OXYGEN-DEPENDENT METABOLISM OF ORGANIC XENOBIOTICS

IN MARINE FISH AND MUSSEL

by

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Studies were undertaken to examine the processes of oxygen-dependent organic xenobiotic metabolism in fish and mussel. The antioxidant enzymes superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), selenium-dependent glutathione peroxidase (SeGPX; EC 1.11.1.9), glutathione reductase (EC 1.6.4.2), DT Diaphorase (quinone oxidoreductase; EC 1.6.99.2), total GPX activity (sum of SeGPX and Se-independent GPX activities) and levels of cytochrome P450 (CYP) were determined in 13500g supernatants of embryos, 3-day and 11-day post-hatch larvae of turbot Scophthalmus maximus. The early life stages of S. maximus demonstrated the potential to detoxify reactive oxygen species (ROS), organic peroxides and redox cycling compounds. The in vitro capacity of larval 13500g supernatants to generate ROS was quantified and basal rates were stimulated up to 3 fold by redox cycling compounds. Exposure to benzo[a]pyrene (BaP) increased CYP1A protein levels and 7-ethoxyresorufin O-deethylase (EROD) activity in larval and juvenile life stages. BaP was metabolised by 4-day post-hatch larvae to phenols (54%), dihydrodiols (32%) and diones (quinones, 14%) and DNA adducts were formed when juvenile S. maximus were exposed to 25 ppb BaP. EROD and antioxidant enzyme activities were measured in feral sardine Sardina pilchardus larvae from the north coast of Spain.

Western analysis of microsomes and partially purified cytochrome P450 (CYP) from digestive gland of mussel Mytilus edulis was undertaken using polyclonal antibodies to hepatic perch Perca fluviatilis CYP1A, rainbow trout Oncorhynchus mykiss CYP3A and rat CYP2B, CYP2E and CYP4A isoforms. The apparent molecular weights in kD (mean ± range or SD; n = 2-4) for partially purified CYP were 42.5 ± 0.5 and 48.1 ± 0.3 (2 bands, anti-CYP1A); 67.4 ± 0.7, 52.8 ± 0.6 and 44.5 ± 2.5 (3 bands, anti-CYP3A); 52.8 ± 0.7, 48.1 ± 1.1 and 43.9 ± 1.1 (3 bands, anti-CYP2B); 52.7 ± 0.8 and 47.2 ± 0.2 (2 bands, anti-CYP2E); 50.9 ± 0.3 and 44.1 ± 0.2 kD (2 bands, anti-CYP4A). Digestive gland microsomes of indigenous M. galloprovincialis from a polluted compared to a clean field site showed higher levels of bands recognised by anti-CYP1A, anti-CYP2E and anti-CYP4A, but not anti-CYP2B and anti-CYP3A (P < 0.05), indicative of independent regulation of different CYP forms. Three weeks after transplantation from a clean to a polluted site, levels of only the CYP1A-immunopositive protein were determined to be higher (63 %) than levels for the clean site (P < 0.05), indicating that the anti-CYP1A antibody showed greater specificity for a contaminant-inducible CYP form than the other antibodies. Overall, the apparent molecular weight and field studies indicate at least five different digestive gland CYP forms.

Hepatic flavin-dependent monoxygenase (FMO; EC 1.14.13.8) dimethylamine N-oxidase activity was characterised in S. maximus. The presence of FMO1 and FMO2 were indicated by immunorecognition of single bands (c55 kD) using anti-pig liver FMO1 and anti-rabbit lung FMO2 antibodies. Tissue specific differential expression and activity were determined in the euryhaline flounder Platichthys flesus and a correlation between salinity and branchial FMO activity but not cytochrome P450 content was observed and indicated a potential osmoregulatory role for FMO.
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### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>'NO</td>
<td>Nitric oxide radical</td>
</tr>
<tr>
<td>'OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>'O₂</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>AHH</td>
<td>Aryl hydrocarbon hydroxylase</td>
</tr>
<tr>
<td>BaP</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>CYP</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DMA</td>
<td>N,N-Dimethylalanine</td>
</tr>
<tr>
<td>DMA ox.</td>
<td>N,N-Dimethylalanine N-oxidase</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EROD</td>
<td>7-Ethoxyresorufin O-deethylase</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide (oxidised form)</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin-containing monooxygenase</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Gluathione (reduced form)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione (oxidised form)</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCB</td>
<td>Hexachlorobiphenyl</td>
</tr>
<tr>
<td>HO₂⁻</td>
<td>Hydroperoxyl radical</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hyperchlorous acid</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>L·</td>
<td>Alkyl radical</td>
</tr>
<tr>
<td>LO·</td>
<td>Alkoxy radical</td>
</tr>
<tr>
<td>LOO·</td>
<td>Alkylperoxyl radical</td>
</tr>
<tr>
<td>MFO</td>
<td>Mixed function oxidase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>NADPH and NADH</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide (oxidised form)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADP+</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidised form)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NC-IUBMB</td>
<td>Nomenclature committee of the international union of biochemistry and molecular biology.</td>
</tr>
<tr>
<td>NO₂·</td>
<td>Nitrogen dioxide radical</td>
</tr>
<tr>
<td>non-SeGPX</td>
<td>Selenium-independent glutathione peroxidase</td>
</tr>
<tr>
<td>O₂·⁻</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>O₃</td>
<td>Ozone</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite ion</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PCBs</td>
<td>Polychlorobiphenyls</td>
</tr>
<tr>
<td>PCDD</td>
<td>Pentachlorodibeno-p-dioxin</td>
</tr>
<tr>
<td>RLS</td>
<td>Rate limiting step</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RO·</td>
<td>Alkoxy radical</td>
</tr>
<tr>
<td>ROO·</td>
<td>Alkylperoxyl radical</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SeGPX</td>
<td>Selenium-dependent glutathione peroxidase</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TCB</td>
<td>Tetrachlorobiphenyl</td>
</tr>
<tr>
<td>TCDD</td>
<td>Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCDF</td>
<td>Tetrachlorodibenzofuran</td>
</tr>
<tr>
<td>Thiourea ox.</td>
<td>Thiourea oxidase</td>
</tr>
<tr>
<td>TMA</td>
<td>N.N.N-Trimethylamine</td>
</tr>
<tr>
<td>TMA ox.</td>
<td>N.N.N-Trimethylamine N-oxidase</td>
</tr>
<tr>
<td>TMAO</td>
<td>N.N.N-Trimethylamine N-oxide</td>
</tr>
</tbody>
</table>
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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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External contributions to this thesis:

Section 2.4 Experimental details concerning *Scophthalmus maximus* larval metabolism of radiolabelled benzo[a]pyrene were undertaken and described by the co-author of the paper, S.C.M O'Hara. Manuscript preparation and all other experimental details were undertaken by L.D.Peters.

Section 2.5 Experimental details concerning $^{32}$P postlabelling of *S. maximus* DNA adducts were undertaken and described by the co-authors of the paper, R.H. Morse and R.Waters. Manuscript preparation and all other experimental details were undertaken by L.D.Peters.

Section 2.6 Experimental details concerning the chemical analysis of the frozen mixed zooplankton were undertaken and described by the co-authors of the paper, C. Porte and J. Albigés. Manuscript preparation and all other experimental details were undertaken by L.D.Peters.

Section 3.2 The transplantation of *Myltilus galloprovincialis* in the Venice lagoon was performed by the co-author, C. Nasci. Indigenous and transplanted *M. galloprovincialis* were collected by L.D. Peters and the tissue was dissected by the both of us. All subsequent analysis was undertaken at Plymouth Marine Laboratory. Manuscript preparation and all other experimental details were undertaken by L.D.Peters.

Section 4.2, 4.3 and 4.4 Experimental details concerning the FMO Northern blot analysis of fish tissue were undertaken and described by the co-authors of the paper, S. Shenin-Johnson and R.N. Hines. FMO Western blot analysis was performed by L.D.Peters and the enzyme activities were determined by D. Schlenk and L.D. Peters. Manuscripts were prepared by the first author, viz.
Regular scientific seminars and conferences were attended at which work was often presented; external institutions were visited and at least 12 papers have been submitted or accepted for publication in peer reviewed journals. The publications, abstracts at meetings, meetings attended and presentations made relating to this thesis are as follows:

Publications: 12


Meetings attended: 11

9th meeting of Pollution Responses in Marine Organisms (PRIMO), Bergen Norway, 1997
Biomar Workshop, Ischia, Italy, 1997.
Aquatic life cycles strategies, Plymouth, UK, 1997
Institute of Fisheries Management, York, UK, 1996.
Biological markers of pollution, Chinon, France, 1995.
Marine Biology: Molecular and Genetic Advances, Plymouth, UK, 1995
15th meeting of European Society for Comparative Physiology and Biochemistry (ESCPB), Genova, Italy, 1994.
7th meeting of Pollution Responses in Marine Organisms (PRIMO) Göteborg, Sweden, 1993.

Presentations: 9


At Teaching workshops: 3

Determination of 7-ethoxyresorufin O-deethylase (EROD) activity in fish. TACIS Black Sea training workshop, Plymouth, UK, 1996.


I. The mixed function oxygenase (MFO) system in mammals and fish: laboratory and field studies. II. Oxidative stress and its relevance to disease in marine species.

Invited seminars and lectures: 2

Fish larval biomarkers of pollution, Biologische Anstalt Helgoland, Hamburg, Germany, 1993.

Biochemical indicators of pollution. Institute of Fisheries Management, York, UK, 1996.

At meetings where papers were presented: 3


Signed... ........................................
Date... 1st Dec. 1997
CHAPTER 1 INTRODUCTION

1.1 ORGANIC XENOBIOTICS IN THE MARINE ENVIRONMENT

The number of organic compounds synthesised by man totals over 1.8 million and is growing at a rate of at least 250,000 new formulations per year (Zhou et al., 1996). Global production of these anthropogenic organics has been estimated to be 100-200 million tons per year and up to one third of this production may enter into the environment (Stumm & Morgan 1981). Via airborne and aquatic distribution, many of these chemicals have the potential to exert deleterious biological effects on individual organisms, communities or ecosystems (Walker & Livingstone 1992; Walker et al., 1996a). Of the 28,000 water pollution incidents reported in England and Wales during 1991, over 22,000 of them had significant effects on aquatic systems, 23% relating to oil discharges and 12% from industrial sources (Abel 1996). Of the numerous contaminants entering the marine environment, some organic xenobiotics are regarded as posing an important hazard to aquatic systems (Scientific symposium on the North Sea Quality Status Report, 1993). Polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and insecticides are just a few examples of a diverse group of chemicals identified in the marine environment and are currently the subject of many studies concerning the biological effects of pollutants (Varanasi 1989; Walker & Livingstone 1992; Walker et al., 1996a). Such lipophilic compounds are readily taken up into the tissues of aquatic invertebrates (Livingstone 1992), fish (Varanasi et al., 1989; Varanasi et al., 1992), marine mammals (Boon et al., 1992; Tanabe & Tatukawa 1992) and birds (Elliott et al., 1992; Renzoni 1992) either directly from the water column (Livingstone 1992; Randall et al., 1996) or from their diet (James & Kleinow 1994).
Of the numerous investigations reviewed above, several have described the biochemical
effects of exposure to xenobiotics using both marine molluscs, *Mytilus* sp. and teloest fish
have particularly been exploited in both laboratory and field studies (Moore *et al.*, 1989) since they are sedentary intertidal or subtidal filter feeders (Gosling 1992) and consequently readily accumulate lipophilic contaminants into their tissues (Livingstone 1992).

The fate of specific lipophilic xenobiotics - once taken up by the organism, may depend
upon many processes including the metabolism to hydrophilic metabolites (George 1994;
Livingstone 1991; Stegeman & Hahn 1994; Walker & Livingstone 1992) and hence, the
mechanisms of xenobiotic metabolism are described in the following sections.

### 1.2 OVERVIEW OF ORGANIC XENOBIOTIC METABOLISM

The fundamental theme of xenobiotic metabolism is the biotransformation of the parent
compound by existing metabolic pathways, to more polar, hydrophilic metabolites that can
be eliminated by the organism (Gibson & Skett 1994; Walker *et al.*, 1996a). The metabolic
pathways of xenobiotic transformation are varied and include oxidation, reduction,
hydrolysis, hydration and conjugation reactions (Gibson & Skett 1994). In general, these
reactions may be separated into 2 types, Phase 1 (functionalisation reactions) and Phase 2
(conjugation reactions). Phase 1 metabolism includes oxidation, reduction, hydrolysis and
hydration reactions and renders a chemically active group e.g. hydroxyl group, sulphahydroyl group or an epoxide into the xenobiotic so there is a new functional group available for Phase 2 reactions. Phase 2 reactions are characterised as conjugation reactions.
and are generally responsible for the addition of polar molecules e.g. glucuronides or sulphates to either the xenobiotic or the metabolites produced from phase 1 reactions. These two phases lead to a progressive increase in water solubility from a lipohilic xenobiotic to a water soluble polar conjugate.

The mixed-function oxidase (MFO) system is comprised of 3 components, cytochrome P450 (see Section 1.4), lipids (including phospholipids) and nicotinamide adenine dinucleotide phosphate reduced form (NADPH)-cytochrome P450 reductase and is located in the endoplasmic reticulum of many cells including those of the liver and kidney (Gibson & Skett 1994). MFO utilises molecular oxygen to catalyse many phase 1 hydroxylation and oxidation reactions including aromatic and aliphatic hydroxylation, O-, N- and S-dealkylation, epoxidation, S- and N-oxidation and alcohol oxidation. There have been many studies examining the endogenous functions of the MFO system (see Section 1.4.2) e.g. fatty acid and steroid synthesis or detoxication of plant allelochemicals however many anthropogenic xenobiotics have the potential to be metabolised via cytochrome P450-dependent phase 1 pathways. Another phase 1 oxidation system is the flavin-containing monooxygenase (FMO) system also located in endoplasmic reticulum (Ziegler 1993). Although the substrates of FMO have been characterised as soft nucleophiles e.g. dimethylamine, few studies have elucidated the endogenous functions of this oxidation system (see Section 1.11).

In the majority of cases, phase 1 and phase 2 metabolism leads to reduction of toxicity however, in a small but significant number of cases, metabolism can lead to an increase in toxicity, termed bioactivation (Gibson & Skett 1994; Varanasi et al., 1992; Walker et al., 1996a). In some marine organisms cytochrome P450-dependent oxidation bioactivates the PAH procarcinogen benzo[a]pyrene to the carcinogen benzo[a]pyrene-7,8-dihydr0diol-9,10-epoxide (see Figure 1.1) and FMO can activate the carbamate pesticide aldicarb to
Figure 1.1 The P450-dependent bioactivation of benzo[a]pyrene.
aldicarb S-oxide which exerts its toxicity by inactivating the enzyme acetylcholinesterase (Nunn et al., 1996; Schlenk 1995). In both these examples of bioactivation, factors that regulate or affect the oxidation pathways in these aquatic organisms also have the potential to affect the toxicity and fate of the xenobiotic. Of particular relevance to this thesis is the influence of life stage on fish cytochrome P450 activities (Buhler & Williams 1989); characterisation of cytochrome P450 in Mytilus sp. and a potential salinity-dependent regulation of fish FMO, (FMO catalyses the formation of the osmolyte trimethylamine N-oxide see Chapter 4).

Many anthropogenic and natural xenobiotics of enviromental relevance exert toxicity via the generation of free radicals and the formation of reactive oxygen species - ROS (Berenbaum 1995; Stohs 1995). A free radical is defined as molecule with an unpaired electron in a valance orbital (Davies 1995) and these highly reactive molecules (plus ROS) are capable of interacting with numerous cellular macromolecules (Cadenas 1995a; Rice-Evans et al., 1995). The deleterious effects of ROS are held in check by a suite of antioxidant processes however exposure to xenobiotics capable of stimulating ROS production may overwhelm the antioxidant processes and generate the condition of oxidative stress (see Section 1.7.1).

The following sections describe further relevant information concerning cytochrome P450 (Section 1.3), ROS generation, prooxidant and antioxidant processes (Section 1.6) and the FMO system (Section 1.10).
1.3 CYTOCHROME P450 SUPERFAMILY

Cytochrome P450 [EC 1.14.14.1] is the terminal component of the microsomal mixed function oxidase system (Cooper et al., 1965) and catalyses the oxidation of a wide variety of structurally diverse compounds by inserting a single atom from molecular oxygen into the substrate (Gibson & Skett 1994). The first references to mammalian P450 were published in 1958 (Garfinkel 1958; Klingenberg 1958) and report a microsomal - carbon monoxide complex with a Soret absorbance maxima at 450 nm. Sato and Omura (1961) determined that the microsomal protein contained a haem component and proposed the name cytochrome P450. The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) classify P450s as “heme-thiolate proteins” rather than cytochromes however the cytochrome phraseology remains (Nelson et al. 1996).

Since P450 enzymes are important in the oxidative metabolism of endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, retinols, lipid hydroperoxides and a wide range of anthropogenic xenobiotics; the subject of P450 research has rapidly developed following the initial studies in the late 1950s. Up to October 1995, at least 481 P450 genes have been sequenced in 85 eukaryote and 20 prokaryote species (Nelson et al. 1996). Over 260 of these sequences were determined since 1993 (Nelson et al. 1993).

It is now accepted that the P450 superfamily is very old with an ancestral gene proposed to exist prior to prokaryote/eukaryote divergence with an estimated origin more than 1.5 - 3 billion years ago (Nelson et al. 1993). One of the oldest P450 isoforms may be the bacterial camphor-metabolising P450 from Pseudomonas putida. This isoform has been crystallised
(Poulos et al. 1986) and studied in an attempt to elucidate the catalytic cycle of substrate oxidation (Poulos & Ragg 1992).

1.4 PROPERTIES AND CHARACTERISTICS OF P450

1.4.1 Nomenclature and classification of P450s

The sequencing of the P450 genes and proteins has enabled the development of a classification system based on the degree of homology between determined or inferred P450 amino acid sequences. P450 proteins with amino acid sequence homologies less than or equal to 40% are usually defined as originating from different gene families. If P450s have greater then 46-55% homology then they are classified in the same subfamily (Nelson et al. 1996) and sequences have to be greater than 97% homologous to be classified as being identical isoforms (Nebert et al. 1991). Several phylogenetic dendrograms have been published (see Degtyarenko & Archakov 1993; Nebert & Gonzalez 1987; Nebert et al. 1989) however the most recent is presented in Figure 1.2.

The accepted naming of a P450 gene include the italicised root symbol 'CYP' denoting cytochrome P450 ('Cyp' for mouse and Drosophila) followed by an arabic number designating the P450 family, a letter indicating subfamily and then an arabic numeral representing the specific gene e.g. CYP1A1. If no subfamily or second gene exists in a family, then the subfamily and gene number may be omitted e.g. CYP10. No italics should be used when referring to the gene products e.g. CYP2B mRNA, CYP3A cDNA or CYP4A1 protein.
Figure 1.2 P450 phylogenetic tree, (excluding CYP101-like sequences). Group I are present in vertebrates (except CYP18 which is expressed in insects); both Group II and VI exist in plants; Group III are found in both vertebrates and invertebrates; Group IV appear to catalyse fatty acid hydroxylation in eukaryotes and prokaryotes; Group V encode mitochondrial P450s; Group VII are expressed in fungi (Nelson et al. 1996).
1.4.2 The P450 molecule, structure and reaction mechanisms

The general equation of the monooxygenation reaction may be described as follows:

\[ \text{NADPH} + \text{H}^+ + \text{O}_2 + \text{RH} \rightarrow \text{NADP}^+ + \text{H}_2\text{O} + \text{ROH} \]  
(eq. 1)

where RH is the substrate and ROH the oxidised metabolite. The generalised distribution of the CYP families/subfamilies in various species and some of their related catalytic functions are summarised in Table 1.1. As the terminal oxidase component of the mixed function oxidase system, P450 is located chiefly in the membranes of the endoplasmic reticulum (Burke & Orrenius 1982) and has a monomeric molecular weight of approximately 45 - 60 kD (Boon et al. 1992). The prosthetic group consists of iron (Fe^{3+}) protoporphyrin IX and this planar structure is co-ordinated in the P450 molecule by 2 axial bonds above and below the plane of the porphyrin ring (Castro 1982). One bond is with the sulphahydryl group of a cysteine residue and the second may be with either water or tyrosine. The cysteine - haem binding region is one of the most highly conserved segments in the P450 superfamily (Porter & Coon 1991).

The following is a description of the events that take place during the P450 catalytic cycle as described by Coon et al. (1992), Gibson & Skett (1994), Guengerich & MacDonald (1990), Poulos & Ragg (1992). The binding of the substrate to the active site of P450 induces a conformational change in the protein. Prior to substrate binding, the usual coordination of the Fe^{3+} haem iron is with 6 ligands (4 with nitrogens from the porphyrin ring and 2 with the P450) forming an octahedral complex (see Figure 1.3). In this configuration the lone pairs of electrons from the nitrogens of the ligands can subject differential electron repulsion of the d-orbital electrons of Fe^{3+} ion inducing the separation of the d-orbitals
<table>
<thead>
<tr>
<th>CYP family/subfamily</th>
<th>Distribution and function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vertebrates (dioxin-inducible) metabolism of PAHs, halogenated and heterocyclic hydrocarbons, and aromatic amines.</td>
</tr>
<tr>
<td>2</td>
<td>Vertebrates and invertebrates, metabolism of drugs and environmental chemicals.</td>
</tr>
<tr>
<td>3</td>
<td>Vertebrates, metabolism of drugs and environmental chemicals.</td>
</tr>
<tr>
<td>4</td>
<td>Vertebrates, fatty acid hydroxylation; invertebrates, unknown function.</td>
</tr>
<tr>
<td>5</td>
<td>Vertebrates, thromboxane synthase.</td>
</tr>
<tr>
<td>6</td>
<td>Insects, metabolism of plant products and pesticides.</td>
</tr>
<tr>
<td>7A</td>
<td>Vertebrates, cholesterol 7α-hydroxylase.</td>
</tr>
<tr>
<td>7B</td>
<td>Vertebrates, unknown functions.</td>
</tr>
<tr>
<td>8</td>
<td>Vertebrates, prostacyclin synthase.</td>
</tr>
<tr>
<td>9</td>
<td>Insects.</td>
</tr>
<tr>
<td>10</td>
<td>Molluscan mitochondrial enzyme.</td>
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<tr>
<td>11</td>
<td>Vertebrates, cholesterol side chain cleavage, steroid 11β-hydroxylase and aldosterone synthase (mitochondrial enzyme).</td>
</tr>
<tr>
<td>12</td>
<td>Insect mitochondrial enzymes.</td>
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<td>13-14</td>
<td>Nematodes.</td>
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<td>15</td>
<td>Insects.</td>
</tr>
<tr>
<td>16</td>
<td>Nematodes.</td>
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<td>Insects.</td>
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<td>Vertebrates, aromatisation of androgens.</td>
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<td>20</td>
<td>Vertebrates, steroid 21-hydroxylase.</td>
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<tr>
<td>21</td>
<td>Vertebrates, steroid 24-hydroxylase (mitochondrial enzyme).</td>
</tr>
<tr>
<td>22</td>
<td>Vertebrates, steroid 27-hydroxylase (mitochondrial enzyme).</td>
</tr>
<tr>
<td>51</td>
<td>Animals, filamentous fungi, yeast and plants, sterol biosynthesis.</td>
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<td>52</td>
<td>Yeast, alkane hydroxylase.</td>
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<td>53-62</td>
<td>Fungi.</td>
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<td>71-92</td>
<td>Plants.</td>
</tr>
<tr>
<td>73</td>
<td>Plants, cinnamic acid hydroxylase.</td>
</tr>
<tr>
<td>101-118</td>
<td>Bacteria.</td>
</tr>
</tbody>
</table>

**Table 1.1** A summary of P450 families/subfamilies and their enzyme functions in various species (Nelson *et al.* 1996).
Figure 1.3  A. $d$- Orbital electron distribution in the low and high spin states of cytochrome P450. B. Hexa-co-ordinated low spin haem iron configuration and penta-co-ordinated high spin P450 (Gibson & Skett 1994).
into 2 higher energy states and 3 lower energy states (see Figure 1.3). Although the exact mechanisms are unclear, following substrate binding to the P450 active site, the electron configuration of the Fe$^{3+}$ ion is altered and electrons from the low spin orbitals are promoted to the high spin state. The consequences of this promotion of electrons is 2 fold. Firstly the redox mid - point potential of the P450 molecule is made more positive (favouring the transfer of electrons from an electron donor such as a flavoprotein NADPH-cytochrome P450 reductase to P450) and secondly there is an observed shift in the P450 spectral properties resulting with an absorption peak peak at 385 - 390 nm and a trough at approximately 420 nm. The substrates that induce these changes are termed Type I ligands.

In contrast, Type II ligands (mainly nitrogenous bases) are thought to ligate to the haem iron directly resulting in a 6-co-ordinated low spin haemoprotein. Type II ligands also have a characteristic absorbance spectra with a peak at 425 - 435 nm and trough at 390 - 405 nm. A third spectral shift in absorbance may be observed following substrate-P450 interaction, termed Reverse Type I with an absorbance peak at 420 nm and a trough at 388 - 390 nm.

Following substrate binding, 1 of the 2 electrons associated with NADPH and H$^+$ is transferred by the flavoprotein NADPH-cytochrome P450 reductase to the haem-substrate complex reducing Fe$^{3+}$ to Fe$^{2+}$. NADPH-cytochrome P450 reductase is also located in the endoplasmic reticulum and contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) prosthetic groups, the former acting to accept electrons whist the latter donates them to the P450 complex (Black 1992). There are other proteins capable of presenting electrons to P450, one of the oldest systems in prokaryotes utilises a ferridoxin (Fe$_2$S$_2$) protein, putidaredoxin (and its associated reductase) whereas some mitochondrial P450s use the Fe$_2$S$_2$ protein adrenodoxin (Nebert et al. 1989). After the first electron reduction of the P450 - substrate complex, molecular oxygen binds and putative electron rearrangement occurs following the addition of a second electron from either NADPH-
cytochrome P450 reductase or cytochrome b$_5$. Cytochrome b$_5$ may be reduced by both cytochrome P450 reductase or cytochrome b$_5$ reductase (an NADH-dependent process) prior to presenting the electron to the P450 complex. Although the events following the binding of molecular oxygen and subsequent monooxygenation of substrate are not fully elucidated, a proposed outline of the catalytic cycle as described by Poulos & Ragg (1992) are presented in Figure 1.4. The binding of molecular oxygen to the complex is a critical point at which catalysis may proceed or interrupted resulting in the release of reactive oxygen species including O$_2^-$ (Coon et al. 1992; Cross & Jones 1991).

1.4.3 Regulation

Although the mechanisms of P450 regulation appear diverse (see Figure 1.5), the most common means of regulation appears to be at the level of gene transcription (Porter & Coon, 1991). The most extensively characterised system relates to the expression of the CYP1A1 isoform which may be regulated via a cytosolic receptor that binds to aromatic hydrocarbons - Ah receptor (Landers & Bunce 1991). The receptor - ligand complex crosses the nuclear membrane binds to the DNA and enhances the transcription of CYP1A1 (Nebert & Jones 1989) and regulates the expression/activity of up to 26 other proteins (Sutter & Greenlee 1992). The Ah receptor - ligand complex binds to a regulatory region upstream of the CYP1A1 gene and the transcription event may be controlled by at least 3 regulatory elements; the first (approximately 1000 to 800 base pairs upstream of CYP1A1) may comprise of a xenobiotic response element (XRE) and/or dioxin response element (DRE); the second regulatory region (approximately 600 to 400 base pairs upstream) may
Figure 1.4 The P450 catalytic cycle (Poulos & Ragg 1992)
Figure 1.5 Mechanisms of P450 regulation with examples of isoforms subject to the regulation.
be an endogenous *CYP1A1* control element; and thirdly a TATAA box promoter between 20 and 30 base pairs upstream of the transcriptional initiation site (Fuji-Kuriyama *et al.* 1992; Nebert & Jones 1989).

Other mechanisms of P450 regulation include altering the efficiency of mRNA processing i.e. excision of the introns and the splicing of the exons (Silver & Krauter 1990), changing the stability of the *de novo* mRNA (Paine 1991), availability of the haem prosthetic group (Gibson & Skett 1994), cAMP-dependent post-translational modification (Goldfarb 1990) and P450 protein stabilisation (Song *et al.* 1989).

1.5 CYTOCHROME P450 IN AQUATIC ORGANISMS

The diversity of aquatic organisms reporting the expression of P450s or at least exhibiting an *in vitro* mixed function oxidase activity (proposing the expression of cytochrome P450) include ceolentrates (Heffeman *et al.* 1996), anelids (Fries & Lee 1984), molluscs (Yawetz *et al.*, 1992; Porte *et al.*, 1995), crustaceans (James *et al.*, 1996), echinoderms (Besten *et al.* 1993) as well as vertebrates including fish - chondrichthyes (Barnes & Miller 1993) and osteichthyes (Andersson & Förlin 1992), mammals - pinnipeds (Boon *et al.* 1992) and cetaceans (Boon *et al.* 1992) and birds (Walker 1992). Although many of these studies imply the presence of P450 isoforms due to substrate / activity relationships, only 7 P450 gene families have been cloned in aquatic organisms, 5 in teleost fish, CYP1, 2, 11, 17 and 19 (Stegeman & Hahn 1994) and 2 unique isoforms cloned from aquatic invertebrates; CYP2L (James *et al.* 1996) and CYP10 (Teunissen *et al.* 1992).
<table>
<thead>
<tr>
<th>P450 family / subfamily</th>
<th>Prominent microsomal activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oncorhynchus mykiss</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A1</td>
<td>EROD, AHH *</td>
<td>Williams &amp; Buhler 1984; Miranda et al. 1990; Lorenzana et al. 1988; Berndston &amp; Chen 1994</td>
</tr>
<tr>
<td>1A3</td>
<td></td>
<td>Berndston &amp; Chen 1994</td>
</tr>
<tr>
<td>2B</td>
<td>Lauric acid (ω) hydroxylase</td>
<td>Miranda et al. 1989; Miranda et al. 1990</td>
</tr>
<tr>
<td>2K1</td>
<td>Lauric acid (ω-1) hydroxylase and aflotoxin B1 activation</td>
<td>Miranda et al. 1989; Miranda et al. 1990; Buhler et al. 1995</td>
</tr>
<tr>
<td>2M1</td>
<td></td>
<td>Yang et al. 1995</td>
</tr>
<tr>
<td>3A</td>
<td>Pregnenolone-6β-hydroxylase and estrodiol hydroxylase</td>
<td>Miranda et al. 1989; Miranda et al. 1991</td>
</tr>
<tr>
<td>11</td>
<td>Side-chain cleavage</td>
<td>Takahashi et al. 1993</td>
</tr>
<tr>
<td>17</td>
<td>17 α-hydroxylase 17,20-lyase</td>
<td>Sakai et al. 1992</td>
</tr>
<tr>
<td>19</td>
<td>Aromatase</td>
<td>Tanaka et al. 1992</td>
</tr>
<tr>
<td><strong>Stenotomus chrysops</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A1</td>
<td>EROD, AHH *</td>
<td>Klotz et al. 1986; Elskus et al. 1992; Smolowitz et al. 1991</td>
</tr>
<tr>
<td>2B</td>
<td></td>
<td>Klotz et al. 1983; Klotz et al. 1986</td>
</tr>
<tr>
<td>3A</td>
<td>Testosterone 6β-hydroxylase</td>
<td>Klotz et al. 1983; Klotz et al. 1986</td>
</tr>
</tbody>
</table>

*EROD, AHH * indicates promiant microsomal activity.

Table 1.2 continued overleaf
<table>
<thead>
<tr>
<th>Species</th>
<th>Subfamilies</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fundulus heteroclitus</em></td>
<td>2K2</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td></td>
<td>2N1</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td></td>
<td>2P1</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td></td>
<td>2P2</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td></td>
<td>2P3</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td><em>Ictalurus punctatus</em></td>
<td>19</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td><em>Gadus morhua</em></td>
<td>1A</td>
<td>Goksoyr 1985</td>
</tr>
<tr>
<td><em>Perca fluviatilis</em></td>
<td>1A</td>
<td>Zhang et al. 1991</td>
</tr>
<tr>
<td><em>Opsanus tau</em></td>
<td>1A1</td>
<td>Morrison et al. 1995</td>
</tr>
<tr>
<td><em>Pagrus major</em></td>
<td>1A1</td>
<td>Mizukami et al. 1994</td>
</tr>
<tr>
<td><em>Chaetodon capistratus</em></td>
<td>1A1</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td><em>Pleuronectes plateussa</em></td>
<td>1A1</td>
<td>Leaver et al. 1993</td>
</tr>
<tr>
<td><em>Microgadus tomcod</em></td>
<td>1A1</td>
<td>Roy et al. 1995</td>
</tr>
<tr>
<td><em>Dicentrarchus labrax</em></td>
<td>1A1</td>
<td>Nelson et al. 1996</td>
</tr>
<tr>
<td><em>Raja erinacea</em></td>
<td>1A</td>
<td>Ball et al., 1980</td>
</tr>
</tbody>
</table>

Table 1.2 Purified or cloned P450 families and subfamilies from fish species. * EROD = 7ethoxyresorufen O-deethylase activity; AHH = aryl hydrocarbon hydroxylase activity.
1.5.1 Cytochrome P450s in fish

Cytochrome P450 forms have been studied in one or more species in over 10 families of fish with some of the P450s showing catalytic activity with multiple substrates (Stegeman & Hahn 1994). The microsomal P450s prominent in the metabolism of xenobiotics are in gene families 1 to 4 and demonstrate broad substrate specificity e.g. reconstituted rainbow trout *Oncorhynchus mykiss* CYP2K metabolises estradiol, progesterone, testosterone, lauric acid, benzphetamine and aflatoxin B₁ (Miranda et al. 1989; Miranda et al. 1990; Stegeman & Hahn 1994). Novel substrates of CYP1 - 4 are described each year (Stegeman 1993). Protein purification and cloning studies have identified the expression of CYP1A1, 1A3; CYP2B, 2E, 2K1, 2K2, 2M, 2N, 2P1, 2P2 & 2P3; CYP3A, CYP17, and CYP19 (see Table 1.2) however comparisons of substrate / activity relationships infer the expression of other forms e.g. CYP11 a mitochondrial enzyme (Stegeman 1993). At least 35 specific isoforms have been immunoidentified, purified (partially or fully) or cloned; the majority from liver tissue although 1 form has been purified from kidney and 3 steroidogenic forms from ovary tissue (Stegeman 1995).

1.5.1.1 CYP1A family in fish

The CYP1A subfamily and in particular CYP1A1 plays a significant role in phase 1 metabolism and catalyses the monooxygenation of a diverse group of xenobiotics including PAHs, planer PCBs, dioxins and polychlorinated dibenzofurans. Although there are many novel *in vitro* substrates for CYP1A (Burke *et al.* 1995), the endogenous substrate of this enzyme has not yet been determined (Gonzalez 1989). Generally, fish CYP1A *in vitro* catalytic activity is measured by either aryl hydrocarbon hydroxylase (AHH) activity or 7-ethoxyresorufen *O*-deethylase (EROD) activity (see Figure 1.6), (Andersson & Förlin
Figure 1.6 7-Ethoxyresorufin O-deethylase activity
Figure 1.5 Mechanisms of P450 regulation with examples of isoforms subject to the regulation.
During the phase 1 metabolism of benzo[a]pyrene, fish CYP1 catalyses the stereospecific oxidation at the 7,8- and 9,10- positions (see Figure 1.1). Metabolic attack at these positions is associated with the activation of this compound to a carcinogen (see following section) and hence CYP1A may be an important factor in environmental carcinogenesis in fish (Stegeman 1993). CYP1A forms have been cloned or purified from 11 species of fish (see Table 1.2) however antibody recognition studies have indicated CYP1A expression and / or induction in at least 38 fish species (Peters & Livingstone 1995; Stegeman & Hahn 1994). Regulation of CYP1A expression may be affected by many factors (see Figure 1.7) including, xenobiotic exposure, temperature, hormones and developmental stages.

1.5.1.1 Regulation by xenobiotics

Fish hepatic CYP1A1 may be induced by a wide variety of organic molecules (see Table 1.3) and over 75 field studies have been published (Bucheli & Fent 1995; Goksøyr 1995; Livingstone 1993). Reviews include Andersson & Förlin (1992), Bucheli & Fent (1995), Goksøyr (1995), Livingstone (1993), Stegeman (1995), Stegeman & Hahn (1994). When an inducer binds to the Ah receptor in the cytosol, it is proposed that a 90 kD heat shock protein previously coupled to the receptor dissociates (Wilhelmsson et al. 1990) and the receptor - ligand complex then moves into the nucleus via a nuclear translocation factor. The receptor - ligand complex binds with the xenobiotic regulatory element located upstream of CYP1A and initiates transcription (Landers & Bunce 1991).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity</th>
<th>Protein</th>
<th>mRNA</th>
</tr>
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<tr>
<td>B-Naphthoflavone</td>
<td>+</td>
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<td>3-Methylcholanthrene</td>
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<td>Benzo[a]pyrene</td>
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<tr>
<td>2,3,3',4,4'-PCB (CB-105)</td>
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<td>2,2',4,4',5,5'-HCB (CB-153)</td>
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<td>2,3,3',4,4',5-PCB (CB-156)</td>
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<td>2,2',4,4'-TCB (CB-47)</td>
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<tr>
<td>2,3,7,8-TCDD</td>
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Table 1.3 continued overleaf
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<tr>
<td>2,3,7,8-TCDF</td>
<td>+</td>
</tr>
<tr>
<td>2,3,6,8-TCDF</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4,7,8-PCDF</td>
<td>+</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>-</td>
</tr>
<tr>
<td>p,p'-DDE</td>
<td>-</td>
</tr>
<tr>
<td>Pregnenolone-16α-carbonitrile</td>
<td>-</td>
</tr>
<tr>
<td>Isosafrole</td>
<td>+</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>+</td>
</tr>
<tr>
<td>Mirex</td>
<td>-</td>
</tr>
<tr>
<td>Kepone</td>
<td>-</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>-</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>-</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>-</td>
</tr>
<tr>
<td>Indole-3-carbinol</td>
<td>-</td>
</tr>
<tr>
<td>Pulp mill effluents</td>
<td>+</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>+</td>
</tr>
<tr>
<td>Cyclopropenoid fatty acids</td>
<td>+</td>
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</table>

Table 1.3 Aromatic and chlorinated hydrocarbons evaluated as inducers of CYP1A in fish (Stegeman & Hahn 1994; Goksøyr 1995). + = elevation, - = no observed change. DDT, dichlorodiphenyltrichloroethane; HCB, hexachlorobiphenyl; PCB, pentachlorobiphenyl; TCB, tetrachlorobiphenyl; TCDD, tetrachlorodibenzo-p-dioxin; PCDD, pentachlorodibenzo-p-dioxin; TCDF, tetrachlorodibenzofuran.
1.5.1.1.2 Temperature and hormonal regulation of CYP1A1

Both field and laboratory studies indicate that temperature acclimation can effect regulation of CYP1A1 (Kloepper-Sams & Stegeman 1993; Lange et al., 1992). Laboratory exposure studies using known inducers have determined cold-acclimated fish produce CYP1A mRNA at comparable rates to warm acclimated fish however the CYP1A protein and enzyme activity in the cold acclimated fish increases slowly over time following induction (Kloeppep-Sams & Stegeman 1993). Estradiol treatment can suppress the induction of fish CYP1A mRNA as well as protein (Elskus et al. 1992) although the mechanisms by which this effects CYP1A are not known (Stegeman 1995). Studies using *O. mykiss*, where hepatic CYP1A1 activities were determined over a reproductive cycle observed increases in male aryl hydrocarbon hydroxylase (AHH) activity paralleled increases in plasma androgen levels whereas in females where both plasma testosterone and estradiol levels increase, AHH activity decreased during the late reproductive cycle (Förlin & Haux 1990).

1.5.1.1.3 Life-stage regulation of CYP1A1

The developmental life stage of the fish appears to affect CYP1A activity (Andersson & Förlin 1992; Stegeman & Hahn 1994) particularly during embryonic and larval life stages where increased CYP1A activity is observed post-hatching (see Table 2.1).

1.5.1.2 CYP2 family in fish

Numerically, the CYP2 family contains the highest number of mammalian CYP genes (Nelsen et al. 1996) however only 6 subfamilies (CYP2B, 2E, 2K, 2M, 2N and 2P) have been indicated in fish species (Nelson et al., 1996; Stegeman 1995). Buhler et al. (1995) have sequenced a full length cDNA from the liver of *O. mykiss* that has been
assigned as CYP2K1. Northern blots of total RNA indicated higher levels of expression in the trunk kidney of males compared to females. Northern blot studies also indicated the expression of a CYP2E-like sequence when hepatic mRNA from guppy Poeciliopsis sp. hybridised with a rat CYP2E1 probe (Kaplan et al. 1991). Southern blot analysis probing with a rat CYP2B1 cDNA indicated the presence of a CYP2B-like sequence in \textit{O. mykiss} (Haasch et al. 1990; Kleinow et al. 1990). Purification and immunorecognition studies also propose CYP2B expression in \textit{O. mykiss} (Miranda et al. 1990), scup Stenotomus chrysops (Klotz et al. 1983) and butterfly fish \textit{Chaetodon capistratus} (Vrolijk et al. 1995). Recently, novel forms of CYP2 have been cloned from killifish \textit{Fundulus heteroclitus} classified as CYP2K2, CYP2M1, CYP2N1, CYP2N2, CYP2N3, however little is known of their functions (Nelson et al. 1996).

Some of the mammalian CYP2 forms are inducible following treatment with phenobarbitol however this induction response is absent in phenobarbitol exposed fish (Elskus & Stegeman 1989; Stegeman & Hahn 1994; Stegeman & Kloepper-Sams 1987). Since evidence exists for CYP2 in several fish species, it is proposed by Stegeman & Hahn (1994) that fish have a different regulatory mechanism for CYP2 expression compared to mammals, possibly due to different levels of exposure to dietary xenobiotics from terrestrial plants.

### 1.5.1.3 CYP3A in fish

The evidence indicating CYP3A expression in fish comprises of immunorecognition and substrate / activity studies of purified hepatic P450 forms from \textit{O. mykiss} (Miranda et al. 1989; Miranda et al. 1991) and \textit{S. cyrysops} (Klotz et al. 1983; Klotz et al. 1986).
Microsomal activities attributed to the purified CYPs of both *O. mykiss* (LMC5) and *S. cyrysops* (P450A) include steroid 6β-hydroxylase, an activity catalysed in mammals by 3A proteins (Halvorson *et al.* 1990). Hepatic rat and human 3A forms were immunorecognised by antibodies to LMC5, whereas antibodies to mammalian CYP3A forms recognised the LMC5 protein (Miranda *et al.* 1991) and a hepatic microsomal protein from *C. capistratus* (Vrolijk *et al.* 1995).

1.5.1.4 CYP11A in fish

Although the majority of P450s are located in the endoplasmic reticulum, a P450 cholesterol side chain cleavage enzyme has been identified and cloned in the mitochondria from *O. mykiss* ovarian tissue (Takahashi *et al.* 1993). The inferred sequence homology places it in the CYP11A subfamily.

1.5.1.5 CYP17 in fish

The protein product expressed by the CYP17 gene family catalyses both 17 α-hydroxylase and 17,20-lyase reactions (Nelson *et al.* 1996). Both activities are involved in the formation of testosterone whereas the hydroxylase activity alone is necessary in the formation of 17 α-hydroxyprogesterone. A cDNA sequence has been isolated, encoding a CYP17 sequence from the ovary of *O. mykiss* (Sakai *et al.* 1992). The inferred 522 amino acid sequence was 64% homologous with chicken CYP17 but showed lower homology (46-48%) to mammalian CYP17 sequences.
1.5.1.6 CYP19 in fish

A cDNA sequence has been identified encoding a P450 aromatase (oestrogen synthase) from the ovary of *O. mykiss* (Tanaka *et al.* 1992). The inferred amino acid sequence was more than 50% identical with human, rat, mouse and chicken aromatases however the N terminus of the protein was more hydrophobic than other vertebrate proteins (Stegeman 1993). Increased expression of CYP19 appears to be closely linked to the production of estradiol and CYP19 may be regulated by transcriptional activation consistent with mechanisms reported in mammalian species (Hickey *et al.* 1990).

1.5.2 Cytochrome P450s in *Mytilus* sp.

The cytochrome P450-dependent monooxygenase - MFO system, or its components, have been identified in at least 21 species of mollusc (Cheah *et al.*, 1995; Livingstone 1991) however only 1 form, CYP10 has been cloned from the molluscan pond snail *Lymnaea stagnalis* (Teunissen *et al.*, 1992). Mytiloid tissue distribution studies indicate P450 in digestive gland, gill and other tissues (Livingstone & Farrar 1984) and estimated levels of microsomal P450 content vary from 3 - 134 pmol/mg protein for the blue mussel *Mytilus edulis* and *M. galloprovincialis*.
1.5.2.1 Spectroscopic properties

Cytochrome P450 content has been determined in digestive gland microsomal preparations of various *Mytilus* species either as the dithionite difference spectra of the carbon monoxide-liganded samples, (Stegeman 1985) or the carbon monoxide difference spectra of the dithionite-reduced samples (Ade *et al.* 1982; Gilewicz *et al.*, 1984; Livingstone & Farrer 1984; Livingstone *et al.*, 1989). Both methods report a peak between 447 and 452 nm and a second peak between 416 and 424 nm. The size of the lower wavelength peak may depend upon both the previous exposure history of the organism and the reproductive cycle; the quantification of the P450 peak may be difficult when the P420 peak is large (Livingstone *et al.*, 1989). Ligand-P450 spectral interaction studies using *M. edulis* P450 revealed clotrimazole, ketoconazole, metyrapone and pyridine as typical Type II binding spectra whereas ligands such as 7-ethoxycoumarin, testosterone, SKF-525A and α-naphthaflavone (predicted to give Type I) gave Reverse Type I spectra following binding to the P450 complex (Livingstone *et al.*, 1989).

1.5.2.2 Catalytic activity

At least 13 MFO activities have been described in tissue from *Mytilus* sp. (see Table 1.4) with potential *in vivo* metabolic activities including xenobiotic metabolism and steroid synthesis. Levels of mytiloid microsomal P450 were determined to be highest in digestive gland tissue and this is paralleled with higher levels of the associated MFO components as well as the oxidative activities (Livingstone & Farrar 1984). Microsomal yields have also been observed to be highest in digestive gland tissue (Livingstone *et al.*, 1989).
<table>
<thead>
<tr>
<th>Species</th>
<th>Mixed function oxidase activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mytilus edulis</em></td>
<td>Benzo[a]pyrene hydroxylase</td>
<td>Lemaire &amp; Livingstone 1993; Sole <em>et al.</em>, 1996</td>
</tr>
<tr>
<td></td>
<td>Dimethylaniline N-demethylase</td>
<td>Livingstone <em>et al.</em>, 1989</td>
</tr>
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<td></td>
<td>Biphenyl hydroxylase</td>
<td>Willis &amp; Addison 1974</td>
</tr>
<tr>
<td></td>
<td>7-Ethoxycoumarin <em>O</em>-deethylase</td>
<td>Kirchin <em>et al.</em>, 1988</td>
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<td></td>
<td>Benzphetamine <em>N</em>-demethylase</td>
<td>Kirchin <em>et al.</em>, 1988</td>
</tr>
<tr>
<td></td>
<td>Testosterone hydroxylase</td>
<td>Kirchin <em>et al.</em>, 1988</td>
</tr>
<tr>
<td></td>
<td>7-Ethoxyresorufin <em>O</em>-deethylase</td>
<td>Stegeman 1985</td>
</tr>
<tr>
<td></td>
<td>Aldrin epoxidase</td>
<td>Moore <em>et al.</em>, 1980</td>
</tr>
<tr>
<td></td>
<td>Arachidonic acid hydroxylase</td>
<td>Kirchin <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>Mytilus galloprovincialis</em></td>
<td>Benzo[a]pyrene hydroxylase</td>
<td>Livingstone <em>et al.</em>, 1997</td>
</tr>
<tr>
<td></td>
<td>Dimethylaniline N-demethylase</td>
<td>Ade <em>et al.</em>, 1982</td>
</tr>
<tr>
<td></td>
<td>Aminopyrene <em>N</em>-demethylase</td>
<td>Gilewicz <em>et al.</em>, 1984</td>
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<tr>
<td></td>
<td>7-Ethoxycoumarin <em>O</em>-deethylase</td>
<td>Galli <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>Mytilus californianus</em></td>
<td>Aldrin epoxidase</td>
<td>Krieger <em>et al.</em>, 1979</td>
</tr>
<tr>
<td></td>
<td>Antipyrine hydroxylase</td>
<td>Krieger <em>et al.</em>, 1979; Krieger <em>et al.</em>, 1979</td>
</tr>
<tr>
<td></td>
<td><em>p</em>-Nitroanisole <em>O</em>-demethylase</td>
<td>Krieger <em>et al.</em>, 1979</td>
</tr>
</tbody>
</table>

**Table 1.4** Putative mixed function oxidase activities indicated in *Mytilus* sp.
Mytilus sp. digestive gland microsomes metabolise BaP in the presence of NADPH to the 7,8-, 9,10- and 4,5-dihydrodiols as well as phenols (Livingstone et al., 1997; Michel et al., 1994; Stegeman 1985) however the major microsomal metabolites formed (47 -65 % of the total polar metabolites) are the 1,3-, 6,12- and 3,6-quinones (Lemaire & Livingstone 1993; Livingstone et al., 1997; Michel et al., 1992; Porte et al., 1995). In vitro metabolism of BaP leading to the formation of quinones, diols and phenols has also been observed in the absence of added NADPH (Lemaire & Livingstone 1993; Livingstone et al., 1989), and other apparent NADPH-independent MFO activities e.g. 7-ethoxycoumarin O-deethylase, testosterone hydroxylase, N,N-dimethylaniline demethylase and benzphetamine demethylase activities have been described in M. edulis digestive gland microsomes (Kirchin 1988; Livingstone et al., 1988a). P450 inhibitor studies indicated sensitivity to α-naphthoflavone and SKF525A (Livingstone & Farrer 1984; Michel et al., 1992; Moore et al., 1989) for both NADPH-dependent and NADPH-independent BaP metabolism and suggests that the in vitro metabolism of BaP involves more than one metabolic pathway (Livingstone et al., 1989; Livingstone et al., 1997; Stegeman 1985). The mechanism of the NADPH-independent oxidation pathway has not been elucidated however Livingstone et al. (1989) have proposed that either an endogenous source of reducing equivalents or an activated oxygen from an endogenous peroxide (lipid peroxide) have the potential to support the catalysis of the oxidation of BaP. The one electron oxidation of BaP may be undertaken by a peroxidase activity inherent to cytochrome P450 (O’Brien 1984) and this pathway has been proposed as a mechanism for PAH dione formation where BaP-cation radicals are generated (Cavaliere et al., 1993). In some studies it was observed that NADPH can inhibit BaP metabolism and one mechanism involving the direct re-protonation of the BaP-cation radical may account for the observed reduction of BaP metabolism (Livingstone et al., 1989).
1.5.2.3 Evidence of multiple CYP forms

As reported above, at least 13 MFO activities have been described in tissue from *Mytilus* sp. (see Table 1.4) undertaking a variety of catalytic epoxidation, hydroxylation and deethylation reactions using structurally diverse substrates. Although the association of a CYP isoform to a specific catalytic activity may be speculative since homologous CYPs of related species may catalyse different reactions (Stegeman & Hahn 1994), the diverse structures of the substrates (fatty acids and aromatic rings) would indicate multiple CYPs or a single mytiloid CYP with uniquely broad-substrate specificity.

A polyclonal antibody raised against perch *Perca fluviatilis* hepatic CYP1A immunoreacted with a CYP1A-like epitope of a partially purified preparation of *M. edulis* digestive gland CYP (Porte et al., 1995). A similar epitope was also immunoidentified in digestive gland microsomal preparations from *M. edulis* and *M. galloprovinialis* (Livingstone et al., 1997; Solé et al., 1996). The presence and expression of CYP1A, 3A, 4A and 11A was investigated in the digestive gland of *M. edulis* using both Northern and Southern blot techniques (Wootton et al., 1995). cDNA probes for CYP1A1 (*O. mykiss*), CYP3A and 11A (human) and CYP4A (rat) hybridised to *M. edulis* mRNAs of c2.1 kb length under low stringency hybridisation conditions, indicating the expression of multiple CYP forms. Hybridisation of Southern blots using genomic DNA gave further evidence of CYP3A- and CYP11A -like genes in *M. edulis* digestive glands.

1.5.2.4 Regulation

Microsomal CYP content, BaP metabolism and 7-ethoxycoumarin O-deethylase activity vary seasonally with an observed decrease in CYP, metabolism and catalytic activity during late spring corresponding with the spawning of gametes and lower seawater temperatures.
(Kirchin et al., 1992; Livingstone et al., 1989; Solé et al., 1995a; Wootton et al., 1996). In an early study BaP hydroxylase activity was observed to be higher in female mussels than males (Livingstone & Farrer 1984) but more recent studies have observed no sex differences with other MFO activities (Kirchin et al., 1992; Livingstone et al., 1989).

Although no mechanism accounting for seasonal variation of CYP or MFO activities has been published, the reported seasonal variation of the levels of CYP1A, CYP3A and CYP4A mRNA would indicate regulation at the CYP gene level (Wootton et al., 1996).

