BIOMONITORING OF TOXIC ORGANIC POLLUTANTS IN MULLET LIVER MICROSOMES BY CYP1A LEVEL AND 7- ETHOXYRESORUFIN O-DEETHYLASE (EROD) ACTIVITY IN THE WEST BLACK SEA REGION OF TURKEY

by
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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality as a thesis for the degree of Master of Science.

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ABSTRACT

BIOMONITORING OF TOXIC ORGANIC POLLUTANTS IN MULLET LIVER MICROSOMES BY CYP1A LEVEL AND 7- ETHOXYRESORUFIN O-DEETHYLASE (EROD) ACTIVITY IN THE WEST BLACK SEA REGION OF TURKEY

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The Black Sea has been increasingly threatened by chemical pollutants over the past decades as a result of industrial, anthropogenic, and agricultural activities. Industrial areas, rivers and coastal cities have tendency to create local pollution in the Black Sea. In this study, the extent of PAHs, PCBs and dioxins type pollution was determined and monitored in Zonguldak Harbour, Ereğli Harbour, Sakarya River Mouth, Melen and Gülüç Stream Mouths and Amasra by measuring CYP1A associated 7-ethoxyresorufin O-deethylase (EROD) activity and immunochemical detection of CYP1A protein level in mullet liver microsomes in August 2005, 2006 and 2007. The lowest EROD activities were measured in mullet samples caught from Amasra. This region was considered as reference site. Mullets collected from

Zonguldak city harbour, a highly urbanized and industrial city in the West Black Sea Region of Turkey, had about 6-9 times higher EROD activities than those obtained from reference site, Amasra. The highly elevated EROD activities were also detected in mullets captured from Ereğli Harbour which were about 7-9 times higher with respect to the value obtained from Amasra. Gülüç Stream was the other highly polluted station in this study. EROD activities of mullet samples caught from this station were about 6 times higher than Amasra. EROD activities of mullets caught from Melen Stream Mouth were about 2-2.5 times higher than those obtained from Amasra. EROD activities in mullets sampled from Sakarya River Mouth were 2-4 times higher than those caught from Amasra. CYP1A protein levels were also determined in mullet liver microsomes by immunochemical analysis. CYP1A protein level measurements were highly correlated with EROD activity measurements. Besides these biochemical parameters, total PAH concentrations were measured in fish liver tissues. Total PAH concentrations of mullets caught from Zonguldak Harbour, Ereğli Harbour, Sakarya River Mouth, Gülüç and Melen Stream Mouths were higher than those obtained from reference site, Amasra.

In this study, the induced CYP1A protein level and associated EROD activities, and high total PAH concentrations in mullet livers showed the presence of PAHs, PCBs and dioxins type organic pollutants in Zonguldak Harbour, Ereğli Harbour, Sakarya River, Melen Stream and Gülüç Stream. These results indicate that these sites have tendency to create local pollution in the West Black Sea Region of Turkey.

Keywords: Black Sea, CYP1A, EROD, Mullet, PAH, Pollution.

ÖZET

TÜRKİYE'NİN BATI KARADENİZ BÖLGESİ'NDE, KEFAL BALIĞI KARACİĞER MİKROZOMUNDA, CYP1A SEVİYESİ VE 7-ETOKSİREZORUFİN O-DEETİLAZ (EROD) AKTİVİTESİ İLE TOKSİK ORGANİK KİRLETİCİLERİN İZLENMESİ

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Karadeniz, son yıllarda endüstriyel, evsel ve tarımsal aktiviteler sonucunda ortaya çıkan kimyasal kirleticilerin tehdidi altındadır. Endüstriyel alanlar, nehirler ve kıyı şehirleri Karadeniz'de yerel kirlilik yaratma eğilimindedir. Bu çalışmada, PAH, PCB ve dioksin türü kirliliğin miktarı Zonguldak Limanı, Ereğli Limanı, Sakarya Nehir Ağzı, Melen ve Gülüç Dereleri Ağzı ve Amasra'da, Ağustos 2005, 2006 ve 2007'de, kefal balığı karaciğer mikrozomlarında CYP1A proteinine bağlı 7-etoksirezorufin O-deetilaz (EROD) aktivitesi ölçülerek ve CYP1A protein miktarı immünokimyasal yöntemler ile belirlenerek tayin edildi ve izlendi. En düşük EROD aktivitesi Amasra'dan yakalanan kefal balığı örneklerinde ölçüldü. Bu bölge referans bölgesi olarak düşünüldü. Türkiye'nin Batı Karadeniz Bölgesi'nde bulunan,

kalabalık nüfusa ve endüstriyel alanlara sahip olan Zonguldak ilinin limanından yakalanan kefal balıklarında Amasra'dan 6-9 kat fazla EROD aktivitesi bulundu. Ereğli Limanı'ndan yakalanan kefal balıklarında da Amasra'dan yakalanan balıklardan 7-9 kat fazla EROD aktivitesi bulundu. Gülüç Deresi bu çalışmadaki oldukça kirli diğer bir istasyondu. Bu istasyondan yakalanan kefal balıklarının EROD aktiviteleri Amasra'dan yakalanan balıkların EROD aktivitelerinden yaklaşık 6 kat fazla bulundu. Melen Deresi Ağzı'ndan yakalanan kefal balıklarının EROD aktiviteleri Amasra'dan 2-2.5 kat daha fazla bulundu. Sakarya Nehir Ağzı'ndan yakalanan kefal balıklarının EROD aktiviteleri Amasra'dan yakalanan balıkların 2-4 katı bulundu. Kefal balığı karaciğer mikrozomlarında CYP1A protein seviyesi immünokimyasal yöntemler ile belirlendi. Elde edilen sonuçlarda CYP1A protein seviyesi ölçümleri, EROD aktivite ölçümleri ile oldukça bağlantılı bulundu. Bu biyokimyasal yöntemlerin yanı sıra, balık karaciğer dokularında toplam PAH konsantrasyonu ölçüldü. Zonguldak Limanı, Ereğli Limanı, Sakarya Nehir Ağzı, Melen ve Gülüç Dereleri Ağzı'ndan yakalanan kefal balıklarının toplam PAH konsantrasyonu referans istasyonu, Amasra'dan daha yüksek bulundu.

