BIOCHEMICAL MONITORING OF TOXIC AND CARCINOGENIC ORGANIC POLLUTANTS ALONG THE İZMİR BAY AFTER THE GREAT CANAL PROJECT AND POSSIBLE HEALTH EFFECTS

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ABSTRACT

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The induction of hepatic cytochrome P4501A1 and its monooxygenase activity 7-ethoxyresorufin O- deethylase, (EROD) in fish by PAHs, PCBs and dioxins has been suggested as an early warning system "most sensitive biochemical response" for assessing environmental contamination conditions. In this study, the degree of induction of cytochrome P4501A1 protein as determined immunochemically and CYP1A1 associated EROD activity in fish were utilized as biomarkers of exposure to PAHs, PCBs and related organic pollutants along the İzmir Bay on the Aegean Sea Coast after the Great Canal Project. Three different fish species were used throughout this study, namely leaping mullet (*Liza saliens*), annular seabream (*Diplodus annularis*) and common sole (*Solea vulgaris*) which were representatives of pelagic, benthopelagic and benthic fish, respectively. Fish were sampled in November 2002 and October 2003 from different sites of the Bay. The mullet caught from Harbor, Üçkuyular port site, and Pasaport region displayed highly elevated EROD activities which were 2258±840 (n=15), 2011±490 (n=4), 1813±287 (n=11) pmole /min/mg protein, respectively and were 104, 80 and 79 fold higher than that of fish obtained from the reference point (25±9 pmole/min/mg

protein; n=4). Mullet caught along the pollutant gradient at three other sites (Hekim Island, İnciraltı, and Zeytinalanı) exhibited less but highly significant induced EROD activity. EROD activities of common sole sampled from Foça open sites (107±20 pmol/min/mg protein, n=5) and site16A (80±12 pmol/min/mg protein, n=9) were found to be very low and the latter was accepted as reference site. The highest EROD activity were seen in fish captured from İnciraltı which was about 6.3 times higher than those obtained from reference site. Common sole caught from the mouth of Gediz River and Hekim Island exhibited also highly elevated EROD activities. Annular seabream was tested to monitor CYP1A inducing chemicals for the first time in this study. The highest EROD activity (1376±279 pmol/min/mg protein, n=8) were detected in fish samples collected from Harbor region. An inverse relationship was found between distance to the harbor region and EROD activities of annular seabream captured from other sampling sites. In this study for the first time, major cytochrome P450 dependent mixed function oxidase activities such as benzphetamine N- demethylase, ethylmorphine N-demethylase and aniline 4-hydroxylase, were characterized in annular seabream. Changes in the P450 1A1 protein level were determined by immunochemical analysis to monitor the pollutant based induction in all fish species and good correlation was obtained between EROD activity and CYP1A protein content. Fish from polluted sites had both highly induced EROD activity and cytochrome P450 1A content. Chemical analysis of total PAH concentration in sediment and liver tissues of some fish sample were also carried out.

Although, İzmir Great Canal Project has been active since 2000 to treat and protect the İzmir Bay from the contamination of domestic and industrial wastes this study clearly demonstrated that the level of PAH, PCB and dioxin type persistent organic contaminants are still very high especially in the Inner and Middle Bay. This has implications for human fish consumption from contaminated areas, as well as for the health status of aquatic organisms.

Keywords: Cytochrome P4501A1, EROD, PAH, PCB, dioxins, İzmir Bay, pollution, procarcinogen, ecotoxicology.

ÖZ

TOKSİK ve KARSİNOJENİK ORGANİK KİRLİLİĞİN BÜYÜK KANAL PROJESİ'NDEN SONRA İZMİR KÖRFEZİ BOYUNCA BİYOKİMYASAL OLARAK İZLENMESİ ve SAĞLIĞA OLABİLECEK ETKİLERİ

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Balıklarda, karaciğer sitokrom P4501A1 ve buna bağımlı 7-etoksiresorufin O-deetilaz aktivitesinin PAH, PCB ve dioksinlerle indüklenmesi bu tip organik kirleticilerin varlığının belirlenmesinde erken uyarı sinyali görevi yaparak, "en sensitif biyomarker " olarak kullanılmaktadır. Bu çalışmada immunokimyasal olarak tayin edilen P4501A1 protein ve P450 1A1 bağımlı EROD aktivitelerindeki artış derecesi, PAH, PCB ve buna benzer kirliliğin boyutlarının belirlenmesinde balıklarda biyomarker olarak kullanıldı. Çalışmada yüzey, dip ve yüzey-dip balık çeşitlerini temsil eden üç farklı tür balık kullanıldı, bunlar sırası ile kefal, dil ve isparozdur. Balık toplama çalışması Kasım 2002 ve Ekim 2003' de körfezin değişik noktalarında yapıldı. Liman Bölgesi , Üçkuyular vapur iskelesi ve Pasaport' dan yakalanan kefal balıklarında çok fazla yükselmiş EROD aktiviteleri bulundu, bunlar sırası ile 2258±840 (n=15), 2011±490 (n=4) ve 1813±287 (n=11) pmol/dak/mg ve referans bölgesi EROD aktivitelerine (25±9 pmol/dak/mg, n=4) göre 104, 80 ve 79 kez daha yüksekti. Kirlilik boyunca 3 farklı mevkiden (Hekim adası, İnciraltı ve Zeytinalanı) yakalanan kefallerde düşük ancak oldukça önemli oranda yükselmiş EROD aktiviteleri bulunmuştur. Foça açıkları ve mevki 16A'dan yakalanan dil balıklarında

EROD aktiviteleri sırasıyla 107±20 (n=5) ve 80±12 (n=9) pmol/ dak/mg bulundu. Buna göre mevki 16A referans bölgesi olarak seçildi. Dil balıklarında en yüksek EROD aktivitesi İnciraltı bölgesinden yakalanan balıklarda görüldü bu sonuçlar referans bölgesinden yakalanan balıklara göre 6.3 kez daha yüksekti. Ayrıca Gediz nehri ağzı ve Hekim adasından yakalanan dil balıklarında da oldukça yüksek EROD aktiviteleri gözlendi. İlk defa bu calışmada isparoz balığı CYP1A indükleyici kimyasalları takip etmek için test organizması olarak kullanıldı. En yüksek EROD aktivitesi (1376±279 pmol/dak/mg, n=8) Liman bölgesinden yakalanan isparozlarda görülürken diğer 3 farklı bölgeden toplanan isparoz balıklarında EROD aktivitesinin Liman bölgesinden uzaklaşıldıkça düştüğü gözlendi. Bunlara ek olarak isparoz da ilk kez en önemli sitokrom P450 bağımlı karışık fonksiyonlu oksidaz aktivitelerinden olan benzfetamin N-demetilaz, etilmorfin N- demetilaz ve anilin 4- hidroksilaz aktiviteleri karakterize edildi. Üç balık türünde de P4501A1 protein düzeyindeki değişiklikler, kirlilik esaslı artışı izlemek için immunokimyasal olarak analiz edildi. Buna göre kirlenmiş bölgelerden yakalanan balıklarda hem CYP1A1 protein düzeyinde hemde EROD aktivitelerinde artış gözlendi. Ayrıca farklı bölgelerden toplanan sedimanlarda ve balık karaciğerinde toplam PAH derişimi kimyasal yöntemlerle analiz edildi.

İzmir Kanal Projesi, körfezi evsel ve endüstriyel atıklardan korumak ve arındırmak amacıyla 2000 yılından beri aktif olarak çalışmaktadır. Buna rağmen bizim sonuçlarımız özellikle İç ve Orta Körfezde PAH, PCB ve dioksin tipi kalıcı organik kirliliğin hala çok yüksek olduğunu açıkça göstermektedir. Bu, kirli bölgelerden yakalanan balıkları tüketen insanlar kadar bu bölgelerde yaşayan tüm deniz canlıların sağlığı açısından önemli bir tehdittir.

Anahtar Kelimeler: Sitokrom P4501A, EROD, PAH, PCB, dioksin, , İzmir Körfezi, kirlilik, prokarsinojen, ekotoksikoloji.

Dedicated To My Sister

Serap ODABAŞI

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ε-ACA	ε-Amino caproic acid
3-MC	3-Methylcholanthrene
Ah	Aromatic hydrocarbon receptor
ALP	Alkaline phosphatase
Arnt	Ah receptor nuclear translocator
B(a)P	Benzo(a)pyrene
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BIS	N,N'-Methylene bisacrylamide
BSA	Bovine serum albumin
BTE	Basal transcriptional elements
CO	Carbon monoxide
CYP	Cytochrome P450
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EROD	7-Ethoxyresorufin O-deethylase
HEPES	N-2-Hydroxyethylpiperazine-N'-2, ethane sulfonic acid
KCL	Potassium chloride
Kpi	Potassium phosphate
MFO	Mixed-Function oxidase
mRNA	Messenger ribonucleic acid
MROD	Methoxyresorufin O-demethylase
MTs	Metallothionenins
NADH	Nicotinamide adenine dinucleotide reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form

NBT	Nitroblue tetrazolium
Pab	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAH	Polyaromatic hydrocarbons
PB	Phenobarbital
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxins
PCDF	Polychlorinated dibenzofurans
PMSF	Phenylmethylsulfonyl fluoride
SDS	Sodium dodecyl sulfate
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEMED	N,N,N',N'-Tetramethylethylene diamine
XRE	Xenobiotic responsive element

CHAPTER I

INTRODUCTION

With the growth of civilization, the environment is continuously loaded with foreign chemicals (xenobiotics) released by urban communities and industries. Chemical wastes have increased to dangerously high levels in some areas. At present, estimates indicate that more than 60000 chemicals are in common use and about 500 new chemicals are said to enter to the commercial market annually. The marine environment is the ultimate sink for many of those chemicals as the end stage collectors due to direct discharges of domestic and industrial wastes, urban and agricultural runoff, discharges from ships or hydrological and atmospheric process. The marine environment, covers 70% of the earth, can neutralize some chemical wastes; however, many of the chemicals introduced into the environment during the last 50 years are highly persistent compounds such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), pesticides, alkyltin compounds and heavy metals (i.e. Pb, Hg, Cd etc.). Because of their chemical stability and persistency, they tend to accumulate in the different compartment of the marine environment. The result is the exposure of the marine organisms to highly toxic chemicals.

Marine environment supports many different forms of life, from viruses to mammals. Alarming number of toxic chemicals is a major threat for the health of those organism inhabiting the seas as well as human consumer of such organisms. (Payne *et al*., 1987; Dave, 1990). Because of the effects of chemicals in the marine environment, it

has become more important to monitor the extent of pollution and its impact on marine species.

1.1 Biological Response of Organism to Environmental Contaminants

Toxic organic contaminants such as PAHs, PCBs and dioxins found in the marine ecosystem, can be taken up by the organisms through the direct uptake from water by gills or skin (bioconcenration), through the uptake of suspended particles (ingestion) and through the consumption of contaminated food (biomagnifications).Those chemicals are highly lipophilic in nature, and can easily enter through the cell.

The first stage of the pollutant effect on the organism is the interaction with endogenous molecules. These interactions can be divided into three main groups. In the first group, the contaminants can be sequestrated and then neutralized in the neutral lipid fraction. In the second group, contaminants can bind specifically to some cellular molecules such as receptors for example, some xeno- estrogenic chemicals like DDT, a kind of organochlorine pesticides largely used in agricultural purposes, bind to estrogen receptor and show estrogenic effects (Harrison *et al*., 1995; Kupfer, 1995) that can cause finally feminization of male species (Gimeno *et al.,* 1990). In the third group, contaminants can interact with the biotransformation enzymes. These enzymes either metabolize contaminants into nonreactive hydrophilic compounds which are easily excreted from the body or convert them into reactive metabolites which are more toxic than the parent compounds. All these interaction can result in accumulation of foreign chemicals in the body burdens of the organisms, or direct and indirect (via its metabolites) toxic effects or excretion of the parent compound and/or its metabolites.

Pollutants which are bioaccumulated in the organism, first cause changes at the molecular and cellular level. This may lead to adverse effect in the organism which in turn may cause changes at the population and the community level in the years to come. (Arınç *et al.,* 2000). As stated above, for example xeno-estrogenic compounds first bind

to the estrogen receptor at the cellular level and cause feminization of male species at the individual level and finally this may be threaten the existence of susceptible species at the population even community level. The sequential order of responses to pollutant stress within a biological system is given in Figure 1.1.

Growing number of toxic organic chemicals in the aquatic environment may cause deleterious effects on populations. For this reason it is very important to detect the relationship between contaminant level (exposure) and their biological effects before any detrimental effect has been established at higher organization level.

1.2. Biomarkers

Conventional tools of monitoring are based on measurement of a selected group of chemicals (chemical analyses) in different environmental compartments such as water, sediment and body burdens of the organisms. However chemical analyses are often unable to predict biological consequences of contaminants. Because, xenobiotic chemicals, in the forms found in the environment, often do not by themselves constitute a hazard to organisms. Once exposure has occurred and substances entered into body (first into the cell), a sequence of biological responses may take place (section 1.1). Biological responses depend on many intrinsic (i.e. age, sex, health and nutritional status of the organisms) and extrinsic (i.e. dose, duration and route of exposure, presence of other chemicals) factors that present barriers to assessment of contaminant level (exposure) and their biological effects. However, biomarker responses can circumvent these problems to a large extent by focusing on relevant molecular events that occur after exposure.

The term **biomarker** can be defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) which can be related to exposure to or toxic effects of environmental chemical

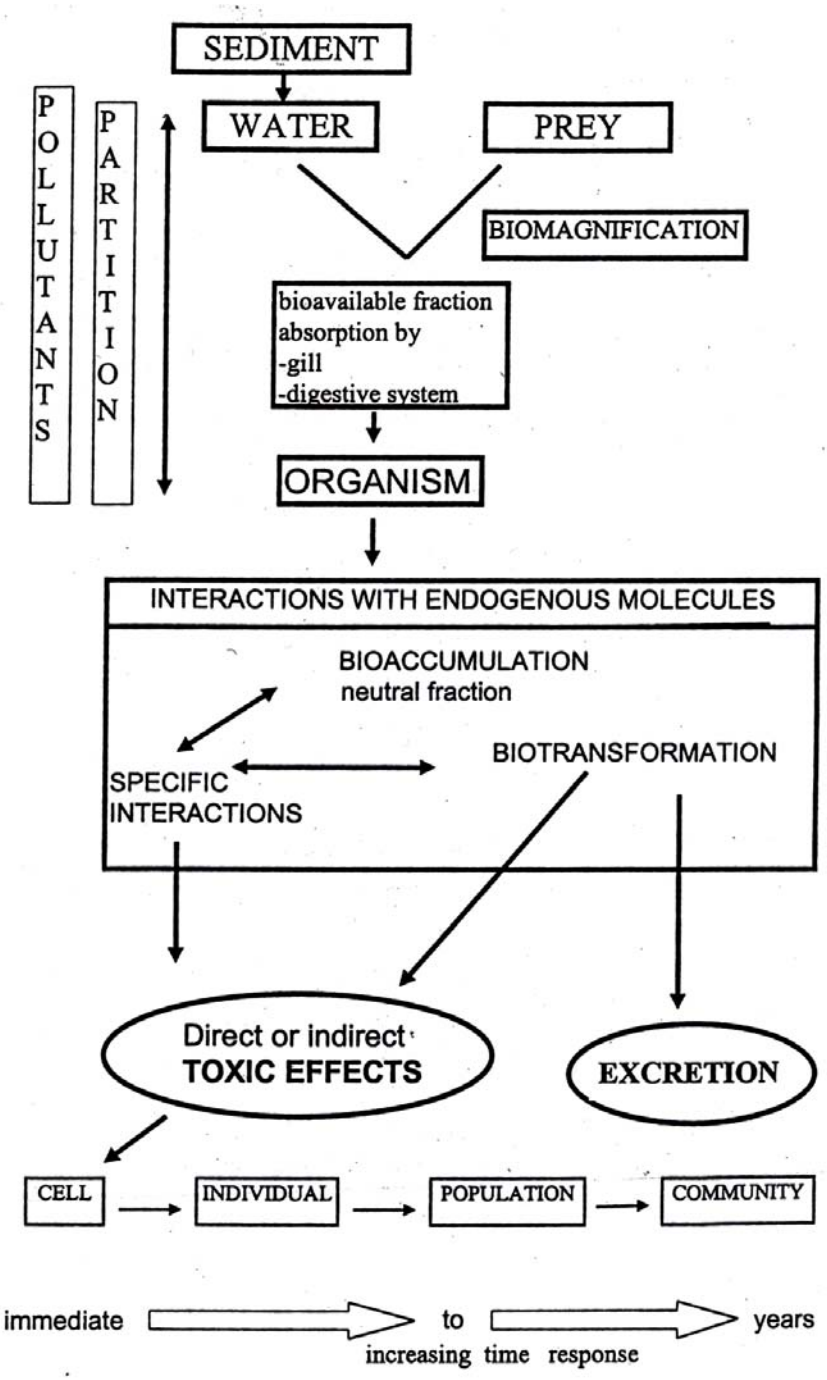


Figure 1.1 The relationship between pollutants in the aquatic environment and biological effects (taken from Narbonne, 1992)

(Peakall, 1994). Generally this term can be defined at any biological level of organization each of which has its own merits. Table 1.1. shows the biomarkers at different level of organization. With increasing level of organization sensitivity, specificity and accuracy may decrease but their ecological relevance increase (Bucheli and Fent, 1995)

Biomarker responses are, broadly speaking of two kinds: those that measure only exposure and those that measure both exposure and toxic effects of environmental chemicals (Peakall, 1992; Walker, 1998). Biomarkers at the biochemical level which are related with the changes in gene expression, alterations in specific protein content and enzyme activity in response to contaminants, measure both exposure and toxic effects of contaminants. By this way they bridge the gap between contaminant level and their biological effects. In addition, biochemical markers are usually first detectable and quantifiable responses to environmental changes (Stegeman *et al*., 1992). For these reasons they are often referred to as an early warning system and they are most widely used for assessing environmental health (Payne *et al.,* 1987; Haux and Forlin, 1988)

Employment of biochemical marker, for assessing the level of toxic compounds in the marine environment and possible effects on the marine organisms, has been mainly based on fish. Fish may provide the best model to serve this purpose (Powers, 1989). They can be found virtually everywhere in the aquatic environment from fresh to salt water, from shallow surface waters to the intense pressure of the depths. They play a major ecological role in the aquatic food webs because of their function as a carrier of energy from lower to higher tropic level (Beyer 1996). In addition they provide a very important protein source as well as essential ω-3, ω-6 unsaturated fatty acids for the nutrition of humans. Therefore, the health and protection of these organisms are of pivotal importance.

Table 1.1 Biomarkers at different biological levels (taken from Bucheli and Fent, 1995)

Biological level	Example of biomarker
*Molecules	Enzyme content and/ or activity
	Specific mRNAs
	DNA adducts
Cells	Structural and functional alteration of organelles
	Proliferation of endoplasmic reticulum
	Chromosomal aberrations
	Histopathological alterations
Organs	Liver condition index (LSI)
	Gonadosomatic Index
	Immune parameters
	Reproductive parameters
Individuals	Body Condition Index
	Fertility
	Maturation retardation
Population	Gene frequency
	Age structure
	Size distribution
Ecosystem	Diversity indices
	Functional parameters

*Biomarkers at the biochemical levels

1.3 Fish Biomarkers

It is possible to analyze the early adverse effects of toxic pollutants on fish by using the various types of biochemical markers. Some of the most important and specific ones are given below.

1.3.1 The Oxidative Stress

Many environmental contaminants (or their metabolites) exert their toxic effects related to oxidative stress. (Winston and Di Giulio, 1991). Oxidative stress can be defined as injurious effects of reactive oxygen species (ROS), which are the reduction products (i.e. $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$) of molecular oxygen. ROS are continually produced in the biological systems by a number of process involving foreign compounds and they may react with cellular macromolecules leading to enzyme inactivation, lipid peroxidation, DNA damage and ultimately cell death (Winston, 1991).

The antioxidant defenses of the organisms, which serve to remove the ROS include various low molecular weight free radical predators (antioxidant), such as glutathione, β-carotene, vitamin A, C, E and a number of antioxidant enzymes, superoxide dismutase (catalyzes $O_2^{\bullet -}$ reduction) and catalase (H_2O_2 reduction). In laboratory experiments with fish, the model compound benzo(a)pyrene, as well as PAHs and PCBs cause significant alterations in the activities of antioxidant enzymes (Livingstone *et al*., 1990; Cajaraville *et al*., 1992; Otto and Moon, 1995).

The measurement of the level and the activities of antioxidant enzymes and concentration of certain antioxidants can be potential markers of oxidative stress in marine organisms exposed to pollutants (Wenning *et al*., 1988; Ribera *et al*., 1989; Livingstone, 1990; Malins *et al*., 1990).

1.3.2 The Metallothioneins

Metallothioneins (MTs) constitute a family of low molecular weight cysteine rich proteins. They act as intracellular regulators of endogenous metals such as Cu and Zn, donators of metals to metalloproteins, and play a pivotal role in the detoxification of excess levels of these and other toxic metal ions such as Cd and Hg (Kagi and Kojima, 1987). In fish the formation of MTs can be induced by heavy metals at the transcriptional level. It has been proposed that the amount of MTs in tissues may serve as an indicator of metal exposure in aquatic species (Olafson *et al*., 1979). Many studies have substantiated this hypothesis. Laboratory exposure of rainbow trout (*Salmo gairdneri*) to a metal mixture (Zn: Cu: Cd, 400:20:1) resulted in a highly significant correlation between hepatic MT levels and water levels of mixture (Roch and McCarter, 1984). In addition, a field study of perch (*Perca fluviatilis*); living in a habitat contaminated with Cd showed that increased liver MT levels correlated strongly with increased levels of Cd (Olsson and Haux, 1986).

Measurement of MTs induction can be used as a sensitive biomarker for metal contamination of the environment (Haux and Forlin 1988).

1.3.3 The Neurotoxicity

Many organophosphate and carbamate pesticides are very effective inhibitors of acetylcholinesterase (ACHE) which is an essential enzyme in the transmission of nerve flux. ACHE inhibition has been used to assess the nature and extent of the exposure of wildlife to agricultural and forest spray. (Zinkl *et al.,* 1991) A significant depression of ACHE activity in fish from organophosphate polluted sites was observed in muscle tissue of brown trout and flounder in Newfoundland, Canada (Payne *et al.,* 1996). The measurement of ACHE inhibition in marine organism can be used as a very specific biomarker to assess the organophosphate-type pesticide contamination.

1.3.4 The DNA Adducts

Some process involved in the contaminant metabolism result in the production of highly reactive radical intermediate by products. For example, oxidative metabolism of PAHs proceeds via highly electrophilic intermediates, which bind covalently to cellular macromolecules such as DNA and cause formation of DNA adducts. If these adducts are not correctly repaired by the nuclear repair mechanism enzymes, it can result to a misexpression of the genome. This is the first step in chemical carcinogenesis (Dunn, 1991; Arınç *et al*., 2000). A significant increase in the hepatic levels of DNA adducts was observed in most of the field studies with various fish species, brown bullhead (Dunn *et al*., 1987), English sole (Varanasi *et al*., 1987), flounder (Baan *et al*., 1994), cod (Ericson *et al*., 1996), shorthorn sculpin (Stephenson *et al*., 2000) from PAH polluted environment. The measurement of the DNA adducts (especially in liver which is the key organ for biotransformation of xenobiotics), can be considered as sensitive and specific biomarker for the assessment of PAH exposure as well as for the assessment of potentially genotoxic effects.

1.3.5 The Biotransformation Enzymes

Biotransformation of relatively insoluble organic chemicals to more water soluble compounds is a requisite for their detoxification and excretion. The first step in biotransformation is usually the oxidative step, catalyzed by the microsomal cytochrome P450 dependent mixed function oxidase (MFO) system (also called monooxygenases, drug metabolizing enzymes). This 'Phase I' metabolism is usually followed by Phase II in which oxygenated groups of xenobiotics are conjugated with glutathione sulfate or glucuronate by different families of transferase enzymes. Thus, the resulting polar and water soluble end product can be excreted from the organisms through bile or urine (Nebert and Gonzales, 1987).

Many chemically different compounds induce de novo synthesis of cytochrome P450 dependent mixed function oxidases (Nebert and Gonzales, 1987; Nebert *et al*., 1989). In fish, some of the cytochrome P450 dependent monooxygenase activities are found to be highly inducible by certain organic contaminants such as PAHs, PCDD, PCDF and PCBs (Arınç *et al.*, 1978; Payne *et al.,* 1987). For example induction of benzo(a)pyrene hydroxylase and 7-ethoxyresorufin O-deethylase activities in response to PAH treatment is observed in various fish species (Arınç *et al.*, 1978; Stegeman and Koepper-Sams, 1987; Goksoyr and Forlin, 1992; Arınç and Şen, 1994b). From the toxicokinetic point of view induction of the biotransformation enzymes should result in a faster rate of elimination and decreased toxicity. However, cytochrome P450 dependent mixed function oxidation is also responsible for the activation of foreign chemicals to the reactive intermediates that ultimately results in toxicity, carcinogenicity and mutagenecity (Conney and Burns, 1972; Pelkonen and Nebert, 1982; Arınç and Sen, 1994 a, b). Many PAHs are metabolized by cytochrome P450 dependent MFO system to reactive metabolites. As an example, benzo(a)pyrene , a member of PAHs, is metabolized by cytochrome P450 dependent MFO system in the presence of epoxide hydrolase result in the formation of the ultimate carcinogen, benzo(a)pyrene 7,8 dihydrodiol 9,10- epoxide (BPDE), which forms DNA- adducts (Parke *et al*.,1991). Induction of cytochrome P450s may result in high levels of activated carcinogens, and consequently to higher degree of persistent DNA-adduct formation or to enhanced oxidative damage (Stegeman, 1995; Arınç *et al.,* 2000). Therefore induction of cytochrome P450 may play a dual role.

Fish population living in highly polluted areas often have high incidences of gross pathological lesions and neoplasm associated with elevated levels of toxic chemicals in the sediments (Payne *et al*., 1987). Malins *et al*. (1985) reported high levels of neoplasm in fish collected from a creosote (mixture of petroleum products) polluted Puget Sound, U.S.A. Kocan *et al*. (1985) observed that much of the cellular toxicity associated with the extracts of sediment from Puget Sound requires “metabolic

activation". DNA, isolated from neoplastic nodules of hepatic tissues of English sole exposed to creosote pollution in Puget Sound was shown to contain modified guanine, 2, 6-diamino-4-hydroxy-5-formamido pyrimidine (Fapy Gua).

In this respect, studies of cytochrome P450 enzymes in aquatic organisms hold a central role in assessing the toxic effects of chemicals both from the point of view of organism and from that of chemical. Furthermore, inducibility of the enzyme system by only certain class of chemicals in fish could provide a valuable indication of the presence of such compounds in the field and an assessment of early toxic effects on living organisms. This part will be covered in detail in section 1.7

Phase II enzymes are also inducible by exposure to certain foreign compounds (Hammock and Ota, 1983; Anderson et al., 1985; George 1994). However as compared with cytochrome P450 MFOs, the induction response of phase II enzymes are generally less pronounced (Anderson et al., 1985; George, 1994) so that they may be masked by natural variability factors such as sex, maturity, nutrition, season, temperature, etc. For these reasons they have more restricted use as biomarker.

1.4 General Aspects of Cytochrome P450 Dependent MFO System

Cytochrome P450 dependent mixed function oxidases catalyze the following generalized reaction given in Figure 1.2. In this reaction one atom of molecular oxygen is incorporated into substrate (RH) so-called monooxygenases while the other is being reduced to water in the presence of reducing equivalents in the form of NADPH, H^+.

$$RH + NADPH + H^+ + O_2 \rightleftharpoons R\text{-}OH + NADP^+ + H_2O$$

Figure 1.2 Generalized reaction catalyzed by the cytochrome P450 dependent monooxygenases

MFO can catalyze wide range different types of reactions. These includes oxidative hydroxylation of aromatic and aliphatic hydrocarbons, O-, N, and S-dealkylation reactions, N- oxidation, sulfoxidation, O-oxidation, epoxidations and deamination reactions. For detail see Table 1.2.

Substrates for MFO are of two categories: Endogenous and exogenous. Endogenous substrates include saturated and unsaturated fatty acids, eicasanoids, steroids, bile acids, vitamin D_3 derivatives, retinoids and uroporphyrinogens. Exogenous substrates includes drugs, carcinogens, precarcinogens, industrial solvents (benzene, pyridine, toluene, acetone, ethanol), environmental pollutants including PAHs such as benzo(a)pyrene and biphenyl, halogenated hydrocarbons such as PCB, insecticides (phorate, thicarb), pesticides (DDT, carbamate), herbicides (dichlorobenil), ingredients in soap and detergent, certain fungal toxins and antibiotics, amino azo dyes, and food additives. (Conney and Burns, 1972; Lu and Levin, 1974; Arınç and Philpot, 1976; Pelkonen and Nebert, 1982; Nebert and Gonzales, 1987; Porter and Coon, 1991; Arınç, 1993; Okita and Masters, 1997; Hasler 1997)

Cytochrome P450 dependent monooxygenases function as a multicomponent electron transport system and mainly present in the endoplasmic reticulum consisting of cytochrome P450, NADPH cytochrome P450 reductase and lipid (Lu and Levin 1974).

Table 1.2 Generalized reactions catalyzed by cytochrome P450s with representative substrates (primarily based on Guengerich, 1993 and Schenkman, 1991).