Elevation of total CYP levels or related catalytic activities was reported following laboratory exposure to oil (Livingstone 1987), 3-methylcolanthrene (Livingstone et al., 1988b; Michel et al., 1994), polychlorobyphenyls (Livingstone et al., 1997; Michel et al., 1993). Similarly, field studies show positive correlations between microsomal BaP hydroxylase activity or other CYP-related measurements and environmental exposure to PAHs (Livingstone 1988; Michel et al., 1994; Solé et al., 1995b; Solé et al., 1996).

Recognition of the CYP1A-immunopositive protein was observed in the 13 400g supernatants of M. galloprovincialis digestive glands following exposure to BaP (Canova et al., 1997) however levels of microsomal immunopositive protein were elevated after exposure to the polychlorobiphenyl congeners, CB-138 or Arochlor 1254 (Livingstone et al., 1997). Positive correlations between Mytilus sp. microsomal CYP1A-immunopositive protein and field exposure to PAHs have been variable; both good and poor correlations have been reported (respectively Solé et al., 1996; Solé et al., 1997) even when MFO activities such as BaP hydroxylase activity appear to be elevated in response to exposure. Little or nothing is known of the mechanisms of this apparent regulation of CYP1A-immunopositive protein since the Ah receptor is proposed to be absent following photoaffinity ligand binding studies (Hahn et al., 1992; Hahn et al., 1994).
1.6 ROS TOXICITY, PROOXIDANT AND ANTIOXIDANT PROCESSES

The concerted 4 electron reduction of $O_2$ to $H_2O$ by aerobic organisms is a co-ordinated process involving enzymes and trans-membrane proteins of the the mitochondrial electron transport system and confers an energetic benefit to organisms utilising oxidative metabolism compared to anaerobic metabolism (Davies 1995). However incomplete reduction of $O_2$ gives rise to oxidation states of ROS (see Table 1.5) which possess reactivity deleterious to aerobic cell constituents; and as a consequence, an array of cellular antioxidant processes have developed to counter ROS toxicity (Cadenas 1995a). Under regulated conditions, ROS may be enzymically generated and utilised to perform a diverse variety of oxidation reactions including the cytotoxic killing of phagocytosed pathogens by neutrophils or the formation of functional membranes in sea urchin embryos (Cross & Jones 1991). In addition to aerobic metabolism and enzymically regulated ROS production, other processes including exposure to xenobiotics and ionizing radiation may affect the cellular steady-state concentrations of ROS. When ROS production is not countered by antioxidant defenses, deleterious reactions with proteins, lipids and nucleic acids may lead to molecular alterations in cell functions and as such oxidative stress has been implicated in many mammalian diseases and degenerative processes including mutagenesis, cancer, inflammatory disease and neurotoxicity (Davies 1995).

1.7 PROPERTIES OF OXYGEN AND ITS RADICALS

Gound state molecular oxygen exists as diatomic molecule maintained by a single bond $O=O$, and therefore exists with two unpaired valence electrons in parallel spin. Since there are 2 unpaired electrons in valence orbitals, molecular oxygen is a bi-radical and because the
<table>
<thead>
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<th>Non-radical species</th>
</tr>
</thead>
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<tr>
<td>Superoxide</td>
<td>$O_2^-$</td>
</tr>
<tr>
<td>Hydroperoxyl</td>
<td>$HO_2^-$</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>$'OH$</td>
</tr>
<tr>
<td>Nitrogen dioxide</td>
<td>$NO_2^-$</td>
</tr>
<tr>
<td>Peroxyl</td>
<td>$ROO^'/LOO^'$</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>$'NO$</td>
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<tr>
<td>Alkoxyl</td>
<td>$RO^'/LO^'$</td>
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<td></td>
<td>Hydrogen peroxide</td>
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<td>Ozone</td>
</tr>
<tr>
<td></td>
<td>$O_3$</td>
</tr>
<tr>
<td></td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td></td>
<td>$ONOO^-$</td>
</tr>
<tr>
<td></td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td></td>
<td>$^1O_2$</td>
</tr>
</tbody>
</table>

Table 1.5 Radical and non-radical reactive oxygen species, ROS (Pardini 1995). R = any organic molecule, L = unsaturated lipid molecule.
electrons are in parallel spin, molecular oxygen is paramagnetic existing in a triplet state written as $^3\text{O}_2$ (Cadenas 1989). The strong O-O bond may be weakened either by 1 electron reduction leading to the formation of a superoxide anion mono-radical, or the spin of one of the unparied valence electrons is inverted via an energetic process (photochemical or thermal) relieving the spin restriction and forming the less stable O=O bond termed singlet molecular oxygen, written as $^1\text{O}_2$ (Cadenas 1989; Pardini 1995).

Molecular oxygen can undergo sequential, non-enzymic univalent reduction to several reactive intermediates. The first 1 electron reduction of $\text{O}_2$ generates the superoxide radical ($\text{O}_2^-$) according to eq. 2.

$$\text{O}_2 + e^- \rightarrow \text{O}_2^- \quad \text{(eq. 2)}$$

Cadenas (1995a) described 3 important types of reactions undertaken by $\text{O}_2^-$: firstly $\text{O}_2^-$ is a weak base undergoing protonation to the hydroperoxyl radical, $\text{HO}_2^-$ according to eq. 3, secondly $\text{O}_2^-$ can behave as a 1 electron reductant of acceptors such as transition metals and cytochrome $c$ and finally $\text{O}_2^-$ behaves as a 1 electron oxidant of molecules with acidic protons e.g. ascorbate. Whereas $\text{O}_2^-$ displays moderate reactivity $\text{HO}_2^-$ is capable of reacting with hydrogen atoms from polyunsaturated hydrocarbons and lipid peroxides initiating lipid autoxidation (Gebiki & Bielski 1981). Since $\text{HO}_2^-$ has a pKa of 4.8 it is possible that limited concentrations of this radical may be achieved in the acidic environments of mitochondrial membranes.

$$\text{O}_2^- + \text{H}^+ \rightarrow \text{HO}_2^- \quad \text{(eq. 3)}$$
The capacity of O$_2^-$ to undertake all 3 types of reaction is exemplified by the dismutation of O$_2^-$ in the presence of protons (eq. 4).

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  
(eq. 4)

In this reaction 1 O$_2^-$ molecule acts as an electron donor forming ground state oxygen, the second O$_2^-$ acts as an electron acceptor and is reduced to hydrogen peroxide, H$_2$O$_2$ (Davies 1995). Other uncatalysed dismutation/disproportionation reactions have been determined (eq. 5 and 6) with variable rate constants (respectively eq. 4, $<1 \times 10^2$ M$^{-1}$ sec$^{-1}$; eq. 5, $8.5 \times 10^7$ M$^{-1}$ sec$^{-1}$; eq. 6, $7.6 \times 10^5$ M$^{-1}$ sec$^{-1}$) but common to all the reactions is the formation of H$_2$O$_2$ and O$_2$. N.B. The rate constant for eq. 4 may be increased to $1.9 \times 10^9$ M$^{-1}$ sec$^{-1}$ by the enzyme superoxide dismutase, E.C. 1.15.1.1.

\[ \text{O}_2^- + \text{HO}_2^+ + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  
(eq. 5)

\[ \text{HO}_2^- + \text{HO}_2^- + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  
(eq. 6)

Although H$_2$O$_2$ is an ROS and is the product of 2 electron reduction of molecular oxygen, it is not a free radical but since the source of the second electron can be donated from the product of 1st electron reduction, Davies (1995) proposed that whenever O$_2^-$ (or HO$_2^-$) is generated, there will always be a resultant production of H$_2$O$_2$. In addition, H$_2$O$_2$ may also be generated by a direct 2 electron reduction of ground state oxygen catalysed by many peroxisomal flavin enzymes such as glucose oxidase, urate oxidase and D-amino oxidase (Pardini 1995).
H$_2$O$_2$ unlike O$_2^{.-}$, can cross biological membranes (Davis 1995; Pardini 1995) and as a consequence may oxidise macromolecules un-related to the site of O$_2^{.-}$ generation e.g. oxyferryl complexes (Fe$^{IV}$ = O) may be generated following interraction with metal chelate and hemoproteins (Cadenas 1995a). Another important feature of H$_2$O$_2$ is that it can undergo further reduction to more potent oxidants such as the hydroxyl radical, 'OH by the interraction with O$_2^{.-}$ (eq. 7) known as the ‘Haber-Weiss’ reaction. The 'OH is the most reactive of the oxyradicals and will oxidise proteins, lipids and nucleic acids (see below).

$$\text{H}_2\text{O}_2 + \text{O}_2^{.-} \rightarrow \cdot\text{OH} + \cdot\text{OH} + \text{O}_2$$  \hspace{1cm} (eq. 7)

The rate of this reaction can be enhanced via a ‘Fenton-like’ reaction involving an iron- or copper-chelate (Halliwell & Gutteridge 1990) according to eqs. 8 and 9 where the chelate-metal complex both maintains the metal in solution and lowers the redox potential of the transition metal.

$$\text{O}_2^{.-} + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{O}_2$$  \hspace{1cm} (eq. 8)

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH}$$  \hspace{1cm} (eq. 9)

Another proposed mechanism for the production of both 'OH and the nitrogen dioxide radical NO$_2^{.-}$, involves the interraction of O$_2^{.-}$ with the endothelial -derived relaxing factor nitric oxide NO. First, O$_2^{.-}$ reacts with NO to form the peroxynitrite ONOO$^-$ ion which may be protonated and homolytically cleaved to NO$_2^-$ and 'OH (Cadenas 1995a, Pardini 1995).
1.7.1 Deleterious reactions of ROS

1.7.2 Lipid peroxidation

Lipid peroxidation is a free radical chain reaction predominantly initiated by the interaction of \('OH, \:^1O_2\ or \:HO_2^- with polyunsaturated fatty acids resulting in the abstraction of a susceptible hydrogen atom and the formation of a lipid or organic radical (L' or R'). The carbon-centered radical is subsequently quenched by molecular oxygen and a peroxyl radical is formed (see eq. 10 and 11). Once initiated, the free radical reaction is propagated via a second interaction with an unsaturated fatty acid producing a lipid peroxide and another lipid radical (eq. 12).

\[
\text{LH + 'OH} \rightarrow \text{L'} + \text{H}_2\text{O} \quad \text{(eq. 10)}
\]

\[
\text{L'} + \text{O}_2 \rightarrow \text{LOO}^- \quad \text{(eq. 11)}
\]

\[
\text{LOO}^- + \text{LH} \rightarrow \text{LOOH} + \text{L'} \quad \text{(eq. 12)}
\]

The process of lipid hydroperoxide and radical formation continues until a termination reaction occurs via a bi-radical reaction leading to the formation of stable nonradical products (see eq. 13, 14 and 15). Limited oxygen availability may favour termination reactions as described by eq. 13 (Pryor 1976).

\[
\text{L'} + \text{L'} \rightarrow \text{L - L} \quad \text{(eq. 13)}
\]

\[
\text{LOO}^- + \text{L'} \rightarrow \text{LOOL} \quad \text{(eq. 14)}
\]
Pardini (1995) proposed that an early event in membrane phospholipid peroxidation injury is the formation of fatty acid acyl hydroperoxides. This leads to the stimulation of phospholipase $A_1$ and $A_2$ and phospholipase $C$ activities and the resultant formation of lysophospholipid and free fatty acyl hydroperoxide. Lysophospholipid is a detergent and its formation can damage the integrity of the membrane if it is not rapidly reacylated or degraded. If not removed, phospholipid hydroperoxides degrade to aldehyde products with deleterious cross-linking properties inhibiting enzymes involved in DNA and RNA synthesis, protein synthesis, mitochondrial respiration and glycolysis (Pardini 1995).

### 1.7.3 Oxidative modifications of proteins

Proteins are vulnerable to oxidative damage (Davies 1987) either by the interaction with lipid peroxidation products or via ROS generation by metal-protein prosthetic groups of enzymes (Stadtman 1990; Stadtman 1991). From Table 1.6 it is observed that many important enzyme activities are sensitive to the presence of ROS and cell function may be impaired when ROS is not checked by the antioxidant processes.

### 1.7.4 Oxidative damage to nucleic acids

Both DNA and RNA bases may be readily peroxidised by analogous processes to lipid peroxidation (Davies 1995; Wiseman & Halliwell 1996). Under normal physiological conditions it is estimated that in nuclear DNA one base out of 224 000 bases is oxidatively modified (Ames et al., 1991) and in a single liver cell there may be 1 million oxidatively modified bases (Ames et al., 1993). Oxidative DNA damage has been demonstrated in vivo.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl CoA hydrolase</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>Acetylcholine esterase</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Lactoperoxidase</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Leucyl-t-RNA-synthetase</td>
</tr>
<tr>
<td>Catalase</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>Enolase</td>
<td>6-Phosphogluconate dehydrogenase</td>
</tr>
<tr>
<td>Fructose diphosphatase</td>
<td>Phosphoglucomutase</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>α-1-Proteinase inhibitor</td>
</tr>
<tr>
<td>Glutamine dehydrogenase</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>Ribonuclease A and B</td>
</tr>
<tr>
<td>Glyceraldehyde dehydrogenase</td>
<td>Superoxide dismutase</td>
</tr>
</tbody>
</table>

**Table 1.6** Enzymes that are oxidatively inactivated (See Pardini 1995).
(Adelman et al., 1988; Fridovich 1978; Kasai et al., 1986; Richter 1988; Richter et al., 1988; Simic et al., 1989) and include single strand breaks, double strand breaks, sister chromatid exchange, DNA-DNA cross links, DNA-protein cross links, base modifications e.g. hydroxylations and damage to the sugar phosphate backbone (Simic et al., 1989; Teebor et al., 1988).

1.7.5 Biological sources of ROS

Since the mitochondrial electron-transport chain is a known source of H$_2$O$_2$ from disproportionating O$_2$•⁻, via interactions with complex I, II and III (Cadenas et al., 1977; Loshen et al., 1971) it is likely that all aerobic cells produce, or have the potential to produce ROS. Specific processes for ROS generation have been identified in several vertebrate tissues and organs, utilising electrons from either NADPH (phagocytes, reproductive tissue) or NADH (heart, phagocytes) although the function of the ROS have not been fully characterised in some tissues / organs e.g. heart (Cross & Jones 1991; Nohl 1987).

Many enzyme-dependent reactions have the potential to generate and release ROS following the oxidation of substrates, these include monoamine oxidase, urate oxidase, acetyl CoA oxidase, xanthene oxidase, aldehyde oxidase and tryptophan dioxygenase activities (Cadenas 1995a). In addition, other enzymic sources of ROS in cells are the reductases associated with the microsomal cytochrome P450 monoxygenase system which can utilise NAD(P)H to generate O$_2$•⁻ (Cross & Jones 1991; Kappus 1993). Quinones or hydroquinones either from dietary sources or the metabolites produced by the MFO system have the capacity to redox cycle via flavoprotein reductases to produce ROS (see Figure 1.8). Partial 1 electron reduction of these quinoid compounds gives rise to a semiquinone
Figure 1.8 The one and two electron redox cycling properties of quinoid compounds (Cadenas 1995b).
radical capable of transferring the electron to molecular oxygen reverting back to the initial quinone, this results in a net loss of NAD(P)H and the formation of $O_2^{-}$ (Dutton et al., 1989). Some methyl substituted naphthoquinones with glutathionyl substituents undergo 2 electron oxidation to the hydroquinone via the enzyme DT Diaphorase [quinone oxidoreductase; EC 1.6.99.2] however as a consequence of the glutathionyl substituent, these hydroquinones may auto-re-oxidise to the quinone liberating their 2 electrons sequentially to produce and $O_2^{-}$ and $H_2O_2$ (Buffington et al., 1989). As reported above transition metal-chelates may stimulate ROS production via 'Fenton-like' reactions and exposure to thermodynamic energy in the form of uv light or radiation can generate $^{1}O_2$.

1.8 ANTIOXIDANT PROCESSES

Currently two major groups of antioxidants have been described, firstly compounds that interrupt the propagation of the free radicals, and secondly enzymes that catalyse the detoxification of ROS (Cadenas 1995a). Since oxidative stress may cause damage to cellular macromolecules then repair mechanisms may be included as antioxidant processes as they protect against the deleterious effects of non-regulated oxidation (Davies 1995). Evidence of genetic regulation of antioxidant processes has been identified in both prokaryotes and eukaryotes where oxidised transcription factors upregulate a battery of antioxidant enzymes (Eisenstark et al., 1995; Rushmore et al., 1991; Sen & Packer 1996; Storz et al., 1990).

1.8.1 The antioxidant molecules

Generally antioxidant molecules (A) donate a single electron or hydrogen atom to a radical species, transferring the radical characteristic to the antioxidant molecule itself (eq. 16). The antioxidant is subsequently recovered via interactions with other antioxidants e.g. ascorbic
acid (forming an ascorbyl radical) or other radicals (eq. 17 and eq. 18) or spontaneously degrades.

\[ AH + X^- \rightarrow A^- + XH \]  
(eq. 16)

\[ A^- + \text{Ascorbic acid} \rightarrow \text{Ascorbyl radical} + XH \]  
(eq. 17)

\[ A^- + O_2^- \rightarrow A + O_2 \]  
(eq. 18)

Ascorbic acid is regenerated by the enzyme ascorbyl free radical reductase (Choi & Rose 1989). From eq. 17 it is observed that ascorbic acid undergoes a 1 electron transfer to form a radical, however ascorbic acid can also undergo a 2 electron reaction with \( \text{H}_2\text{O}_2 \) to dehydroascorbate with the production of water (eq. 19). The enzyme dehydroascorbate reductase regenerates ascorbic acid (Summers & Felton 1993). It is apparent from the above equations that a suite of reductases and reducing equivalents (NADPH and reduced glutathione) are necessary for the regeneration of the antioxidants and as such may also be regarded as essential for the preservation of the antioxidant status of the cells.

\[ \text{H}_2\text{O}_2 + \text{Ascorbic acid} \rightarrow 2 \text{H}_2\text{O} + \text{dehydroascorbate} \]  
(eq. 19)

Examples of important naturally occurring non-protein antioxidant molecules include vitamin E (\( \alpha \)-tocopherol), ascorbic acid, ubiquinol, caeruloplasmin, uric acid, thiols, carotenoids and polyphenols (Cadenas 1995a, Davies 1995, Halliwell 1995; Krinski 1995; Pardini 1995; Rice-Evans 1995).
1.8.2 Antioxidant enzymes

Antioxidant enzymes are important preventative antioxidants that act to reduce the formation of ROS and increase cell survival (Michiels et al., 1994). The key enzymes are superoxide dismutase (SOD)[EC 1.15.1.1; catalyses eq. 4]; catalase [EC 1.11.1.6; catalyses eq. 20]; selenium-dependent glutathione peroxidase (SeGPX) [EC 1.11.1.9; catalyses eq. 21 and 22 where GSH and GSSG are respectively reduced and oxidised glutathione, and ROOH is organic hydroperoxide]; selenium-independent glutathione peroxidase [catalyses eq. 22]; glutathione reductase [EC 1.6.4.2; catalyses eq. 23] and DT-diaphorase (catalyses eq. 24 where Q and QH2 are respectively quinone and hydroquinone).

\[
\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad \text{(eq. 20)}
\]

\[
\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG} \quad \text{(eq. 21)}
\]

\[
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG} \quad \text{(eq. 22)}
\]

\[
\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2\text{GSH} \quad \text{eq. 23)}
\]

\[
\text{NADPH} + \text{H}^+ + \text{Q} \rightarrow \text{NADP}^+ + \text{QH}_2 \quad \text{(eq. 24)}
\]

SOD was first described by McCord & Fridovich (1969) and is proposed to occur in all aerobic cells (Pardini 1995). It contributes an important role in antioxidant processes and in some organisms, genetic deletion of this enzyme has been shown to be a lethal mutation (Fridovich 1989). In mammals 2 forms exist, a cytosolic copper-zinc enzyme and a mitochondrial form utilising manganese. The Mn-dependent enzyme is also present in bacteria and plants along with Fe-SOD (Davies 1995; Fridovich 1989). The catalytic
product of SOD is \( \text{H}_2\text{O}_2 \) and to avoid further ROS toxicity, catalase and SeGPX attempt to reduce \( \text{H}_2\text{O}_2 \) concentrations by the rapid reduction to water (Flohé 1982; Halliwell & Gutteridge 1990). Catalase is a tetrameric haemoprotein located primarily in peroxisomes where \( \text{H}_2\text{O}_2 \) production is high (Pardini 1995). SeGPX utilises the reducing power of the sulphahydryl moiety of GSH (a tripeptide containing \( \gamma\)-glutamyl-cysteiny1-glycine) to reduce both \( \text{H}_2\text{O}_2 \) and organic peroxides whilst forming the disulphide-bonded compound GS-SG, oxidised glutathione during the reaction. Glutathione reductase uses NADPH to reduce GSSG permitting the continuous action of GPX. In order for SeGPX to attack lipid peroxides, the LOOHs have to be removed from the membranes, hence phospholipase C facilitates GPX activity. Selenium-independent GPX activities have also been identified and attributed to both a dimeric \( \alpha \)-class glutathione S-transferase enzyme (George 1994) and a phospholipid hydroperoxidase (Weitzel et al., 1990). These non-SeGPX do not appear to react with \( \text{H}_2\text{O}_2 \) but are capable of reducing lipid peroxides, the phospholipid hydroperoxidase enzyme acting directly on membrane-bound lipids. From Figure 1.8 it may be observed that DT diaphorase may be considered to initiate a prooxidant process since some hydroquinones formed by DT diaphorase have the potential to redox cycle. For other quinones however, e.g. methyl substituted naphthoquinones (see Section 2.4) that undergo 1 electron redox cycling, the hydroquinone produced by the action of DT diaphorase is redox stable and therefore DT diaphorase acts to reduce ROS generation (Cadenas 1995a; Cadenas 1995b).

1.9 ROS IN AQUATIC ORGANISMS

Literature reviews have described prooxidant and antioxidant processes in many aquatic phyla with observed or proposed mechanisms of ROS generation involving redox cycling,
the microsomal electron transport system and transition metal-catalysed reactions (Di Giulio et al., 1989; Lemaire & Livingstone 1993; Livingstone 1991; Livingstone et al., 1994; Winston 1991; Winston & Di Giulio 1991). Specific examples of regulated ROS generation by fish and bivalves include the cytotoxic killing of pathogens by phagocytes (Coles et al., 1995; Pulsford et al., 1994) and both antioxidant enzyme activities and ROS scavengers have been identified in hepatic, digestive gland or equivalent tissue and extra-hepatic tissue from both aquatic vertebrates and invertebrates (Di Giulio 1991; Lemaire & Livingstone 1993; Livingstone 1991). Evidence that the antioxidant processes do not sufficiently protect against the deleterious effects of ROS in fish tissue include oxidative damage to both DNA (Nishimoto et al., 1991) and lipids (Di Giulio et al., 1993; Livingstone et al., 1993).

1.9.1 Fish prooxidant processes

Although there have been some studies examining non-xenobiotic-dependent ROS production e.g. age-dependent increase in $O_2^-$ production in the mitochondria of chinook salmon *Oncorhynchus tschawytscha* (Sawada et al., 1993), the majority of studies in fish species focus on the *in vitro* xenobiotic stimulation of microsomal ROS production (Winston & Di Giulio 1991). Since the liver can accumulate xenobiotics and it is a site of MFO-dependent electron transport, the liver is the major organ of study (Lemaire & Livingstone 1993). The basal and stimulated rates of ROS production are dependent upon NAD(P)H (Lemaire & Livingstone 1993; Lemaire et al., 1994) and both redox cycling (Di Giulio et al., 1989; Lemaire & Livingstone 1993; Lemaire et al., 1994; Winston & Di Giulio 1991) and metal or metal-chelates (Dimitrova et al., 1994; Kennish et al., 1989) have been implicated in the *in vitro* stimulation of ROS. For example, it was observed that nitroaromatics stimulated the hepatic microsomal $O_2^-$ production in channel catfish *Ictalurus punctatus*, largemouth bass *Micropterus salmoides* and *O. mykis* (Washburn & Di
Giulio 1989) whilst menadione, nitrofurantoin, PAH metabolites (PAH quinones) and pollutant quinones stimulated $\cdot$OH production in flounder *Platichthys flesus* and *P. fluviatilis* (Lemaire & Livingstone 1997; Lemaire *et al.*, 1994).

### 1.9.2 Antioxidant processes in fish

In the review by Lemaire & Livingstone (1993), the existence of antioxidant scavenger molecules such as vitamin E, ascorbic acid, glutathione and total carotenoids were reported in 14 species of fish. Although inter-species variability in scavenger tissue concentrations were observed, the levels of scavenger molecules were generally greater in fish liver, kidney, gills and brain compared to spleen or muscle when expressed per gram wet weight of tissue (see Table 1.7). The antioxidant enzymes SOD, catalase and GPX have also been identified in hepatic and extra-heptic tissue preparations from at least 14 families (25 species) of teleosts and elasmobranchs (Lemaire & Livingstone, 1993) and some investigations have been undertaken to determine if the regulation of these enzymes can be altered by prooxidant processes such as xenobiotic exposure. Environmental factors such as temperature, hypoxia and seasonal variation have been reported to affect antioxidant enzyme activities in both freshwater and marine teleosts (Livingstone *et al.*, 1995; Rady 1993; Vig & Nemcsók 1989).

Following exposure to sediments contaminated with PAHs, PCBs and other chemicals, increases in hepatic SOD and catalase activities were observed in *I. punctatus* (Di Giulio *et al.*, 1993) and dab *Limanda limanda* (Livingstone *et al.*, 1993) but not *P. flesus* (Bergman *et al.*, 1994). Whereas exposure to kraft mill effluent increased the hepatic catalase activity of *I. punctatus* (Mather-Mihaich & Di Giulio 1991) this complex mixture had no effect on either hepatic catalase or SOD activities of *O.mykiss* (Otto *et al.*, 1994). When *O. mykiss* were exposed to aflatoxin B1 however an increase in DT diaphorase activity was observed.
<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Tissue</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione (µmol/g wet weight)</td>
<td>Liver</td>
<td>0.91 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Gills</td>
<td>0.45 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>Gas gland</td>
<td>0.136</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.78 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>0.219</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>0.265</td>
</tr>
<tr>
<td></td>
<td>Larvae</td>
<td>0.907</td>
</tr>
<tr>
<td>Vitamin E (µmol/g wet weight)</td>
<td>Liver</td>
<td>0.273 ± 0.119</td>
</tr>
<tr>
<td></td>
<td>Gill</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Gas gland</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>Whole body</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>Vitamin C (µmol/g wet weight)</td>
<td>Liver</td>
<td>0.33 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Gills</td>
<td>0.142</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.22 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Brain</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>Larvae</td>
<td>0.70</td>
</tr>
<tr>
<td>Total carotenoids (µg/g wet weight)</td>
<td>Whole fish</td>
<td>10275 ± 6900</td>
</tr>
</tbody>
</table>

**Table 1.7** Glutathione, vitamins E and C and carotenoid concentrations in different species of fish (see Lemaire & Livingstone 1993 for identities of fish species).
(Parker et al., 1993). It should be noted that although the studies described in this paragraph report apparent elevation of enzyme activities following exposure to xenobiotics, the exposure and sampling times vary greatly from 2 days to 12 months and therefore the above studies indicate that regulation of fish antioxidant enzymes may in influenced by exposure to organic contaminants however these responses are variable and may be both species- and xenobiotic-dependent. Species-dependent differences in the levels of hepatic catalase, DT diaphorase, glutathione reductase, glutathione $S$-transferase and total glutathione but not SOD in $I. punctatus$ and brown bullhead $Ameriurus nebulosus$ may account for observed species differences in susceptibility to oxidative stress (Hasspieler et al., 1994).

Differences in the levels of antioxidant enzyme activities have also been observed in several field studies. Hepatic GPX, SOD, catalase but not glutathione reductase activities were higher in mullet $Mugil$ sp. sampled from an estuary impacted with metals and organics compared to a reference (less polluted) site (Rodríguez-Ariza et al., 1993). SOD activity was determined to be higher in the livers of spot $Leiostomus xanthurus$ sampled from polluted areas (Roberts Jr et al., 1987) whilst both SOD and catalase activities were higher in $L. limanda$ caught near oil production rigs in the North Sea (Livingstone et al., 1992). Catalase and DT diaphorase activities were higher in male goby $Zosteriosser ophiocephalus$ from polluted sites in the Venice Lagoon however seasonal or other factors could have influenced the determined activities (Livingstone et al., 1995). Both tissue- and enzyme-specific changes in antioxidant enzyme activities were observed in Nile tilapia $Oreochromis niloticus$ sampled from a polluted reservoir compared to a control site; respectively gill (decrease in catalase activity), liver (increase in SOD activity and decreases in catalase and glutathione reductase activities) and erythrocytes (elevated SOD and GPX activity but a decrease in catalase activity) (Bainy et al., 1996). An altered hepatic
SOD isoform profile (identified by isoelectrofocussing) using *Mugil* sp. sampled from polluted sites has also been reported (Pedrajas *et al.*, 1993).

1.10 THE FLAVIN-CONTAINING MONOOXYGENASE (FMO)

The flavin-containing monooxygenase [FMO; EC 1.14.13.8] is located in the smooth endoplasmic reticulum and catalyses the monooxygenation of a unique variety of xenobiotics (Hodgson *et al.*, 1995). Using NADPH as a cofactor and bound FAD as the prosthetic group, this microsomal protein monooxygenates substrates with diverse chemical structures categorised as soft nucleophiles including inorganic ions and organic chemicals containing nitrogen, sulphur, phosphorous or selenium heteroatoms - see Table 1.8 (Jakoby & Ziegler 1990; Hines *et al.*. 1994; Ziegler 1993). Although the isoforms of cytochrome P450 may be regarded as preferring hard nucleophiles, many substrates for FMO are also P450 substrates but different products and stereochemistry may differentiate the 2 metabolic pathways (Hodgson *et al.*, 1995).

The FMO was first reported by Pettit *et al.* (1964) and following purification from pig liver was initially termed the microsomal mixed function amine oxidase (Ziegler & Mitchell 1972). Williams *et al.* (1984) and Tynes *et al.* (1985) demonstrated the existence of multiple FMO forms and this was later supported by amino acid sequence data (Ozols 1989). Currently up to 5 FMO homologues have been DNA sequenced in 5 mammalian species (Gasser 1996; Lawton *et al.*, 1994) with apparent molecular weights of c 60-63 kD (Rettie *et al.*, 1995), however a unique FMO has also been sequenced in the yeast *Sacharomyces cerevisiae* with an apparent molecular weight of 42.4 kD (Suh *et al.*, 1996). The general characteristics of FMOs regardless of tissue or animal source are that they
<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen-containing organics</td>
<td></td>
</tr>
<tr>
<td>Primary amines</td>
<td><em>n</em>-Octylamine</td>
</tr>
<tr>
<td>Secondary amines</td>
<td></td>
</tr>
<tr>
<td>Acyclic</td>
<td><em>N</em>-methylanaline</td>
</tr>
<tr>
<td>Cyclic</td>
<td>Desmethyltrifluperazine</td>
</tr>
<tr>
<td>Tertiary amines</td>
<td></td>
</tr>
<tr>
<td>Acyclic</td>
<td>Chlorpromazine</td>
</tr>
<tr>
<td>Cyclic</td>
<td>Nicotine</td>
</tr>
<tr>
<td>Hydroxylamines</td>
<td><em>N</em>-Hydroxylaminoazobenzene</td>
</tr>
<tr>
<td>Hydrazines</td>
<td></td>
</tr>
<tr>
<td>Monosubstituted</td>
<td>Methylhydrazine</td>
</tr>
<tr>
<td>Disubstituted (1,1)</td>
<td>1,1-dimethylhydrazine</td>
</tr>
<tr>
<td>Monosubstituted (1,2)</td>
<td>1,2-dimethylhydrazine</td>
</tr>
<tr>
<td>Sulphur-containing organics</td>
<td></td>
</tr>
<tr>
<td>Thiols</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>Sulphides</td>
<td>Phorate</td>
</tr>
<tr>
<td>Disulphides</td>
<td>Butyl disulphide</td>
</tr>
<tr>
<td>Thiocarbamides</td>
<td>Thiourea</td>
</tr>
<tr>
<td>Thioamides</td>
<td></td>
</tr>
<tr>
<td>Dithioacids and dithiocarbonate</td>
<td>Dithiobenzoate</td>
</tr>
<tr>
<td>Mercaptopurines and mercaptopyrimidines</td>
<td></td>
</tr>
<tr>
<td>Phosphorous-containing organics</td>
<td></td>
</tr>
<tr>
<td>Phosphines</td>
<td>Diethylphenylphosphine</td>
</tr>
<tr>
<td>Phosphonothioates</td>
<td>Phonophos</td>
</tr>
<tr>
<td>Selenium-containing organics</td>
<td>2-Selenylbenzanilide</td>
</tr>
<tr>
<td>Boronic acids</td>
<td>Butylboronic acid</td>
</tr>
</tbody>
</table>

*Table 1.8* Examples of organic and inorganic substrates for FMO (Jakoby & Ziegler 1990; Hodgson et al., 1995).
form kinetically stable 4α-hydroperoxy flavin intermediates (see Section 1.11.2) in the presence of NAD(P)H and oxygen, independent to substrate binding (Ziegler 1993).

1.11 PROPERTIES AND CHARACTERISTICS OF FMO

1.11.1 Nomenclature and classification of FMOs

As different FMO forms were identified, multiple nomenclature systems developed including the appointment of trivial species-dependent names (see Table 1.9). In 1994 Lawton et al. proposed a naming system based upon inferred or known amino acid sequence homologies. Known sequence homologies for 5 orthologues have been characterised to vary between 82-87% whereas non-orthologous forms vary between 51-57% (see Table 1.10). The mammalian FMO system is represented by ‘FMO’ and the 5 known orthologues are distinguished by Arabic numerals (FMO1 to FM05). Genes and cDNA designations are italicised and mRNA and protein designations are not. From a calculated rate of evolution of FMOs it is proposed that individual members of the FMO gene family arose by duplication of a common ancestral gene approximately 250-300 million years ago (Phillips et al., 1995).

1.11.2 The FMO molecule, structure and reaction mechanisms

FMO activities have been detected or characterised in a variety of organisms including Acinbacter (Ryerson et al., 1982) yeast (Suh et al., 1996), marine invertebrates (Schlenk &
<table>
<thead>
<tr>
<th>Proposed name</th>
<th>Trivial name</th>
<th>Species</th>
<th>Source</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMO1</td>
<td>Liver</td>
<td>Pig</td>
<td>cDNA</td>
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Table 1.9 Summary of the trivial FMO names, species distribution and nature of the determined amino acid sequences for the 5 FMO forms (Lawton et al., 1994; Overby et al., 1995).
Table 1.10 Comparisons of primary structures of FMOs (Lawton * et al.*, 1994; Overby *et al.*, 1995; Phillips *et al.*, 1995). Interspecies comparisons are shaded and ortholog comparisons are in bold. (* = 52-57 % sequence homology as reported by Overby *et al.*, 1995).
Buhler 1989; 1990a), elasmobranchs (Goldstein & Dewitt-Harley 1973), marine and freshwater teleost fish (Ágústsson & Støm 1981; Schlenk & Buhler 1991a; Schlenk & Buhler 1993) and terrestrial mammals (Lee et al., 1995). In mammals the highest levels of microsomal FMO activities have been measured in liver, kidney and lung preparations with some specific FMO forms exhibiting differential expression with developmental stage - FMO1 is expressed in human fetal liver but does not appear to be expressed in adult hepatic tissue (Gasser 1996; Ziegler 1993). FMOs appear to demonstrate several amino acid sequences conferring a primary protein structure including 2 Gly-Xaa-Gly-Xaa-Xaa-Gly motifs characteristic of FAD-pyrophosphate- and NADPH-pyrophosphate-binding sites, putative FAD- and NADPH-binding sites, the latter forming a $\beta\alpha\beta$ secondary structure known as the Rossmann fold (Phillips et al., 1995).

The catalytic cycle in Figure 1.9 is based on the kinetic and spectral studies using purified FMO1 as reviewed by Ziegler (1993) and Poulsen & Ziegler (1995). The FAD prosthetic group first reacts with NADPH and then molecular oxygen to form the 4α-hydroperoxyflavin bond, responsible for the oxidation of soft nucleophiles. The formation of the stabilised hydroperoxy bond occurs in the absence of substrate and it is proposed that the enzyme predominantly exists in the high energy reactive state (Hodgson et al., 1995). Any nucleophile that can be oxidised by the hydroperoxide is a potential substrate and a single point of contact between substrate and the terminal oxygen may sometimes be all that is required for product formation (Ziegler 1993; Jakoby & Ziegler 1990). The substrate is converted to product by the nucleophilic attack on the terminal oxygen followed by heterolytic cleavage of the peroxide. The product immediately leaves the active site and then the enzyme-FAD-OH complex is converted to enzyme-FAD with the release of water. The release of water is proposed to be the rate-limiting step (RLS) for the catalytic cycle (Poulsen & Ziegler 1995). The enzyme-FADH$_2$ complex is regenerated following the loss
Figure 1.9 The FMO catalytic cycle of FMO1. $E_{\text{FAD-OOH}}$ = hydroperoxyflavin, $E_{\text{FAD-OH}}$ = hydroxyflavin (Poulsen & Ziegler, 1995; Ziegler 1993).
of water via a series of reversible reactions involving the exit of the NADP⁺ cofactor, addition of NADPH and H⁺ and the reduction of the FAD prosthetic group. As a consequence of the RLS being independent of substrate binding or product leaving, the observed \( V_{\text{max}} \) for many substrates appears to be similar (Ziegler 1993). Many studies have been undertaken to investigate the steric properties of the FMO substrates in an attempt to identify homologue-specific substrates (Ziegler 1993). Nagata et al. (1990) observed that access to the hydperoxyflavin was different for FMO1 and FMO2 using both organic nitrogen and sulphur nucleophilies. Substrates with substituents within 3Å of the nucleophilic heteroatom and more than 8Å in their longest axis were totally excluded by FMO2 but not FMO1. Steric hinderence determining substrate presentation at the active site was also observed by Guo et al. (1992) when thiourea and other thiocarbamides of increasing molecular surface area were incubated with microsomal FMOs from different mammalin tissues. A recent study examining the stereochemical composition of FMO substrates has enabled further differentiation of selective FMO homologues by the characterisation of the chirality of alky p-tolyl sulfoxides generated following FMO-dependent S-oxidation (Rettie et al., 1995).

1.1.3 Regulation

There is some evidence to suggest that FMO may be regulated via dietary input of one or more organic nitrogen or sulphur xenobiotic soft nucleophiles since both FMO protein and activity were altered by diet (Kaderlik et al., 1991). Although little is known of the endogenous functions of FMO, naturally occurring endogenous substrates have the potential to regulate FMO levels. Marine molluscs for example may utilise FMO to oxidise dimethysulphide (present in phytoplankton) (see Stegeman & Hahn 1994) whereas many
organisms are exposed to, or derive naturally occurring endogenous substrates including thio-ether conjugates, methionine, cysteamine and TMA (Duescher et al., 1994; Elfarra 1995; Ziegler 1988; Ziegler 1993).

Primary amines are not usually substrates for FMO but lipophilic primary amines can increase the $V_{\text{max}}$ of FMO1 two fold by binding to effector sites distinct from the catalytic site (Ziegler & Mitchel 1972; Ziegler et al., 1973;). Activation appears to be dependent upon the protonated primary amine and the subsequent changes in $V_{\text{max}}$ are due to increases in the rate of dehydration of the enzyme-FAD-OH complex (see Poulsen & Ziegler 1995).

FMO regulation via gene expression has recently been undertaken using cDNA probes and induction of FMO homologues have been partially characterised. Expression of $FMO1$ and $FMO2$ apppears to be independent of a TATAA regulatory box or DNA methylation status (Lou & Hines 1996; Shehin-Johnson et al., 1996) and it is proposed that tissue specific transcription factors contribute to the regulation of both $FMO1$ and $FMO2$ expression (Lou & Hines 1996; Shehin-Johnson et al., 1996). Regulation of rabbit lung FMO activity by glucocorticoids was first observed by Devereux & Fouts (1975) however Lee et al. (1993) demonstrated glucocorticoid-dependent induction of male lung $FMO2$. A correlation between levels of plasma progesterone or cortisol with $FMO2$ expression in mid- and late term pregnancy indicate both developmental and hormonal regulation (Lee et al., 1995). Further evidence of hormonal regulation of FMO was presented by Lee et al. (1995) where both progesterone and dexamethasone induced $FMO2$, and a 4-fold increase of $FMO1$ mRNA was also observed following progesterone and glucocorticoid administration.
1.12 FMOs IN AQUATIC ORGANISMS

The expression of FMO enzyme activity, proteins or genes has been examined in many aquatic organisms (Schlenk 1993; Schlenk 1997). Studies of FMO catalytic activity have utilised substrates including methimazole, dimethylaniline, trimethylaniline, 2-aminoanthracene, 2-aminofluorene and 2-acetylaminofluorene and in vitro activities have been determined in the sub-cellular fractions from digestive gland, hepatopancreas or liver tissue from mussel, *M. edulis, M. galloprovincialis* (Kurelec 1985; Kurelec and Krca 1987; Livingstone et al., 1990); oyster *Crassostrea gigas* (Schlenk & Buhler 1989; Schlenk & Buhler 1990b); clam *Mercenaria mercenaria* (Anderson & Doos 1983); chiton *Chryptochiton stelleri* (Schlenk & Buhler 1990a); crab *Carcinus maenius* (Marsh et al., 1992); sponges (whole animals) *Geodia cydonium, Tethya aurantium, Verongia aerophoba, Pellina semitubulosa* (Kurelec et al., 1987); the copepod *Calanus finmarchicus* (whole animals) (Strøm 1980), at least 25 species of fishes (Schlenk 1997).

Studies using antibodies to mammalian FMOs have indicated conserved FMO epitopes using hepatic proteins from alligator *Alligator mississippiensis*, turtle *Chryemys pictapicter* and 6 species of fish (Schlenk 1997) with estimates of molecular weight ranging 50 - 59 kD.

1.12.1 FMOs in fishes

Although some studies using fish have been undertaken to examine the distribution of either FMO or FMO-dependent metabolites for inter-species comparisons (Baker et al., 1963; Schlenk 1993), other studies have been interested in the distribution of the specific
FMO metabolite \( N.N.N \)-trimethylamine \( N \)-oxide (TMAO) (Ágústsson & Strøm 1981; Daikoku et al., 1988; Goldstein & Dewitt-Harley 1973). The regulation of tissue TMAO levels by marine organisms is a relatively common biochemical mechanism of osmoregulation (Pang et al., 1977). In one study, both tissue levels of TMAO and \( N.N.N \)-trimethylamine (TMA) oxidase activities were elevated in salinity- and TMA-challenged eel \textit{Anguilla japonica} and guppy \textit{Poecilia reticulata} (Daikoku et al., 1988) implicating a potential osmoregulatory function for FMO in fish (Schlenk 1997).

Microsomal FMO activities (See Table 1.11 for substrates) have been determined in liver, kidney, gill and intestine of \textit{O. mykiss} (Schlenk & Buhler 1991a; Schlenk & Buhler 1991b, Schlenk 1995); gill, liver and kidney of \textit{P. flesus} (Schlenk et al., 1995); muscle, liver and kidney of \textit{A. japonica} (Daikoku et al., 1988); liver and gill of turbot \textit{Scophthalmus maximus} and Japanese medaka \textit{Oryzias latipes} (Schlenk 1997; Schlenk et al., 1995); kidney of \textit{L. limanda} (Ágústsson & Strøm 1981); whole body homogenates of \textit{P. reticulata} (Daikoku et al., 1988) and liver homogenates or sub-cellular fractions from at least 19 other teleost or elasmobranch species (See Table 1.11). Prior to the characterisation of the hepatic FMO of \textit{S. maximus} submitted in this thesis, only 3 other studies have described FMO characterisation in nurse shark \textit{Ginglymostoma cirratum} (Goldstein & Dewitt-Harley 1973), \textit{O. mykiss} (Schlenk 1995; Schlenk & Buhler 1991a; Schlenk & Buhler 1991b) and cod \textit{Gadus morhua} (Ágústsson & Strøm 1981). In general, fish FMO activities are optimal at pH 7-9 and are inactivated by temperatures above 40 °C. Unlike mammalian FMO, no sex differences have been reported for enzyme activities (Schlenk & Buhler 1993) and \textit{in vitro} FMO activity appears not to be increased by co-incubation of substrate with primary amines implying a lack of amine effector sites (Daikoku et al., 1988).

Antibodies to mammalian FMO1 and FMO2 have been employed to examine the expression of fish microsomal proteins with epitopes similar to the mammalian forms.
<table>
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<th>Species and tissue</th>
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<th>mRNA (probe)</th>
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<td>Cashman &amp; Olsen 1989</td>
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<td></td>
<td></td>
<td>1.4 kb</td>
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<td>Agüstsson &amp; Srtom 1981</td>
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Table 1.11 Determined FMO activities (including specific activities), apparent molecular weights and mRNA hybridisation (including estimated sequence lengths) in fish sub-cellular fractions (adapted from Schlenk 1997). FMO activities are TMA ox. = trimethylaniline oxidase; DMA ox. = dimethylaniline oxidase; thiourea oxidase; methimazole oxidase; thiobencarb S-oxygenase.
Immunorecognition of microsomal proteins of apparent molecular weights 50 kD (smooth dogfish shark *Squalus acanthias*; silky shark *Carcharinus falciformus*), 57 & 61 kD (*O. mykiss*), 57 kD (*O. latipes*), 56 - 59 kD (*P. flesus*) and 55 kD (*S. maximus*) were determined in gill, liver or kidney tissue from 6 species of fish (respectively Schlenk & Lipshen 1994; Schlenk & Buhler 1993; Schlenk et al., 1995; Peters et al., 1995). In many of the Western blot studies, bands of identical apparent molecular weight were visualised using either antibody and as a consequence it is was not possible to differentiate between a single FMO with epitopes to both antibodies or 2 immuno-distinct FMOs with similar apparent molecular weights. However, kidney and gill of *P. flesus* expressed two FMO-immunopositive proteins while the liver expressed only one indicating multiple FMO forms (Schlenk et al., 1995).

Northern blot analysis of total RNA using a mammalian 1.6 kb cDNA probe for FMO1 has indicated the expression of FMO1-like mRNA ranging between 1.1 - 3 kb in length in *S. acanthias*, *O. mykiss*, striped bass *Morone saxatilis*, *P. flesus* and *S. maximus* under non-stringent conditions (review Schlenk 1997).

In contrast to the above studies, other fish species appear not to express FMO activities (Baker et al., 1963; Schlenk 1995), for example hepatic microsomes from *I. puntatus* failed to exhibit any *N,N*-dimethylanaline (DMA) *N*-oxidase activity or FMO-like microsomal proteins (Schlenk et al., 1993). Schlenk (1997) summarised that the fish species which do not express FMO are a) predominantly of freshwater origin, b) possess apparently low levels of TMA or TMA oxide and c) unable to tolerate changes in salinity.
In the previous sections CYP-dependent and flavin-dependent monooxygenase processes and ROS generation have been outlined with respect to aquatic organisms. The early life stages of fish are highly sensitive to exposure to anthropogenic xenobiotics (Black 1988; Walker et al., 1996b) however compared to adult life stages, little is known concerning fish larval CYP expression and regulation, less is understood about the processes of xenobiotic metabolism and almost nothing has been published concerning ROS toxicity and larval antioxidant processes. Whereas multiple forms of CYP have been identified and sequenced in vertebrates, there have been fewer investigations using invertebrate species. Specifically the presence or expression of multiple CYP forms (CYP1A, 3A, 4A and 11A) have been indicated in *Mytilus* sp. (Wootton et al., 1995) but previous Western blot studies of digestive gland microsomal preparations have utilised only the anti-CYP1A antibody (Livingstone et al., 1997). Levels of *Mytilus* CYP1A-immunopositive protein were determined to be higher in mussels exposed to Ah ligands both in laboratory and field studies (respectively Solé et al., 1996; Livingstone et al., 1997) however studies of differential CYP protein levels have not been undertaken using either indigenous or transplanted mussel populations. Another important monooxygenase system is the FMO system expressed in fish liver. Although little is understood of its endogenous functions or endogenous regulation, FMO may catalyse the oxidation of a diverse group of substrates including the osmolyte precursor TMA (Schlenk 1993; Schlenk 1995).

The objectives of this study were to elucidate the processes of oxygen-dependent xenobiotic metabolism in marine fish and mussel and were specifically to examine levels of CYP1A (via EROD activity and Western blot determinations), mechanisms of toxicity (ROS generation and P450-dependent bioactivation) and antioxidant processes in the sensitive life stages of fish (Chapter 2); undertake immunochemical investigations of CYP
forms/epitopes in mussel partially purified CYP and digestive gland microsomes prepared from mussels sampled from indigenous and transplanted populations (Chapter 3); characterise a fish FMO and examine the differential expression of activity following salinity challenges (Chapter 4).

Each chapter contains an explanatory introduction followed by published or “in press” papers. The results are discussed within each paper and each chapter concludes with a summary of the findings relating to the chapter. The format of this thesis has been constructed following advice from the University of Plymouth Research Support Unit.
CHAPTER 2

FISH CYTOCHROME P450-DEPENDENT METABOLISM, PROOXIDANT AND ANTIOXIDANT PROCESSES

2.1 INTRODUCTION

Most of the previous studies concerning CYP-dependent xenobiotic metabolism (described in section 1.5.1) and prooxidant / antioxidant processes (described in section 1.9.1 and 1.9.2) in fish, have been undertaken using adult life stages. The early life stages of fish are highly sensitive to exposure to a broad selection of xenobiotics e.g. sewage sludge, inorganic and organic contaminants (Black et al., 1988; Buhl & Hamilton 1991; Costello & Gamble 1992; Walker et al., 1996b; Wright 1988) and fish embryo and larval toxicity studies have been extensively used to assess the toxicological impact of anthropogenic contaminants on aquatic biota (Black 1988; Fujimara et al., 1991; Groth et al., 1993; Middaugh et al., 1993). Impairment of fish reproductive success as a consequence of exposure to anthropogenic contaminants has the potential to influence year-class recruitment of susceptible species by reducing embryo hatching success (Westernhagen et al., 1987). Toxicity studies following embryo and larval exposure to either contaminants at environmental concentrations (Wright 1988), the sea surface microlayer (Cleary et al., 1993; Westernhagen et al., 1987), polluted sediments (Cooper et al., 1993) and sediment extracts (Wilson & Tillet 1996) and field in situ water column exposure (Hall Jr. et al., 1993), indicate the survival of the early life stages of fish in the field may be influenced by
<table>
<thead>
<tr>
<th>Species</th>
<th>MFO activity or CYP levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fundulus heteroclitus</em></td>
<td>Elevation of AHH activity</td>
<td>Binder &amp; Stegeman, 1980; Binder &amp; Stegeman, 1984; Binder <em>et al.</em>, 1985</td>
</tr>
<tr>
<td><em>Salvinus namaycush</em></td>
<td>Elevation of AHH activity</td>
<td>Binder and Lech, 1984</td>
</tr>
<tr>
<td><em>Gadus morhua</em></td>
<td>Increase in CYP1A1 protein</td>
<td>Goksøyr <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><em>Scophthalmus maximus</em></td>
<td>Elevation of EROD activity</td>
<td>Peters <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Sparrus aurata</em></td>
<td>Elevation of EROD activity</td>
<td>Peters L.D. &amp; Nasci C. (personal communication)</td>
</tr>
<tr>
<td><em>Salvinus alpinus</em></td>
<td>Elevation of EROD activity</td>
<td>Monod <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Coregonus lavaretus</em></td>
<td>Elevation of EROD activity</td>
<td>Monod <em>et al.</em>, 1996</td>
</tr>
<tr>
<td><em>Thymallus thymallus</em></td>
<td>Elevation of EROD activity</td>
<td>Monod <em>et al.</em>, 1996</td>
</tr>
</tbody>
</table>

**Table 2.1** Elevation of cytochrome P450-dependent catalytic activity or CYP1A protein following the hatching of fish embryos.
anthropogenic contaminants. Incidences of developmental defects have been determined in pelagic fish embryos sampled from the southern North sea (Cameron et al., 1992) and the western Baltic (Westernhagen et al., 1988) with elevated malformation rates near river estuaries and near-shore waters. Although these higher rates of observed embryonic malformation (from demersal fish species) appear correlated to previously identified sites with contaminated sediments, little is known concerning the mechanisms involved concerning the malformations. A recent study however has demonstrated the levels of hydrophobic DNA adducts (identified by $^{32}$P postlabelling techniques, see Section 2.5) in $G. \text{morhua}$ embryos and larvae from the Baltic were 19 times higher than levels observed in the equivalent life stages sampled from a relatively unpolluted population in the north east arctic (Ericson et al., 1996) suggesting that DNA damage may be occurring in the field. The accumulation of lipophilic contaminants in the gonads of female fish and their deleterious effects on egg development (Hose et al., 1981; Binder & Lech 1984; Westernhagen 1988) indicate that maternal transfer of organic hydrocarbons may affect embryo and larval development and survival in addition to the uptake of xenobiotics by the embryos and larvae directly from the aquatic environment.

The presence of a CYP-dependent MFO system has been determined in both marine and freshwater fish embryos (Binder & Stegeman, 1980; Binder & Stegeman, 1984; Binder et al., 1985; Binder and Lech, 1984; Goksoyr et al., 1988; Monod et al., 1996) and thus the early life stages of fish have the capacity to metabolise and eliminate organic xenobiotics (observed by Kocan & Landolt 1984) but also have the ability to bioactivate procarcinogens to carcinogens. Regulation of CYP1A appears to be life stage dependent since elevation of CYP protein or catalytic activity (determined as EROD activity or arylhydrocarbon hydroxylase (AHH) activity) has been observed in 8 species of fish following hatching (see Table 2.1). Evidence of xenobiotic-dependent regulation has been
### Table 2.2 Elevation of cytochrome P450-dependent catalytic activity or CYP1A protein in fish embryos and larvae following exposure to organic contaminants during their embryonic development.