Bu çalışmada, kefal balığı karaciğerlerinde bulunan indüklenmiş CYP1A protein seviyeleri ve CYP1A proteinine bağlı EROD aktiviteleri ve yüksek toplam PAH seviyeleri, PAH, PCB ve dioksin türü organik kirleticilerin Zonguldak Limanı, Ereğli Limanı, Sakarya Nehri, Melen Deresi ve Gülüç Deresi'nde var olduğunu gösterdi. Bu sonuçlar, bu bölgelerin Türkiye'nin Batı Karadeniz Bölgesi'nde yerel kirlilik yaratma eğiliminde olduğunu göstermektedir.

Anahtar Kelimeler: Karadeniz, CYP1A, EROD, Kefal Balığı, PAH, Kirlilik.

DEDICATED TO MY FAMILY

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
ε-ACA	ε-Amino caproic acid
Arnt	Aromatic hydrocarbon receptor nuclear translocator
AhR	Aromatic hydrocarbon receptor
AHH	Aryl hydrocarbon hydroxylase
BCIP	5-bromo-4-chloro-3-indolylphosphate
BNF	β-naphthoflavone
BPH	Benzo[*a*]pyrene hydroxylase
BPDE	Benzo[*a*]pyrene 7,8 dihydrodiol 9,10-epoxide
BSA	Bovine serum albumin
BSEP	Black Sea Environmental Programme
CAT	Catalase
CO	Carbon monoxide
CYP	Cytochrome P450
DDD	Dichlorodiphenyltrichloroethane
DDE	Dichlorodiphenyltrichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEA	Diethanolamine
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EROD	7-Ethoxyresorufin O-deethylase
FAD	Flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FMN	Flavin mononucleotide
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid
H_2O_2	Hydrogen peroxide
HSP90	Heat shock protein of 90 kDa
MFO	Mixed-Function oxidase
MO	Monooxygenases
MTs	Metallothionenins

NADH	Nicotinamide adenine dinucleotide reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NBT	Nitro blue tetrazolium chloride
OP	Organophosphate
PAH	Polyaromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
PMS	Phenazine methosulfate
PMSF	Phenylmethanesulfonyl fluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBST	Tris Buffered Saline plus Tween 20
TEMED	N, N, N', N' tetrametylethylene diamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	Uridine diphosphate
UNEP	United Nations Environment Programme
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
Tween 20	Polyxyethylene sorbitan monolaurate
XAP2	X-associated protein 2
XRE	Xenobiotic responsive element

INTRODUCTION

The number of chemicals produced and released into the environment has been continuously increasing in industrialized countries. The ultimate sink for many of the chemical pollutants is the marine environment (Stegeman and Hahn, 1994). The marine environment can neutralize some chemicals. However, many of the chemicals introduced into the environment 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.). Contaminating chemicals (pollutants) in the aquatic environment can be harmful not only by their direct effects on the organisms but also indirectly producing some diseases and developmental abnormalities in humans living in these polluted environments. Therefore it is very important to monitor the levels of xenobiotics (foreign chemicals) and their effects on marine organisms.

1.1. Bioaccumulation, Bioconcentration and Biomagnification

Xenobiotics enter marine environment in a variety of ways. Uptake of them by marine organisms occurs from the sediment, water-column and other biota, and increases with increasing bioavailability/lipophilicity/hydrophobicity and concentration of chemicals. Since some of xenobiotics are not readily degraded by

physical, chemical or biological processes, they persist in the environment. **Bioaccumulation** can be defined as the tendency of substances to accumulate in the body of exposed organism with over time or with age. Bioaccumulation occurs when an organism absorbs a toxic substance at a rate greater than that at which the substance is eliminated. The degree of accumulation depends on the level of exposure and the mechanisms by which the organism expels, stores, or metabolically inactivates these chemicals (Wright and Welbourn, 2002). Since the chemicals must pass through the lipid bilayer of membranes to enter the body, bioaccumulation potential of them is directly proportional to lipid solubility (lipophilicity). In general, persistent chemicals have low water solubility, high lipid solubility and high molecular masses (Leblanc, 2004). So they have high bioaccumulation potential.

The phenomenon whereby a living organism contains higher concentrations of a given substance than the concentration in its immediate source of that substance is known as **bioconcentration** (Wright and Welbourn, 2002).

Chemicals can also be transferred along food chains from prey organism to predator (trophic transfer). **Biomagnification**, also known as bioamplification or biological magnification, is the increase in concentration of a substance that occurs in a food chain as a consequence of food chain energetics. Figure 1.1 illustrates biomagnification. Organisms at lower trophic levels accumulate small amounts of xenobiotics; when eaten by organisms at the next higher levels, they accumulate larger amounts of pollutants. At the highest trophic levels, the increased concentrations of xenobiotics in tissues may become toxic (Leblanc, 2004).

Bioconcentration and bioaccumulation occur within an organism, and biomagnification occurs across trophic (food chain) levels therefore biomagnification is an important concept in ecology, environmental science, and ecotoxicology.

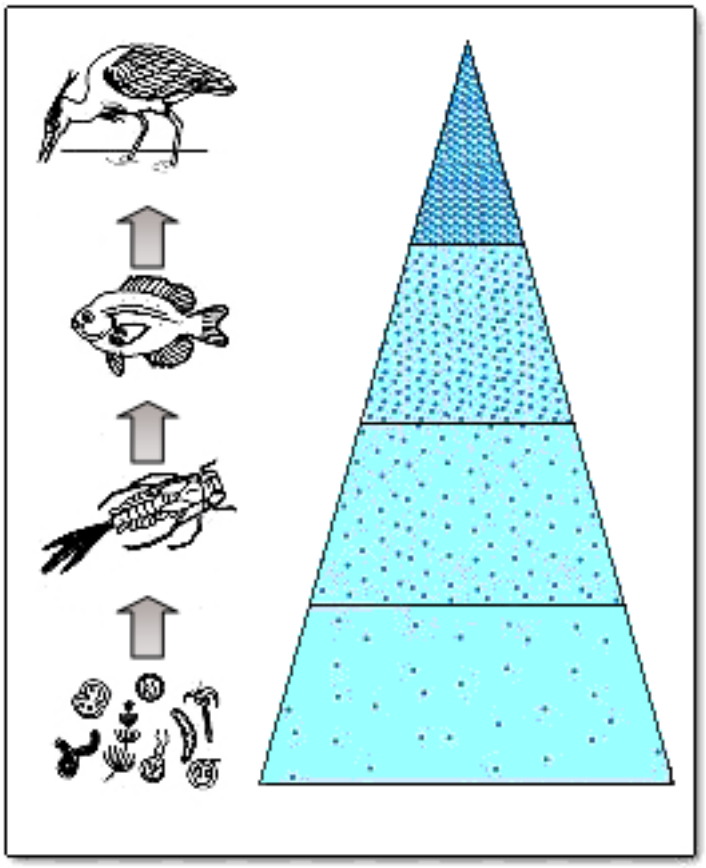


Figure 1.1. Biomagnification (modified from Wright and Welbourn, 2002).