1. Hydroxylations	
A) Carbon	
1. Aromatic	: benzo(a)pyrene, benz(a)anthracene, acetanilid, benzene, naphthalene
2.Aliphatic	: n-propylbenzene, palmitic acid, pentobarbital
B) Nitrogens	: N-acetylaminofluorene
2. Heteroatom oxidants	
A) Nitrogen	: sulfonilamide, aniline, amphetamine, trimethylamine, indole, quinolone
B) Sulfur	: chlorpromazine, insecticides, phenylbutazone thio derivatives
C) Oxygen	: testosterone, coumarin
3. Dealkylations	
A) Nitrogen	: aminopyrene, ethylmorphine, benzphetamine, dimethyaniline, Nitrosodimethylamine
B) Oygen	: ethoxyresorufin, p-nitroanisole, ethoxycoumarin, phenacetin, codeine
C) Sulfur	: 6-methylmercaptopurine, S-methylthiobenzole
4. Reductions	
A) Dehalogenations	: halothane
B) Azo	: amaranth
C) Nitro	: P-nitrobenzoate
5. Deaminations	: amphetamine
6. Desaturations	: testosterone
7. Desulfurations	: parathion, malathion, thiobarbital, phenylthio urea

1.4.1 General Properties of Cytochrome P450s

Cytochrome P450 exists as a superfamily of structurally and functionally related isozymes, and are composed of single polypeptide chain with iron- protoporphyrin IX loosely bound by hydrophobic forces, electrostatic and covalent bonds. Molecular weights of the isozymes are in the range of 45.000 to 60.000 (Philpot and Arınç, 1976; Lu and West, 1980; Arınç and Adalı, 1983; Black and Coon, 1986). Four of the iron ligands are included in the planar porphyrin ring, the fifth is a thiolate group from a cysteine residue in the peptide backbone, and the sixth is the site of oxygen binding during the monooxygenase reaction cycle (given in section 1.4.3). The molecule is embedded in the endoplasmic reticulum membrane by one or two segments leaving the active site in the cytoplasm (Brown and Black, 1989; Werck and Fayereisen, 2000).

This group of protein has a unique absorbance spectrum that is obtained by adding reducing agent such as sodium dithionate to a microsomal preparation by carbon monoxide (CO) bubbling. CO is bound to the reduced heme protein and produce an absorbance spectrum with a peak at 450 nm, thus name P450 for "pigment with an absorbance at 450 nm" (Omura and Sato, 1964 a, b)

Even though the general features of monooxygenase reactions catalyzed by different isoforms of cytochrome P450s are the same, there are often large differences in the chemistry of both substrate and end products, from large and bulky molecules to small and planar ones, from complex steroids and polyunsaturated fatty acids to simple benzenes. Some of the cytochrome P450s are quite specific for particular substrates while the others are relatively non-selective. The most well known example is 7-ethoxyresorufin which is catalyzed mainly by CYP1A1 and named as ethoxyresorufin O-deethylase (EROD) (Burke *et al*., 1985; Lubet *et al*., 1990). It is used to measure the amount of CYP1A1 in any sample. It is suggested that the specificity of cytochrome P450 tends to increase with the increasing size and complexity of the substrate, which fit into the catalytic site of cytochrome P450 (Lewis et al., 1987; Guengerich, 1987).

Certain chemicals can 'induce' or increase the level of some cytochrome P450s in tissues of organism. The inducers may selectively induce one cytochrome P450 or several different P450s at the same time. Inducers can be substrates of the P450s or not. The most well known and potent inducers of the cytochrome P450s are the PAHs and phenobarbital which selectively induce the level of CYP1A and CYP2B isoforms of P450s, respectively.

1.4.1.1 Classification and Nomenclature of Cytochrome P450s

In 1987, Nebert *et al.* proposed a system of a nomenclature of all cytochrome P450 genes and proteins based on the relatedness of primary amino acid sequences of proteins. In this system, P450s are grouped into a gene superfamily that is further subdivided into gene subfamily. According to this, the root symbol CYP (the abbreviation for cytochrome P450) is followed by an arabic number for families (generally a group of protein with more than 40 % amino acids sequence identity) than a capital letter comes for subfamilies (greater than 55% identity) and lastly an arabic number comes for the gene, i.e. *CYP1A1* by italicized letters. At the mRNA and protein level nonitalicized root symbol 'CYP' are recommended (CYP1A1) and the term P450 1A1 is also allowed for the protein.

At least 3043 P450 genes (1277 animals, 207 lower eukaryotes, 1098 plant and 461 bacteria) have been named as of 19 January 2004. (http://drnelson.utmem.edu./cytochromeP450.html). These genes are encoding structurally and functionally similar cytochrome P450 isoforms.

1.4.2 General Properties of NADPH -Cytochrome P450 Reductase

NADPH dependent cytochrome P450 reductase is the second component of the P450 system and functions in the transfer of electron from NADPH to cytochrome P450 (terminal oxidase). It is a membrane bound amphipathic protein contains both hydrophobic peptide and hydrophilic peptide. Its monomer molecular weight determined to be 78000 (Gum and Strobel, 1981; Black and Coon, 1982; İşcan and Arınç, 1986 and 1988)**.** Hydrophilic peptide having molecular weight of 71000 contains 1 mol of each of FAD and FMN. Hydrophobic peptide is responsible for proper interaction of reductase with cytochrome P450 and anchoring the reductase to endoplasmic reticulum. In contrast cytochrome P450s, reductase does not exhibit multiplicity of enzymatic form. It is a single enzyme and has no substrate specificity or no significant inducibility by chemicals except phenobarbital (Arınç, 1995).

1.4.3. Mechanism of Cytochrome P450 dependent Mixed Function Oxidases

The mechanism postulated for the hydroxylation of organic substances by microsomal P450 dependent MFO is illustrated in figure 1.4 (taken from Lehninger, 1975). The reducing equivalents from NADPH are transferred through NADPH-cytochrome P450 reductase to cytochrome P450 during hydroxylation of various compounds. The substrate RH first combines with Fe^{+3}form. The latter is oxygenated and a second electron from NADPH converts bound oxygen to $O_2^{\bullet -}$ radical. An internal oxidoreduction takes place by the formation of hydroxylated substrate and H_2O, which contained the oxygen atom introduced as O_2. Free cytochrome P450 is regenerated in its Fe^{+3}form. (Figure 1.3 A)

It has been proposed that lipid facilitate the transfer of electron from NADPH-cytochrome P450 reductase to cytochrome P450 (Lu and Levin, 1974) .This is shown in Figure 1.3.B.

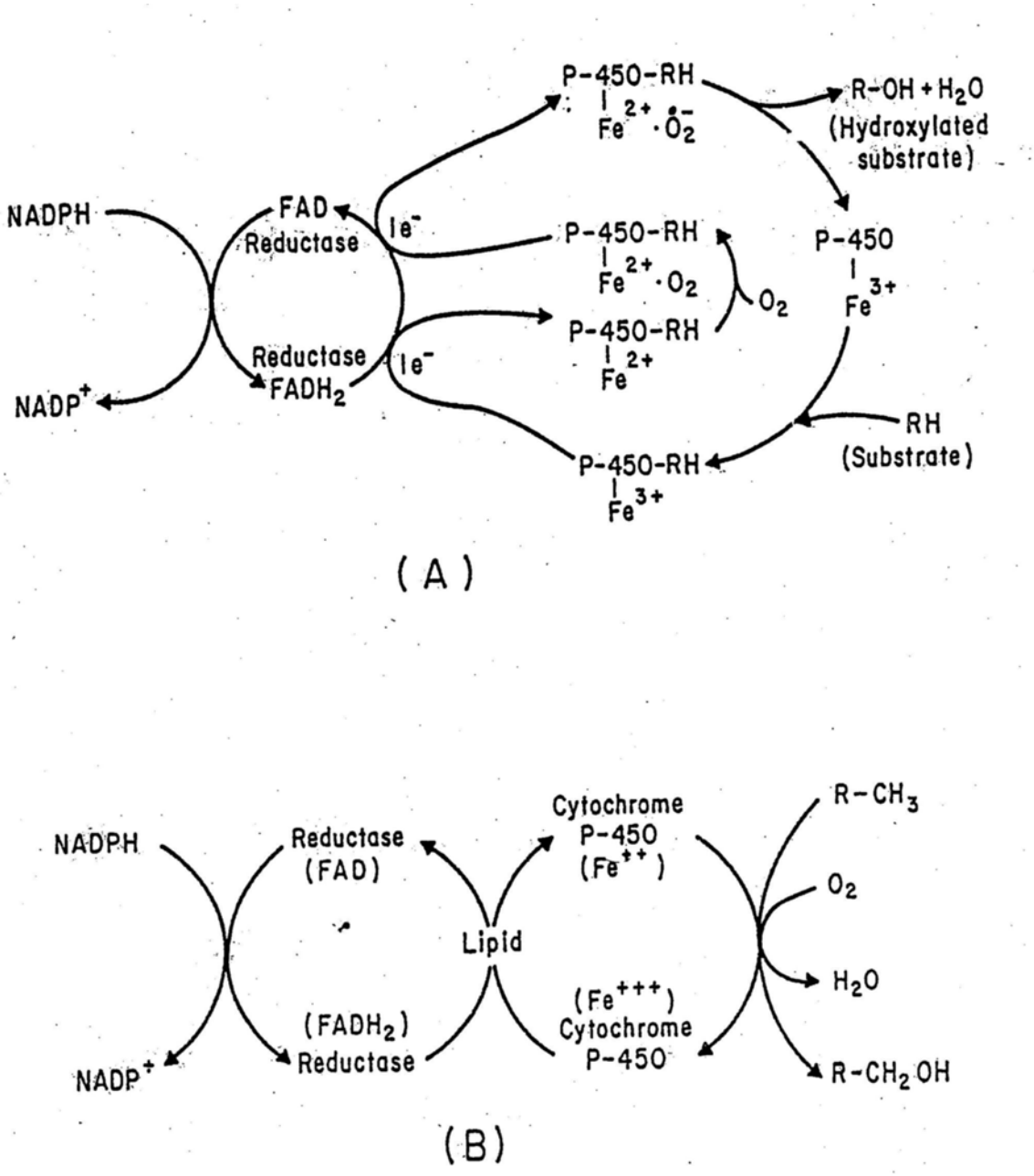


Figure 1.3 Mechanism postulated for the hydroxylation of organic substrates by the liver microsomal cytochrome P450 dependent MFO system.

A. Oxygenated intermediates (Taken from Lehninger 1975)

B. The role of lipid fraction

1.5 Cytochrome P450 MFO System in Fish

Although, Brodie and Maickel (Brodie and Maickel, 1962) in 1962 suggested that fish lacked the required enzymes for the metabolism of xenobiotics, it is now well established that a liver MFO system, with the capability of metabolizing a variety of chemicals exist in both freshwater and marine fish. (Pohl *et al.,* 1974; Bend and James, 1978; Stegeman and Kaplan, 1981; Lech *et al.,* 1982; Arınç and Adalı, 1983; Goksoyr *et al.,* 1987; Arınç and Şen, 1993a; Buhler, 1995; Stegeman, 1995). Resolution of the fish liver microsomal MFO system was first described by Arınç *et al.* in 1976 (Arınç *et al*., 1976). Little skate liver microsomal MFO system was resolved into three components: cytochrome P450, NADPH cytochrome P450 reductase and lipid.(Arınç *et al*., 1976; Bend *et al*., 1977; Arınç *et al*., 1978). Thus MFO system in fish appears to be multicomponent systems similar to the microsomal cytochrome P450 dependent electron transport system in mammals.

As in mammals multiple forms of cytochrome P450s belonging to the families of CYP1A, CYP2B, CYP2E, CYP2M, CYP2N, CYP2K, CYP3A, CYP11A, CYP17, CYP19, CYP26, CYP46, CYP51 and many others are found in fish species. Very recently, Nelson 2003 reported that at the family level 17 of 18 mammalian families were found in pufferfish (fugu). Only one P450 family, CYP39, was missing in this fish species (Nelson, 2003).

Besides the structural similarities, the catalytic functions of the fish liver microsomal P450 MFO system are very similar to mammals. Substrates metabolized by fish MFO system include exogenous compounds such as PAHs, PCBs and dioxins type toxic environmental contaminants, as well as endogenous compounds like testosterone, estrodiol, progesterone, and arachidonic acid. These substrates metabolized by different isoforms of fish cytochrome P450 MFO system via epoxidation, hydroxylation, dealkylation, and oxidation reactions. For example, CYP1A1 catalyze the epoxidation of benzo(a)pyrene, CYP2K1 catalyze the hydroxylation of lauric acid at the ω-1 position

(Williams *et al.,* 1984; Buhler *et al.,* 1997), and CYP3A27 catalyze the hydroxylation of progesterone at 6β position (Miranda *et al.,* 1991). A novel P450 subfamily, the CYP2N1 and CYP2N2 catalyze the epoxidation of arachidonic acid to epoxyecosatrienoic acids (Oleksiak *et al.,* 2000).

Among the fish cytochrome P450 isoforms, P4501A holds the priority due to its role in metabolism of carcinogens, mutagens and environmental pollutants. So the occurrence and functions of CYP1A forms in fish species are being investigated vigorously (Williams and Buhler, 1982, 1984; Klotz *et al*., 1983, 1986; Goksoyr, 1985; Miranda *et al*., 1989 and 1991; Zhang *et al*., 1991; Şen and Arınç 1997, 1998 a,b).

1.6 Cytochrome P450 1A

Two cytochrome P450 proteins, P450 1A1 and P450 1A2 have been identified from in all mammalian species studied up to now, including humans. These forms are previously called as cytochrome P448. They are involved in oxidative metabolism and activation of aromatic hydrocarbons; PAHs and other toxic chemicals. A number of planar compounds including 3-methyl-cholanthrene (3-MC), benzo(a)pyrene B(a)P, 2,3,7,8-tetra chlorodi benzo-dioxin (TCDD), dibenzofurans, PCBs are known to be potent common inducers of these two forms of cytochrome P450. In each group, induction appears to be proceeded via aryl hydrocarbon (Ah) receptor mediated mechanism. These two forms of cytochrome P450 subfamily share 75% identical amino acid sequences, a very similar gene organization and chemical properties (Sogawa *et al*., 1985; Gonzales *et al*., 1990). However their catalytic properties for carcinogenic chemicals and mechanism of transcriptional regulation show clear differences. For example, in animals cytochrome P450 1A1 had markedly high specific B(a)P hydroxylase activity which resulted in carcinogenicity, while P450 1A2 had a limited activity (Yamazoe and Kato, 1993; Ioannidis and Parke, 1993). Expression of cytochrome P4501A1 had been correlated with development of polycyclic aromatic hydrocarbon- associated cancers and other disorders in rodents (Nebert and Jones,

1989). The formation of the B(a)P 7, 8 diol 9,10 epoxide and BPDE adduct of deoxyguanosine monophosphate were demonstrated (Tang et al., 1976).

1.6.1 Cytochrome P4501A1 in Fish

In all fish species studied so far a single gene or purified protein with the properties related to the CYP1A subfamily in the mammals have been found. (Williams *et al*, 1982; Klotz *et al*., 1983; Williams *et al*.,1984; Goksoyr *et al*., 1985; Heilman *et al*,1988; Zhang *et al*., 1991; Morrison *et al*, 1995; Şen and Arınç,1998b). This gene and protein classified as CYP 1A1 based on the sequence comparison with the mammalian counterparts. In 1988 Heilmann *et al*. showed a 57-59 % identity between trout P450 and mammalian CYP1A1 and 51-53 % identity between trout P450 and mammalian CYP1A2, and concluded that trout P450 is a CYP1A1 (Heilmann *et al*., 1988).

CYP1A1 has been studied extensively in fish. It was purified from several teleosts species, from freshwater species rainbow trout (Williams and Buhler 1984, Miranda *et al*., 1989; 1990; Calender *et al*., 1989; Andersson, 1992), and perch (Zhang *et al*., 1991) and from marine species scup (Klotz *et al*., 1983; 1986 Stegeman *et al*., 1990), cod (Goksoyr, 1985; Goksoyr *et al*., 1986) and leaping mullet (Şen and Arınç, 1998a). Their molecular properties, biocatalytic activities, immunological properties and the gene regulation appear to be similar to those of mammalian CYP1A1. All purified P450 1A1, from different teleost fish species have the CO-reduced maxima at 447-448 nm. They are the major catalyst for B(a)P hydroxylation and 7-ethoxyresorufin O-deethylation (EROD) reactions. They are strongly inducible by 3-MC and B(a)P, however they are at barely detectable level in unexposed or untreated fish. Their molecular weights around 58.000 and show strong cross reactivity with antibodies to mammalian CYP1A1 and antibodies to each other CYP1A1(For detail see Arınç and Şen, 1999; Şen, 1997).

CYP1A1 are highly inducible by various PAH, PCB, TCDD and other halogenated compounds. Table 1.3 shows the xenobiotics that induce fish cytochrome P4501A1. Several fold induction of cytochrome P4501A1 content and P4501A1 associated B(a)P hydroxylase activities up on treatment of fish by 3-MC and related compounds has been demonstrated (Buhler and Williams, 1989; Forlin and Calender, 1993; Bucheli and Fent, 1995).

In one of the study (Bend *et al*., 1977), the effect of administration of 3-methylcholanthrene (3-MC) and TCDD on the hepatic microsomal MFO activities of little skate (Raja erinacea) was displayed. It was found that B(a)P hydroxylase activity of skate hepatic microsomal was increased when skate was treated twice with 3-MC (50mg/kg body weight), orally. Similar results were also obtained with TCDD. B(a)P hydroxylase activity of little skate hepatic microsomes was markedly increased about 15-18 fold after administration of two separate doses of TCDD (4.5 μg/kg body weight) intraperitonially. However, no significant changes were observed in aniline hydroxylase, 7- ethoxycoumarin O-deethylase, and cytochrome P450 content of the fish liver microsomes with TCDD treatment.

In 1994, Arınç and Şen demonstrated that B(a)P treatment of gilthead seabream (25mg/kg, intraperitonially, for 5 consecutive days) caused 2-3 fold increase in EROD activity of gilthead seabream liver microsomes. However same treatment did not cause any significant changes in ethylmorphine N-demethylase and aniline 4-hydroxylase activities of liver microsomes (Arınç and Şen, 1994b) as in the above case, indicating that CYP1A1 enzyme induction by PAH type inducers are highly specific and sensitive in fish populations.

Table 1.3 Examples of xenobiotics that induce cytochrome P450 1A1 in fish

Benzo(a)pyrene
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
3-Methylcholanthrene
Polychlorinated biphenyls
Ethoxyquin
β-Naphthoflavone
Hexabromobenzene
Endosulfan, Butylatedhydroxytoluene
Pyrene, Chrysene
Butylated hydroxyanisole
7,12- dimethylbenz(a)-anthracene
Tert-Butylhydroxyquinone

1.7. Hepatic Cytochrome P4501A Induction in Fish as an Environmental Biomarker

Liver CYP1A1 induction in fish by certain classes of chemicals described above has been applied extensively as a biomarker in field studies. In 1975 Payne and Penrose (Payne and Penrose, 1975) showed that brown trout taken from a small urban lake in Newfoundland, Canada contaminated with petroleum hydrocarbons had increased cytochrome P450 1A1 associated B(a)P hydoxylase activity. Payne then in 1976 suggested that the use of B(a)P hydroxylase activity of fish liver as an environmental monitor for the first time (Payne , 1976). Subsequent studies showed that fish caught in waters contaminated with petroleum oil hydrocarbon, paper mill effluents, and industrial and municipal wastes exhibited elevated levels of CYP1A1 and associated enzyme activity (Edward and Addison, 1988; Goksoyr and Forlin, 1992; Livingstone, 1993; Stegeman, 1995; Addison *et al*., 1996; Buhler *et al*., 1998; Arınç and Şen, 1999; Arınç *et al*., 2000; Arınç *et al*., 2001; Flammarion *et al*., 2002).

The marine environment today is loaded with perhaps 60000 different chemical. Even though chemical analyses are able to measure a wide range of pollutants quantitatively and accurately, such a complex mixture of chemical pollutants can not be fully assessed. Furthermore it does not reveal the impact of chemical pollutants on the aquatic environment (Arınç *et al*., 2000). On the other hand the use of CYP1A induction as biomarker in fish, only by certain type of environmental contaminants such as PAH, PCB, may give an integrated information about levels of these chemicals, their bioavailability, co-acting (synergistic or antagonistic) effects and biological response of the organisms (Goksoyr and Forlin ,1992).

CYP1A1 response measured as EROD or AHH activities has already been incorporated into some major monitoring programs such as the National Status and Trends Program in United States (Collier et al., 1992) and North Sea Task Force Monitoring Master Plan of the North Sea Nations in Europe (Goksoyr and Forlin, 1992). The general strategy in the field studies is to compare CYP1A1 associated enzyme activity in fish from suspected sites with those in fish from reference site. Most of the earlier field studies employed the induction of liver B(a)P hydroxylase activity in biomonitoring. The use of this assay has been declining because of carcinogenic property of substrate; benzo(a)pyrene as well as possibility of substrate cross-reaction with other CYP isozymes (Arınç *et al*., 2000). Nowadays CYP1A1 associated enzyme activity determined by using 7-ethoxyresorufin as a substrate. The measurement of 7-ethoxyresorufin O-deethylase activity appears to be most sensitive and most widely used catalytic probe for determining induction response of CYP1A1 in fish.

In addition to CYP1A1 associated monooxygenase activity, CYP1A1 induction can be measured at CYP1A1 protein level and also CYP1A1 mRNA level. Table 1.4 shows the common methods for determination of CYP1A1 induction in biomonitoring studies.

After purification of CYP1A1 proteins from different fish species, the antibodies against these proteins have been successfully produced. Using the immunochemical techniques such as ELISA and Western Blot, these antibodies have been used to demonstrate correlation between CYP1A1 protein level and contaminants load in deep – sea fish from the Northern Atlantic (Stegeman *et al*., 1986), in English sole from Puget Sound, WA, U.S.A (Varanasi *et al*., 1986), in winter flounder from Notheastern US areas (Elskus et al., 1989; Stegeman et al., 1987), and in flounder from Frierfjorden in Norway (Stegeman et al., 1988), in leaping mullet from İzmir Bay in Turkey (Arınç and Şen, 1999)

Table 1.4 Common Methods for determination of CYP1A induction as a biomarker

Level	Nomenclature	Marker
DNA	→ CYP1A1	
▼		
mRNA	→ CYP1A1	DNA probe
▼		
protein	→ P450 1A1	Antibody
▼		
enzyme	→ EROD/ AHH	Catalytic activity

After the cloning of the first fish CYP1A1 gene (Heilmann *et al.,* 1988) a cDNA probe has become available for the determination of CYP1A1 transcript (mRNA) i.e. the initial phase of induction. Procedures to study the induction at mRNA level require isolation of RNA from tissue of interest (mostly liver tissue used for this purpose) and designing a specific probe with a nucleotide sequences complementary to *CYP1A1*. Northern blot analysıs is carried out to determine the level CYP1A1 transcript. Studies have indicated that P4501A1 mRNA level correlate with increases in P450 1A1 enzyme activity and P4501A1 protein levels (Renton and Addison, 1992). For example, Arınç, Kocabıyık and Su (2001) had demonstrated that P4501A mRNA expression, CYP1A protein levels and associated enzyme activity, EROD, were naturally induced in feral leaping mullet caught from contaminated sites of the İzmir Bay, Turkey. In this study induction mRNA was measured using a nucleic acid hybridization technique. For the hybridization studies, a new 33-mer oligonucleotide probe 5'-d CTC ATC CAG CTT CCT GTC CTC GCA GTG ATC AAT-3' was designed, which is corresponded to the totally conserved amino acid motif of CYP1A protein from position 291 to 301 among the various fish species. Results of Northern blot analysis revealed that RNA isolated from the liver of mullet collected from highly contaminated region of the İzmir Bay with a dissolved and dispersed petroleum hydrocarbon content of 12.45 μg l^{-1} gave a strong hybridization signal, whereas only a weak hybridization signal was detected in the liver RNA of fish caught from the reference site containing less than 1 μg l^{-1} of petroleum hydrocarbons. Similarly, fish from the contaminated site had approximately 80 times more EROD activity than the feral fish captured from the reference site. Studies using polyclonal antibodies produced against purified mullet CYP1A also showed the similar trend. In conclusion, this study clearly showed that, feral leaping mullet caught from contaminated water displayed induction of CYP1A at three levels of expression, namely, mRNA, apoprotein and catalytic activity (Arınç *et al*., 2001).

As a result, all these studies have shown that the induction of CYP1A1 and its catalytic activity, EROD in fish provide important information about the environmental

pollution and have been used as an "early warning signal" of possibly more serious pathologies (Payne *et al*, 1987; Arınç *et al*., 2000).

1.8. CYP 1A1 Regulation

Regulation of induction of CYP1A is the best studied and well characterized among the cytochrome P450 families. There are at least two types of regulatory DNA sequences found in the cell. The first one is designated as xenobiotic responsible elements (XRE) that works as an inducible enhancer in response to inducers. This cis-acting regulatory DNA element is important in terms of inductive response (Fujisava-Sehara *et al*., 1987; Whithlock *et al*., 1996). The other regulatory element is named as basal transcription element (BTE) that is involved in the constitutive expression of the gene (Yanagida *et al*., 1990; Fuji-Kuriyama *et al*., 1992). It has been shown that these regulatory elements exist in human CYP1A1 gene. XRE works via a trans acting regulatory factor which is found in the cytosol. It is identified and named as XRE-binding factor or Ah (aromatic hydrocarbon) receptor (Jhonson and McKnight, 1989; Fujisawa-Sehera *et al*., 1988; Denison *et al*., 1988; Elferink *et al*., l990; Bradfield *et al*., 1990). It was found that it exists as cryptic form in the cytoplasm of the untreated cells and associates with the inducers when it is available (Hapgood *et al*., 1989, Denison *et al*., 1986; Cuthill *et al*., 1987). The cryptic form of the Ah receptor is bound to with a heat shock protein, HSP90 found in the cytoplasm, within the presence of inducer, the Ah receptor binds to inducers and dissociates from the HSP90 and translocates to nuclei. Another protein called as Arnt (Ah receptor nuclear translocating protein) is required for the CYP1A1 induction (Goffmann *et al*., 1991; Ryes *et al*., 1992; Probst *et al*., 1993; Whitelaw *et al*. 1993; Lif *et al*., 1994). It is a nuclear protein dimerizing with liganded Ah receptor, providing the binding of Ah receptor to DNA (Whitlock *et al*., 1996).

The model mechanism for the induction of CYP1A1 is given in Figure 1.4. The lipophilic inducer passively diffuses into the cell and binds to Ah receptor which is bound to heat shock protein Hsp 90, the latter is subsequently released upon binding.

The Ah receptor and xenobiotic complex translocate into the nucleus where it heterodimerizes with Arnt. The dimer generates a functional, XRE-binding transcription factor and bind to XRE sites on the enhancer. General Transcription factors now have more efficiently access to promoter region of CYP1A1 gene. Consequently, messenger RNA synthesis is increased, resulting in elevated protein level (Stegman and Hahn, 1994).

Studies with teleost species (Haasch *et al*., 1989; Kloepper-Sams and Stegeman, 1989; Lorenzon and Okey, 1990) showed that regulation of CYP1A1 is a receptor mediated system as seen in mammals. An Ah receptor similar to the mammalian Ah receptor has been found in cytosol of several fish (Heilmann *et al*., 1988; Lorenzon and Okey, 1990; Hahn *et al*., 1992). Moreover, XRE occurs in inducible gene CYP1A1 gene in fish but it is nature is unknown (Devaux and Pesonen, 1992). On the other hand, while the general mechanism seems to be similar, the process of the CYP1A1 induction in fish has several distinctions when compared to mammals such as greater lag time between mRNA increase and translation, posttranslational suppression by estradiol (Stegeman, 1993; 1995).