<table>
<thead>
<tr>
<th>Life stage and Species</th>
<th>Exposure conditions</th>
<th>MFO activity or CYP1A levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Embryo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fundulus heteroclitus</em></td>
<td>Polychlorobiphenyls</td>
<td>DMSO, Elevation of AH activity</td>
<td>Binder and Stegeman, 1980; Binder et al., 1985</td>
</tr>
<tr>
<td></td>
<td>No 2 fuel oil</td>
<td>DMSO, Elevation of AH activity</td>
<td>Binder and Stegeman, 1980</td>
</tr>
<tr>
<td><strong>Larvae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fundulus heteroclitus</em></td>
<td>Polychlorobiphenyls</td>
<td>DMSO, Elevation of AH activity</td>
<td>Binder and Stegeman, 1980; Binder et al., 1985</td>
</tr>
<tr>
<td><em>Gadus morhua</em></td>
<td>Water soluble fraction of North Sea cruse oil</td>
<td>Increase in CYP1A1 protein (polyclonal antibody)</td>
<td>Gokseýr and Solberg, 1987; Gokseýr et al., 1991; Gokseýr et al., 1988</td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>Polychlorobiphenyls</td>
<td>DMSO, Elevation of EROD activity</td>
<td>Norrgren et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Polychlorinated naphthalenes</td>
<td>DMSO, Elevation of EROD activity</td>
<td>Norrgren et al., 1993</td>
</tr>
<tr>
<td><em>Salvinus alpinus</em></td>
<td>ß-naphthoflavone</td>
<td>Acetone, Elevation of EROD activity</td>
<td>Monod et al., 1996</td>
</tr>
</tbody>
</table>

EROD = 7-ethoxyresorufin O-deethylase activity, AH = arylhydrocarbon hydroxylase activity, DMSO = dimethylsulphoxide.
demonstrated following exposure to *Ah* ligands; embryonic exposure to PCB mixtures, PAHs and model contaminants induced CYP1A protein or elevated catalytic activity in both embryonic and larval tissue preparations (see Table 2.2). Larval expression of CYP1A is also inducable as indicated by elevated EROD and AHH activity or elevation of CYP1A immunopositive protein following exposure to PCB mixtures, individual PCB congeners, PAHs, lindane and model contaminants (see Table 2.3).

Section 2.2, 2.3 and 2.5 of this thesis describe studies examining the CYP1A expression and potential bioactivation of BaP in larval and juvenile life stages of *S. maximus*. Section 2.3 also examines the capacity of *S. maximus* larvae to generate ROS whose deleterious effects may be protected against by a suite of antioxidant enzymes present in the embryonic and larval life stages (Section 2.4). Finally a field study examining CYP1A and antioxidant enzyme expression in the clupeid sardine *Sardina pilchardus* is submitted as section 2.6.
<table>
<thead>
<tr>
<th>Species</th>
<th>Exposure conditions</th>
<th>MFO activity or CYP levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salvinus namaycush</em></td>
<td>Polychlorobiphenyls</td>
<td>Elevation of AHH activity</td>
<td>Binder and Lech, 1984; Binder et al. 1985</td>
</tr>
</tbody>
</table>
| *Oncorhynchus mykiss*| Polychlorobiphenyls and polyaromatic hydrocarbons | Elevation of AHH activity  
Elevation of EROD activity  
Elevation of aminopyrene-N-demethylase activity | Vigano et al., 1995 |
|                     | Polychlorobiphenyl congener CB-126, PAHs    | Elevation of EROD activity  
Elevation of EROD activity  
Elevation of EROD activity | Engwall et al., 1994 |
|                     | Polychlorobiphenyl extracts from sediments |                                                            |                                  |
| *Scophthalmus maximus*| BaP                                        | Elevation of EROD activity                                    | Peters and Livingstone 1995      |
|                     | Lindane                                    | Elevation of EROD activity                                    | Peters and Livingstone 1995      |
| *Sparrus aurata*    | BaP                                        | Elevation of EROD activity                                    | Peters L.D. and Nasci C. (personal communication) |
| *Salvinus alpinus*  | β-naphthoflavone                           | Elevation of EROD activity                                    | Monod et al., 1996               |

Table 2.3 Elevation of cytochrome P450-dependent catalytic activity in fish larvae following exposure to organic contaminants during larval development. EROD = 7-ethoxyresorufin O-deethylase activity, AHH = arylhydrocarbon hydroxylase activity, DMSO = dimethylsulphoxide, DMF = dimethylformamide.
2.2 STUDIES ON CYTOCHROME P4501A IN EARLY AND ADULT LIFE STAGES OF TURBOT (*SCOPHTHALMUS MAXIMUS* L.).
Studies on Cytochrome P4501A in Early and Adult Life Stages of Turbot (Scophthalmus maximus L.)

Laurence D. Peters & David R. Livingstone

NERC Plymouth Marine Laboratory, Citadel Hill, Plymouth, UK. PL1 2PB

ABSTRACT

Cytochrome P4501A (CYP1A) has been investigated extensively in the liver of adults of many different fish species. This study reports the presence and elevation of CYP1A measured as 7-ethoxyresorufin O-deethylase (EROD) activity and protein amount (Western blot — semi-quantified by image analysis) in larval, juvenile and adult turbot (Scophthalmus maximus) after exposure to organic contaminant and model inducers. Basal EROD activity was not detectable in embryos, but increased from 3 day larvae (whole body 13 500g supernatants) through 90 day juveniles (hepatic 13 500g supernatants) to adults (hepatic microsomes), respectively in pmol/min/mg protein (± SEM), 0.57±0.06, 10.8±2.4 and 12.3±4.7. Exposure to 5 ppm benzo[a]pyrene (B[a]P), 1 ppm γ-hexachlorocyclohexane (lindane) and 25 ppm B[a]P caused respectively, 3-, 6- and 2-fold elevation of EROD activity in 4 and 9 day larvae and juvenile stages. Image analysis of Western blots of juvenile samples detected a 3-fold difference in CYP1A protein whereas EROD activity of the same sample varied 4-fold. The results confirm the potential of using CYP1A induction as a biomarker for impact by organic pollution on early developmental stages of fish.

The early life stages of fish appear to be particularly sensitive to anthropogenic contaminants (Black, 1988; Buhl & Hamilton, 1991). The embryonic, larval and juvenile stages of both marine and fresh-water species may be affected by polynuclear aromatic hydrocarbons (PAHs) (Stene & Lonning, 1984; Goksøyr et al., 1991) and polychlorobiphenyls (PCBs) (Binder & Stegeman, 1980; Binder & Lech, 1984). Such contaminant exposure is indicated to induce cytochrome P4501A (CYP1A) in these early life-stages and has the potential to be developed as a biomarker of impact by organic contaminants for use in pollution monitoring (Adams et al., 1989; Livingstone, 1993). This study reports the presence and apparent inducibility of CYP1A in early and adult life stages of turbot (Scophthalmus maximus L.), and the development of 7-ethoxyresorufin O-deethylase (EROD) activity and CYP1A protein as biomarkers for use with fish larvae.

Live larvae and juveniles were obtained from a commercial hatchery (Golden Sea Produce, Hunterston, West Kilbride, Scotland), and adults were caught
locally off the coast of Plymouth, UK. Larvae were exposed to contaminants (see Table I) added in a minimal volume of dimethylformamide (DMF) to static, aerated sea-water. Adults were injected with β-naphthoflavone (20 mg/kg in corn oil, i.p.) and held for 24 h before sacrifice. Larval tissue was frozen whole under N₂(1) and stored at −70°C prior to analysis. Juvenile livers were assayed immediately for enzyme activity, whilst adult hepatic microsomes were prepared (100 000 g spin. 1.5 h) in 0.15M KCl-KOH, pH 7.5, 20% glycerol and stored under N₂(1). Pooled larval samples or juvenile livers were homogenised by sonication and centrifuged for 5 min at 13 500 g in 0.15M KCl-KOH, pH 7.6, 20% ethylenediaminetetraacetic acid. EROD activity was determined by incubation of 90μl of 13 500 g supernatant with NADPH, 7-ethoxyresorufin and phosphate buffer pH 7.6 (final concentrations, 0.225mM, 3.74μM and 59mM respectively) for 60 min at 30°C (Peters et al., 1992). Reactions were stopped by the addition of 200μl acetone, centrifuged (as above) and resorufin fluorescence measured against an 85 nM resorufin standard. Aliquots of adult microsomes were assayed for EROD activity by the above method.

SDS-PAGE was carried out by the method of Laemmli (1970) using 10% SDS gels, and semi-dry blotted on to nitrocellulose. Blots were analysed by the method of Towbin et al. (1979) with rabbit anti-perch (Perca fluviatilis) polyclonal P4501A antibody (gift from L. Forlin, University of Göteborg, Sweden) and then goat anti-rabbit Ig G (whole molecule) alkaline phosphatase conjugate. Bands were visualised and then semi-quantified by image analysis. Values are given as mean ± SEM (n = 3–6) and were compared by one a tail t-Test (p < 0.05).

The variation in levels of turbot cytochrome P4501A measured as EROD activity, with development and exposure to model inducers of CYP4501A (benzo[a]pyrene [BaP], β-naphthoflavone) and lindane is presented in Table I. There was no detectable activity in embryos (prior to hatching), but basal levels were measurable in all other life stages examined, increasing from 0.45 to 0.57 pmol/min/mg protein in whole body-tissue preparations of 3 and 11 day larvae to 10.8 and 12.3 pmol/min/mg protein in livers of 90 day juveniles and sexually mature adults. Microsomal CYPIA activity, however, has been indicated in both the embryonic and yolk sac stages of other marine teleosts measured as aryl hydrocarbon (BaP) hydroxylase (AHH) activity (Binder & Lech, 1984), with an approximate 9-fold increase in AHH activity being seen with hatching (Binder & Stegeman, 1984; Binder et al., 1985). Whole body EROD activity increased approximately 3-fold in 4 day old turbot larvae following 24 h exposure to 5 ppb Ba[α]P, increasing from 0.23 to 0.64 pmol/min/mg protein. PAH mixtures, such as the water-soluble fraction of North Sea crude oil, elevated both EROD activity and P4501A protein amount in cod (G. morpha) larvae, following 1–6 weeks exposure (Goksøyr et al., 1991).

Turbot larvae (7 day) exposed to γ-hexachlorocyclohexane (lindane) for 48 h showed a 6-fold increase in whole body EROD activity, increasing from 0.41 to 2.29 pmol/min/mg protein. Lindane is an inducer of mammalian cytochrome P4501A (Videla et al., 1990) and 3-fold increases in AHH activity with organochlorines were observed in larvae of killifish (F. heteroclitus) with exposure to the PCB mixture Arocolor 1254 (Binder et al., 1985). Hepatic EROD activity in 90 day turbot juveniles was elevated only 2-fold by exposure to 25 ppb Ba[α]P for 48 h, viz. from 10.8 to 22.5 pmol/min/mg protein. whereas in adults it increased up
TABLE I
7-Ethoxyresorufin O-Deethylase (EROD) Enzyme Activities in Turbot (*Scophthalmus maximus*) at Different Developmental Stages and With Exposure to Xenobiotics. *(p < 0.05 Control Versus Exposed)*

<table>
<thead>
<tr>
<th>Turbot</th>
<th>Age</th>
<th>EROD activity (pmol/min/mg protein)</th>
<th>Condition</th>
<th>Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td>3 day</td>
<td>not detected</td>
<td>Basal activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 day</td>
<td>0.57 ± 0.06</td>
<td>Basal activity</td>
<td></td>
</tr>
<tr>
<td>Larvae</td>
<td>9 day</td>
<td>0.23 ± 0.05</td>
<td>Carrier control (DMF)</td>
<td>24 hours; 3 day larvae</td>
</tr>
<tr>
<td></td>
<td>11 day</td>
<td>0.64 ± 0.10*</td>
<td>5 ppb B[a]P</td>
<td>24 hours; 3 day larvae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.41 ± 0.05</td>
<td>Carrier control (DMF)</td>
<td>24 hours; 7 day larvae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.29 ± 0.24*</td>
<td>Carrier control (DMF)</td>
<td>48 hours; 7 day larvae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.45 ± 0.08</td>
<td>Basal activity</td>
<td></td>
</tr>
<tr>
<td>Juveniles</td>
<td>90 day</td>
<td>10.8 ± 2.40</td>
<td>Carrier control (DMF)</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.5 ± 6.40</td>
<td>1 ppb Lindane</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3 ± 4.70</td>
<td>25 ppb B[a]P</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td></td>
<td>164.3 ± 6.00*</td>
<td>Carrier (Corn oil i.p.)</td>
<td>24 hours</td>
</tr>
</tbody>
</table>

| Adult    |      | 164.3 ± 6.00*                        | β-naphthoflavone 20 mg/kg i.p.| 24 hours               |

*Mean ± SEM (n = 3-6) DMF, dimethylformamide.

*Whole body 13 500g supernatant.

*Liver 13 500g supernatant.

*Liver microsomes.
to 13-fold following injection of β-naphthaflavone, viz. from 12.3 to 164.3 pmol/min/mg protein.

Increases in CYPIA protein were demonstrated by Western blotting for 3 day larvae and 90 day juveniles exposed to BaP, and for adults injected with β-naphthaflavone. The results for liver of juveniles and adults are presented in Fig. 1. (Note: only a faint band [not shown] was evident for control adult hepatic microsomes.) There was a 12-fold increase in immunoreactive protein from 3-day larvae exposed to 5 ppb B[A]P, semi-quantified by image analysis compared to control tissue. A 3-fold increase in immunoreactive protein was observed for juveniles (3.32 increased to 9.11 image analysis arbitrary units), compared to a 4-fold increase in EROD activities of the samples used in the analysis (5.1 increased to 19.2 pmol/min/mg protein).

The results demonstrate the presence and putative induction of CYPIA throughout the life stages of turbot, concurring with findings for other fish species (Binder & Stegeman, 1984; Binder et al., 1985). The responses were measurable at the level of EROD activity and immunoreactive protein detected by Western blotting in 3 day through to 11 day larvae, indicating the suitability of such

Fig. 1. Western blot of adult and juvenile turbot (S. maxima) hepatic CYPIA protein reacted with anti-P4501A from perch (P. fluviatilis). From left to right, pre-stained markers (lane 1); 5 μg microsomal protein from β-naphthaflavone induced adult (EROD activity 164.3 pmol/min/mg protein) (lane 2); 17.6 μg protein from 13,500g supernatant of juvenile treated with DMF-carrier (EROD activity 5.1 pmol/min/mg protein) (lane 3); 17.6 μg protein from 13,500g supernatant of juvenile treated with 25 ppb benzo[a]pyrene for 48 h (EROD activity 19.2 pmol/min/mg protein) (lane 4).
Studies on cytochrome P450IA in turbot

measurements for use as biomarkers of organic contaminant exposure in early developmental stages of fish.

ACKNOWLEDGEMENT

This work was funded by the UK Department of the Environment contract PECD 7/7/359.

REFERENCES

2.3 BENZO[A]PYRENE METABOLISM AND XENOBIOTIC-STIMULATED REACTIVE OXYGEN SPECIES GENERATION BY SUBCELLULAR FRACTION OF LARVAE OF TURBOT (*SCOPHTHALMUS MAXIMUS* L.)
Benzo[a]pyrene Metabolism and Xenobiotic-Stimulated Reactive Oxygen Species Generation by Subcellular Fraction of Larvae of Turbot (Scophthalmus maximus L.)

L. D. Peters, S. C. M. O'Hara and D. R. Livingstone
NERC Plymouth Marine Laboratory, Citadel Hill, Plymouth, Devon PL1 2PB, U.K.

ABSTRACT. NADPH-dependent \(^3\)H-benzo[a]pyrene (BaP) metabolism and basal and xenobiotic-stimulated NADPH-dependent reactive oxygen species (ROS) production were investigated in 11,600 g supernatants of 4-day-old yolksac larvae of turbot (Scophthalmus maximus L.). BaP metabolites were resolved by HPLC and detected radiochemically. ROS were quantified by the iron(II)-EDTA mediated production of hydroxyl radical (·OH) that was detected by its oxidation of 2-keto-4-methylbutyric acid (KMB) to yield ethylene. BaP metabolism produced phenols, dihydrodiols and dienes (quinones) (respectively, 54, 32, and 14% of free metabolites) and putative protein adducts. Metabolites identified by retention time included the 7,8-dihydrodiol, the 1,6-, 3,6- and 6,12-diones and the 3- and 9-phenols. Pre-exposure of turbot larvae to 5 ppb BaP for 24 hr caused an approximate 2-fold increase in both BaP metabolism and 7-ethoxyresorufin O-deethylase activity, indicative of the induction of cytochrome P4501A and its involvement in BaP metabolism. Basal KMB oxidation rates were similar for NADH and NADPH. Inhibition studies indicate that OH was formed via the production of superoxide anion radical and hydrogen peroxide. Basal ROS production was stimulated up to 3-fold by a range of redox cycling aromatic hydrocarbon quinones and indicated to be stimulated by other xenobiotics, including nitroaromatics. The results indicate biotransformation and ROS production as potential mechanisms of toxicity in larval fish. COMP BIOCHEM PHYSIOL 114C:221–227, 1996.

KEY WORDS. Fish larvae, turbot, Scophthalmus maximus, benzo[a]pyrene metabolism, 7-ethoxyresorufin O-deethylase activity, reactive oxygen species, quinones, nitroaromatics

INTRODUCTION

Many potentially toxic anthropogenic organic xenobiotics enter the marine environment and are taken up by aquatic organisms at different trophic levels (44) and life stages, including larval fish (11,15,18,24). Studies of mechanisms of toxicity are necessary both for the prediction of pollutant effect and the design of molecular biomarkers for environmental monitoring and impact assessment (25,28,30). Potential mechanisms of pollutant toxicity in fish include biotransformation to reactive metabolites and resultant damage to DNA, particularly involving the cytochrome P450 monooxygenase system (12,41) and pro-oxidant processes involving the generation of reactive oxygen species (ROS) such as the superoxide anion radical (O\(^2\)-), hydrogen peroxide (H\(_2\)O\(_2\)) and the hydroxyl radical (·OH) (21,26,40,51).

Fish early life stages are very sensitive to environmental change (8), including pollutant exposure as observed both in the field (14,49) and experimentally (7,45,53). Effects of aromatic hydrocarbons (AHS), polychlorobiphenyls (PCBs) and dioxins on fish embryos and larvae include induction of cytochrome P4501A (10,13,24,43), DNA-adduct formation, oncogene activation, carcinogenicity and other pathologies (3,11,39). Metabolism of the AH benzo[a]pyrene (BaP), including formation of the highly mutagenic BaP-7,8-dihydrodiol-9,10-epoxide, have been extensively characterised in adult fish systems (38,42), but much less is known in fish larvae (5,6,18). The stimulation of basal ROS production by a wide range of xenobiotics, including components of pulp mill effluents and BaP metabolites (23), has been demonstrated for hepatic subcellular fractions of adult fish (51), and evidence exists for a role for pro-oxidants in chemically caused carcinogenesis via damage to DNA (29,32). Again, little is known of such mechanisms of toxicity in early life stages of fish, but antioxidant enzymes that protect against ROS are present in larvae of several fish species (35).

Previous studies have demonstrated the presence of an inducible cytochrome P4501A (34), antioxidant enzymes
(33) and lipid peroxides (Peters L. D. and Livingstone D. R., unpublished data) in larvae of turbot (Scophthalmus maximus). The present study was carried out to determine the in vitro potential of 4-day-old (yolk sac) S. maximus larvae to metabolise a model AH, namely BaP, and to catalyse basal and xenobiotic-stimulated NAD(P)H-dependent ROS production as possible mechanisms of pollutant toxicity in early life stages of fish. Proposed mechanisms of larval exposure to lipophilic anthropogenic contaminants include maternal transfer to the egg (48), uptake from food (35) and exposure to the sea surface microlayer (19). BaP metabolism was determined on untreated larvae and larvae pre-exposed to BaP as an inducer of CYP1A. Induction of CYP1A was measured in terms of the characteristic 7-ethoxyresorufin O-deethylase (EROD) activity (41). ROS production was measured as the iron-EDTA-mediated formation of -OH in the presence of a wide range of 19 model and pollutant xenobiotics, including AH-quinones, nitroarenes, PCBs and pesticides.

MATERIALS AND METHODS

Chemicals

Biochemicals including 7-ethoxyresorufin, resorufin, NADH, NADPH, BaP, 2-keto-4-methylbutyric acid (KMBA), acetylaminofluorene, 1-hexachlorocyclohexane (lindane), diethyl phthalate and the enzymes superoxide dismutase (SOD; EC 1.15.1.1) and catalase (EC 1.11.1.6) were obtained from Sigma Chemical Company Ltd. (Poole, Dorset, U.K.). Standard laboratory chemicals including EDTA (disodium salt) were AnalR grade from Merck Ltd. (Lutterworth, Leicestershire, U.K.). Chemicals for HPLC were from Merck Ltd. or Packard (Pangbourne, Berkshire, U.K.) and were HPLC or Scintran grade. 3H-BaP was from Amersham (Little Chalfont, Buckinghamshire, U.K.) and authentic BaP metabolites were obtained from the NCI Chemical Carcinogen Repository (Kansas City, MO, U.S.A.). All quinones and nitrocompounds were from Aldrich Chemical Company Ltd. (Poole, Dorset, U.K.), except nitrotartrato (N-(5-nitro-2-furanyliden)-1-amino-hydantoin), which was from Sigma Chemical Company Ltd. Aroclor 1254 was from Chem Service (PA, U.S.A.).

Animals, Exposure Conditions and Sample Preparation

S. maximus larvae (2–3 days posthatch) were obtained from a commercial hatchery (Golden Sea Produce, West Kilbride, Scotland) and maintained in 3-L glass bowls containing filtered aerated seawater at 18°C. Seawater was either treated with a minimal volume of dimethylformamide (DMF) or 3 ppm BaP (dissolved in DMF), and the larvae kept for 24 hr under a 12:12 hr day-night cycle as described above. Larvae were collected by filtration, immediately frozen in liquid N2 and stored at −70°C before analysis.

Aliquots of approximately 50 larvae (0.3–0.5 g total wet weight) were sonicated in 1.6 ml of 0.15M KCl (pH adjusted to 7.5 by KOH) containing 1 mM EDTA at 4°C and then centrifuged at 11,600 g for 3 min in a microcentrifuge. The resulting supernatants were used for the determination of BaP metabolism, EROD activity and ROS (-OH) production. Assays were carried out in duplicate on multiple biological samples (see text for details).

\[ ^3\text{H}-\text{BaP Metabolism} \]

In vitro BaP metabolism was determined radiometrically as described in Lemaire et al. (20). Contained in a final volume of 1.5 ml were 50 mM Tris–HCl (pH 7.8), 3 mM MgCl2, 0.2 mM NADPH, 60 μM \[ ^3\text{H}-\text{BaP} \] (185 μCi per μmol) in DMF and larval supernatant (1.9–3.2 mg protein). Incubations were performed in a shaking water bath, under yellow light at 25°C. Reactions were initiated by the addition of the \[ ^3\text{H}-\text{BaP} \], run for 30 min and stopped by the addition of 1.5 ml aceton. Controls were taken at time zero. BaP metabolites were extracted under argon (three times) with ethyl acetate containing 0.1 mM butylated hydroxytoluene, dried with anhydrous sodium sulphate and taken up in a reduced volume of methanol (MeOH). Metabolites were separated by reverse-phase HPLC, quantified by on-line radioactivity monitoring (Reeve, Glasgow, Strathclyde, U.K.) and identified by retention time using authentic BaP metabolite standards detected spectrophotometrically at 254 nm. HPLC separation was effected by a Waters C18 μBondpak 3.9 mm × 30 cm column, flow rate 1 ml/min, MeOH–water gradient starting at 60% MeOH, increasing to 100% after 40 min; the column temperature was 40°C. Putative protein adducts were quantified as follows: the precipitate formed after ethyl acetate extraction was washed four times with 4 ml MeOH, solubilised in 1M NaOH, pH adjusted to neutral and the radioactivity measured by scintillation counting. \[ ^3\text{H}-\text{BaP} \] was purified before use by HPLC separation as described above. Traces of metabolites and a number of unidentified peaks were removed from the radio-substrate by this method.

In vitro -OH Production (KMBA Oxidation)

ROS production was quantified by the iron-EDTA-mediated formation of -OH from other ROS and possible radicals: a major component of the production in the presence of iron–EDTA is generated from O2•− and H2O2 via the Haber-Weiss reaction (O2•− + H2O2 → -OH + OH− + O2) (52). The NAD(P)H-dependent generation of -OH was detected by the oxidation of the scavenging agent KMBA to ethylene as described in Winston et al. (50). Incubations were carried out in sealed 25-ml conical flasks in a shaking water bath at 23°C. Contained in a final volume of 1 ml were 100 mM K2HPO4–KH2PO4, pH 7.4, 10 mM MgCl2, 1 mM sodium azide, 500 μM NAD(P)H, 75 μM FeCl3 in 150 μM EDTA and larval supernatant (0.60–0.85 mg pro-
tein). Preliminary studies demonstrated that ethylene production was linear over time up to at least 48 min. Ethylene (1 ml air samples taken directly from the headspace of the sealed flasks) was routinely sampled at zero and 30 min of incubation. Air samples were injected into a gas chromatograph (Varian) coupled with flame ionisation detector. Separation was achieved using a 2-m Porapak Q-column (80–100 mesh) at 60°C with the detector set at 200°C. The carrier gas (nitrogen) flow was 30 ml/min, and quantification was by reference to ethylene standards prepared at identical temperature and pressure to the samples. Incubations were carried out in the absence and presence of a range of redox cycling and non-redox cycling (see Discussion) xenobiotics (see Table 2), which were added in a minimal volume DMF to give a final concentration of 1 mM. Incubations were also carried out containing all of the above reagents, including NADPH, but lacking larval supernatant; to determine xenobiotic-mediated chemical rate of KMBA oxidation. Xenobiotic-stimulated rates of KMBA oxidation were calculated from the (rate in the presence of xenobiotic) minus (the rate in the absence of xenobiotic + the chemical rate). The involvement of O2•− and H2O2 in the basal production (i.e., absence of xenobiotic) of -OH was investigated by the addition of respectively, SOD (100 SOD units per flask; inhibits O2•− formation) and catalase (100 units per flask; no sodium azide present; inhibits H2O2 formation).

Other Methods

EROD activity was determined fluorometrically at 30°C as described in Peters et al. (35). Contained in a final volume of 150 µl were 59 mM K2HPO4, KH2PO4, pH 7.6, 0.23 mM NADPH, 3.74 µM 7-ethoxycoumarin and larval supernatant (0.57–0.81 mg protein). Resorufin fluorescence was measured against an 85 mM resorufin standard. Protein was determined by the method of Lowry et al. (27). All values are presented as means ± SEM. Groups of values were compared by one-tailed t-test (P < 0.05).

RESULTS

The results of BaP metabolism by 11,600 g supernatants of S. maximus larvae are given in Table 1 and Fig. 1. Larval supernatants from both control and BaP-exposed conditions metabolised BaP to dihydrodiols (diols), quinones (diones), phenols and putative protein adducts; putative terrois were formed by BaP-exposed larval samples only (Table 1). Figure 1 shows a typical HPLC metabolite trace, after correction for zero time metabolites, for a BaP-exposed larval sample. The identity of the first metabolite to elute from the column was concluded to most likely be a tetrol or triol, because it emerged well before any diol standard. Several metabolites were detected in the diol elution region, but only one was identifiable as the BaP-7,8-dihydrodiol. None of the other presumed diol peaks (labelled diols a, b and c) co-eluted with either BaP-4,5-dihydrodiol or BaP-9,10-dihydrodiol standards. The other identifiable major metabolites were the BaP-3,6-dione and BaP-9- and BaP-3-phenols. Although not present in the sample shown in Fig. 1, the BaP-1,6- and BaP-6,12-diones were also formed (Table 1).

Exposure of larvae to 5 ppb BaP for 24 hr increased, or was indicated to increase, the rates of formation of all metabolite groups of BaP by up to 2-fold; phenols constituted 54 and 49% of the total free BaP metabolites formed by, respectively, control and BaP-exposed larval samples (Table 4). Total rates of BaP metabolism (including protein adduct formation) and EROD activity increased by similar amounts (1.8- to 1.9-fold) with BaP-exposure (Table 1). Increases in rates of production with BaP-exposure were seen, or indicated, for all individual metabolites, including BaP-dihydrodiols a and c (6-fold and 5-fold, respectively) and BaP-3-OH phenol (1.9-fold) (Table 1).

The results of the studies on ROS production by 11,600 g supernatants of S. maximus as detected by the iron–EDTA-mediated oxidation of KMBA are shown in Table 2. Larval samples produced ROS at similar rates using either NADH or NADPH as electron donor (Table 2, basal rate). KMBA

| TABLE 1. Changes in rates of in vitro BaP metabolism and EROD activity of 11,600 g supernatants of whole body 4-dav-old S. maximus larvae after exposure to 5 ppb waterborne BaP for 24 hr |
|---------------------------------|-----------------|-----------------|
| Control (n = 6) | Exposed (n = 1) | Fold increase |
| Putative terrois | | |
| Diols | | |
| a | 0.82 ± 0.02 | 4.90 ± 1.57* | 6 |
| b | 0.87 ± 0.07 | 1.23 ± 1.23 | |
| c | 0.93 ± 0.06 | 4.50 ± 2.32* | 4.8 |
| 7,8 | 7.48 ± 2.55 | 5.73 ± 0.52 | |
| Total diols | 10.1 ± 3.5 | 16.4 ± 4.2 | |
| Diones | | |
| 1,6 | 1.35 ± 0.35 | 2.30 ± 2.30 | |
| 3,6 | 1.52 ± 0.31 | 4.50 ± 2.52 | |
| 6,12 | 1.45 ± 0.74 | 1.20 ± 0.79 | |
| Total diones | 4.3 ± 1.6 | 8.4 ± 0.7 | |
| Phenols | | |
| 9 | 3.02 ± 0.14 | 4.07 ± 1.28 | |
| 3 | 11.73 ± 2.02 | 21.70 ± 2.12* | 1.8 |
| Total phenols | 16.8 ± 2.7 | 25.8 ± 2.9* | 1.5 |
| Total free metabolites | 31.2 ± 5.1 | 52.8 ± 7.7* | 1.7 |
| Putative protein adducts | | |
| EROD activity (pmol/min/mg protein) | 0.27 ± 0.03 | 0.50 ± 0.03* | 1.9 |

Values are means ± SEM, unless indicated otherwise, rates are in pmol/30 min/mg protein. *P < 0.05 one-tailed t-test comparing exposed to control.
Mechanisms of organic pollutant-mediated toxicity in liver and other tissues of adult fish include CYP1A-catalysed bioactivation of xenobiotics to form macromolecular adducts and enhanced production of ROS leading to oxidative stress (see Introduction). The same potential mechanisms of toxicity have been shown to be also present in larval stages of S. maximus by this in vivo study. First, the AH BaP was metabolised to potentially toxic metabolites, including BaP 7,8-dihydrodiol (the precursor of carcinogetic BaP 7,8-diol 9,10-epoxide) and redox cycling BaP-diones (23). Second, NAD(P)H-dependent ROS production was stimulated by a number of AH-quiones and indicated to be stimulated by a variety of organic xenobiotics, including nitroaromatics.

The presence of an inducible CYP1A capable of metabolising BaP in fish larvae has been indicated from previous studies on enzyme activity, immunorecognition and BaP metabolism (6,13,34,43). The same situation is indicated oxidation inhibited by SOD (25–44% inhibition) and catalase (50–60% inhibition), indicating the involvement of, respectively, O₂⁻ and H₂O₂, in the NAD(P)H-dependent formation of •OH. NAD(P)H-dependent ROS production was stimulated up to 3-fold the basal rate by several AH-quiones, in particular 1,4-naphthoquinone and 9,10-phenanthrenequinone. Much less stimulation was indicated for other AH-quiones, nitroaromatics and non-redox cycling xenobiotics such as lindane and diethyldithphalate, but in none of these cases was the elevation over basal rate statistically significant at P < 0.05. Determined or indicated stimulation was not consistently higher for NADH or NADPH, but generally somewhat similar patterns of stimulation were seen for the different xenobiotics for the two coenzymes (e.g., maximal rates for 9,10-phenanthrenequinone and 1,4-naphthoquinone and minimal rates for 1,4-benzoquinone and 1,2-naphthoquinone).

**TABLE 2. Effects of catalase, SOD and xenobiotics on NAD(P)H-dependent ROS production (iron-EDTA-mediated oxidation of 2-keto-4-methylbutyric acid to ethylene) by 11,600 g supernatants of whole body 3-day-old S. maximus larvae**

<table>
<thead>
<tr>
<th></th>
<th>NADH</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal rate</strong></td>
<td>22.92 ± 1.07</td>
<td>19.12 ± 2.39</td>
</tr>
<tr>
<td>+100 SOD units/ml</td>
<td>17.10 ± 1.65*</td>
<td>10.80 ± 0.44*</td>
</tr>
<tr>
<td>+100 catalase units/ml no acid</td>
<td>11.58 ± 0.54*</td>
<td>7.61 ± 0.25*</td>
</tr>
</tbody>
</table>

**Effects of xenobiotics (stimulated rate minus basal rate)**

<table>
<thead>
<tr>
<th>Quinones</th>
<th>NADH</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-benzoquinone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>duroquinone</td>
<td>11.05 ± 0.62*</td>
<td>6.30 ± 0.61</td>
</tr>
<tr>
<td>1,2-naphthoquinone</td>
<td>0.86 ± 0.86</td>
<td>0</td>
</tr>
<tr>
<td>1,4-naphthoquinone</td>
<td>46.63 ± 4.30*</td>
<td>22.21 ± 0.11*</td>
</tr>
<tr>
<td>menadione</td>
<td>4.74 ± 2.39</td>
<td>14.19 ± 1.33*</td>
</tr>
<tr>
<td>anthraquinone</td>
<td>2.93 ± 1.19</td>
<td>0</td>
</tr>
<tr>
<td>9,10-phenanthrenequinone</td>
<td>62.94 ± 3.33*</td>
<td>58.58 ± 2.08*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrocompounds</th>
<th>NADH</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>nitrocyclopentane</td>
<td>0.94 ± 0.94</td>
<td>0</td>
</tr>
<tr>
<td>nitrobenzene</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-nitroanphthalene</td>
<td>0.15 ± 0.15</td>
<td>0.70 ± 0.05</td>
</tr>
<tr>
<td>nitroanthrone</td>
<td>3.95 ± 0.76</td>
<td>2.19 ± 0.95</td>
</tr>
<tr>
<td>2-nitrofluorene</td>
<td>2.96 ± 1.53</td>
<td>2.06 ± 2.06</td>
</tr>
<tr>
<td>9-nitroantracene</td>
<td>0</td>
<td>0.07 ± 0.07</td>
</tr>
<tr>
<td>1-nitropyrene</td>
<td>1.42 ± 0.71</td>
<td>0.93 ± 0.93</td>
</tr>
</tbody>
</table>

**Nonredox cycling xenobiotics**

| benzaldehyde         | 1.62 ± 0.72 | 1.37 ± 0.83 |
| acetylaminofluorene  | 0.49 ± 0.49 | 1.78 ± 0.48 |
| lindane              | 0.91 ± 0.46 | 0.78 ± 0.57 |
| 1-acrolein 1254q   | 0          | 0.30 ± 0.1  |
| phthalic acid diethyl ester | 0.04 ± 0.04 | 1.83 ± 1.49 |

Values are means ± SEM in nmol ethylene/min/g wet wt (n = 3–5).

*P < 0.05 two-tailed t-test comparing addition of SOD, catalase of xenobiotic with control for same reduced coenzyme-dependent reaction.
for larvae of *S. maximus* from the parallel increases in the rate of total BaP metabolism and EROD activity with exposure to waterborne BaP. The 2-fold elevation in these two measurements compares well with a 3–4-fold elevation in EROD activities (31). CYP1A protein observed in previous BaP-exposure studies on *S. maximus* (34) and also to results of other fish larval studies, viz., 2.6-fold increase in BaP hydroxylase activity in *F. heteroclitus* exposed to PCBs (6) and a 3.5–8.6-fold higher BaP hydroxylase activity in lake trout (*Salvelinus namaycush*) from polluted sites compared with farmed-reared animals (4).

The demonstration of metabolism of BaP by 11,600 g supernatants of larvae of *S. maximus* is consistent with earlier in vitro (see below) and in vivo (18) studies on fish larvae. In the latter case, the formation of free metabolites, glucuronides and sulphated conjugates was indicated in rainbow trout (*S. gairdneri*). The formation of the 7,8-diol, 1,6-, 3,6- and 6,12-diones and the 3- and 9-phenols by *S. maximus* was also observed for whole body microsomes of *F. heteroclitus* larvae (6). However, in contrast, the formation of the 9,10-diol was observed for *F. heteroclitus* but not *S. maximus*. For both larval species, total diols comprised about 30% of total free metabolites. More significantly, the metabolite profiles (i.e. composition of diols, dienes, phenols) for both species were found to be little different, indicating a moderate induction of putative CYP1A, indicating either a relatively modest or moderate induction of CYP1A (about 2-fold; see above) or a similarity in catalytic specificity between the constitutive and induced P450 forms. A possible exception was the appearance of putative tetrols after the exposure of *S. maximus* larvae to BaP. No information is available on this aspect for fish larvae, but studies on hepatic microsomes of uninduced adult fish detected metabolites eliciting before diols on an HPLC gradient for brown bullhead (*Ictalurus nebulosus*), carp (*Cyprinus carpio*) (38) and sea bass (*Dicentrarchus labrax*) (31) but not for little skate (*Raja erinacea*) (2), California killifish (*Fundulus parvipinnis*) and speckled sanddab (*Citharichthys snyderi*) (16). Overall, the composition of diols (31–32%), dienes (14–16%) and phenols (49–54%) found for larvae of *S. maximus* fall within the range reported for adult hepatic microsomes, viz. respectively, 17–47, 3–8 and 30–65% of total metabolites for trout (*Salmo trutta fetus*) (1), mullet (*Mugil cephalus*) (37), *R. erinacea* (2), *F. parvipinnis* and *C. snyderi* (16), *I. nebulosus* and *C. carpio* (38) and *D. labrax* (31). The levels of dienes produced by 11,600 g supernatant of *S. maximus* larvae are slightly higher than those reported for microsomes of adult fish, which could be related to the involvement of enzymes present in the larval cytosolic fraction (e.g., a proposed mechanism of BaP-dione formation is 1-electron oxidation which may be catalyzed by peroxidases and prostaglandin H synthase, in addition to cytochrome P450) (9). Such a mechanism, which produces a cation radical of BaP, could also lead to the observed formation of putative protein adducts.

Basal and xenobiotic-stimulated NAD(P)H-dependent ROS production have been demonstrated for subcellular fractions of adult fish and other aquatic organisms (21, 26, 51). Mechanisms that could contribute to such ROS production include uncoupling of enzymes in electron-transport systems, auto-oxidation of cytochrome P450 and redox cycling of xenobiotics catalysed by flavoprotein reductases (22, 51). The KMBA oxidation studies demonstrate that *S. maximus* larvae also have the potential to generate ROS using both NADH and NADPH as electron donors. Basal rates of ROS production by 11,600 g larval supernatants were similar for NADH and NADPH; however, studies of other aquatic organisms, including invertebrates (50) and fish (17, 23) reported higher rates of ROS production using NADH rather than NADPH as cofactor, although these were for microsomal preparations rather than 11,600 g supernatants. The inhibition of basal rates of ROS production by addition of SOD and catalase indicates the involvement of respectively, O$_2^-$ and H$_2$O$_2$ in OH formation, probably via a Haber-Weiss reaction (see Materials and Methods), as has been observed in other studies on aquatic organisms (23, 51).

The demonstrated or indicated stimulation of NAD(P)H-dependent ROS production of larval *S. maximus* by a variety of both redox and non-redox cycling xenobiotics indicates a widespread auto-oxidation of cytochrome P450 and redox-damaged processes and possible oxidative stress in early life stages, particularly in relation to products of AH metabolism. The observations with the different types of xenobiotics indicate ROS production occurs both by redox cycling and other free radical interactions, also possibly via bio-transformation to redox cycling metabolites (i.e., dienes) in the case of BaP. Stimulation of NAD(P)H-dependent ROS production by quinones and nitrocompounds has also been observed for hepatic microsomes of adult fish, viz. flounder (*Platichthys flesus*) (menadione and nitrofurantoin), *P. flesus* and perch (*Perca fluviatilis*) (d duroquinone) (23) and channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*) and rainbow trout (*Oncorhyncus mykiss*) (6-nitrobenzoic acid, m-dinitrobenzoic acid and nitrofurantoin) (47).

The different degrees of stimulation of ROS production of *S. maximus* larval samples by different xenobiotics is indicated to be dependent on the redox properties of the chemical but probably mainly on the substrate specificity of the enzymes involved in the redox cycling process. Considering the 1-electron reduction potential (E) of A/A$^-·$ (where A is the xenobiotic and A$^-·$ is the univalent-reduced anion radical) and O$_2$/O$_2^-$ ($E = -325$ mV), the more positive is the value of A/A$^-·$ relative to $-325$ mV, the less likely is the xenobiotic radical to reduce O$_2^-$ (see 46). Thus, the high E values for benzoquinone (+78 mV) and 1,2-naphthoquinone (+89 mV) are consistent with the observed low rates of ROS production with these compounds. In contrast, the E values for 9,10-phenanthrenequinone (−124 mV) and 1,4-naphthoquinone (−140 mV) compared with mena-
dione (−203 mV) are inconsistent with the much higher rates of ROS production for the former compounds. Similarly, the E value for anthraquinone (−390 mV) is inconsistent with the observed low rate of ROS production for this compound. These comparisons argue for the substrate specificity of the larval flavoprotein reductases being a determinant in the redox cycling potential of the xenobiotic. In this respect, it is also important to note that the studies were carried out at a fixed concentration (1 mM) of xenobiotic that may not be optimal (saturating) for each compound tested.

The present study demonstrates a potential for pollutant-mediated toxicity in larval fish both by biotransformation to reactive metabolites and via ROS production. Both processes in adult fish can lead to damage to DNA (26,32,40). Such processes may be of particular concern in environments such as the sea surface microlayer where early life stages can come in contact with relatively high concentrations of pollutants (19) and photooxidation (e.g., of PAHs) to pro-oxidant products can increase pollutant toxicity (36).

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Larval BaP Metabolism and ROS Generation

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2.4 ANTIOXIDANT ENZYME ACTIVITIES IN EMBRYOLOGIC AND EARLY LARVAL STAGES OF TURBOT.
Antioxidant enzyme activities in embryologic and early larval stages of turbot

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The antioxidant enzymes superoxide dismutase (SOD: EC 1.15.1.1), catalase (EC 1.11.1.6), selenium-dependent glutathione peroxidase (SeGPX: EC 1.11.1.9), glutathione reductase (EC 1.6.4.2) and DT-diaphorase (EC 1.6.99.2), plus total GPX activity (sum of SeGPX and Se-independent GPX activities), were studied in 13 500 g supernatants of embryos and 3-day and 11-day post-hatch larvae of turbot Scopthalmus maximus L. SOD activity decreased progressively during development from embryos to 11-day-old larvae, indicative of a decreased need to detoxify superoxide anion radical (O₂⁻). In contrast, catalase, SeGPX and glutathione reductase activities increased progressively from embryos to 11-day-old larvae, indicative of an increased need to metabolize hydrogen peroxide (H₂O₂) and organic peroxides. Consistent with the latter changes, levels of lipid peroxides (i.e. thiobarbituric acid reactive substances) increased 13-fold from embryos to 3-day-old larvae, whilst total peroxidizable lipid was indicated to decrease. Increases were seen for NADPH-dependent DT-diaphorase (after hatching) and total GPX (between 3 and 11 days post-hatch) activities, whilst no change was found in NADH-dependent DT-diaphorase activity. Overall, the results demonstrate a capacity for early life-stages of S. maximus to detoxify reactive oxygen species (O₂⁻ and H₂O₂) and other pro-oxidant compounds (organic peroxides, redox cycling chemicals). Furthermore, qualitative and quantitative antioxidant changes occur during hatching and development, possibly linked to such events as altered respiration rates (SOD changes) and tissue reorganization and development (catalase, SeGPX, lipid peroxidation).

Key words: Scopthalmus maximus; development; embryo; antioxidant enzymes; lipid peroxide.

INTRODUCTION

Eukaryotic aerobic organisms use the mitochondrial electron-transport system to couple the tetravalent reduction of molecular oxygen to water, with the oxidative phosphorylation of adenosine diphosphate to adenosine triphosphate. Electrons are transferred to oxygen through a series of electron carriers and the concerted reduction may be described by equation (1).

\[ \text{O}_2 + 4\text{H}^+ + 4e^- \rightarrow 2\text{H}_2\text{O} \quad (1) \]

The paradox that organisms face when living in an aerobic environment is that molecular oxygen may undergo univalent reduction to produce harmful intermediates, termed reactive oxygen species (ROS), and these appear to be largely responsible for oxygen toxicity (Davies, 1995).

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The product of one electron reduction of molecular oxygen is the superoxide anion radical \( \text{O}_2^- \), equation (2).

\[
\text{O}_2 + e^- \rightarrow \text{O}_2^-
\]  

(2)

The \( \text{O}_2^- \) may be formed enzymically (Cross & Jones, 1991), or via a phenomenon termed redox cycling, which occurs when the product of an enzymic reduction immediately auto-re-oxidizes, generating \( \text{O}_2^- \) from molecular oxygen (Dutton et al., 1989). This anion radical may undergo a dismutation reaction to produce hydrogen peroxide \( (\text{H}_2\text{O}_2) \) which is the product of bivalent reduction of oxygen [equation (3)].

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]  

(3)

The trivalent reduction of oxygen produces the highly reactive hydroxyl radical \( (\cdot\text{OH}) \) which may be generated by the interaction of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) via the Haber–Weiss reaction [equation (4)].

\[
\text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \cdot\text{OH} + \text{OH}^- + \text{O}_2
\]  

(4)

The rate of *OH formation is greatly enhanced in the presence of iron (and other transition metals) which catalyses the Fenton reaction according to equations (5) and (6) (Aust et al., 1985).

\[
\text{Fe(III)} + \text{O}_2^- \rightarrow \text{Fe(II)} + \text{O}_2
\]  

(5)

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \cdot\text{OH} + \text{OH}^-
\]  

(6)

In the aquatic environment, marine organisms balance the production of ROS with a wide variety of cellular antioxidant defences, that serve to detoxify free radicals (Di Giulio et al., 1989). Increased cellular levels of ROS are proposed to occur in marine organisms when the pro-oxidant processes are not equalled by the antioxidant defences (Winston, 1991), and as a consequence, oxidized DNA, proteins and lipids (lipid peroxides) may result (Lemaire & Livingstone, 1993). These oxidized macromolecules are deleterious to the organism; however, cellular ROS generation may be stimulated also under regulated conditions in specific cell types e.g. ROS production after phagocytosis of a pathogen by fish and molluscan phagocytes (Pulsford et al., 1994; Coles et al., 1995) or the generation of \( \text{H}_2\text{O}_2 \) by echinoid eggs to cross-link the glycoprotein coat and harden the egg surface to form the fertilization membrane (Cross & Jones, 1991).

The systems that protect aquatic organisms against ROS toxicity include antioxidant enzymes and low molecular weight scavengers (Winston & Di Giulio, 1991). Antioxidant enzyme activities have been identified in eight marine phyla including 19 families of fish, whereas low molecular weight scavengers (glutathione, vitamins \( \text{E} \) and \( \text{C} \), and total carotenoids) have been determined in six marine phyla including at least 15 species of fish (Lemaire & Livingstone, 1993). Few studies have been undertaken to determine the levels of antioxidant enzymes in the early life stages of fish (Aceto et al., 1994; Peters et al., 1994) and little or nothing is known of these enzyme activities in embryonic and larval stages of turbot \( \text{Scophthalmus maximus} \) L.

During development, fish embryos and larvae are sensitive to the environment that surrounds them (Hoar & Randall, 1988); oxygen availability, for example, is
vital for embryo development where cell division and de novo tissue formation require increased metabolic rates and stimulation of oxygen consumption in both fish embryos (Rombough, 1988) and larvae (Walsh et al., 1989). Increased uptake of exogenous oxygen may have the potential to affect pro-oxidant processes in the early life stages of fish. During embryogenesis, the metabolic energy substrates change between glucose, free amino acids and lipids depending upon species and developmental stage (Hemming & Buddington, 1988; Finn et al., 1991), and these metabolic fuels may provide endogenous sources of ROS production. Field studies indicate that both exogenous and/or endogenous/dietary factors may influence larval antioxidant enzymes (Peters et al., 1994), inferring that pro-oxidant processes are phenomena experienced during fish larval development. In accordance with this, a study has demonstrated that S. maximus larval tissue can generate ROS in vitro (Peters et al., 1996) indicating that fish larvae have the potential to generate ROS in vivo.

This study describes the development of antioxidant enzyme activities and lipid peroxidation processes in the embryo, yolk sac (3 days post-hatch) and post-first feed (11 days post-hatch) larvae of turbot. The enzyme activities studied comprised superoxide dismutase (SOD) [EC 1.15.1.1; catalyses equation (3)], catalase [EC 1.11.1.6; catalyses equation (7)], selenium-dependent glutathione peroxidase (SeGPX) [EC 1.11.1.9; catalyses equation (8) where GSSG and GSH are respectively oxidized and reduced glutathione], total GPX [i.e. the sum of SeGPX and selenium-independent GPX activities, measured by catalysis of equation (9) where ROOH is organic hydroperoxide], glutathione reductase [EC 1.6.4.2; catalyses equation (10)], and NAD(P)H-dependent DT-diaphorase (quinone oxidoreductase) [EC 1.6.99.2; catalyses equation (11) where Q and QH₂ are respectively quinone and hydroquinone].

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad (7) \\
\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG} \quad (8) \\
\text{ROOH} + 2\text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG} \quad (9) \\
\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2\text{GSH} \quad (10) \\
\text{NAD(P)H} + \text{Q} + \text{H}^+ \rightarrow \text{QH}_2 + \text{NAD(P)}^+ \quad (11)
\]

SOD, catalase and SeGPX detoxify ROS (O₃⁻ and H₂O₂), while SeGPX and total GPX activities detoxify organic peroxides produced by, for example, pro-oxidant processes such as lipid peroxidation. Glutathione reductase is required to regenerate GSH for the GPX reactions. DT-diaphorase is a flavoprotein reductase which catalyses the two electron reduction of redox cycling quinones and related compounds to hydroquinones, so preventing their univalent reduction to quinone anion radicals leading to ROS production via autoxidation (Cadenas, 1995). Such quinones may be present endogenously or in diet of larvae (Lemaire & Livingstone, 1993). DT-diaphorase is inhibited by dicumarol (Cadenas, 1995), and the activity in embryos and larvae of S. maximus was measured as dicumarol-inhibitable dichlorophenolindophenol (DCPIP) reductase activity. Total DCPIP reductase activity (sum of activities catalysed by all flavoprotein reductases) is reported in this study also to indicate the development of an enzyme activity not related to pro/antioxidant processes.
The development of *S. maximus* larvae includes the formation of an oil droplet that persists even after the yolk sac has been resorbed (Al-Maghazachi & Gibson, 1984). Fish larval oil globules are composed primarily of free lipids, which on a caloric basis are the most important energy reserve for developing fish (Hemming & Buddington, 1988). Polyunsaturated fatty acids (PUFA) in particular, are proposed to be significant in fish embryogenesis and larval development (Tocher et al., 1987). However, PUFA are readily oxidized by ROS to lipid peroxides (Niki, 1987). In this study, lipid peroxidation processes were examined in terms of levels of lipid peroxides and total peroxidizable lipid (essentially PUFA), both determined as thiobarbituric acid reactive substances. The overall results are discussed in relation to possible pro-oxidant and antioxidant processes occurring in *S. maximus* during hatching and larval development.

**MATERIALS AND METHODS**

**MATERIALS**

Sodium azide, GSSG and GSH, ethylenediaminetetraacetic acid-disodium (EDTA), horse heart cytochrome c, hypoxanthine, H₂O₂, cumene hydroperoxide, β-nicotinamide adenine dinucleotide phosphate oxidized form (NADP⁺) and reduced form (NADPH), adenosine-5'-diphosphate (ADP), dicumarol, glutathione reductase, xanthine oxidase (EC 1.1.3.22) and DCPIP were obtained from Sigma Chemical Co. Ltd., U.K. All other chemicals were analar grade from Merck Ltd., U.K.