1.2. Biomarkers

Many biochemical techniques have been developed to examine and monitor environmental pollution. 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 chemicals is called biomarker

(biochemical markers) (Peakall, 1994; Van der Oost et al., 2003). Even though chemical analyses are able to measure a wide range of pollutants quantitatively and accurately, the complex mixture of chemical pollutants cannot be fully assessed. Furthermore, it does not reveal the impact of chemical pollution on the aquatic environment. The use of biochemical markers fulfills this purpose (Arınç et al., 2000).

Xenobiotics may cause changes at all levels of biological organizations. Table 1.1 illustrates the biomarkers at different levels of organization. Biological markers can be classified in three categories as biomarkers of exposure, biomarkers of effects, or biomarkers of susceptibility (WHO, 1993; Van der Oost et al., 2003). Biomarker of exposure covers an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism (WHO, 1993; Van der Oost et al., 2003). Table 1.2 summaries the major biomarkers of exposure. The measurement of aniline or its *p*-aminophenol metabolite in blood or urine may be given as an example to a biomarker of human exposure to aniline (Figure 1.2) (Manahan, 2003).

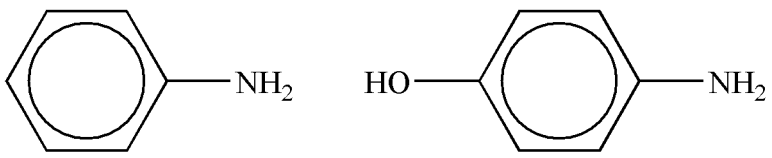


Figure 1.2. Chemical structure of aniline and its *p*-aminophenol metabolite.

Table 1.1. Biomarkers at different levels of organization (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
Individuals	Body Condition Index Fertility Maturation retardation
Population	Gene frequency Age structure Size distribution
Ecosystem	Diversity indices Functional parameters

Biomarker of effect comprises a measurable biochemical, physiological, behavioral or other alteration within an organism that, depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease (WHO, 1993; Van der Oost et al., 2003). In addition to measuring aniline or its *p*-aminophenol metabolite in blood or urine, exposure to aniline can also be measured by its production of blood methemoglobin (a product of hemoglobin useless for carrying oxygen in blood in which the iron(II) in hemoglobin has been oxidized to iron(III)) (Manahan, 2003).

Biomarker of susceptibility is an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance (WHO, 1993; Van der Oost et al., 2003). The most obvious biomarkers of susceptibility are those associated with weakened immune systems, which may make organisms more defenseless to cancer, infectious diseases, or parasites (Manahan, 2003).

Table 1.2. Summary of major biomarkers of exposure (modified from McCarthy and Shugart, 1990; Benson and DiGiulio, 1992; Peakall, 1992; Bucheli and Fent, 1995; Mitchelmore and Chipman, 1998; Arınç et al., 2000; Hyne and Maher, 2003; Van der Oost et al., 2003).

Biomarker	Tissue	Use
CYP1A	Liver	Indicator of exposure to organic chemicals such as PAHs and PCBs
Glutathione S-transferases	Liver	Indicator of exposure to pesticides and metalloids
Cellulase/carbohydrase	Stomach	Indicator of exposure to pesticides
Acetylcholinesterase	Brain	Indicator of exposure to organophosphorus or carbamate pesticides
Carboxylesterase	Various	Indicator of exposure to pyrethroid and carbamate pesticides
DNA strand breakage, adduct formation, chromatid exchange	Various	Indicator of exposure to alkylating or arylating agents and oxidative stress
Aminolevulinic acid dehydratase	Blood	Indicator of exposure to lead
Metallothionein	Various	Indicator of exposure to metals
Retinoids	Liver	Indicator of exposure to dioxin and furans
Adenylate energy change, ATP/ADP ratio	Various	Indicator of exposure to stress
Stress proteins	Various	Indicator of cells experiencing stress
Glutathione	Liver	Indicator of oxidative stress

1.2.1. Fish Biomarkers

Biochemical markers have been extensively used in aquatic pollution assessment programs. Fish plays an increasingly important role in these studies. Many studies demonstrated that it responds to changes in the aquatic environment with great sensitivity (Klontz, 1995; Bresler et al., 1999; Arınç et al., 2000; Flammarion et al., 2002). Various biochemical parameters in fish have been tested for their responses to toxic substances and their potential use as biomarkers of exposure or effect. In fish, most extensively investigated biomarkers are oxidative stress and antioxidant enzymes, metallothioneins, DNA adducts, acetylcholinesterase (AChE) and biotransformation enzymes (enzymes involved in the detoxication of xenobiotics and their metabolites).

1.2.1.1. The Oxidative Stress and Antioxidant Enzymes

Oxidative stress can be defined as the production and accumulation of reduced oxygen intermediates such as superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals (Lesser, 2006). The reactive oxygen species (ROS) are generated by either the endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria or the detoxification reactions involving the liver cytochrome P-450 enzyme system or exogenous sources include exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to ionizing radiation, and bacterial, fungal or viral infections. Table 1.3 shows some examples of reactive oxygen species and oxygen centered

radicals of biological intrest. The reactive oxygen species may produce damage when they react with important cellular components such as DNA, or the cell membrane. ROS such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical ($^{\bullet}OH^{-}$) can produce lipid peroxidation, enzyme inactivation and DNA adducts (Davies, 1995; Jifa et al., 2006). If this occurs, cells function may decrease or cells may die.

Table 1.3. Reactive oxygen species and oxygen centered radicals of biological interest (taken from Lackner, 1998).