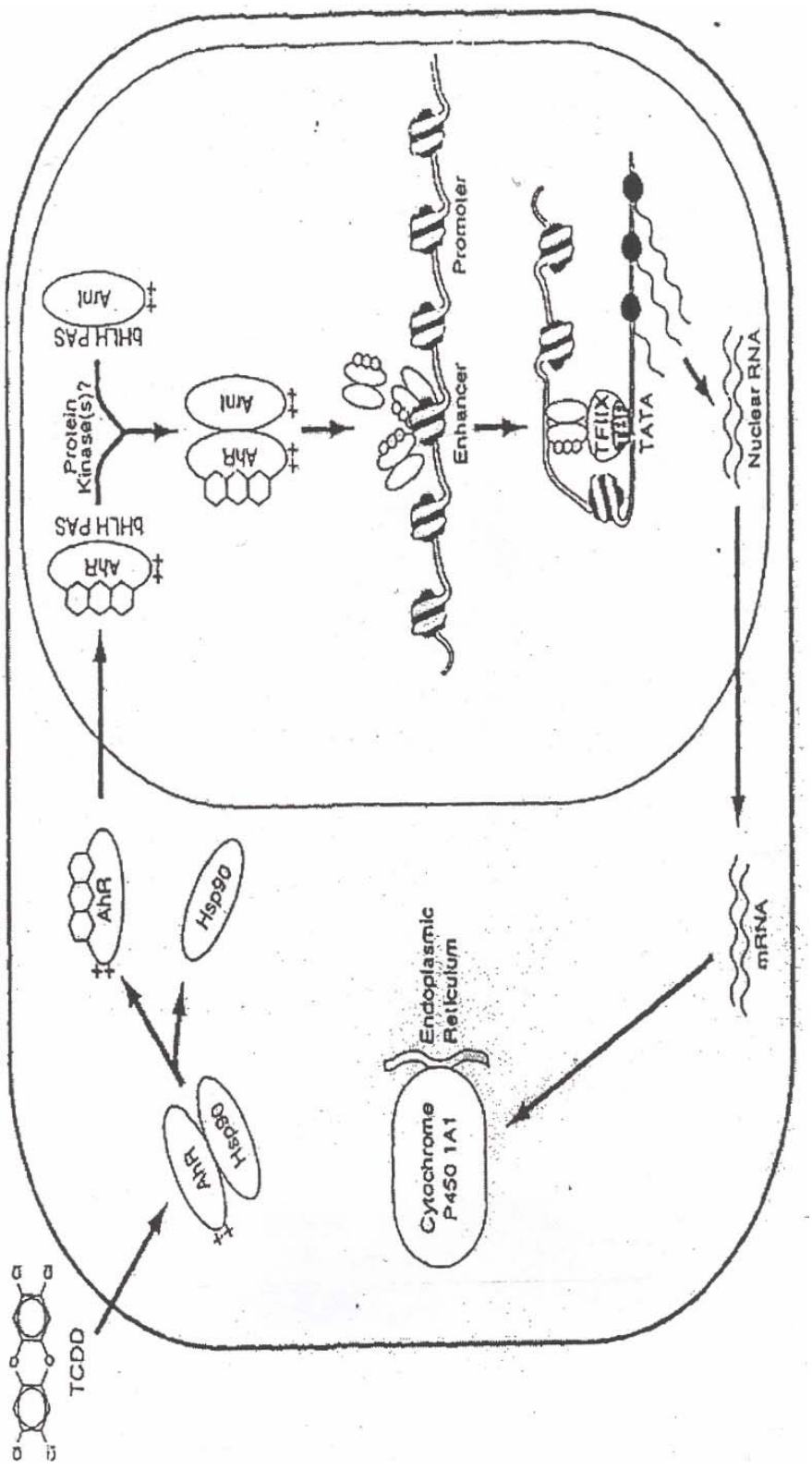


Figure 1.4 Diagram showing the basic steps of cytochrome P450 1A1 induction (taken from Whitlock *et al.*, 1996)

1.9 The İzmir Bay

The İzmir Bay is the greatest natural bay of the Turkey located along eastern Aegean Sea of the Mediterranean. The Bay extends approximately 24 km in east- west direction with an average width of 5 km. The Bay is naturally divided into three parts according to hydrology and topography: Inner, Middle and Outer Bays. The Inner Bay extends from the head of the Bay to the Yenikale light house, the Middle Bay extends to Kokola point and the outer bay extends from Kokola point to Karaburun. See Figure 1.5

The city of İzmir is built around the perimeter of the Inner Bay. This metropolitan area has experienced a population explosion during last three decades from 450.000 in 1965 to 3.000.000 in 1997. The rapid population increase is the main source of the urbanization and proliferation of various size industries and intense agricultural activities which impact heavily on the environment of the Bay. Industrial activities cover a large range of industries, including food processing, tanneries, paint, paper and pulp factories, chemical and textile factories, vegetable oil and soap production, and a petroleum refinery. The wastes of these industries which include heavy metals as well as PAH, PCB, PCDD and PCDF type persistent organic contaminants are discharged directly into the Inner Bay through domestic sewage network or a few small river. In addition, domestic waste waters of nearly 3 million inhabitants are also discharged through sewage network, by this way a large amounts of phosphorous and nitrogen, are introduced into the Inner Bay. The İzmir Harbor is the second largest port in Turkey. As indicated by Balkas and Juhasz (1993), 25% of Turkey's export and 55% of her import pass through the port and commercial ships visiting the harbor each year is approximately 2000. Discharges of petroleum hydrocarbons from these ships are a major source of PAH type pollution in the Inner Bay.

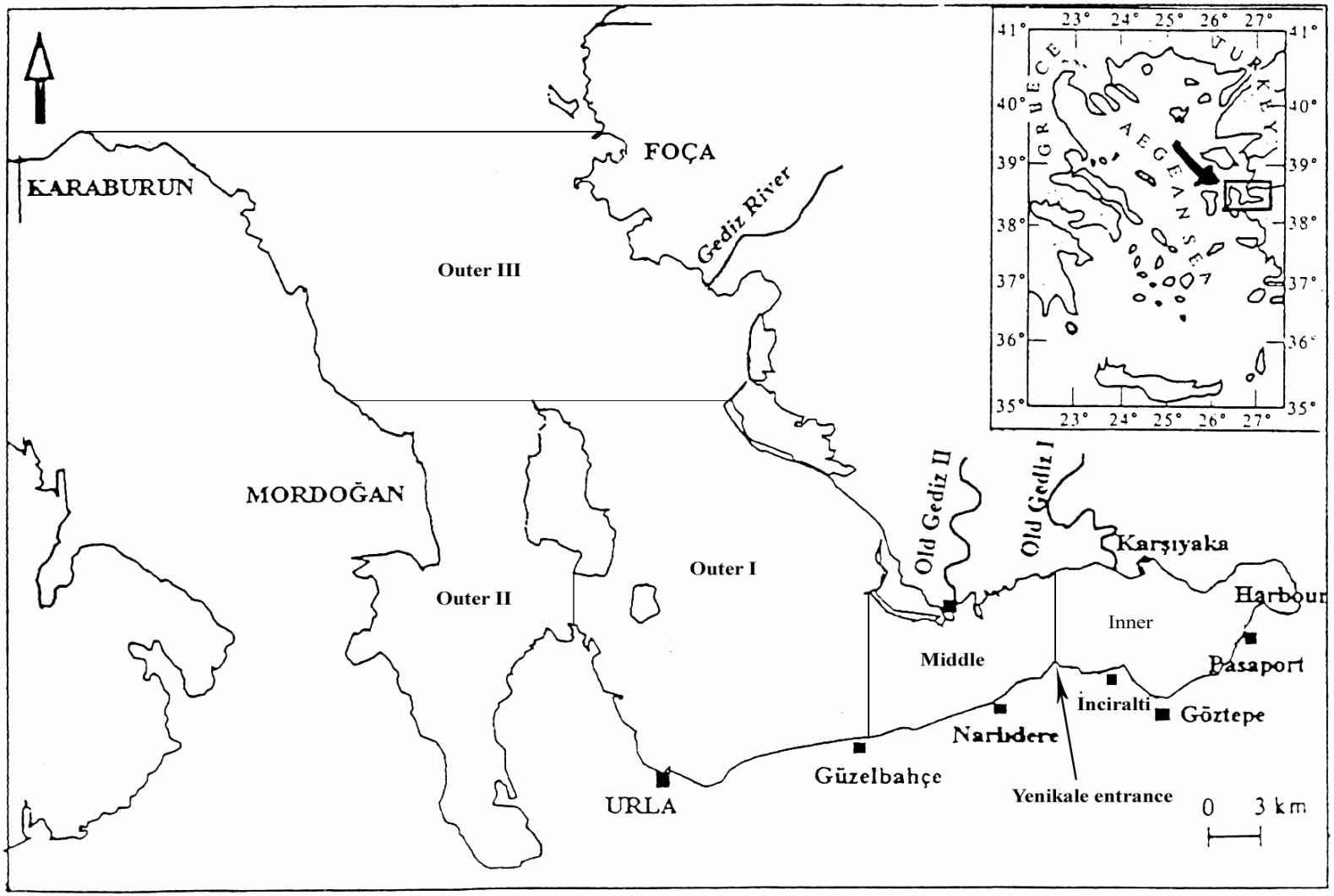


Figure 1.5 The Map of İzmir Bay

In summary, industrial and domestic wastes, discharges from ships as well as contaminated waters of several small rivers heavily pollute the Inner Bay (Arınç and Şen, 1999). The middle Bay is a transition zone with pollutant concentration intermediate between the Outer and Inner Bays, and the pollution in the Outer Bay is not considered significant (Balkas, Juhasz, Yetis and Tuncel, 1992).

Quantitative metal analyses in İzmir Bay have demonstrated that concentration of heavy metals such as chromium, copper, lead, cadmium and mercury in sediments and surface waters of the Inner Bay were 6-15 times higher than those of the Outer Bay (Balcı and Türkoğlu, 1993).

Aksu *et al*., in 1998 studied the extent of heavy metal and organic pollution from the surface sediments of the İzmir Bay, in detail. The concentration of 42 elements in 84 samples (collected from 84 different point along the İzmir Bay), established that surface sediments in the Inner Bay displayed significant enrichments in Ag, As, Cd, Cr, Hg, Mo, P, Pb, Sn, V and, Zn. Heavy metal concentrations are highest in the Harbor region and sharply decrease near Yenikale Entrance. 14 samples from the Inner Bay showed that these sediments also exhibit significant enrichment in PCDD, PCDF and PAH concentrations. Total PCDD in concentration ranged from >0.95 ng/g (ppb) in the Harbor region of the Inner Bay, to 0.09-0.07 ng/g at the Yenikale Entrance to >0.04 ng/g in the Middle Bay. Total PCDF concentration was notably less, ranging from 0.06 ng/g in the Inner Bay to 0.04 ng/g at the Yenikale Entrance to 0.03 ng/g in the Middle Bay. Total PAH concentration are highest in the Inner Bay (9.27 µg/g or ppm) dramatically decreasing to 1.25 µg/g at the Yenikale Entrance and to 0.42 µg/g in the Middle Bay. They also determined the individual concentrations of various PAH isomers. According to this İzmir Bay surface sediments are dominated by 4- and 5- ring PAHs which are carcinogenic and mutagenic (Saed *et al*.,1995) with lesser contribution from the 2-,3-ring compounds which are relatively non-toxic. In all İzmir Bay surface samples, benz(a)anthracene and pyrene are dominant PAH isomers. Samples from the inner most

harbor region contained major quantities of benzo(a)pyrene, benzo(b)fluoranthene and benzo(ghi)perylene. (For details see Aksu *et al*., 1998)

Except for two regions, the Outer and Middle Bay show low level of heavy metal contamination (Aksu *et al*., 1998). Moderate levels of heavy metal contamination observed around Hekim Island dump site is probably caused by the continuous dumping of sediments excavated from the most polluted harbor region of the Inner Bay between 1976 and 1990. Moderate pollution is also observed in the south of the mouth of Gediz River which is located in the Outer Bay. This river is a recipient of municipal and industrial waste water and moderate pollution observed in the Outer Bay surface sediment is correlated with the pollution associated with the discharge of the Gediz River. In addition to Gediz, several small rivers entering the Inner Bay loaded with the untreated sewage and industrial wastes. For example until 1994, the leather tanning plants which use large quantities of arsenic and chromium salts in the tanning procedure discharged their untreated wastes products directly into the Inner Bay via Melez River (Aksu *et al*.,1998). In a local study Müezzinoğlu and Şengül (1987) reported notable enrichment of Cr in bottom sediments of some small rivers discharged into the Inner Bay.

Boyacıoğlu, investigated the genotoxic and mutagenic effect in the sediment samples of the İzmir Bay collected between October-December 1995 from the stations on the rivers and sewages draining to the İzmir Bay and in the inner, middle and outer parts of the Bay by Ames mutagenecity test using S.typhimurium TA98 and TA100 strains. According to the results, the rivers and sewage draining to the inner parts of the Bay are important sources of the mutagenic activity of due to their pollution load (Boyacıoğlu 1999).

İzmir Great Canal Project has been active to prevent the Bay from the direct discharges of domestic and industrial wastes since 2000. Project aims to collect and

refine 100 % of industrial and 70% domestic waste waters discharged into the Inner Bay from various sources. Accordingly, refined waters are planned to be introduced into the Outer Bay from Çiğli Refining System. Great Canal Project has a biological system in which nitrogen and phosphorous containing nutrient elements can be refined. In the scope of Great Canal Waste Water Monitoring Project (Büyük Kanal Atık Sularının İzmir Körfezi'nde İzlenmesi Projesi), chemical analyses of nutrient elements and heavy metals have been carried out since 1996. According to the 2001 final report, although there was a decrease in the concentration of phosphorous element in the Inner Bay, no significant change was observed in the concentration of heavy metals both in the Middle and Inner Bay (For details see, "Büyük Kanal Atık Sularının İzmir Körfezi'nde İzlenmesi Projesi" 2001 Final Report, Project No: DBTE-134, supported by İzmir Büyükşehir Belediyesi İzmir Su ve Kanalizasyon İdaresi Genel Müdürlüğü and Dokuz Eylül Üniversitesi Deniz Bilimleri ve Teknolojisi Enstitüsü). However, no data were available about the concentration of PAH, PCDD and PCDF type persistent organic chemicals after Canal Project.

1.10 Previous Biomonitoring Studies along the İzmir Bay

Biomonitoring of toxic and carcinogenic organic pollutants along the İzmir Bay have been going on since 1995 in our laboratory. In these studies the degree of induction of cytochrome P450 1A1 associated 7-ethoxyresorufin O-deethylase (EROD) activity, immunochemical detection of P4501A1 and P450 1A1 mRNA level in leaping mullet were used as biomarker for assessment of PAH, PCB and dioxin type organic pollutants. In addition EROD activities in common sole, a benthic fish species were also determined to evaluate the levels of persistent toxic organic contaminants in the sea basins. (Arınç and Şen 1999, Arınç *et al*., 2000; 2001)

First biomonitoring studies were carried out between years 1995 –1996. Two different fish species- a pelagic fish leaping mullet (*Liza saliens*) and a benthic fish

common sole (*Solea vulgaris*) were sampled from different sites of the Bay by following a pollution gradient. Leaping mullet from the highly urbanized and industrial section of the bay, Pasaport (site1) showed highly elevated enzyme activities (1293±292pmol/min/mg protein, n=208), which were about 62 times higher than the value at the reference site (25± 9 pmol/ min /mg protein, n= 4).Leaping mullet caught along a pollutant gradient at three other sites, Karsıyaka (site 2), İnciralti (site3) and Tuzla (site 4), also had highly elevated activities, namely 761±139 n=13, 417±39 n=12, 334±40 n=12 pmol/min /mg protein, respectively. These were 36, 18, and 15- fold higher than those obtained from the reference region, site 10 (Arınç *et al*., 1999) See Figure 1.6. In addition, polyclonal antibodies (Pab) raised (Arınç *et al*., 1999) against purified mullet P450 1A (Şen and Arınç, 1998b) were used to detect the degree of induction of liver P450 1A of fish in response to pollutant in İzmir Bay. These results obtained from that study clearly indicated that mullet caught from the polluted sites had highly induced EROD activity and cytochrome P4501A1 contents.

The benthic fish, common sole, captured in İnciralti (site 3) had EROD activity of 2000±115 pmol/min/mg protein n=13, indicating that sediments from that area were highly contaminated with CYP1A-inducing chemicals generally PAH, PCDD, PCDF. This result was 5 times higher than the EROD activities of leaping mullet that caught from same site, indicating that PAH type contaminants mostly accumulated in the sediment rather than the sea water. Common sole captured from in Outer Bay (mouth of the Gediz) had higher EROD activity (300±10 pmol/min/mg protein n=5) than that of the fish caught from Tuzla (113±14 pmol/min/mg protein n=49) (Arınç *et. al*., 1999).

In biomonitoring studies between 1996 and 1997, mullet that caught from Pasaport again exhibited highly elevated EROD activity (883±172 pmol/min/mg protein n=13) which was approximately 80 fold higher than those obtained from reference site. EROD activities (11±2 pmol/min/mg protein). Western blot analyses were also in good correlation with EROD activity results. In that study, in addition to those studies,

CYP1A1 mRNA levels were also quantified in leaping mullet liver from contaminated areas and from the reference site by using molecular biology techniques (details of the study were given in section 1.7). The results of that study were found to be parallel to EROD activity measurement and Western blot analysis. (Arınç *et. al.*, 2001)

Biomonitoring studies that carried out in January 1999 showed highly elevated EROD activities of leaping mullet from Pasaport (1028±287 pmol/min/ mg protein n=4). Gray mullet (Mugil cephalus) caught from the harbor (site 1A) also had highly increased enzyme activity (1398±410 pmol/min/mg protein). Leaping mullet caught from Narlıdere (site 6) and from Urla (site 11) displayed moderately elevated EROD activities that were 105±26 (n=4) and 310±21 (n=4) pmol/min /mg protein respectively (Arınç *et. al.*, 2000).

In the scope of present study, we monitor the PAH, PCDD and PCDF type toxic organic pollutants along the İzmir Bay after the Great Canal Project, by examining the hepatic CYP1A and its monooxygenase activity (EROD) and CYP1A protein level by Western blotting in three different fish species, leaping mullet, common sole and annular seabream.

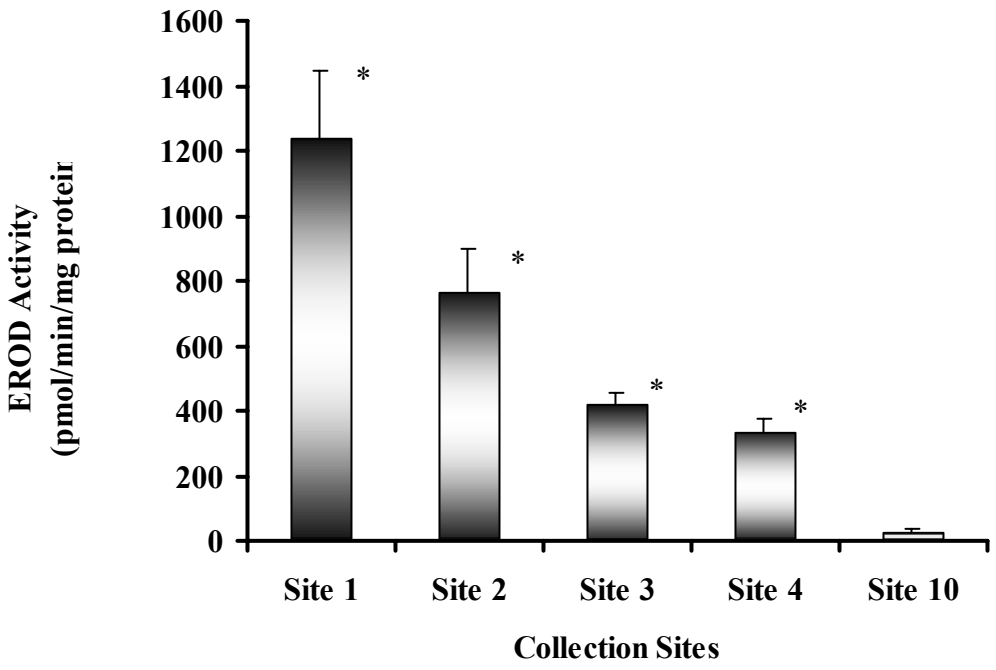


Figure 1.6 Ethoxyresorufin O-deethylase (EROD) activities of liver microsomes of mullet (Liza saliens) caught at five different sampling sites from the Izmir Bay in 1995 and 1996 (**Taken from Arınç and Şen 1999**). Site 1 -Pasaport, Site 2 – Karsıyaka, Site 3- İnciraltı, Site 4- Tuzla and Site 10 –Outer Bay (reference point). Enzymes activities were given as mean±SE. *Significantly different from the reference point value $p < 0.001$.

1.11 Test Species

In this study three kinds of fish species were used: Leaping mullet (*Liza saliens*), common sole (*Solea vulgaris*), and annular seabream (*Diplodus anularis*).

Leaping mullet, a pelagic fish belong to the family of Mugilidae of the class osteichtyes. It is an economically important marine fish inhabiting usually inshores, entering the lagoons and estuaries along the Atlantic coasts northward of Bay of Biscay, also whole of the Mediterranean, Black sea, and Sea of Azov. It is an omnivorous fish and its main food consists of planktonik organisms such as small crustoceans, blue-green algae, diatoms and detritic matter. It is resistant and tolerant to pollutants and various environmental conditions so it could live in polluted areas (Balık et al., 1992), and it is also consumed in large quantities in Turkey. It is suitable for field studies because it inhabits along all coasts of Turkey. It is the choice of test species because being a surface fish it gives information about the quality of sea water.

Common sole, a benthic (sediment) fish belong to the family of Soleidae (Soles) of the class Actinopterygii (ray-finned fishes). It is found in the Eastern Atlantic: southward from Trondheim Fjord (including North Sea and western Baltic) and Mediterranean Sea (including Sea of Marmara, Bosphorus and southwestern Black Sea). It burrows into sandy and muddy bottoms and retreats to deeper water during winter. It feeds on worms, molluscs and small crustaceans at night . It is an economically important marine fish due to marketing of their meats. It is the choice of test organism because it is a benthic fish and gives information about the contaminant level of sediment. (http://www.fishbase.org/Summary/SpeciesSummary)

Annular seabream , a benthopelagic fish belong to the class Actinopterygii family of Sparidae. It is found in the Eastern Atlantic: Madeira and Canary islands. Also found along the coast of Portugal northward to the Bay of Biscay, Mediterranean, Black

Sea and Sea of Azov. It inhabits chiefly sandy bottoms, rarely on rocky bottoms. It is carnivorous, feeds on worms, crustaceans, molluscs, echinoderms and hydrozoans. It is the choice of test organism because it is widely distributed along the different region of the İzmir Bay. (http://www.fishbase.org/Summary/SpeciesSummary)

1.12 The aim of the Study

In the marine environment, organisms are often exposed to complex mixtures of pollutants, including PAHs, PCBs, and dioxins type persistent organic chemicals. These chemicals induce one of the P450 isozyme, P4501A1 in fish liver. P4501A1 oxidatively metabolizes these toxic compounds and precarcinogens/carcinogens to their epoxide and other oxygenated metabolites which in turn bind to DNA and form DNA adducts leading to membrane impairment, cellular toxicity, mutation and even carcinogenesis. The induction of hepatic CYP1A1 and its monooxygenase activity 7-ethoxyresorufin O-deethylase (EROD) in fish by PAHs, PCBs and dioxins has been suggested as an early warning signal and most sensitive biochemical response for assessing environmental contamination conditions. This has implications for human fish consumption from contaminated area as well as reproduction success and survival of fish species.

In this study one of the our aim was to determine the level of PAH, PCB and dioxins after the Great Canal Project, in several locations along the Izmir Bay by measuring the induction of cytochrome P4501A1 associated EROD activity and immunochemical detection of P4501A1 protein level in the liver of three different fish species (leaping mullet, common sole and annular seabream) and to compare these results with the previous studies (Arınç *et al*, 1999,2000 and 2001). Here we provide the first measurement of the level of CYP1A1 inducing chemicals in the İzmir Bay after the Great Canal Project.

Among the available environmental monitoring techniques, the integrated use of chemical analysis and biochemical response to pollutants is an effective procedure for detecting the impact of contaminants in the aquatic systems. In this context we aimed to quantify total PAH concentrations in fish liver tissues and sediment that were collected from different section of the Bay along a pollutant gradient.

Leaping mullet and common sole have been successfully used in our laboratory as test species to monitor the level of CYP1A inducing chemicals along the Izmir Bay. However, in the present study, annular seabream was used as a test species for the first time. Accordingly, during this study we also aimed to find out if annular seabream would be convenient as a monitoring species for evaluating CYP1A inducing chemicals in the marine environment. In addition, another purpose of this study was to characterize major cytochrome P450 dependent MFO activities such as benzphetamine N-demethylase, ethylmorphine N-demethylase and aniline 4-hydroxylase in annular seabream which have not been addressed before.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acetic acid glacial (0056), aluminum oxide 90 mesh size active neutral (101077), benzene (101782), chrysen (814075), cupper (II)- sulfate-pentahydrate ($CuSO_4.5H_2O$ 02787), ethylene diamine tetra acetic acid disodium salt (EDTA; 08421), D-glucose-6-phosphate dehydrogenase type XI (11645) glycerol (04093), hexane (104368), potassium hydroxide (KOH) potassium chloride (KCl; 04935), magnesium chloride ($MgCl_2$; 05833), methanol (02500), sodium hydroxide (NaOH; 06462), sodium dodecyl sulfate (SDS; 822050), zinc chloride ($ZnCl_2$; 08515) were purchased from E. Merck, Darmstadt, Germany.

Acrylamide (A8887), ammonium acetate (A7672), ε-amino caproic acid (ε-ACA; A2504), bovine serum albumin (BSA; A7511 or A7888), 5-bromo-4-chloro-3-indolyl phosphate (BCIP; B8503), ethoxyresorufin (E3763), N,N-dimethylformamide (D8654), D-glucose-6-phosphate monosodium salt (G7879), glycine (G7126), N-2-hydroxyethylpiperazine-N-2, ethane sulfonic acid (HEPES; H3375), β-mercaptoethanol (M6250), N,N'-methylene bisacrylamide (BIS; M7256), β-nicotinamide adenine dinucleotide, reduced form (NADH; N8129), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$; N0505), nitroblue tetrazolium (NBT; N6876), phenylmethane sulfonyl fluoride (PMSF), phenazine methosulfate (PMS; P9625), polyxyethylene sorbitan monolaurate (Tween 20; P1376), sodium potassium tartrate (Rochell salt; S2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris,

T1378), anti-rabbit IgG-ALP conjugate (A3687) were purchased from Sigma Chemical, Company, Saint Louis, Missouri, USA.

Ammonium persulfate (161-0700), N, N, N', N' tetrametylethylene diamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, California, USA. Aniline (A-0759) was obtained from Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, USA.

Benzphetamine-HCl was kindly provided by Dr. J. F. Stiver of UpJohn Co., USA. Ethylmorphine-HCl was purchased from Tarım ve Köy İşleri Bakanlığı, Toprak Mahsülleri Ofisi, Ankara, Türkiye.

All the other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.1.2 Fish Collection

The fish were captured in November 2002 and October 2003 from the different sites of İzmir Bay, on the Aegean Coast of Turkey. Three different fish species were used throughout the study namely leaping mullet (*Liza saliens*), annular seabream (*Diplodus annularis*) and common sole (*Solea vulgaris*) which were representative of pelagic, benthopelagic, and benthic fish, respectively. Leaping mullets, each weighing 300-400 g, were caught by fish net at eight different sites (site 1: Pasaport, site 1B: Liman –Yalıçapkını, site 1C: Liman -Alsancak, site 3: İnciraltı, site 3A: Üçkuyular port site, site 11A: Zeytinalanı, site 19: Hekim Island, site 10: Outer Bay). Annular seabream, each weighing 60-70 g, were caught by fish net at four different sites (site1C: Liman -Alsancak, site 1: Pasaport, site 3A: Üçkuyular and site 11A: Zeytinalanı). Common sole, each weighing 35-45, were caught by beam trawl at six different sites (site 16A, site 10A: Foça open site, site 5: Mouth of Gediz, site3: İnciraltı, site 19: Hekim Island, site 7A: Foça coastal line). The map of İzmir Bay and the collection sites of sample for three different fish species were given in Figure 2.1. Pasaport (site1), Harbor –Liman (site1B and 1C),

Üçkuyular (site 3A), and İnciraltı (site 3) were in the Inner Bay, Hekim Island (site 19), Zeytinalanı (site 11A), mouth of Gediz (site 5), site 16A, Foça coastal line (site 7A), Foça open site (site 10A) and site 10 were in the Outer Bay.

2.1.3 Sediment Collection

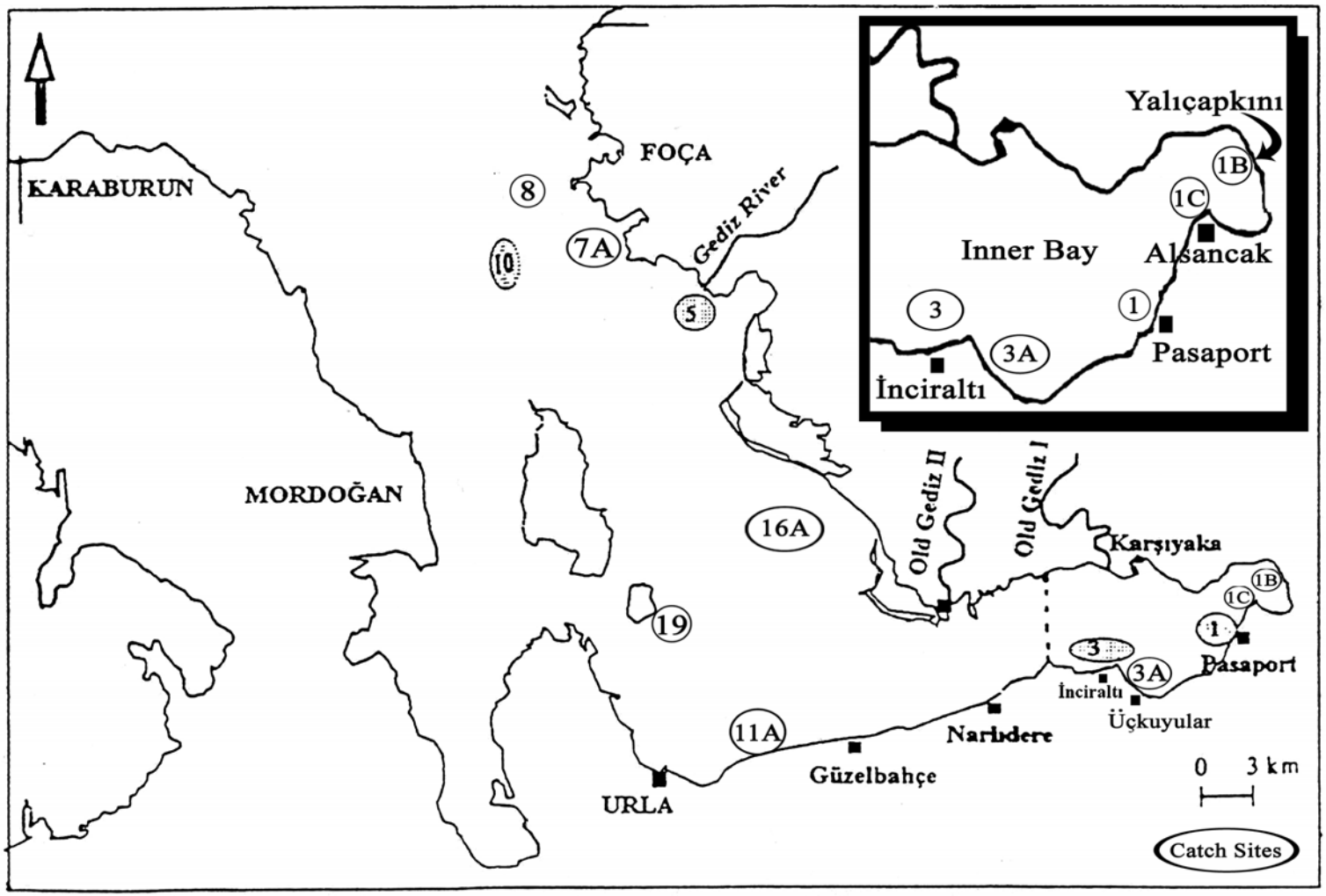
Sediment samples were collected from three different sites (site 1C-Liman Alsancak, site 3- İnciraltı and site 10-Outer Bay) in İzmir Bay by the help of RV Koca Piri Reis of the 9 Eylül University Institute of Marine Sciences and Technology by using Van Veen Grab sampler (see appendix for schematic representation).