**SAMPLING PROCEDURES**

Turbot embryos and larvae were obtained from Golden Sea Produce, Hunterston, West Kilbride, Scotland and were collected from routine commercial spawns. The embryos studied were at flatfish embryo developmental stage III (Cameron et al., 1992) and 3-day and 11-day post-hatch larvae were at developmental stages 1d and 2b/c respectively (Al-Maghazachi & Gibson, 1984). Enriched rotifers *Brachionus plicatilis* and *Artemia* sp., nauplii were available as a food source 3 and 9 days after hatching according to established commercial techniques at the hatchery. Embryos and larvae were frozen under liquid nitrogen and stored for up to 8 weeks prior to biochemical analysis. Aliquots of larvae were weighed (c. 0.1 g) and homogenized by sonication in 500 μl of 0.15 M KCl-KOH, pH 7.5, 1 mM EDTA at 4°C. Embryos were ground to powder under liquid nitrogen by pestle and mortar, the nitrogen boiled off at ambient room temperature and the frozen tissue preparation weighed. Embryonic tissue (c. 0.15 g) was resuspended in 500 μl of the above buffer. All samples were centrifuged at 13,500 g for 3–4 min and the resulting supernatants (protein concentration 0.2–7 mg ml⁻¹) kept at 4°C before analysis.

**ENZYME ANALYSIS**

Enzyme activities were measured in duplicate using a Varian Cary 1 spectrophotometer at 25°C and were linear with respect to time and protein concentration. SOD activity was measured by the degree of inhibition of the reduction of cytochrome c by O₂⁻ generated by the xanthine oxidase-hypoxanthine reaction, monitored at 350 nm (McCord & Fridovich, 1969). One unit of SOD activity is defined as the amount of sample causing 50% inhibition of cytochrome c under the standard conditions of the assay. The reaction volume was 3 ml and contained 43 mM KH₂PO₄/K₂HPO₄ buffer pH 7.8, 0.1 mM EDTA, 50 μM hypoxanthine, 1.8 μM ml⁻¹ xanthine oxidase and 10 μM cytochrome c. Different sample volumes were incubated with reagents to achieve 50% inhibition and the reaction
started by the addition of the enzyme xanthine oxidase. Catalase activity was measured in quartz cuvettes by the decrease in absorbance at 240 nm due to H₂O₂ consumption (ext. coeff. ε₂₄₀nm = 40 mM⁻¹ cm⁻¹) according to Aebi (1974). In a final volume of 2 ml were 50 mM KH₂PO₄/K₂HPO₄ pH 6.5, 50 mM H₂O₂ and a 50-mL sample. The reaction was started by the addition of the sample. Glutathione reductase activity was measured by the decrease in absorbance at 340 nm due to NADPH consumption using the ext. coeff. ε₃₄₀nm = 6.22 mM⁻¹ cm⁻¹ (Ramos-Martinez et al., 1983). Contained in final reaction volume of 1 ml were 100 mM KH₂PO₄/K₂HPO₄ pH 7.5, 1 mM oxidized glutathione, 50 µM NADPH and a 25-µl sample.

SeGPX and total GPX activities were determined spectrophotometrically by a coupled assay with glutathione reductase (Gunzler & Flohe, 1976). Hydrogen peroxide and cumene hydroperoxide were the substrates for SeGPX and total GPX activity respectively, with the rate monitored by the decrease of NADPH consumed during the re-formation of GSH from GSSG at 340 nm. The reaction was initiated by the addition of the peroxide, the chemical rate determined in the absence of sample being subtracted from the total rate. The SeGPX activity was determined under the conditions of 50 mM KH₂PO₄/K₂HPO₄ buffer pH 7.0, 3.5 mM GSH, 0.8 mM H₂O₂, 1 mM sodium azide, 4 units of glutathione reductase, 0.12 mM NADPH, 75 µl sample and water to give a final reaction volume of 1 ml. The reaction conditions for the total GPX assay were 65 mM KH₂PO₄/K₂HPO₄ buffer pH 7.5, 2 mM GSH, 4 mM cumene hydroperoxide, 4 units of glutathione reductase, 0.12 mM NADPH and a 20-µl sample. NADPH- and NADH-dependent DT-diaphorase activities were measured respectively as the dicumarol-inhibitable part of NADPH- and NADH-dependent DCPIP reductase activities (Benson et al., 1980). NAD(P)H-DCPIP reductase activity was determined at 600 nm (ε₆₀₀nm = 21 mM⁻¹ cm⁻¹) with final reagent concentrations of 50 mM Tris-HCl buffer pH 7.6, 40 µM DCPIP and 0.3 mM NAD(P)H in a final reaction volume of 1 ml. DT-diaphorase was calculated by measuring the rates of DCPIP reduction in the presence and absence of dicumarol (0.1 mM in 0.15% NaOH). Protein was measured by the method of Lowry et al. (1951).

LIPID PEROXIDE AND PEROXIDIZABLE LIPID ANALYSIS

Lipid peroxide and peroxidizable lipid levels were measured in tissue supernatants in terms of thiobarbituric acid reactive species, and expressed as malonaldehyde equivalents (Livingstone et al., 1990). Aliquots (0.013 g) were added to 35% trichloroacetic acid (TCA) and 1% butylated hydroxytoluene (BHT) and centrifuged at 3000 rpm for 5 min. The supernatants were mixed with 0.75% thiobarbituric acid and 0.012% BHT and heated at 80°C for 20 min. After cooling, 70% TCA was added and lipid peroxide quantified as malonaldehyde-like equivalents at 535 nm using the extinction coefficient (ε₅₃₅nm = 156 mM⁻¹ cm⁻¹). Peroxidizable lipid levels were determined in sample supernatants by the incubation of aliquots (equivalent to 0.003 g wet weight) with 20 µM FeSO₄/1 mM ADP and 0.5 mM ascorbic acid for 2 h at 25°C. After incubating the samples, 35% TCA was added and lipid peroxide determination was performed as described above. The thiobarbituric acid method, although commonly used, may be limited since other reactive aldehydes may not be detected and malonaldehyde produced may react with other cellular constituents (see Lemaire et al., 1993).

STATISTICAL ANALYSIS

Statistical analysis of the enzyme activities of embryo, 3-day and 11-day-old larvae were performed by analysis of variance (ANOVA). When comparison was made between two groups, the two-tailed t-test was used. Differences were considered statistically significant at P<0.05. Details concerning number of samples used are given in the text.
SOD activity was determined to be higher in embryos than larval stages of development [Fig. 1(a)], however both catalase and glutathione reductase activities increased during the development from embryo to the free swimming larvae—11 days post-hatch [Fig. 1(b) and (c)]. SeGPX activity was indicated to increase after hatching and was higher in 11-day-old larvae compared to the other developmental stages [Fig. 1(d)]. Total GPX activity was also indicated to increase after hatching; however, in 11-day-old larvae, Total GPX activity was lower than the activities detected in embryos and 3-day-old larvae (Table 1). NADPH-dependent DT-diaphorase activity decreased after hatching whereas NADH-dependent DT-diaphorase activity did not change between developmental stages (Table 1). NADPH-dependent DCPIP reductase activity was lower after hatching than activities measured at other life stages studied whilst NADPH-dependent DCPIP reductase activity increased after hatching (Table 1).
TABLE I. Changes in total glutathione peroxidase, NAD(P)H-dependent DT diaphorase and NAD(P)H-DCPIP* reductase activities in the early life stages of *Scophthalmus maximus*

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Embryo Mean ± s.E.</th>
<th>3 days post-hatch Mean ± s.E.</th>
<th>11 days post-hatch Mean ± s.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glutathione peroxidase</td>
<td>65·0 ± 14·7</td>
<td>95·0 ± 9·1</td>
<td>14·5 ± 5·2§§</td>
</tr>
<tr>
<td>(nmol min⁻¹ mg⁻¹ protein)</td>
<td>n=8</td>
<td>n=4</td>
<td>n=4</td>
</tr>
<tr>
<td>NADPH-dependent DT diaphorase†</td>
<td>20·0 ± 2·5</td>
<td>4·3 ± 0·8§</td>
<td>7·7 ± 1·5§</td>
</tr>
<tr>
<td>(nmol min⁻¹ mg⁻¹ protein)</td>
<td>n=8</td>
<td>n=5</td>
<td>n=4</td>
</tr>
<tr>
<td>NADH-dependent DT diaphorase†</td>
<td>5·7 ± 1·5</td>
<td>6·4 ± 3·0</td>
<td>2·9 ± 1·3</td>
</tr>
<tr>
<td>(nmol min⁻¹ mg⁻¹ protein)</td>
<td>n=8</td>
<td>n=4</td>
<td>n=4</td>
</tr>
<tr>
<td>NADPH-dependent DCPIP reductase‡</td>
<td>24·9 ± 2·8</td>
<td>8·5 ± 1·0§</td>
<td>19·7 ± 3·3†</td>
</tr>
<tr>
<td>(nmol min⁻¹ mg⁻¹ protein)</td>
<td>n=8</td>
<td>n=5</td>
<td>n=4</td>
</tr>
<tr>
<td>NADH-dependent DCPIP reductase‡</td>
<td>10·0 ± 1·9</td>
<td>17·3 ± 3·6§</td>
<td>18·3 ± 0·9§</td>
</tr>
<tr>
<td>(nmol min⁻¹ mg⁻¹ protein)</td>
<td>n=8</td>
<td>n=4</td>
<td>n=4</td>
</tr>
</tbody>
</table>

*DCPIP: dichlorophenolindophenol.
†Dicumarol inhibitable part of NAD(P)H-dependent DCPIP reductase activity.
‡Total NAD(P)H-dependent DCPIP reductase activity.
§P<0·05 v. embryo by Fisher PLSD test: analysis of variance.
$P<0·05$ v. 3-day-old larvae by Fisher PLSD test: analysis of variance.

TABLE II. Changes in lipid peroxide and peroxidizable lipid in the early life stages of *Scophthalmus maximus*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Embryo Mean ± s.E.</th>
<th>3 days post-hatch Mean ± s.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxide*</td>
<td>5·0 ± 0·9</td>
<td>65·5 ± 2·0†</td>
</tr>
<tr>
<td>(nmol g⁻¹ wet weight)</td>
<td>n=4</td>
<td>n=4</td>
</tr>
<tr>
<td>Peroxidizable lipid*</td>
<td>277·4 ± 114·6</td>
<td>72·9 ± 11·4</td>
</tr>
<tr>
<td>(nmol g⁻¹ wet weight)</td>
<td>n=4</td>
<td>n=4</td>
</tr>
</tbody>
</table>

*Thiobarbituric acid reactive substances (malonaldehyde equivalents).
†P<0·05 v. embryo by two-tail t-test.

Comparing levels of lipid peroxide and peroxidizable lipid in embryos and 3-day larvae, a 13-fold increase in lipid peroxide was detected after hatching and was mirrored by an indicated 3·8-fold decrease in peroxidizable lipid (Table II).

DISCUSSION

Very few studies have been undertaken to examine pro-oxidant and anti-oxidant processes in fish larvae (Aceto et al., 1994; Peters et al., 1996). In this study, the activities of the antioxidant enzymes SOD, catalase, glutathione reductase, DT-diaphorase and GPX were determined in the early life stages of *S. maximus*. Both SOD and NADPH-dependent DT-diaphorase activities decreased after hatching whilst total GPX activity decreased between 3 and 11 days post-hatch. Catalase, glutathione reductase and SeGPX activities increased...
(or were indicated to increase) during development from embryos to 11-day-old larvae. However, no change in NADH-dependent DT-diaphorase activity was measured between the three developmental stages. NADH-dependent DCPiP reductase activity increased after hatching whereas the NADPH-dependent DCPiP reductase activity was lower in 3-day-old larvae compared to the other life stages studied.

SOD activity was higher in embryos compared to the larval stages and may be indicative of a decreasing need to detoxify $O_2^-$ during development from embryo to 11-day-old larvae. Following fertilization, the oxygen uptake by fish embryos increases (Finn et al., 1991) and peaks after the embryo hatches (Serigstad, 1987). During S. maximus embryo development, rates of oxygen consumption increased 3-fold from fertilization to hatching and then reached a maximum between 3–4 days post-hatch (Rønnestad et al., 1992; Finn et al., 1995). The increased rate of oxygen uptake may increase pro-oxidant processes such as $O_2^-$ production, which in S. maximus, are counteracted by SOD activity present in the embryos and larvae. Oxygen availability has been demonstrated to be one of the factors affecting embryo hatching. Hypoxia or agents that cause hypoxia, e.g. low seawater dissolved concentrations of cyanide or high concentrations of dissolved hydrogen, stimulate hatching whereas hyperoxia may suppress fish hatching (Yamagami, 1988). If this phenomenon is physiologically relevant to the hatching of S. maximus then embryologic SOD may act to reduce elevated tissue concentration of $O_2^-$ which may result during respiratory chain and/or trans-membrane electron transfer (Loschen et al., 1971; Cross & Jones, 1991) during hatching.

A simultaneous increase in catalase, glutathione reductase and SeGPX activities with development of embryos to larvae would indicate a progressive need to remove H$_2$O$_2$ and lipid peroxides from the tissues. The latter concurs with an observed increase in lipid peroxide which rose from 2 to 90% of the peroxizable lipid after hatching (calculated from the data in Table II). During S. maximus larval development, such proposed increases in H$_2$O$_2$ production are not derived from dismutation of $O_2^-$ since larval SOD activity declined after hatching. In vitro studies have demonstrated that S. maximus larval tissue preparations can generate both $O_2^-$ and H$_2$O$_2$ using either NADH or NADPH as reducing equivalents (Peters et al., 1996).

Increasing glutathione reductase and SeGPX activities with respect to larval developmental stages may indicate also an increased requirement for glutathione both for SeGPX activity and as a free radical scavenger for radicals derived from lipid peroxidation and oxidation of other macromolecules. In this study, the developmental changes of SeGPX and total GPX activities differed, i.e. an increase in SeGPX activity with larval development contrasted with decreasing total GPX activity in 11-day-old larvae. This indicates that SeGPX is not likely to be the only source of larval GPX activity. GPX activity has been associated with isoforms of the enzyme glutathione transferase (GST: Lawrence & Burk, 1976); regulation of GST expression in fish may occur via factors other than ROS production (George, 1994).

S. maximus larval NADPH-dependent DT-diaphorase activities were lower than the activity determined in embryo preparations and may reflect a decreased potential for ROS generation via redox cycling endogenous or dietary...
compounds, e.g. quinones (O'Brien, 1991). Purification of mammalian DT-diaphorase demonstrated that the pure enzyme utilized either NADH or NADPH as an electron donor (Ernster, 1987). Development of larval NADH- and NADPH-dependent DT-diaphorase activities occurred independently of each other, and indicated that the NAD(P)H-dependent activities may be attributed to two separate enzymes or that the regulation of the NAD(P)H-dependent activities are different during the early life stages of S. maximus. DT-diaphorase is an important enzyme in vitamin K synthesis (Ernster, 1987) and it is also possible that during S. maximus larval development, vitamin K-dependent processes, i.e. synthesis and utilization may affect larval DT-diaphorase activity.

In this study, embryo and larval NAD(P)H-dependent DCPIP reductase activities indicated that hatching may influence the in vitro activities of larval enzymes. However, it was observed that catalase, glutathione reductase and SeGPX activities increased at each developmental stage studied. As a consequence of this development, the early life stages of turbot have the potential to counter pro-oxidant processes that may occur during larval development.

Aceto et al. (1994) studied the ontogeny of antioxidant enzymes in the embryos and yolk-sac larvae of Oncorhynchus mykiss (Walbaum). Trout glutathione reductase and catalase activities were found to increase after hatching (as was observed in S. maximus), however both SeGPX and total GPX activities were higher in O. mykiss embryos than fry, the opposite being observed in S. maximus embryos and 3-day-old larvae. SOD activity was significantly higher in S. maximus embryos compared to its larval stages, which is the converse to that found in the study of Aceto et al. (1994). Plant allelochemicals have been demonstrated to inhibit SOD activity of aquatic larval invertebrates (Nivsarkar et al., 1991) and dietary components can affect levels of antioxidant enzymes in the larval and adult life stages of organisms (Pritos et al., 1990; Davies, 1995). Such dietary factors may be influencing the enzyme activities in larval S. maximus, particularly when they change their metabolic energy source from endogenous lipids (Korsgaard, 1991; Ronnestad et al., 1992) to exogenous food between 3 and 11 days post-hatch.

Cousin et al. (1987) reported the development of specific digestive tract enzymes in S. maximus larvae at the time of (or approximately 1 day prior to) feeding. Non-specific proteases, amylase and esterases all developed in the organs of the digestive system in S. maximus larvae 3 days post-hatch, at a time of larval development when the mouth is open and the organism may be hunting for prey but the yolk sac (or at least the oil globule) is still present (Al-Maghazachi & Gibson, 1984; Cousin et al., 1987). In this study, the presence of antioxidant enzyme activities was determined in both the embryological and larval stages of S. maximus and may reflect a requirement to nullify pro-oxidant processes in the early life stages of S. maximus.

In summary, this study has demonstrated that S. maximus embryos contain enzyme activities that (a) metabolize $O_2^-$ (SOD) and $H_2O_2$ (catalase and SeGPX); (b) detoxify organic hydroperoxides (SeGPX and total GPX); and (c) reduce the potential of redox cycling by endogenous and foreign compounds (DT-diaphorase). These activities presumably act as antioxidant defences against pro-oxidant processes in the developing embryo. When the embryos
hatched, larval SOD activity decreased whilst catalase, glutathione reductase and SeGPX activities increased, suggesting that the enzymes were being regulated differentially. This may reflect changes in sources of oxidative stress as also indicated by an increase in the levels of lipid peroxides.

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References


ONTOGENY OF TURBOT LARVAL ANTIOXIDANT ENZYMES


2.5 RESPONSES OF HEPATIC CYTOCHROME P4501A AND FORMATION OF DNA-ADDUCTS IN JUVENILES OF TURBOT (*SCOPHTHALMUS MAXIMUS* L.) EXPOSED TO WATER-BORNE BENZO[A]PYRENE.
Responses of hepatic cytochrome P450 1A and formation of DNA-adducts in juveniles of turbot (Scophthalmus maximus L.) exposed to water-borne benzo[a]pyrene

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Abstract

The time-course of the induction of hepatic cytochrome P450 1A (CYP1A) and the formation of DNA-adducts in liver and surrounding tissues were studied in juvenile turbot (Scophthalmus maximus) (size range 6.0 ± 0.5 cm) exposed to a single water-column dosage of 1 or 25 ppb benzo[a]pyrene (BaP) for up to 48 h. CYP1A induction was measured in terms of 7-ethoxyresorufin O-deethylase (EROD) activity and CYP1A-immunopositive protein (Western blotting using polyclonal antibody to hepatic CYP1A of perch, Perca fluviatilis). The formation of BaP-DNA-adducts was determined by 32P-postlabelling analysis of extracted DNA. Hepatic EROD activity was not elevated by exposure to 1 ppb BaP at any time but increased 2- to 3-fold, 24 and 48 h after exposure to 25 ppb BaP, indicating induction of CYP1A at the higher BaP exposure concentration. Western blot analysis identified major immunopositive bands of apparent molecular weight 58 kDa (consistent with the presence of a CYP1A protein) and 48.5 kDa. Although levels of the 58kDa CYP1A-immunopositive protein were higher at 25 ppb than 1 ppb BaP 48 h after exposure, no overall consistent meaningful correlation between EROD activity and CYP1A-immunopositive protein could be discerned, probably owing to the relatively low levels of CYP1A induction and the sensitivity of the Western blot analysis. Consistent with the results for EROD activity, formation of DNA-adducts was indicated at 1 ppb BaP, but a 6-fold increase after 16 h was seen at 25 ppb. The latter level of DNA-adducts remained high at 24 h but decreased by 50% after 48 h. The marked formation of DNA-adducts at 16 h, before the increase in EROD activity, indicates a constitutive capacity for the metabolism of BaP to DNA-adducts. The maximal levels of DNA-adducts observed in BaP-exposed juveniles were similar to those in adult S. maximus.

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Abbreviations: benzo[a]pyrene. BaP; benzo[a]pyrene dial epoxide, BaPDE; deoxyribonucleoside 3'-monophosphate, dNp; dimethylsulphoxide, DMSO; 7-ethoxyresorufin O-deethylase, EROD; polynuclear aromatic hydrocarbons, PAH; relative adduct labelling, RAL

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injected intraperitoneally with 20 mg kg$^{-1}$ BaP, respectively, 583 ± 131 and 650 ± 106 attomoles adducts µg$^{-1}$ DNA. Co-chromatography of extracted adducted-bases with (+) and (-)-anti-benzo[a]pyrene diol epoxide-N$^0$-guanine and N6-adenine nucleic acid standards failed to identify any specific adducts.

Keywords: Turbot liver; Scophthalmus maximus; Cytochrome P450 1A; DNA-adducts; Benzo[a]pyrene; Juvenile

1. Introduction

Marine fish readily take up lipophilic organic contaminants from the environment, and possess a variety of molecular and cellular mechanisms to protect them against the deleterious effects of such chemicals (Walker and Livingstone, 1992). These mechanisms include biotransformation enzymes which convert organic contaminants to water-soluble, excretable metabolites (Buhler and Williams, 1989; Foureman, 1989; George, 1994). Of central importance in the metabolism of many contaminants is cytochrome P450 1A (CYPIA—for nomenclature see Stegeman, 1992) which converts polynuclear aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (BaP), both to non-toxic products and to reactive electrophilic species capable of binding covalently to DNA (Stegeman and Lech, 1991; Goksøyr and Förlin, 1992; Stegeman and Hahn, 1994). Exposure to specific contaminants, including certain PAHs results in the induction of CYPIA and the formation of DNA-adducts, both of which have been implicated in the etiology of chemically caused carcinogenesis in fish (Stein et al., 1990; Stegeman and Lech, 1991; Stegeman and Hahn, 1994).

Hepatic BaP metabolism and CYPIA induction have been demonstrated in many adult fish species, both in the laboratory and the field (Goksøyr and Förlin, 1992; Livingstone, 1993; Stegeman and Hahn, 1994). Less is known of DNA-adduct formation, but it is indicated to be a general phenomenon in fish liver, both from radiolabelled substrate and more specific $^{32}$P-postlabelling studies (Varanasi et al., 1992; Livingstone, 1993; Maccubbin, 1994). DNA-adduct formation has been seen, or indicated, following administration of PAHs by intramuscular injection (English sole, Pleuronectes vetulus—previously Parophrys vetulus) (Stein et al., 1993), intraperitoneal injection (bluegill sunfish, Lepomis macrochirus) (Shugart et al., 1987) and via the diet (winter flounder, Pseudopleuronectes americanus) (McElroy et al., 1991), water-column (mosquito fish, Gambusia affinis) (Batel et al., 1985) and sediment (Stein et al., 1990). Dose-dependent hepatic BaP-DNA-adduct formation was observed for P. vetulus, and the adducts persisted for days to months (Varanasi et al., 1989; Stein et al., 1993). Dose-dependent relationships have also been observed in the field, e.g. positive correlations were seen between levels of bulky, hydrophobic DNA-adducts in liver of eel (Anguilla anguilla) and levels of PAHs in sediments (Van Schooten et al., 1995). Species differences in BaP metabolism and adduct formation are also indicated, different patterns of metabolism and lower levels of hepatic BaP-DNA-adducts being seen in the starry flounder (Platichthys stellatus) compared with P. vetulus (Stein et al., 1990).
Much less is known of CYPIA induction and DNA-adduct formation in juvenile life stages, compared with adults (Andersson and Förlin, 1992; Livingstone, 1993). The marine teleost turbot (*Scophthalmus maximus* L.) is a farm-reared fish of commercial importance. It is bottom-dwelling, and has been shown to readily take up organic contaminants from both sediments and food-sources (Courtney and Langston, 1980). Its larvae are sensitive to BaP exposure (Peters et al., 1992), and molecular studies have shown the presence of an inducible CYPIA in whole larvae, and liver of both juveniles and adults (Peters and Livingstone, 1995). The present study examines the time-course of putative induction of hepatic CYPIA in juveniles with water-borne exposure to BaP, and the formation of DNA-adducts in body cavity whole tissues (dermis, gastro-intestinal tract, liver, etc). CYPIA was studied in terms of 7-ethoxyresorufin O-deethylase (EROD) activity and CYPIA protein levels (Western blotting), and DNA-adduct formation by the $^{32}$P-postlabelling technique (Randerath et al., 1981). The results are discussed in relationship to findings for other aquatic organisms.

2. Methods

2.1. Chemicals

Proteinase K and calf spleen phosphodiesterase were obtained from Boehringer Männheim Ltd UK. Resorufin, 7-ethoxyresorufin, β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), adenosine 5'-triphosphate (ATP), BaP, *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, RNAase (Type II-A from bovine pancreas), micrococcal nuclease, potato apyrase, α-amylase (Type 1-A from porcine pancreas) and goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, and all other biochemicals were from Sigma Chemical Company Ltd, UK. Nitrocellulose, T4 polynucleotide kinase, $[^{32}$P]-ATP ($> 185$ TBq mmol$^{-1}$) and Hyperfilm β-max were from Amersham International plc., UK. Phenol/chloroform was from Applied Biosystems, UK. Chloroform/isoamylcohol and plastic backed PEI-cellulose TLC plates (Machery–Nagel) were from Camlab UK. Isomeri nuclear acid adduct standards of BaP were prepared by reaction of (+) or (−)-anti-benzo(a)pyrene diol epoxide (BaPDE) with deoxyguanosine and adenosine 3'-monophosphates as described in Morse et al. (1996). The standards produced were cis- and trans-(+)-anti-BaPDE-N$^2$-guanine, cis- and trans-(−)-anti-BaPDE-N$^2$-guanine, cis- and trans-(+)-anti-BaPDE-N6-adenine and, cis- and trans-(−)-anti-BaPDE-N6-adenine. All other chemicals (AnalaR grade) were from Merck Ltd, UK. Rabbit polyclonal antibody to hepatic CYPIA of perch (*Perca fluviatilis*) was a kind gift from L. Förlin, University of Göteborg, Sweden.

2.2. Animal treatments

Sexually immature *S. maximus* (length $6.0 ± 0.5$ cm) were obtained from a commercial hatchery (Golden Sea Produce, Hunterston, West Kilbride, Scotland UK)
and maintained at 15 ± 2°C in 1500 l recirculating seawater. Feeding of fresh shrimp ceased 7 days before commencement of the exposure experiment; and 12 h prior to dosing, the fish were moved into 20 l aerated static-seawater tanks. The seawater was treated with BaP (12 o'clock) dissolved in a minimal volume of dimethyl sulphoxide (DMSO) to achieve 1 and 25 ppb BaP in the water, and fish were sampled at 8, 16, 24 and 48 h after dosage. Fish were also sampled from control (no additions to the seawater) and vehicle control (DMSO only, final carrier concentration 10 ppm) seawater tanks. Details of the number of samples used are given in the text. Fish were stunned by a blow to the head and killed by severing the spinal chord. Livers (weight: 34.3 ± 2.4 mg) were processed immediately for the determination of EROD activity. Processed samples were also frozen for the determination of CYPIA protein. For determination of DNA-adducts, the whole body cavity was used (dermis, gastro-intestinal tract, liver etc), weighed (1.3 ± 0.1 g), frozen in liquid nitrogen and stored at −70°C prior to analysis. For comparison, adult S. maximus were injected intraperitoneally with BaP dissolved in corn oil (20 mg kg⁻¹), or corn oil only (control), and kept in aerated 100 l static-seawater tanks for 48 h. Fish were fed a diet of Merlangius merlangus L. fillets until 7 days prior to the exposure and then starved up to the injection of the contaminant/corn oil. After exposure they were sacrificed as above and their livers dissected, frozen in liquid nitrogen and stored at −70°C prior to DNA-adduct analysis. Adult S. maximus were also injected intraperitoneally with a single dose of β-naphthoflavone dissolved in corn oil (20 mg kg⁻¹) and maintained in aerated 100 l static-seawater tanks for 24 h. Hepatic microsomal preparations of whole livers from these animals were used as positive controls for Western blot analysis. All fish were selected at random for respective treatments and all exposure conditions included a seawater temperature at 15 ± 2°C and 12/12 h day night cycle.

2.3. Enzyme sample preparation and EROD activity determination

Sample preparation procedures were carried out at 4°C. Individual livers were homogenised by sonication in 500 ml 0.15 M KCl/1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, and the homogenates centrifuged at 13 500 g for 5 min. The resultant supernatant was used to measure EROD activity and CYPIA protein (see below). EROD activity was determined fluorometrically as described in Peters and Livingstone (1995). Contained in a final volume of 150 μl were 90 μl sample (approximately 0.5 mg total protein), 0.23 mM NADPH, 3.74 μM 7-ethoxyresorufin and 60 mM KH₂PO₄/K₂HPO₄ pH 7.6. The reaction was initiated by the addition of NADPH, incubated for 45 min at 30°C, and stopped by the addition of 200 μl acetone. Following centrifugation at 13 500 g for 1 min, fluorescence (Ex 537 nm, Em 583 nm) was determined against resorufin standards (0.34, 0.68 or 1.36 μM). Controls consisting of incubation mixtures as above but stopped at time zero by addition of acetone, and then incubated for 45 min, were subtracted from the sample time 45 min fluorescence. Protein was measured by the method of Lowry et al. (1951).
13500 g supernatants, processed as above, were diluted with homogenisation buffer to a protein concentration of 5 mg ml⁻¹ and then boiled with equal volumes of 0.125 M Tris-HCl pH 6.8, 4% sodium dodecyl sulphate (SDS w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 0.001% bromophenol blue (w/v) for 2–3 min. Aliquots of 25 µg protein were loaded on 10% SDS gels and electrophoresis was performed by the method of Laemmli (1970). Gels were semi-dry blotted on to nitrocellulose following the method of Towbin et al. (1979). Blots were washed with a solution of 0.5% gelatin (w/v), 0.2% Tween 20 (v/v), 0.1% sodium azide (w/v), 10% 2-mercaptoethanol (v/v), 0.001% bromophenol blue (w/v) for 12–14 h overnight at room temperature. After washing with a solution of 0.2% Tween 20 (v/v), 10 mM Tris-HCl pH 8.0 and 0.15 M NaCl, the blots were incubated with rabbit anti-perch CYP1A antibody in the above buffer (1:1000 v/v) for 12–14 h overnight at room temperature. After washing with a solution of 0.2% Tween 20 (v/v), 10 mM Tris-HCl pH 8.0 and 0.15 M NaCl, the blots were incubated with excess alkaline phosphatase-labelled goat anti-rabbit IgG (1:3000 v/v) for 1 h. Finally, the nitrocellulose was washed as above and the blots visualised by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both 0.2 mM) in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂. Samples were semi-quantified by image analysis using a Kontron image analyzer (Image Processing System, UK) and results expressed in arbitrary units. Apparent molecular weights of immunopositive bands were determined from simultaneously run pre-stained molecular weight markers as described in Peters and Livingstone (1995).

2.5. DNA extraction and ³²P-postlabelling analysis

Frozen tissue samples from juvenile or adult S. maximus were cut into small pieces and incubated in prewarmed polypropylene tubes containing 500 µl phosphate buffered saline (0.14 M NaCl, 2.68 mM KCl, 8 mM Na₂HPO₄·2H₂O, 1.5 mM KH₂PO₄ pH 7.3), 100 µl RNase (1 mg ml⁻¹) and 500 µl lysis buffer (100 mM Tris-HCl pH 8.0, 4 M urea, 0.5% n-lauroylsarcosine, 10 mM trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid) for 1 h at 37°C, with shaking; to break up the tissue. Forty units of proteinase K in 20 mM Tris-HCl pH 8.5 was then added and the mixture incubated at 37°C, with shaking, for 16 to 20 h. The resulting digest was serially extracted with 7:3 (v:v) phenol/chloroform, 17.5:31.5:1 (v:v:v) phenol/chloroform/isoamylalcohol and 24:1 v:v chloroform/isoamylalcohol. The DNA was precipitated by the addition of 0.1 volume of 3M CH₃COONa pH 5.2 and 2 volumes of −20°C ethanol. Samples were washed once with 70 and 100% ethanol and then dissolved in 0.5 ml 100 mM NaCl, 20 mM EDTA. 50 mM Tris-HCl pH 8.0 overnight at 4°C. Glycogen contamination of the DNA was removed by the addition of 7 µl a-amylase at 37°C for 1 h. DNA was recovered by precipitation with 2 volumes of −20°C ethanol and then washed with 70% and 100% ethanol. The samples were then dissolved in water and the DNA concentration determined at 260/280 nm. Sample DNA concentration was adjusted to 1 µg µl⁻¹.

The DNA samples were analysed by ³²P-postlabelling as described in Morse et al. (1996). Briefly 3 µg DNA were digested to deoxyribonucleoside 3'-monophosphates
(dNp's) by incubation with micrococcal nuclease and calf spleen phosphodiesterase. The nuclease PI mediated enhancement procedure was used to enrich the concentration of adducts in the digest (Reddy and Randreth, 1986).

Adducted nucleotides were then 32P-labelled by addition of 8 µl [32P]-ATP (> 5000 Ci mmol⁻¹, > 185 TBq mmol⁻¹) in the presence of 0.5 µl T4 polynucleotide kinase (10 U). Labelled adducts were subsequently separated on 20×20 cm plastic backed PEI-cellulose TLC plates. The chromatographic conditions used were specifically optimised for the separation of BaP-DNA adducts, namely; D1-1M Na₂HPO₄/NaH₂PO₄ pH 6.8; D2-2.5 M ammonium formate pH 3.5; D3-4 M lithium formate pH 3.2, 8.5 M urea; D4-0.8 M lithium chloride, 0.5 M Tris-HCl pH 9, 8.5 M urea and D5-1.7 M Na₂HPO₄/NaH₂PO₄ pH 6.0.

Areas of radioactivity on the chromatograms were located by autoradiography. Hyperfilm β-max was exposed to plates at -70°C with intensifying screens for 72 h. Radioactivity in adduct spots was quantified by excising the spots from the TLC plates followed by liquid scintillation counting. The level of radioactivity in adjacent blank areas was also determined and subtracted when relative adduct labelling (RAL) was calculated (Gupta, 1985). RAL values were translated into attomole adducts per µg DNA by assuming that 1 µg DNA is equal to 3.24×10¹² attomole nucleotides.

Standard BaPDE-nucleic acid adducts were 32P-postlabelled and separated by TLC under identical conditions as for the adducts from S. maximus DNA, in order to identify specific adducts as described in Morse et al. (1996). The limits of DNA adduct quantitation were estimated to be 1 adduct in 10⁸ or 10⁹ normal nucleotides.

2.5.1. Statistical analysis

Values are presented as means±SEM (n=3-5) or ± range (n=2). Groups of values for different conditions, or over time, were compared by ANOVA (P<0.05).

3. Results

The results for hepatic EROD activity of juvenile S. maximus with exposure to water-borne BaP are given in Fig. 1. After 48 h exposure to DMSO alone, EROD activity was lower (4.8±0.5 pmol min⁻¹ mg⁻¹) than time zero controls (10.8±2.4 pmol min⁻¹ mg⁻¹). After exposure to 1 ppb BaP, no change in hepatic EROD activity was seen over time, and no elevation in activity was seen compared with either time zero untreated or 48 h vehicle control animals. In contrast, at 25 ppb BaP hepatic EROD activity increased markedly 24 h after exposure, i.e. respectively 2.1- and 3.1-fold higher than time zero untreated and 1 ppb BaP animals, and remained high 48 h after exposure.

Examples of Western blots of hepatic samples of juvenile and adult S. maximus using polyclonal antibody to hepatic CYP1A of P. fluviatilis are shown in Fig. 2. Strong recognition of a protein band of approximate 58kDa apparent molecular weight was seen in 13500 g supernatants of juvenile S. maximus exposed to 25 ppb
BaP for 48 h. An immunopositive band of similar apparent molecular weight was evident in hepatic microsomes of BaP-treated adult *S. maximus*, but not in 1 ppb BaP-exposed juveniles. A second major immunopositive band of lower apparent molecular weight (48.5 kDa) was also seen in most blots. Semi-quantification of putative hepatic CYP1A (58 kDa band) of juveniles by image analysis (*n* = 3) confirmed elevation of the immunopositive protein in 25 ppb relative to 1 ppb BaP-exposed animals 48 h after exposure, respectively 24.6 ± 2.5 compared with 16.9 ± 1.7 arbitrary units. However, although levels of CYP1A of both 1 and 25 ppb BaP-exposed animals after 48 h were higher than those of time zero control animals (12.3 ± 1.3), neither was significantly higher than 48 h vehicle control fish.

Fig. 1. Time-course of the responses of 7-ethoxyresorufin O-deethylase (EROD) activity in juvenile turbot (*S. maximus*) exposed to 1 ppb (•••) or 25 ppb (●●●) benzo[a]pyrene (BaP): control (■ ■). Values are means ± SEM, *n* = 3; *P* < 0.05 compared with vehicle control or 1 ppb BaP.

Fig. 2. Western bolts of 13000 g supernatant of liver of juvenile turbot (*S. maximus*) probed with polyclonal antibody to hepatic CYP1A of perch (*P. fluviatilis*). From left to right lanes A-C, 25 μg total protein from juveniles exposed to 1 ppb BaP (48 h); lanes D-F, 25 μg total protein from juveniles exposed to 25 ppb BaP (48 h); lanes G-H, 5 μg hepatic microsomal protein from β-naphthoflavone induced adult turbot (see Materials and Methods for details).
Fig. 3. Representative autoradiograms of $^{32}$P-postlabelled DNA-adducts in liver and surrounding tissue of juvenile turbot (S. maximus) after exposure to water-borne benz[a]pyrene (BaP). (a) 48 h after exposure to vehicle control; (b) 8 h after exposure to 1 ppb BaP; (c) 48 h after exposure to 25 BaP.

(21.3 ± 0.4). No differences in CYPIA levels were evident between animals exposed to 1 ppb and 25 ppb BaP after 8, 16 and 24 h (data not shown).

Representative chromatographic profiles of adducted $^{32}$P-postlabelled nucleotides
from liver and/or surrounding tissues of vehicle control and BaP-treated juvenile *S. maximus* are presented in Fig. 3. Fig. 3(a) is an autoradiogram from a fish after 48 h exposure to the vehicle DMSO, whereas Fig. 3(b) and (c) are autoradiograms from fish exposed to respectively 1 and 25 ppb BaP. All autoradiograms derived from BaP-exposed *S. maximus* revealed discrete chromatographic spots of varying intensity and size (greater than for unexposed control fish), indicative of the formation of multiple BaP-DNA-adducts. Fish exposed to DMSO alone (vehicle solvent) gave several faint spots (of considerable lower intensity than those observed for BaP-exposed animals) in the upper right hand quarter of the autoradiogram (see Fig. 3(a)). Individual putative adducts (i.e. spots on the autoradiograms) were quantified and the results are presented in Fig. 4. The level of DNA-adducts in unexposed juvenile *S. maximus* was 102 ± 41 attomol µg⁻¹ DNA and no elevation was detected 8 or 48 h after exposure to DMSO alone. At 1 ppb BaP, levels of total DNA-adducts were indicated to increase 2.5-fold after 8 h but then decline rapidly back to control levels by 16 h exposure. In contrast, at 25 ppb BaP, levels of total DNA-adducts increased markedly 16 h after exposure (i.e. 5.7-fold increase up to 583 ± 131 attomol µg⁻¹ DNA) and then declined by about 50% over the next 32 h.

Table 1
Formation of DNA-adducts in liver of adult turbot (*S. maximus*) following 48 h exposure to a single intraperitoneal injection of benzo[a]pyrene (BaP) in corn oil (20 mg kg⁻¹ wet wt.)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adduct level a</th>
<th>Adduct No. b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil alone</td>
<td>nd a</td>
<td>0</td>
</tr>
<tr>
<td>BaP</td>
<td>650 ± 106</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± range (*n* = 2); a attomol µg⁻¹ DNA. b number includes non-quantifiable adducts; * not detected, below limit of quantitation (see Methods).
BaP-DNA-adduct formation in liver and/or surrounding tissues of juvenile *S. maximus* was compared with the same process in liver of adult fish, although the route of exposure to BaP was different, i.e. respectively water-borne compared with intraperitoneal injection. Autoradiograms of the adducted $^{32}$P-postlabelled nucleotides obtained from adult liver are shown in Fig. 5. No DNA-adducts were detected in corn oil-injected fish; however many discrete spots (up to 17 or more—see Table 1) were observed on the autoradiograms from the BaP-treated animals, indicative of BaP-DNA-adduct formation. The levels of adducts in liver of adult *S. maximus* (48 h after BaP injection) were similar to the maximal levels found in liver and/or surrounding tissues of juveniles 16 h after exposure to 25 ppb water-borne BaP, respectively 650 compared with 583 attomol adducts g$^{-1}$ DNA (Table 1). The DNA samples were $^{32}$P-postlabelled concomitantly with the BaP-treated calf thymus DNA and the isomeric adduct standards of (+)- or (-)-anti-BaPDE-N$^2$-guanine and (+)- or (-)-anti-BaPDE-N$^6$-adenine. These standards failed to identify any of the adducts induced in either the adults or the juveniles.
4. Discussion

The metabolism of PAHs to reactive intermediates has been demonstrated in marine mammals (Boon et al., 1992), fish (Sikka et al., 1990a) and invertebrates (Winston et al., 1993). Fish have metabolic pathways that biotransform BaP via phase I metabolism to highly reactive metabolites (Nishimoto et al., 1992) that may undergo phase II conjugation to polar metabolites (George, 1994), or form adducts with macromolecules such as DNA (Varanasi et al., 1989). Metabolic pathways involving either 1- or 2-electron oxidation have been proposed as possible pathways of BaP bioactivation with the formation of intermediates, e.g., BaP radicals or diol epoxides, that bind to DNA bases (Zaleski et al., 1991; Cavalieri et al., 1993; Rogan et al., 1993). The processes of bioactivation may involve multiple forms of cytochrome P450, however the CYP1A family has specifically been linked to the conversion of BaP to the carcinogen BaP-7,8-dihydrodiol-9,10-epoxide-in conjunction with the enzyme epoxide hydrolase, (Schoor and Srivastava, 1984).

In this study, the levels of CYP1A (assayed via EROD activity and Western Blot analysis) and the formation of DNA adducts (determined by $^{32}$P-postlabelling) were measured in tissue of juvenile S. maximus exposed for up to 48 h to 1 and 25 ppb water-borne BaP. Induction of CYP1A as indicated by EROD activity was seen 24 to 48 h after exposure at 25 ppb BaP, but at no time over the 48 h at 1 ppb BaP, presumably because of insufficient uptake of BaP. The latter interpretation is supported by the absence of DNA-adduct formation at 1 ppb BaP, other than an indicated increase at 8 h exposure only. The time-course of induction observed for hepatic CYP1A in fish varies with the route and dosage of exposure, but elevation after two days is consistent with other studies on juvenile (Peters and Livingstone, 1995) and adult (Celander et al., 1993) fish. In contrast to EROD activity, no clear picture emerged for the measurement of hepatic CYP1A protein in juvenile S. maximus with exposure to BaP. Although a CYP1A-immunopositive band of the correct apparent molecular weight of 58 kDa was detected (compared with 55 to 59 kDa for other species of teleost fish Goksøyr et al., 1991), and higher levels of CYP1A protein were evident in 25 ppb than 1 ppb BaP-exposed fish (48 h), no differences were discerned between the two BaP-exposure conditions after 24 h (unlike EROD activity), or surprisingly between the 25 ppb BaP-exposed and vehicle (DMSO) control animals after 48 h. The elevation of hepatic CYP1A protein in species of adult fish has been observed to occur slightly before, or simultaneously with, increases in EROD activity, consistent with a transcriptional activation mechanism of induction (Kloepper-Sams and Stegeman, 1989; Celander et al., 1993). The apparent elevation of CYP1A protein in juvenile S. maximus by DMSO is in contrast to previous exposure studies with dimethylformamide as vehicle solvent where no such effect was observed (Peters and Livingstone, 1995). The explanation for the lack of a clear elevation of CYP1A protein in parallel with changes in EROD activity is most likely owing to the relatively low levels of CYP1A induction with exposure to 25 ppb BaP, and the limitations of the Western blot assay using cross-species antibody (Goksøyr et al., 1991). Thus, although the amount of
CYP1A protein as a percentage of total cytochrome P450 can increase from 20 up to over 70% in liver of fish with exposure to CYP1A-inducers (Kloepfer-Sams and Stegeman, 1994), this reflects increases in EROD activity of 8-fold or more (Kloepfer-Sams and Stegeman, 1989), compared with the maximal 3.1-fold observed for juvenile S. maximus in these studies.

No statistical increases were seen in levels of 32P-postlabelled adducts in liver and/or surrounding tissues of juvenile S. maximus tissue 48 h after exposure to 1 ppb BaP, consistent with a low uptake of BaP and/or lack of induction of CYP1A as indicated by EROD activity (see before). Such water-column concentrations of total PAHs and BaP are seen environmentally (Neff, 1979; Livingstone, 1983), but presumably greater bioaccumulation of BaP and subsequent formation of DNA-adducts could occur over longer time-courses of exposure. The formation of 32P-postlabelled adducts (several faint spots) was indicated after juvenile S. maximus were exposed to DMSO however they were of considerable lower intensity than those observed for BaP-exposed animals. Such faint spots have been seen before in larvae of African clawed frog (Xenopus laevis) exposed to similar concentrations of DMSO (H. R. Morse and R. Waters, unpublished data). In contrast to 1 ppb BaP, a 5.7 fold increase in DNA-adducts 16 h after exposure, followed by a 50% decline in adduct levels by 48 h, was observed in juvenile S. maximus with exposure to 25 ppb BaP. Again these are environmentally realistic water-column concentrations of total PAHs, particularly following oil spills (Neff, 1979; Livingstone, 1983). The pattern of formation of DNA-adducts from BaP will be dependent upon the tissue concentration of bioavailable BaP, the constitutive levels of CYP1A and other cytochrome P450 and non-cytochrome P450 mechanisms of metabolism, and the induction of CYP1A as a major catalyst (Gokseyr and Förlin, 1992; Walker and Livingstone, 1992; Maccubbin, 1994; Stegeman and Hahn, 1994). The maximal elevation of DNA-adduct levels at 16 hours, prior to the elevation of EROD activity at 24 h, indicates that either the basal levels of CYP1A determined in control fish contributed to BaP bioactivation (Vähäkangas et al., 1989; Bjelogrlic et al., 1993) and/or the adducts were formed by other processes, such as 1-electron oxidative (Cavalieri et al., 1993) and peroxidative (O'Brien, 1978; Marnett, 1985) pathways. There was no further increase in the levels of DNA adducts formed after the elevation of EROD activity and this may reflect either a decrease in the bioavailability of BaP in the experimental conditions or an increased capacity to metabolise the contaminant via CYP1A phase I and/or phase II conjugation processes (Foureman, 1989; George, 1994).

The rapid formation of DNA-adducts and their subsequent decrease was observed when P. vetulus was exposed to BaP and the mammalian hepatocarcinogen 7H-dibenzo[c,g]carbazole (Stein et al., 1993). Levels of BaP-DNA-adducts (predominantly anti-B[a]P diol epoxide) were elevated 2 h after injection and increased an estimated 100-fold after 2 days. A relatively rapid decrease in detectable adducts followed between 2 and 28 days (approximately 80% of total adducts were lost), the remaining adducts persisting to the end of the experiment at 84 days. In a similar study using brown bullhead (Ictalurus nebulosus), a significant decrease in the levels of BaP-adducts was also observed (74% of maximum level), 70 days after intraperitoneal injection of BaP (Sikka et al., 1990b). In this study using juvenile S. max-
inus the trend of rapid DNA-adduct formation (6-fold) followed by a subsequent decrease in adduct levels was indicated over a much shorter time course (48 h) and may be a consequence of developmental processes of the juvenile life stages of S. maximus. Although BaP formed persistent DNA-adducts in P. vetulīs and I. nebulosus, the decrease in the levels of adducts was perhaps as a consequence of DNA repair, which equally may be applicable to this study of juvenile S. maximus.

Repair processes via excision of adducted bases followed by resynthesis of complimentary DNA using the undamaged/unpaired DNA strand (unscheduled DNA synthesis, UDS) as a template has not been extensively studied in fish species, however initial results propose that levels of UDS are low in fish tissues (Maccubbin, 1994). Low levels of UDS may make the tissue susceptible to the effects of mutagens and account for the persistence of the DNA-adducts in 32P-postlabelling studies. Other repair mechanisms, such as error prone repair, are known to occur in eukaryotes (Venitt and Parry, 1984), but little is understood of these systems in fish species.

Both in vitro and in vivo studies of the interactions of BaP-metabolites with DNA in fish liver have identified the major adduct as anti-BaP diol epoxide/deoxyguanosine (anti BPDE/dG) (Varanasi and Gmur, 1980; Nishimoto and Varanasi, 1985; Varanasi et al., 1989). The identity of the adducts produced by either S. maximus juveniles or adults could not be confirmed using 8 BaPDE-DNA standards. Morse et al. (1996) reported that co-chromatography of the standards demonstrated migration to the same general area of the plate, such that absolute assignments of adducts in biological samples should be made with caution. The application of 32P-postlabelling techniques in field and laboratory exposure studies has indicated the production of many covalently bound DNA-adducts in liver tissue and isolated cells (Maccubbin et al., 1990; Nishimoto et al., 1992). In this study up to 19 putative DNA-adducts were resolved from adult S. maximus tissue after intraperitoneal injection of BaP or juvenile S. maximus exposed to water-treated BaP.

In summary these results indicate that juvenile S. maximus exposed to BaP have the capacity to induce CYP1A and form bulky hydrophobic DNA-adducts at environmentally realistic water-column concentrations of PAHs. These adducts may be formed prior to the elevation of hepatic EROD activity and processes are present in juvenile S. maximus that act to reduce levels of DNA adducts. Future experiments may identify if these processes are species and/or life-stage specific.

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2.6 7-ETHOXYRESORUFIN O-DEETHYLASE (EROD) AND ANTIOXIDANT ENZYME ACTIVITIES IN LARVAE OF SARDINE (SARDINA PILCHARDUS) FROM THE NORTH COAST OF SPAIN.
7-Ethoxyresorufin O-deethylase (EROD) and Antioxidant Enzyme Activities in Larvae of Sardine (Sardina Pilchardus) from the North Coast of Spain

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Sardine larvae (Sardina pilchardus) and their major food source (mixed zooplankton) were sampled from four sites along a transect out of the Bilbao estuary and a fifth (reference) site further along the north coast of Spain (23.5.92-10.6.92). Whole body sardine larvae were assayed for 7-ethoxyresorufin O-deethylase (EROD) activity (measure of CYP1A) as a biomarker of exposure to organic pollution, and the antioxidant enzymes catalase (EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) as potential biomarkers of oxidative stress. EROD activity was lowest at a site closest to the Bilbao estuary, and increased along the transect until it reached levels recorded from samples for a reference site. Both catalase and SOD activities were highest at the inshore site of the transect decreasing offshore before increasing again at the site furthest from the coastline. Levels of organic contaminants (polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls and ppDDE) in mixed zooplankton were generally similar at the different sites, with the exception of higher PAHs at the extremes of the transect and at the reference site. The results are discussed in relation to the biomarker application of fish larvae, CYP1A and antioxidant enzymes.

There are many areas of the coastline with elevated levels of anthropogenic contaminants because of the efflux of industrially polluted river water, offshore shipping and other activities (Walker & Livingstone, 1992). Contaminants such as polychlorobiphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) have been measured in the tissues of many marine species in estuaries (Spies et al., 1985) and also offshore (Boon et al., 1992a) where fresh water plumes are mixed with tidal flows (Lohse, 1991). This study addresses a potential contaminant gradient near to the port of Bilbao (North Spain) and compares the results with an off-shore reference site. High levels of PAHs (Swindelhurst & Johnston, 1991) and heavy metals (Seebold et al., 1982; Ruíz de la Rosa & Saiz Salinas, 1993) have been reported in the sediments of Bilbao estuary. Dilution of these contaminants has been observed off-shore with the mixing with Atlantic Ocean water (Perez et al., 1987).

Mechanisms of toxicity by PAHs and PCBs in fish include the induction of hepatic cytochrome P4501A (CYP1A) leading to DNA-duct formation and carcinogenesis (Stegeman & Leeth, 1991; Goksøyr & Forlín, 1992). Induction of CYP1A measured at the level of enzyme activity, 7-ethoxyresorufin O-deethylase (EROD) activity, enzyme amount and mRNA has been reported in fish as a biomarker of exposure to organic contaminants (Adams et al., 1990; Goksøyr & Forlín, 1992; Livingstone, 1993). Other potential mechanisms of contaminant-mediated toxicity in aquatic organisms include the enhanced generation of reactive oxygen species (oxyradicals), such as the superoxide anion (O2-) and hydrogen peroxide (H2O2), leading to oxidative damage to DNA and other molecules (Livingstone et al., 1990; Livingstone 1991; Winston & Di Giulio, 1991). Hepatic oxidative DNA lesions have been observed in fish both experimentally (Nishimoto et al., 1991) and from contaminated environments (Malins et al., 1990). Antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1) (converts O2- to H2O2) and catalase (EC 1.11.1.6) (converts H2O2 to water) are likely to play an important role in protection against contaminant-mediated oxyradical generation.

Early life stages of fish are very sensitive to environmental change (Cameron et al., 1992), including contaminant exposure as observed both experimentally (Buhl & Hamilton, 1991; Fent, 1991; Goksøyr et al., 1991; Schoor et al., 1991) and in the field (Westernhagen et al., 1988). Whereas induction of CYP1A has been demonstrated or indicated in fish larvae (Binder et
al., 1985; Binder & Stegeman, 1980; Goksøyr & Solberg, 1987). Little is known of the presence or responses of antioxidant enzymes, although SOD and catalase have recently been detected in larvae of turbot (Scophthalmus maximus) and sprat (Sprattus sprattus) (Peters et al., 1992). In the current study, EROD, SOD and catalase activities were measured in whole larvae of sardine (Sardina pilchardus) and compared with levels of contaminants (PAHs, PCB congeners and pp’-DDE) in their food source (mixed zooplankton). The results are considered in relation to the specific field sites and the potential of the enzyme measurements as biomarkers for exposure to pollution and contaminant-mediated oxidative stress (Stegeman et al., 1992).

