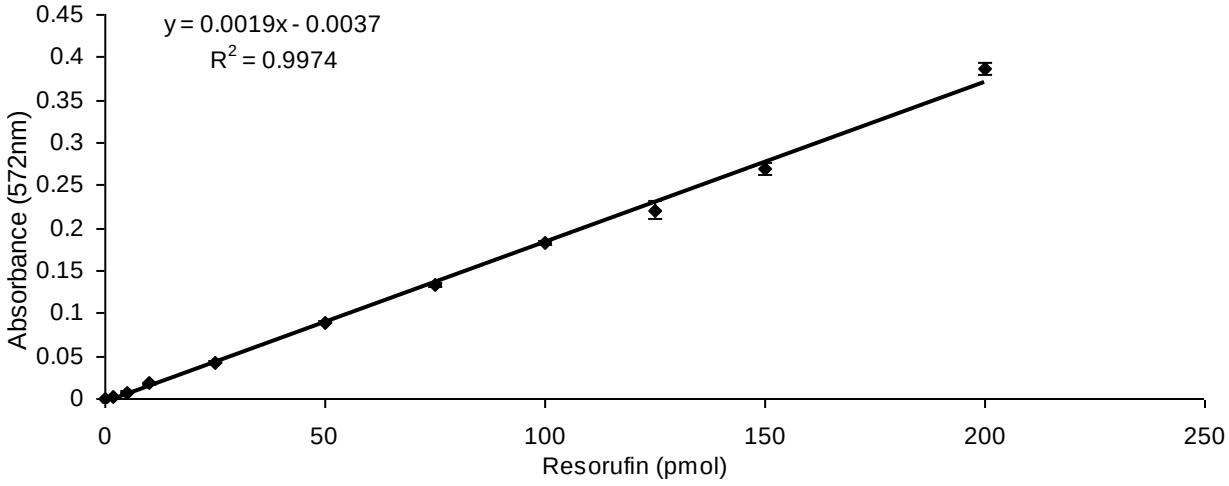


Figure I.V: The EROD assay standard curve produced for spectrophotomeric studies at varying resorufin (pmol) concentrations. The standard curve was analyzed at 572nm and genereated in the computer package Microsoft Excel (SD± 3).

Appendix II: Chemicals and suppliers

2-[4-(hydroxyethyl)-1-piperazinyl-ethanesulfonic acid (HEPES)	Merck (Cat. No. 1.10110.0250)
7-ethoxyresorufin	Sigma (Cat. No. E3763)
Acetic acid	Merck (Cat. No. 1031148)
Acetone	Merck (Cat. No. 8.22251.2500)
Acrylamide	Sigma (Cat. No. A8887)
Ammonium persulfate(APS)	Sigma (Cat. No. A3678)
Aprotinin	Sigma (Cat. No. A1153)
Atlantic salmon vtg	Biosense lab (Cat. No. V01002301-001)
BM chemiluminescence western blotting kit (mouse/rabbit)	Roche (Cat. No. 11 520 709001)
Bovine serum albumin (BSA)	Sigma (Cat. No. A7906)
Bradfords reagent	Sigma (Cat. No. B6916)
Bromophenol blue	Sigma (Cat. No. B8026)
Coomassie brilliant blue R-250	Merck (Cat. No. 112553)
Dicumarol	Sigma (Cat. No. M1390)
DL- isocitric acid trisodium salt	Sigma (Cat. No. I1252)
DL-Dithiothreitol	Sigma (Cat. No. D9163)
Ethanol	Merck (Cat. No. 1.00971.2500)
Ethylenediamine tetracetic acid disodium salt (EDTA)	Merck (Cat. No. 223.60.20)
Glycerol	Merck (Cat. No. 1032361)
Glycine	Merck (Cat. No. 1.04169.1000)
Heparin sodium salt	Sigma (Cat. No. H5515)
Hydrochloric acid	Merck (Cat. No. H1758)

Magnesium sulphate	Merck (Cat. No. 412.40.00)
Methanol	Merck (Cat. No. 8.22283.2500)
N,N,N',N'- tetramethylethylene- diamine (TEMED)	Sigma (Cat. No. T9281)
Nicotinamide adenine dinucleotide phosphate (NADPH)	Calbiochem (Cat. No. 481973)
Nitrocellulose membrane	Sigma (Cat. No. N8267)
Page Silver^{TM} silver staining kit	Fermentas (Cat. No. K0681)
Phenylmethanesulfonyl fluoride (PMSF)	Fluka (Cat. No. 78830)
Polyethyleneglycol 20 000 (PEG)	Merck (Cat. No. 8.18897.5000)
Ponceau S red	Sigma (Cat. No. P3504)
Rabbit anti-fish CYP1A peptide	Biosense lab (Cat. No. C02401201-500)
Rabbit anti-salmon vtg	Biosense lab (Cat. No. V01402201-100)
Resorufin	Sigma (Cat. No. R3257)
Sodium chloride	Merck (Cat. No. 1.06404.1000)
Sodium deoxycholate monohydrate	Sigma (Cat. No. 238392)
Sodium dodecyl sulfate(SDS)	Merck (Cat. No. 8.17034.1000)
Sodium hydroxide	Merck (Cat. No. 1.06469.1000)
Trichloroacetic acid	Merck (Cat. No. 1.00807.0250)
Tris (hydroxymethyl)- aminomethane	Merck (Cat. No. 1.08382.1000)
Tween 20	Merck (Cat. No. 8.22184.0500)
β- mercaptoethanol	Fluka (Cat. No. 63700)