2.2 Methods

2.2.1 Preparation of Fish Liver Microsomes

The method described by Arınç and Şen (1993a) was used for the preparation of liver microsomes. Fish were killed by decapitation and the livers, weighing approximately 2-5 grams for leaping mullet, 0.4-1 grams for common sole and 0.5-2 grams for annular seabream were removed immediately. The gall bladder was removed carefully with scissors in order to avoid the spillage of its contents that are known to be inhibitory to monooxygenase activities. The livers were first wrapped by freezing bags and covered by aluminum foil and frozen by putting into liquid nitrogen. Freshly frozen fish livers were transported in liquid nitrogen from İzmir to university laboratory in Ankara.

Microsomes were prepared from one fish liver at a time separately. In the laboratory, the livers were taken from liquid nitrogen and thawed on ice. All subsequent steps were carried out in 0-4°C ice bath. Livers were washed first with cold distilled water, and then with cold 1.15% KCl solution to remove as much blood as possible. After draining on a paper towel, tissues were weighed and cut into small


KARABURUN
FOÇA
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pieces with scissors. The resulting tissue mince was homogenized in 1.15% KCl solution containing 10mM EDTA pH 7.7, 0.25 mM ε-ACA, 0.1 mM PMSF at a volume equal to 2.5 times the weight of liver tissue using Potter-Elvehjem glass homogenizer packed in crushed ice, coupled motor (Black & Decker, V850 multispeed drill)-driven Teflon pestle at 2400 rpm, twelve passes were made for homogenization.

The homogenate was centrifuged at 13300×g (Sigma 3K30 Refrigerated Centrifuge, Saint Louis, Missouri, USA) by using 12156 rotor for 40 minutes to remove cell debris, nuclei and mitochondria. The supernatant fraction containing endoplasmic reticulum and other fractions of the cell was filtered through two layers of cheesecloth. The microsomes were sedimented from the supernatant solution by centrifugation at 45000 rpm (70000×g) for 60 minutes using Sorvall-Kombi ultracentrifuge, Ivan Sorvall Inc.,Newton, Connecticut, 06740 USA with T-880 rotor. The packed microsomal pellet was suspended in 1.15% KCl solution containing 10 mM EDTA and resedimented by ultracentrifugation at 45000 rpm (70000×g) for 50 minutes. The supernatant fraction was discarded. Then the washed microsomal pellet was resuspended in 10% glycerol containing 10 mM EDTA pH 7.7 at a volume of 0.5 ml for each gram of liver tissue for leaping mullet and annular seabream. For common sole pellet was resuspended at a volume of 1 ml for each gram of liver tissue. Resuspended microsomes were homogenized manually using the Teflon-glass homogenizer in order to obtain homogenous suspension.

Microsomes were separated into small aliquots and put into eppendorf tubes, stored in liquid nitrogen after gassing with nitrogen.

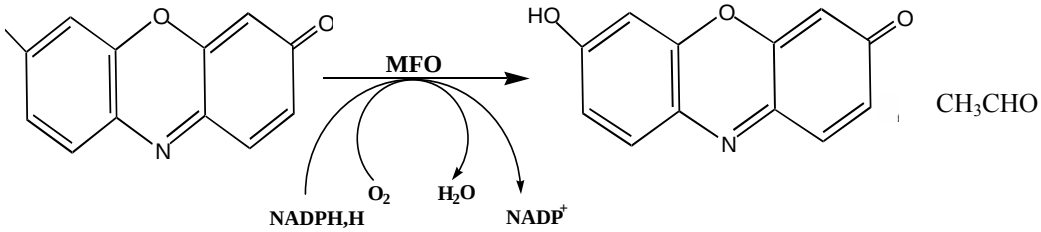
2.2.2 Protein Determination

Concentrations of microsomal proteins were determined by method of Lowry *et al*. (1951). Bovine serum albumin was used as standard. Annular seabream and common sole liver microsomes were first diluted to 1:100 with distilled water

whereas leaping mullet liver microsomes was diluted to 1:200. An aliquot of 0.1, 0.25 and 0.5 mL of microsomes was mixed with distilled water in order to complete the volume to 0.5 mL in test tubes. After that, they were mixed with 2.5 mL of alkaline copper reagent which was prepared freshly by mixing 2% copper sulfate, 2% sodium potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH in the written order and incubated at room temperature for 10 minutes. Then, 0.25 mL of 1.0 N Folin-Phenol reagent was added to the tubes and incubated at room temperature for 30 minutes. The resulting color intensity was measured at 660 nm. The standard curve of BSA from 20 to 200µg was plotted and used for determination of protein concentration of samples.

2.2.3 Determination of 7-Ethoxyresorufin-O-deethylase Activity of Liver Microsomes

Cytochrome P450 1A (CYP1A) associated 7-Ethoxyresorufin –O-deethylase activity of fish liver microsomes were determined by the method of Burke and Mayer (1974) with some modifications. Assay conditions optimized for gilthead seabream liver microsomes by Arınç and Şen (1994b) was also used for fish species in this study. Figure 2.2 shows the dealkylation reaction of ethoxyresorufin catalyzed by monooxygenases in the presence of molecular oxygen and NADPH.



Ethoxyresorufin **7-Ethoxyresorufin**

Figure 2.2 Ethoxyresorufin O-deethylase reaction

A 0.5 mM stock substrate solution was first prepared by dissolving appropriate amount of 7-ethoxyresorufin in DMSO. Then 10 μM daily solution was prepared by diluting 1:50 with 0.2 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl. 1 mM stock resorufin standard was prepared by dissolving appropriate amount of resorufin in DMSO. Then 5 μM daily solution was prepared by diluting 1:200 with 0.2 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl. As it is shown in Table 2.2, a typical reaction mixture contained 0.1 M potassium phosphate buffer pH 7.8, 0.1 M NaCl, 2.4 mg BSA, 1.5 μM 7-ethoxyresorufin, 50 or 100 μg microsomal protein, 0.5 mM NADPH generating system (constituents of generating system the were given in Table 2.1) in a final volume of 2.0 ml in a fluorometer cuvette. The reaction was initiated by the addition of substrate and followed for three minutes in spectrofluorometer (Hitachi F2000, Hitachi ltd., Tokyo, Japan) at 535 nm (excitation) and 585 nm (emission) wavelengths. Finally a known amount of resorufin was added as an internal standard to the reaction mixture and the increase in fluorescence was recorded.

Table 2.1 Preparation of NADPH generating system.

Constituents	**Stock Solutions**	**Volume to be taken (ml)**	**Final concentration in 0.5ml reaction mixture**
Glucose-6-phospate	100 mM	0.0125	2.5 mM
$MgCl_2$	100 mM	0.0125	2.5 mM
HEPES, pH:7.8	200 mM	0.0375	14.6 mM
$NADP^+$	20 mM	0.0125	0.5 mM
Glucose-6-phosphate dehydrogenase	700 U/ml	0.00071	0.5 Units
Distilled water		to 0.075	

Table 2.2 The constituents of the reaction mixture for the determination of EROD activity in fish liver microsomes.

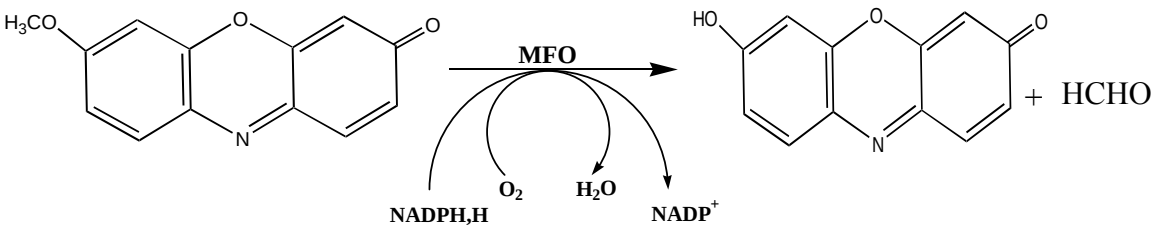
Constituents	**Stock Solutions**	**Volume to be taken (ml)**	**Final concentration in 2 ml reaction mixture**
Fish liver microsomes	------	Depending on the protein concentration of microsomes.	50 μg or 100 μg of microsomal protein.
Potassium phosphate buffer, at pH 7.8 containing 0.4 M NaCl	400 mM	0.5	100 mM
BSA	12 mg/ml	0.2	1.2 mg/ml
Ethoxyresorufin	10 μM	0.30	1.5 μM
NADPH Generating System[a]		0.3	0.5 mM
Distilled water		to 2.0 ml	

[a]NADPH generating system will be prepared as described in Table 2.1

2.2.4 Determination of 7-Methoxyresorufin-O-demethylase Activity

Besides ethoxyresorufin, methoxyresorufin is also highly specific substrate for CYP1A enzyme. The O-dealkylation of methoxyresorufin (MROD) was measured in annular seabream (*Diplodus annularis*) liver microsomes by using same spectrofluorometric assay of EROD activity measurement with some modifications. Figure 2.3 shows the methoxyresorufin O-demethylase reaction catalyzed by monooxygenases in the presence of molecular oxygen and NADPH.

A 1.5mM stock substrate solution was first prepared by dissolving appropriate amount of 7-methoxyresorufin in DMSO. Then 30 μM daily solution was prepared by diluting 1:50 with 0.2 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl. The preparation of resorufin was same as explained in EROD activity measurement. As it is shown in Table 2.3, a typical reaction mixture contained 0.1 M potassium phosphate buffer pH 7.8, 0.1 M NaCl, 2.4 mg BSA, 4.5 μM 7-methoxyresorufin, 50 μg microsomal protein, 0.5 mM NADPH generating system in a final volume of 2.0 ml in a fluorometer cuvette. The following procedure used for measuring the methoxyresorufin O-demethylase (MROD) activity was identical with that used for ethoxyresorufin O-deethylase (EROD) except that 4.5 μM of substrate concentration was used in the activity measurements.



Methoxyresorufin **7-hydroxyresorufin**

Figure 2.3 Methoxyresorufin O-demethylase (MROD) reaction

Table 2.3 The constituents of the reaction mixture for the determination of MROD activity in fish liver microsomes.

Constituents	**Stock Solutions**	**Volume to be taken (ml)**	**Final concentration in 2 ml reaction mixture**
Fish liver microsomes	------	Depending on the protein concentration of microsomes.	50 μg of microsomal protein.
Potassium phosphate buffer, at pH 7.8 containing 0.4 M NaCl	400 Mm	0.5	100 mM
BSA	12 mg/ml	0.2	1.2 mg/ml
Methoxyresorufin	30 μM	0.30	4.5 μM
NADPH Generating System[a]		0.3	0.5 mM
Distilled water		to 2.0 ml	

[a]NADPH generating system will be prepared as described in Table 2.1

2.2.5 Determination of Benzphetamine N-Demethylase Activity

Benzphetamine is a specific substrate for the phenobarbital inducible forms of CYP isozymes. N-demethylation of benzphetamine is found to be associated with cytochrome P450 2B isozymes (Adalı and Arınç, 1990, Antonovic et al., 1999) Benzphetamine is N-demethylated by mixed function oxidases in the presence of

molecular oxygen and NADPH. At the end of the reaction norbenzphetamine, $NADP^+$, water and formaldehyde are produced (Figure 2.4).

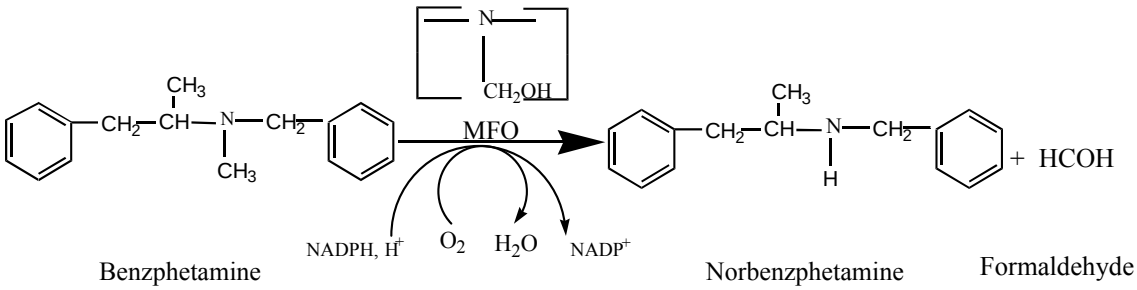


Figure 2.4 Benzphetamine N-demethylase reaction

Benzphetamine N-demethylase activity of annular seabream and leaping mullet liver microsomes were determined colorimetrically by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). As it is shown in Table 2.4, a typical assay mixture contained 100 mM HEPES buffer pH 7.8, 1.5 mM benzphetamine, 1.5 mg microsomal protein and 0.5 mM NADPH generating system in a final volume of 0.5 ml.

NADPH generating system was composed of 2.5 mM glucose-6-phosphate 2.5 mM MgCl2, 14.6 mM HEPES pH 7.8, 0.5 mM $NADP^+$ and 0.5 units of glucose-6-phosphate dehydrogenase (Table 2.3). All the constituents were incubated at 37°C for 5 minutes.

Benzphetamine N- demethylation reaction was initiated by the addition of 0.075 ml of NADPH generating system to incubation mixture and to zero time blanks in which 0.5ml of 0.75 N perchloric acid was added before the addition of cofactor, and incubated at 25°C for 10 minutes aerobically with moderate shaking in water bath. At the end of incubation period, the enzymatic reaction was stopped by addition of 0.5 ml 0.75 N perchloric acid. The contents of the tubes were transferred into eppendorf tubes and were centrifuged at 14000xg for 15 minutes for the removal of denatured microsomal proteins using Sigma 1-15 centrifuge (Sigma Laborzentrifugen GmbH An der Unteren Söse 50 D-37520 Osterode am Harz) in cold room. Finally, 0.5 ml aliquots of supernatant solution were transferred to test tubes and were mixed freshly prepared 0.375ml Nash reagent (prepared by the addition of 0.1 ml of acetylacetone, just before use, to 25 ml solution containing 7.7 g ammonium acetate and 0.15 ml of glacial acetic acid). The mixture was incubated at 50°C for 10 minutes in a water bath and the intensity of yellow color developed was measured at 412 nm using Schimadzu UV-1201 spectrophotometer (Shimadzu Co., Analytical Instruments Division, Kyoto, Japan).

A 0.5 mM freshly prepared formaldehyde solution was used as standard. The tubes were containing standards at four concentrations (12.5, 25, 50 and 100 nmoles) as well as containing other incubation constituents mixed by Nash reagent and incubated at 50 °C for 10 minutes to give same color reaction. A standard formaldehyde calibration curve was constructed and used for calculation of enzyme activities.

Table 2.4 The constituents of the reaction mixture for the determination of Benzphetamine N-demethylase activity in fish liver microsomes.

Constituents	**Stock Solutions**	**Volume to be taken (ml)**	**Final concentration in 0.5 ml reaction mixture**
Fish liver microsomes	------	Depending on the protein concentration of microsomes.	1.5 mg of microsomal protein in 0.5 ml.
HEPES buffer, at pH 7.8	400 mM	0.125	100 mM
Benzphetamine-HCl	7.5 mM	0.1	1.5 mM
NADPH Generating System[a]		0.075	0.5 mM
Distilled water		to 0.5 ml	

[a]NADPH generating system was prepared as described in Table 2.1

2.2.6 Determination of Ethylmorphine N-Demethylase Activity

Ethylmorphine is N-demethylated by several isozymes of cytochrome P450 dependent microsomal monooxygenases. Figure 2.5 shows the N-demethylation reaction catalyzed by mixed function oxidases.

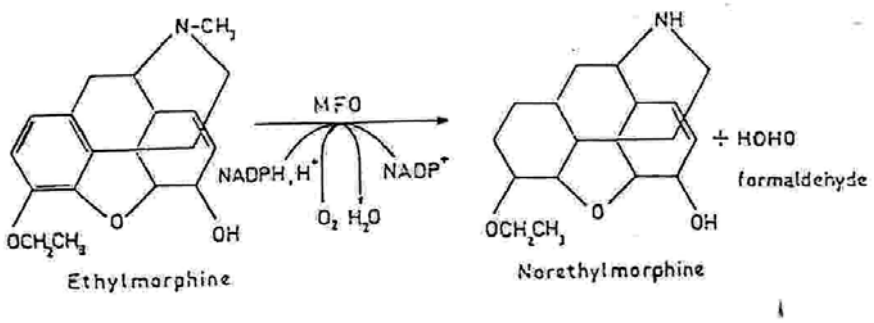


Figure 2.5 Ethylmorphine N-demethylation Reaction

Ethylmorphine N-demethylase activity of leaping mullet and annular seabream liver microsomes were determined calorimetrically by measuring the quantity of formaldehyde formed during the reaction according to the method of Nash (1953) as modified by Cochin and Axelrod (1959) with minor modifications. The assay conditions were optimized for lung and liver microsomal ethylmorphine N-demethylase by Arınç (1985).

As it is shown in Table 2.5, a typical assay mixture contained 100 mM HEPES buffer pH 7.8, 15 mM ethylmorphine, 1.5 mg microsomal protein and 0.5 mM NADPH generating system in a final volume of 0.5 ml. NADPH generating system was prepared as described in Table 2.1.

The procedure used for measuring the ethylmorphine N-demethylase activity was identical with that used for benzphetamine N-demethylase activities as given in section 2.2.5 except that the incubation period was 15 minutes.

Table 2.5 The constituents of the reaction mixture for the determination of Ethylmorphine N-demethylase activity in fish liver microsomes.

Constituents	**Stock Solutions**	**Volume to be taken (ml)**	**Final concentration in 0.5 ml reaction mixture**
Fish liver microsomes	------	Depending on the protein concentration of microsomes.	1.5 mg of microsomal protein in 0.5 ml.
HEPES buffer, at pH 8.0	400 mM	0.125	100 mM
Ethylmorphine-HCl	75 mM	0.1	15 mM
NADPH Generating System[a]		0.075	0.5 mM
Distilled water		to 0.5 ml	

[a]NADPH generating system was prepared as described in Table 2.1

2.2.7 Determination of Aniline 4-Hydroxylase Activity

Hydroxylation of aniline is mainly associated with CYP2E1 isozyme of cytochrome P450 enzyme family. Figure 2.6 shows the reaction catalyzed by mixed function oxidases.

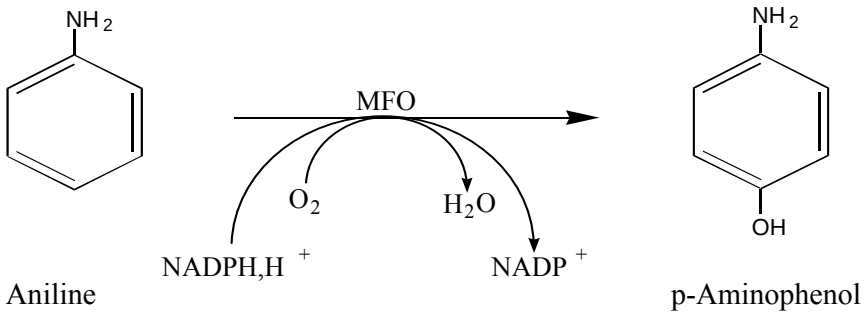


Figure 2.6 Aniline 4-hydroxylation reactions catalyzed by mixed function oxidases

Aniline 4-Hydroxylase activity of leaping mullet and annular seabream liver microsomes were determined by measuring the quantity of p-aminophenol (pAP) formed according to the method of Imai *et al.* (1966). In general, NADPH generating system is used as cofactor. The assay conditions were optimized for lung and liver microsomal aniline 4-hydroxylase by Arınç and İşcan (1983).

The typical assay mixture contained 10 mM aniline, 100 mM HEPES buffer pH 7.6, 0.5 mM NADPH generating system and 3 mg microsomal protein in a final volume of 1.0 ml as it was given in Table 2.6. The reaction was initiated by the addition of 0.15 ml NADPH generating system to incubation mixtures and to zero time blanks in which 0.5 ml of 20 % trichloroacetic acid was added before addition of cofactor, and was incubated at 25°C for 25 minutes under the air with moderate shaking in water bath. At the end of incubation period, the reaction was stopped by the addition of 0.5 ml of 20 % trichloroacetic acid. The denatured protein was collected by centrifugation at 14.000xg for 40 minutes, by using Sigma 1-15 centrifuge (Sigma Laborzentrifugen GmbH An der Unteren Söse 50 D-37520 Osterode am Harz) in cold room. A 1.0 ml aliquot of the supernatant solution containing p-aminophenol was removed and was mixed with 0.5 ml 20 % Na_2CO_3

and with 0.5 ml 4% phenol in 0.4 N NaOH. The mixture was incubated at 37°C for 30 minutes in a water-bath and the intensity of blue color was measured at 630 nm.

p-Aminophenol (pAP) solution was used as standard. Four different standard concentrations (2.5, 5.0, 12.5, and 25 nmoles) containing aniline and other incubation constituents were run under the same conditions as for reaction mixture. A standard calibration curve was constructed and used for calculation of enzyme activities which was expressed as nmol of pAP/min/mg.

Table 2.6 The constituents of the reaction mixture for the determination of Aniline 4-Hydroxylase activity in fish liver microsomes.

Constituents	**Stock Solutions**	**Volume to be taken (ml)**	**Final concentration in 1.0 ml reaction mixture**
Fish liver microsomes	------	Depending on the protein concentration of microsomes.	3 mg of microsomal protein in 1.0 ml.
HEPES buffer, at pH 7.6	400 mM	0.250	100 mM
Aniline	100 mM	0.1	10 mM
NADPH Generating System[a]		0.150	0.5 mM
Distilled water		to 1.0 ml	

[a]NADPH generating system was prepared as described in Table 2.1

2.2.8 Determination of NADPH-Cytochrome P450 Reductase Activity

NADPH-dependent cytochrome P450 reductase activity of leaping mullet and annular seabream was measured spectrophotometrically, by the method of Masters *et al.* (1967) except that the reaction was carried out in 0.3 M potassium phosphate buffer, pH 7.7 at room temperature.

The assay depends on the measurement of the rate of reduction of artificial substrate, cytochrome *c*, at 550 nm. Reaction mixture contained 0.7 ml of cytochrome *c* (85 nmol; 1.1 mg cytochrome c per ml in 0.3 M potassium phosphate buffer, pH 7.7), 0.025 ml NADPH (130 nmoles; freshly prepared 3.3 mg NADPH per ml) solution and appropriate amounts of cytochrome P450 reductase sample (0.01 to 0.05 ml). Before the addition of NADPH, baseline was recorded. The reaction was initiated by the addition of NADPH and followed for 120 seconds at 550 nm at room temperature using Hitachi 220A double beam spectrophotometer with cuvettes of 1.0 cm light path. The enzyme activities were calculated using the extinction coefficient of 19.6 $mM^{-1}cm^{-1}$ for the difference in absorbance between reduced minus oxidized form of cytochrome *c* at 550 nm as described by Yonetani (1965). One unit of reductase is defined as the amount of enzyme catalyzing the reduction of one μmole of cytochrome c per minute under the above conditions

2.2.9 Western Blot Analysis – Protein Blotting

Cytochrome P4501A protein level was determined using the polyclonal antibodies produced in our laboratory against purified leaping mullet liver cytochrome P4501A (Arınç and Şen, 1999).

First microsomal proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS, in a discontinuous buffer system as described by Laemmli (1970) by using 4% stacking gel and 8.5% separating gel. The separating and stacking gel solutions were prepared just before use as given in Table 2.7 in a given order.

Table 2.7 Components of separating and stacking gel solutions

Constituents	**Separating Gel (8.5%) (0.375 M Tris, pH 8.8)**	**Stacking Gel (4%) (0.125 M Tris, pH 6.8)**
Gel solution (ml)	8.5	1.3
Distilled water (ml)	13.55	6.1
Separating gel buffer (ml)	7.5	-
Stacking Gel buffer (ml)	-	2.5
10% SDS (ml)	0.3	0.1
Ammonium persulfate (ml)	0.15	0.05
Temed (ml)	0.015	0.01
Total volume	30	10

After preparing gel setup, liver microsomes were diluted 1:3 (3 part sample and 1 part buffer) with 4× sample dilution buffer containing 0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.01% bromophenol blue and were boiled in a boiling water bath for 1 minutes and 10 seconds.

After application of the samples, gel set up, together with cooling core were placed in Bio-Rad Protean II Cell. The Cell was connected to the power supply Bio-Rad model 2 (Bio-Rad Laboratories, Richmond, California, USA) and electrophoresis was run overnight at 6mA and 60V. When electrophoresis was completed, gel was removed from the cell for western blot analyses which was carried out as described by Towbin et al. (1979) with some modifications. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 15 minutes with constant shaking in order to adjust the final size of gel and remove

the buffer salts and SDS which were used in the SDS-Polyacrylamide gel electrophoresis. Nitrocellulose membrane was cut 1 cm larger then the dimension of the gel and two pieces of filter paper (Whatman #1) were cut to a dimension a little bit larger than the membrane. Nitrocellulose membrane, two filter paper and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Western blot sandwich was prepared as seen in Figure 2.7. A test tube was rolled gently over the sandwich in order to remove air bubbles between the layers. This step is very critical because any air bubbles between gel and membrane will block the transfer of proteins present at this point. Later, the sandwich was put into the Bio-Rad Trans-Blot Cell and the cell was filled with cold transfer buffer. Voltage and current were set to 90V and 400 mA, respectively. Transfer process was carried out at cold room (4°C) for 90 minutes. At the end of this period, the membrane having the transferred protein on it, i.e. "blot" was obtained and taken from the cell and placed into a plastic dish in such a way that protein side facing up and washed with TBST (Tris Buffered Saline plus Tween 20: 20mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 0.05 % Tween 20) for 10 minutes in order to remove the salts and buffers of transfer medium. Then the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBST) for 60 minutes in order to fill the empty spaces between bound proteins by this way prevent the non-specific binding of antibodies on the membrane.

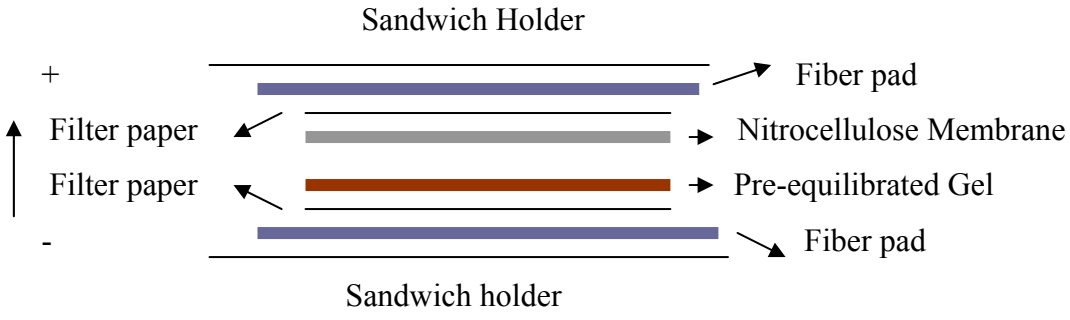


Figure 2.7. Cross-section of the Western blot sandwich.

The blot was incubated with primary antibody for 2 hours. As primary antibody, polyclonal antibodies produced against purified leaping mullet liver cytochrome P4501A in rabbits were used and dissolved in blocking solution 1:5000 ratio. Then the blot was washed 3 times with 100 ml TBST for 5 minutes each. The washing steps are necessary to remove excess antibody from the membrane. The blot was then incubated with secondary antibody conjugated to marker enzyme-alkaline phosphatase (anti-rabbit IgG-ALP conjugate, 1:10.000 ratio) for 1 hour. The blot was washed three times with TBST for 5 minutes each to remove excess antibody. Since the excess antibody will give reaction with substrate solution nonspecifically the complete removal of the excess antibody between each washing steps are extremely important.Finally, blot was incubated with substrate solution given in Table 2.8 as described by Ey and Ashman (1986) to visualize the specifically bound antibodies. The final images were then dried under air, covered by filter paper and stored at dark.

Table 2.8 Preparation of substrate solution for immunodetection

Solution A: 2.67 ml of 1.5 M Tris-HCl, pH 8.8 4.0 ml of 1M NaCl 0.82 ml of 100 mM $MgCl_2$ 0.04 ml of 100 mM $ZnCl_2$ 0.096 ml of DEA 12.2 mg NBT Distilled water to 40 ml (pH of the solution was adjusted to 9.55 with saturated Tris before completing to final volume)
Solution B: 2 mg/ml phenazine methosulfate in distilled water
Solution C: 5.44 mg/0.136 ml N, N-dimethyl formamide
Finally NBT/BCIP substrate solution was prepared by mixing solution A with solution C and 0.268 ml of solution B.