Materials and Methods

Sample collection

Zooplankton samples were collected from four sites along a transect out of Bilbao (P1 to P4), and at a fifth site further along and away from the shore (station 44; termed the ‘reference site’) (Fig. 1). Material was collected during a cruise of the RRS Challenger (23.5.92-10.6.92) as part of a European Sardine Anchovy Recruitment Programme (SARP) by double oblique hauls with a 200 μm bongo net (tow speed ~3 knots, haul time ~10 min). Fish larvae were sorted in clean, glass trays and S. pilchardus (approx. 7-14 mm in length) identified by light microscopy (Russell, 1976). The latter were immediately frozen in liquid nitrogen until return to the laboratory where they were stored in a -10°C freezer. The mixed zooplankton which remained (predominantly copepods) after removal of fish larvae was frozen and stored in aluminium foil lined cryo-preservation vials (Northumbria Biologicals Ltd) in liquid nitrogen. Additionally a number of sardine larval samples were sonicated in homogenization buffer and stored as homogenates in liquid nitrogen to ascertain the effects of different storage conditions on enzyme activities (see below).

Chemicals and biochemicals

Biochemicals including β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate and commercial enzymes were obtained from Sigma Chemical Co. (Dorset); nitrocellulose was from Amersham, UK and rabbit polyclonal antibody to hepatic cytochrome P4501A of perch (Perca fluviatilis) was a kind gift from Dr L. Forlin, University of Göteborg, Sweden. The solvents, n-hexane, diethylether and iso-octane, were of pesticide grade (SDS, Peypin, France) and Florisil (100-200 mesh) was from Merck (Darmstadt, Germany). Analytical grade PCB standards (IUPAC No.: 28, 52, 101, 118, 138, 153, 187) were supplied by Promochem (Wessel, Germany). All other chemicals were AnalaR grade from Merck, UK.

Biochemical analyses

Aliquots of about 25 sardine larvae were weighed (c. 0.07 g) and homogenized in 500 μl 0.15 M KCl containing 1 mM ethylenediaminetetraacetic acid and 20% glycerol (v/v), pH 7.5 at 4°C using an MSE sonicator, medium setting (2×10 s). Homogenates were centrifuged at 13 500 g for 5 min and the resulting supernatants (protein concentration 2.5-9.3 mg per ml) either used immediately for determination of enzyme activities, or stored at -20°C prior to total protein and Western blot measurements.

Enzyme analysis

Enzyme activities were measured in duplicate (3 to 16 samples per site) as described in Peters et al. (1992) and were linear with respect to time and protein concentration. Catalase (2H₂O₂ = 2H₂O + O₂) and SOD (2H⁺ + O₂⁻ = H₂O₂) activities were assayed.
spectrophotometrically at 25°C in respective ly 3 ml and semi-micro cuvettes in a dual beam Varian Cary 1 spectrophotometer. Catalase activity was measured by the decrease in H$_2$O$_2$ at 240 nm (Ext. coeff. $c_{280}=40$ M$^{-1}$ cm$^{-1}$) and SOD activity by the inhibition of xanthine oxidase/hypoxanthine generated O$_2^-$ production (cytochrome c. reduction at 550 nm; 1 SOD unit defined as the amount of sample producing 50% inhibition under the conditions of the assay). EROD activity was assayed fluorometrically at 30°C using a micro-assay adaptation of the method of Burke & Mayer (1974). Supernatant (90 µl) was incubated in a final volume of 150 µl containing 59 mM K$_2$HPO$_4$/KH$_2$PO$_4$, pH 7.4, 0.23 mM NADPH and 3.74 µM 7-ethoxyresorufin for 60 min, before addition of 300 µl acetonitrile to stop the reaction. Following centrifugation (13 500 x g 5 min), fluorescence of the supernatant was measured in a Perkin-Elmer 300 fluorometer (500 µl well; Ex 537 nm; Em 583 nm) and quantified by reference to resorufin standards. Catalase and EROD activities were the same in larvae frozen whole or as homogenates, whereas SOD activity was 2.2-fold lower in the former ($p<0.05$, n=5). This correction factor was applied to the SOD results for the five sites.

**Western blotting**

SDS-PAGE was performed by the method of Laemmli (1970). Denaturing gels were run (10% SDS) for an hour and the proteins transferred to nitrocellulose using semi-dry conditions. Visualization of CYP1A was performed by the method of Towbin et al. (1979), using rabbit anti-perch CYP1A serum and goat anti-rabbit Ig G (whole molecule) alkaline phosphatase conjugate. Hepatic microsomes of β-naphthoflavone-induced adult S. maximus (i.p. 20 mg kg$^{-1}$; 24 h exposure) were used as positive control for CYP1A. Protein was measured by the method of Lowry et al. (1951).

**Chemical analysis**

Frozen mixed zooplankton samples (three samples per site) were lyophilized to constant mass and treated with 5 ml 6N NaOH for 18 h at 30°C. The hydrolyzed mixture was extracted with diethylether (4 × 5 ml) and evaporated to dryness. The residue was dissolved in n-hexane (500 µl) and further purified by liquid chromatography with Florisil. Glass columns (150 × 5 mm i.d.) were filled with 2 g of Florisil, previously activated overnight at 150°C and sample extracts placed on the top of the column were eluted with 20 ml of n-hexane.

**Results and Discussion**

Zooplankton contaminant body-burden was taken as a measure of S. pilchardus larval contaminant exposure. The former readily bioaccumulate contaminants such as PCBs and PAHs (Livingstone, 1991) and are the major food source for fish larvae. Also, correlations between contaminant levels in food and tissues of the feeding organisms are well documented for fish (Varanasi et al., 1992). The zooplankton contaminant levels are given in Table 1. Differences between sites were generally not great for PCBs and pp'DDE, but somewhat more marked for PAHs. Most PCB congeners tended to decrease from sites P1 to P2, with a maximum reached

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>P1</th>
<th>P2</th>
<th>Site</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB CB-28/31</td>
<td>12.1 ± 3.6</td>
<td>9.9 ± 0.2</td>
<td>23.4 ± 3.8</td>
<td>8.6 ± 2.4</td>
<td>15.2 ± 4.3</td>
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<td>PCB CB-52</td>
<td>11.6 ± 5.8</td>
<td>5.1 ± 0.9</td>
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<td>4.8 ± 0.3</td>
<td>6.1 ± 0.6</td>
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</tr>
<tr>
<td>PCB CB-101</td>
<td>3.2 ± 1.6</td>
<td>2.2 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.1 ± 0.4</td>
<td>1.7 ± 0.4</td>
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<tr>
<td>PCB CB-118</td>
<td>3.8 ± 0.3</td>
<td>2.8 ± 0.0</td>
<td>4.7 ± 0.2</td>
<td>4.5 ± 0.6</td>
<td>1.7 ± 0.3</td>
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<td>PCB CB-123</td>
<td>3.8 ± 0.8</td>
<td>2.6 ± 0.1</td>
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<td>PCB CB-138</td>
<td>3.4 ± 1.3</td>
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<td>PCB CB-187</td>
<td>1.2 ± 0.2</td>
<td>0.9 ± 0.0</td>
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<td>1.3 ± 0.2</td>
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</tr>
<tr>
<td>PCB total</td>
<td>39.1 ± 10.9</td>
<td>25.5 ± 1.2</td>
<td>50.7 ± 3.5</td>
<td>29.0 ± 1.6</td>
<td>27.1 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>pp'DDE</td>
<td>2.0 ± 0.2</td>
<td>1.4 ± 0.0</td>
<td>2.3 ± 0.2</td>
<td>1.9 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Total PAH</td>
<td>43.1 ± 15.2</td>
<td>15.8 ± 1.0</td>
<td>26.8 ± 0.5</td>
<td>34.7 ± 0.8</td>
<td>244.7 ± 165.6</td>
<td></td>
</tr>
</tbody>
</table>

*See Fig. 1. for sites.

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*See Materials and Methods for identity of PCB congeners, PCBs and pp'DDE in ng g$^{-1}$ dry wt; Total PAHs in µg chrysene equiv. g$^{-1}$ dry wt.
at site P3. Levels at P4 were similar to those at P2. The same trend was indicated for total PCBs and pp'-DDE, but none of the differences were statistically significant. In contrast, total PAHs along the transect were indicated to be highest at P1, the site nearest to Bilbao. This is consistent with observations on water-column metal (Zn, Cd, Pb) levels which were markedly higher at the entrance to the Bilbao harbour than at sites further out (Perez et al., 1987). At the reference site (station 44; along the coast and further off-shore than the transect) levels of PCBs and pp'-DDE were similar to those at site P4, the site on the transect furthest from Bilbao. However, somewhat surprisingly total PAHs were indicated to be markedly higher at station 44 than at any of the transect sites, although the variability at station 44 was high. This high level could presumably be the result of some localized incident, such as release of oil from a ship, although high levels of PAHs in seawater have been observed previously in this region (J. J. Gonzalez, pers. comm.). Rates of metabolism and elimination of PAHs are greater than for PCBs in most organisms, including copepod zooplankton (Livingstone, 1991; Walker & Livingstone, 1992), which is consistent with PAH tissue levels reflecting shorter rather than longer term events. Compared to other studies of mixed zooplankton contaminant body burdens, the total levels of PCBs (seven congeners) were similar to those for the North Sea (Peters et al., 1992; seven congeners), Adriatic Sea (Fossato et al., 1991; Cl sub 14) and the fresh water lakes of southern Ontario (Taylor et al., 1991; five congeners). They were, however, one to two orders of magnitude lower than those recorded off the Belgian coast (Delbeke et al., 1990; Arochlor 1254 standard) or in earlier studies of the North Sea (Knickmeyer & Steinhart, 1989). The levels of pp'-DDE were similar to those in the zooplankton of the North Sea (Peters et al., 1992), but somewhat lower than those for the lakes of southern Ontario (Taylor et al., 1991). No information is available on total PAHs in zooplankton, but levels in whole body crabs (Carcinus maenas, Uca Pugnax) varied from 0.2 mg g sub -1 (clean site) to 280 (oil spill) mg g sub -1 dry wt (Livingstone, 1991), compared to 16 to 245 (mg g sub -1 dry wt for the zooplankton, indicating a considerable degree of PAH exposure in the latter.

The enzyme activities in larvae of S. pilchardus are presented in Fig. 2. SOD and catalase activities were similar to those observed for 8–12 mm larvae of S. sprattus and 3 day old S. maximus (Peters et al., 1992). Both antioxidant enzyme activities declined through sites P1–P3, and were similar at the reference site compared to site P1 (Fig. 2A and B). However, whereas SOD activity was low at site P4 (Fig. 2B), catalase activity increased and was equal at that site P1 (Fig. 2A). Similar profiles of decreasing SOD and catalase activities away from coastal contamination (mainly PCBs and PAHs) were observed in liver of dab (Limanda limanda) from the German Bight of the North Sea (Livingstone et al., 1992). These hepatic activities in L. limanda also increased again at the sites most distant from the coast and showed the additional consistency with the S. pilchardus larval data that the profiles for SOD and catalase were not exactly the same (Livingstone et al., 1992). The latter difference is perhaps not surprising, given that O sub 2 dismutation (SOD-catalysed reaction) is not the only source of H sub 2O sub 2 (substrate for catalase), e.g. H sub 2O sub 2 produced from amino acid or cytochrome P450 oxidase activities. Correlations between both larval antioxidant activities and zooplankton contaminants were variable for PCBs and pp'-DDE (corr. coeff 0.34 to 0.70). There was a higher correlation between total PAH and SOD activity (corr. coeff 0.76). Elevated hepatic activities of SOD, and aryl hydrocarbon hydroxylase (indicative of PAH, PCB or similar exposure), were seen in spot (Leiostomus xanthurus) from contaminated field sites (Roberts et al., 1987).

Potential sources of contaminant-mediated oxygen radical production include interactions with various organic compounds, including PAHs via metabolism to redox cycling quinones or to free organic radicals (Livingstone et al., 1990; Livingstone, 1991). Increases have been observed in hepatic catalase activity in
could represent a species difference in level of constituent activity to produce the transect profile include a general deterioration in larval condition towards Bilbao (indicated to be unlikely from the antioxidant enzyme data), endogenous factors affecting EROD activity such as temperature (Lange et al., 1992) and development (Peters et al., 1992), and other sources of contaminants such as parental contribution (Westernhagen et al., 1987). Fish larvae are particularly sensitive to food availability, with periods of starvation affecting markers of general larval condition such as RNA/DNA ratios (Clemmesen, 1988). Such nutritional stress could impair detoxication enzyme systems (e.g. CYP1A), thus enhancing contaminant-mediated effects. Laboratory studies to investigate the interaction between nutrition and detoxication should therefore be carried out, e.g. with hatchery-reared turbot (S. maximus) (Peters et al., 1992).

Overall the studies indicate that factors other than PAH and PCB loadings alone are affecting CYP1A catalytic activity in larvae of S. pilchardus in the field, and that more studies are required before application as a biomarker is possible. They reinforce the necessity to measure both EROD activity and CYP1A protein levels in environmental monitoring (Livingstone, 1993), probably in the latter case by immunocytochemical techniques, given the small size of larval samples. Biomarker application of CYP1A in fish larvae could be used as a sentinel of contaminant impact on early life stages, or where the interest is in a particular species per se. Wider use could be limited by the practical difficulties of sample collection.

This work was partially funded by UK Department of the Environment contract PECD 7/7/359 and European Community contract number MA196.

Binder, R. L., Stegeman, J. J. & Lech, J. J. (1985). Induction of cytochrome P-450-dependent monoxygenase system in embryos and elutriate-hybrids of the killfish Fundulus heteroclitus and cod (Gadus morhua). Similar levels of PAHs and PCBs in zooplankton, but much higher EROD activities in larvae of S. sprattus, were detected in the North Sea (Peters et al., 1992), but the latter could represent a species difference in level of constitutive or inducible cytochrome P4501A activity.  
Correlations between EROD activity, P4501A protein and PAH/PCB levels have been observed in numerous field studies involving many species of adult fish (Goksøyr & Forlin, 1992; Stegeman et al., 1992; Livingstone, 1993). However, in some highly polluted situations induction of P4501A protein has not been accompanied by elevated EROD activity (Fabacher & Baumann, 1985; Lockhart & Menner, 1992), possibly due to inhibition or denaturation of the enzyme by contaminants such as certain PCB congeners (Boon et al., 1992a,b), hepatotoxins (Jimenez et al., 1990; Oikari & Jimenez, 1992), metals such as Cd (Fair, 1986; George, 1989), and organotin (Fent & Stegeman, 1993). The zooplankton body-burden data indicate that interference by PCBs is unlikely to have produced the EROD activity transect profile in S. pilchardus larvae, but other contaminants could have been factors causing inhibition at the in-shore sites, e.g. water-column Cd levels were, respectively, 3.32 and 0.05 μg l⁻¹ at the entrance to and distant from Bilbao harbour (Perez et al., 1987). Unfortunately, despite running larval protein loadings of up to 80 μg protein per lane, cytochrome P4501A protein was either not detectable, or at the limit of detection and not quantifiable, by Western blotting: the positive control (induced S. maximus hepatic microsomes) gave a single band of 58 kDa at a loading of 1 μg protein (data not shown).

Other possibilities which might have affected larval EROD activity to produce the transect profile include a general deterioration in larval condition towards Bilbao (indicated to be unlikely from the antioxidant enzyme data), endogenous factors affecting EROD activity such as temperature (Lange et al., 1992) and development (Peters et al., 1992), and other sources of contaminants such as parental contribution (Westernhagen et al., 1987). Fish larvae are particularly sensitive to food availability, with periods of starvation affecting markers of general larval condition such as RNA/DNA ratios (Clemmesen, 1988). Such nutritional stress could impair detoxication enzyme systems (e.g. CYP1A), thus enhancing contaminant-mediated effects. Laboratory studies to investigate the interaction between nutrition and detoxication should therefore be carried out, e.g. with hatchery-reared turbot (S. maximus) (Peters et al., 1992).

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2.7 SUMMARY OF THE CHAPTER FINDINGS

The results from Chapter 2 indicate the early life stages of *S. maximus* and *S. pilchardus* express proteins with similar catalytic properties (EROD activity) to CYP1A determined in hepatic microsomal preparations of adult fish. Western blot analysis indicated a putative CYP1A protein in larval and juvenile *S. maximus* subcellular fractions. The apparent molecular weight was similar to the immunopositive band detected in hepatic microsomes prepared from *S. maximus* adults injected with a CYP1A inducer (β-naphthoflavone). *S. maximus* larval and juvenile CYP1A levels appeared to be inducible following water-borne exposure to BaP however a field study using *S. pilchardus* larvae did not indicate a correlation between larval EROD activity and determined contaminant loadings in the diet of the larvae. *In vitro* metabolism studies demonstrated that larvae have the potential to metabolise BaP to phenols, diols and quinones and also generate ROS. PAH metabolism may be elevated by larval pre-exposure to CYP1A inducers and the *in vitro* ROS generation could be stimulated by co-incubation with aquatic contaminants. Fish embryos and larvae differentially express a suite of antioxidant enzymes which have the potential to oppose *in vivo* oxidative stress.

To summarise the findings of Chapter 2 following the discussions presented in each paper;

- EROD activity and CYP1A-immunopositive protein can be determined in larval, juvenile and adult life stages of *S. maximus* and may be elevated following exposure to Ah receptor ligands (Section 2.2; 2.3; 2.5).

- The apparent molecular weight of *S. maximus* CYP1A was estimated to be 58 kD via Western blot determination (Section 2.5).
S. maximus larvae metabolised BaP to phenols, dihydrodiols and quinones. Pre-exposure of larvae to BaP caused a 2 fold increased in both BaP metabolism and EROD activity (Section 2.3).

A single exposure to 25 ppb BaP resulted in the formation of hydrophobic DNA adducts in tissue of juvenile S. maximus. Elevation of EROD activity coincided with a decrease in adducts. Basal levels of CYP1A may have been sufficient to bioactivate BaP however contrary to other studies (see Livingstone 1993) the hydrophobic adducts did not persist (Section 2.5).

In vitro studies demonstrated S. maximus larval tissue can generate ROS (including O$_2^{•-}$ and H$_2$O$_2$) and the ROS formation may be stimulated by organic xenobiotics. Fish larvae have the potential to generate ROS in vivo (Section 2.3).

S. maximus embryos and larvae express antioxidant enzymes to metabolise O$_2^{•-}$, detoxify H$_2$O$_2$ and organic hydroperoxides and protect against redox cycling. The enzymes appear to be differentially regulated (Section 2.4).

Antioxidant enzymes and EROD activity were determined in field sampled clupeid larvae S. pilchardus. Enzyme activities were highly variable between sites. Factors other than determined organic hydrocarbon contamination of mixed zooplankton appear to affect S. pilchardus larval enzyme activity (Section 2.6).
CHAPTER 3

**MYTILUS SP. CYTOCHROME P450**

3.1 INTRODUCTION

The multiple forms of CYP catalyse a wide variety of monooxygenation reactions that contribute to cellular oxidative metabolism in both prokaryotes and eukaryotes (Gibson & Skett 1994; Nelson *et al.*, 1996). The products of the CYP super gene family undertake the oxidation of endogenous substrates e.g. fatty acid and steroid hydroxylation however some CYP gene families also demonstrate the ability to catalyse the oxidation of xenobiotics e.g. vertebrate CYP1, CYP2 and CYP3 (Nelson *et al.*, 1996). Regulation of these CYPs may be affected by exposure to xenobiotics and some families e.g. hepatic CYP1 may be induced in organisms exposed to specific aromatic and chlorinated hydrocarbons (Table 1.3). Elevation of CYP1A levels may indicate exposure to these inducers and thus CYP1A has the potential to be exploited as biomarker of exposure to xenobiotics.

Studies have indicated the presence and / or expression of multiple CYP forms in *Mytilus* sp. (Wootton 1995). Using Northern blot techniques, cDNA probes hybridised with CYP1A1-, CYP3A-, CYP4A- and CYP11A-like mRNA from mussel (Wootton *et al.*, 1995; Wootton *et al.*, 1996). Southern blot analysis of genomic DNA presented further evidence for *CYP3A* and *CYP11A*-like genes (Wootton 1995). Seasonal variation of CYP-like mRNA profiles through the year were determined to be CYP family specific indicating
potential differential regulation of mussel CYP genes (Wootton 1995; Wootton et al., 1996). In 1995 Porte et al. published a Western blot demonstrating partially purified *M. edulis* CYP of apparent molecular weight of 48 kD with an epitope recognised by an anti-fish CYP1A antibody. Using this partially purified CYP as a molecular weight marker (positive control) of the digestive gland microsomal CYP-immunopositive proteins, laboratory and field studies were undertaken to examine the CYP1A-like epitope response (Livingstone et al., 1997; Solé et al., 1996; Solé et al., 1997). Under laboratory exposure conditions PCB congener CB-138 and Arochlor 1254 appeared to elevate levels of CYP1A-immunopositive protein (Livingstone et al., 1997) however field studies have reported both good and poor correlations between estimated CYP1A-immunopositive protein and PAH body burden (Solé et al., 1996; Solé et al., 1997).

At least 75 field studies have been undertaken using the induction of fish hepatic CYP1A levels as a biomarker of exposure (Bucheli & Fent 1995; Goksøyr 1995; Livingstone 1993) but less is understood concerning the response of the CYP1A-like gene or protein product in mussel tissue following xenobiotic exposure or endogenous regulation. The objectives of the studies reported in Chapter 3 were to undertake immunochemical investigations of mussel CYP using antibodies to a suite of CYP families and to examine the specificity of the CYP1A response using indigenous and transplanted populations of mussels from reference and polluted sites.

The following 2 papers in Section 3.2 and 3.3 examine the interactions between anti-CYP polyclonal antibodies and either partially purified *M. edulis* P450 or digestive gland microsomal preparations from *M. galloprovincialis* sampled from the Venice Lagoon, Italy. Section 3.2 is an invited paper “in press” in a special edition of the journal Comparative Biochemistry and Physiology. Section 3.3 is a paper also “in press” in a special edition of Marine Environmental Research following the 9th meeting of “Pollution Responses In
Marine Organisms", Bergen, Norway, 1997. The references for each paper appear following the text in the same section and may not be included in Chapter 6. The short papers submitted to the special edition of Marine Environmental Research (1000 words) are permitted to include data that may be published in full in other journals and as such Figure 5 of the paper in Section 3.2 is repeated as Figure 1 of the paper in Section 3.3.
3.2 IMMUNOCHEMICAL INVESTIGATIONS OF CYTOCHROME P450 FORMS/EPITOPES (CYP1A, 2B, 2E, 3A & 4A) IN DIGESTIVE GLAND OF MYTILUS SP.
Immunochemical investigations of cytochrome P450 forms/epitopes
(CYP1A, 2B, 2E, 3A & 4A) in digestive gland of *Mytilus* sp.

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Running title: Cytochrome P450 isoforms in mussel

*Abbreviations:* App. mol. wt., apparent molecular weight; CYP, cytochrome P450; MFO, mixed-function oxygenase; PCB, polychlorobiphenyl; PAH, polycyclic aromatic hydrocarbon; RT-PCR, reverse transcriptase polymerase chain reaction.

Key words: CYP forms, cytochrome P450, digestive gland, immunochemical analysis, mussel, *Mytilus edulis, Mytilus galloprovincialis*, Venice Lagoon
Western blot analysis of microsomes and partially purified cytochrome P450 (CYP) from digestive gland of *Mytilus edulis* was carried out using polyclonal antibodies to hepatic *Perca fluviatilis* CYP1A, *Oncorhynchus mykiss* CYP3A and rat CYP2B, CYP2E and CYP4A isoforms. Multiple CYP bands were detected in partially purified CYP compared to single bands for microsomes for anti-CYP1A, anti-CYP2B, anti-CYP2E and anti-CYP3A. In contrast, anti-CYP4A showed two distinct bands for both. The apparent molecular weights in kD (mean ± range or SD; n = 2-4) for partially purified CYP were 42.5 ± 0.5 and 48.1 ± 0.3 (2 bands, anti-CYP1A); 67.4 ± 0.7, 52.8 ± 0.6, 44.5 ± 2.5 (3 bands, anti-CYP3A); 52.8 ± 0.7, 48.1 ± 1.1 and 43.9 ± 1.1 (3 bands, anti-CYP2B); 52.7 ± 0.8 and 47.2 ± 0.2 (2 bands, anti-CYP2E); 50.9 ± 0.3 and 44.1 ± 0.2 kD (2 bands, anti-CYP4A). Digestive gland microsomes of *Mytilus galloprovincialis* from a polluted compared to a clean field site showed higher levels of bands recognised by anti-CYP1A, anti-CYP2E and anti-CYP4A, but not anti-CYP2B and anti-CYP3A (P < 0.05), indicative of independent regulation of different CYP forms. Overall, the apparent molecular weight and field studies indicate at least five different digestive gland CYP forms.
INTRODUCTION

The cytochrome P450 (CYP)-dependent monooxygenase or mixed function oxygenase (MFO) system, or its components, have been identified in at least 21 species of mollusc (15, 4) performing a range of in vitro catalytic activities including; benzo[a]pyrene hydroxylation, N,N-dimethylaniline and aminopyrene N-demethylation, aldrin expoxidation, 7-ethoxycoumarin O-deethylation (see 15), 7-ethoxyresorufin O-deethylation (4, 38,) and testosterone 6-β and arachidonic acid hydroxylation (12). The association of a particular CYP to a specific catalytic activity may be speculative since homologous CYPs of related species may catalyse different reactions (33). However, the diverse nature of the MFO activities described above would at least indicate multiple CYPs, or unusually a single molluscan CYP with uniquely broad-substrate specificity.

There are currently 481 CYP gene sequences identified that may be subdivided into 74 gene families (25), five of which (CYP1, 2, 11, 17 and 19) have been cloned from fish (33). To date only two aquatic invertebrate CYPs have been completely sequenced, namely CYP2L from the crustacean spiny lobster Panulirus argus (10, 11) and CYP10 from the molluscan pond snail Lymnaea stagnalis (34). Studies using Northern, Southern and Western blot techniques indicate that multiple CYPs are expressed in digestive gland of the bivalve Mytilus sp. (16). A polyclonal antibody raised against perch (Perca fluviatilis) hepatic CYP1A immunoreacted with a CYP1A-like epitope in a partially purified preparation of M. edulis CYP (28). The epitope was also detected in digestive gland microsomal preparations from M. edulis (30) and Mytilus galloprovincialis (20). Other evidence for the expression of multiple CYP genes in digestive gland is provided by Northern blot studies of M. edulis digestive gland RNA which showed hybridisation with CYP1A, 3A, 4A1 and 11A cDNA probes (sequences from respectively, rainbow trout Oncorhynchus mykiss, human, rat and human genes) (37). The expression of CYP forms is
indicated to be regulated since a 6-fold variation in CYP1A-like putative mRNA was determined in the digestive gland of *M. galloprovincialis* sampled from 6 sites in the Venice Lagoon (37), and levels of *M. edulis* CYP1A-, CYP3A- and CYP4A-like putative mRNAs varied seasonally (36). The existence of specific or multiple CYP isoforms in digestive gland of *M. edulis* is further supported by reverse transcriptase PCR (RT-PCR) studies using oligonucleotide primers to a conserved region of *O. mykiss* CYP1A1 which produced a single 280 bp band recognised by the *O. mykiss* CYP1A1 cDNA, (36), and Southern analysis of genomic DNA using human CYP3A and CYP11A cDNAs (37).

Western blot immunorecognition analysis has been used in many studies to indicate the presence and induction of different CYPs or CYP-epitopes in fish (8, 24, 33), crustaceans (2) and molluscs (29, 28, 20). Since both microsomes and/or partially purified CYP of digestive gland *M. edulis* and *M. galloprovincialis* demonstrated an epitope to hepatic CYP1A using *P. fluviatilis* anti-CYP1A antibody (28, 20), this study was undertaken to determine if epitopes of other CYPs were also present in *Mytilus* sp., and by comparison of band mobilities or apparent molecular weights (app. mol. wts.), to obtain evidence for multiple CYPs at the protein level. Western blot analysis of digestive gland microsomes and partially purified CYP of *M. edulis* were performed using a range of polyclonal antibodies to hepatic CYPs of major gene subfamilies present in vertebrates, including lower vertebrates such as fish (33, 25), viz. CYP1A (*P. fluviatilis*), CYP3A (*O. mykiss*) and CYP2B, 2E and 4A (rat). Additionally, in order to examine the variation in the presence of different CYP forms/epitopes in digestive gland, microsomes were compared from two populations of *M. galloprovincialis* from clean and polluted environments in Venice Lagoon, Italy. The latter site contained high levels of contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs), which are indicated (30) or have been shown (3, 20) to elevate levels of CYP1A-immunopositive protein in
digestive gland microsomes of *Mytilus* sp. The results are discussed in relation to the existence and regulation of multiple, specific forms of CYP in *Mytilus* sp.

**MATERIALS AND METHODS**

**Chemicals**

Tris[hydroxymethyl]aminomethane (TRIS), dithiothreitol (DTT), sodium cholate, polyethylene glycol (MW 8 000), reduced glutathione (GSH), secondary anti-IgG (whole molecule) alkaline phosphatase conjugate antibodies and all other biochemicals were from Sigma Chemical Co. Ltd, UK. Glycerol, tetrasodium ethylenediaminetetraacetate (EDTA), sucrose, KCl, K2HPO4 and KH2PO4 were AnalR grade from Merck Ltd. (Leicestershire, UK). Octyl-sepharose was from Pharmacia-LKB Ltd., UK and nitrocellulose from Amersham International plc., UK. See Table 1 for the sources and details of the primary antibodies.

**Mussel sampling and microsomal preparation**

*M. edulis* (shell length 4-5 cm) were collected from a clean site, Whitsand Bay, Cornwall, UK, approximately 4 months after their spawning period. *M. galloprovincialis* (4-6 cm) were collected from clean (Punta Lido) and urban-polluted (Salute) sites in the Venice Lagoon, Italy (27). The latter site has been shown to have elevated levels of PCBs, PAHs and other contaminants in, viz. PCBs (sum of 8 congeners) were 7.2 times higher and PAH levels (sum of 12 individual forms) were 4.8 times higher in *M. galloprovincialis* digestive gland tissue sampled from Salute compared to Lido (Lio Grande) (6). Digestive glands were dissected, damped dry with a paper tissue and the crystalline style removed. Digestive glands were frozen in liquid nitrogen and stored at -70°C for up to 1 month prior to microsomal preparation or P450 purification. Microsomes were prepared by the method of
Livingstone (14). Aliquots of digestive gland were weighed and homogenised on ice (1:4 w/v) in 20 mM TRIS-HCl pH 7.6, 0.5 M sucrose, 0.15 M KCl. Homogenates were centrifuged (4°C) at 500g for 20 minutes and the supernatant centrifuged at 10 000g for 45 minutes. The floating lipid layer was discarded and the supernatant was centrifuged at 100 000g for 90 minutes. The pellet was re-suspended in homogenisation buffer (see above) and then centrifuged at 100 000g for 30 minutes. The washed microsomal pellet was re-suspended in 20 mM TRIS-HCl pH 7.6, 20% glycerol (w/v) to give a protein concentration of about 8 mg ml⁻¹. Total protein was measured by the method of Lowry et al. (21).

**CYP partial purification**

The partial purification of *M. edulis* CYP was undertaken following the method of Porte et al. (28). All procedures were undertaken at 4°C. Approximately 80g of digestive gland was homogenised (1:4 w/v) in 20 mM TRIS-HCl pH 7.6, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA, 1 mM DTT in a chilled stainless steel blender. The homogenate was centrifuged at 500g for 15 minutes and then the supernatant was centrifuged at 12 000g for 45 minutes. The floating lipid layer was removed and the supernatant was centrifuged at 100 000g for 1 hour 45 minutes. The microsomal pellet was re-suspended in 40 ml of 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol (w/v). Microsomes were solubilised with 1.3% sodium cholate and 0.1% GSH for 1 hour in a N₂ saturated atmosphere. The solubilised microsomes were centrifuged at 100 000g for 50 minutes and the supernatant was frozen overnight at -70°C. The supernatant was thawed and stirred in a N₂ saturated atmosphere at 4°C with 4% polyethylene glycol (v/v) for 30 minutes and then centrifuged at 100 000g for 20 minutes. The supernatant was then stirred with 11% polyethylene glycol (v/v) as described above and centrifuged at 70,000g for 20 minutes. The pellet was re-suspended in 20 ml of 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol (w/v), 0.05% Na cholate (w/v) and loaded on to an octyl-Sepharose affinity chromatography column (200 x 15 mm) pre-equilibrated with 10 mM
K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol (w/v), 0.05% Na cholate (w/v). Following approximately 2 column volumes washing of the column to remove unbound protein, CYP was eluted by the equilibration buffer containing 0.2% Emulgen 911 (w/v). Eluted fractions containing haemoprotein (measured by absorbance at 417 nm) were pooled and concentrated by ultrafiltration (mol. wt. cut-off > 30 kDa; Amicon, UK) to yield a CYP specific content of 80 - 100 pmol mg$^{-1}$ protein compared to 30 pmol mg$^{-1}$ for crude microsomes. The partially purified CYP preparation was stored frozen in aliquots at -70 °C.

**Western blot analysis**

Microsomal and partially purified CYP preparations were boiled with an equal volume of 0.125 M TRIS-HCl pH 6.8, 4% sodium dodecyl sulphate (SDS-w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 0.001% bromophenol blue (w/v) for 2-3 minutes. Aliquots of partially purified CYP protein (8-40 µg) and microsomal protein (16-40 µg) were loaded on 10% SDS gels and electrophoresis was performed by the method of Laemmli (13). Gels were semi-dry blotted on to nitrocellulose following the method of Towbin et al. (35). Blots were washed with a solution of 0.5% gelatin (w/v), 0.2% Tween 20 (v/v), 0.1% sodium azide (w/v), 10 mM TRIS-HCl pH 8.0, 0.15 M NaCl for 30 minutes. They were then incubated with primary antibody (see Table I) in the above buffer for 12-14 hours overnight at room temperature. After washing with a solution of 0.2 % Tween 20 (v/v), 10 mM TRIS-HCl pH 8.0, 0.15 M NaCl, the blots were incubated with alkaline phosphatase-conjugate secondary antibody for 1 hour (see Table I). Finally, the nitrocellulose was washed as above and the blots visualised by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly1 phosphate (both 0.2 mM) in 100 mM TRIS-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl$_2$. Samples were semi-quantified by image analysis using a Kontron image analyzer (Image Processing System, UK) and results expressed in arbitrary units. The relationship between protein amount and immunorecognition (semi-quantified by
image analysis) was determined to be linear for proteins electrophoresed in this study. Apparent molecular weights of immunopositive bands were determined from simultaneously run pre-stained molecular weight markers.
RESULTS

Western blot analysis of *M. edulis* digestive gland microsomes and partially purified CYP

Representative Western blots of microsomes or partially purified CYP from *M. edulis* digestive gland incubated with the various antibodies and visualised by alkaline phosphatase-conjugated secondary antibodies are shown in Figures 1 (anti-CYP1A, anti-CYP4A) and 2 (anti-CYP3A, anti-CYP2B, anti-CYP2E). The app. mol. wts. of the discrete bands (determined by comparison with pre-stained mol. wt. markers) of partially purified CYP and the relative staining intensities of the bands are summarised in respectively Figures 3 (values are means ± range, n = 2 or SD n = 4) and 4 (typical pattern shown). Anti-CYP1A recognised two bands in partially purified CYP (Fig. 1) of app. mol. wts. 48.1 ± 0.3 and 42.5 ± 0.5 kD (Fig. 3). The relative degree of staining of the 48.1 kD band was generally about 6-fold higher than the 42.5 kD band (Fig. 4). In contrast, reaction with anti-CYP1A revealed one band only in microsomes (Fig. 1) of approximate app. mol. wt. 48 kD (Fig. 3). Incubation with anti-CYP4A recognised two bands in partially purified CYP (Fig. 1) of app. mol. wts. 50.9 ± 0.3 and 44.1 ± 0.2 kD (Fig. 3). Microsomal preparations also indicated the presence of two immunopositive bands in the range of 44 to 51 kD; however they were not easily distinguishable in the photographs of the Western blot (Fig. 1). The relative staining of the two anti-CYP4A bands in partially purified CYP was typically of equal intensity (Fig. 4).

Partially purified CYP demonstrated at least three bands when incubated with anti-CYP3A antibody (Fig. 2), the lower mol. wte. band showed considerable variation in app. mol. wt. (44.5 ± 2.5 kD) for replicate analyses of the same preparation whereas this was not observed for the higher molecular weight (52.8 ± 0.6 and 67.4 ± 0.7) bands (Fig. 3). A fourth band of higher app. mol. wt. to CYP (c90 kD) was also observed. In contrast, the
presence of a single anti-CYP3A band of variable app. mol. wt. (44 to 52 kD) was determined in microsomes (Fig. 2). Both anti-CYP2B and CYP2E recognised single immunopositive bands of similar 51 kD app. mol. wt. in microsomes (Fig. 2). Similarly, both showed two major bands in partially purified CYP of similar app. mol. wt., viz. 52.8 ± 0.7 and 48.1 ± 1.1 kD (anti-CYP2B) and 52.7 ± 0.8 and 47.2 ± 0.2 kD (anti-CYP2E); a third faint band of 43.9 ± 1.1 kD was also seen for anti-CYP2B (Figs. 2 & 4). The staining with low molecular weight proteins (app. mol. wt. < 28 kD) (see Figs. 1 and 2) was shown to be due to non-specific binding of the secondary antibody, i.e. visualisation of bands was seen in the absence of the primary anti-CYP antibodies; in contrast, no cross-reactivity between secondary antibody and high molecular weight bands was ever observed (data not shown).

**Western blot analysis of M. galloprovincialis digestive gland microsomes from two populations**

The immunorecognition of anti-CYP1A, anti-CYP4A, anti-CYP2B, anti-CYP2E and anti-CYP3A antibodies against *M. galloprovincialis* digestive gland microsomes from two populations in Venice Lagoon, Italy was investigated. Similar qualitative band patterns were obtained for both populations of *M. galloprovincialis* (data not shown) as for digestive gland microsomes of *M. edulis* (see Figs. 1 and 2). The bands corresponding to the major bands in partially purified CYP of *M. edulis* digestive gland were quantified for both populations and compared. Levels of immunopositive proteins detected by anti-CYP1A (48 kD band), anti-CYP4A (51 and 44 kD bands) and anti-CYP2E (53 kD bands) were higher in *M. galloprovincialis* from Salute (polluted site) compared to Punta Lido (relatively clean site); however, no differences between populations were seen for immunopositive proteins detected by anti-CYP2B (53 kD) and anti-CYP3A (45 kD) (Fig. 5).
Western blot analysis of digestive gland microsomes and partially purified CYP from *M. edulis* and digestive gland microsomes from *M. galloprovincialis* were performed using polyclonal antibodies to CYPs from fish and rat liver representing five major CYP subfamilies (1A, 2B, 2E, 3A, 4A) (25). Immunopositive bands were detected in both *Mytilus* sp. for all antibodies examined, indicating the presence of epitopes or sequences from the respective CYP subfamilies, although clearly the indicated (sequence) similarity may not be sufficient to classify the protein as a member of that subfamily. The degree of immunorecognition of the bands in partially purified CYP of *M. edulis* digestive gland varied 11-fold between the different antibodies (calculated from Fig. 4), presumably reflecting both differences in epitope similarity/antibody recognition and the relative amounts of the different CYPs either in the original microsomes, or altered by the partial purification procedure. Incubation with anti-CYP1A, anti-CYP2B, anti-CYP2E or anti-CYP3A antibodies resolved two or three bands in partially purified CYP, whereas only single bands were identified in the microsomes, possibly indicating better electrophoretic separation of CYPs in the former compared to the more complex mixture of proteins present in microsomes. Although the difference in CYP specific content between microsomes and the partially purified enzyme was not great (i.e. purification factor of about 3), the limited purification procedure did result in much clearer Western blots for partially purified CYP, including removal of the non-specific 'immunopositive' low molecular weight bands. The app. mol. wts. of all the CYP immunopositive bands ranged from approximately 42 to 53 kD and were typical of CYPs in general (25), including Western blot analysis estimates of CYPs in crustaceans (30 - 52 kD) (2) and anthazoans (~41 kD) (9).
The results with anti-CYP1A are consistent with previous molecular biological and immunochemical molluscan studies which indicate the existence of a CYP isoform with sequence similarities to CYP1A in digestive gland of *Mytilus* sp. (see Introduction) and other molluscs (29). This includes detection of a 280 bp fragment produced by RT-PCR from digestive gland RNA of *M*. edulis, using primers to amplify a sequence around the haem binding site of *O*. *mykiss* CYP1A1 (36). CYP1A epitopes have also been indicated in other marine invertebrate groups by Western blot analysis using antibodies to hepatic CYP1A of several fish species (app. mol. wts. in parenthesis), viz. in digestive gland of the chiton *Cryptochiton stelleri* (60 kD) (29), pyloric caeca of the starfish *Asterias rubens* (I) and column or whole body of five sea anemone species (~70 kD) (9). In contrast, no CYP1A-immunopositive bands were detected in digestive gland of the bivalve *Donax trunculus* or gastropod *Patellan caerulea* (38).

Whereas the CYP2 family and its subfamilies, particularly CYP2B, are highly diversified and present in many isoforms in terrestrial mammals, much less is known of their existence in lower vertebrates and marine invertebrates (25). Gene sequences of seven CYP2 forms from 4 subfamilies (CYP2K, CYP2L, CYP2N, CYP2P) have been determined in two species of fish, namely *O*. *mykiss* and/or the killifish *Fundulus heteroclitus* (25), and one form (CYP2L1) in the crustacean *P*. *argus* (2). Other CYP subfamilies (CYP2B, CYP2C, CYP2D, CYP2F) have also been indicated in *F*. *heteroclitus* (26). However, little is known of the presence of CYP2 subfamilies in *Mytilus* sp. No hybridisation was observed when Northern blots of RNA from *M*. *edulis* digestive gland were incubated with mammalian cDNA probes to CYP2E (32) or CYP2B (31). However, in contrast, in this study both rat anti-CYP2B and anti-CYP2E clearly recognised multiple epitopes in *M*. *edulis* and *M*. *galloprovincialis* digestive gland preparations, with similar app. mol. wts. for the two antibodies for two of the recognised bands (48 and 53 kD). This compares with
immunorecognition antibody studies on other marine invertebrates which detected epitopes with scup (*Stenotomus chrysops*) hepatic anti-CYP2B in pyloric caeca of *A. rubens* (~ 46 kD)-(1); with *O. mykiss* hepatic anti-CYP2K1 in digestive gland of *C. stelleri* (54 and 60 kDa; NB trivial name for *O. mykiss* CYP2K1 was P450 LM2) (29) and whole body or column of five sea anemone species (~ 40 kD) (9); and with *P. argus* hepatopancreas anti-CYP2L in hepatopancreas of closely related slipper lobster (*Scyllarides nodifer*) (52.5 kD) (2).

Previous studies have indicated the catalytic activity and expression of CYP3A and CYP4A isoforms in digestive gland of *Mytilus* sp. Testosterone 6-β hydroxylation and arachidonic acid hydroxylation are catalysed by mammalian CYP3A and CYP4A respectively and both activities have been detected in *M. edulis* digestive gland microsomes (12). Lauric acid hydroxylase activity, which is also catalysed by mammalian CYP4A, has been measured in digestive gland microsomes of *M. galloprovincialis* (22). Hybridisation of human CYP3A and rat CYP4A cDNA probes by Northern or Southern analysis has been seen with RNA and DNA (CYP3A only) from *Mytilus* sp digestive gland (37, 36). Consistent with all these findings is the demonstration by Western blot analysis of epitopes of both CYP3A and CYP4A in digestive gland microsomes and partially purified CYP of *Mytilus* sp.. Multiple forms of CYP3A are known in mammals, and studies with hepatic anti-CYP3A of *S. chrysops* and *O. mykiss* have shown immunopositive proteins in respectively pyloric caeca of *A. rubens* ~ 53 kD) (1) and whole body or column of five sea anemone species (~ 30 kD) (9). Multiple CYP4A isoforms are also known to exist in organisms (25), e.g. four isoforms of CYP4A (CYP4A1, CYP4A2, CYP4A3 and CYP4A8) are present in rat (25). Consistent with this, two immunopositive bands were detected with rat anti-CYP4A in both microsomes and partially purified CYP of *Mytilus* sp. digestive gland. The antibody to hepatic CYP4A of rat used in this study is known to recognise principally CYP4A1, but also to show a much reduced recognition of the
INTRODUCTION

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Oncorhynchus mykiss, human, rat and human genes) (37). The expression of CYP forms is indicated to be regulated since a 6-fold variation in CYP1A-like putative mRNA was determined in the digestive gland of M. galloprovincialis sampled from 6 sites in the Venice Lagoon (37), and levels of M. edulis CYP1A-, CYP3A- and CYP4A-like putative mRNAs varied seasonally (36). The existence of specific or multiple CYP isoforms in digestive gland of M. edulis is further supported by reverse transcriptase PCR (RT-PCR) studies using oligonucleotide primers to a conserved region of O. mykiss CYP1A1 which produced a single 280 bp band recognised by the O. mykiss CYP1A1 cDNA, (36), and Southern analysis of genomic DNA using human CYP3A and CYP11A cDNAs (37).

Western blot immunorecognition analysis has been used in many studies to indicate the presence and induction of different CYPs or CYP-epitopes in fish (8, 24, 33), crustaceans (2) and molluscs (29, 28, 20). Since both microsomes and/or partially purified CYP of digestive gland M. edulis and M. galloprovincialis demonstrated an epitope to hepatic CYP1A using P. fluviatilis anti-CYP1A antibody (28, 20), this study was undertaken to determine if epitopes of other CYPs were also present in Mytilus sp., and by comparison of band mobilities or apparent molecular weights (app. mol. wts.), to obtain evidence for multiple CYPs at the protein level. Western blot analysis of digestive gland microsomes and partially purified CYP of M. edulis were performed using a range of polyclonal antibodies to hepatic CYPs of major gene subfamilies present in vertebrates, including lower vertebrates such as fish (33, 25), viz. CYP1A (P. fluviatilis), CYP3A (O. mykiss) and CYP2B, 2E and 4A (rat). Additionally, in order to examine the variation in the presence of different CYP forms/epitopes in digestive gland, microsomes were compared from two populations of M. galloprovincialis from clean and polluted environments in Venice Lagoon, Italy. The latter site contained high levels of contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorobiphenyls (PCBs), which are indicated (30)
or have been shown (3, 20) to elevate levels of CYP1A-immunopositive protein in digestive gland microsomes of *Mytilus* sp. The results are discussed in relation to the existence and regulation of multiple, specific forms of CYP in *Mytilus* sp.

MATERIALS AND METHODS

**Chemicals**

Tris[hydroxymethyl]aminomethane (TRIS), dithiothreitol (DTT), sodium cholate, polyethylene glycol (MW 8 000), reduced glutathione (GSH), secondary anti-IgG (whole molecule) alkaline phosphatase conjugate antibodies and all other biochemicals were from Sigma Chemical Co. Ltd, UK. Glycerol, tetrasodium ethylenediaminetetraacetate (EDTA), sucrose, KCl, K2HPO4 and KH2PO4 were AnalR grade from Merck Ltd. (Leicestershire, UK). Octyl-sepharose was from Pharmacia-LKB Ltd., UK and nitrocellulose from Amersham International plc., UK. See Table 1 for the sources and details of the primary antibodies.

**Mussel sampling and microsomal preparation**

*M. edulis* (shell length 4-5 cm) were collected from a clean site, Whitsand Bay, Cornwall, UK, approximately 4 months after their spawning period. *M. galloprovincialis* (4-6 cm) were collected from clean (Punta Lido) and urban-polluted (Salute) sites in the Venice Lagoon, Italy (27). The latter site has been shown to have elevated levels of PCBs, PAHs and other contaminants in, viz. PCBs (sum of 8 congeners) were 7.2 times higher and PAH levels (sum of 12 individual forms) were 4.8 times higher in *M. galloprovincialis* digestive gland tissue sampled from Salute compared to Lido (Lio Grande) (6). Digestive glands were dissected, damped dry with a paper tissue and the crystalline style removed. Digestive
glands were frozen in liquid nitrogen and stored at -70°C for up to 1 month prior to microsomal preparation or P450 purification. Microsomes were prepared by the method of Livingstone (14). Aliquots of digestive gland were weighed and homogenised on ice (1:4 w/v) in 20 mM TRIS-HCl pH 7.6, 0.5 M sucrose, 0.15 M KCl. Homogenates were centrifuged (4°C) at 500g for 20 minutes and the supernatant centrifuged at 10 000g for 45 minutes. The floating lipid layer was discarded and the supernatant was centrifuged at 100 000g for 90 minutes. The pellet was re-suspended in homogenisation buffer (see above) and then centrifuged at 100 000g for 30 minutes. The washed microsomal pellet was re-suspended in 20 mM TRIS-HCl pH 7.6, 20% glycerol (w/v) to give a protein concentration of about 8 mg ml⁻¹. Total protein was measured by the method of Lowry et al. (21).

**CYP partial purification**

The partial purification of *M. edulis* CYP was undertaken following the method of Porte et al. (28). All procedures were undertaken at 4°C. Approximately 80g of digestive gland was homogenised (1:4 w/v) in 20 mM TRIS-HCl pH 7.6, 0.5 M sucrose, 0.15 M KCl, 1 mM EDTA, 1 mM DTT in a chilled stainless steel blender. The homogenate was centrifuged at 500g for 15 minutes and then the supernatant was centrifuged at 12 000g for 45 minutes. The floating lipid layer was removed and the supernatant was centrifuged at 100 000g for 1 hour 45 minutes. The microsomal pellet was re-suspended in 40 ml of 10 mM K₂HPO₄/KH₂PO₄ pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol (w/v). Microsomes were solubilised with 1.3% sodium cholate and 0.1% GSH for 1 hour in a N₂ saturated atmosphere. The solubilised microsomes were centrifuged at 100 000g for 50 minutes and the supernatant was frozen overnight at -70°C. The supernatant was thawed and stirred in a N₂ saturated atmosphere at 4°C with 4% polyethylene glycol (v/v) for 30 minutes and then centrifuged at 100 000g for 20 minutes. The supernatant was then stirred with 11% polyethylene glycol (v/v) as described above and centrifuged at 70,000g for 20 minutes.
The pellet was re-suspended in 20 ml of 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol (w/v), 0.05% Na cholate (w/v) and loaded on to an octyl-Sepharose affinity chromatography column (200 x 15 mm) pre-equilibrated with 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$ pH 7.6, 0.1 mM EDTA, 0.1 mM DTT, 20% glycerol (w/v), 0.05% Na cholate (w/v). Following approximately 2 column volumes washing of the column to remove unbound protein, CYP was eluted by the equilibration buffer containing 0.2% Emulgen 911 (w/v). Eluted fractions containing haemoprotein (measured by absorbance at 417 nm) were pooled and concentrated by ultrafiltration (mol. wt. cut-off > 30 kDa; Amicon, UK) to yield a CYP specific content of 80 - 100 pmol mg$^{-1}$ protein compared to 30 pmol mg$^{-1}$ for crude microsomes. The partially purified CYP preparation was stored frozen in aliquots at -70 °C.

**Western blot analysis**

Microsomal and partially purified CYP preparations were boiled with an equal volume of 0.125 M TRIS-HCl pH 6.8, 4% sodium dodecyl sulphate (SDS-w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 0.001% bromophenol blue (w/v) for 2-3 minutes. Aliquots of partially purified CYP protein (8-40 µg) and microsomal protein (16-40 µg) were loaded on 10% SDS gels and electrophoresis was performed by the method of Laemmli (13). Gels were semi-dry blotted on to nitrocellulose following the method of Towbin et al. (35). Blots were washed with a solution of 0.5% gelatin (w/v), 0.2% Tween 20 (v/v), 0.1% sodium azide (w/v), 10 mM TRIS-HCl pH 8.0, 0.15 M NaCl for 30 minutes. They were then incubated with primary antibody (see Table 1) in the above buffer for 12-14 hours overnight at room temperature. After washing with a solution of 0.2 % Tween 20 (v/v), 10 mM TRIS-HCl pH 8.0, 0.15 M NaCl, the blots were incubated with alkaline phosphatase-conjugate secondary antibody for 1 hour (see Table 1). Finally, the nitrocellulose was washed as above and the blots visualised by the addition of nitroblue tetrazolium and 5-
bromo-4-chloro-3-indolyl phosphate (both 0.2 mM) in 100 mM TRIS-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂. Samples were semi-quantified by image analysis using a Kontron image analyzer (Image Processing System, UK) and results expressed in arbitrary units. The relationship between protein amount and immunorecognition (semi-quantified by image analysis) was determined to be linear for proteins electrophoresed in this study. Apparent molecular weights of immunopositive bands were determined from simultaneously run pre-stained molecular weight markers.
RESULTS

_Western blot analysis of M. edulis digestive gland microsomes and partially purified CYP_

Representative Western blots of microsomes or partially purified CYP from _M. edulis_ digestive gland incubated with the various antibodies and visualised by alkaline phosphatase-conjugated secondary antibodies are shown in Figures 1 (anti-CYP1A, anti-CYP4A) and 2 (anti-CYP3A, anti-CYP2B, anti-CYP2E). The app. mol. wts. of the discrete bands (determined by comparison with pre-stained mol. wt. markers) of partially purified CYP and the relative staining intensities of the bands are summarised in respectively Figures 3 (values are means ± range, n = 2 or SD n = 4) and 4 (typical pattern shown). Anti-CYP1A recognised two bands in partially purified CYP (Fig. 1) of app. mol. wts. 48.1 ± 0.3 and 42.5 ± 0.5 kD (Fig. 3). The relative degree of staining of the 48.1 kD band was generally about 6-fold higher than the 42.5 kD band (Fig. 4). In contrast, reaction with anti-CYP1A revealed one band only in microsomes (Fig. 1) of approximate app. mol. wt. 48 kD (Fig. 3). Incubation with anti-CYP4A recognised two bands in partially purified CYP (Fig. 1) of app. mol. wts. 50.9 ± 0.3 and 44.1 ± 0.2 kD (Fig. 3). Microsomal preparations also indicated the presence of two immunopositive bands in the range of 44 to 51 kD; however they were not easily distinguishable in the photographs of the Western blot (Fig. 1). The relative staining of the two anti-CYP4A bands in partially purified CYP was typically of equal intensity (Fig. 4).