Compound	Source
Ozone (O_3)	Electric discharges and radiation, disinfection
Singlet oxygen ($^1O_2$; also $^1\Delta_g O_2$, O_2^*)	Excited state of oxygen, 22 kcal/mol above ground state, radiation, nonenzymatic dismutation of $O_2^{\cdot -}$ and H_2O_2
Superoxide anion radical ($O_2^{\cdot -}$; also protonated as $HO_2^{\cdot}$)	One-electron reduction product of oxygen, O_2
Hydrogen peroxide (H_2O_2)	Dismutation of $O_2^{\cdot -}$
Hydroxyl radical ($OH^{\cdot}$; also $HO^{\cdot}$)	Fenton reaction, metal (iron)-catalysed Haber-Weiss reaction out of $O_2^{\cdot -}$ and H_2O_2
Alkoxy radical ($RO^{\cdot}$)	Lipid peroxidation
Peroxy radical ($ROO^{\cdot}$)	Lipid peroxidation
Organic hydroperoxide (ROOH)	Lipid peroxidation
Excited carbonyl (^3RO; also RO^*)	Excited state of organic compound, may also be produced by $^1O_2$

Antioxidants help prevent cellular damage caused by metabolically and environmentally produced reactive oxygen species (ROS) (Speers-Roesch and Ballantyne, 2005). Antioxidants can be enzymes such as superoxide dismutase (SOD), which catalyzes the dismutation of O_2^- to water and oxygen, catalase (CAT),

which reduces H_2O_2 to water and oxygen, and glutathione reductase (GR), which regenerates reduced glutathione (GSH) used as a direct scavenger of ROS or as a substrate for the antioxidant enzyme glutathione peroxidase (GPX), which catalyzes the reduction of H_2O_2 derived from oxidative metabolism as well as peroxides from oxidation of lipids, and non-enzymatic compounds such as reduced glutathione (GSH) and oxidized glutathione (GSSG) or molecules such as vitamins E and C, beta carotene (Wilhelm Filho et al., 2001; Speers-Roesch and Ballantyne, 2005; Trenzado et al., 2006).

1.2.1.2. The Metallothioneins

Metallothioneins (MTs) is a family of cysteine-rich, low molecular weight proteins. They have a capacity to bind both endogenous (Zn, Cu, Se etc.) and exogenous (Cd, Hg, Ag etc.) heavy metals through the thiol group of their cysteine residues, which represents nearly the 30% of their amino acidic residues. Metallothioneins play important roles in intracellular regulation of the essential metals and detoxification of excess levels of these and pollutant metals. The induction of metallothioneins in response to increased concentrations of metal ions forms the basis of its use as a biomarker for metal exposure (Livingstone, 1993).

1.2.1.3. Acetylcholinesterase

Many organophosphate (OP) and carbamate pesticides and certain heavy metals are inhibitors of acetylcholinesterase (AChE) which terminates the transmission of nerve impulses in cholinergic synapses by hydrolyzing the

neurotransmitter acetylcholine to acetic acid and choline (Sanchez-Hernandez et al., 1998; Kraut et al., 2000). Monitoring studies of neurotoxic chemicals are mostly based on the inhibition of AChE activity. Several studies on AChE levels in mussels and fish have confirmed the potential use of AChE as biomarker of organic pollution (Kabeer Ahammad Sahib et al., 1980; Kopecka et al., 2004).

1.2.1.4. The DNA Adducts

Many organic xenobiotics are metabolically activated to electrophilic metabolites that bind to nucleic acids and proteins and form covalent adducts (Livingstone, 1991; Østby and Krøkje, 2002). The covalent modification of DNA bases by chemicals can alter the structure of DNA. The presence of adducts may cause crucial mutations and stimulate a cascade of subsequent events leading to cell death, uncontrolled cell replication, and other cell dysfunctions through DNA replication or erroneous repair (Venier, 2001; Pisoni et al., 2004). DNA adduct formation can be used as a biomarker of organic contaminant exposure for aquatic organisms. An increase in the levels of DNA adducts was observed in either field studies with different aquatic organisms such as flounder (*Platichthys flesus*) (Lyons et al., 2004), eelpout (*Zoarces viviparus*) (Frenzilli et al., 2004) and mussel (*Mytilus galloprovincialis*) (Pisoni et al., 2004) or laboratory studies on various fish species such as flounder (*Platichthys flesus*) experimentally exposed to benzo[*a*]pyrene (Malmström et al., 2000) and Atlantic cod (*Gadus morhua*) chronically exposed to crude oil (Aas et al.,2000).

1.2.1.5. The Biotransformation Enzymes

Biotransformation can be defined as an enzyme-catalyzed conversion of a xenobiotic compound into a more water-soluble form, which can be excreted from the body more easily than the parent compound (Lech and Vodicnik, 1985; Van der Oost et al., 2003). Biotransformation of xenobiotics occurs in two stages. In Phase I, relatively insoluble organic chemicals are catalyzed through oxidation, reduction and hydrolysis. Phase II involves conjugation of the chemical or its metabolites with polar compounds such as glucuronic acid, glutathione, sugars, sulfates and phosphates to form water-soluble compounds which are readily excreted.

In mammalian and some nonmammalian groups, cytochrome P450-dependent mixed function oxidase system (monooxygenases, MFO) catalyzes most of the oxidative biotransformation of xenobiotics. The cytochrome P450 (CYP)-dependent monooxygenase system is a membrane-bound, multi-component, electron transport system in which the monooxygenation of a wide range of xenobiotics such as organochlorins, dioxins and aromatic hydrocarbons and endogenous molecules such as prostaglandins, steroids and fatty acids are catalyzed (Lu and Levin, 1974; Arınç and Philpot, 1976; Gonzalez, 1988; Guengerich, 1990; Arınç et al., 2005; Sole´ and Livingstone, 2005; Arınç et al., 2007).

Glutathione S-transferases, sulfotransferases, UDP-glucuronyl transferases, N-acetyl transferases are the most important Phase II enzymes that have a role in biotransformation of xenobiotic compounds via conjugation reactions. Conjugations are addition type of reactions in which large and often polar chemical groups or

compounds such as sugars and amino acids are covalently added to xenobiotics. The majority of the phase II type enzymes catalyze these synthetic conjugation reactions, thus facilitating the excretion of chemicals by the addition of more polar groups to the molecule.

Some xenobiotic compounds possess the requisite functional groups (such as ---COOH, ---OH or ---NH_2) for direct metabolism by conjugative phase II enzyme systems, while others are metabolized by an integrated process involving prior action of the phase I enzymes. Phase II enzymes can play an important role in homeostasis as well as in detoxification and clearance of many xenobiotic compounds. Figure 1.3 summarizes biotransformation reactions of xenobiotics with several reaction examples.