2.2.10 Spectrofluorometric Measurement of Total Polyaromatic Hydrocarbon in Fish Liver Tissue

Spectrofluorometric measurement of the total PAHs concentration in fish liver tissue described by UNEP, 1992 is very rapid and practical method. For PAHs UV light is required to excite the emission of visible light. When UV light is passed through a sample, the sample emits light (flouresces) proportional to the concentration of the fluorescent molecule (in this case PAHs). The principle of the method is based on the extraction of PAHs by suitable pure solvent. PAHs are highly hydrophobic compounds cannot be dissolved in water or other polar solvents for this reason, highly hydrophobic and nonpolar solvents such as benzene or hexane can be used to dissolve PAHs. Extraction of desired compounds from a biological tissue generally requires a pretreatment step to get red rid of unwanted, or interfering substances. Saponification is usually employed for the pretreatment of biological samples prior to extraction. This is not only for isolation of fats but also removing sulfur and sulfur containing compounds which can be interfere with spectrofluorometric measurements. For the saponification, sample is immersed in a KOH- ethanol or (NaOH- ethanol) solution and refluxed 1 or 2 hours. Then from this solution desired molecules are extracted by using a hydrophobic solvent.

In this method first livers were dried in an oven at 40 °C overnight. Dried tissue was homogenized in a mortar. 0.2 g of dried and homogenized tissue put into a round bottomed flask and refluxed with 20 ml of ethanol containing 0.75 g of KOH for 120 min by constant heating (for saponification process). Then a 20 ml of PAH-free hexane was added to flask, (to extract the PAHs from saponification solution) mixed very well and waited until the flask reached to room temperature. When flask was attained to room temperature, it was poured into separatory funnel and sufficient amount of distilled water was added. Ethanol is more soluble in water than in hexane so addition of water causes separation of hexane and ethanol into two phases. For better separation funnel should be shaken vigorously. The upper, hexane phase containing PAHs was seen as a very clear transparent solution. Approximately 20 ml upper hexane phase was collected by a glass pipette. The lower aqueous phase was

extracted further two times with 20 ml of PAH- free hexane. All hexane phases collected from these three extractions were pooled and the volume was recorded. The fluorescence intensity of the pooled sample was compared with the fluorescence of series of reference solutions, which was chrysene standard.

100 ppm stock solution was prepared for chrysene in hexane. This stock solution was diluted for 0.1, 0.25, 0.50, 0.75, 1,2 and 5 ppm solution and intensity of them were measured with spectrofluorometer at 310 nm excitation and 360 nm emission wavelengths. A standard calibration curve was constructed by using these data.

The following equations were used for the calculations.

$C_g \times V_f = C_s \times W_s$

Where:

C_g: Petroleum hydrocarbon concentration of residue obtained from graph (μg/l and /or μg/ml)

V_f: Final volume of the sample extract (l and /or ml)

C_s: Concentration of sample which was dissolved /dispersed petroleum hydrocarbons in fish (μg/g)

W_s: Weight of sample

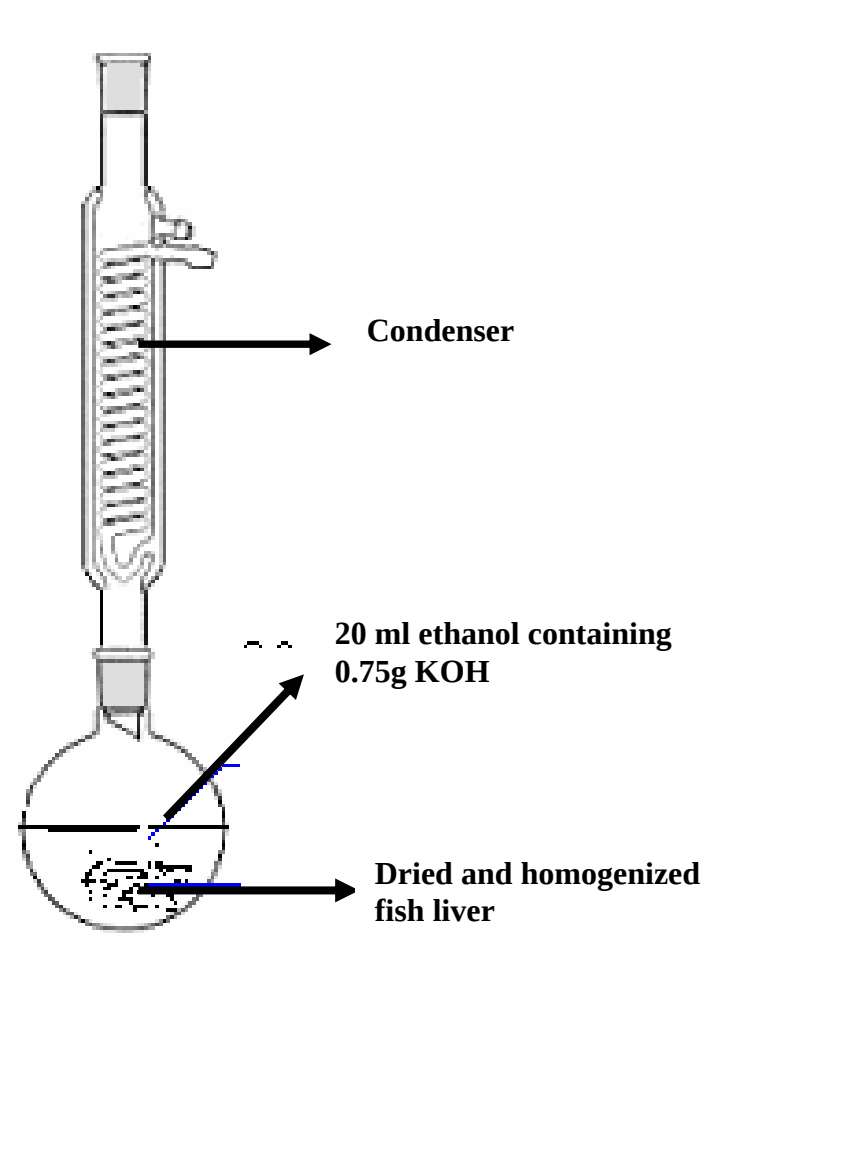


Figure 2.8 Reflux set- up for total PAH extraction from dried fish liver tissue

2.2.11 Spectrofluorometric Measurement of Total Polyaromatic Hydrocarbons in Sediment

The method described by UNEP, 1986 is based on extraction of the PAHs from the sediment by using a suitable organic solvent. For this purpose a mixture of two solvent with different polarities should be used. One of the solvent should be

highly non polar like hexane and or benzene while the other is relatively polar like ethanol or methanol. PAHs are highly soluble in the nonpolar ones on the other hand undesired molecules such as lipids are soluble in relatively polar solvent. In principle analysis of PAHs from sediment can be divided into three step: Extraction of the PAHs using a solvent system by soxhlet apparatus, separation of the solvent that contains desired molecules, concentration and clean-up processes.

In this method about 20-30 grams (wet weight) of sediments were weighed into a celulosic extraction thimble and this was extracted for eight hours with 250 ml 1:1 benzene: methanol solution for about 48 cycles in soxhlet apparatus. In soxhlet apparatus solvent is vaporized, when it condenses it drops on the sediment contained in the thimble and extracts soluble compounds including PAHs. When the liquid level fills the body of extractor, it automatically siphons into the flask. Figure 2.9 shows the soxhlet apparatus. This process continues repeatedly about 48 times in nearly eight hours. At the end of this period, the extract was transferred into separatory funnel. Benzene and methanol phases were separated. Sometimes small amount of distilled water was added to this extract for better separation, because methanol dissolves in benzene but it is more soluble in water.

The benzene phase which contain PAHs was collected into a flask and it was evaporated to dryness (concentration) by mild heating about 30-40 C^{o} using rotary evaporator. After evaporation residue dissolved in 10-15 ml hexane and for further clean-up processes from the undesired molecules this was passed through the activated alumina column (Merck activated neutral). Activated alumina holds lipophilic substances that may remain after separation and cleans up the final extract.

The eluent was collected and analyzed at spectrofluorometer at 310 nm excitation and 360 nm emission wavelengths. The unknown concentration of the sample was calculated using the standard calibration curve of chrysene. The calculation of total PAH concentration in sediments was same as the calculation of total PAH in fish liver tissue.

2.2.11.1 Preparation of Alumina Column

A Pasteur pipette nearly 5 cm long was used as column. Its narrowing end was blocked by a piece of glass wool and then filled with 90 mesh size activated neutral alumina obtained commercially from Merck. Then column was washed with about 10 ml PAH- free hexane before the sample application. Then extract was passed from the column and the volume of total elute was recorded.

2.2.12 Statistical Analysis

Data were presented as the mean ± standard error of mean (SEM). Statistical analyses were performed by using Minitab statistical software package. Student's t-test was used in statistical interpretations of results.

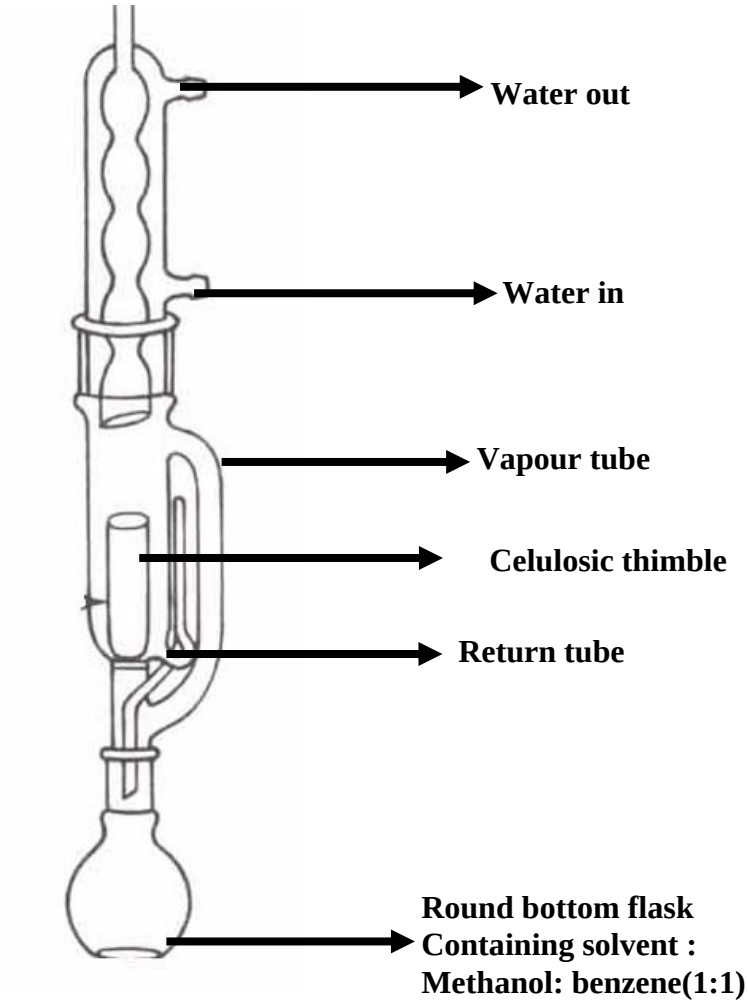


Figure 2.9 Soxhlet apparatus for the extraction of total PAHs from sediment

CHAPTER 3

RESULTS

In this study, the degree of induction of cytochrome P4501A1 protein as determined immunochemically and of CYP1A1 associated EROD activity of fish liver microsomes were used for the assessment of PAH or /PCB type organic pollutants along the İzmir Bay after the Great Canal Project. Three different fish species were used throughout this study, namely leaping mullet (*Liza saliens*), annular seabream (*Diplodus annularis*) and common sole (*Solea vulgaris*) which were representatives of pelagic, benthopelagic and benthic (sediment) fish, respectively. In addition, total PAH concentrations were determined in surface sediments of the Bay and also in liver tissues of the some fish samples. Furthermore, biocatalytic properties of annular seabream liver microsomal cytochrome P450 dependent mixed function oxidases were determined according to their ability to catalyze O-demethylation of methoxyresorufin, *p*-hydroxylation of aniline and N-demethylation of ethylmorphine and benzphetamine.

3.1 Liver microsomal 7-Ethoxyresorufin O-Deethylase Activity of Leaping Mullet (*Liza saliens*) caught from different sites of İzmir Bay.

Microsomes were prepared from one fish liver at a time separately. A sample number was given to each of the microsomal preparation.

EROD activities of P4501A1 from liver microsomes were determined by using 7-ethoxyresorufin O-deethylase reaction. This reaction is based on the conversion of 7-ethoxyresorufin into the resorufin which is measured spectrofluorometrically at 585 nm (Burke and Mayer *et al*., 1974).

For the present study, 54 leaping mullet were caught from eight different sites (1, 1B, 1C, 3, 3A, 11A, 19, 10) of the İzmir Bay. Site 1 (Pasaport), site 1B (Liman-Yalıçapkını), site 1C (Liman-Alsancak), site 3 (İnciraltı) and site 3A (Üçkuyular) are in the Inner Bay which has been considered the most polluted part of the Bay. Site 11A (Zeytinalanı) and site 19 (Hekim Island) are located in the Outer Bay. Site 10, which is located in the outermost section of the Bay, has appeared to be good reference site since the fish caught from this area in previous biomonitoring study had very low EROD activities which were 25±9 pmol/min/mg protein, n=4 (Arınç and Şen, 1999). Table 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, and 3.9 show microsomal EROD activities obtained from the livers of individual fish caught from the seven different sites along the İzmir Bay. All activities were measured in duplicates.

The leaping mullets from harbor region displayed highly elevated EROD activities. Along the Harbor, fish were caught from two different sites: site 1B which is called as Liman-Yalıçapkını and site 1C which is called as Liman –Alsancak portside. Mullets that caught from Alsancak (site 1C) showed the highest EROD activities (2590±340 pmole/min/mg protein, n=7) among the other sampling sites which were about 104 times higher with respect to the value at the reference site. Fish sampled from the harbor region of site 1B (Liman-Yalıçapkını) had also very high EROD activities (1969±252 pmole/min/mg protein, n=8) which were about 79 times higher than those obtained from the reference site (site 10). Table 3.1 and 3.2 show the microsomal EROD activities obtained from individual fish livers (from site 1B and site 1C respectively). EROD activities of leaping mullets along the harbor, (site 1B and site 1C as total) were given in Table 3.3.

Leaping mullets that caught from Üçkuyular (site 3A) which is another port side in the Inner Bay, had also very high EROD activity which was 2011±490 pmol/min/mg protein (n= 4, for individual fish liver microsomal activities see Table 3.4). This value was 80 times higher than those obtained from the reference site. Fish sampled from the highly urbanized and industrial section of the Bay, Pasaport region (site 1) also showed highly elevated enzyme activities both in November 2002 (1813± pmol/min/mg protein) and October 2003 (1493± pmol/min/mg protein) which were about 73 and 60 times higher with respect to the value at reference site. Tables 3.5 and 3.6 show EROD activities of leaping mullet sampled from Pasaport region in November 2002 and October 2003 respectively.

The EROD activities of mullets were found to be decreased at site 11A (Zeytinalanı) and site 3 (İnciraltı) which were 843±100 pmol/min/mg protein n=4 and 582±72 pmol/min/mg protein n=4 respectively (see Table 3.7 and 3.8). Although Hekim Island located at the Outer Bay, EROD activities of leaping mullet caught from this area were found to be significantly high (1169±100 pmol/min/mg protein n=8) which were about 47 times higher with respect to reference value. The reason of high EROD activities obtained in leaping mullet liver microsomes from this area will be discussed in next chapter. The EROD activities of mullets from Hekim Island were given in Table 3.9.

The average liver microsomal EROD activities of leaping mullet caught from seven different sites along a pollution gradient in the İzmir Bay were summarized in Table 3.10 and presented in Figure 3.1 as a graph. Each value was represented as mean ± standard error of mean of 4-15 individual fish.

Table 3.1 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from Site1C, Liman -Alsancak region. (Nov2002)

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min /mg)
148	2147-2105	2126
149	2387-2382	2384
150	3032-2832	2932
151	1684-1763	1724
152	3642-3784	3713
153	1473-1553	1513
154	3710-3763	3736
Average activity± standard error n = number of fish liver		2590±340*** n= 7

***Indicates that the value is significantly different from that of reference sites $p < 0.001$

Table 3.2 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from Site 1B, Liman- Yalıçapkını region. (Nov2002)

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
161	2558-2574	2566
162	2710-2832	2771
163	1695-1737	1716
164	1639-1661	1650
165	842-850	846
166	1553-1532	1542
173	1710-1763	1736
174	2895-2947	2921
Average activity± standard error n= number of fish liver		1969±252*** n=8

***Indicates that the value is significantly different from that of reference sites $p < 0.001$

Table3.3 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught along the Harbour, (site1C, Liman -Alsancak region and site 1B, Liman-Yalıçapkını as total) Nov-2002

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
148	2147-2105	2126
149	2387-2382	2384
150	3032-2832	2932
151	1684-1763	1724
152	3642-3784	3713
153	1473-1553	1513
154	3710-3763	3736
161	2558-2574	2566
162	2710-2832	2771
163	1695-1737	1716
164	1639-1661	1650
165	842-850	846
166	1553-1532	1542
173	1710-1763	1736
174	2895-2947	2921
Average activity ±standard error n=number of fish liver		2258±840*** n= 15

***Indicates that the value is significantly different from that of reference sites $p < 0.001$

Table 3.4 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from Site 3A, Üçkuyular region. (Nov2002)

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
160	3237-3237	3237
167	1368-1395	1382
170	2268-2421	2344
172	1105-1053	1079
Average activity ±standard error n= number of fish liver microsomes		2011±490* n=4

*Indicates that the value is significantly different from that of reference sites $p < 0.05$

Table 3.5 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from Site1, Pasaport region. (Nov2002)

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
137	2339-2329	2234
138	1486-1421	1454
139	580-580	580
140	2579-2618	2599
141	908-897	902
142	1653-1605	1629
143	3453-3560	3507
144	3158-3237	3198
145	1400-1395	1398
146	1416-1405	1411
147	1003-1060	1032
Average activity ± standard error n=number fish liver		1813±287*** n= 11

***Indicates that the value is significantly different from that of reference sites $p< 0.001$

Table 3.6 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from site 1, Pasaport region. (Oct-2003)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
206	1815-1763	1789
207	1410-1394	1402
208	3395-3342	3368
209	1579-1605	1592
210	737-737	737
211	895-895	895
212	1184-1105	1144
213	1026-1026	1026
Average activity± standard error n= number of fish liver		1494±296** n= 8

**Indicates that the value is significantly different from that of reference sites $p < 0.01$

Table 3.7 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from Site11A, Zeytinalanı region. (Nov2002)

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
157	1053-1131	1092
158	605-605	605
159	820-842	831
246	840-844	842
Average activity ± standard error n=number of fish liver		843±100** n= 4

**Indicates that the value is significantly different from that of reference sites $p < 0.01$

Table 3.8 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from site 3, İnciraltı region. (Nov2002)

Sample No	Activty (pmol/min/mg)	Average Activity (pmol/min/mg)
168	395-400	398
169	745-750	748
171	579-579	579
247	600-608	604
Average activity ± standard error n=number of fish liver		582±72** n=4

**Indicates that the value is significantly different from that of reference sites $p < 0.01$

Table 3.9 EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from site 19, Hekim Island Region. (Oct-2003)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
214	1605-1605	1605
215	1079-1052	1066
216	1158-1211	1184
217	842-842	842
218	921-815	868
219	1500-1500	1500
220	1052-921	986
221	1342-1263	1302
Average activity± standard error n=number of fish liver		1169±100*** n= 8

***Indicates that the value is significantly different from that of reference sites $p< 0.001$

Table 3.10 The average EROD activities of liver microsomes of leaping mullet (*Liza saliens*) caught from different sites of the Bay.

Sampling Sites & Date of sampling	Number fish (n)	EROD Activities (pmol/min/mg) Average ± standard error
Site 1C- Liman-Alsancak Nov 2002	7	2590±340
Site 3A-Üçkuyular Nov 2002	4	2011±490
Site 1B- Liman-Yalıçapkını Nov 2002	8	1969±252
Site1-Pasaport Nov 2002	11	1813±287
Site 1-Pasaport Oct 2003	8	1494±296
Site 19- Hekim Island Oct-2003	8	1169±100
Site 11A-Zeytinalanı Nov-2002	4	843±100
Site 3- İnciraltı Nov-2002	4	582±72
Site 10- Outer Bay	4	25±9

Figure 3.1 illustrated the graphical representation of EROD activities with standard error among different collection sites. It is apparent that the average activity of the EROD from site 1B, 1C, 3A and 1 were highly elevated which were located in the most polluted part of the Bay.

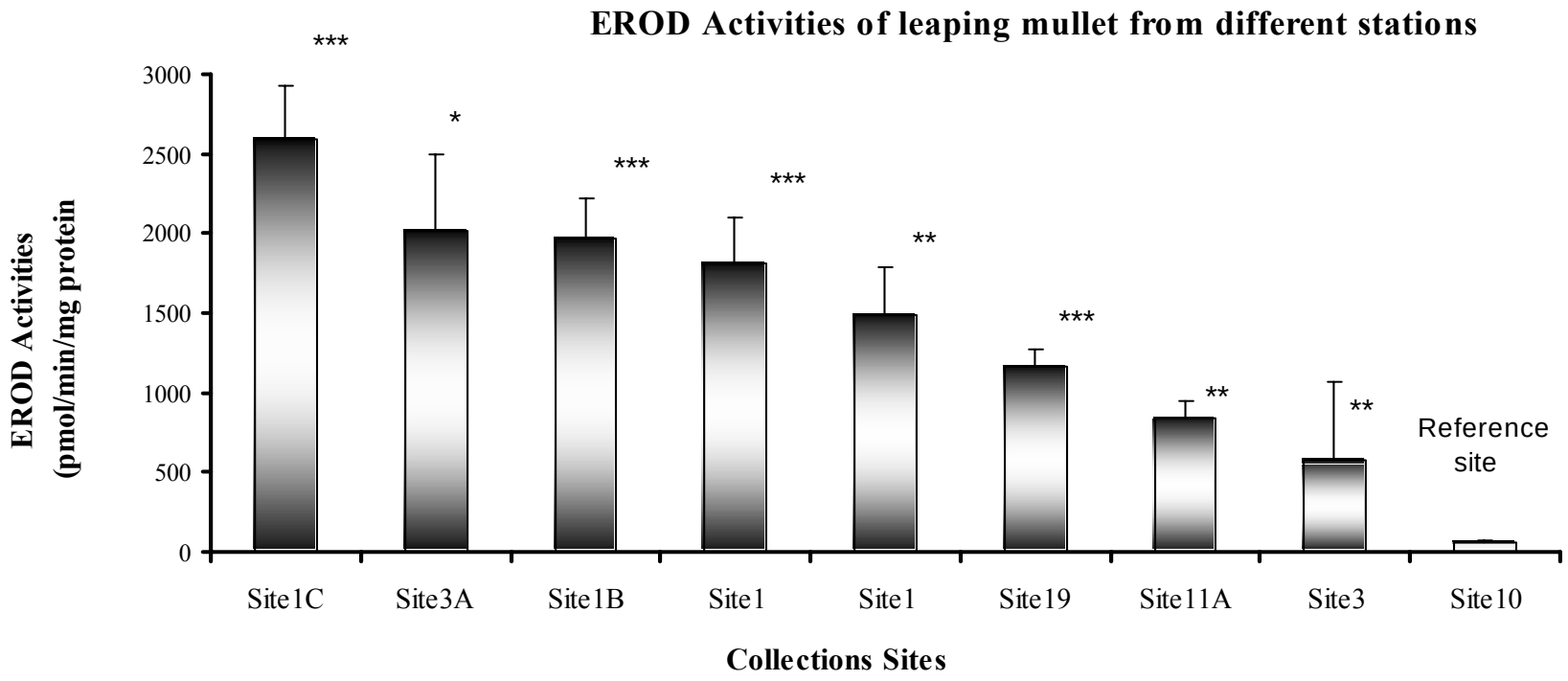


Figure 3.1: EROD activities of leaping mullet sampled from different sites: Site 1C: Liman –Alsancak, site 3A: Üçkuyular, site 1B: Liman-Yalıçapkını, site 1: Pasaport, site 19: Hekim Island, site 11A: Zeytinalanı, site 3: İnciraltı, site 10: Outer Bay

‡ Fish were collected at 2003. All the others were collected in 2002.

*** Significantly different from the lowest values (reference site) with $P<0.001$

** Significantly different from the lowest value (reference site) with $p<0.01$

* Significantly different from the lowest value (referen cesite) with $P<0.05$

3.2 Liver microsomal 7-Ethoxyresorufin O-deethylase Activity of Common Sole (Solea vulgaris) caught from different sites of İzmir Bay.

For this study, 82 common sole were caught from six different sites (3, 19, 7A, 5, 10A, 16A) of the İzmir Bay. Only site 3 (İnciraltı) were in the Inner Bay. The other sites, site 19 (Hekim Island), site 7A (Foça coastal line), site 5 (mouth of Gediz), site 10A and site 16A were in the Outer Bay. Table 3.11, 3.12, 3.13, 3.14, 3.15, 3.16, and 3.17 show microsomal EROD activities obtained from fish livers. Each microsomal sample was prepared from an individual common sole that caught from sites 5, 7A, 10A, and 16A. However for samples from site 3 and site 19, microsomes were prepared from two or three fish livers. All EROD activities were measured in duplicates.

The lowest liver microsomal EROD activity (80± 12 pmol/min/mg protein n=9, was obtained from common sole that sampled from site 16A which was considered as the reference site (Table 3.11). Common sole captured from three sites: site 19 (Hekim Island), site 5 (mouth of Gediz), and site 7A (Foça coastal line), along the Outer bay displayed elevated EROD activities which were 340±22 (n=24), 267±47 (n=5), and 290±71 (n=4) pmol/min/mg protein, respectively (for detail see Table 3.12, 3.13 and 3.14) and were 4.3-, 3.3-, and 3.6 times higher than those obtained from the reference site (site 16A). These higher EROD activities of benthic fish species, common sole, indicate that sediments in this area were contaminated with CYP1A inducing chemicals. The EROD activity of common sole that caught from site 10A which was another sampling site in the Outer Bay was very close to those obtained from reference site (site 16A). The average EROD activity of common sole captured from site 10A was found 107±20 pmol/min/mg for n=5 (Table 3.15 indicates activities).

The highest EROD activities were detected in samples captured from site 3 (İnciraltı) which were 385±49 (n=11) in November 2002 and 503±89 (n=24) in October

2003 (See Tables 3.16 and 3.17). These were 4.8 and 6.3 times higher respectively than those obtained from reference site (site 16A).

The average liver microsomal EROD activities of common sole caught at six different sites along a pollution gradient in the İzmir Bay summarized in Table 3.18 and presented in Figure 3.2 as a graph. Each value was presented as mean ± standard error of mean.

Table 3.11 EROD activities of liver microsomes of Common sole (*Solea vulgaris*) caught from site 16A. (Nov 2002)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
108	120-122	121
109	25-27	26
110	54-73	64
111	40-44	42
112	79-86	82
113	134-128	131
114	89-82	86
115	103-103	103
121	68-72	70
Average activity ± standard error n=number of fish liver		80 ± 12 n= 9

Each microsomal sample was prepared from an individual fish liver.
Site 16A considered as a reference point.

Table 3.12 EROD activities of liver microsomes of Common sole (*Solea vulgaris*) caught from site 19, Hekim Island Region. (Oct-2003)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
230	263-289	276
231	342-342	342
232	421-421	421
233	289-289	289
234	368-368	368
235	421-421	421
236	263-263	263
237	342-342	342
Average activity± standard error n= number of fish liver		340±22*** n= 24

Each sample prepared from three fish liver.

***Indicates that the value is significantly different from that of reference sites $p<0.001$.

Table 3.13 EROD activities of liver microsomes of Common sole (*Solea vulgaris*) caught from site 5, Gediz Region. (Nov 2002)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
116	342-377	360
117	107-109	108
118	345-359	352
119	306-292	299
120	221-208	215
Average activity ± standard error n= number of fish liver		267±47* n=5

Each sample prepared from individual fish liver.

*Indicates that the value is significantly different from that of reference sites ($p<0.05$)

Table 3.14 EROD activities of liver microsomes of Common sole (*Solea vulgaris*) caught from site 7A, Foça coastal line (Nov 2002).

Sample number	Activity Pmol/min/mg	Average Activity pmol/min/mg
133	180-187	184
135	151-149	150
129	412-425	419
128	398-413	406
Average activity ± standard error n= number of fish liver		290±71* n=4

Each sample prepared from one fish liver.

*Indicates that the value is significantly different from that of reference sites, $p<0.05$

Table 3.15 EROD activities of liver microsomes of Common sole (*Solea vulgaris*) caught from site 10A (Nov 2002).

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
130	31-32	32
131	105-97	101
132	141-143	142
134	128-126	127
136	132-134	133
Average activity ± standard error n= number of fish liver		[a]107±20 n=5

Each sample was prepared from one fish liver.

[a]The average activity was very close to the value that obtained from reference site (site 16A).