Partially purified CYP demonstrated at least three bands when incubated with anti-CYP3A antibody (Fig. 2), the lower mol. wte. band showed considerable variation in app. mol. wt. (44.5 ± 2.5 kD) for replicate analyses of the same preparation whereas this was not observed for the higher molecular weight (52.8 ± 0.6 and 67.4 ± 0.7) bands (Fig. 3). A
fourth band of higher app. mol. wt. to CYP (c90 kD) was also observed. In contrast, the presence of a single anti-CYP3A band of variable app. mol. wt. (44 to 52 kD) was determined in microsomes (Fig. 2). Both anti-CYP2B and CYP2E recognised single immunopositive bands of similar 51 kD app. mol. wt. in microsomes (Fig. 2). Similarly, both showed two major bands in partially purified CYP of similar app. mol. wt., viz. 52.8 ± 0.7 and 48.1 ± 1.1 kD (anti-CYP2B) and 52.7 ± 0.8 and 47.2 ± 0.2 kD (anti-CYP2E); a third faint band of 43.9 ± 1.1 kD was also seen for anti-CYP2B (Figs. 2 & 4). The staining with low molecular weight proteins (app. mol. wt. < 28 kD) (see Figs. 1 and 2) was shown to be due to non-specific binding of the secondary antibody, i.e. visualisation of bands was seen in the absence of the primary anti-CYP antibodies; in contrast, no cross-reactivity between secondary antibody and high molecular weight bands was ever observed (data not shown).

Western blot analysis of M. galloprovincialis digestive gland microsomes from two populations

The immunorecognition of anti-CYP1A, anti-CYP4A, anti-CYP2B, anti-CYP2E and anti-CYP3A antibodies against M. galloprovincialis digestive gland microsomes from two populations in Venice Lagoon, Italy was investigated. Similar qualitative band patterns were obtained for both populations of M. galloprovincialis (data not shown) as for digestive gland microsomes of M. edulis (see Figs. 1 and 2). The bands corresponding to the major bands in partially purified CYP of M. edulis digestive gland were quantified for both populations and compared. Levels of immunopositive proteins detected by anti-CYP1A (48 kD band), anti-CYP4A (51 and 44 kD bands) and anti-CYP2E (53 kD bands) were higher in M. galloprovincialis from Salute (polluted site) compared to Punta Lido (relatively clean site); however, no differences between populations were seen for
immunopositive proteins detected by anti-CYP2B (53 kD) and anti-CYP3A (45 kD) (Fig. 5).

DISCUSSION

Western blot analysis of digestive gland microsomes and partially purified CYP from M. edulis and digestive gland microsomes from M. galloprovincialis were performed using polyclonal antibodies to CYPs from fish and rat liver representing five major CYP subfamilies (1A, 2B, 2E, 3A, 4A) (25). Immunopositive bands were detected in both Mytilus sp. for all antibodies examined, indicating the presence of epitopes or sequences from the respective CYP subfamilies, although clearly the indicated (sequence) similarity may not be sufficient to classify the protein as a member of that subfamily. The degree of immunorecognition of the bands in partially purified CYP of M. edulis digestive gland varied 11-fold between the different antibodies (calculated from Fig. 4), presumably reflecting both differences in epitope similarity/antibody recognition and the relative amounts of the different CYPs either in the original microsomes, or altered by the partial purification procedure. Incubation with anti-CYP1A, anti-CYP2B, anti-CYP2E or anti-CYP3A antibodies resolved two or three bands in partially purified CYP, whereas only single bands were identified in the microsomes, possibly indicating better electrophoretic separation of CYPs in the former compared to the more complex mixture of proteins present in microsomes. Although the difference in CYP specific content between microsomes and the partially purified enzyme was not great (i.e. purification factor of about 3), the limited purification procedure did result in much clearer Western blots for partially purified CYP, including removal of the non-specific ‘immunopositive’ low molecular weight bands. The app. mol. wts. of all the CYP immunopositive bands ranged
from approximately 42 to 53 kD and were typical of CYPs in general (25), including Western blot analysis estimates of CYPs in crustaceans (30 - 52 kD) (2) and anthozoans (~ 41 kD) (9).

The results with anti-CYP1A are consistent with previous molecular biological and immunochemical molluscan studies which indicate the existence of a CYP isoform with sequence similarities to CYP1A in digestive gland of *Mytilus* sp. (see Introduction) and other molluscs (29). This includes detection of a 280 bp fragment produced by RT-PCR from digestive gland RNA of *M. edulis*, using primers to amplify a sequence around the haem binding site of *O. mykiss* CYP1A1 (36). CYP1A epitopes have also been indicated in other marine invertebrate groups by Western blot analysis using antibodies to hepatic CYP1A of several fish species (app. mol. wts. in parenthesis), viz. in digestive gland of the chiton *Cryptochiton stelleri* (60 kD) (29), pyloric caeca of the starfish *Asterias rubens* (1) and column or whole body of five sea anemone species (~ 70 kD) (9). In contrast, no CYP1A-immunopositive bands were detected in digestive gland of the bivalve *Donax trunculus* or gastropod *Patella caerulea* (38).

Whereas the CYP2 family and its subfamilies, particularly CYP2B, are highly diversified and present in many isoforms in terrestrial mammals, much less is known of their existence in lower vertebrates and marine invertebrates (25). Gene sequences of seven CYP2 forms from 4 subfamilies (CYP2K, CYP2L, CYP2N, CYP2P) have been determined in two species of fish, namely *O. mykiss* and/or the killifish *Fundulus heteroclitus* (25), and one form (CYP2L1) in the crustacean *P. argus* (2). Other CYP subfamilies (CYP2B, CYP2C, CYP2D, CYP2F) have also been indicated in *F. heteroclitus* (26). However, little is known of the presence of CYP2 subfamilies in *Mytilus* sp. No hybridisation was observed when Northern blots of RNA from *M. edulis* digestive gland were incubated with mammalian
cDNA probes to CYP2E (32) or CYP2B (31). However, in contrast, in this study both rat anti-CYP2B and anti-CYP2E clearly recognised multiple epitopes in *M. edulis* and *M. galloprovincialis* digestive gland preparations, with similar app. mol. wts. for the two antibodies for two of the recognised bands (48 and 53 kD). This compares with immunorecognition antibody studies on other marine invertebrates which detected epitopes with scup (*Stenotomus chrysops*) hepatic anti-CYP2B in pyloric caeca of *A. rubens* (~ 46 kD) (1); with *O. mykiss* hepatic anti-CYP2K1 in digestive gland of *C. stelleri* (54 and 60 kDa; NB trivial name for *O. mykiss* CYP2K1 was P450 LM2) (29) and whole body or column of five sea anemone species (~ 40 kD) (9); and with *P. argus* hepatopancreas anti-CYP2L in hepatopancreas of closely related slipper lobster (*Scyllarides nodifer*) (52.5 kD) (2).

Previous studies have indicated the catalytic activity and expression of CYP3A and CYP4A isoforms in digestive gland of *Mytilus* sp. Testosterone 6-β hydroxylation and arachidonic acid hydroxylation are catalysed by mammalian CYP3A and CYP4A respectively and both activities have been detected in *M. edulis* digestive gland microsomes (12). Lauric acid hydroxylase activity, which is also catalysed by mammalian CYP4A, has been measured in digestive gland microsomes of *M. galloprovincialis* (22). Hybridisation of human CYP3A and rat CYP4A cDNA probes by Northern or Southern analysis has been seen with RNA and DNA (CYP3A only) from *Mytilus* sp digestive gland (37, 36).

Consistent with all these findings is the demonstration by Western blot analysis of epitopes of both CYP3A and CYP4A in digestive gland microsomes and partially purified CYP of *Mytilus* sp.. Multiple forms of CYP3A are known in mammals, and studies with hepatic anti-CYP3A of *S. chrysops* and *O. mykiss* have shown immunopositive proteins in respectively pyloric caeca of *A. rubens* ~ 53 kD) (1) and whole body or columnn of five sea anemone species (~ 30 kD) (9). Multiple CYP4A isoforms are also known to exist in
CYP4A3 isoform (G. G. Gibson, *pers. comm.*). However, assignment of particular bands to particular CYP forms is not possible given likely species differences in isoform structure and app. mol. wt.

Elevated levels of CYP1A-, CYP4A- (44.1 and 50.9 kD bands) and CYP2E-, but not CYP2B- and CYP3A-, immunopositive bands were seen in digestive gland microsomes of *M. galloprovincialis* from a population containing high levels of PAHs and PCBs compared to a more clean population. These differences could be due to exogenous factors such as exposure to the organic contaminants, or to endogenous factors such as seasonal variation, or for other reasons (18). The 1.5-fold higher level of CYP1A-immunopositive protein in the contaminated population is consistent with previous laboratory and field studies on *Mytilus* sp. and indicative of induction by such classical inducers of CYP1A as certain PAHs and PCBs (25). Thus, 1.6- to 3.5-fold increases in levels of CYP1A-immunopositive protein in digestive gland of *Mytilus* sp. were seen with laboratory exposure to PCBs (20) and field exposure to aliphatic and aromatic hydrocarbons (30), whereas about 4-fold higher levels of putative CYP1A mRNA were seen in digestive gland of *M. galloprovincialis* from an industrial site in Venice Lagoon compared to a cleaner reference site in the Adriatic Sea (19, 17). Little is known of the inducibility of other immunopositive-forms by xenobiotics but contradictory results have been obtained for CYP2B-inducers, an increase in total cytochrome P450 content of *M. galloprovincialis* digestive gland being seen with exposure to sodium phenobarbital by some authors (7) but not others (23). An increase in lauric acid hydroxylase activity of digestive gland microsomes of *M. galloprovincialis* was seen with laboratory exposure to the PAH 3-methylcholanthrene (22), which is consistent with the elevated CYP4A-immunopositive proteins seen in the field study. Endogenous factors could also play a part in the population differences as different patterns of seasonal variation has been seen in levels of putative CYP1A-like, CYP3A-like and CYP4A-like mRNAs in digestive gland of *M. edulis* (36).
Immunochemical cross-reactivity of antibodies to CYPs has been extensively used to study the presence and phylogenetic distribution of CYP isoforms/epitopes in aquatic organisms (8, 33, 2, 9). However, caution is required with interpretation to the number of forms present, because a common epitope may be recognised by antibodies to several CYPs, and a single CYP may have epitopes which may be recognised by a range of antibodies. Thus, for example, considering Figure 3, it is therefore not possible to discern if the CYP1A 42.5 kD, CYP4A 44.1 kD, CYP3A 44.5 kD and CYP2B 43.9 kD bands are four unique proteins CYPs of similar app. mol. wts., or one CYP form with epitopes recognised by the four antibodies. However, considering all the apparent molecular weight data, the most conservative interpretation is that at least four CYP isoforms exist corresponding to the ranges of non-overlapping app. mol. wts., viz. ~ 42 to 47 kD, 47 to 49 kD, 51 to 53.5 kD and 67 kD (see Fig. 3). Considering the *M. galloprovincialis* microsomal field data of Figure 5, at least two CYP forms are indicated, one containing epitopes to anti-CYP1A, anti-CYP4A and anti-CYP2E which was elevated in the Salute population, and one containing epitopes to anti-CYP2B and CYP3A which was not elevated. The field differences thus indicate that the CYP2E- and CYP2B-epitopes discerned in microsomes reside on distinct proteins, and similarly so do the CYP1A- and CYP3A-epitopes. Neither of these distinctions could have been concluded from the app. mol. wt. data of Figure 3. Therefore, combining the immunorecognition results of both Figures 3 and 5, a conservative assessment indicates the presence of at least five CYP forms in digestive gland of *Mytilus* sp..

In conclusion, multiple epitopes recognised by polyclonal antibodies to five major CYP gene subfamilies (CYP1A, CYP2B, CYP2E, CYP3A, CYP4A) have been detected in *M. edulis* digestive gland microsomes and partially purified CYP, and *M. galloprovincialis* digestive gland microsomes. The results indicating CYP1A, CYP3A and CYP4A epitopes are
consistent with previous immunorecognition and/or molecular biological studies of *Mytilus* sp. The differences in epitope profiles between the two populations of *M. galloprovincialis* indicate the independent regulation of different CYP forms, consistent with the observations from seasonal variation of putative CYP mRNAs levels in *M. edulis* (36). Overall, the data indicate at least five CYP forms in digestive gland of *Mytilus* sp., which may be similar to known CYP subfamilies, but more likely could be unique, as has already been found for a number of recently discovered invertebrate CYPs (5, 25).

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Figure 1. Western blots of partially purified CYP (Lanes 1 & 4) and microsomes (Lanes 2 & 3) of digestive gland of *M. edulis* incubated with anti-CYP1A or anti-CYP4A antibody. See Table 1 for source of antibodies. The app. mol. wts. of the bands for partially purified CYP are shown on the figure.

Figure 2. Western blots of partially purified CYP (Lanes 1, 4 & 6) and microsomes (Lanes 2, 3 & 5) of digestive gland of *M. edulis* incubated with either anti-CYP3A, anti-CYP2B or anti-CYP2E antibody. See Table 1 for source of antibodies. The app. mol. wts. of the bands for partially purified CYP are shown on the figure.

Figure 3. App. mol. wts. of immunopositive bands of partially purified CYP of digestive gland of *M. edulis* detected by Western blot analysis with anti-CYP1A, anti-CYP4A, anti-CYP3A, anti-CYP2B or anti-CYP2E antibodies. See Table 1 for source of antibodies. Values were determined from simultaneously run pre-stained molecular weight markers (n = 2-4; mean ± range or SD).

Figure 4. Relative staining intensity of immunopositive bands detected by Western blot analysis of partially purified CYP of digestive gland of *M. edulis* with anti-CYP1A, anti-CYP4A, anti-CYP2B or anti-CYP2E antibody. See Table 1 for source of antibodies. Immunorecognition expressed as arbitrary units from a single representative run.

Figure 5. Levels of immunopositive bands detected by Western blot analysis with anti-CYP1A, anti-CYP4A, anti-CYP2E, anti-CYP2B or anti-CYP3A antibody in digestive gland microsomes of *M. galloprovincialis* from clean (Punta Lido) and chemically contaminated (Salute) sites in Venice Lagoon, Italy. See Table 1 for source of antibodies.
Quantification was performed on bands of similar app. mol. wts. to simultaneously run *M. edulis* digestive gland partially purified CYP. Individual microsomal preparations are from the pooled tissues of 4-6 mussels (mean ± SEM; n = 5). * = P < 0.05 2 tail, t-Test.
TABLE 1. Origins and working dilutions of primary and secondary polyclonal antibodies.

<table>
<thead>
<tr>
<th></th>
<th>CYP1A</th>
<th>CYP4†A</th>
<th>CYP3A</th>
<th>CYP2B</th>
<th>CYP2E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibody</strong></td>
<td>Rabbit anti-<em>P. fluviatilis</em> CYP1A</td>
<td>Sheep anti-rat CYP4A</td>
<td>Rabbit anti-<em>O. mykiss</em> CYP3A</td>
<td>Goat anti-rat CYP2B</td>
<td>Goat anti-rat CYP2E</td>
</tr>
<tr>
<td><strong>Dilution</strong></td>
<td>1:1 000</td>
<td>1:2 000</td>
<td>1:1 000</td>
<td>1:100</td>
<td>1:50</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td>Gift, Dr L Förlin, University of Göteborg, Sweden</td>
<td>Gift, Dr G Gibson, University of Surrey, UK</td>
<td>Gift, Dr L Förlin, University of Göteborg, Sweden</td>
<td>Oxford Biomedical Research Inc., UK</td>
<td>Oxford Biomedical Research Inc., UK</td>
</tr>
<tr>
<td><strong>Secondary antibody</strong> *</td>
<td>Sheep anti-rabbit</td>
<td>Donkey anti-sheep</td>
<td>Sheep anti-rabbit</td>
<td>Rabbit anti-goat</td>
<td>Rabbit anti-goat</td>
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<tr>
<td><strong>Dilution</strong></td>
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<td>1:3 000</td>
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<td>1:3 000</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
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<td>Sigma UK.</td>
<td>Sigma UK.</td>
<td>Sigma UK.</td>
<td>Sigma UK.</td>
</tr>
</tbody>
</table>

* alkaline phosphatase conjugate; † 90% response to rat CYP4A1 and 10% response to CYP4A3. (Personal communication, Prof. G. Gibson, University of Surrey, UK)
Figure 3

Apparent molecular weight (kD)

1A  4A  3A  2B  2E
Apparent molecular weight
Figure 5

- CYP1A
- CYP4A (50.9kD band)
- CYP4A (44.1 kD band)
- CYP2E
- CYP2B
- CYP3A

Arbitrary units

Lido Salute
3.3 VARIATION IN LEVELS OF CYTOCHROME P4501A, 2B, 2E, 3A AND 4A-IMMUNOPOSITIVE PROTEINS IN DIGESTIVE GLAND OF INDIGENOUS AND TRANSPLANTED MUSSEL *MYTILUS GALLOPROVINCIALIS* IN VENICE LAGOON, ITALY.
VARIATION IN LEVELS OF CYTOCHROME P450A, 2B, 2E, 3A AND 4A-IMMUNOPOSITIVE PROTEINS IN DIGESTIVE GLAND OF INDIGENOUS AND TRANSPLANTED MUSSEL *MYTILUS GALLOPROVINCIALIS* IN VENICE LAGOON, ITALY.

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A short paper submitted to Marine Environmental Research following the 9th meeting of Pollution Responses in Marine Organisms, Bergen, 1997.
Mytilus galloprovincialis digestive gland microsomes were prepared from i) indigenous populations sampled from a clean reference (Lido) and an urban-contaminated site (Salute) and ii) mussels transplanted for up to 3 weeks from Lido to an industrial-contaminated site (CVE) in Venice Lagoon, Italy. Western analysis was performed using antibodies to 5 mammalian or fish CYP forms (1A, 2B, 2E, 3A, 4A). Simultaneously run M. edulis digestive gland partially purified CYP aided identification of immunopositive bands. Levels of CYP1A, CYP2E, and CYP4A-immunopositive proteins were 50 to 300 % higher in indigenous M. galloprovincialis from Salute compared to Lido (P<0.05). Three weeks after transplantation to CVE, levels of only the CYP1A-immunopositive protein were determined to be higher (63 %) than levels for Lido (P < 0.05), indicating that anti-CYP1A shows greater specificity for a contaminant-inducible CYP form than the other antibodies.
Previous studies using genetic probes have indicated the expression and regulation of multiple forms of cytochrome P450 (CYP) in digestive gland of mussel *Mytilus* sp. with sequence similarities to vertebrate CYP1A, CYP3A and CYP4A (Wootton *et al.*, 1995, 1996). Porte *et al.* (1995) showed that partially purified CYP from *Mytilus edulis* digestive gland was immunopositive to anti-fish CYP1A; more recently, recognition by antibodies to other vertebrate CYPs (CYP2B, CYP2E, CYP3A, CYP4A) has been recorded (Peters *et al.*, 1997). Studies in Venice Lagoon, Italy over several years have shown that *Mytilus galloprovincialis* from different sites contain different levels of polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and other contaminants (Livingstone *et al.*, 1995; Fossato *et al.*, 1997). The present study was undertaken to obtain greater incite into antibody specificity (for possible biomarker use) and the differential regulation of multiple CYPs by comparing levels of CYP-immunopositive proteins in digestive gland of *M. galloprovincialis* from relatively clean and contaminated indigenous populations and with transplantation from clean to a contaminated environment.

*M. galloprovincialis* (4-6 cm) were sampled from indigenous populations at Lido (relatively clean reference site) and Salute (urban-contaminated site) and from caged animals transplanted from Lido to CVE,(industrial-contaminated site) for 1 and 3 weeks in May 1996. Previously determined levels of Σ12 PAHs and Σ8 PCBs in the digestive glands of *M. galloprovincialis* in ng/g wet wt. were respectively 43 and 38 (Lido), 208 and 272 (Salute) and 120 and 221 (CVE) (see Fossato *et al.*, 1997). Digestive gland microsomes were prepared and Western analysis performed (maximum 50 µg protein/lane) as described in Peters *et al.* (1997), using *M. edulis* digestive gland partially purified CYP as a positive control in band indentification. Blots were incubated with polyclonal anti-*Perca fluviatilis* CYP1A (1:1000 dilution), anti-rat CYP4A (1:2000 dilution), anti-rat CYP2E (1:100 dilution), anti-rat CYP2B (1:50 dilution) and anti-*Oncorhynchus mykiss*
CYP3A (1:1000 dilution) antibodies followed by visualisation with alkaline phosphatase conjugate secondary antibody. Quantification was performed on bands of similar apparent molecular weights using a BioRad Imaging densitometer. Total protein was measured by the method of Lowry et al. (1951).

Similar results were obtained for Western analysis of digestive gland microsomes of *M. galloprovincialis* as seen for *M. edulis* (Peters et al., 1997), including the detection of two major CYP4A-immunopositive bands (data not shown). Levels of CYP1A(c48 kD band)-, CYP4A(50.9 kD band)-, CYP4A(44.1 kD band)- and CYP2E(c52 kD band)-immunopositive proteins in digestive gland microsomes were respectively 50, 60, 275 and 300% higher in *M. galloprovincialis* from Salute compared to Lido (*P* < 0.05), whereas no differences were detected in CYP2B(c52 kD band)- and CYP3A(c47 kD band)-immunopositive protein levels (Fig. 1). Three weeks after transplantation of *M. galloprovincialis* from Lido to CVE, there was no change in the levels of digestive gland microsomal CYP4A- and CYP2E-immunopositive proteins (Fig. 2). However levels of CYP1A-immunopositive protein at CVE increased 63% compared to Lido (*P* < 0.05) and approached concentrations similar to those for indigenous (i.e. long-term exposed) *M. galloprovincialis* at Salute (Fig. 2). After 1 week transplantation, the mean level of CYP1A-immunopositive protein was intermediate between the mean levels at Lido and after 3 weeks at CVE (Fig. 2), indicative of a time-dependent increase in CYP1A-immunopositive protein.

The higher levels of CYP1A-, CYP2E- and CYP4A- (but not CYP2B- or CYP3A-) immunopositive proteins at Lido compared to CVE are consistent with the differential regulation of multiple forms of CYP in digestive gland of *Mytilus* sp. (Wootton et al., 1996; Peters et al., 1997) however it is unclear if the differential regulation reflects endogenous or xenobiotic metabolism. The preferential elevation of only CYP1A-
immunopositive protein with 3 weeks transplantation from a relatively clean to a site containing 3-to 6-fold higher tissue levels of PAHs and PCBs is consistent with the observations of increased digestive gland CYP1A-immunopositive protein in *Mytilus* sp. with laboratory exposure to PCBs (Livingstone *et al.*, 1997) or field exposure to PAHs (Solé *et al.*, 1996). Additionally, it is indicative of greater specificity of anti-CYP1A for a contaminant-inducible CYP form in *Mytilus* sp. than the other antibodies.

ACKNOWLEDGEMENTS

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REFERENCES


Figure 1. Determined levels of *M. galloprovincialis* digestive gland microsomal immunopositive bands following Western blot analysis with anti-CYP1A, anti-CYP4A, anti-CYP2E, anti-CYP2B and anti-CYP3A antibodies. *M. galloprovincialis* were sampled from Lido (reference site) and Salute (urban-contaminated site). Microsomal preparations (*n* = 5) were from pooled tissues (4-6 digestive glands); levels of immunopositive protein (arbitrary units) expressed as mean ± SEM; * = P < 0.05 2 tail t-Test.
Figure 2. Determined levels of *M. galloprovincialis* digestive gland microsomal immunopositive bands following Western blot analysis with anti-CYP1A, anti-CYP4A and anti-CYP2E antibodies. *M. galloprovincialis* were sampled from indigenous populations from Lido (reference site), Salute (urban-contaminated site) and mussels transplanted from Lido to CVE (industrial-polluted site) for 1 and 3 weeks. Microsomal preparations (n = 5-10) were from pooled tissues (4-6 digestive glands); levels of immunopositive protein (arbitrary units) expressed as mean ± SEM; * = P < 0.05 2 tail t-Test vs Lido; † = P < 0.05 2 tail t-Test vs Lido and CVE 1 week.
3.4 SUMMARY OF THE CHAPTER FINDINGS

The results from Chapter 3 demonstrated that antibodies raised against mammalian and fish CYP families immunoreacted with epitopes present on both partially purified *M. edulis* CYP and digestive gland microsomes of *M. galloprovincialis*. The studies indicated the presence of multiple CYP forms (at least 5 immunoidentified forms) however since no sequence data is available it is not possible to assign CYP families or sub-families to the immunopositive proteins. The multiple epitopes were recognised by polyclonal antibodies to five major CYP gene subfamilies (CYP1A, CYP2B, CYP2E, CYP3A and CYP4A).

To summarise the findings of Chapter 3 following the discussions presented in each paper;

The apparent molecular weights of the partially purified CYP proteins with epitopes to the polyclonal antibodies were estimated by Western blot analysis (Section 3.2 & 3.3). The apparent molecular weights were respectively, CYP1A (48.1 and 42.5 kD), CYP2B (52.8, 48.1 and 43.9 kD), CYP2E (52.7 and 47.2 kD), CYP3A (67.4, 52.8 and 44.5 kD) and CYP4A (50.9 and 44.1 kD).

*M. galloprovincialis* sampled from an indigenous population, chronically exposed to urban pollution reported higher levels of CYP1A, CYP4A and CYP2E epitopes compared to mussels sampled from a cleaner reference site. This could be due to differential regulation either by endogenous substrates or xenobiotics. Following the transplantation of *M. galloprovincialis* from a clean to a polluted site for 3 weeks, an increase in the determined levels of only the CYP1A-immunoidentified isoform was observed (Section 3.3). These results would indicate that regulation of the CYP1A-immunopositive protein may be occurring however increased specificity of the antibody probe and knowledge of protein
sequence of the immunopositive proteins would enhance the understanding of the CYP1A response.
4.1 INTRODUCTION

The FMO system demonstrates broad substrate specificity and is capable of oxidising nitrogen, sulphur and phosphorus heteroatoms, generally termed "soft nucleophiles" (Jakoby & Ziegler 1990). Although dietary intake of organic nitrogen or sulphur nucleophiles may influence FMO activity (Kaderlik et al., 1991), there is little or no evidence of FMO induction by xenobiotics. Although the endogenous functions of FMO have not been defined, it is proposed that FMO can oxidise endogenous substrates such as thio-ether conjugates and thio-amines produced by the organism or taken up in the diet (Ziegler 1993).

FMO has been determined in numerous aquatic invertebrate and vertebrate species including fish (Schlenk 1993; Schlenk 1995; Schlenk 1997). Similar to mammalian FMO, fish can utilise the FMO system to both detoxify xenobiotics and bioactivate others to more reactive products (Schlenk 1993). The pesticide aldicarb* for example may be oxidised to the more toxic aldicarb sulfoxide by the FMO system present in *O. mykiss* hepatic microsomes (Schlenk & Buhler 1991b) and it is estimated that *O. mykiss* is one hundred times more sensitive to aldicarb toxicity compared to *I. punctatus*, the latter species not expressing an FMO activity (Schlenk 1995). This increased risk may be due in part, to the
bioactivation of the pesticide as well the xenobiotic affecting (or potentially competing with) the endogenous functions of the FMO system. As a consequence, organisms that up-or down-regulate FMO activity during their development or life cycle potentially present variable risk of FMO-dependent xenobiotic mechanisms of toxicity.

The osmolyte TMAO is found in the tissues of many aquatic organisms and marine fish generally have higher levels of TMAO or its precursor TMA. The N-oxidation of TMA to the osmolyte TMAO is catalysed by FMO and indicates a potential osmoregulatory function for FMO during salinity adaptation by marine organisms (Pang et al., 1977). Hepatic TMA N-oxidase activity of *A. japonica* and *P. reticulata* increased following either transfer from freshwater to seawater or treatment with TMA (Daikoku et al., 1988). Freshwater fish experience different osmoregulatory challenges compared to marine fish - and freshwater fish predominantly do not express FMO (Schlenk 1997). This study examines the expression of FMO in the marine stenohaline teleost *S. maximus* and the euryhaline teleost *P. flesus*. Although both are demersal flatfish, *P. flesus* is predominantly an estuarine fish and migrates to the marine environment to reproduce. Since *P. flesus* osmoregulates in both estuarine and marine environments it may have the potential to regulate TMAO levels via the expression of FMO.

The objectives of this study were to characterise hepatic FMO in the stenohaline species *S. maximus* (Section 4.2); compare branchial and hepatic FMO expression and catalytic activity determined in both a stenohaline (*S. maximus*) and euryhaline (*P. flesus*) species (Section 4.3); determine changes of *P. flesus* FMO activity following an osmoregulatory challenge (Section 4.4).
4.2 CHARACTERISATION OF HEPATIC FLAVIN-CONTAINING MONOOXYGENASE FROM THE MARINE TELEOST TURBOT (*SCOPEHTHALMUS MAXIMUS* L.).
Characterization of hepatic flavin monooxygenase from the marine teleost turbot (Scophthalmus maximus L.)

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1. The presence and properties of flavin monooxygenase (FMO) in liver of the marine teleost, turbot (Scophthalmus maximus) were examined in relation to organic xenobiotic metabolism and osmoregulation.

2. Hepatic microsomes of sexually mature fish contained NADPH-dependent FMO as evidenced by the conversion of N,N-dimethylaniline (DMA) to DMA-N-oxide, and immunorecognition of single bands (approximate apparent molecular weight of 55 kDa) by antibodies to mammalian FMO 1 and FMO 2. Additionally, Northern analysis using a full-length cDNA probe to mammalian FMO 1 revealed a single hybridizing band of approximately 2·5 kb.

3. No significant differences were seen between male and female turbot FMO with respect to DMA N-oxidase activity, levels of immunoreactive protein (with anti-FMO 1 or anti-FMO 2) and gene expression (hybridizing mRNA).

4. Hepatic microsomal DMA N-oxidase activity was inhibited by methimazole (an FMO substrate) and trimethylamine (TMA), but not by piperonyl butoxide (a P450 inhibitor). Inhibition by TMA is indicative of a role for FMO in osmoregulation, catalysing the conversion of TMA to TMA-N-oxide. DMA N-oxidase activity was optimal at pH 8·8 and 25°C, and displayed Michaelis-Menten kinetics with respect to DMA (apparent Km = 88 µM).

Introduction

Hepatic monooxygenation, catalysed by cytochrome P450 or flavin monooxygenase (FMO), is an important biotransformation pathway for the metabolism of many organic xenobiotics, including pollutants. Whereas P450 has been studied extensively in fish and other aquatic organisms (Livingstone 1991, Stegeman and Hahn 1994), much less is known of the exogeneous or endogenous functions of FMO (Schlenk 1993). Like mammals, FMO in fish can both detoxify xenobiotics and activate certain compounds to more toxic products, e.g. conversion of aldicarb to aldicarb sulphoxide by hepatic microsomes of rainbow trout (Oncorhynchus mykiss) (Schlenk and Buhler 1991a). FMO requires NADPH and O2 for catalysis, and exists in a stable activated, high-energy state independent of substrate binding (Ziegler 1993). The 4-α-hydroperoxy side-chain on the flavin molecule of FMO (formed by the addition of electrons and O2) is susceptible to attack by weak nucleophiles such as various tertiary amines and thioethers, so defining its substrate specificity (Ziegler 1988). Thus, a characteristic catalytic property of FMO in both mammals and fish

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is oxidation of the xenobiotic N,N-dimethylaniline (DMA) to the N-oxide (Ziegler and Pettit 1964, Schlenk 1993).

Little is known of the endogenous functions of FMO in fish, but an osmoregulatory role has been indicated in salinity adaptation (Pang et al. 1977). Specifically, FMO is proposed to catalyse the metabolism of trimethylamine (TMA) to the osmolyte TMA N-oxide (Schlenk 1993). The latter N-oxide accounts for some 1–3% of the body dry weight of marine teleost fish, but is very low in freshwater fish (Strøm 1980). Induction of the enzyme and an osmoregulatory role, are indicated from TMA and salinity exposure studies. Thus, TMA N-oxidase activity in liver of eel (Anguilla japonica) and guppy (Poecilia reticulata) was markedly increased with transfer of the fish from freshwater to seawater (Daikoku et al. 1988). Further elevation of the TMA N-oxidase activity occurred on subsequent exposure to TMA by injection or through the diet.

FMO exists in multiple forms in mammals. To date, five isoforms have been identified by their amino acid sequences (termed FMO 1, FMO 2, etc., Hines et al. 1994), and are distributed in organs such as liver, kidney and lung. The occurrence of some of these isoforms has been indicated in marine fish (Schlenk 1993), e.g. antibodies to mammalian FMO 1 and FMO 2 recognized proteins of similar molecular weight in microsomes of various tissues, including liver, of O. mykiss (Schlenk 1989). Similarly, using mammalian cDNA probes, the expression of both FMO 1 and FMO 2 mRNA was indicated in liver of dogfish shark (Squalus acanthus) and striped bass (Morone saxatilis) (Schlenk 1993).

The aim was to characterize FMO in liver of the marine teleost turbot (Scophthalmus maximus), a species available both farm reared (larvae through to mature adults) and from the wild, which offers potential for xenobiotic exposure and osmoregulatory studies. FMO was characterized from male and female fish in terms of gene expression, multiple forms (using a number of mammalian cDNA and antibody probes) and catalytic properties.

Materials and methods

Animals
Sexually mature (length 24.5–47.0 cm) feral S. maximus, caught in local waters off Plymouth, UK, were maintained in large tanks with continuous flowing seawater at 12–15°C for up to 3 months before biochemical analysis. They were fed on a selection of freshly killed local fish, but were starved for 3 days prior to killing. Fish were killed instantaneously by a blow to the head, and the livers immediately dissected and used.

Chemicals
DMA was obtained from Aldrich Chemical Co. (Milwaukee, USA) and recrystallized by the method of Schlenk and Buhler (1989). All biochemical and most molecular biology reagents, including β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), phenylmethylsulphonylfluoride (PMSF), sodium dodecyl sulphate (SDS), molecular weight markers for SDS-polyacrylamide gel electrophoresis (PAGE), methimazole, piperonyl butoxide, diethyl pyrocarbonate (for RNA extraction and Northern blotting), and anti-sheep and anti-goat IgG alkaline phosphatase conjugates were from Sigma Chemical Co. (Poole, UK). Other chemicals, including ethylenediaminetetraacetic acid (EDTA), were of AnalR grade, or equivalent, and obtained from Merck (Harlow, UK). Nitrocellulose paper was from Amersham (Little Chalfont, UK), and RNAzol® from Biogenesis (Poole, UK). [α-32P]-deoxycytosine triphosphate (dCTP) was obtained from NEN-Dupont (Boston, USA).

Microsomal preparation for enzyme studies
All sample procedures were carried out at 4°C. Dissected liver was homogenized in a 1:4 wet weight:buffer volume ratio of 100 mM Tris–HCl, pH 7.6, containing 0.15 M KCl, 1 mM EDTA, 0.1 mM PMSF (added in minimal volume of ethanol), using an electrically driven Potter–Elvehjem homogenizer. The homogenate was centrifuged at 20000 g, for 20 min, and the resulting supernatant spun at 100000 g.
Flavin monooxygenase of turbot liver

for 90 min. The microsomal (100 000g) pellet was re-suspended in 100 mM K_2HPO_4/KH_2PO_4, pH 8.0, containing 20% w/v glycerol and 1 mM EDTA. This was used as the source of enzyme activity immediately, or stored at -70°C prior to Western blotting analysis. Microsomes from individual livers were used for the sexual comparison studies, whereas pooled microsomes from mixed sexes were used for the enzyme kinetic and inhibitor studies (see below).

**Enzyme activity studies**

DMA N-oxide activity was assayed by the method of Schlenk and Buhler (1991b), which was a modification of the original method of Ziegler and Pettit (1964). Contained in a final reaction volume of 1 ml were 50 mM Tris-HCl, pH 8.4, 0.5 mM NADPH, 2 mM DMA and 1 mg microsomal protein. The reaction was initiated by the addition of DMA, and the reaction mixture incubated at 20°C for 30 min. DMA N-oxide was indirectly quantified at 420 nm using an extinction coefficient of 8.2 mM cm^{-1} using a Varian Cary 1 spectrophotometer. Enzyme assays were carried out in duplicate, and experiments were performed varying DMA concentrations, temperature, and pH (see text). The apparent Michaelis constant (K_m) for DMA was calculated by Lineweaver-Burk plot using a weighted, least-squares regression (Enzpack 3, Biosoft, UK). Additionally, the effects of competing FMO substrates (methimazole, TMA) and an inhibitor of P450 (piperonyl butoxide) were examined. Methimazole, TMA and piperonyl butoxide were directly added prior to initiation of the reaction by DMA (5 min pre-incubation). Incubations contained inhibitor concentrations of 2 mM. Protein was measured by the method of Lowry et al. (1951).

**Western blot analysis**

SDS-PAGE was carried out according to the method of Laemmli (1970). Microsomal preparations were diluted with distilled water to a protein concentration of 5-7 mg/ml and boiled for 2-3 min with an equal volume of 0.125 M Tris-HCl, pH 6.8, containing 4% SDS (w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v) and 0.001% bromophenol blue (w/v). Aliquots (43 μg protein) of the mixture were then loaded onto a 10% SDS polyacrylamide gel and run using an Atto mini-gel system (Genetic Research Instrumentation, Dunmow, UK) for 40 min, followed by semi-dry transfer onto nitrocellulose paper (Towbin et al. 1979). Immunoreactive microsomal proteins were visualized using polyclonal sheep anti-pig liver FMO 1 IgG fraction (1:1000 v/v) and polyclonal goat anti-rabbit lung FMO 2 IgG fraction (1:1000 v/v) with subsequent incubation with anti-sheep or anti-goat IgG (whole molecule) alkaline phosphatase conjugate (1:3000 v/v). Purification and subsequent raising of antibodies to each of the purified proteins were performed using previously published methods (Ziegler and Mitchell 1972, Williams et al. 1985). Purified proteins (FMO 1 and FMO 2) were used as positive controls for immunoblots. Molecular weights of S. maxima immunoreactive proteins were determined from molecular weight markers transferred to the membrane. Protein bands were semiquantified using a Kontron image analyser (Image Processing System, Watford, UK).

**Northern blot analysis**

Total RNA was extracted from individual fresh livers by a single-step method of Chomczynski and Sacchi (1987) using guanidium thiocyanate/phenol/chloroform extraction (RNAzo+) according to the manufacturer’s (Biogenes Ltd.) instructions, and electrophoretically fractionated on a 1.5% agarose gel. RNA was then transferred to nitrocellulose under vacuum (VacuGene pump, Pharmacia-LKB, St. Albans, UK) and hybridized overnight at 47°C with a full-length cDNA probe encoding FMO 1, radiolabeled by random primer extension to a specific activity of >10^6 cpm/μg with [α-32P]-dCTP (Feinberg and Vogel 1983, Feinberg and Vogel 1984). The FMO 1 cDNA probe was synthesized using polymerase chain reaction amplification. Both sense and anti-sense primers based on the sequence reported by Lawton et al. (1990) were used to amplify the FMO 1 cDNA from a total cDNA library made from 1.0 μg total liver rabbit liver RNA. The resulting FMO 1 cDNA fragment spanning from nucleotides 34 to 1674 was cloned into the SmaI site of pUC19 and the identity of the FMO cDNA verified by complete DNA sequence analysis.

After incubation with the FMO 1 cDNA probe the membrane was washed three times at 47°C with 0.5× SSC containing 0.1% SDS and exposed to Kodak XAR film for 2 weeks. Bands were semi-quantitated using BioRad GS870 imaging densitometer (Biorad Inc., New York, USA). Each sample was normalized by dividing the area of the 18S RNA band visualized by ethidium bromide staining on the membrane prior to hybridization.

**Statistics**

Values are presented as mean ± SEM. Differences between groups of values were compared using a student’s t-test, and effects of various inhibitors were compared using Dunnet’s multiple range test, which compares multiple samples to a single control. p < 0.05 was accepted as statistically significant. The correlation matrix was determined using StatView 512+®.
Results

Microsomal DMA N-oxide activity, microsomal immunoreactive protein and whole-tissue hybridizing RNA were detected in the liver of both male and female *S. maximus* (table 1). No statistically significant differences were observed for any of these measurements between male and female fish. Polyclonal antibodies raised against mammalian FMO 1 and FMO 2 recognized single proteins of approximately 55 kDa apparent molecular weight (figure 1). The level of recognition, or protein amounts, were indicated to be greater for anti-FMO 2 than anti-FMO 1, but the differences were not statistically significant (data not shown). Single bands of similar apparent molecular weight were also seen for the two positive controls, pig liver FMO 1 (figure 1A, lane 8) and rabbit lung FMO 2 (figure 1B, lane 1). Hybridization of total hepatic RNA with a full-length cDNA for FMO 1 revealed a single band of approximately 2.5 kb (figure 2). Expression of the 55 kDa protein recognized by anti-FMO 1 did not correlate with that of the 55 kDa protein recognized by anti-FMO 2 individual males (table 2). However, expression of the 55 kDa protein recognized by anti-FMO 1 did correlate with FMO activity and the 2.5 kb transcript hybridizing to FMO 1 cDNA. Conversely, expression of the 55 kDa protein recognized by anti-FMO 2 did not correlate with FMO activity or the 2.5 kb transcript hybridizing to FMO 1 cDNA. In females, expression of proteins recognized by both FMO 1 and FMO 2 positively correlated with each other as well as FMO activity and the 2.5 kb mRNA transcript (table 2).

DMA N-oxidase activity characterization studies were carried out on the pooled hepatic microsomes of male and female *S. maximus*. The activity showed Michaelis-Menten kinetics with respect to dependence on DMA concentration, with a correlation of 0.97 observed for the Lineweaver-Burk plot (figure 3). The apparent $K_m$ for DMA was 88 $\mu$M. Somewhat sharp profiles were observed for the dependence of DMA N-oxidase on both temperature (figure 4) and pH (figure 5) with optima of respectively 25°C (sharp decline at 37°C) and pH 8.8 (range 8.5–9.2). DMA N-oxidase activity was inhibited by trimethylamine and methimazole, 43 and 75%, respectively, but stimulated 45% by piperonyl butoxide (table 3).

Discussion

FMO catalyses the monooxygenation of tertiary amines and a variety of other heteroatom-containing ‘soft’ nucleophilic compounds. In fish tissues, such as liver, it is indicated to have a role in both exogenous and endogenous metabolism respectively, organic xenobiotic metabolism (e.g. N-oxidation of DMA), and osmoregulation (N-oxidation of TMA) (see Introduction).

The present study was carried out to improve the understanding of FMO presence and function in a marine fish species, the turbot *S. maximus*. The liver was chosen as a major organ of organic xenobiotic metabolism in fish (Varanasi et al. 1989, Walker and Livingstone 1992, Schlenk 1993) and one in which FMO activity is affected by salinity (Charest et al. 1988, Daikoku et al. 1988). The presence and expression of a functional FMO gene/enzyme in hepatic microsomes of *S. maximus* was shown by the detection of hybridizing mRNA species, immunoreactive protein, and enzyme activity with DMA as substrate. The specific activity of hepatic FMO in *S. maximus* (0.2–0.3 nmol min$^{-1}$ mg$^{-1}$ microsomal protein) was lower than that of other fish (≈0.740 for *O. mykiss* (Schlenk and Buhler 1991b), 1.2 for the smooth dogfish (*Squalus acanthias*) (Schlenk and Li-Schlenk 1995), and 0.05–1.15 for four species of elasmobranch (Goldstein and Dewitt-Harley 1973)). Catalytic activity
Flavin monooxygenase of turbot liver

Table 1. Levels of N,N-dimethylaniline (DMA) N-oxidase activity and anti-FMO 1 and anti-FMO 2 immunoreactive protein in hepatic microsomes, and FMO 1 mRNA in whole liver of male and female Scophthalmus maximus.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA N-oxidase activity (pmol/min/mg protein)</td>
<td>254.0 ± 65.4</td>
<td>280.0 ± 142.9</td>
</tr>
<tr>
<td>FMO 1 protein†</td>
<td>23.7 ± 6.3</td>
<td>17.9 ± 5.2</td>
</tr>
<tr>
<td>FMO 2 protein†</td>
<td>30.8 ± 4.2</td>
<td>26.4 ± 5.1</td>
</tr>
<tr>
<td>FMO 1 mRNA†</td>
<td>67 ± 10</td>
<td>80 ± 15</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 3); no differences observed in any measurement between male and female (p > 0.05).
† Arbitrary units (see Materials and methods for details of probes).

Figure 1. Western blot analysis of hepatic microsomes from individual male and female Scophthalmus maximus using polyclonal antibodies to mammalian FMO 1 (A: lanes 1–3, male; 4–6, female; 7, mol. wt. markers; and 8, pure pig FMO 1) and FMO 2 (B: lanes 1, pure rabbit FMO 2; 2, mol. wt. markers; 3–5, female; and 6–8, male); 43 μg protein was loaded per lane.

Figure 2. Northern blot analysis of total hepatic RNA from individual male and female Scophthalmus maximus probed with FMO 1 cDNA: lanes 1–3, hepatic RNA from female; and 4–6, hepatic RNA from male; 10 μg RNA was loaded per lane. Molecular weight was determined using ethidium bromide-stained RNA markers (Promega) 0.36–9.49 kb.
Table 2. Correlation coefficients for the intercomparison of hepatic microsomal anti-FMO 1 and anti-FMO 2 immunoreactive protein amount, \(N,N\)-dimethylaniline \(N\)-oxidase activity and mRNA hybridizing to FMO 1 cDNA in male and female *Scophthalmus maximus*.

<table>
<thead>
<tr>
<th>Sex</th>
<th>FMO 1</th>
<th>FMO 2</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male FMO 1</td>
<td>×</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>Male FMO 2</td>
<td>-0.09</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td>Male enzyme activity</td>
<td>1.00</td>
<td>-0.08</td>
<td>×</td>
</tr>
<tr>
<td>Male FMO 1 mRNA</td>
<td>0.99</td>
<td>-0.14</td>
<td>0.99</td>
</tr>
<tr>
<td>Female FMO 1</td>
<td>×</td>
<td></td>
<td>×</td>
</tr>
<tr>
<td>Female FMO 2</td>
<td>0.99</td>
<td>×</td>
<td></td>
</tr>
<tr>
<td>Female enzyme activity</td>
<td>0.99</td>
<td>0.99</td>
<td>×</td>
</tr>
<tr>
<td>Female FMO 1 mRNA</td>
<td>0.99</td>
<td>0.99</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Data from table 1.

\(\times\) Invalid comparison.

---

Figure 3. Lineweaver-Burke plot of the dependence of \(N,N\)-dimethylaniline \(N\)-oxidase activity on \(N,N\)-dimethylaniline substrate concentration in hepatic microsomes of *Scophthalmus maximus*. Each value is the mean of three assays (± SEM; not shown if less than symbol size). Other reaction conditions: 0.5 mM NADPH, 50 mM Tris-HCl, pH 8.4, 20°C. Activities were determined from pooled samples from male and female.

towards TMA, and a possible role in osmoregulation, is indicated from the inhibition of microsomal DMA \(N\)-oxidase activity by TMA. Hepatic microsomal TMA \(N\)-oxidase activity has been measured directly in a number of teleost fish species, including *A. japonica* and *P. reticulata* (Daikoku *et al.* 1988) and cod (*Gadus morhua*) (Agústsson and Strom 1981).

No marked differences in DMA \(N\)-oxidase activity were seen between male and female *S. maximus*, consistent with most previous fish studies (Schlenk 1993), but differing from mammalian liver, which had higher FMO levels in the male than female rat (Dannan *et al.* 1986) and the reverse in mouse (Duffel *et al.* 1981).
Figure 4. Dependence of N,N-dimethylaniline N-oxidase activity of hepatic microsomes of *Scophthalmus maximus* on incubation temperature. Each value is the mean of two assays (± range; not shown if less than symbol size). Other assay conditions: 2 mM N,N-dimethylaniline, 0.5 mM NADPH, 50 mM Tris-HCl, pH 8.4.

Figure 5. Dependence of N,N-dimethylaniline N-oxidase activity of hepatic microsomes of *Scophthalmus maximus* on pH (50 mM Tris-HCl). Each value is the mean of two assays (± range; not shown if less than symbol size). Other assay conditions: 2 mM N,N-dimethylaniline, 0.5 mM NADPH, 20°C.
Table 3. Effects of competitive FMO substrates (methimazole, trimethylamine) and inhibitor of P450 (piperonyl butoxide) on N,N-dimethylaniline N-oxidase activity of hepatic microsomes of *Scophthalmus maximus*.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Methimazole</th>
<th>Trimethylamine</th>
<th>Piperonyl butoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activities</td>
<td>100 ± 11</td>
<td>25 ± 5†</td>
<td>57 ± 12†</td>
<td>145 ± 21†</td>
</tr>
</tbody>
</table>

Activities expressed as % of the control DMA N-oxidase activity (240 ± 26 pmol/min/mg protein), values are mean ± SEM (n = 3).

† p < 0.05 compared with control.

No differences in FMO protein levels with sex were seen in *S. maximus*. However, the correlation of protein recognized by anti-FMO 1 and anti-FMO 2 in the female, and the absence of the same correlation in the male, indicates that sexual differences may be present. In studies with *O. mykiss*, hepatic isoform differences of FMO with sex have been indicated (Schlenk and Buhler 1993).

The physico-chemical and enzyme kinetic properties of the *S. maximus* hepatic microsomal FMO are similar to those observed for other fish and mammalian species. The relatively sharp pH profile for DMA N-oxidase activity of 8.5–9.2 (optimum pH 8.8) compares well with that of 8.4–9.2 for *O. mykiss* (Schlenk and Buhler 1991b) and a pH optimum of 8.4 for the porcine enzyme (Ziegler 1988).

Similar high pH optima of 8.2 and 9.0 were seen for TMA N-oxidase activity for respectively *G. morhua* (Agústsson and Strem 1981) and the nurse shark (*Ginglymostoma cirratum*) (Goldstein and Dewitt-Harley 1973). The marked temperature sensitivity of DMA N-oxidase activity of *S. maximus* (optimum of 25°C decreasing by 77% at 37°C) is very similar to that found for *O. mykiss* (optimum of 25°C decreasing by 87% at 35°C (Schlenk and Buhler 1991b). High sensitivity to heat inactivation is a characteristic of mammalian FMO 1 (Ziegler 1988), possibly indicating a structural homology of this isoform with fish FMO(s).

Hepatic microsomes of *S. maximus* showed Michaelis–Menten kinetics with respect to DMA N-oxidation, consistent with results for *O. mykiss* (Schlenk and Buhler 1991b), but with a much lower apparent *K_m* for DMA, viz. at the same pH (8.4) and similar NADPH concentration (88 μM) (*S. maximus*) compared with 1.2 mM (*O. mykiss*). However, the apparent *K_m* for hepatic FMO activity in the elasmobranch *S. acanthus* (44 μM) (Schlenk and Li-Schlenk 1995) was two-fold lower than *S. maximus*. The marked intra- and interspecies variation in both specific activities and/or apparent *K_m*’s for FMO in fish is striking and may indicate an enzyme that is regulated in relation to both endogenous (TMA) and exogenous factors (salinity, temperature, etc.). For example, hepatic TMA N-oxidase activity was enhanced seven and 224 times in *A. japonica* exposed to TMA and increased salinity respectively (Daikoku et al. 1988).

DMA can be metabolized by both P450-dependent (demethylation) and FMO-dependent (N-oxidation) pathways in mammalian hepatic microsomes (Houdi and Damani 1985). The absence of inhibition of *S. maximus* DMA N-oxidase activity by the P450 inhibitor piperonyl butoxide is therefore consistent with other vertebrate studies, including fish (Schlenk et al. 1991b, Schlenk 1993), and indicates that the reaction is not catalysed by P450. The fact that piperonyl butoxide stimulated *S. maximus* DMA N-oxidase activity is most likely due to inhibition of the competing P450-dependent pathway, so directing DMA towards increased FMO-dependent metabolism. In contrast with this result, other P450 inhibitors
Flavin monoxygenase of turbot liver

SKF-525A, carbon monoxide, N-benzylimidazole did not stimulate FMO activity in other fish species (Goldstein and Dewitt-Harley 1973, Schlenk and Buhler 1991b). The inhibition of $S. maxima$us DMA N-oxidase activity by two substrates of FMO (methimazole and TMA) indicate that the reaction is FMO-catalysed. Studies on DMA N-oxidase activity in $O. mykiss$ (Schlenk and Buhler 1991b) and $S. acanthus$ (Schlenk and Li-Schlenk 1995) have demonstrated that the inhibition by methimazole is non-competitive, whereas that of TMA is competitive. It is unclear how TMA and methimazole inhibit hepatic FMO activity in $S. maxima$us, but the occurrence of competitive inhibition by TMA and non-competitive inhibition by methimazole in several other fish species suggests structural similarities in the active sites for fish FMOs.