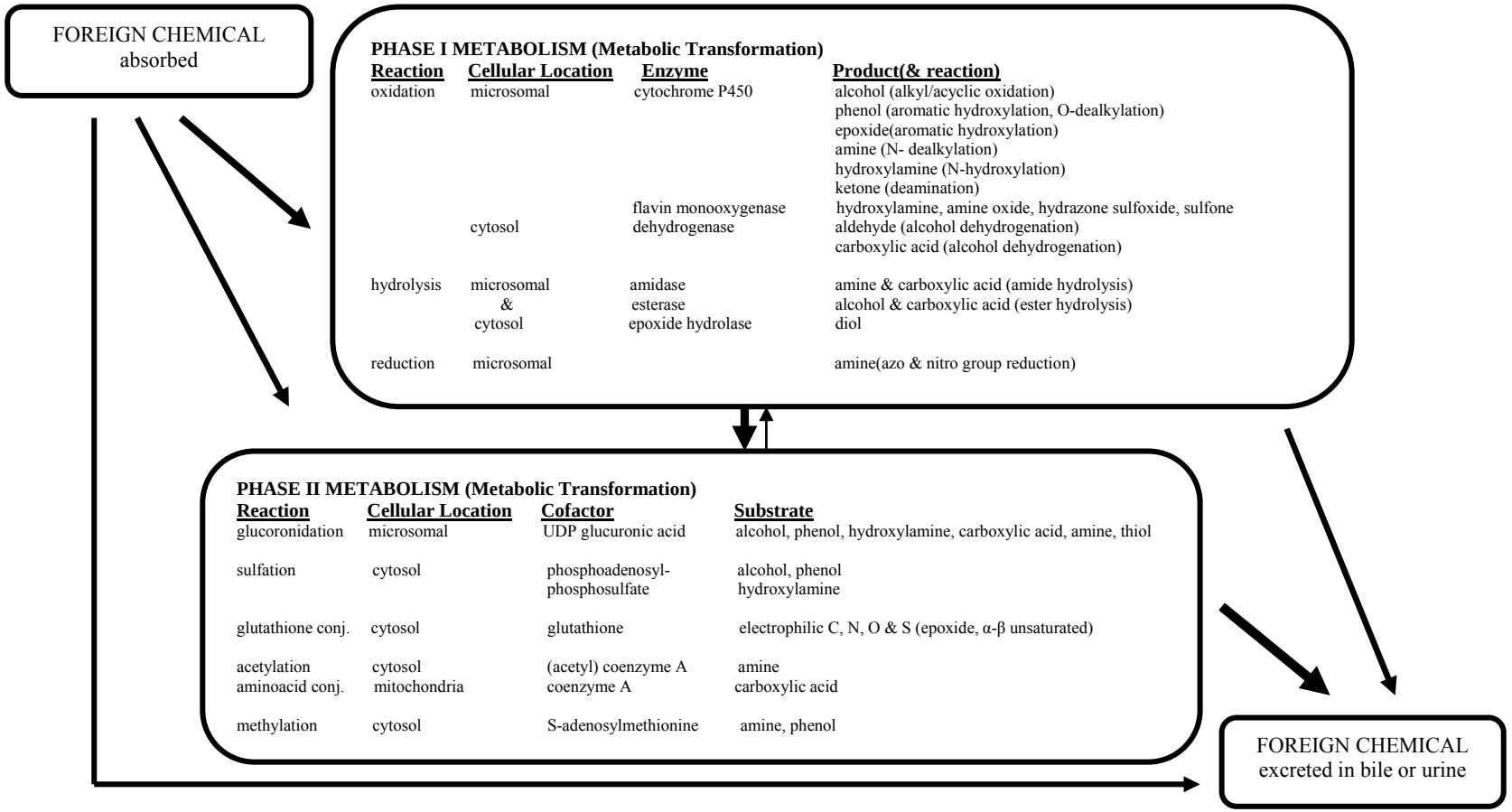


Figure 1.3. Biotransformation reactions of xenobiotics (taken from Franklin and Yost, 2000).

1.3. General Properties of Cytochrome P450 System

The cytochrome P450-dependent monooxygenases (MO) or mixed function oxidases (MFO) belong to a superfamily of heme-containing enzymes with more than 7700 distinct CYP sequences with 866 families (as of September 2007, Dr. Nelson's Cytochrome P450 homepage; http://drnelson.utmem.edu). At present, there are 2740 animals, 2675 plants, 813 bacteria, 1231 fungi and 244 lower eukaryotes for which CYP sequences were named. Cytochromes are present, at high levels, in the liver, accounting for 1 to 2% mass of hepatocytes (Lester et al. 1993; Lewis, 2001; Siroka and Drastichova, 2004) and also found in the intestine, kidney, lungs, brain, skin, prostate gland, placenta, etc. (Arınç and Philpot, 1976; Adalı and Arınç, 1990; Anzenbacherova and Anzenbacher, 2001; Arukwe, 2002; Ortiz-Delgado et al., 2002; Siroka and Drastichova, 2004; Arınç et al., 2005; Arınç et al., 2007).

The name cytochrome P450 is derived from the characteristic Soret peak formed by absorbance of light at wavelengths near 450 nm when the heme iron is reduced with sodium dithionate and complexed to carbon monoxide (Omura and Sato, 1964 a, b).

A system of nomenclature of all cytochrome P450 genes and proteins were proposed in 1987 by Nebert and coworkers. They proposed a numenclature based on the presence of common amino acid sequence. The accepted guidelines from nomenclature designate cytochrome P450 genes as CYP (or cyp in the case of mouse genes). The CYP designation is followed by an Arabic numeral to denote the gene family which share more than 40% amino acid sequence identity. The subfamily is

designated by a capital letter after the family designation and protein sequences within the same subfamily have greater than 55% amino acid sequence similarity in the case of mammalian proteins, or 46% similarity in the case of nonmammalian proteins (Figure 1.4). The individual isoforms is then identified using a second Arabic numeral following the subfamily designation. The name of the gene is italicized, whereas the protein (enzyme) is not (Rose and Hodgson, 2004).