Table 3.16 EROD activities of liver microsomes of Common Sole (*Solea vulgaris*) caught from site 3, İnciraltı Region. (Nov 2002)

Sample Number	Activity (Unit/mg)	Average Activity (Unit/mg)
122	618-597	608
123	389-383	386
124	345-342	344
125	252-241	247
126	387-386	386
127	336-340	338
Average activity± standard error n= number of fish liver		385±120** n=11

** Indicates that the value is significantly different from that of reference sites $p<0.01$. Each sample prepared from two fish liver except number 123 which was prepared from one fish liver

Table 3.17 EROD activities of liver microsomes of Common Sole (*Solea vulgaris*) caught from site 3, İnciraltı Region. (Oct-2003)

Sample Number	Activity (Unit/mg)	Average Activity (Unit/mg)
238	196-196	196
239	263-263	263
240	500-500	500
241	658-658	658
242	1026-1000	1013
243	421-421	421
244	447-447	447
245	526-526	526
Average activity± standard error n= number of fish liver		503±89*** n= 24

*** Indicates that the value is significantly different from that of reference sites $p<0.001$. Each sample prepared from three fish liver.

Table 3.18 The average EROD activities of liver microsomes of Common sole (*Solea vulgaris*) from different collection sites.

Sampling Sites & Date of sampling	Number fish (n)	**EROD Activities (pmol/min/mg)** Average ± standard error
Site 16A- Reference site Nov 2002	9	80±12
Site 10A-Foça open site Nov 2002	5	107±20
Site 5 –Gediz Nov 2002	5	267±47
Site 7A- Foça coastal line Nov 2002	4	290±71
Site 19– Hekim Island Oct 2003	24	340±22
Site 3-İnciraltı Nov 2002	11	385±49
Site 3- İnciraltı Oct 2003	24	503±89

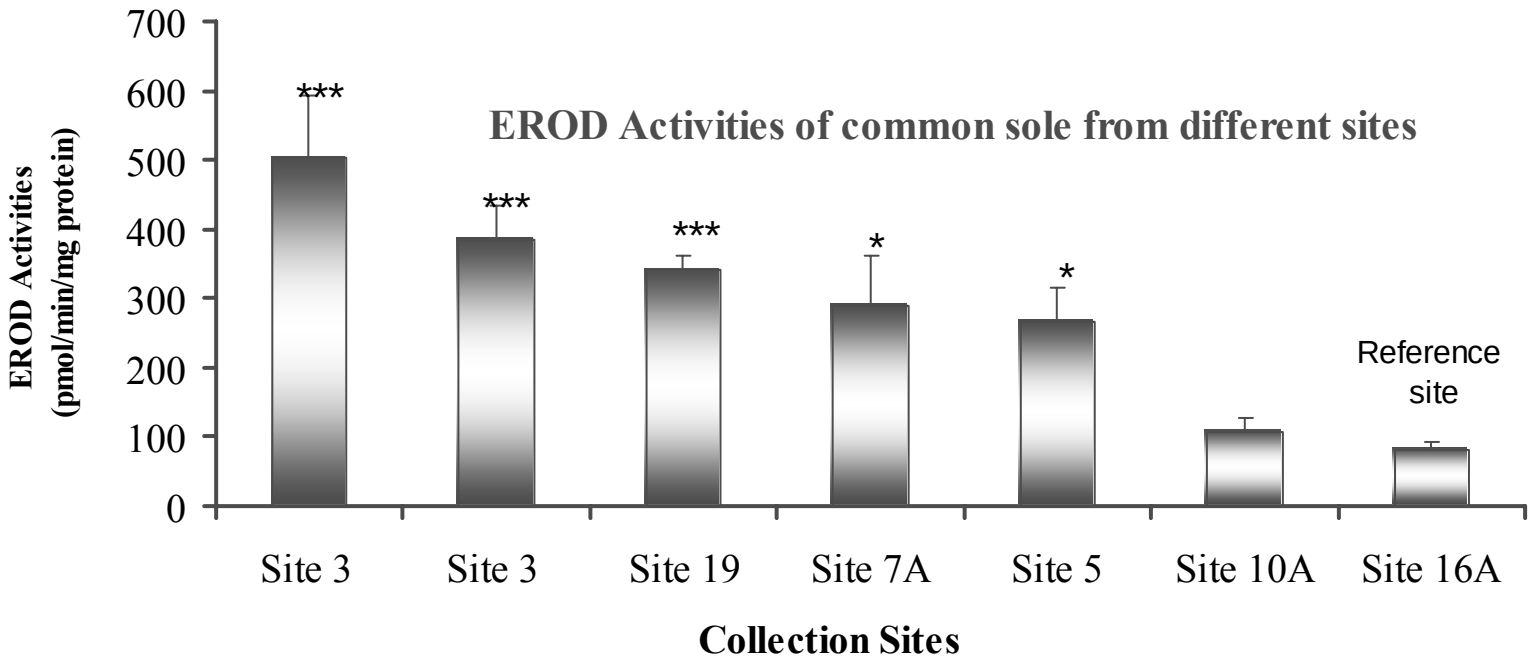


Figure 3.2: EROD activities of common sole sampled from different sites: Site 3: İnciraltı, site 19: Hekim Island, site 7: Foça coastal line Site 5: Gediz, site 10A: Foça open site, site 16A

‡ Fish were collected in 2003. All the others were collected in 2002.

*** Significantly different from the lowest values (reference site) with P<0.001

** Significantly different from the lowest value (reference site) with p<0.01,

* Significantly different from the lowest value (reference site) with P<0.05

3.3 Liver Microsomal 7-Ethoxyresorufin O-Deethylase Activity of Annular Seabream (*Diplodus annularis*) caught from different sites of İzmir Bay.

Microsomes were prepared from one fish liver at a time separately. EROD activities of all samples were measured in duplicates.

For this study, 32 annular seabream caught from four different sites (site 1C, site 1, site 3A and site 11A) of the Bay. Site 1C (Liman-Alsancak), site 1 (Pasaport) and 3A (Üçkuyular) were in the Inner Bay whereas site 11A (Zeytinalanı) located in the Outer Bay.

Table 3.19, 3.20, 3.21, 3.22 show microsomal EROD activities that obtained from individual fish livers. The average liver microsomal EROD activities of annular seabream caught at four different sites along a pollution gradient in the Izmir Bay summarized in Table 3.23 and presented in Figure 3.3 as a graph. Each value is the ± standard error of mean of eight individual fish.

Annular seabream sampled from harbor region, site 1C showed the highest EROD activity which was 1376±279 pmol/min/mg protein, n=8 (see Table 3.19). Fish caught at two other sites Pasaport (site1) and Üçkuyular (site 3A) also displayed highly elevated EROD activities which were 758±119 (n=8), and 706±132 (n=8) pmol/min/mg protein, respectively (Tables 3.20 and 3.21). The lowest EROD activity (182±42 pmol/min/mg protein, n=8 (see Table 3.22) was observed from annular seabream that caught from Zeytinalanı (site 11A).

Table 3.19 EROD activities of liver microsomes of annular seabream *(Diplodus annularis)* caught from site 1C, Alsancak region. (Oct-2003)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
222	3070-3070	3070
223	1786-1788	1787
224	846-846	846
225	822-822	822
226	1210-1210	1210
227	932-987	960
228	684-684	684
229	1632-1632	1632
Average activity± standard error n= number of fish liver		1376±279 n= 8

Table 3.20 EROD activities of liver microsomes of annular seabream *(Diplodus annularis)* caught from site 1, Pasaport Region. (Nov 2002)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
175	689-684	686
176	710-702	706
177	510-500	505
178	1315-1395	1355
179	916-895	906
180	376-395	386
181	1052-1105	1078
182	445-432	438
Average activity± standard error n= number of fish liver		758±119 n=8

Table 3.21 EROD activities of liver microsomes of annular seabream *(Diplodus annularis)* caught from site 3A, Üçkuyular Region. (Nov 2002)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
191	1131-1158	1144
192	789-832	810
193	1184-1236	1210
194	289-289	289
195	895-853	874
196	266-276	271
197	368-347	358
198	689-697	693
Average activity± standard error n= number of fish liver		706±132 n=8

Table 3.22 EROD activities of liver microsomes of annular seabream *(Diplodus annularis)* caught from site11A, Zeytinalanı region. (Nov 2002)

Sample Number	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
183	105-104	104
184	98-97	98
185	142-139	140
186	263-263	263
187	429-444	436
188	153-153	153
189	74-76	75
190	187-189	188
Average activity± standard error n= number of fish liver		182 ±42 n=8

Table 3.23 The average EROD activities of liver microsomes of annular seabream *(Diplodus annularis)* caught from different collection sites.

Sampling Sites & Date of sampling	Number fish (n)	EROD Average Activities (pmol/min/mg) Mean± SEM
Liman-Oct 2003	8	1376± 279
Pasaport-Nov 2002	8	758 ±119
Üçkuyular-Nov 2002	8	706 ±132
Zeytinalanı-Nov 2002	8	182 ±42

As can be seen from Figure 3.3 an inverse relationship was found between the distance to the Inner Harbor (site 1C, Liman-Yalıçapkını), and EROD activities of fish caught along the İzmir Bay (site 1, site 3A and site 11A).

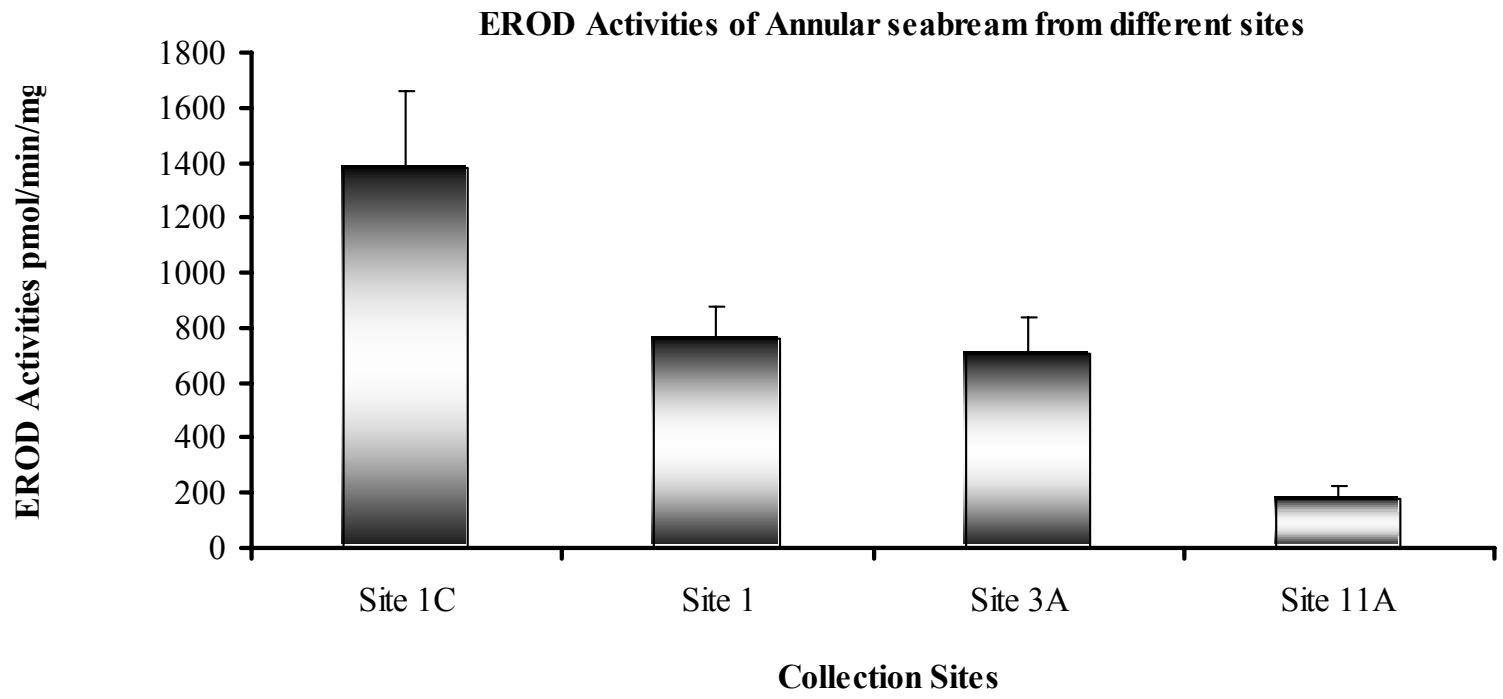


Figure 3.3 EROD activities of annular seabream liver microsomes from different sites. Site1C: Liman-Alsancak, Site1: Pasaport, Site 3A: Üçkuyular, Site 11A: Zeytinalanı

‡ Fish were collected in 2003. All the others were collected in 2002.

3.4 Liver Microsomal Methoxyresorufin O-demethylase Activity of Annular Seabream (*Diplodus Annularis*) caught different sites of İzmir Bay.

Methoxyresorufin O-demethylase (MROD) reaction is associated with cytochrome P4501A1 isozyme which are induced by PAH, PCB and dioxin types chemicals. MROD activity of annular seabream liver microsomes was determined by the same method used for EROD activity measurement except that 4.5 μM of substrate concentration was used. Thirteen different microsomal preparations, which were also used in EROD activity measurements, were used in MROD activity determination. The aim was to demonstrate a relationship between hepatic MROD and EROD activities in annular seabream. MROD activities of annular seabream captured from site 11A (Zeytinalani) were found 101±12 pmol/min/mg protein, n=4 (given in Table 3.24) which was very similar but a little less than those obtained from EROD activity measurement (182±42 pmol/min/mg protein, n=8). Annular seabream caught from site 3A and site 1 displayed highly elevated MROD activities which were 624±54 (n=5) and 624±34 (n=4) pmol/min/mg protein respectively (Tables 3.25 and 3.26).

Table 3.24 MROD activities of liver microsomes of annular seabream (*Diplodus annularis*) caught from Site 11A, Zeytinalanı Region. (Nov-2002)

Sample No	Activity (pmol/min/mg)	Average Activity (pmol/min/mg)
184	119-108	114
183	64-66	65
185	118-121	120
188	103-103	103
Average activity ± standard error		101 ± 12 n=4

Table 3.25 MROD activities of liver microsomes of annular seabream (*Diplodus annularis*) caught from site 3A, Üçkuyular Region. (Nov-2002)

Sample No	Average (pmol/min/mg)	Average Activity (pmol/min/mg)
198	526-526	526
192	632-658	645
193	684-684	684
191	632-652	642
Average activity± standard error		624 ± 34 n=4

Table 3.26 MROD activities of liver microsomes of annular seabream (*Diplodus annularis*) caught from site 1, Pasaport Region. (Nov-2002)

Sample No	Activity (pmol/min/mg/mg)	Average Activity (pmol/min/mg)
175	553-553	553
176	526-537	532
179	539-553	546
181	666-679	673
178	797-839	818
Average activity ± standard error		624 ± 55 n=5

Table 3.27 The average MROD and EROD activities of *Diplodus annularis* (annular seabream) liver microsomes from different collection sites.

Sampling Sites & Date of sampling	**MROD Activities (pmol/min/mg)** Mean± SEM	**EROD Activities (pmol/min/mg) Mean±SEM**
Zeytinalanı-Site 11A Nov 2002	101 ±12 n=4	182±42 n=8
Pasaport-Site1 Nov 2002	624±34 n=5	758±119 n=8
Üçkuyular-Site 3A Nov 2002	624±55 n=4	706±132 n=8

The results tabulated in Tables 3.24, 3.25 and 3.26 were highly compatible with the EROD activities of same microsomal preparations. Table 3.27 summarizes the average MROD and EROD activities in annular seabream sampled from three different sites of the Bay and they were presented in Figure 3.4. A significant correlation was found between EROD and MROD activities suggesting a similar response of both enzymes to PAH, PCB and dioxin types environmental contaminants.

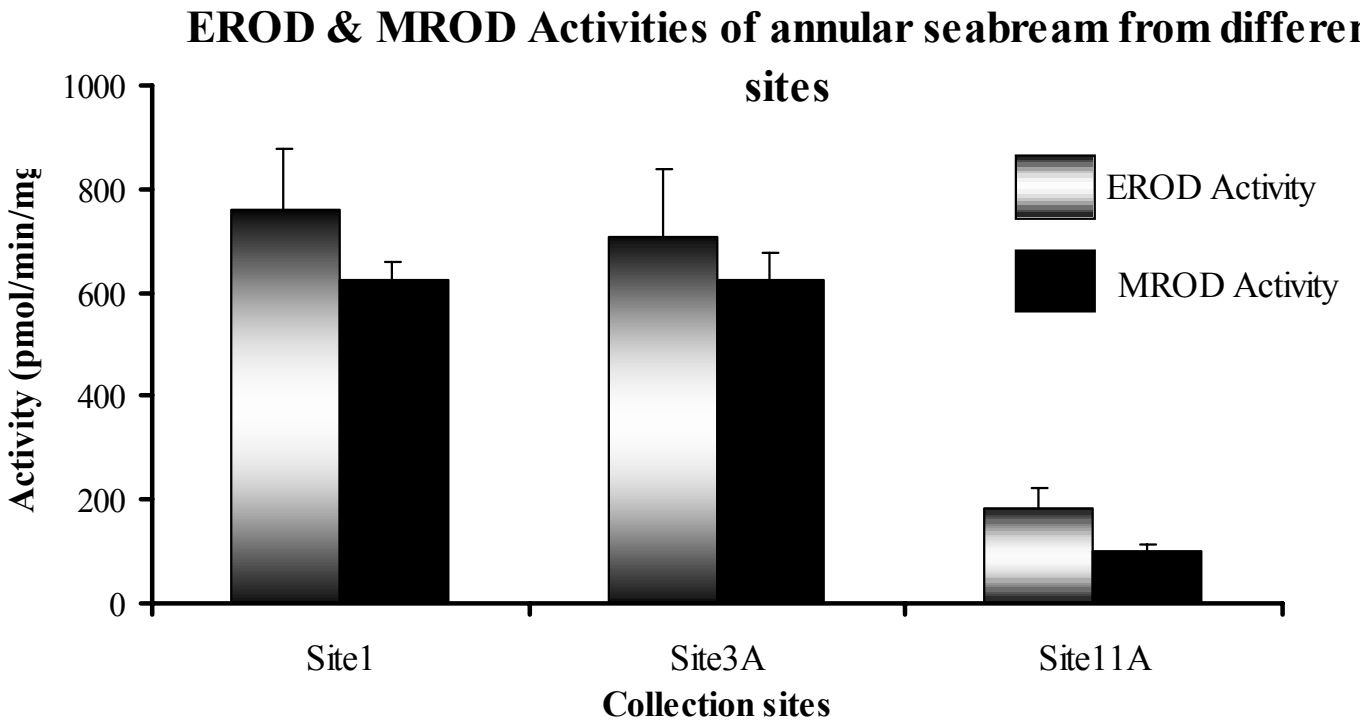


Figure 3.4 EROD and MROD activities of Annular seabream liver microsomes from different sites of the Bay, site 1 (Pasaport), site 3A (Üçkuyular), site 11A (Zeytinalanı)

3.5 Changes in Cytochrome P4501A1 Protein Levels as Determined by Western Blot Analysis

Changes in the cytochrome P4501A1 protein levels in hepatic microsomes of the samples, due to pollution based induction were determined by Western blotting coupled with immunodetection. For the analysis, a polyclonal anti-mullet P4501A IgG and anti-rabbit IgG-ALP conjugate were used as primary and secondary antibodies respectively. Polyclonal anti –mullet P4501A, prepared in our laboratory (Arinc and Sen, 1999), shows a cross reactivity with liver microsomes of three fish species, leaping mullet, common sole, and annular seabream.

3.5.1 Western Blot Analysis of Leaping Mullet (*Liza saliens*)

The Western blot analysis of leaping mullet was illustrated in Figure 3.5. Intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A) per mg microsomal protein by Scion Image software program. Results were represented for each lane in Table 3.28. Polyclonal anti-mullet P4501A1, showed strong cross-reactivity with liver microsomes of the leaping mullet caught from site 1-Pasaport (lane 5,6,9 and 10) and site 1C- Liman-Yalıçapkını (lane 7-8), indicating that P4501A1 was highly induced as a result of presence of P450-1A inducing chemicals in these areas. Liver microsomes of mullet from reference area, site 10 (see lane 1 and 2), showed very low cross reactivity (233 R.P.A/mg microsomal protein) with polyclonal anti-mullet P4501A1. The band intensity (the degree of cross-reactivity) was decreased in a pollutant related manner with the decreasing EROD activities of the samples. For example the most intense band (1374 R.P.A/mg microsomal protein) was seen in leaping mullet microsomes that had highly elevated EROD activity (3198 pmol/min/mg). The band intensities were moderate in the samples (lane 3 and 4) that displayed moderate EROD activities. The results obtained for leaping mullet clearly indicated that feral fish

sampled from the polluted sites had both highly induced EROD activity and cytochrome P4501A1 protein level.

3.5.2 Western Blot Analysis of Common Sole (*Solea vulgaris*)

The Western blot analysis of common sole was illustrated in Figure 3.6. Intensity of each band was quantified as arbitrary unit, relative peak area (R.P.A) per mg microsomal protein by Scion Image software program. Results were represented for each lane in Table 3.29. Polyclonal anti-mullet P4501A, showed strong cross-reactivity with liver microsomes of the common sole caught from site 3-İnciraltı (lane 7-8) and site 5-mouth of Gediz (lane 5 and 6), indicating that P4501A was highly induced as a result of presence of P450-1A inducing chemicals in the sea basins of these areas. Liver microsomes of common sole from reference area site 16A (lane1-2), showed very weak cross reactivity with polyclonal anti-mullet P4501A1. The band intensities (the degree of cross-reactivity) were decreased in a pollutant related manner and they show strong relation with the EROD activities of the samples. For example the most intense band (1360 R.P.A/mg) was observed in common sole microsomes (lane 7-8) that had highly elevated EROD activity (608 pmol/min/mg). The band intensity was moderate in the sample (lane 5) that displayed also moderate EROD activity. The results obtained for common sole clearly indicated that feral fish sampled from the polluted sites had both highly induced EROD activity and cytochrome P4501A1 protein level.

3.5.3 Western Blot Analysis of Annular seabream (*Diplodus annularis*)

The Western blot analysis of annular seabream was illustrated in Figure 3.7. Intensity of each band was quantified as arbitrary unit, relative peak area (R.P.A) per mg microsomal protein by Scion Image software program. Results were represented for each lane in Table 3.30. Polyclonal anti-mullet P4501A1, showed a strong cross-reactivity with liver microsomes of the annular seabream caught from site1-Pasaport

(lane 6-7), which is the most polluted part of the Bay. Liver microsomes of mullet from site 11A (lane 1-3) showed very weak cross reactivity with polyclonal anti-mullet P4501A1. The band intensities (the degree of cross-reactivity) were decreased in a pollutant related manner with decreasing EROD activities of the samples. For example the most intense band (860 R.P.A/mg) was observed in annular seabream microsomes (lane 7-8) that had highly elevated EROD activity (1355 pmol/min/mg). The band intensities were moderate in the samples (lane 4-5) that displayed moderate EROD activities. These results clearly demonstrated that annular seabream collected from the polluted site has highly induced EROD activity and P4501A1 protein content.

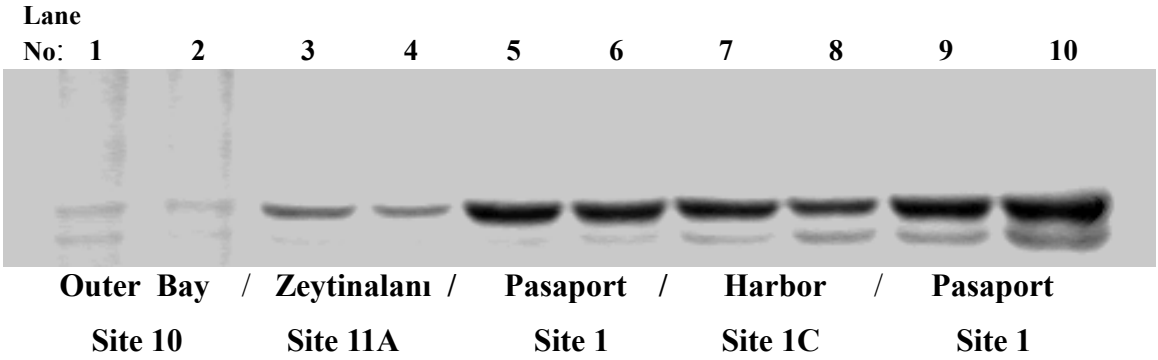


Figure 3.5 Immunochemical detection of liver microsomal cytochrome P4501A of leaping mullet (*Liza saliens*) caught from the different sites of the Bay. A 75µg microsomal protein was applied to each slot.

Table 3.28 EROD activities and band intensities (presented as arbitrary unit of R.P.A/mg microsomal protein) of each sample in the western blot analysis of leaping mullet

Lane No:	**EROD Activity (pmol/min/mg protein)**	**Relative Peak Area (R.P.A/mg microsomal protein)**
Lane 1&2	34	233
Lane 3	831	573
Lane 4	605	440
Lane 5&6	975	1097
Lane 7	2599	1120
Lane 8	2566	913
Lane 9	2932	1207
Lane 10	3198	1374

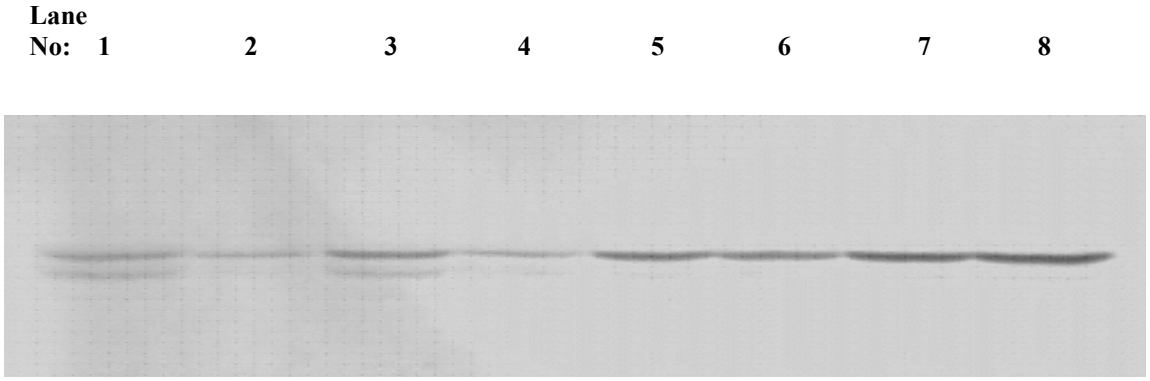


Figure 3.6 Immunochemical detection of liver microsomal cytochrome P4501A of common sole (*Solea vulgaris*) caught from different sites of the Bay.
A 100 µg microsomal protein was applied to each slot.

Table 3.29 EROD activities and band intensities (presented as arbitrary unit of R.P.A/mg microsomal protein) of each sample in the western blot analysis of sole

Lane No:	**EROD Activity (pmol/min/mg protein)**	**Relative Peak Area (R.P.A/mg microsomal protein)**
Lane 1	70	310
Lane 2	86	200
Lane 3	127	480
Lane 4	142	270
Lane 5	299	800
Lane 6	352	770
Lane 7	608	1120
Lane 8	608	1360

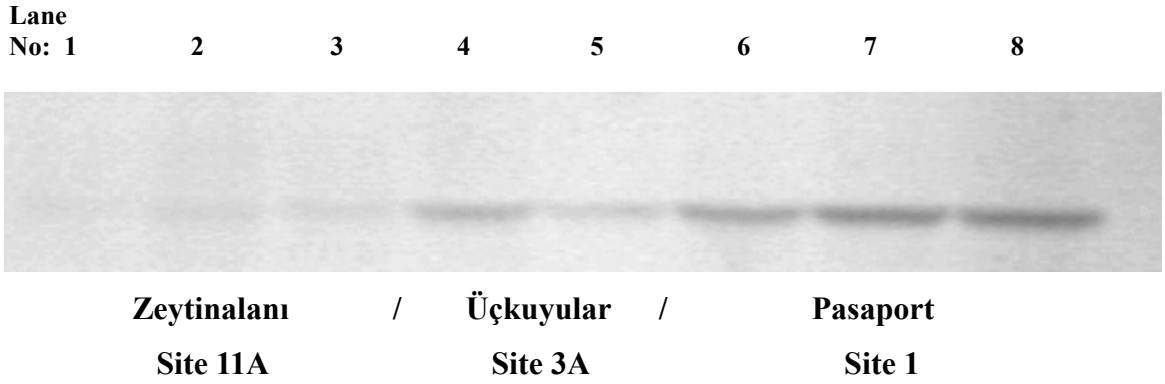


Figure 3.7 Immunochemical detection of liver microsomal cytochrome P4501A of annular seabream (*Diplodus annularis*) caught from different sites of the Bay.
A 100 µg microsomal protein was applied to each slot.

Table 3.30 EROD activities and band intensities (presented as arbitrary unit of R.P.A/mg microsomal protein) of each sample in the western blot analysis of annular seabream

Lane No:	EROD Activity (pmol/min/mg protein)	Relative Peak Area (R.P.A/mg protein)
Lane 1	75	194
Lane 2	104	240
Lane 3	140	270
Lane 4	810	470
Lane 5	874	390
Lane 6	1078	670
Lane 7	1355	870
Lane 8	1355	860

3.6 Total Polyaromatic Hydrocarbon Concentrations of Fish Liver Tissue

Total PAH concentrations in the liver tissues of fish species were determined by using a spectrofluorometric method described by UNEP 1992. Total PAH concentration determined from the individual fish liver at a time separately. Table 3.31 shows the results.

The maximum total PAH concentration which were 33.6±15 μg/g dried liver weight (n=5) was observed in leaping mullet that captured from site1, Pasaport region. The total PAH concentration of leaping mullet caught from site 1C, Liman-Alsancak region was found 16±9.9 μg/g dried liver weight (n=5). Common sole that sampled from site 3 (Inciralti) and site 19 (Hekim Island) displayed very close total PAH concentrations which were respectively, 6.7±1.9 (n=3) and 8.9±1.8 (n=3) μg/g dried liver weight. Annular seabream caught from site 1C (Liman-Yalıçapkını) and site1 (Pasaport) demonstrated nearly same total PAH concentrations those were 5.6±0.59 (n=3) and 6.4±1.0 (n=5) μg/g dried liver weight respectively.