In addition to functional similarities, recognition of $S. maxima$us microsomal proteins by antibodies raised against FMO 1 and FMO 2 (Hines et al. 1994). Indicate structural homology between the $S. maxima$us and mammalian FMOs. Each antibody recognized a single band of protein with similar apparent molecular weight (55 kDa for $S. maxima$us) as the purified pig liver or rabbit lung enzymes. Although temperature sensitivity studies indicated a functional homology of the $S. maxima$us enzyme to FMO 1, antibodies to FMO 2 were indicated to have a slightly higher degree of recognition than antibodies to FMO 1. In $O. mykiss$ and $S. acanthus$, antibodies to FMO 1 and 2 recognized hepatic proteins of the same apparent molecular weight with equal intensity (Schlenk 1993), whereas in rabbit, FMO 1 and 2 are immunochemically distinct (Ziegler 1988).

The order of FMO isozyme evolution is not known, but the occurrence of FMO 1 is tissues of all mammalian species examined to date suggests that this form may be one of the early mammalian forms of FMO. Thus, a full-length cDNA coding for FMO 1 was used to probe mRNA isolated from $S. maxima$us liver. The presence of a single band of approximately 2.5 kb, concomitant with the presence of FMO protein and activity in individual animals, indicates that the identity of the hybridizing band is FMO mRNA. The Western and Northern analyses both gave single bands, possibly indicating the presence of a single gene and enzyme. However, in the former case recognition by both FMO 1 and FMO 2 antibodies could indicate either a single isoenzyme with both epitopes, or two isoenzymes of very similar molecular weight. The lack of correlation between isoforms recognized by anti-FMO 1 and anti-FMO 2 in the males suggests that isoform differences may, in fact, be present in male $S. maxima$us. In the case of hepatic microsomes of $O. mykiss$, using the same mammalian antibodies, two bands of different apparent molecular weight (61 and 57 kDa) were detected (Schlenk and Buhler 1993).

In summary, molecular biological and enzymological studies have shown the presence of FMO in hepatic microsomes of the marine teleost $S. maxima$us. Inhibition of DMA N-oxidase activity by methimazole and TMA indicate a wide substrate specificity for the enzyme, consistent with known FMO properties and indicative of roles in biotransformation and osmoregulation. Recognition of the $S. maxima$us FMO by mammalian cDNA and protein probes suggests structural and genetic homology between the piscine and mammalian enzymes.

Acknowledgements

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Reference


Flavin monooxygenase of turbot liver.


4.3 DIFFERENTIAL EXPRESSION AND ACTIVITY OF FLAVIN-CONTAINING MONOOXYGENASE IN EURYHALINE AND STENOHALINE FLATFISHES INDICATES POTENTIAL OSMOREGULATORY ROLE.
Differential expression and activity of flavin-containing monooxygenases in euryhaline and stenohaline flatfishes indicates potential osmoregulatory role

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N,N-Dimethylaniline (DMA) N-oxidase activity indicative of flavin-containing monooxygenase (FMO) was examined in four tissues (liver, gill, muscle, and kidney) of the flounder (Platichthys flesus). Gill microsomes had the highest levels of activity (456 ± 343 pmol/min/mg), while kidney (121 ± 109) and liver (67 ± 26) had levels just above detection. A single faint band of a 56 kD protein was observed in liver and gill microsomes following Western blot analyses with polyclonal antibodies to FMO1. DMA N-oxidase activity in gill and liver directly correlated with the expression of the 56 kD protein recognized by polyclonal antibodies against FMO form 1. Likewise a mRNA band of approximately 2.5 kilobases was higher in gill than a 3.0 kb band in liver following hybridization with an FMO1 cDNA probe. Gill and liver microsomal DMA N-oxidase from the euryhaline P. flesus was compared with that of the stenohaline turbot (Scophthalmus maximus). DMA oxidase activity, FMO protein and mRNA were significantly greater in the gill of P. flesus, while S. maximus had higher levels of enzyme activity in the liver, but also significant levels in gill. Comparison of the enzymatic properties of the P. flesus gill and S. maximus liver enzymes indicated dramatic differences in K_m between gill and liver, but were both inhibited by equimolar concentrations of trimethylamine (TMA). Gill microsomal activity in each species was unaffected by the mammalian FMO2 substrate (competitive inhibitor) n-octylamine. Differential expression of FMO in tissues from stenohaline and euryhaline fish suggests a functional relationship between FMO and osmoregulation.

Key words: Flavin-containing monooxygenase; N,N-Dimethylaniline; Trimethylamine; Gill; Liver; Kidney; Flounder; Turbot.


Introduction

Flavin-containing monooxygenases are a class of endoplasmic reticulum enzymes requiring NADPH and molecular oxygen for catalysis and are involved in the oxidation of numerous heteroatom-containing xenobiotics (Ziegler, 1988; 1993). Five forms of FMO have been cloned and sequenced in mammalian species. Expression levels vary depending upon the animal species and tissue (Ziegler, 1993; Hines et al., 1994). Two of the more dominant
mammalian isoforms, FMO 1 and FMO 2, are differentially expressed in liver and lung, respectively (Tynes and Philpot, 1987). Initial characterization studies with each form identified primarily nitrogen and sulfur-containing drugs and pesticides as substrates with differences in specificity and temperature resistance (Ziegler, 1988). Recent reports have shown that endogenous chemicals such as thioether conjugates (Sausen et al., 1993) and trimethylamine (TMA) (Ziegler, 1993) are also substrates for one or more isoforms.

Oxidation of TMA to the N-oxide has been shown to play an osmoregulatory function in terrestrial (Avison et al., 1991) and marine (Pang et al., 1977; Charest et al., 1988; Van Waarde, 1988) organisms. Comparison of hepatic TMA oxidases in several piscine species with various forms of mammalian FMO indicates shared substrate specificities, pH optimums, cofactor requirements, and amino-acid sequences (Schlenk, 1993). TMA N-oxidase activity in liver of eel (Anguilla japonica) and guppy (Poecilia reticulata) was markedly increased with transfer of the fish from freshwater to seawater or treatment with TMA (Daikoku et al., 1988). In addition, the occurrence of FMO activity in marine fish and its absence in many freshwater fish (Baker et al., 1963; Schlenk, 1993) provides further evidence that FMO may serve an important osmoregulatory function.

In order to test this hypothesis, the occurrence and tissue distribution of FMO was examined in a species of fish that readily migrates from salt to brackish water, the flounder (Platichthys flesus) and in a species that resides in saltwater for its entire life history, the turbot (Scophthalmus maximus).

Materials and Methods

Animals

Juvenile P. flesus (length 21-30 cm) and S. maximus (length 24.5-47 cm) were caught in local waters off Plymouth, U.K., and maintained in 1000-liter tanks with continuous flowing seawater at 12-15°C for up to 3 months before biochemical analysis. They were fed on a diet of fresh fish but were starved for 3 days prior to sacrifice. Fish were killed instantaneously by a blow to the head. Tissues were immediately dissected and used for enzyme, protein and RNA analyses.

Chemicals

DMA was obtained from Aldrich Chemical Co., Milwaukee, Wl, U.S.A. and recrystallized by the method of Schlenk and Buhler (1989). All biochemical and most molecular biology reagents, including β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), phenylmethylsulphonylfluoride (PMSF), sodium dodecylsulphate (SDS), molecular weight markers for SDS-polyacrylamide gel electrophoresis (PAGE), methimazole, piperonyl butoxide, diethyl pyrocarbonate (for RNA extraction and Northern hybridization), and anti-sheep and anti-goat IgG alkaline phosphatase conjugates were from Sigma Chemical Co., Ltd., Poole, U.K. Other chemicals, including ethylenediaminetetraacetic acid (EDTA), were obtained from Merck Ltd., Harlow, U.K. Nitrocellulose paper was from Amersham International, Little Chalfont, U.K., and RNAzol™ from Biogenesis Ltd., Poole, U.K. [α-32P]-Deoxy­cytosine triphosphate (dCTP) was obtained from NEN-Dupont, Boston, MA, U.S.A.

Microsomal preparation for enzyme studies

All sample procedures were carried out at 4°C. Dissected tissues from three fish were pooled and homogenized in 100 mM Tris–HCl pH 7.6 containing 0.15 M KCl, 1 mM EDTA, 0.1 mM PMSF (added in minimal volume of ethanol), using either an electrically driven Potter-Elvehjem (liver, kidney) or a Polytron (muscle, gill) homogenizer. The homogenate was centrifuged at 20,000 g for 20 minutes, and the resulting supernatant fraction spun at 100,000 g for 90 minutes. The microsomal (100,000 g) pellet was resuspended in 100 mM K2HPO4/KH2PO4 pH 8.0 containing 20% w/v glycerol and 1 mM EDTA. This microsomal suspension was used as the source of enzyme activity immediately, or stored at −70°C prior to Western blot analysis.

Enzyme activity studies

DMA N-oxidase activity was assayed by the method of Schlenk and Buhler (1991), which was a modification of the original method of Ziegler and Pettit (1964). Contained in a final reaction volume of 1 ml were 50 mM Tris–HCl pH 8.4, 0.5 mM NADPH, 2 mM DMA (added in minimal volume of 100 μl of distilled water) and 1 mg of microsomal protein. The reaction was initiated by the addition of DMA and the reaction mixture incubated at 20°C for 30 minutes. DMA N-oxide was indirectly quantified at 420 nm using an extinction coefficient of 8.2 μM−1 cm−1 on a Varian Cary 13 spectrophotometer. Enzyme assays were carried out in duplicate, and experiments performed varying DMA concentration. The apparent Michaelis constant (app. Km) for DMA was calculated by Lineweaver-Burk plot using a weighted least squares regression (Enzpack 3, Biosoft, U.K.). Additionally the effects of the competing FMO substrates
TMA and n-octylamine were examined. TMA was dissolved in distilled water and directly added prior to initiation by DMA. Incubations contained inhibitor concentrations of 2 mM. Protein was measured by the method of Lowry et al. (1951).

Western blot analysis

SDS-PAGE was carried out according to the method of Laemmli (1970). Microsomal preparations were diluted with distilled water to a protein concentration of 5.7 mg/ml and boiled for 2-3 minutes with an equal volume of 0.125 M Tris--HCl pH 6.8 containing 4% SDS (w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v) and 0.001% Bromophenol Blue (w/v). Aliquots (43 μg protein) of the mixture were then loaded onto a 10% SDS polyacrylamide gel and ran using an Atto mini gel system (Genetic Research Instrumentation Ltd., Dunmow, U.K.) for 40 minutes, followed by semi-dry transfer onto nitrocellulose paper (Towbin et al., 1979). Immunoreactive microsomal proteins were visualized using polyclonal sheep anti-pig liver FMO 1 IgG fraction and polyclonal goat anti-rabbit lung FMO 2 IgG fraction with subsequent incubation with anti-sheep or anti-goat IgG (whole molecule) alkaline phosphatase conjugate. Purification and subsequent raising of antibodies to each of the purified proteins were performed using previously published methods (Ziegler and Mitchell, 1972; Williams et al., 1985). Purified FMO 1 and FMO 2 were used as positive controls for immunoblots. Molecular weight determinations of piscine proteins were based on migration patterns of purified FMO 1 (56,000 daltons) or FMO 2 (59,000 daltons) transferred to the membranes as well as stained molecular weight standards (BIORAD, U.K.). Individual protein bands were semi-quantified by image analysis using a Kontron image analyzer (Image Processing System, Watford, U.K.).

Northern blot analysis

Total RNA was extracted from individual fresh livers by the single step method of Chomczynski and Sacchi (1987) using guanidinium thiocyanate/phenol/chloroform extraction (RNAzol™) according to the manufacturer's Biogenesis Ltd. instructions, and electrophoretically fractionated on a 1.5% agarose gel (Formaldehyde/MOPS). RNA was then transferred to nitrocellulose under vacuum (VacuGene pump, Pharmacia-LKB, St. Albans, U.K.) and hybridized overnight at 47°C with a cDNA probe encoding FMO 1, radiolabelled by random primer extension to a specific activity of >10^6 cpm/μg with [α-32P]-dCTP (Feinberg and Volgelstein, 1984). The FMO 1 cDNA probe was synthesized using polymerase chain reaction amplification. Briefly, sense and antisense primers based on the sequence reported by Lawton et al. (1990) were used to amplify the FMO 1 cDNA from a total cDNA library made from 1.0 μg of total rabbit liver RNA. The resulting FMO 1 cDNA fragment spanning from 34 to 1674 was cloned into the SMAl site of pUC19 and the identity of the FMO cDNA was verified by complete DNA sequence analysis.

After hybridization, the membrane was washed three times at 47°C with 0.5 X SSC containing 0.1% SDS and exposed to Kodak XAR film for 2 weeks. Bands were semi-quantitated using a BioRad GS670 imaging densitometer (BioRad Inc., New York, U.S.A.). Each sample was normalized by dividing the area of the 18S RNA band visualized by ethidium bromide staining on the membrane prior to hybridization.

Statistics

Values are presented as mean ± SEM. Differences between groups of values were compared using the Student's t-test. The effects of various inhibitors were compared using Dunnet's multiple range test, which compares multiple samples to a single control. P < 0.05, and was accepted as statistically significant.

Results

P. flesus tissue distribution

Microsomal DMA N-oxidase was examined in muscle, liver, kidney and gill of P. flesus. No detectable activity was observed in muscle with limits of detection of 50 pmol/min/mg. Gill microsomes had the highest levels of activity (456 ± 343 pmol/min/mg), while kidney (121 ± 109) and liver (67 ± 26) had levels just above detection. A single faint band of a 56 kD protein was observed in liver and gill microsomes following Western blot analyses with polyclonal antibodies to FMO 1 (Figure 1A). However, of the samples incubated with anti-FMO 2, kidney possessed the highest concentration of a FMO 2 cross-reactive protein, which was 55 kD. An additional band of 57 kD was also recognized by FMO 2 antibodies. No detectible FMO 2 cross-reactive protein was observed in gill microsomes (Figure 1B). Densitometric measurement of the FMO 1 cross-reactive bands in gill and liver indicated that the protein was expressed nearly twice as high in gill as liver (Table 1). Additional bands were also observed on Western blots, but the majority of these were artifacts brought about by the secondary antibodies conjugated to alkaline phosphatase. This was
verified by omitting incubations with the primary antibody to FMO 1 and incubating the membrane only in the secondary system. Northern blot analysis of total RNA from gill using a FMO 1 cDNA probe showed a band of mRNA of approximately 2.5 kb that was of 4.1 times greater optical density than a band in liver of approximately 3.0 kb (Figure 2).

Discussion

Although FMO activity has been identified in various species of fish and mammals, its endogenous function and evolution to xenobiotic metabolizing enzymes has yet to be explored. Several studies have demonstrated that FMOs catalyze the oxidation of thio-ether conjugates in the kidney (Sausen et al., 1993) and the choline byproduct, TMA (Higgins et al., 1972; Ayesh and Smith, 1990). TMA is used by terrestrial and marine organisms as an osmoregulator and may stabilize proteins in elasmobranchs that contain high levels of urea (Pang et al., 1977; Van Waarde, 1988; Avison et al., 1991). Our laboratory has been examining the relationship of TMA and FMO by surveying and characterizing the enzyme in marine and freshwater fish (Schlenk, 1993). In contrast to earlier findings by Baker et al. (1963), more recent investigations in our laboratory have shown that hepatic FMO activity has only been observed in saltwater and anadromous fish raised in freshwater (Schlenk, 1993). To date, hepatic FMO activity has not been observed in at least ten freshwater species found in the Southeastern United States (Schlenk, unpublished data). Consequently, the purpose of this study was to examine whether a euryhaline species of

Fig. 1A. Western blot analysis of microsomal protein from P. flesus tissues using polyclonal antibodies raised against pig liver FMO 1. Lanes 1–3, liver; lane 4, gill; lane 5, muscle; lane 6, kidney; lane 7, MW markers; lane 8, purified pig liver FMO 1; lane 9, purified rabbit lung FMO 2.

Fig. 1B. Western blot analysis of P. flesus tissues using polyclonal antibodies raised against rabbit lung FMO 2. Lane 1, purified rabbit lung FMO 2; lane 2, purified pig liver FMO 1; lane 3, MW markers; lane 4, kidney; lane 5, muscle; lane 6, gill; lanes 7–9, liver.
fish that typically resides in brackish water environments possessed active FMO(s).

The flounder (*Platichthys flesus*) was chosen because of earlier studies, which characterized FMO activity in another species of flatfish that was stenohaline, the turbot (*Scophthalmus maximus*) (Peters et al., 1995). Initial investigations of *P. flesus* liver indicated extremely low levels of FMO activity, which prevented kinetic and substrate characterization in this tissue. Examination of TMA oxidase in crude hepatic homogenates of other euryhaline flatfish by Baker et al. (1963) also failed to show measurable activity. However, it should be noted that the microsomal method of analysis used in the present study is approximately 10–20 times more sensitive than crude liver homogenates (Schlenk and Buhler, 1991).

The relatively low levels of hepatic FMO activity correlated with low levels of a 56 kD protein recognized by polyclonal antibodies to FMO 1 and a 3.0 kb band of mRNA that hybridized to a FMO 1 cDNA under moderate stringency. Polyclonal antibodies to mammalian FMOs have been used to characterize FMOs in several fish species such as rainbow trout (*Oncorhyncus mykiss*) (Schlenk and Buhler, 1993), dogfish shark (Schlenk and Li-Schlenk, 1994) and *S. maximus* (Peters et al., 1995). Transcripts hybridizing with mammalian FMO 1 cDNA have been previously observed in *S. maximus* liver (2.5 kb), striped bass (*Morone saxatilis x chrysops*) liver (1.4 kb) and smooth dogfish shark (*Squalus acanthias*) liver (1.1 kb) (Peters et al., 1995; Schlenk, 1993). The higher molecular weight of the hepatic FMO 1 cross-reactive transcript relative to the gill indicates potential tissue specificities in expression. Although modest levels of FMO activity were present in *P. flesus* liver, further examination of other *P. flesus* tissues, such as the gill, revealed relatively high levels of activity. FMO 1 cross-reactive protein (relative to liver) and the 2.5 kb FMO 1 cross-reactive mRNA. In contrast to freshwater-reared rainbow trout and saltwater-reared *S. maximus*, the gill of *P. flesus* possessed higher levels of FMO 1 cross-reactive protein, enzyme activity and FMO 1 cross-reactive mRNA compared to hepatic tissue. Although FMO activity and two proteins of approximately 56–59 kD recognized by anti-FMO 1 and anti-FMO 2 have been observed in gill microsomes of rainbow trout, liver and trunk-kidney microsomes had much higher levels of activity and protein (Schlenk and Buhler, 1989; Schlenk and Buhler, 1991; Schlenk, 1993). Likewise, hepatic DMA N-oxidase was shown to exceed gill activity in *S. maximus* further suggesting tissue specific expression of FMO.

![Fig. 2. Northern blot analysis of liver and gill RNA of *P. flesus* using a full-length FMO 1 cDNA probe. Lanes 1–2 10 μg of total RNA from gill; lanes 3–4 10 μg of total RNA from liver. Molecular weight determined using ethidium bromide-stained RNA markers (Promega) 0.36–9.49 kb.](2.5kb)
(Schlenk and Buhler, 1991). The 14-fold increase in $K_m$ of *S. maximus* liver versus gill argues that liver may be the primary organ for TMAO formation in saltwater fish. Studies performed in salmon and kelp bass also support this conclusion, although gill was not examined in any of these species (Charest et al., 1988). The relatively high apparent $K_m$ of FMO activity from *P. flesus* gill suggests that enzyme activity can occur with exposure to relatively large (mM) levels of substrate. Large amounts of TMAO (the oxidation product of TMA) might be needed in an osmoregulatory capacity if the animal were rapidly changing salinity regimes. The presence of significant FMO activity in an organ that plays a critical role in osmoregulation, especially in an animal that must adapt to salinity changes brought about by storms or large tidal surges (such as the 10–15-foot change during summer in Plymouth estuaries) indicates a potential osmoregulatory function for this group of enzymes. Indeed, salinity changes and the administration of TMA in euryhaline eel and guppies were shown to stimulate hepatic FMO activity (Daikoku et al., 1988). However, attempts to induce FMO activity or FMO cross-reactive proteins in liver of stenohaline rainbow trout with injections of TMA were unsuccessful (Schlenk and Buhler, 1993). Each of these studies failed to examine gill tissue for FMO activity or protein. Recent

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**Table 2. Effects of temperature and competitive substrates for FMO 1 (TMA) and FMO 2 (n-octylamine) on gill and hepatic DMA N-oxidase (pmol/min/mg) in *S. maximus* and *P. flesus***

<table>
<thead>
<tr>
<th></th>
<th>P. flesus Gill</th>
<th>S. maximus Gill</th>
<th>S. maximus Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>456 ± 343</td>
<td>168 ± 15</td>
<td>240 ± 26</td>
</tr>
<tr>
<td>TMA</td>
<td>214 ± 9.8*</td>
<td>35.3 ± 1.0*</td>
<td>136 ± 16*</td>
</tr>
<tr>
<td>n-octylamine</td>
<td>487 ± 82</td>
<td>173 ± 22</td>
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</tr>
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</table>

Values are mean ± SD (*n* = 3 replications of pooled microsomes). *P* 

< 0.05 compared to control. --- Not determined.
studies exploring the effect of salinity on gill and hepatic FMO activity in *P. flesus* revealed that salinity reduction from seawater to brackish water led to a 98% reduction in branchial FMO activity within 2 weeks (Schlenk *et al*., in press).

Since this was the first attempt to characterize gill FMO activity from any species of fish, gill FMO was compared to mammalian FMOs from lung tissue. Comparisons to FMO 2, which is the predominant form in the lung, suggest that gill FMO(s) may be more homologous to FMO 1 than FMO 2. In contrast to the substrate specificity of FMO 2, N-octylamine does not appear to be a substrate for the gill enzyme in *P. flesus* or *S. maximus* because it did not reduce DMA N-oxidase. N-Octylamine has been shown to be a substrate for FMO 2 and would have been expected to inhibit DMA N-oxidase levels in gill had it been a substrate (Williams *et al*., 1985; Ziegler, 1988). However, the apparent *K*ₐ for N-octylamine in fish FMOs is unknown and is relatively large in mammalian FMO 2 (6.7 nM) (Tyner *et al*., 1986). Consequently, it is possible that 2 mM of n-octylamine may not have been sufficient to cause inhibition of the enzyme. Clearly, reconstitution studies with purified enzyme could help determine appropriate inhibitor concentrations for future studies.

Although only one band was observed in Western blots probed with antibodies to both FMO 2 and FMO 1, it should be noted that multiple isoforms of FMO may be present in *P. flesus* gill and may preferentially oxidize DMA and not N-octylamine. Semi-quantitation of the FMO 2 cross-reactive bands of protein in muscle, kidney, liver and gill demonstrated that kidney showed the highest levels of expression and may possess a unique form more homologous to FMO 2 than FMO 1. Since gill and liver forms of the enzyme have different catalytic properties and DMA N-oxidase activity does not appear to correlate with FMO 2 cross-reactive protein expression, gill FMO(s) in fish may be unique from the lung isoform in mammals and the piscine liver isoform. However, further characterization, especially regarding substrate specificity, will be necessary to validate this hypothesis.

In summary, branchial FMO activity, FMO 1 cross-reactive protein and mRNA of the euryhaline flatfish, *P. flesus*, was higher than that of the liver. Conversely, in a stenohaline flatfish, *S. maximus*, FMO activity was lower in gill than liver. Gill FMO activity from each species was kinetically unique from liver activity of *S. maximus* as well as other marine fish. In addition, gill FMO(s) were more structurally related to FMO 1 than FMO 2 while kidney FMO(s) may be more related to mammalian FMO 2. The presence of significant TMA-inhibited FMO activity, protein, and mRNA in the gill of a euryhaline fish suggests a possible role of FMO in osmoregulation.

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References


products of related but distinctly different genes. J. Biol. Chem. 265, 5855-5861.
4.4 CORRELATION OF SALINITY WITH FLAVIN-CONTAINING MONOOXYGENASE ACTIVITY BUT NOT CYTOCHROME P450 ACTIVITY IN EURYHALINE FISH (*PLATICHTHYS FLESUS*).
SHORT COMMUNICATION

Correlation of Salinity with Flavin-Containing Monooxygenase Activity but Not Cytochrome P450 Activity in the Euryhaline Fish (Platichthys flesus)

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ABSTRACT. To test the association between flavin-containing monooxygenases (FMOs) and osmoregulation, saltwater-adapted euryhaline flounder (Platichthys flesus) were acclimated to 34 (ambient), 25, and 15 parts per thousand (%) salinity for 1 or 2 weeks. FMO activity (thiourea S-oxide) was assayed in gill and liver microsomes in P. flesus. Branchial FMO activity was reduced dramatically (98%) in fish exposed to a salinity of 15‰ as compared with control, while hepatic FMO activity was reduced by 60%. Reduction of FMO activity in response to reduced salinity (15‰) appeared to occur within 1 week or less in both liver and gill of the flounder. Although hepatic FMO activity continued to fall and was not detected after 2 weeks at 15‰, branchial FMO activity was still present. A dose-response relationship in FMO reduction was present in liver, but there was no difference observed between 25 and 15‰ salinity in FMO activity of flounder gill. Serum osmolality and hepatic cytochrome P450 content were unchanged by salinity. In an attempt to determine whether trimethylamine (TMA) plays a role in piscine FMO, the effect of TMA on hepatic and branchial FMO activity was examined. Intraperitoneal injections of TMA failed to induce activity. Thus, an association between osmoregulatory function and FMO expression was observed in a species of euryhaline fish, indicating that alterations by salinity may affect xenobiotic biotransformation in euryhaline animals. BIOCHEM PHARMACOL 52:5:815-818, 1996.

KEY WORDS. flavin-containing monooxygenases; osmoregulation; trimethylamine; salinity; euryhaline.

FMOs are a polymorphic family of enzymes, found in the smooth endoplasmic reticulum of the cell, that primarily catalyze the oxidation of soft nucleophilic heteroatom-containing substrates to their respective oxides [1, 2]. Five gene families have been partially characterized in mammals, which appear to correspond to at least five unique isoforms that are differentially expressed in various tissues [3]. For example, form 2 of FMO (designated FMO2) is the predominant form in rabbit lung, whereas expression of FMO1 is greater in the liver [4, 5]. The differential expression of FMO isoforms in various tissues suggests distinct functional roles for each isoform. Although a role in the biotransformation of xenobiotics has been established for FMOs, endogenous functions have not been identified clearly for the isoforms. Thus, it has been difficult to determine how FMOs evolved into significant xenobiotic oxidases.

One reason why endogenous functions have not been assigned to FMOs is that few endogenous substrates for the isoforms have been identified [1, 2]. Early work demonstrated that FMO1 in pig liver was responsible for the oxidation of cysteamine [1]. Recent studies have shown that FMO3 in the rabbit kidney catalyzes the S-oxidation of methionine [6], as well as other cysteine conjugates [7]. Lastly, it has been reported that FMOs may be responsible for the oxidation of choline-derived TMA to TMAO. Studies in humans afflicted with "fish odor syndrome" or trimethylaminuria, have indicated a genetic deficiency in certain individuals who are unable to convert odorless TMA to the non-odorous TMAO [8, 9]. In vitro studies have shown that one or more forms of FMO are responsible for this reaction and that individuals with this disease also possess low FMO activity [10].

Although TMAO appears to have no known function in mammals, it serves an osmoregulatory function in marine elasmobranchs [11]. Initial studies of sharks, who possess tissue levels of TMAO as high as 70 mM, indicated that these organisms have relatively high levels of FMO activity [12]. Recently, we have shown that at least two shark and one marine teleost species possess significant FMO activity and a microsomal protein from the liver that reacts with antibodies generated against mammalian FMO1 and FMO2 [12-14]. Examination of freshwater fish species that do not possess TMA or TMAO, such as the channel catfish, showed that catfish do not have detectable FMO activity or...
homologous protein in liver, gill, or kidney [15]. However, anadromous freshwater species such as the rainbow trout have FMO activity in liver, kidney, and gill, as well as two homologous proteins found in each tissue [16, 17]. Recent studies with a saltwater-adapted euryhaline fish, the flounder (Platichthys flesus), showed that the gill possesses the highest amounts of activity, followed by the kidney, whereas the liver had negligible activity [18]. The differential expression of FMO activity and protein in the gill, which is the predominant osmoregulatory organ of this euryhaline species, and the lack of expression in TMA-deficient species were consistent with the hypothesis that piscine FMOs serve an osmoregulatory function. Consequently, the purpose of this study was to examine the role of FMO in osmoregulation by examining the effect of change in salinity on FMO expression in a euryhaline teleost.

MATERIALS AND METHODS

Animals

Sexually mature flounder (P. flesus, length 25–30 cm) were caught in local waters off Plymouth, U.K., and maintained in 1000-L tanks with continuous flowing seawater at 12–15°C for 1 month before analysis. Four fish were sampled randomly and placed in one 40-L aquarium containing seawater at 34‰ (100% seawater) for 2 weeks. A second group of four fish were also placed in another 40-L aquarium containing seawater at 25‰ (1:4 dilution with dechlorinated tap water) for 2 weeks. Lastly, two groups of four fish were placed in two separate 40-L aquaria (four fish/container) containing seawater at 15‰ (1:4 dilution). One of these 15‰ groups was maintained for 1 week while the other was maintained for 2 weeks. As observed in previous studies with trout [16] and turbot [14], no sexual differences in branchial or hepatic FMO activity were observed between male and female flounder (data not shown); thus, data from animals of both sex were combined. Each static system was aerated and changed daily, and fish were fed a diet of fresh fish daily. Following exposure (1 or 2 weeks), fish were removed from the aquarium, blood was sampled from the caudal vein, and then the fish were euthanized. Tissues were dissected immediately and used for enzyme analyses. Blood osmolality was measured using a Wescor 5100C Vapor Pressure Osmometer.

To determine the effects of TMA on FMO expression, four mature male flounder in flow-through tanks were given intraperitoneal injections of 100 mg/kg TMA on days 0 and 2 and were killed on day 3. Control animals received an equal volume of the carrier, nanopure water. Livers were dissected on day 3 and frozen at −80°C for enzyme analysis.

Chemicals

Thiocholine was the gift of Dr. Daniel Ziegler. Most of the remaining biochemical reagents, including NADPH, and phenylmethylsulfonyl fluoride (PMSF) were from the Sigma Chemical Co. Ltd., U.K. and U.S.A.

Microsomal Preparation for Enzyme Studies

All sample procedures were carried out at 4°C. Dissected tissues from three individual fish were homogenized in 100 mM Tris–HCl, pH 7.6, containing 0.1 M KCl, 0.1 mM PMSF (added in a minimal volume of ethanol), using either an electrically driven Potter-Elvehjem (liver) or a Polytron (gill) homogenizer. The microsomal (100,000 g) pellet was resuspended in 100 mM potassium phosphate, pH 8.0, containing 20% (v/v) glycerol. This microsomal suspension was used immediately as the source of enzyme activity.

FMO activity was assessed in flounder by measuring the thiourea-dependent oxidation of thiocholine [19]. Briefly, in a total volume of 1.0 mL, containing 0.1 mM potassium phosphate, pH 8.8, 0.1 to 1.0 mg of microsomal protein, 0.15 mM thiocholine, 0.1 mM NADPH, and 1.2 mM thiourea was added. After 30 min, 0.8 mL was removed, placed in a tube containing 0.1 mL of 3 M trichloroacetic acid, and centrifuged for 10 min at 10,000 g. The resulting supernatant was then transferred to a tube containing 1 mL of 1.0 M potassium phosphate, pH 7.5, 0.6 mL of water, and 0.05 mL of dithionitrobenzoate (DTNB) (10 mM). Incubation conditions were determined from previous studies [12, 14, 20]. The concentration of thiocholine was measured by using a millimolar absorptivity of 13.6 cm-1 M-1 for 5-thio-2-nitrobenzoate at 412 nm and compared against incubations that did not contain NADPH or thiourea. Hepatic cytochrome P450 content was measured by the method of Omura and Sato [21]. Protein was measured by the method of Lowry et al. [22].

RESULTS AND DISCUSSION

Utilizing organisms that reside in different osmoregulatory "media" (i.e. teleost fish), studies have shown that hepatic FMO activity is present primarily in saltwater fish or freshwater fish, such as the rainbow trout (Oncorhynchus mykiss), that are able to adapt to salinity conditions [13]. In addition, examination of FMO activity, protein, and mRNA in the euryhaline species of flatfish used in the current study, P. flesus, showed that expression of FMO and its activity were higher in the osmoregulatory organs, gill and kidney, than in liver, further supporting an association between FMOs and osmoregulation [18]. Previous studies examining the euryhaline American eel and guppy found that TMA oxidase was induced by enhanced salinity as well as intraperitoneal injections of TMA [23]. Consequently, the effect of salinity on FMO expression was examined in a euryhaline fish to test the hypothesis that FMOs have a role in osmoregulation.

To determine whether daily water changes impaired osmoregulatory function, the serum osmolality of flounder was examined after 2 weeks, prior to liver and gill dissection (Table 1). No significant differences in serum osmolality...
were observed between fish held in a 1000-L container with recirculating seawater (untreated fish) and those that resided in 40-L containers receiving daily water changes and salinity treatments. Earlier studies have shown that excessive handling of fish can impair osmoregulatory function and cause temporary laboratory diuresis [24]. To prevent this type of phenomenon in this study, fish were not handled during the entire duration of exposure. Water was changed by siphon, with daily salinity measurements. Serum osmolality was unaltered by any of the salinity treatments, indicating no impairment of osmoregulatory function. To determine whether salinity reduced biochemical function in general, hepatic cytochrome P450 content in flounder and was also unchanged by salinity was examined in flounder and was also unchanged by salinity.

As shown in earlier FMO studies with N,N-dimethyl-aniline, thiourea oxidase in flounder gill, although extremely variable, was significantly higher in gill than liver (Table 2). In contrast, FMO activity was found to be higher in the liver than in the kidney or gill in the stenohaline flatfish Scophthalmus maximus [14]. FMO activity and protein were also higher in the liver than in kidney and gill, respectively, in freshwater-raised rainbow trout [25]. Thus, hepatic FMO activity and protein appear to be relatively greater in freshwater-raised euryhaline fish than in saltwater-raised euryhaline fish. It should be noted, however, that FMO activity has only been examined in one species of saltwater-raised euryhaline fish. Clearly, more species need to be examined to see whether other species of saltwater-raised euryhaline fish have profiles similar to that of the flounder.

To determine whether a functional relationship between FMO and osmoregulation was present, saltwater-adapted flounder were housed in chambers with decreased salinity. FMO activity in both gill and liver microsomes was directly correlated with salinity exposure (Table 2). Reduction in activity appeared to occur within a 1-week period in both tissues of flounder. Preliminary studies in the euryhaline medaka showed increases in a 57 kDa branchial FMO-like protein within 24 hr after increasing salinity. In flounder, changes in FMO activity appeared to occur within 1 week. During salinity changes, dramatic histological and biochemical changes occur in euryhaline fish such as the up-regulation of Na+/K+-ATPase and the production of chloride cells [24]. How these well-characterized changes affect FMO expression and the time-course of FMO expression after altering salinity is currently being explored.

Increases in TMAO have been observed in freshwater-raised euryhaline fish as they migrate to higher salinity [26]. Thus, flounder were treated with TMA to determine whether this osmoregulatory compound had any effect on FMO activity. Treatment of flounder with various doses of TMA did not alter FMO activity in flounder (data not shown). This is consistent with previous studies in freshwater-raised rainbow trout and channel catfish, which showed that intraperitoneal injection of TMA did not change levels of FMO activity or protein [15, 17]. However, these results contrast with the studies performed by Daiskoku et al. [23], who treated freshwater-raised eel and guppy with an intraperitoneal dose of TMA and increased salinity and observed a significant induction of hepatic TMA oxygenase and TMAO in the treated animals. Thus, regulation of FMO does not appear to be consistently controlled by TMA. Although species differences may explain this phenomenon, it is likely that other mechanisms are responsible for enzyme regulation. Examinations of the effects of osmoregulatory hormones on enzyme expression is currently underway and should provide better insight into the mechanism of FMO regulation, function, and evolution.

**TABLE 1. Effect of salinity on serum osmolality and hepatic P450 content in the flounder (P. flesus)**

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>Hepatic P450 content (nmol/mg)</th>
<th>Osmolality (mosmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>0.090 ± 0.075</td>
<td>307 ± 8</td>
</tr>
<tr>
<td>15</td>
<td>0.076 ± 0.003</td>
<td>303 ± 4</td>
</tr>
<tr>
<td>15</td>
<td>0.071 ± 0.003</td>
<td>302 ± 2</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD of four individuals.

**TABLE 2. Effect of time and salinity on hepatic and branchial FMO activity in the flounder (P. flesus)**

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>Duration</th>
<th>FMO activity (nmol thiourea oxidized/mg/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hepatic</td>
</tr>
<tr>
<td>Un treated</td>
<td></td>
<td>1.35</td>
</tr>
<tr>
<td>34</td>
<td>1 week</td>
<td>2.12</td>
</tr>
<tr>
<td>15</td>
<td>1 week</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.28</td>
</tr>
<tr>
<td>34</td>
<td>2 weeks</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.29</td>
</tr>
<tr>
<td>25</td>
<td>2 weeks</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.12</td>
</tr>
<tr>
<td>15</td>
<td>2 weeks</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

Each value is the measurement of enzyme activity in an individual fish.

* ND - not detected (<10 pmol/mg/min).
+ Not measured.

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* Schlenk D. manuscript submitted for publication.
References

In the studies of Chapter 4, FMO expression and microsomal *in vitro* pathways of FMO-dependent oxidative metabolism were determined in different tissues sampled from *S. maximus* and *P. flesus*. The *in vitro* substrates, thiourea and methylamines (and other soft nucleophiles) may be available in the diets of these fish and have the potential to be *in vivo* FMO substrates. The physico/chemical properties of the characterised FMO activities were observed to be similar to other published studies of fish FMO e.g. alkaline pH optimum and heat sensitivity (Schlenk & Buhler 1991a; Ágústsson & Strom 1981), and at least 2 FMO forms were indicated in both *S. maximus* and *P. flesus*. Interspecies differences were observed however in the tissue distribution of FMO: the euryhaline *P. flesus* reported the highest determined FMO activity in branchial microsomes whereas the highest FMO activity of the stenohaline *S. maximus* was observed in hepatic microsomal preparations.

A reduction in *P. flesus* hepatic and branchial microsomal FMO activity appeared to occur within 1 week in response to maintenance of the fish in reduced salinity and this may suggest that during salinity compensation, euryhaline fish may have altered capacities for FMO-dependent xenobiotic metabolism.

To summarise the findings of Chapter 4 following the discussions presented in each paper;

Hepatic microsomal DMA oxidase activity was identified and characterised in the stenohaline teleost *S. maximus* with an *in vitro* temperature and pH optima of respectively 25 °C and pH 8.8. The apparent molecular weights of hepatic FMO1 and FMO2 proteins determined by Western blot were 55 kD and the expression of FMO1 mRNA was indicated. DMA oxidase activity was sensitive to co-incubation with other FMO substrates.
Hepatic, renal and branchial microsomal DMA oxidase activity was determined in the euryhaline *P. jlesus* with the highest-tissue specific activity measured in tissue from gills. The apparent molecular weight of hepatic FMO1 and FMO2 proteins determined by Western blot were 56 kD (FMO1) and, 55 & 57 kD (FMO2) and the expression of FMO1 mRNA was indicated. Gill microsomes had the highest levels of DMA oxidase activity.

Hepatic and branchial microsomal thiourea S-oxidase activity was determined in *P. flesus*. *P. flesus* maintained in estuarine salinity conditions reported lower FMO activity than fish at seawater salinities.
Oxygen-dependent metabolism has the potential to detoxify or bioactivate organic xenobiotics and these processes have been examined in marine fish and mussels. In Chapter 2 it was observed that the larval stages of fish appear to have an inducible MFO system (Section 2.2) and have the capacity to biotransform PAHs to reactive metabolites with carcinogenic potential (Section 2.3). It was observed that after a water-borne exposure to BaP for only 16 hours, juvenile fish can form bulky hydrophobic DNA adducts (Section 2.5). Since elevation of EROD activity was observed after 24 hours exposure to Ah ligands in larval, juvenile and adult fish, it is possible that the early life stages have mechanisms of CYP regulation similar to those described in adult fish (Stegeman & Hahn 1984).

Oxidative stress may be an inherent condition common to aquatic organisms, however in vitro prooxidant processes have been described in fish tissue (Lemaire & Livingstone 1993; Winston & Di Giulio 1991). Studies with adult fish tissue have demonstrated that such processes may be stimulated by xenobiotics (Lemaire & Livingstone 1993; Lemaire & Livingstone 1997) but in Section 2.3 it was observed that xenobiotics can similarly stimulate fish larval tissue ROS production. The addition of antioxidant enzymes into the in vitro system indicated the presence of O$_2^-$ and H$_2$O$_2$ and thus antioxidant enzymes may contribute to larval antioxidant processes. A suite of antioxidant enzyme activities were
quantified in both embryonic and larval life stages indicating that during the early life stages of fish, there is a requirement to counter ROS toxicity.

Fish embryo and larval sensitivity to xenobiotics has been the subject of many studies in recent years however relatively few of them have addressed the mechanisms of toxicity that occur in the early life stages. Binder & Stegeman (1980) were the first to report arylhydrocarbon hydroxylase activity in fish embryos and yolk sac larvae indicating that BaP may be bioactivated to carcinogens. In Section 2.3, larval MFO-dependent metabolites of BaP (putative tetrals, diols, phenols, quinones) were resolved confirming the presence of reactive metabolites. Pre-exposure of the larvae to PAHs altered the metabolite profile in favour of diols and phenols but not quinones indicative of CYP1A involvement. Elevation of fish larval EROD activity following exposure to BaP and the insecticide lindane further suggest that fish larval biotransformation pathways can be influenced by xenobiotic exposure, either enhancing or reducing toxicity. Lipophilic contaminants may be readily taken up from the marine environment by fish larvae (Cleary et al., 1993; Cooper et al., 1993; Hall Jr. et al., 1993) and the bioactivated metabolites may interact with DNA possibly accounting for observed variations in DNA adducts determined in feral larvae (Ericson et al., 1996). Fish embryos and larvae may be highly sensitive to DNA lesions produced either by direct interactions with bulky organic molecules (Varanasi et al., 1992) or as a consequence of ROS-DNA interactions (Wiseman & Haliwell 1996) since these early life stages are undergoing periods of cell division and cell growth requiring high rates of DNA turnover (Espina & Weis 1995). Cell division is an important process in mutagenesis and carcinogenesis and factors increasing cell division can increase rates of mammalian cancer (Ames & Gold 1996).

Correlation between larval EROD activity and PAHs or PCBs in their food was not indicated in the study reported in Section 2.6. In contrast, Stagg & McIntosh (1996)
observed a significant regression of larval EROD activity with water-column hydrocarbon concentrations sampled from the North Sea. This correlation may reflect alternative routes of uptake of xenobiotics into the larvae as well as differences in sample size, the responsiveness of the sentinel species as well as the methods of regression calculations. Since the inheritance of lipophilic xenobiotics can induce larval MFO activity (Binder & Lech 1984) the previous exposure history of the mother may have a significant influence upon feral larval EROD activity determinations. When hatchery-reared larvae are used for laboratory investigations, maternal exposure is usually standardised (eggs are from a single fish) and thus hatchery-reared larvae have the potential to be used in field monitoring studies. Gillet & Monod (1997) placed "caged" fish embryos in several freshwater tributaries of Lake Geneva and observed elevation of larval EROD activity at some sites compared to unpolluted laboratory reared larvae indicating field exposure to environmental Ah ligands. In the Lake Geneva study, it is noteworthy that the fertilised eggs from a particular female were divided: a sub-sample was reared in the laboratory as the control group, whilst other sub-samples were transplanted into the environment. Under such experimental conditions, elevation of EROD activity compared to controls is more likely due to environmental rather than maternal factors.

In contrast to the above studies concerning fish CYP-dependent monooxygenation, the presence of multiple CYP forms was investigated in molluscan bivalves. Immunodetection using mammalian and fish polyclonal antibodies indicated CYP1A, 2B, 2E, 3A and 4A epitopes in digestive gland microsomal preparations and partially purified CYP from Mytilus sp.. Studies of indigenous and transplanted mussels proposed differential regulation of the CYP epitopes, and in conjunction with estimates of apparent molecular weights, the presence of at least 5 isoforms were indicated. Wootton (1995) also concluded
that differential regulation of multiple CYP forms could occur seasonally, following comparisons of CYP1A, 3A and 4A mRNA levels over a 12 month period.

Following transplantation of mussels from a clean to a polluted site for 3 weeks, levels of the CYP1A epitope were higher in transplanted mussels than levels determined from the clean site, and were similar to those from an indigenous population of mussels sampled from a 2nd polluted site. The mechanism by which the CYP1A epitope is regulated is unclear however, exposure to PCBs in laboratory studies, (Livingstone et al., 1997) and PAHs in the field (Solé et al., 1996) have also elicited an apparent increase in Mytilus sp. CYP1A-immunopositive protein. Expression of this protein has the potential to be used as a biomarker of exposure to organic xenobiotics in marine and esturine environments once a) the regulatory processes that affect CYP1A-immunopositive protein expression have been characterised, and b) the specificity of the molecular probes employed to estimate its expression are improved.

Fish CYP-dependent metabolism of BaP can produce diols and diol epoxides however the major metabolites of mussel BaP metabolism are BaP quinones (Lemaire & Livingstone 1993). Quinones have the potential to redox cycle, generating ROS and the Appendix to this thesis contains firstly a paper describing oxidative DNA damage in mussel tissue following exposure to BaP and secondly a paper demonstrating that partially purified mussel CYP catalyses the formation of BaP quinones.

Differential expression was also observed when the presence of multiple forms of FMO were examined in different fish tissues. The expression of both FMO1 and FMO2 proteins were indicated in the liver of a euryhaline flatfish however only the FMO1 form was determined in branchial tissue (section 4.3). In vitro xenobiotic metabolism of amines and thiols were identified in flatfish (Section 4.2, 4.3 & 4.4). Tissue distribution studies and
inter-species comparisons of FMO-dependent \( N \)- or \( S \)-oxidase activities suggested a potential role of FMO in the synthesis of the osmolyte trimethylamine \( N \)-oxide. A decrease in FMO activity was observed when a euryhaline species was adapted to freshwater (Section 4.4). Since freshwater adaption by euryhaline species may reduce FMO activity then these organisms may consequently have a lower capacity to bioactivate carbamate pesticides however, it is also possible that exposure to carbamates may interfere with the production of TMAO and thus affect osmoregulation. Whereas this thesis examines the specific effects of pollutants at the molecular level, there are many examples of contaminants exerting deleterious physiological effects e.g. impaired reproductive success and reduced scope for growth either at the tissue, organ or whole-organism level (Taylor 1996; Walker et al., 1996a). Linking pollutant-mediated subcellular effects with whole organism responses, and indeed linking organisms with populations and communities are important processes in evaluating the biological effects of pollutants and as such require a detailed understanding of the biological organisation of the determined responses (Attrill & Depledge 1997).

The bioactivation of organic xenobiotics by CYP- or FMO-dependent mechanisms have the potential to cause deleterious changes in the cells of marine organisms either by the direct action of the metabolites with macromolecules or by the interference of endogenous functions of the monooxygenase metabolic pathways. Similarly xenobiotics e.g. plant allelochemicals or anthropogenic contaminants, or their metabolites, can also elicit damage to macromolecules such as proteins, lipids, RNA and DNA via the stimulation of ROS (Berenbaum 1995; Lemaire & Livingstone 1997; Stohs 1995). These molecular alterations have been implicated in disease progression, impairment of health, ageing and carcinogenesis (Ames & Gold 1996; Cand & Verdetti 1989; Davies 1995).
Future studies:

- Complete the purification of *Mytilus edulis* CYP and determine the amino acid sequence.

- Characterise the catalytic activity of the purified enzyme.

- Use oligonucleotide probes (determined from the above amino acid sequence) to determine CYP genes.

- Construct specific cDNA probes to examine regulation in *Mytilus* and other invertebrate species.

- Examine assays for determining DNA repair and apply to fish larval life stages as a potential biomarker of effect (a useful tool as a prognostic biomarker).
APPENDIX 1: OTHER PUBLICATIONS

The following two papers are additional to those included in Chapters 2, 3 and 4. The first is a published paper titled “Partial purification and properties of cytochrome P450 from the digestive gland microsomes of the common mussel, *Mytilus edulis* L.” The second paper is an “in press” manuscript titled “Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive proteins in mussels exposed to waterborne benzo[a]pyrene” and will appear in *Mutation Research.*
Partial Purification and Properties of Cytochrome P450 from Digestive Gland Microsomes of the Common Mussel, Mytilus edulis L.

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ABSTRACT

Cytochrome P450 from the digestive gland of M. edulis was partially purified by sodium cholate solubilization, 4–15% polyethylene glycol fractionation, and octyl-Sepharose affinity, DEAE-Sepacel ion-exchange and hydroxylapatite chromatography (yields of up to 7–10%). Three peaks were resolved by DEAE-Sepacel chromatography (termed peaks 1–3). P450 specific content was increased from 26 to 800 pmol per mg protein, and the ratio of P450 content to NADPH-cytochrome c (P450) reductase activity reduced by a factor of 250. Oxidised spectrum λmax of P450 was 410.5 ± 1.5 nm. Type II difference spectra were seen with both type II (clotrimazole, metyrapone) and type I (α-naphthoflavone, 7-ethoxycoumarin) compounds. Western blotting with polyclonal anti-P450/A from perch (Perca fluviatilis) gave a single band of approximately 54 kDa molecular weight. A reconstituted system containing peak 2 or 3, rat liver P450 reductase, lipid and NADPH metabolised benzo[a]pyrene to diones, diols, phenols and putative protein adducts. Peak 2 plus cumene hydroperoxide was indicated to produce diones and protein adducts only. Peak 2 alone was indicated to produce diones and phenols. The major free metabolites in all cases were diones (53–100%). The results indicate the existence of a P4501A-like enzyme in M. edulis, possibly with unusual properties as indicated by the difference spectra, metabolism in absence of NADPH and added P450 reductase, and predominance of diones.

Cytochrome P450 is the terminal component of the mixed-function oxygenase (MFO) system. It is indicated to exist as a multi-gene family throughout the animal kingdom (Livingstone, 1990; Wootton et al., 1995) and is of central importance in the metabolism of many endogenous and exogenous substrates, including organic pollutants such as polynuclear aromatic hydrocarbons (PAHs). In mussel it is primarily localised in the endoplasmic reticulum (microsomes) of...
the digestive gland and shows the unusual properties of MFO activity in the absence of added NADPH, and metabolism of the PAH benzo[a]pyrene (BaP) to predominantly diones (Livingstone et al., 1989). In order to investigate these phenomena and further characterise the molluscan MFO system, cytochrome P450 was partially purified and studied using modifications of a previous purification scheme (Kirchin et al., 1987; Livingstone et al., 1989).

Mussels (4–5 cm length) were collected from a clean, rocky shore population near Plymouth. Partially purified P450 was prepared from digestive gland microsomes by sodium cholate solubilization (1.3% w/v with 0.1% glutathione under nitrogen), 4–15% polyethylene glycol (PEG) fractionation, octyl-Sepharose affinity chromatography (elution with 0.2% emulgen), DEAE-Sephascel ion-exchange chromatography (elution with linear KCl gradient) and hydroxylapatite chromatography (elution with 300 mM KH2PO4/K2HPO4 pH 7.6/20% glycerol/0.1 mM EDTA). Spectrophotometric measurements were carried out on a Varian Cary I dual-beam spectrophotometer at 25°C. Cytochrome P450 and the contaminating ‘418-peak’ (haemprotein of unknown function — possibly denatured P450) were measured by the carbon monoxide (CO)-difference spectrum of sodium dithionite reduced samples (Livingstone, 1988; Livingstone et al., 1989). NADPH-cytochrome c (P450) reductase activity, total haemprotein (absorbance at 417 nm), total protein (Lowry method) and ligand-binding difference spectra were measured by standard procedures (Livingstone, 1988; Livingstone et al., 1989). SDS-PAGE was carried out according to Laemmli (1970) and Western blotting according to Towbin et al. (1979) using polyclonal anti-P4501A from perch (Perca fluviatilis) (gift from L. Förlin, University of Göteborg, Sweden) and alkaline phosphatase visualization. Reconstitution studies with 3H-benz[a]pyrene (BaP) as substrate were performed at 25°C according to Wolf et al. (1979) using 30 μg L-α-dilauroylphosphatidyl choline per ml and rat liver cytochrome P450 reductase (~ 70 pmol per pmol P450) (gift from C.R. Wolf, ICRF, Edinburgh, UK). BaP metabolites and putative protein adducts were analysed respectively by HPLC and protein precipitation as described in Lemai et al. (1993). Values are given as means ± SEM.