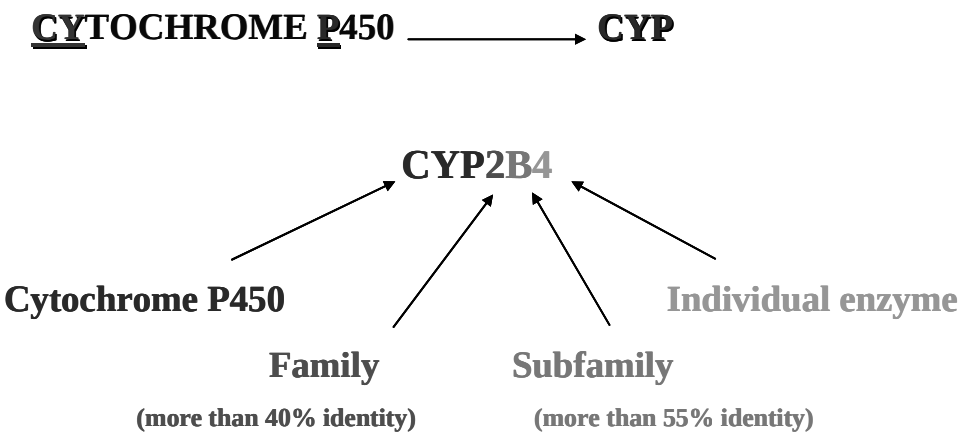


Figure 1.4. Nomenclature of cytochrome P450 gene.

1.4. Mechanisms and Reactions of Cytochrome P450 System

The cytochrome P450 system participates in a large number of different oxidation reactions, including hydroxylation, *O*-, *N*-, and *S*-dealkylation reactions, *N*-, *S*- and *P*-oxidation, dehalogenation, epoxidation, deamination etc. as a catalyst. Table1.4. shows the general reactions catalyzed by the cytochrome P450-dependent mixed function oxidase system.

Table 1.4. Reactions catalyzed by the cytochrome P450-dependent mixed function oxidase system (modified from Rose and Hodgson, 2004).

Reactions	**Examples**
Epoxidation/hydroxylation	Aldrin, benzo[*a*]pyrene, aflatoxin, bromobenzene
N-, *O*-, *S*- Dealkylation	Ethylmorphine, atrazine, *p* -nitroanisole, methylmercaptan
N-, *S*-, *P*- Oxidation	Thiobenzamide, chlorpromazine, 2-acetylaminofluorene
Desulfuration	Parathion, carbon disulfide
Dehalogenation	Carbon tetrachloride, chloroform
Nitro reduction	Nitrobenzene
Azo reduction	*O*- Aminoazotoluene

The common reaction catalyzed by cytochrome P450 is a monooxygenase reaction, in which one oxygen atom is incorporated into an organic substrate (RH) while the other oxygen atom is reduced to water (Figure 1.5).

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

Figure1.5. General reaction catalyzed by the cytochrome P450-dependent mixed function oxidase system.

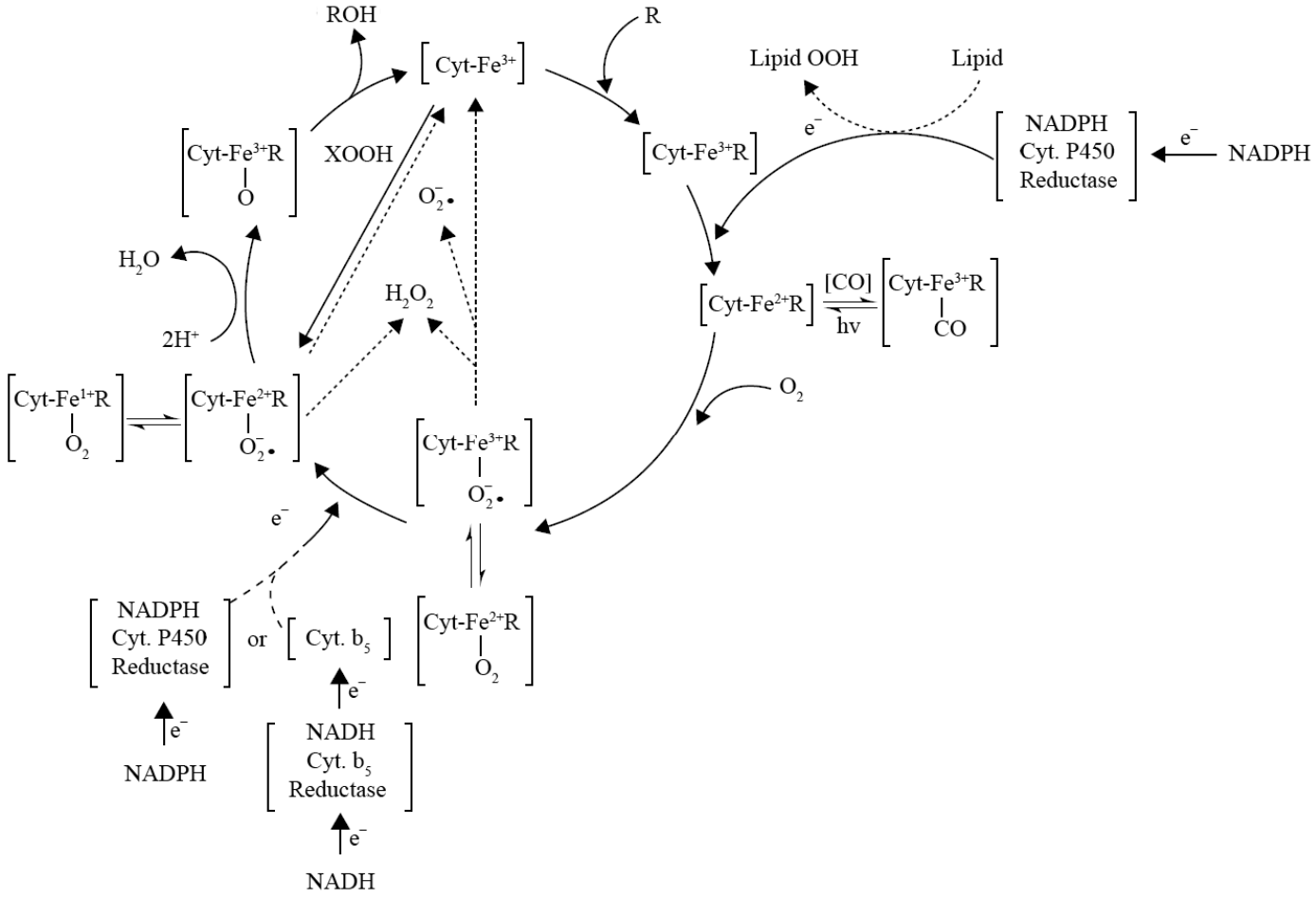


Figure 1.6. Generalized scheme showing the sequence of events for P450 monooxygenation (taken from Rose and Hodgson, 2004).