3.7 Total Polyaromatic Hydrocarbon Concentrations in the Sediment

Total PAH concentrations were determined from the sediment samples collected from three different sites of the Bay by using a spectrofluorometric method described by UNEP 1986. The maximum total PAH concentration was observed in sediment collected from harbor region which is the innermost section of the Bay. The lowest total PAH concentration was observed in sediment collected from site 10 located in the outermost section of the Bay. Table 3.32 shows the results.

Table 3. 31 Total PAH concentration in the livers of fish that caught from different sites

Fish Species	Sites	Total PAH conc. in liver µg /g dried weight ± SEM n=number of fish liver
Common sole	Site 19 Hekim adası	7.8
		11
		8
		Average ± SEM 8.9±1.8 n=3
Common sole	Site 3 İnciraltı	7.9
		7.8
		4.5
		Average ± SEM 6.7±1.9 n=3
Leaping mullet	Site 1 Pasaport	53
		16
		61
		20
		18
		33.6±9.8 n=5
Leaping mullet	Site 1C Liman	9.8
		12
		9.0
		33
		14
		Average ± SEM 16±9.9 n=5

Continue of Table3.31

Fish Species	Sites	Total PAH conc. in liver μg /g dried weight ± SEM n= number of fish liver
Annular seabream	Site 1 Pasaport	9.7
		5.4
		4.8
		3.9
		9.4
		Average ± SEM 6.4 ± 1 n=5
Annular seabream	Site 1C Liman	5.4
		5.6
		4.8
		Average ± SEM 5.6 ± 0.59 n=3

3.32 Total PAH concentration in sediment collected from different sites of the Bay

Region	**Total PAH conc. In sediment μg/g wet weight**
Site 10 Outer Bay	0.22
Site 1C Liman	1.91
Site 3 İnciraltı	0.94

3.8 Biocatalytic Properties of Annular Seabream (*Diplodus Annularis*) Liver Mixed Function Oxidases

Annular seabream (*Diplodus* annularis), a benthopelagic fish, was used as test species for the first time in this study, to monitor the toxic and carcinogenic pollutants along the İzmir Bay, by examining cytochrome P4501A1 associated EROD activity and P4501A1 protein level. This species were found to be very convenient for environmental biomonitoring of CYP1A1 inducing chemicals. Annular seabream has demonstrated a clear contaminant related CYP1A1 induction in their liver microsomes. In addition they can be easily trapped and they were widely distributed along the Izmir Bay from most polluted areas to the relatively clean sites.

Major cytochrome P450 dependent mixed function oxidase activities; methoxyresorufin O-demethylation, benzphetamine N-demethylation, ethylmorphine N-demethylation and aniline 4-hydroxylation, were characterized in the liver microsomes of annular seabream for the first time by this work.

3.8.1 Benzphetamine N-demethylase Activity of Annular Seabream Liver Microsomes

Benzphetamine N-demethylase activity of fish liver microsomes was determined by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). Benzphetamine N-demethylase activity was determined in eight different liver microsomal preparations. The specific activities of different microsomal preparation were given in Table 3.33. The average specific activity was calculated as 1.7±0.4 (n=8) nmoles of formaldehyde formed/min/mg protein (Mean±SD). A comparison of liver microsomal benzphetamine N-demethylase activity in different animal species, including mammals and fish was given in Table 3.34. As can be seen there is a very big differences in the liver

microsomal benzphetamine N-demethylase specific activities among the different species. The specific activity in annular seabream liver microsomes, was significantly higher than those of human, monkey, dog and guinea pig and similar to those of rat, and leaping mullet.

Benzphetamine has high turnover numbers with several CYPs. As the highest affinity was determined for CYP2B, this compound is frequently used as specific substrate for CYP2B (Adalı and Arınç, 1990; Antonovic *et al*., 1999). In mammals cytochrome P4502B is found to be inducible with phenobarbital (PB) type inducers, but fish P450 monoxygenase system is found not to be sensitive to PB-type inducers (Elcombe and Lech, 1979; Kleinow *et al*., 1987; Eiskus and Stegeman, 1989; Arinc *et al*., 1995) however CYP2B related genes are present in fish (Stegeman et al., 1999). The presence of P4502B type protein in leaping mullet was demonstrated in our laboratory very recently with purification and characterization of this protein from liver microsomes (Bozcaarmutlu, 2002).

Table 3.33 Benzphetamine N-demethylase activity of liver microsomes of annular seabream (*Diplodus annularis*) caught from different sites of the İzmir bay.

Sample No	Region	Activity (Unit/mg)	Average Activity (Unit/mg)
184	Zeytinalanı	0.94-0.97	0.96
196	Üçkuyular	1.42-1.50	1.46
180	Pasaport	1.76-2.0	1.88
195	Üçkuyular	1.94-1.92	1.93
181	Pasaport	1.36-1.53	1.44
191	Üçkuyular	2.1-2.4	2.20
193	Üçkuyular	2.15-2.15	2.15
178	Pasaport	1.80-1.94	1.90
Average activity± Standard deviation			1.70 ± 0.4 n=8

Unit shows the nmol formaldehyde that is formed in one minute.

Table 3.34 Liver microsomal benzphetamine N-demethylase specific activities in different species

Species	Microsomal Activity (nmol/min/mg protein)
Rat[a]	2.39±0.64
Guinea pig[a]	0.98±0.096
Dog[a]	0.35±0.078
Monkey[a]	0.94±0.200
Human[a]	0.54±0.427
Rainbow Trout[b]	1.97 nmol/min/nmol P450
Killifish[c]	0.47
Leaping Mullet[d]	1.59±0.951 (n=8)
Annular Seabream[e]	1.7±0.4 (n=8)

References: (a) Shimada *et al*., 1997; (b) Miranda *et al*., 1989; (c) Oleksiak *et al*., 2000; (d) Bozcaarmutlu, 2002; (e) obtained in this study

3.8.2 Ethylmorphine N-Demethylase Activity of Annular Seabream Liver Microsomes

Ethylmorphine N-demethylase activity is mainly associated with CYP3A and CYP2B isozymes**.** In fish liver microsomes, this activity was determined by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). Ethylmorphine N-demethylase activity was determined in eight different liver microsomal preparations. The specific activities of different microsomal preparation were given in Table 3.35. The average specific activity

was calculated as 0.84±0.16 (n=8) nmoles of formaldehyde formed/min/mg protein (Mean±SD). Table 3.36 shows the liver microsomal ethylmorphine N-demethylase specific activities in different species. Ethylmorphine N-demethylase activity of annular seabream was found to be very similar to the activity obtained from guinea pig and leaping mullet liver microsomes.

Table 3.35 Ethylmorphine N-demethylase activity of liver microsomes of annular sea bream (*Diplodus annularis)* caught from different sites of the İzmir bay.

Sample no	Region	Activity (Unit /mg)	Average Activity (Unit/mg)
184	Zeytinalanı-Urla	0.55-0.57	0.56
196	Üçkuyular	1.1-1.1	1.10
180	Pasaport	0.86-0.86	0.86
195	Üçkuyular	0.73-0.78	0.76
181	Pasaport	0.76-0.69	0.73
191	Üçkuyular	1.04-0.98	1.01
193	Üçkuyular	0.88-0.96	0.92
178	Pasaport	0.76-0.83	0.80
Average Activity± Standard Deviation			0.84 ± 0.16 n=8

Unit shows the nmol formaldehyde that is formed in one minute.

Table 3.36 Liver microsomal ethylmorphine N-demethylase specific activities in different species.

Species	Microsomal Activity (nmol/min/mg protein)
Rat [a]	3.37±0.621
Guinea pig[a]	0.76±0.183
Dog[a]	0.273±0.149
Monkey[a]	1.08±0.490
Human[a]	0.423±0.543
Sheep[b]	3.30±1.32 (n=11)
Leaping Mullet[c]	0.75±0.21 (n=4)
Annular Seabream[d]	0.84±0.16 (n=8)

References: (a) Shimada *et al*., 1997; (b) Arınç, 1985; (c) Bozcaarmutlu, 2002; (d) Obtained in this study.

3.8.3 Aniline 4- Hydroxylase Activity of Annular Seabream Liver Microsomes

Aniline 4-Hydroxylase activity is associated with the cytochrome P4502E isozyme. However other forms of cytochrome P450, such as CYP2B of sheep lung was found to catalyze the *p*-hydroxylation of aniline (Adalı et al., 1996).

Aniline 4-hydroxylase activity was measured in nine different microsomal preparations. Table 3.37 shows the specific activities from individual fish liver microsomal preparations. The average specific activity was calculated as 0.128±0.04 (n=9) nmol *p*-aminophenol formed/min/mg protein (mean± SD).

Table 3.38 shows the liver microsomal aniline 4- hydroxylase specific activities in different species. Specific activity was very low in annular seabream when compared with other species but it was very similar to the specific activity of leaping mullet microsomes.

3.8.4 NADPH- Cytochrome P450 Reductase Activity in Annular Seabream Liver Microsomes

NADPH-Cytochrome P450 reductase is the essential component of the mixed function oxidase system and functions in the transfer of electrons from NADPH to cytochrome P450. Cytochrome P450 reductase activity was measured spectrophotometrically, according the procedure of Masters et al., (1967) except that the reaction was carried out in 0.3 M potassium phosphate buffer, pH 7.7 at room temperature.

Cytochrome P450 reductase activity measured in eight different microsomal preparations. Table 3.39 shows the specific activities from individual fish liver microsomal preparations. The average specific activity was calculated as 155±45 (n=8) nmole cytochrome c reduced/min/mg protein (mean± SD).

NADPH-Cytochrome P450 reductase activity was measured in samples caught from contaminated region (Pasaport and Üçkuyular) and also relatively clean sites (Zeytinalani). In this study no significant differences could be observed between fish from polluted sites and relatively uncontaminated sites.

Table 3.37 Aniline 4- hydroxylase activity of liver microsomes of annular seabream (*Diplodus annularis*) caught from different sites of the İzmir bay.

Sample No	Region	Activity (Unit /mg)	Average Activity (Unit /mg)
196	Üçkuyular	0.098-0.080	0.090
197	Üçkuyular	0.091-0.102	0.097
187	Zeytinalanı	0.104-0.106	0.105
182	Pasaport	0.102-0.100	0.101
177	Pasaport	0.228-0.237	0.233
198	Üçkuyular	0.108-0.108	0.108
176	Pasaport	0.175-0.177	0.176
179	Pasaport	0.120-0.118	0.124
193	Üçkuyular	0.091-0.082	0.119
Average Activity ± Standart Deviation			0.128 ± 0.04 n=9

Unit shows the nmol p-aminophenol that is formed in one minute.

Table 3.38 Liver microsomal **a**niline 4-Hydroxylase specific activities in different species.

Species	Microsomal Activity (p-AP/min/mg protein)
Rat [a]	0.403±0.057
Guinea pig[a]	0.565±0.054
Dog[a]	0.280±0.091
Monkey[a]	0.227±0.165
Human[a]	0.696±0.986
Sheep[b]	0.65±022 (n=10)
Leaping Mullet[c]	0.120±0.04 (n=7)
Annular Seabream[d]	0.128±0.04 (n=9)

References: (a) Shimada *et al*., 1997; (b) Arınç and Iscan, 1983; (c) Bozcaarmutlu, 2002; (d) obtained in this study.

Table3.39 NADPH-cytochrome P450 reductase activity of liver microsomes of *Diplodus annularis* (annular sea bream) caught from different sites of the İzmir bay.

Sample No	Region	Activity (Unit /mg)	Average Activity (Unit/mg)
184	Zeytinalanı	78-94	86
196	Üçkuyular	140-144	142
180	Pasaport	159-156	158
175	Pasaport	230-207	219
198	Üçkuyular	134-139	137
195	Üçkuyular	230-197	214
191	Üçkuyular	158-158	158
178	Pasaport	120-123	122
Average Activity± Standard deviation			155±45 n=8

Unit defined as nmole cytochrome c reduced/min

CHAPTER 4

DISCUSSION

It is well accepted that the aquatic environment is becoming threatened by an increasing number of chemicals as a result of high technological and industrial development. The marine environment today is loaded with about 60.000 different chemicals (Goksoyr and Forlin 1992). Especially, estuaries, coastal areas and urban harbors receive large inputs of anthropogenic pollutants through domestic and industrial wastes, urban and agricultural run-off, discharges from ships, hydrological and atmospheric process. As a result, aquatic organisms inhabiting these areas are often exposed to complex mixture of pollutants including PAHs, PCBs, PCDD, PCDF, alkyltin compounds, pesticides and heavy metals. These chemicals are hazardous to aquatic organisms, human consumers and finally to whole ecosystem. Adverse effects of chemical pollutants on the ecosystem can be avoided by taking protective measures.

Chemical analyses are able to measure a wide range of pollutants quantitatively and accurately, however the complex mixture of chemical pollutants cannot be fully assessed. Furthermore such analyses are often unable to predict the impact of chemical pollution on living organisms (Arınç *et al*., 2000). Biochemical markers that are related with changes in gene expression, alteration in enzyme activity or/protein content fulfills this purpose. Biochemical changes that occur in the organisms after exposure to contaminants offer advantages as biomarkers for two reasons. First, biochemical alterations can serve as markers of both exposure and

effect (Peakall, 1992; Walker 1998). Second, biochemical alterations are usually the first detectable and quantifiable responses to chemical contamination.

The best characterized and used biochemical marker so far is the induction of cytochrome P4501A (CYP1A) dependent mixed function oxidases (MFO) or monooxygenases (Bucheli and Fent 1995). Organic contaminants such as PCB, PAH, PCDD and PCDF specifically induce CYP1A in fish liver. CYP1A induction is used as a biomarker of exposure to these types of organic pollutants often serving as an early warning signal of possibly more serious pathologies (Payne *et al.* 1987). P4501A is a subfamily of cytochrome P450 dependent mixed function oxidases which catalyzes oxidation of a number of organic chemicals to more water soluble metabolites that can be further conjugated by Phase II enzymes and excreted. Cytochrome P450 dependent mixed function oxidases are also responsible for the activation of foreign chemicals to reactive intermediate that ultimately result in toxicity, carcinogenicity and mutagenicity. Especially P4501A isozyme is found to be responsible for the activation of PAHs, PCBs and dioxin type pollutants to reactive intermediates.

Benzo(a)pyrene, a member of PAHs is present in petroleum products and in waste materials of industry, and has both mutagenic and carcinogenic properties. (Coney and Burns, 1972; Heidelberger, 1973). This compound causes CYP1A induction at the mRNA level in fish. Oxygenation of benzo(a)pyrene by CYP1A in the presence of epoxide hydrolase results in the formation of the ultimate carcinogen, benzo(a)pyrene 7,8 dihydrodiol 9,10- epoxide (BPDE), which forms DNA- adducts (Parke *et al*.,1991). Greater CYP1A induction may result in high levels of activated carcinogens and consequently to higher degree of persistent DNA-adduct formation or to an enhanced oxidative damage (Stegeman, 1995; Arınç et *al.,* 2000).

Positive correlations have been found between the levels of sediment and tissue contaminants, fish liver CYP1A, liver DNA adducts and liver neoplasm and related lesions. (Varanasi *et al*., 1987; Stein *et al*., 1990; Livingstone, 1993). Malins *et al*. (1985) reported high levels of neoplasm in fish collected from a creosote

(mixture of petroleum products) polluted Puget Sound, U.S.A. Kocan *et al*. (1985) observed that much of the cellular toxicity associated with the extracts of sediment from Puget Sound requires "metabolic activation". DNA isolated from neoplastic nodules of hepatic tissues of English sole exposed to creosote pollution in Puget Sound was shown to contain modified guanine, 2,6-diamino-4-hydroxy-5-formamido pyrimidine (Fapy Gua).

The induction of cytochrome P450 1A and its associated enzyme activities, EROD and AHH in response PAHs, PCB, polybrominated biphenyls, dibenzodioxins and dibenzofurans in fish have been now confirmed in a number field studies (Payne *et al*., 1987; Garrigues *et al*., 1990; Goksoyr and Forlin, 1992; Haasch *et al*., 1993; Stegeman, 1995; Addison *et al*., 1996; Buhler *et al*., 1998; Arınç and Şen, 1999; Arınç *et al*., 2000; Arınç *et al*., 2001). A significant increase in EROD activities was observed in 90% of the 127 investigated field studies while strong increases (> 500% of reference) in EROD activities were observed in 37% of these field studies. In addition, a significant increase in CYP1A levels was observed in 85% of 48 investigated field studies while strong increases (> 500% of reference) were observed in 39 % of these field studies (Oost, Beyer and Vermeluen, 2003).

CYP1A response measured as EROD or AHH activities has already been incorporated into some major monitoring programs such as the National Status and Trends Program in United States (Collier et al., 1992) and North Sea Task Force Monitoring Master Plan of the North Sea Nations in Europe (Goksoyr and Forlin, 1992). The general strategy in the field studies is to compare CYP1A1 associated enzyme activity in fish from suspected sites with those in fish from reference site. Most of the earlier field studies employed the induction of liver B(a)P hydroxylase activity in biomonitoring. The use of this assay has been declining because of carcinogenic property of substrate; benzo(a)pyrene as well as possibility of substrate cross-reaction with other CYP isozymes (Arınç *et al*., 2000). Nowadays CYP1A associated enzyme activity determined by using 7-ethoxyresorufin as a substrate. The measurement of 7-ethoxyresorufin O-deethylase activity appears to be most sensitive and most widely used catalytic probe for determining induction response of CYP1A

in fish. The advantages of using EROD activity as a biomarker are the specificity for CYP1A in fish, high sensitivity, feasibility and simplicity of its measurement (Burke *et al*., 1985; Arınç and Sen, 1994; Bucheli and Fent, 1995). With the development of immunochemical techniques and with the availability of poly- and mono- clonal antibodies for fish CYP1A, the use of both EROD activity and immunoquantification of protein amount is recommended in the field studies.

The İzmir Bay, is one of the largest bay in the Western parts of the Aegean coast of Turkey receives considerable influx of anthropogenic pollutants from diverse point and nonpoint sources including industrial discharges, municipal sewage outfalls, urban and agricultural run-off, influx from the contaminated waters and sediment of rivers, discharges from ships, surface run-off and atmospheric process. Industries located along the shoreline include petroleum refinery, paint production, tanneries, food industries, vegetable oil and soap production, textile industries metal processing, and chemical industries. Industrial and domestic wastes as well as contaminated waters of several small rivers heavily pollute the Inner Bay. Previous reports noted that sediment of Inner bay was contaminated with a variety of heavy metals, organochlorine compounds and polyaromatic hydrocarbons (Balcı and Türkoğlu, 1993; Aksu *et al*., 1998). In addition, the Bay has been monitored since 1995 by measuring the CYP1A induction in leaping mullet and common sole to assess the PAH, PCB and dioxin type contamination in our laboratory (Arınç and Şen, 1999; Arınç *et al*., 2000, 2001). These studies clearly demonstrated that, especially Inner Bay was heavily contaminated with PAH, PCB and other organochlorine type persistent organic precarcinogen/carcinogens chemicals that can disrupt the development, reproduction and survival of aquatic organisms.

İzmir Great Canal Project has started to work actively since 2000 to refine domestic and industrial wastes discharged directly into the Bay. In the present study, our aim was to determine if there exist a change in the levels of PAH, PCB and dioxins type persistent organic chemicals after the Great Canal Project along the İzmir Bay by measuring the degree of induction of cytochrome P4501A associated EROD activity and immunochemical detection of cytochrome P4501A protein level,

and to compare the results obtained in this study with the previous studies (see section 1.11 for detail).

In this study three kinds of fish species, leaping mullet (*Liza saliens*), common sole (*Solea vulgaris*), and annular seabream (*Diplodus annularis*) were used as test organism. Leaping mullet, a pelagic fish, has been used preliminary to monitor the organic pollution along the İzmir Bay in our laboratory. It provides advantages for environmental biomonitoring because its ability to withstand the conditions of highly polluted areas, and its demonstrable CYP1A induction in response to chemical contamination. Common sole, which has been also used in our laboratory for biomonitoring studies along the İzmir Bay, was selected as a benthic fish species. It is a bottom feeding flatfish that bioaccumulates lipophilic hydrocarbon compounds that contaminate the sediment. It has also demonstrable CYP1A induction in response to chemical contamination. Annular seabream a benthopelagic fish, was used for the first time to monitor the level of PAH, PCB and dioxin type contamination. It was tested whether it has a demonstrable CYP1A induction or not in response to chemical contamination.

Monitoring of the induction of P4501A at the enzymatic level was assessed by determination of the ability of fish liver microsomes to convert 7-ethoxyresorufin into the ethoxyresorufin in the presence of NADPH. Liver microsomal EROD activities of leaping mullet caught at seven different sites along the İzmir Bay were given in Figure 4.1. Unfortunately, we were able to capture only one leaping mullet from reference site (site 10- Outer Bay) which had similar EROD activity as before. Since the fish number was not enough to make the comparison, we use the results of previous study (25±9 pmol/min/mg protein n=4 Arınç and Şen, 1999) as the reference value. The highest microsomal EROD activity (2590±340 pmol.min/mg protein n=7) was detected in fish samples captured from harbor region, Alsancak portside (site 1C) which was about 104 times higher than those obtained from the reference site. The harbor region is located in the innermost section of the Bay and it used to receive considerable influx of domestic and industrial wastes. In addition, it has a long-term history as sinks for petroleum hydrocarbons, discharging from ships.

As a result, the highly elevated EROD activity of leaping mullet confirms the presence of high amount of petroleum hydrocarbons in this region. The mullet caught at Üçkuyular port site (site 3A) and Pasaport (site 1) which are also highly urbanized and industrial sections of the Bay, displayed elevated EROD activities, 2011±490 (n=4) and 1813±287 (n=11) pmol/min/mg protein respectively and were 80 and 79 times higher than that of fish obtained from reference site. Mullet caught from İnciraltı (site 3) and Zeytinalanı (site 19) region has less but significantly induced EROD activity which were 582±72 (n=4) and 843±100 (n=4) respectively and were 23 and 34 times higher with respect to the value at the reference site. Although Hekim Island was located in the Outer Bay, the mullet sampled from this site exhibited highly induced EROD activity, 1169±100 (n=8) which was about 47 fold higher than those obtained from reference site. This significantly high EROD activity of mullet sampled from Hekim Island is probably caused by continuous dumping of sediment excavated from the most polluted harbor region of the Inner Bay between 1976 and 1990.

Similar results were obtained in the previous studies carried out in 1995 and 1996 in İzmir Bay (Arınç and Şen, 1999). In that study, the mullet caught from Pasaport (site 1) showed highly elevated EROD activities (1293±292 pmol/min/mg n=208) which were about 62 times higher with respect to that of fish obtained from the reference point, site 10. In another study carried out in 1999 (Arınç et al., 2000) Gray mullet caught from site 1A had highly increased EROD activities (1398±410 pmol/min/mg protein). The EROD activities of mullet caught in İzmir Bay at different sampling sites in the previous studies were given in Figure 4.2. Results of the present study are in good correlation with that of Arınç and Sen, 1999; Arınç et al., 2000 and Arınç et al., 2001 in such a manner that Harbor region and Pasaport (site1) are the most polluted regions of the Bay, and they are still contaminated with PAH and or PCB type carcinogenic compounds.

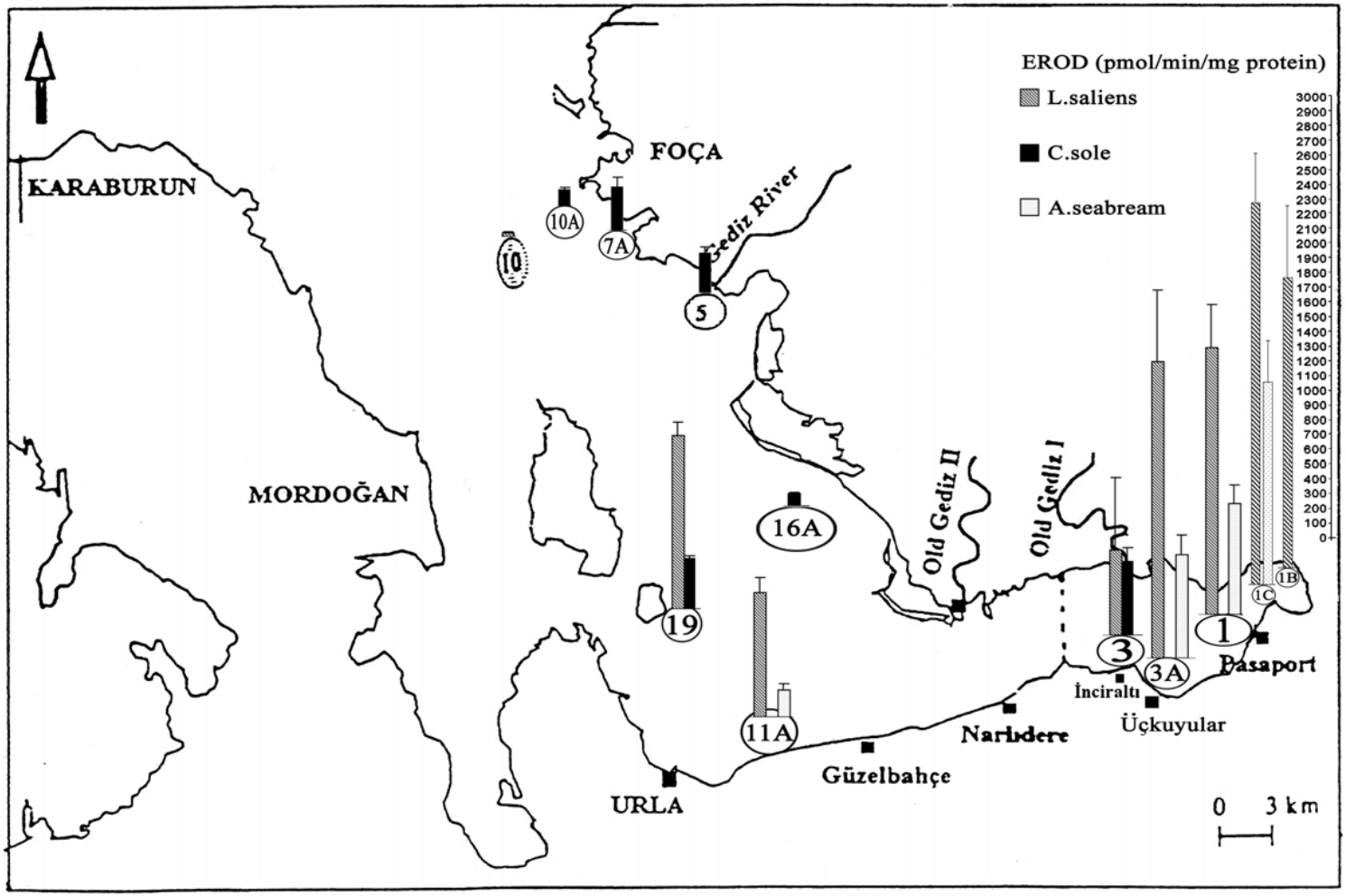


Figure 4.1 EROD activities of leaping mullet (*Liza saliens*), common sole (*Solea vulgaris)* and annular seabream (*Diplodus annularis)* captured in Izmir Bay at different sampling sites (the results of present study).

Liver microsomal EROD activities of common sole caught at six different sites were given in Figure 4.1. The all sampling sites except one region were located in the Outer Bay. EROD activities of common sole from different sites of the Outer Bay extended the results obtained with those of mullets. In addition common sole as a benthic fish species indicates the levels of PAH, PCB and dioxin type persistent organic chemicals in surface sediment of the Bay. The lowest EROD activities were detected in common sole captured from site 10A (Foça open site) and site 16A which were 107±20 (n=5) and 80±12 (n=9) pmol/min/mg protein respectively and the latter accepted as reference site. Common sole caught from İnciraltı (site 3), displayed highest EROD activity (503±89 pmol/min/mg protein, n=24) which were about 6.3 times higher with respect to the value at reference site indicating that sediment in this area were contaminated with CYP1A inducing chemicals. Although site 7A (Foça coastal line), site 19A (Hekim Island) and site 5 (mouth of Gediz) were located in the Outer Bay, the EROD activities of common sole sampled from these areas were significantly higher than those obtained from reference sites.

The average EROD activity of common sole captured from site 7A was 290±71 pmol/min/mg protein (n=4) which was nearly four fold higher than those obtained from reference site. This indicates that site 7A is probably contaminated with PAH, PCB and dioxin type pollutants which cause induction of CYP1A in fish inhabiting this area. The average EROD activity of common sole sampled from site 19A (Hekim Island) was 340±22 pmol/min/mg (n=24) and was about 4.3 times higher with respect to the value at reference site. This induction demonstrated the presence of CYP1A inducing chemicals in the surface sediment of the area, which is caused by continuous dumping of excavated sediments from inner harbor region to Hekim Island. The elevated EROD activity of common sole was confirmed the result obtained with those of mullets from same site.

Common sole captured from site 5 (mouth of Gediz river) in the Outer Bay had elevated EROD activity which was 267±47 pmol/min/mg protein (n=5). The Gediz River runs from the fertile agricultural area treated with herbicides and other

pesticides. It also carries domestic and industrial wastes from the surrounding to the Bay. Elevated EROD activity of common sole captured from this site demonstrated that sediment of the Bay at the mouth of the Gediz River was polluted with CYP1A inducing chemicals. This result is in good correlation with that of Arınç and Sen (1999) in which the EROD activities of common sole sampled from mouth of Gediz River was 300±10 pmol/min/mg protein, n=5 (see Figure 4.2). Therefore there is no change in the concentration of PAH and PCB type carcinogenic organic compounds in this site.