The maximum purification factor and yield obtained were × 34 (specific content increased from 26 to 800 pmol per mg protein c.f. 417 pmol per mg for previous scheme (Kirchin et al., 1987) and 7 to 10%. As observed previously (Livingstone et al., 1989) three peaks were resolved by DEAE-Sephascel chromatography (at 0.75 and 140–330 mM KCl), termed respectively peaks 1 to 3. The ratio of P450 content to NADPH-cytochrome c (P450) reductase activity was reduced by factors of (respectively) 549, 17 and 275 in the three fractions and the ‘418-peak’ was not detectable in peak 2.

Cytochrome P450 λmax (CO-difference spectrum) varied with purification from 449 to 447 nm, similar to that observed for other marine invertebrate P450s (Livingstone, 1991). The oxidised spectrum λmax of purified peak 2 and 3 was 410.5 ± 1.5 nm compared to 417 nm (plus a slight shoulder between 390 to 410 nm) for a previous peak 2 preparation (Kirchin, 1988), indicating that the P450 is mainly in the low-spin state but with a significant high-spin presence. Type II, or modified type II, difference binding spectra (Ks given after compound) were generally seen with both type I compounds (α-naphthoflavone, 8–83 μM; 7-ethoxycoumarin, 154–261 μM) and type II compounds (clotrimazole, 3–29 μM; metyrapone, 630 μM), as was also observed for ammonium sulphate fractionated
Partial purification and properties of cytochrome P450 from mussel

*M. edulis* P450 (Livingstone *et al.*, 1989) and digestive gland microsomes of the pond snail *Lymnaea stagnalis* (type I compounds hexobarbital and 2,2'-dichloro-biphenyl) (Wilbrink *et al.*, 1991). Thus, whatever the mechanistic explanation for the type I compounds showing type II spectra (displacement of endogenous substrate, interaction of substrate functional groups with haem, unique properties of P450) (Livingstone *et al.*, 1989), the phenomenon persists with purification of the P450 and is not restricted to a single molluscan species.

Western blotting of the octyl-Sepharose fraction with anti-P4501A from *P. fluviatilis* gave a single band of 54 kDa apparent molecular weight (Fig. 1), indicative of the existence of a P4501A-like epitope or protein in digestive gland of *M. edulis* and consistent with observations on the presence of P4501A-like DNA (Southern blotting) and mRNA (Northern blotting) sequences in the same tissue (Wootton *et al.*, 1995). The results are also consistent with Western blotting of digestive gland microsomes of the chiton *Cryptochiton stelleri* with anti-P450LM4b (anti-P4501A) from trout (*Oncorhynchus mykiss*) which also revealed a major immunoreactive band at 54 kDa (Schlenk & Buhler, 1989).

Peaks 2 and 3 in a reconstituted system containing rat hepatic cytochrome P450 reductase and NADPH metabolised BaP to diols, diones, phenols and putative tetrols and protein adducts (Table I). No major differences were evident between the patterns of metabolism between the two peaks. Diones were the main products formed (46–71% of total), consistent with observations on crude molluscan microsomes (Livingstone *et al.*, 1989) and indicative that this catalytic feature is a function of the *M. edulis* cytochrome P450. The formation of dihydrodiols is presumably due to the presence of epoxide hydratase in the enzyme preparations. The levels of protein adducts were much lower than for digestive gland microsomes (Lemaire *et al.*, 1993), presumably due to the lower levels of protein present in the assay. The total rates of metabolism of partially purified P450 (24–34 pmol per min per nmol P450) were within the range normally observed for digestive gland microsomes (Lemaire *et al.*, 1993). However, they

![Fig. 1. Western blot of partially purified (Octyl-Sepharose fraction) cytochrome P450 from digestive gland microsomes of *M. edulis*. From left to right: β-naphthoflavone-induced turbot (*Scophthalmus maximus*) hepatic microsomes (lane 1); pre-stained and unstained mol. wt. markers (lanes 2 and 3); mussel P450 preparation at two protein loadings — 19 μg (lane 4), and 9 μg (lane 5).](image-url)
TABLE 1
Metabolism of Benzo[a]pyrene by a Reconstituted System Containing Partially Purified Cytochrome P450 from Digestive Gland Microsomes of M. edulis, L-α-Dilauroylphosphatidyl Choline, NADPH and Rat Hepatic Cytochrome P450 Reductase

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative tetrols</td>
<td>2.49 ± 0.37 (10.6%)</td>
<td>7.66 ± 2.04 (22.7%)</td>
</tr>
<tr>
<td>9,10-diol</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7,8-diol</td>
<td>0.70 ± 0.28 (3.0%)</td>
<td>3.55 ± 1.61 (10.5%)</td>
</tr>
<tr>
<td>1,6-dione</td>
<td>7.36 ± 1.64 (31.2%)</td>
<td>7.27 ± 4.20 (21.6%)</td>
</tr>
<tr>
<td>3,6-dione</td>
<td>7.84 ± 1.43 (33.2%)</td>
<td>6.85 ± 3.80 (20.3%)</td>
</tr>
<tr>
<td>6,12-dione</td>
<td>1.66 ± 0.26 (7.0%)</td>
<td>1.45 ± 1.18 (4.3%)</td>
</tr>
<tr>
<td>3- and 9-phenols</td>
<td>1.42 ± 1.16 (6.0%)</td>
<td>2.83 ± 1.17 (8.4%)</td>
</tr>
<tr>
<td>Putative protein adducts</td>
<td>2.14 ± 0.01 (9.0%)</td>
<td>4.13 ± 0.01 (12.2%)</td>
</tr>
<tr>
<td>Total polar metabolites</td>
<td>23.6 ± 0.1 (100%)</td>
<td>33.7 ± 4.0 (100%)</td>
</tr>
</tbody>
</table>

*Peaks 2 and 3 from DEAE-Sephacel purification. Means ± SEM (n = 3). Units in pmol min⁻¹ nmol⁻¹ P450. Protein adducts are total BaP-derived radioactivity bound to protein fraction. The % of total metabolism of each metabolite is given in parenthesis.

...were much lower than those observed for a similarly reconstituted purified P450 preparation from hepatopancreas microsomes of the spiny lobster, Panulirus argus, viz. 1500 pmol per min per nmol P450 (James, 1990). Single incubations were also carried out with peak 2 and 1 mM cumene hydroperoxide, and peak 2 alone. The former produced only the 1,6-, 3,6- and 6,12-diones and protein adducts (respectively, 13, 32, 50 and 5% of total metabolism). The total rate of metabolism was similar to that in the presence of NADPH, viz. 53 pmol per min per nmol P450, as was also observed for microsomes (Lemaire et al., 1993). Peak 2 alone also produced metabolites, viz. 1,6-, 3,6- and 6,12-diones (73% total) plus 7,8-diol, 3/9-phenol and protein adducts (total rate 76 pmol per min per nmol P450), consistent with observations of NADPH-independent BaP metabolism by mussel cytochrome P450 (Livingstone, 1990; Lemaire et al., 1993).

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Partial purification and properties of cytochrome P450 from mussel

Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive proteins in mussels exposed to waterborne benzo[a]pyrene

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Abstract

A collaborative study was performed on Mediterranean mussels (*Mytilus galloprovincialis*) exposed to a wide dose-range (0.5-1000 ppb) of benzo[a]pyrene (B[a]P). We selected this model polycyclic aromatic hydrocarbon in order to confirm the formation of a specific DNA adduct, previously detected in gill DNA, and to clarify the *in vivo* effects of this mutagenic chemical requiring host-metabolism in mussels.

B[a]P concentration reached consistently higher values in the digestive gland than in other analyzed tissues (49.6-75.8, 342-360, and 47.1-81.5 μg/g wet weight in gills, digestive gland and remaining soft tissues, respectively) of mussels exposed to B[a]P for 2 or 3 days. With the exception of some values at 1000 ppb of B[a]P, DNA adduct levels increased significantly with the dose in gills and digestive gland and ranged from 0.054 to 0.789 adducts per 10^8 nucleotides (mean values per dose-point). Conversely, more complex dose-response relationships were found by detecting in parallel the levels of an oxidative DNA lesion (8-OHdG) and of CYP1A-immunopositive proteins (the latter measured in the digestive gland only).

Overall, the formation of DNA adducts, the evidence of oxidative DNA damage, and changes in CYP1A-immunopositive protein levels support the hypothesis that B[a]P can induce DNA damage in mussels through a number of different molecular mechanisms.

*Key words:* DNA adducts, 8-OHdG, CYP1A-immunopositive protein, *Mytilus galloprovincialis*, benzo[a]pyrene

Abbreviations: BPDE, anti (±)-trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; B[a]P, benzo[a]pyrene; BPQs, benzo[a]pyrene quinones; CYP1A, cytochrome P4501A; dG, deoxyguanosine; dNps, deoxyribonucleoside 3'-monophosphates; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; DMSO, dimethylsulfoxide; MFO, mixed function oxygenases; PAH, polycyclic aromatic hydrocarbons; RAL,
relative adduct labelling; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SW, sea water; TLC, thin layer chromatography; w.w., wet weight.

1. Introduction

For many years bivalve mollusks, such as *Mytilus galloprovincialis* and other mytilid species, have been used as bioindicators of chemical pollution of coastal waters in the so called "Mussel Watch" programmes (1,2). Attention has also been focused on the environmental impact of genotoxic pollutants (3-6) and, more recently, on the application of sentinel organisms, such as mussels, in the biomonitoring of environmental genotoxic agents (7-12).

In addition to the influence of environmental factors on the bioavailability of chemical pollutants, the use of sentinel organisms may be limited by life habits (e.g. only benthic or caged fish may be representative of a local area) as well as physiological and biochemical features of the selected species.

Although mussels appear to be suitable bioindicators (sessile, filter-feeding in the water column, widely distributed in coastal waters and relatively tolerant to environmental changes) they are phylogenetically distanced from man and mammals. Their biochemical pathways may diverge consistently and we still need information on mechanisms underlying xenobiotic uptake and metabolism as well as DNA repair (13-17).

It has been established that cytochrome P450 monooxygenases, or mixed function oxygenase (MFO) are present in mussels, although to a limited extent if compared with fish and mammals (18-22). Despite contrasting opinions on the formation of benzo[a]pyrene (B[a]P)-DNA adducts in marine invertebrates, we have recently demonstrated the dose-related formation of a specific adduct in gill DNA of *M. galloprovincialis* exposed to water-borne B[a]P (23-25). Furthermore, it is interesting that at least half the B[a]P metabolites in mussels are benzo[a]pyrene
quinones (BPQs) which can undergo redox cycling to generate the superoxide anion radical \(\text{O}_2^{-}\) and other reactive oxygen species (ROS) \((20,26,27)\).

In Fig. 1 a scheme, based mainly on mammal cell systems, depicts current opinions on possible molecular mechanisms for \textit{in vivo} activation of B[a]P to DNA-reactive intermediates (the detoxifying role of epoxide hydrolase and conjugating enzymes is not specified).

Pathway 1 refers to the "bay-region theory": two-electron monooxygenation mediated by the NADPH-dependent cytochrome P450 reductase leads to epoxides, phenols, diols and tetrols \((28,29)\). Stereoselective diolepoxides probably represent the most investigated B[a]P metabolites \((30-32)\). In particular, the (+)-anti-B[a]P-7,8-diol-9,10 epoxide forms stable DNA adducts whose mutagenicity and carcinogenicity have been widely studied \((33-37)\).

As discussed by Penning and co., dehydrogenation of 7,8-diols mediated by dihydrodiol dehydrogenase (DD) can divert diols from pathway 1 to yield transient catechols (subject to auto-oxidation), quinones \((1,6-3,6-\) and \(6,12\)-diones) and quinone redox cycling (pathway 2); ultimately, BPQ redox cycling is responsible for amplification of free radicals such as semiquinone anion radicals and ROS \((38)\). Apart from the capacity of BPQs to bind DNA and form unstable adducts, these radicals account for oxidative damage and DNA fragmentation \((39-41)\).

Pathway 3 specifies the direct formation of DNA-reactive radical cations through one-electron oxidation, possibly mediated by the cytochrome 450 or by peroxidative reactions \((42)\).

A further hypothesis for B[a]P-induced DNA damage (pathway 4) postulates endogenous methylation of B[a]P in \textit{meso}-anthracenic position and formation of the ultimate DNA-reactive metabolite, benzylic carbenium ion, through either one-electron oxidation or formation of methyl ester derivatives \((43)\).

Monooxygenation and one-electron oxidation are currently considered the two main reactions for the activation of B[a]P and PAH in general. Since one-electron oxidation and radical cation reactions lead to unstable adducts (such as B[a]P6-
N7Gua, B[a]P6-C8Gua, B[a]P6-N7Ade) which represent the great majority of total DNA adducts in rodents, the mutagenic and carcinogenic role of radical-DNA interactions and DNA depurination is under discussion (42,44).

Taking into account the above evidence and potential pathways, we undertook a collaborative study to evaluate in parallel the tissue B[a]P concentration, the biological effective dose (DNA adducts), a typical DNA oxidation product (8-hydroxy-2'-deoxyguanosine, 8-OHdG) as well as the levels of cytochrome P4501A (CYP1A)-immunopositive proteins in gills and digestive gland of *M. galloprovincialis* exposed to waterborne B[a]P. The levels of CYP1A-immunopositive proteins in mussel digestive gland have been observed to increase with exposure to classical CYP1A-inducers, i.e. polychlorobiphenyls (45) and have been implicated in the metabolism of B[a]P (46,45).

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (B[a]P, CAS No. 50-32-8, Sigma-Aldrich, Milano, Italy) was dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) and added to artificial sea water (SW) at the nominal doses indicated below. All biochemicals for CYP1A-immunopositive protein determination were from Sigma-Aldrich, Poole, UK, and those for DNA adduct detection were from Sigma-Aldrich and Boehringer Mannheim Italia, Milano, I. All other reagents were of an analytical degree or suitable for organic trace analysis.

2.2. Organisms

Mediterranean mussels (*Mytilus galloprovincialis*, Lmk.) were collected from a shellfish farming area of the Venice lagoon in May 1995. Following forced spawning of a few ripe individuals, mussels were maintained and checked as previously reported (25).
2.3. Exposure to B[a]P

Groups of 5 mussels, all grown on the same rope, were selected by size (4.5-5.5 cm shell length) and exposed to 0 (solvent only), 0.5, 5, 50, 100, 500, 1000 ppb of B[a]P in glass tanks (1 liter SW per mussel) for 48 and 72 hours. Final DMSO concentration was 0.005%. Water, B[a]P and food were changed 3 times/day. At the end of the exposure mussels were dissected and gills, digestive gland and remaining soft tissues from each dose-point were pooled and frozen at -76 °C (with the exception of individually frozen pieces of the digestive gland for CYP1A-immunopositive protein determination).

2.4 B[a]P analysis

Pooled tissue samples were analyzed following pentane extraction and HPLC/fluorimetric detection. Briefly, gills, digestive glands and other soft tissues were Polytron-homogenized, samples of 1 ml were saponified with 1.5 ml 3M KOH, sonicated (45' at 60°C) and 1 ml of methanol was added to avoid gel formation during the following step. Three extractions with 3 ml of pentane were performed. Then, the volume of the combined extracts was reduced to 1 ml and the solvent exchanged with acetonitrile under gentle nitrogen stream. After dilution to 10 ml, samples were injected through a syringe-filter (PTFE, 4mm Ø, 0.2 µm pore size) into the chromatograph and isocratic elution (water: acetonitrile 15:85 v/v ratio) at 1.5 ml/min was applied. Chromatograph 800 HRLC equipped with integrator (Bio-Rad, Hercules, CA), column Altex Ultrasphere ODS, 5 µm, 150x4.6 mm (Beckman Instruments Inc., Fullerton, CA) and detector RF-551 (Shimadzu, Kyoto, Japan) set at 264/406 nm were the components of the analytical system. A guard column (Hypersil Green PAH, 10x4.6 mm, Shandon, Runcorn, U.K.) was employed to avoid the introduction of strongly retained compounds into the analytical column. The use of a multi-port valve between the guard and the analytical column allowed on-line back-washing of the former during the analysis through a supplementary pump which provided a flow of
acetonitrile 85%. B[a]P was quantified by single-point calibration while the assessment of B[a]P recovery was based on the internal standard addition method.

2.5 DNA purification

Pools of gills and digestive glands (1 g) were Polytron-homogenized in 10 ml buffer (10 mM EDTA, 20 mM Tris-HCl pH 7.5, 0.5 % SDS) and subjected to DNA purification as previously reported (25). Briefly, samples were digested with RNAse I and proteinase K, adjusted to 1 % SDS, heated at 60°C for 10 min. High molar sodium perchlorate was immediately added and DNA extracted by gently shaking with 24:1 chloroform: isoamyl alcohol for 30 min at room temperature. DNA was then precipitated with cold absolute ethanol, suspended in TE buffer (10 mM Tris-HCl/ 1 mM EDTA, pH 7.4) and precipitated again with sodium acetate and cold ethanol. DNA samples, re-suspended in MilliQ water, were UV quantified and used for DNA adduct and 8-OHdG detection.

2.6 DNA adduct analysis

In gills and digestive gland DNA, adducts were detected by the nuclease P1-enhanced ^32P-postlabeling assay (47,48) following procedural details already reported (25). DNA samples (5 μg) were enzymatically digested to deoxyribonucleoside 3' monophosphates (dNp). After nuclease P1-mediated dephosphorylation of normal dNps, modified dNps (adducts) were labeled with [γ-^32P]ATP (specific activity > 5,000 Ci/mmol) by T4 polynucleotide kinase; free [γ-^32P]ATP was then hydrolysed by incubation with potato apyrase. The molar excess of [γ-^32P]ATP was ascertained by 1-D chromatography (Polygram CEL 300 PEI, 0.12 M sodium phosphate, pH 6.8) of suitable amounts of postlabeled reference DNA.

^32P-postlabeled DNA samples were applied to PEI cellulose plates and bidimensional thin layer chromatography (TLC) developed (buffers: D1, 1 M Na phosphate pH 6.8; D2, 5.3 M Li formate, 8.5 M urea, pH 3.5; D3, 1.2 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 8; D4, 1.7 M Na phosphate pH 6). Adduct spots were
detected by autoradiography, cut out from the TLC plates and quantified by Cerenkov counting.

DNA adduct levels are expressed as relative adduct labeling (RAL, i.e. number of adducts per number of normal dNps) according to:

\[
\text{RAL} = \frac{\text{cpm in adduct spot(s)}}{\text{S.A.} \times \text{total dNp}}
\]

cpm in adduct spot(s) = cpm in adduct spot area after subtraction of an equivalent blank area;
S.A. = specific activity (cpm/pmol) calculated by the \(^{32}\text{P}\) decay factor and the specific activity of \([\gamma-^{32}\text{P}]\text{ATP}\) at the calibration date;
total dNp = pmoles of dNp subjected to TLC.

Reference DNA modified \textit{in vitro} with \(^{3}\text{H}-\text{BPDE} (13.2 \text{ pmol BPDE/mg DNA})\) was included in each postlabeling assay as positive control. Samples were tested in three replicates at least.

2.7 8-hydroxy-2' deoxyguanosine (8-OHdG) quantification

The separation of 8-OHdG and normal deoxyribonucleosides was performed in a LC-18-DB (Supelco, 150x4.6 mm) column equipped with a LC-18 guard column cartridge. Elution was performed with an isocratic mixture of 90% 50 mM potassium phosphate, pH 5.5, and 10% methanol at 1 ml/min flow rate. UV detection was accomplished at 254 nm and electrochemical analysis was by a PED detector (Pulsed Electrochemical Detector, Dionex, Sunnyvale, CA). A calibration curve with known amounts of calf thymus DNA was used for the quantification of deoxyguanosine (dG). The levels of 8-OHdG were referred to the amount of dG detected by UV absorbance at 254 nm and expressed as number of 8-OHdG per \(10^5\) dG bases (8-OHdG/\(10^5\) dG).
8-OHdG standard samples were prepared according to previously published methods (49). Samples were tested in three or more replicates.

2.8 Determination of CYP1A-immunopositive proteins

Immunoreactive CYP1A-like proteins were detected using polyclonal antibody to hepatic CYP1A from *Perca fluviatilis* and the immunopositive protein was identified using partially purified cytochrome P450 from digestive gland of *Mytilus edulis* as a positive control (50, 45). Sub-cellular fractions were prepared at 4°C using individual digestive glands. Each digestive gland was homogenized by sonication in 300 μl 10mM Tris-HCl, pH 7.6 containing 0.15 M KCl, 0.5 M sucrose, 16.8 mU trypsin inhibitor (aprotinin). Homogenates were centrifuged for 30 min at 11600xg and the protein content of the supernatant was determined according to the Lowry method (51). Supernatants were diluted with distilled water to 10 mg/ml and boiled with equal volumes of 0.125 M Tris-HCl, pH 6.8, 4% SDS (w/v), 20% glycerol (v/v), 10% 2-mercaptoethanol (v/v), 0.001% bromophenol blue (w/v) for 2-3 min. Cracked proteins (50 μg) were loaded on 10% SDS gels and electrophoresis was performed (52). Gels were semi-dry blotted on to nitrocellulose and blots were then washed in 10 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl (Tris-buffered saline, TBS), 0.5% gelatin (w/v), 0.2% Tween 20 (v/v), 0.1% Na azide (w/v), for 30 min. Incubation with rabbit anti-perch CYP1A antibody was performed in the buffer (1:1000 v/v) for 12-14 h at room temperature. After two washing steps (15 min each) in TBS containing 0.2% Tween 20 (v/v), blots were incubated with alkaline phosphatase-labelled goat anti-rabbit IgG for 1 h (1:3000 v/v in TBS containing 0.5% gelatin (w/v) and 0.2% Tween 20 (v/v). After secondary antibody incubation and washing, the immuno-reaction was visualized by the addition of 0.48 mM nitroblue tetrazolium and 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.2, 59.3 mM NaCl, 5 mM MgCl2.

Immunopositive bands were semiquantified by image analysis using a Kontron image analyzer (Image Processing System, UK).
3. Results

3.1 Internal B[a]P dose

B[a]P was analyzed separately in pools of gills, digestive glands and remaining soft tissues (mantle, foot, etc.) of the experimental mussel groups.

In contrast with the control group, mussels exposed to 0.5-1000 ppb of B[a]P showed detectable B[a]P concentrations, the internal dose rising parallel with the exposure dose up to 500 and, in some cases, up to 1000 ppb of B[a]P (Table 1). Digestive gland displayed the highest B[a]P accumulation (maximum 342.5 and 360.0 µg/g w.w. at 48 and 72 h, respectively) whereas similar absolute B[a]P values were detected in the gills and other soft tissues (maximum 49.6 and 75.8 in gills and 47.1 and 81.5 in the remaining tissues at 48 and 72 h, respectively).

B[a]P was analyzed in duplicate samples with the exception of the remaining soft tissues. Following the described conditions, B[a]P recovery was 87.2 ± 2.0 % and the dispersion of data for replicated samples was 7.2 %.

Linear regression curves, concerning B[a]P concentration in gills and digestive glands, were statistically significant (p ≤ 0.05) and are reported here in succession (N=14).

Gills at 48 and 72 h: \[ y = 17.88x + 0.04 \text{ and } y = 24.05x + 0.06 \]

Dig. gland at 48 and 72 h: \[ y = 77.29x + 0.25 \text{ and } y = 97.12x + 0.33 \]

3.2 DNA adduct analysis

Fig. 2 shows representative autoradiograms deriving from nuclease P1-enhanced 32P-postlabeling of gill and digestive gland DNA. Whereas control mussel DNA did not show any evident adduct (Fig. 2 a,b) a typical adduct spot appeared in both tissues of exposed mussels (Fig. 2 c,d). Some additional faint spots were also detected in digestive gland samples.

In Table 2 DNA adduct and 8-OHdG levels detected in gills and digestive glands of each experimental mussel group are reported.
Mean adduct levels rose from control values of 0.054-0.119/10^8 adducts per nucleotide (RAL) to 0.559 RAL in gill DNA of mussels exposed for 48 h to increasing B[a]P doses. At exposure time of 72 h, gill DNA adducts reached a maximum (0.789 RAL) at the dose of 100 ppb of B[a]P. In digestive gland DNA, maximum levels were observed at 500 ppb (0.442 RAL) and 1000 ppb of B[a]P (0.643 RAL) after 48 and 72 h of exposure, respectively.

As an overview, internal dose and DNA adduct data referred to gill and digestive gland samples are reported together in Fig. 3.

Compared with the control (ANOVA single-factor), statistically significant adduct increases were found for the doses of 50-1000 ppb and 5-1000 ppb at 48 and 72 h of exposure respectively, both in gill and digestive gland tissues (see symbols in Table 2 and Fig. 3).

Although two experimental conditions (gills/48 h and dig. gland/72 h) produced linear adduct increases over the whole dose-range of exposure, the other two conditions (gills/72 h and dig. gland/48 h) were characterized by decreasing or fluctuating DNA adduct levels within the dose interval of 100-1000 ppb of B[a]P.

Regression analysis, applied to the linear part of the dose-response relationships between B[a]P exposure and DNA adduct levels, provided the following equations.

Gills at 48 and 72 h: \( y = 0.208x + 0.000 \) and \( y = 0.162x + 0.008 \) 
(N=23 and 15, respectively)

Dig. gland at 48 and 72 h: \( y = 0.222x + 0.000 \) and \( y = 0.247x + 0.000 \) 
(N=22 and 29, respectively)

Reference DNA gave the expected patterns of adduct spots, accounting for 269 RAL on average (N=9) and indicating a mean efficiency of 68 % for the whole ^32P-postlabeling procedure.

3.3 8-OHdG quantification
Data concerning 8-OHdG levels in gills and digestive gland of experimental mussels are reported in Table 2 and Fig. 4, the latter representing the 8-OHdG and internal dose together.

Differences in the amount of oxidative DNA damage were detected in the two mussel tissues. Indeed, the occurrence of 8-OHdG was about five times higher in digestive gland (10.85 and 14.01 per 10^3 dG after 48 and 72 h exposure, respectively) than in gill tissue (2.24 per 10^3 dG) of control mussels.

As emphasized by the symbols in Table 2 and Fig. 4, a number of data concerning tissue 8-OHdG values in the exposed mussels were significantly higher in comparison with the control (ANOVA single-factor), particularly for digestive gland DNA at the exposure time of 72 h.

The highest occurrence of 8-OHdG was detected at the doses of 5 (gills/48 h), 100 (gills/72 h), 1000 (dig. gland/48 h) and 100 (dig. gland/72 h) ppb of B[a]P. The corresponding values were 4.28, 7.66, 25.05 and 28.51. Qualitatively, some decrease in 8-OHdG levels was evident at intermediate/high doses of exposure in all four experimental conditions: within the dose interval of 50-500 ppb of B[a]P in gill DNA for the exposure time of 48 h and at 500 ppb of B[a]P in the other cases.

3.4 Determination of CYP1A-immunopositive proteins

The levels of CYP1A-immunopositive proteins in digestive glands of individual mussels were detected by Western blotting and are reported in Table 3.

In only a few cases significant changes in CYP1A protein levels were observed in the exposed versus control mussels (at 50 and 1000 ppb for the 48 h-exposure and at 100 and 500 ppb of B[a]P for the 72 h-exposure) since high interindividual variability impaired the interpretation of data.

4. Discussion
Taking into account the renewal of contaminated SW during exposure, total B[a]P amounts of 0.003-6 mg at 48 h and 0.0045-9 mg at 72 h were delivered for the nominal exposure doses of 0.5-1000 ppb B[a]P per liter per mussel. We did not evaluate the fraction of hydrophobic chemical adsorbed on the surfaces (e.g. inner walls of the glass tanks, byssus and mussel shell) but analysis of tissutal B[a]P indicated dose-related uptake of the chemical in the exposed mussels.

The highest B[a]P accumulation in the digestive gland was expected since the function of this gland parallels that of the vertebrate liver and B[a]P disposition is determined mainly by lipid tissue levels (53). Discrete B[a]P amounts were also detected in the gills (first tissutal barrier during the filtration of suspended and dissolved matter in SW) and in the other soft tissues evaluated together for comparison.

In general, the tissue B[a]P dose increased significantly as the external dose increased. The slopes calculated for the external/internal B[a]P dose relationship at 48 and 72 h of exposure confirmed the greater potential of the digestive gland (slopes: 77.3, 77.1) to accumulate B[a]P in comparison with gills (slopes: 17.9, 24.1) and remaining soft tissues.

Comparison of the internal dose at 48 versus 72 h indicated a divergence in B[a]P accumulation rate starting at 50 ppb and 0.5 ppb B[a]P respectively in gills and digestive glands (emphasizing further the different functionality of the two analyzed tissues) whereas in the other soft tissues the rate of B[a]P accumulation diverged at an intermediate dose (5 ppb).

Furthermore, the internal dose increased rapidly at low levels of exposure (overall 10x at 0.5 versus 5 ppb) whereas, with the exception of the subsequent exposure level (5 versus 50 ppb) for digestive gland samples (10x and 7.4x increases at 48 and 72 h, respectively) B[a]P concentration rose more slowly. This suggests an incoming plateau of B[a]P accumulation at the higher exposure levels. Tissue equilibrium concentration of a hydrophobic chemical such as B[a]P (6.42, log K octanol/water) probably takes more than 48-72 h to be reached; in addition, toxic
effects or physiological counteractions could have occurred at the highest exposure levels. Filtration experiments, performed by measuring the clearance of unicellular algae from SW after 48 h of standard exposure to 1000 ppb of B[a]P, indicated an accelerated filter-feeding activity in response to the noxious stimulus (data not shown).

As regards the biologically effective dose, the ³²P-postlabeling analysis confirmed the appearance of an B[a]P-related adduct in the gills and extended this evidence to the digestive gland.

DNA adduct levels (one specific spot) increased significantly in both tissues of exposed mussels. In this study, the exposure to 50 ppb B[a]P for 48 h represents the lowest effective dose for adduct formation, in agreement with previous results deriving from an independent in vivo experiment (25). On the other hand, more prolonged exposure to B[a]P (72 h) caused a shift of the lowest effective dose to 5 ppb of B[a]P.

Even though adduct increases were not linear over the whole exposure range in 2 out of 4 experimental conditions, DNA adduct levels were generally consistent with dose and exposure time up to 100 ppb of B[a]P (see Fig. 3) and slopes, calculated in the linear part of the dose-response relationships for DNA adduct levels versus B[a]P dose, ranged from 0.162 to 0.247.

Together with low absolute adduct levels, these values indicate that only a small fraction of absorbed B[a]P is metabolically modified and covalently linked to mussel DNA at the end of exposure. It is more difficult to understand why only slight differences in adduct levels were found between gills and digestive gland since the latter showed the highest B[a]P accumulation and it is known to possess higher MFO activity than other tissues (20). Underlying phenomena (such as difference in rates of distinct metabolic reactions as well as DNA repair) between the two tissues, could probably explain these findings. On the other hand, the slight differences in adduct levels between gills and digestive gland agree with in vitro data indicating a saturation of B[a]P metabolism by digestive gland microsomes (Vmax) at 60-100 µM B[a]P (54), since in both tissues the internal dose detected may have exceeded the limiting rate of B[a]P-activating reactions.
With regard to 8-OHdG quantification, basal levels of oxidative DNA lesions were higher in digestive gland than gills possibly emphasizing the existence of structural and functional differences between the two tissues (e.g. lipid content, endogenous peroxidation rate, array of metabolic reactions). Indeed, higher amounts of endogenous oxidation as well as an overestimation of 8-OHdG levels in this lipid-rich tissue could explain the above evidence (37,55).

Even though significant increases in 8-OHdG levels were found in both gill and digestive gland DNA of exposed mussels, the dose-response relationships observed after 48 and 72 h of exposure were not linear and some decrease in 8-OHdG levels appeared at intermediate/high doses.

Likewise, changes in CYP1A-immunopositive protein levels, as detected by Western blotting in digestive gland microsomes, yielded a complex pattern. High interindividuell variability suggest that further study is needed to clarify the meaning of these data.

Although B[a]P quinones represent about half the B[a]P metabolites in mussels, it has not been established whether BPQs redox cycling and radical formation are the only cause of genetic damage in mussels exposed to B[a]P. Indeed, a significant production of chromosomal aberrations (56) and micronuclei (57,58) occurred in gill cells of mussels exposed in vivo to B[a]P and, DNA strand breaks and micronuclei were detected also in haemocytes of B[a]P-exposed mussels (59,58). Such clastogenic effects may be the coherent consequence of radical-DNA interactions (particularly, ROS) and of BPQ reactivity (60,39,61). The increased levels of 8-OHdG detected in this work support the above assertions and contrast with the lack of oxidative DNA damage reported for mussels exposed to menadione and nitrofurantoin (62).

The MFO system appears inducible in mussels since seasonal and sex variations of mussel MFO activity have been reported (63,64) and increased expression of CYP1A-immunopositive proteins in mussel digestive gland was observed following exposure to Aroclor 1254 (45). Nevertheless, the weak spot of the B[a]P-related adduct in gills of exposed mussels did not change in intensity after short and prolonged
in vivo Aroclor pre-treatment and did not co-migrate with the reference spot of BPDE10-N2Gua adduct (25). Chemical identification of the typical mussel adduct is necessary to substantiate this study.

The whole frame of data suggests that B[a]P can be activated to DNA-reactive intermediates in mussels via BPQ formation associated to redox-cycling and, presumably to a lower degree, via diolepoxides. The rates of inducible and non-inducible pathways (i.e. monoxygenation and one-electron oxidation) and the complex changes observed in gill and digestive gland tissues at different exposure conditions need additional study to be correctly understood.

Acknowledgements

A special thanks to Prof. A.G. Levis for its scientific support. We are also grateful to Luciana Ferro, Cristina Pavarin and Maria Manna for their technical and administrative help. Supported by Italian MURST and BIOMAR II (ENV4-CT96-0300).

References


Figure captions

Fig. 1. Possible pathways for activation of B[a]P leading to DNA lesions in vivo (based on Stansbury et al., 1994; Cavalieri and Rogan, 1995, Flowers et al., 1996, Devanesan et al., 1996).

Fig. 2. Representative autoradiograms of gill (a,c) and digestive gland DNA (b,d) obtained from controls (a,b) and from mussels exposed to 500 ppb of B[a]P (c,d). (after 92 h at -76 °C; 1x magnification).

Fig. 3. Mean values of DNA adducts and tissue B[a]P concentration in gills and digestive gland of exposed mussels (*: see Table 2).

Fig. 4. Mean values of 8-OHdG and tissue B[a]P concentration in gills and digestive glands of exposed mussels (*: see Table 2).
benzo[a]pyrene

and/or

monooxygenation

7,8-epoxide hydratation

7,8-diol

or (DD)

monooxygenation catechol

one-electron oxidation

one-electron oxidation

semiquinone anion radical ROS

dielepoxides

quinones

radical cations

benzylic carbenium ions

\[
\begin{align*}
\text{DNA LESIONS} \\
\text{(DNA adducts, oxidative damage, depurination, strand breaks)} \\
\text{TUMOR INITIATION}
\end{align*}
\]

DD: dihydrodiol dehydrogenase; ROS: reactive oxygen species
Table 1

B[a]P concentration in gills, digestive gland and remaining soft tissues of exposed mussels.

<table>
<thead>
<tr>
<th>B[a]P dose (ppb)</th>
<th>Tissue B[a]P concentration (μg/g w.w.)§</th>
<th>48 h exposure</th>
<th>72 h exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>*0</td>
<td>gills</td>
<td>digestive gland</td>
<td>other tissues#</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>50</td>
<td>15.0</td>
<td>14.5</td>
<td>5.7</td>
</tr>
<tr>
<td>100</td>
<td>30.3</td>
<td>147.0</td>
<td>22.7</td>
</tr>
<tr>
<td>500</td>
<td>45.3</td>
<td>207.0</td>
<td>36.0</td>
</tr>
<tr>
<td>1000</td>
<td>49.6</td>
<td>249.2</td>
<td>47.1</td>
</tr>
</tbody>
</table>
| §: HPLC/fluorimetric detection (mean values, N=2); *: 0.005% solvent (DMSO); #: one determination.
Table 2
DNA adducts and 8-OHdG in gills and digestive gland of B[a]P-exposed mussels.

<table>
<thead>
<tr>
<th>B[a]P (ppb)</th>
<th>(48 h exposure)</th>
<th>Gills</th>
<th>Digestive gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adducts/10^6 dNp</td>
<td>8-OHdG/10^5 dG</td>
<td>Adducts/10^6 dNp</td>
</tr>
<tr>
<td>0</td>
<td>0.119 ± 0.031</td>
<td>2.24 ± 0.27#</td>
<td>0.054 ± 0.044</td>
</tr>
<tr>
<td>0.5</td>
<td>0.177 ± 0.026</td>
<td>2.80 ± 0.49</td>
<td>0.099 ± 0.029</td>
</tr>
<tr>
<td>5</td>
<td>0.199 ± 0.062</td>
<td>4.28 ± 0.74#</td>
<td>0.096 ± 0.052</td>
</tr>
<tr>
<td>50</td>
<td>0.219 ± 0.046*</td>
<td>3.59 ± 0.37*</td>
<td>0.411 ± 0.078*</td>
</tr>
<tr>
<td>100</td>
<td>0.327 ± 0.087*</td>
<td>3.28 ± 0.78*</td>
<td>0.369 ± 0.039*</td>
</tr>
<tr>
<td>500</td>
<td>0.513 ± 0.087*</td>
<td>2.60 ± 0.72</td>
<td>0.442 ± 0.052*</td>
</tr>
<tr>
<td>1000</td>
<td>0.559 ± 0.061*</td>
<td>2.86 ± 0.75</td>
<td>0.287 ± 0.106*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>(72 h exposure)</th>
<th>Gills</th>
<th>Digestive gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adducts/10^6 dNp</td>
<td>8-OHdG/10^5 dG</td>
<td>Adducts/10^6 dNp</td>
</tr>
<tr>
<td>0</td>
<td>0.075 ± 0.044</td>
<td>2.24 ± 0.27#</td>
<td>0.063 ± 0.036</td>
</tr>
<tr>
<td>0.5</td>
<td>0.120 ± 0.035</td>
<td>2.75 ± 0.53</td>
<td>0.135 ± 0.058</td>
</tr>
<tr>
<td>5</td>
<td>0.182 ± 0.045*</td>
<td>4.73 ± 0.35*</td>
<td>0.186 ± 0.044*</td>
</tr>
<tr>
<td>50</td>
<td>0.727 ± 0.031*</td>
<td>3.68 ± 0.58*</td>
<td>0.335 ± 0.125*</td>
</tr>
<tr>
<td>100</td>
<td>0.789 ± 0.146*</td>
<td>7.66 ± 0.69*</td>
<td>0.486 ± 0.118*</td>
</tr>
<tr>
<td>500</td>
<td>0.565 ± 0.090*</td>
<td>2.59 ± 0.33</td>
<td>0.537 ± 0.077*</td>
</tr>
<tr>
<td>1000</td>
<td>0.355 ± 0.025*</td>
<td>5.01 ± 0.78*</td>
<td>0.643 ± 0.231*</td>
</tr>
</tbody>
</table>

*: statistically significant (p ≤ 0.05); #: evaluated at 72 h of exposure.
Table 3
CYP1A-immunopositive proteins in gills and digestive gland of B[a]P-exposed mussels.

<table>
<thead>
<tr>
<th>B[a]P (ppb)</th>
<th>CYP1A-immunopositive protein (arbitrary units)</th>
<th>48 h exposure</th>
<th>72 h exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>1.97±0.39</td>
<td>0.50±0.42</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>3.99±3.03</td>
<td>3.49±4.38</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>2.65±3.54</td>
<td>0.55±0.54</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>0.59±0.75*</td>
<td>1.21±0.97</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>1.44±2.07</td>
<td>5.97±3.47*</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>2.27±0.78</td>
<td>2.97±2.10*</td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>3.89±0.50*</td>
<td>ND</td>
</tr>
</tbody>
</table>

*: statistically significant (p ≤ 0.05); ND: not determined.


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VARIATION IN LEVELS OF CYTOCHROME P450 1A, 2B, 2E, 3A AND 4A-IMMUNOPOSITIVE PROTEINS IN DIGESTIVE GLAND OF INDIGENOUS AND TRANSPLANTED MUSSEL, *MYTILUS GALLOPROVINCIALIS* IN VENICE LAGOON, ITALY

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Western blot analysis of cytochrome P450-immunopositive proteins using antibodies to fish (1A, 3A) and mammalian isoforms were performed on digestive gland microsomes of *M. galloprovincialis* from indigenous populations at relatively clean (Lido) and urban-contaminated (Salute) sites, and in animals transplanted from Lido to an industrially-contaminated (CVE) site for 1 or 3 weeks; previous studies have shown high tissue levels of aromatic hydrocarbons and polychlorobiphenyls at Salute and CVE. Levels of CYP1A-, CYP2E-, CYP4A1- and CYP4A3-immunopositive proteins were respectively 53, 276, 75 and 155 % higher at Salute compared to Lido (P < 0.05). No difference between sites was seen in CYP2B- and CYP3A-immunopositive proteins. Levels of CYP1A-immunopositive protein were indicated to increase in a time-dependent manner with transplantation from Lido to CVE, and after 3 weeks were 58 % higher at CVE than Lido (P < 0.05) and reached levels similar to those at Salute. In contrast, no differences were seen in CYP2E-, CYP4A1- and CYP4A3-immunopositive proteins with transplantation from Lido to CVE for 3 weeks. Overall, the results indicate the presence of multiple CYP isoforms which may be differentially altered by environmental factors; additionally that the CYP1A-immunopositive form is responsive to exposure to organic contaminants.
Laboratory exposure of turbot (Scophthalmus maximus L.) larvae to contaminated sea surface microlayer: toxicity and the induction of cytochrome P4501A

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Early life-stages of fish may be exposed to increased levels of pollution through interaction with the sea surface microlayer (SMIC) which bioconcentrates contaminants many-fold compared to subsurface waters. Previous studies have demonstrated potential contaminant-mediated mechanisms of toxicity in larvae of turbot (S. maximus), including the metabolism of benzo(a)pyrene (BaP) to reactive products, the enhanced generation of reactive oxygen species, and the induction of the biotransformation enzyme cytochrome P4501A (CYPIA) which is capable of metabolising contaminants, such as polycyclic aromatic hydrocarbons (PAHs), to mutagenic species.

This study examined lethality and induction of CYPIA in whole-body 3-day old S. maximus larvae following exposure to a) SMIC, or b) organic contaminants from SMIC extracted by dichloromethane (DCM), from both clean and polluted sites (respectively, 10 ppb and 10 ppm total PAHs). Additionally, larvae were exposed to an artificial SMIC containing 20 ppm oleic acid and 1 ppm BaP. The DCM-extracts and artificial SMIC (oleic acid + BaP) dissolved in a minimal volume of dimethylformamide (DMF), were added to clean seawater for the exposure experiments. Control conditions comprised clean seawater alone and seawater plus DMF, and all exposures were carried out at 15 °C with a 12 hour light/ 12 hour dark cycle. Approximately 70% mortality was observed after 56 hours exposure to 10 ppm SMIC compared to less than 5% mortality for any other treatment at 56 hours. However 70 % mortality was observed after 72 hours exposure to DCM-extracted 10 ppm SMIC, indicating that the lipophilic contaminants are toxic but polar contaminants (not removed by DCM) may also be contributing to toxicity. No toxicity was observed with exposure up to 100 hours for any other condition. A 25- to 35-fold induction of CYPIA (measured as 7-ethoxyresorufin-O-deethylase activity) was seen after 48 hours exposure to 10 ppm SMIC, 10 ppm DCM-extracted SMIC, and artificial (1 ppm BaP) SMIC, but not with exposure to controls or 10 ppb SMIC conditions, confirming the presence of CYPIA-inducers in SMIC and indicating the occurrence of sublethal toxic effects not detected in lethality tests. Overall, the results demonstrate the toxic threat of contaminant bioconcentration by SMIC to early life stages of fish with potential consequences for their survival and health.


RECENT PROGRESS ON THE DEVELOPMENT OF BIOMARKERS OF ORGANIC POLLUTION IN MUSSEL (CYTOCHROME P4501A - CYP1A) AND OXIDATIVE STRESS IN FISH

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Plymouth Marine Laboratory, UK

Abstract:

Partially purified mussel digestive gland cytochrome P450 was analysed by immunorecognition studies (Western blotting) for the presence of multiple enzyme forms (isoenzymes) using antibodies to P450 forms of fish (CYP1A, CYP3A) and rat (CYP2B, CYP2E, CYP4A). Catalytic properties (i.e. benzo[a]pyrene (BaP)metabolism) of the partially purified P450 were examined in reconstitution studies. RT-PCR was carried out on RNA from mussel digestive gland, using oligonucleotide primers to amplify a sequence around the conserved haem binding cysteine region of hepatic cytochrome CYP1A1 of trout (collaborative with Univ. Surrey, UK). Analysis of purative CYP1A-catalytic activity (BaP metabolism), CYP1A-like protein and CYP1A-like mRNA was carried out on mussel digestive gland material from PCB-exposure (with Kristineberg Marine St., Sweden & Univ. Padova, Italy) experiments, and field material from the deep sea Skagerrak region (with Kristineberg Marine St., Sweden) and recovery from the "Aegaen Sea" oil spill, Spain (with CSIC, Barcelona, Spain). Antioxidant enzyme and oxydative damage (8-hydroxydeoxyguanosine formation) responses were measured in turbot (Scophthalmus maximus) exposed to pro-oxidant chemicals (nitroaromatics) (with Univ. Birmingham, UK). Field material was collected from the North Sea (dab, Limanda limanda) (with Aquasense) and the Mediterranean (mussel & sea comber) (BIOMAR cruise).
Antibody-recognition (CYP1A, 2B1, 2E1, 4A1) of multiple cytochrome P450 proteins in the digestive gland of the common mussel, *Mytilus edulis* L.

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NERC Plymouth Marine Laboratory, Citadel Hill, Plymouth, PL1 2PB, UK.

Cytochrome P450 (CYP) is a multi-gene family of central importance in the metabolism of organic pollutants and endogenous compounds. Previous genomic and RNA studies have indicated the expression of multiple CYPs in digestive gland of *Mytilus* sp., including putative CYP1A1 and CYP4A1 forms (Wootton *et al.*, Marine Environ. Res., in press). The existence of multiple CYP proteins was investigated in the same tissue of *M. edulis* using polyclonal antibodies to perch (*Perca fluviatilis*) CYP1A, rabbit CYP2B1 and CYP2E1, and sheep CYP4A1 (all hepatic forms). Western blot analysis demonstrated the presence of immunopositive bands for all four antibodies, of the three gene families, in both microsomes and partially purified P450 preparations of *M. edulis*. In the latter case, the position of the bands indicated at least two distinct proteins with epitope similarity to CYP1A and CYP4A1. The detection of the CYP1A-like immunopositive protein in the microsomal fraction indicates potential for use of this method as a biomarker of exposure to organic pollutants.
BENZOA[AR]PYRENE METABOLISM AND REACTIVE OXYGEN SPECIES GENERATION IN LARVAE OF TURBOT
(SCOPHTHALMUS MAXIMUS L.)

Laurence D. Peters and David R. Livingstone
(NERC Plymouth Marine Laboratory, UK)

Bioactivation of organic xenobiotics by enzyme systems has been proposed as an important mechanism of contaminant-mediated toxicity in fish. Cytochrome P450IA1 may be induced by specific contaminants and contribute to the bioactivation of aromatic hydrocarbons (AHs), such as benzo[a]pyrene (BaP) via metabolism to the proximate carcinogen BaP-7,8-dihydrodiol. Other contaminants, or their metabolites such as BaP-diones, may stimulate the formation of reactive oxygen species (ROS) causing oxidative damage to lipids and DNA. Antioxidant enzymes and/or CYP1A1 have been detected in larvae of S. maximus and other fish species, but relatively little is known of these mechanisms of toxicity in early life stages.

Whole 3-day old farm egg-yolk larvae of S. maximus were homogenised by sonication, and aliquots of 13,500g supernatant incubated with 3H-B[ar]P and NADPH. Metabolites were resolved and quantified by reverse phase HPLC (Waters C18 column) with radiometric detection. Total rates of metabolism were 3 to 4 pmol per min per mg protein and comprised predominantly the 7,8-dihydrodiol, phenols and putative protein adducts, with small amounts of diones. The ability of whole-body 13,500g supernatants to support xenobiotic-stimulated NAD(P)H-dependent ROS production was studied using GC by the reaction of hydroxyl radical (·OH) (produced via an iron-catalysed Haber-Weiss reaction: O2^- + H2O2 = ·OH + OH^- + O2) with 2-keto-4-methylbutyric acid (KMBA) to produce ethylene. Basal rates of KMBA oxidation were observed (similar with NADH or NADPH) indicating the presence of electron-transport systems capable of generating ROS in vivo. Addition of superoxide dismutase and catalase inhibited KMBA oxidation by up to 60% indicating the involvement of respectively O2^- and H2O2 in ·OH formation. KMBA oxidation was stimulated by a range of xenobiotics, including 6 AH quinones, 5 nitro-AHs, BaP, 2-acetylaminofluorene (AAF) and lindane. Stimulation was generally greater for NADH than NADPH, the reverse being seen for menadione, 1-nitronaphthalene and AAF. The studies indicate a potential for contaminant-mediated toxicity in fish larvae by both reactive metabolite and ROS mechanisms.

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Cytochrome P4501A1 has been investigated extensively in liver of adults of many different fish species, but much less is known of its presence and regulation in early life stages. Studies were carried out on embryos, larvae, juveniles and adult *S. maximus*. The early life stages were farm-reared (Golden Sea Produce, Scotland) and the adults caught off Plymouth, UK. 7-ethoxyresorufin O-deethylase (EROD) activity, indicative of the presence of cytochrome P4501A1, was measured fluorometrically, using a scaled down micro-assay for larval samples. Immunochemical detection of cytochrome P4501A1 was carried out by Western blotting using polyclonal anti-P4501A1 from perch, *Perca fluviatils* (gift from L. Forlin, Univ. Goteborg, Sweden).

EROD activity was not detectable in newly fertilised embryos, but was present in similar activities in 13,500g supernatants of whole body of 3 day and 11 day old larvae, viz. respectively, 0.57 ± 0.06 and 0.45 ± 0.08 (± SEM) pmol per min per mg protein. The activity increased 550 % in 9 day old larvae following 48 hours exposure to 1 ppb γ-hexachlorohexane (lindane) in the seawater. EROD activity (13,500g supernatant) was considerably higher in liver of 90 day old juveniles than in whole-body larvae, viz. 10.8 ± 2.4 pmol per min per mg protein, and increased approximately 400 % following 24 to 48 hours exposure to 25 ppb benzo[a]pyrene in the seawater. The increase in EROD activity in juveniles was indicated to be accompanied by an increase in the amount of cytochrome P4501A1 protein. Increases in EROD activity and putative cytochrome P4501A1 protein were also seen in liver microsomes of 250g adults following 24 hours exposure to β-naphthoflavone (i.p., 20 mg per kg body wt.), viz. for the former from 12.3 ± 4.7 to 164.3 ± 6.0 pmol per min per mg protein. The results indicate that with the exception of the pre-hatching stage, cytochrome P4501A1 is present and inducible through most, if not all, of the life-cycle of *S. maximus*. 
PARTIAL PURIFICATION AND PROPERTIES OF CYTOCHROME P450 FROM DIGESTIVE GLAND MICROSONOMES OF THE COMMON MUSSEL, *Mytilus edulis* L.

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Mussel digestive gland microsomes show the unusual property of mixed-function oxygenase (MFO) activity in the absence of added NADPH (Livingstone *et al.*, 1989). In order to investigate this and to further characterize the MFO system, cytochrome P450 was partially purified using a modification of a previous scheme (Kirchin *et al.*, 1987; Livingstone *et al.*, 1989). This comprised sodium cholate solubilization, 4-15 % polyethylene glycol fractionation, octyl-Sepharose affinity chromatography (elution with buffer containing 2 % emulgen), DEAE-Sephacel ion exchange chromatography (elution with 0 to 0.5 M linear KCl gradient) and hydroxylapatite chromatography (elution with 300 mM phosphate buffer pH 7.6/20 % glycerol/0.1 mM EDTA). Three peaks were resolved by DEAE-Sephacel chromatography (at 0, 75 and 140-330 mM KCl), termed respectively peaks 1 to 3. The maximum purification factors and yields obtained by the procedure were respectively x 30-34 (specific content increased from 26 to 800 pmol per mg protein) and 7-10 %. The ratio of P450 content to cytochrome c (P450) reductase activity was reduced by a factor of x 250.

Molecular weights of the major bands (SDS-PAGE) varied from 51-54.5 Kd. Cytochrome P450 λmax (CO-difference spectrum) varied with purification from about 449 to 447 nm. Oxidised spectrum λmax was 410.5 ± 1.5. Type II difference binding spectra were generally seen with clotrimazole (app. Ks 3-29 μM), 7-ethoxycoumarin (154-261 μM) and α-naphthoflavone (8-83 μM). Western blotting of the octyl-Sepharose fraction with anti-P4501A1 from perch (*Perca fluviatilis*) (gift from L. Forlin, Univ. Göteborg) gave a single band of ~ 50 Kd molecular weight. A reconstituted system containing peak 2 or 3, NADPH, 30 μg L-α-dilauroyl phosphatidyl choline per ml and rat liver cytochrome P450 reductase (~ 70 pmol per pmol P450) (gift from C.R. Wolf, ICRF, Edinburgh) metabolized benzo[α]pyrene to diols, diones, phenols and putative tetrals and protein adducts. Peak 2 plus 1 mM cumene hydroperoxide produced diones and protein adducts only. Peak 2 in the absence of added P450 reductase or NADPH was indicated to produce metabolites. The major free metabolites in all cases were the 1,6-, 3,6- and 6,12- diones (53-100 % of total).

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