The whole reaction is more complex and takes place in the presence of NADPH reductase (which contains FAD and FMN prosthetic groups involved in transfer of electrons on cytochrome P450) and membrane phospholipids. Cytochrome *b5* can also serve as another source of electrons (Siroka and Drastichova, 2004). The reaction cycle of microsomal cytochrome P450 is shown in a scheme drawing above (Figure 1.6).

The initial step consists of the binding of the xenobiotic to the cytochrome at a substrate binding site on the protein and alters the conformation sufficiently to enable the efficient transfer of electrons to the heme from NADPH via a nearby flavoprotein, NADPH cytochrome P450 reductase. It is followed by one electron reduction catalyzed by NADPH-cytochrome P450 reductase to form a reduced cytochrome-substrate complex. This complex can interact with CO to form the CO-complex, which gives rise to the well-known difference spectrum with a peak at 450 nm and also inhibits monooxygenase activity. The reduction of the heme iron from its normal ferric state to the ferrous state allows a molecule of oxygen (O–O) to bind. The oxidation state of the oxygen and the iron in the ternary complex is not entirely clear, but the oxygen may exist as hydroperoxide (O_2^{2-}) or superoxide (O_2^{-})**.** The ternary complex of xenobiotic, cytochrome, and oxygen receives another electron, either through the same flavoprotein as before or through an alternative path involving a different flavoprotein in which the electron is first passed through cytochrome *b5*, another cytochrome present in the endoplasmic reticulum. This alternate pathway for the second electron can also use NADH as the pyridine nucleotide electron donor. The addition of the second electron to the ternary complex results to the rearrangement with insertion of one atom of oxygen into the substrate

to yield the product while the other atom of oxygen is reduced to water. Figure 1.7 illustrates the role of lipid fraction in the metabolic pathway of cytochrome P450.

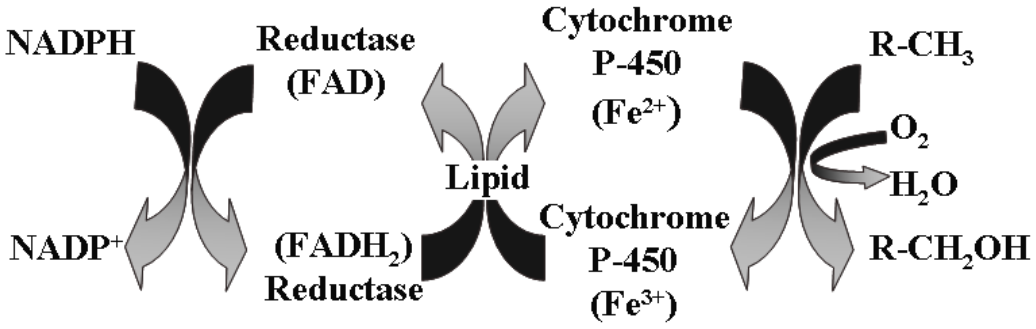


Figure 1.7. The role of lipid fraction in the metabolic pathway of cytochrome P450.

1.5. Cytochrome P450 Mixed Function Oxidase System in Fish

Most of enzymes responsible for biotransformation in mammals have also been found in fish and marine invertebrates. The mixed function oxidase system is one of these enzymes, whose terminal component cytochrome P450 (CYP) exists as a superfamily of proteins capable of oxidizing a wide variety of substrates in numerous aquatic species (Livingstone, 1998; Stegeman and Livingstone, 1998; Doyotte et al., 2001). Arınç et al. were the first to solubilize and isolate cytochrome P450 from a marine elasmobranch, i.e. little skate (*Raja erinacea*) (1976). The first successful purification of cytochrome P450 (CYP1A1) from fish was performed by Williams and Buhler from BNF-treated rainbow trout (Williams and Buhler, 1982). Later, multiple forms have been purified from several fish species- rainbow trout, leaping mullet, scup, cod, killifish, channel catfish and perch (Arınç and Adalı, 1983; Goksøyr, 1985; Klotz et al., 1986; Zhang et al., 1991; Buhler and Wang-Buhler,

1998; Şen and Arınç, 1998; Oleksiak et al., 2000, Oleksiak et al., 2003; Mosadeghi et al., 2007; Bozcaarmutlu and Arınç, 2008).

As in mammals, multiple forms of CYP enzymes are present in fish species. CYP1A, CYP2B, CYP2K6, CYP2K7, CYP2M1, CYP2N1, CYP2N2, CYP2P3, CYP2V1, CYP2X1, CYP3A27 and CYP4T2 are the enzymes/genes that have been purified or cloned from different fish species (Şen and Arınç, 1998; Buhler and Wang-Buhler, 1998; Oleksiak et al., 2000; Şen et al., 2001; Oleksiak et al., 2003; Mosadeghi et al., 2007; Bozcaarmutlu and Arınç, 2008). Similar to mammalian enzymes, fish enzymes involve in metabolism of both endogenous and exogenous compounds.

In fish, CYP1A proteins have been intensively studied because of their role in the metabolism and activation of carcinogens (Gelboin, 1980; Conney, 1982; Stegeman, 1995; Şen and Arınç, 1998) and their potential use as a biomarker for environmental pollution (Payne et al., 1987; Bucheli and Fent, 1995; Addison, 1996; Arınç et al., 2000).

1.6. Cytochrome P4501A (CYP1A)

In mammals two distinct forms of CYP1A have been found, CYP1A1 and CYP1A2. From laboratory animal studies, the gene products of CYP1A1 and CYP1A2 are known to metabolize polyaromatic hydrocarbons and arylamines, respectively, to intermediates that are toxic and/or can form DNA adducts (Dey et al., 1989). Many of the planar PAH compounds induce their own metabolism by

inducing transcription of the aromatic hydrocarbon receptor (Ah receptor). Although expression of CYP1A1 and 1A2 is often coordinately induced, there are clear differences in regulation, not only with respect to substrate specificity but also in their biological expression. For example, CYP1A1 does not appear to be expressed in human liver unless induced, whereas CYP1A2 is endogenously expressed in the liver. CYP1A1, however, is present in many extrahepatic tissues including the lung, where there is a possible association between CYP-mediated activation of benzo[*a*]pyrene and other related chemicals present in cigarette smoke and lung cancer in humans (Rose and Hodgson, 2004). 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 and Buhler, 1982; Klotz et al., 1983; Williams and Buhler, 1984; Goksøyr, 1985; Heilman et al., 1988; Zhang et al., 1991; Şen and Arınç, 1998). This gene and protein classified as CYP1A1 based on the sequence comparison with the mammalian counterparts.