Annular seabream was found to be very convenient test species for biomonitoring of CYP1A inducing chemicals. It demonstrated a clear CYP1A induction in response to contaminants. Liver microsomal EROD activities of annular seabream caught at four different sites along the Bay were given in Figure 4.1. Annular seabream captured from Harbor region (site 1C) exhibited the highest EROD activity which were 1376±279 pmol/min/mg protein (n=8). EROD activities of annular seabream caught along a pollutant gradient at three other sites, Pasaport (site 1), Üçkuyular (site 3A) and Zeytinalanı (site 11A) were 758±119, 706±132, and 182±42 pmol/min/mg protein (n=8 for all) respectively. An inverse relationship was found between the EROD activity of annular seabream and the distance between catch point and discharge region of industrial and domestic wastes into the Harbor. These results confirm that hepatic EROD activity in annular seabream is readily inducible by environmental contaminants.

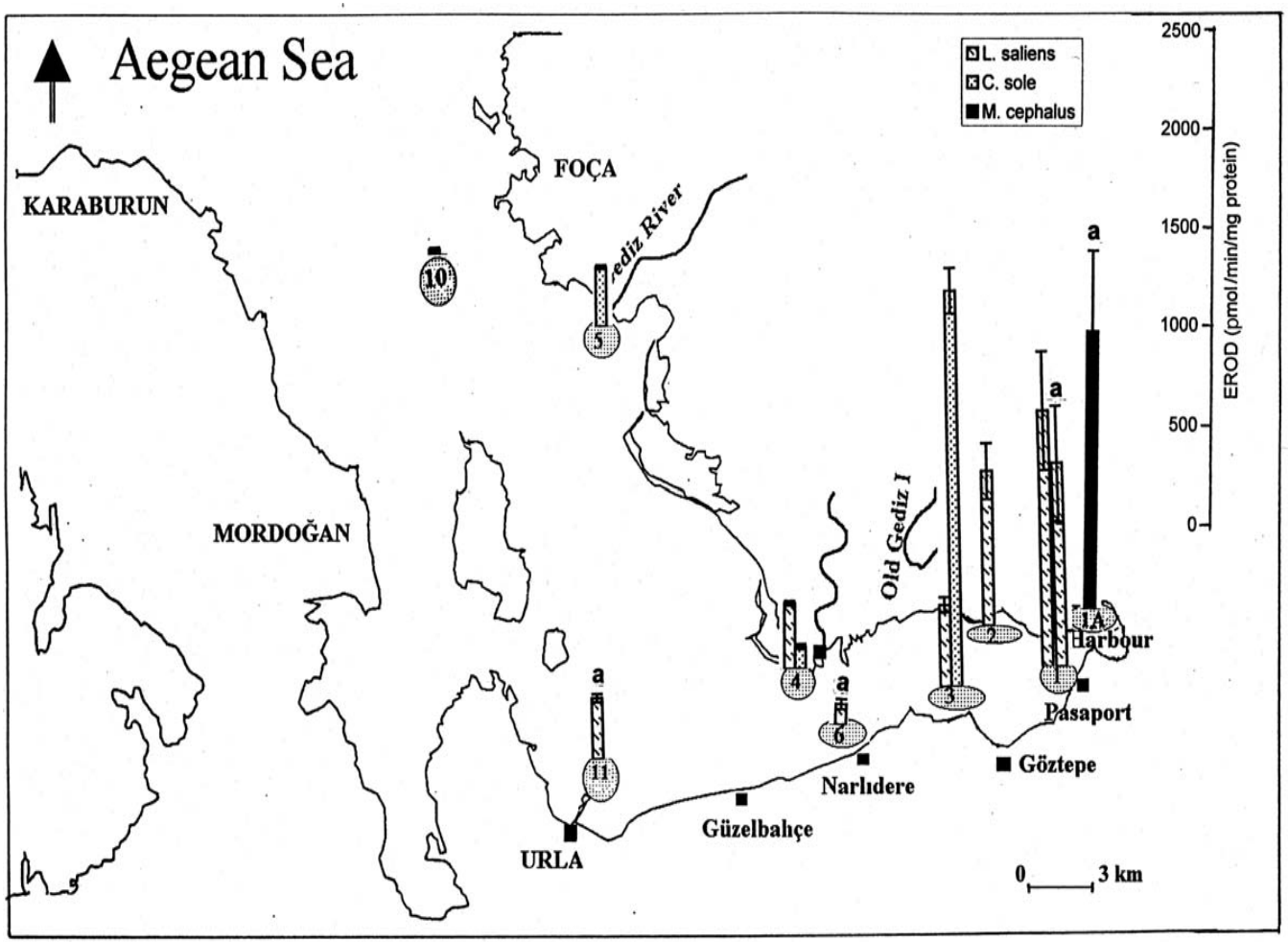


Figure 4.2 EROD activities of liver microsomes common sole (Solea vulgaris) leaping mullet (Liza saliens) and gray mullet (Mugil cephalus) captured in Izmir Bay at eight different sampling sites. The activity bars with "a" represent the field studies carried out in 1999. The others are taken from Arınç and Sen, 1999, showing the results of biomonitoring studies carried out in 1995.

In spite of observed induction in three fish species in response to chemical contamination, there were important variations between species concerning the level of enzyme activities. For example, both annular seabream and mullet displayed induced EROD activity at three locations (site 1C-Alsancak, site 1-Pasaport and site 3A-Üçkuyular), but the EROD activities were generally 2-3 times greater in leaping mullet than in annular seabream, indicating possible differences between metabolizing capacity of two different fish species. This kind of species variations in the induction of CYP1A associated EROD activities were observed in many studies in response to organic contamination. For example, benthic fish, English sole, rock sole and starry flounder were sampled from up to five sites in Puget Sound, WA, USA that were contaminated with PAH, PCB and other organic chemicals. English sole and rock sole caught from the most polluted site had the highest EROD activity while the EROD activity of starry flounder from the same area was only one half of the others (Stein *et al*., 1993). Similar results were obtained in the field studies in Rhone watershed, France. The benthic fish, gudgeon (*Gobio gobio*) was found to be less sensitive than chub (*Leuciscus cephalus*) to the contaminants containing PCB, lindane and hexochlorobenzene (Flammarion *et al*,. 1998).

Chemical pollutants present in natural water displayed great diversity and some compounds may have the potential to inhibit the catalytic activity of cytochrome P450 enzyme system. For example in vivo treatment of benzene was found to reduce both cytochrome P450 content and EROD activity of gilthead seabream (Arınç and Şen 1993b). Metallic elements such as cadmium had profound inhibitory effect on the catalytic activity of cytochrome P450 dependent MFO system. For example reductive effects were observed in fish treated with cadmium (Forlin and Haux, 1986; George, 1989). Cadmium and many other heavy metals have been detected in high concentrations especially in Inner Bay (Bacı and Türkoğlu, 1993). Outer and Middle Bay show low level of heavy metal contamination. Moderate level of heavy metal contamination observed around Hekim Island and mouth of Gediz (Aksu *et al*., 1998). The concentrations of metals present in the Inner Bay were significantly less than the concentration required to cause a significant reduction of EROD activities in fish.

In the present study, major cytochrome P450 dependent mixed function oxidase activities such as methoxyresorufin O-demethylation, benzphetamine N-demethylation, ethylmorphine N-demethylation and aniline 4-hydroxylation were characterized in annular seabream for the first time. A significant correlation has been found between EROD and MROD activities (see Figure 4.3 r=0.94, n=13). Thus MROD activity which has been shown to be catalyzed by CYP1A1 and CYP1A2 in mammals (Burke et al., 1994) appears to be catalyzed by CYP1A in annular seabream. Although Miller *et al*. (2003) reported that MROD activity in untreated fish is very low for this reason it is not useful for biomonitoring studies as EROD activity, our results clearly demonstrated that there is a close relationship between EROD and MROD activity. Both of them are very useful indicators because of high sensitivity specificity and practicability of the reaction.

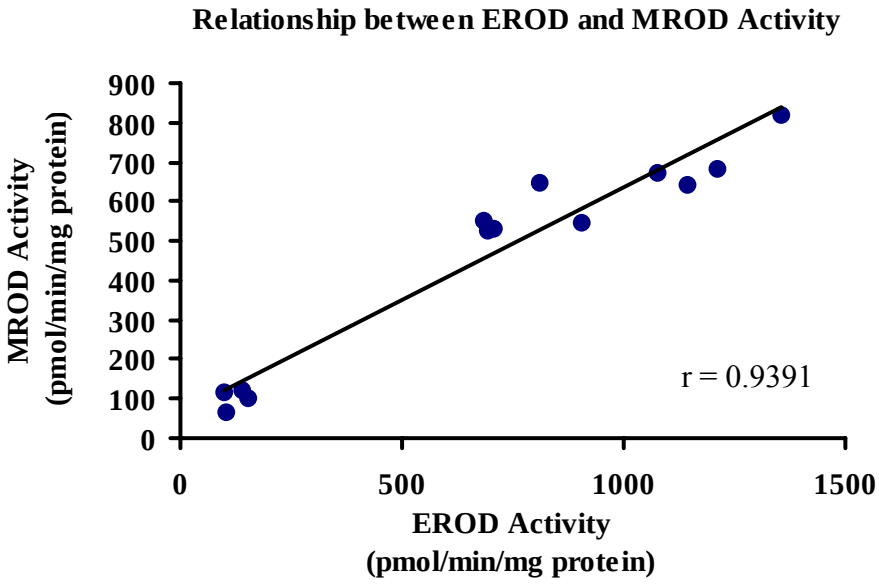
The average specific activity of benzphetamine N-demethylase reaction in annular seabream liver microsomes was found as 1.7±0.4 (n=8) nmoles of formaldehyde formed/min/mg protein (Mean±SD) that was significantly higher than human, monkey, dog and guinea pig and similar to rat and leaping mullet (see Table 3.34). Benzphetamine N-demethylase (BeNZD) activities of annular seabream caught from polluted sites and relatively clean sites did not show a contaminant-related response. In other words there was no induction of the BeNZD activity in response to PAH and /or PCB type pollution. As seen in Figure 4.4 there was also no significant correlation between EROD and BeNZD activity.

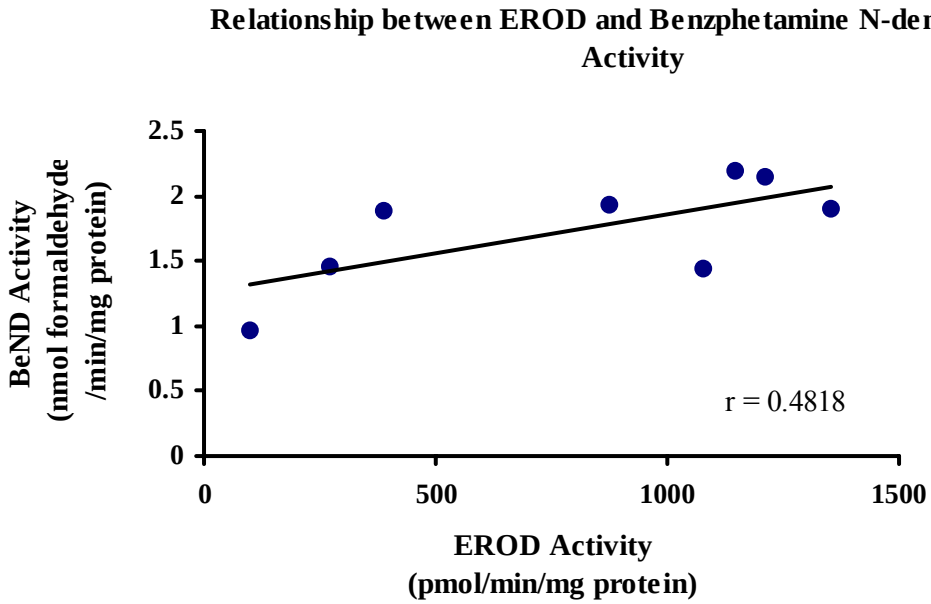
The average specific activity for ethylmorphine N-demethylase reaction in liver microsomes of annular seabream was found as 0.84±0.16 (n=8) nmoles of formaldehyde formed/min/mg protein (Mean±SD). This was found to be very similar to the specific activity obtained from guinea pig and leaping mullet (see Table 3.36). No significant differences have been observed in ethylmorphine N-demethylase activity of annular seabream that caught from polluted sites and relatively uncontaminated sites. This means that there was no induction of ethylmorphine N-demethylase activity in response to PAH and PCB type contamination. Figure 4.5

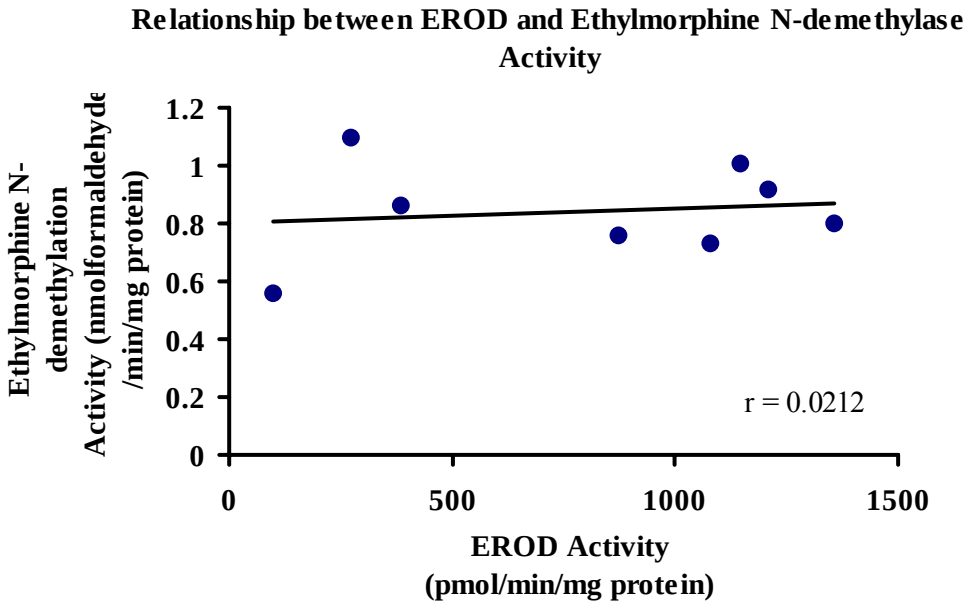
clearly demonstrate that there is no correlation between EROD and ethylmorphine N-demethylase activty.

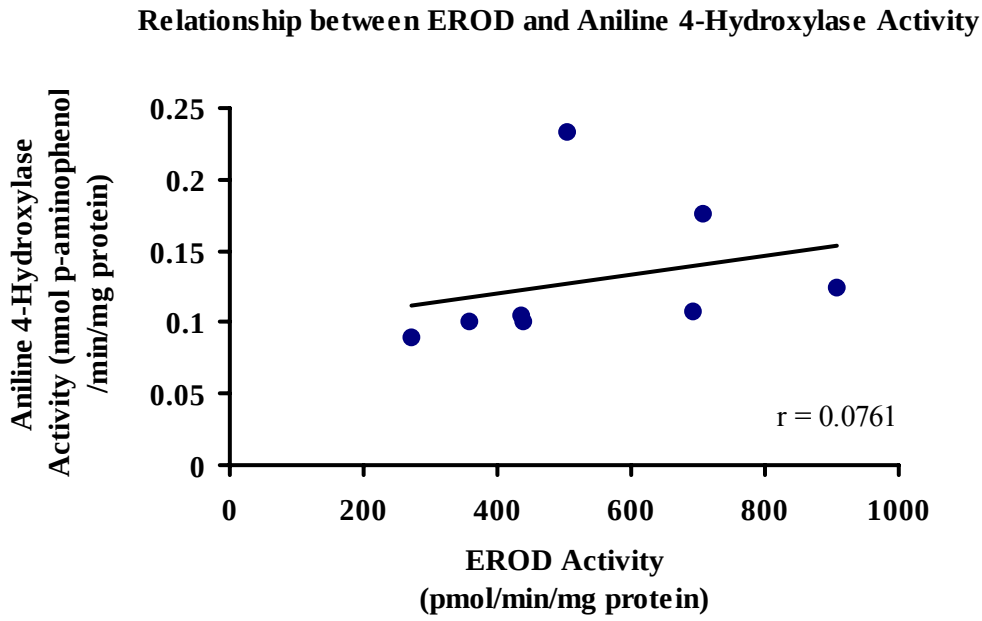
The average specific activity for aniline 4-hydroxylase reaction in liver microsomes of annular seabream was found as 0.128±0.04 (n=9) nmol *p*-aminophenol formed/min/mg protein. This was very low when compared with other species but it was very similar to the specific activity of leaping mullet (see Table 3.38). As in the case of benzphetamine and ethylmorphine N-demethylation reactions aniline 4-hydroxylase activity also did not exhibit a pollution-related trend in annular seabream caught from contaminated and uncontaminated sites. Figure 4.6 shows that there is no correlation between EROD and aniline 4-hydroxylase activity.

NADPH-cytochrome P450 reductase activity was also determined in annular seabream liver microsomes. The average specific activity was calculated as 155±45 (n=8) nmole cytochrome c reduced /min/mg protein. No significant difference was observed in NADPH-cytochrome P450 reductase activity between fish sampled from polluted sites and relatively uncontaminated sites. Although some studies reported a significant increases in hepatic NADPH-cytochrome P450 reductase activity in salmon, bream carp, eel, sunfish and grayling from polluted environment (Seppa Lindstrom and Oikari 1991; Curtis *et al*., 1993; Van der Oost *et al* 1991b; Monod *et al.*, 1988), in most of the cases no significant differences could be observed between fish from control and polluted sites.


Relationship between EROD and MROD Activity
MROD Activity
(pmol/min/mg protein)
900
800
700
600
500
400
300
200
100
0
0
500
1000
1500
EROD Activity
(pmol/min/mg protein)
r = 0.9391


Relationship between EROD and Benzphetamine N-demethylase Activity
BeND Activity
(nmol formaldehyde
/min/mg protein)
2.5
2
1.5
1
0.5
0
0
500
1000
1500
EROD Activity
(pmol/min/mg protein)
r = 0.4818


Relationship between EROD and Ethylmorphine N-demethylase Activity
Ethylmorphine N-demethylation Activity (nmolformaldehyde /min/mg protein)
1.2
1
0.8
0.6
0.4
0.2
0
0
500
1000
1500
r = 0.0212
EROD Activity (pmol/min/mg protein)


Relationship between EROD and Aniline 4-Hydroxylase Activity
Aniline 4-Hydroxylase Activity (nmol p-aminophenol /min/mg protein)
0.25
0.2
0.15
0.1
0.05
0
0
200
400
600
800
1000
r = 0.0761
EROD Activity (pmol/min/mg protein)

In this study changes in the P4501A1 protein level from leaping mullet, common sole, and annular seabream liver microsomes were determined to monitor the pollutant based induction at the protein levels by Western blotting. For analysis, polyclonal anti-mullet IgG prepared from pure mullet P4501A1 (Sen and Arınç, 1999) was used. Polyclonal anti-mullet IgG showed a cross reactivity with liver microsomes of three fish species used in present study.

As can be seen from Figure 3.5 the band intensities of leaping mullet sampled from site 1-Pasaport and site 1C-Harbor region (Lane 5-10) were much higher than the band intensities of samples from site 11A-Zeytinalanı and site 10-Outer Bay. These results showing the induction of cytochrome P4501A1 protein in leaping mullet liver microsomes from the Inner Bay was most probably due to the aquatic pollution of PAH and PCB type. In addition, mullet that had the highest EROD activity (3198 pmol/min/mg protein) displayed also highest cross reactivity (1374 R.P.A./mg microsomal protein: the degree of cross reactivity was expressed as arbitrary unit) with anti- mullet P4501A1 protein. This means that hepatic microsomal EROD activity and CYP1A1 protein level in leaping mullet were highly correlated (Figure 4.7 shows the correlation r=0.75, n=8) supporting the role of CYP1A as primary catalyst of EROD activity. Only in one case this correlation was poor in leaping mullet liver microsomes. Although sample in lane 5 and 6 (same sample was loaded in both lane), had highly elevated P4501A1 protein levels, the EROD activities of this samples were only moderately increased. The reason for this may be the inhibition of catalytic activity by the presence of inhibitor such as metal ions or benzene or catalytic sites of the enzyme may be damaged during preparation or storage.

In previous study (Arınç and Şen, 1999; Arınç *et al*., 2001) obtained similar results that polyclonal antibody showed strong cross reactivity with liver microsomes of mullet from Pasaport indicating the highly induced P4501A1 as a result of presence of PAH and /or PCB type pollutants.

In the present study, as can be seen in Figure 3.6 the band intensities of common sole captured from site 3- İnciralti and site 5-mouth of Gediz were much higher than the band intensities of samples from site 10A (Foça open sites) and site 16A (reference site). This indicates that sediment from İnciralti and Gediz were highly contaminated with carcinogenic CYP1A1 inducing chemicals. As in the case of leaping mullet, the EROD activities and CYP1A1 protein level (expressed as arbitrary unit R.P.A/ mg microsomal protein) of common sole were also well correlated (Figure 4.8 shows the correlation r=0.94, n=7).

In annular seabream the band intensities of samples caught from site 1- Pasaport region were significantly higher than the band intensities of samples from site 11A (see Figure 3.7). There was a good correlation in EROD activities and CYP1A1 protein level of annular seabream (Figure 4.9 shows the correlations r=0.86, n=6). The results obtained for annular seabream clearly indicated that feral fish from polluted sites had both highly induced EROD activity and cytochrome P4501A1 content.

Besides enzymatic and immunochemical markers for the assessment of PAH and PCB type pollutants chemical analysis were also carried out. The total PAH concentration was determined in sediment samples collected from three different sites of the Bay by using a spectrofluorometric method described by UNEP 1986. The maximum total PAH concentrations were found as 1.91 μg/g wet weights in the surface sediment samples collected from Harbor Region. Total PAH concentrations were moderate (0.94 μg/g wet weight) in surface sediments collected from site 3- Inciralti and were lowest (0.22 μg/g wet weight) in sample from Site 10- Outer Bay. These results were in accordance with the CYP1A induction pattern observed at EROD activity and CYP1A1 protein level in three fish species. However total PAH concentration obtained in present study were low when compared with the results of Aksu *et al.*(1998). In this report total PAH concentration from surface sediment samples were determined by using GC-MC method. It was found that total PAH concentration in the Inner Bay was 9.27 μg/g and decreasing to 1.25 μg/g at the Middle Bay (around the Yenikale entrance) and it was 0.42 μg/g in Outer Bay. The

fluorometric technique described by UNEP (1986) gives only approximate results in terms of chrysene equivalents and can not be as sensitive as HPLC and GC techniques but it is easy to employ and provides rapid results.

The present study clearly demonstrated that fish species inhabiting the Izmir Bay especially the Inner regions, are exposed to PAH, PCB and dioxin type toxic organic chemicals that certainly have negative consequences for their fitness and reproduction. Some of these compounds such as B(a)P displayed carcinogenicity through metabolic activation by CYP1A. Some of them are decreased the reproductive capability of fish species that may be considered as the most damaging effects of the persistent pollutants. Spies *et al*. (1988) investigated the relationship between impairment of the reproductive success of starry flounder from Pacific coast of North America and environmental pollution. It was found that CYP1A activity was inversely related to egg viability, the fertilization success and the successful development from fertilization through hatching. In another study it was shown that the induction of CYP1A by xenobiotics resulted in decreased levels of sex steroids, estradiol and testosterone (Munkittrick, 1992, 1994 and 1998). Jhonsen *et al*. (1998), found that female English sole from contaminated areas had depressed levels of plasma estradiol and showed reproductive impairment and elevated arylhydrocarbon hydroxylase activity.

Although, İzmir Great Canal Project has been active since 2000 to prevent the direct discharges of domestic and industrial wastes water into the Bay, the results of present study demonstrated that the level of PAH, PCB and dioxin type persistent organic chemicals are still very high, especially in the Inner Bay. This has implications for the health status and reproduction success of fish inhabiting contaminated areas as well as for the health status of human consumers of such organisms.

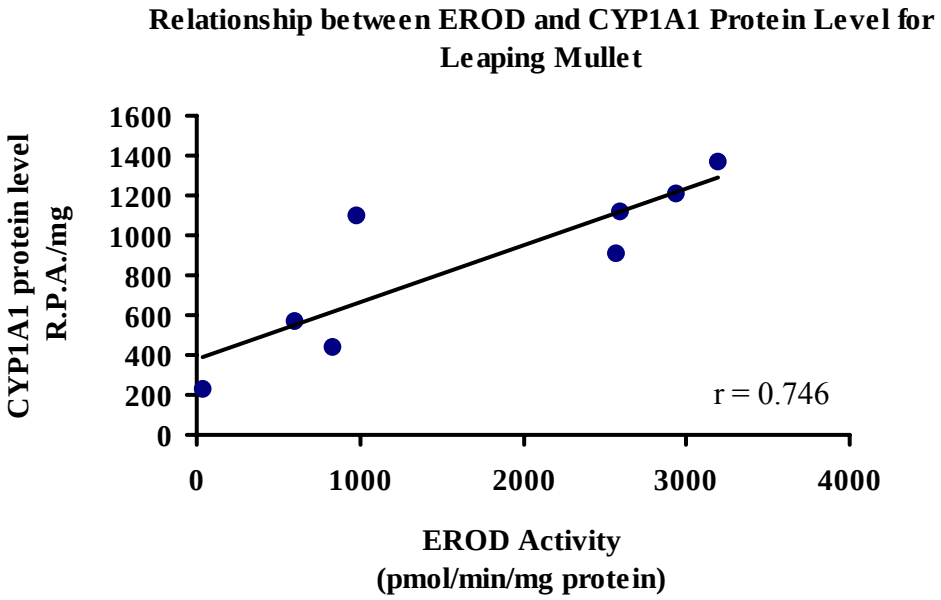


Figure 4.7 Relationship between EROD activity and CYP1A1 protein level (expressed as arbitrary unit; R.P.A. / mg) in leaping mullet liver microsomes n= 8, r = 0.746

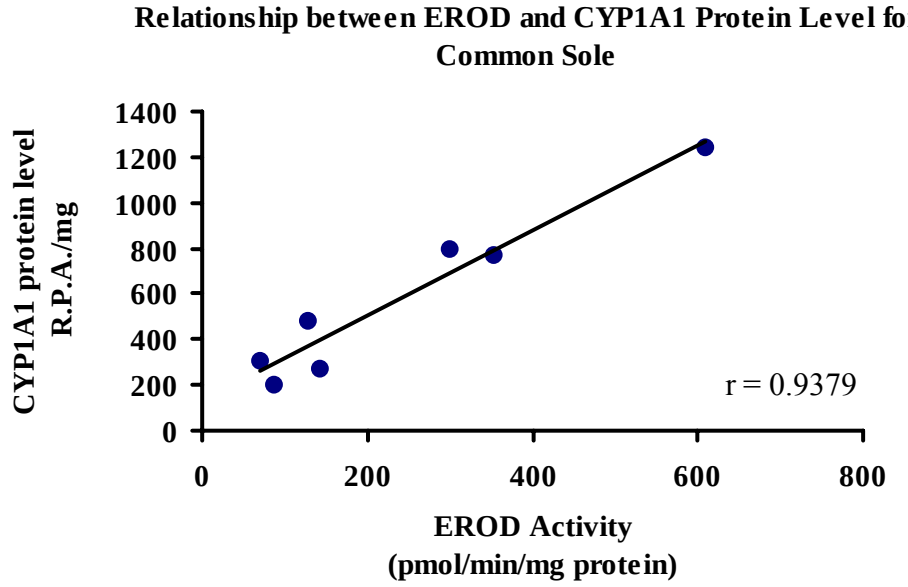


Figure 4.8 Relationship between EROD activity and CYP1A1 protein level (expressed as arbitrary unit; R.P.A. / mg) in common sole liver microsomes n= 7, r = 0.9379

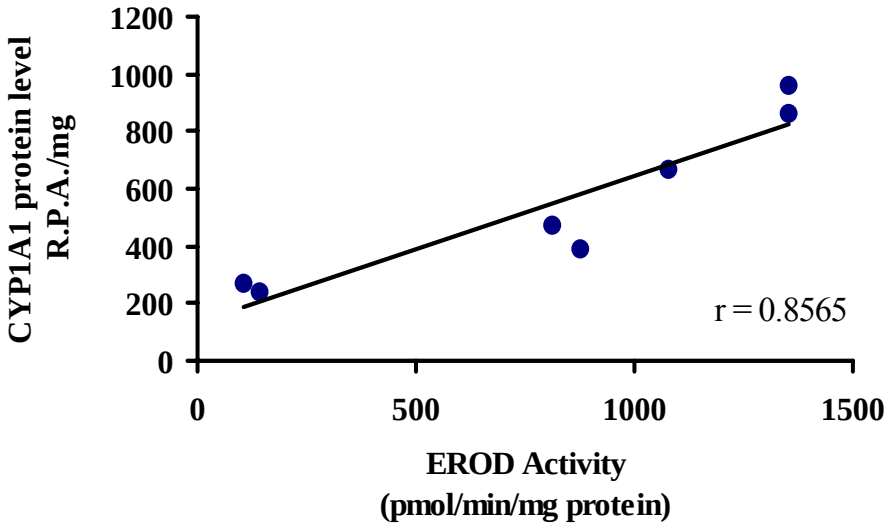


Figure 4.9 Relationship between EROD activity and CYP1A1 protein level (expressed as arbitrary unit; R.P.A. / mg) in annular seabream liver microsomes n= 7, r = 0.8565

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APPENDIX

Van Veen Grab Sampler :