1.6.1. Cytochrome P4501A1 (CYP1A1) in Fish

The occurrence of cytochrome P4501A1 (CYP1A1) in fish has been demonstrated in different studies and P4501A1 was purified from freshwater species rainbow trout (Williams and Buhler, 1982; Williams and Buhler, 1984; Miranda et al., 1989; Miranda et al., 1990; Andersson, 1992), and perch (Zhang et al., 1991) and from marine species scup (Klotz et al., 1983; Klotz et al., 1986; Stegeman et al., 1990), cod (Goksøyr, 1985; Goksøyr et al., 1986) and leaping mullet (Şen and Arınç, 1998) and knowledge about their inducibility by certain xenobiotics has been well established (Murphy and Gooch, 1997; Goksøyr and Husøy, 1998; Ronisz and

Förlin, 1998; Lange et al., 1999; Cousinou et al., 2000; Rotchell et al., 2000; Sarasquete and Segner, 2000; Al-Arabi and Goksøyr, 2002).

The biocatalytic and immunological properties and gene regulation of CYP1A1 in fish appear to be similar to those of mammalian P4501A1 (Stegeman, 1995). All purified P4501A1 isozymes, from different teleost fish species have the CO-reduced maxima at 447-448 nm. Induction of P4501A1 in fish has been observed with various PAHs, PCBs, 2,3,7,8-TCDD, PCDFs and PCDDs, other halogenated compounds (Arınç et al., 1978; Law and Addison, 1981; Spies et al., 1982; Goksøyr and Förlin, 1992; Arınç and Şen, 1994), crude oils (Spies et al., 1982; Vandermeulen, 1990), sediment extracts and bleached kraft mill effluents (Förlin et al., 1985; Andersson et al., 1987; Collier and Varanasi, 1991; Addison, 1996).

Induction of cytochrome P4501A1 and of its associated enzyme activities, namely aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin O-deethylase (EROD) activities, in fish by xenobiotics are commonly used in biomonitoring environmental contaminants (Förlin et al., 1985; Andersson et al., 1987; Payne et al., 1987; Collier and Varanasi, 1991; Goksøyr and Förlin, 1992; Stegeman, 1995; Addison, 1996; Arınç and Şen, 1999; Arınç et al., 2000). The first CYP1A related activity to be proposed as an indicator of pollutant exposure was the aryl hydrocarbon hydroxylase (AHH) activity (Payne, 1976). In 1975, Payne and Penrose showed that brown trout taken from a small urban lake in Newfoundland, Canada contaminated with petroleum hydrocarbons had increased benzo[*a*]pyrene hydroxylase (BPH) activity (Payne and Penrose, 1975). Payne then in 1976, suggested that the use of aryl hydrocarbon hydroxylase activity of fish liver as an

environmental monitor for the first time (Payne, 1976). This method evaluates the hydroxylation of benzo[a]pyrene metabolized by aryl hydrocarbon hydroxylase which is sometimes referred to benzo[a]pyrene hydroxylase (BPH). However, the use of this assay has been declining because of the carcinogenic property of the substrate and the possibility of substrate cross reactions with other CYP isozymes. Therefore the use of the other method, measurement of EROD activity, is mostly used catalytic probe for determining induction response of CYP1A1 in fish (Arınç et al., 2000).

Cytochrome P450 protein level can also be detected by using immunochemical methods, using mono- or polyclonal antibodies to fish CYP1A with western blotting or the enzyme-linked immunosorbent assay (ELISA) (Stegeman et al., 1986; Goksøyr and Solberg, 1987; Goksøyr et al. 1991; Goksøyr and Förlin 1992; Bucheli and Fent, 1995; Goksøyr and Husøy, 1998). Antibodies produced against purified P4501A have been used to assess environmental induction of P4501A in deep-sea fish from Northern Atlantic (Stegeman et al., 1986), in English sole from Puget Sound, USA (Varanasi et al., 1986), in winter flounder and scup from the Northeastern USA (Elksus et al., 1989), in flounder, plaice and dab in Norway (Goksøyr et al.,1991; Goksøyr and Förlin, 1992), in leaping mullet from İzmir Bay, Turkey (Arınç and Şen, 1999), in perch, pike, dab and blenny from waters of Sweden (Förlin and Celander, 1993). Immunoquantitation is generally less quantitative than the EROD assay but the method has the advantage that it can be applied to poor quality (denatured) samples or samples containing inhibitors (Goksøyr et al., 1991; Goksøyr, 1991; Goksøyr and Husøy, 1992).

In addition to cytochrome P4501A1 associated monooxygenase activity and cytochrome P4501A protein level, cytochrome P4501A1 induction can be measured at cytochrome P4501A1 mRNA level. The steps involved in the induction of CYP1A1 include the activation and transcription of the CYP1A1 gene, translation of CYP1A1 messenger RNA to produce CYP1A1 protein, and post-translational modification to produce the catalytically active enzyme (Goksøyr, 1995; Arınç et al., 2001; Anderson and Lee, 2006). Induction stimulates the rate of gene transcription, which results in increased levels of P450 messenger RNA and increased synthesis of P450 protein (Anderson and Lee, 2006). Measurement of CYP1A1 mRNA by Northern blots is becoming an integral part of investigations on CYP1A1 regulation, while several field trials have shown its suitability as a biomarker (Bucheli and Fent, 1995; Arınç et al., 2001; Van der Oost et al., 2003). Cytochrome P4501A1 induction in fish exposed to polluted waters have been examined at mRNA levels in several studies (Kreamer et al., 1991; Renton and Addison, 1992; Courtenay et al., 1993; Haasch et al., 1993; Stegeman, 1995; Buhler and Wang-Buhler, 1998; Arınç et al., 2001). Table 1.5 shows the common methods that are used for the determination of CYP1A induction in biomonitoring studies.

Table 1.5. Common methods for the determination of CYP1A induction.

Level	Nomenclature	Marker
DNA	CYP1A1	
mRNA	CYP1A1	DNA probe
protein	P4501A1	Antibody
enzyme	EROD/ AHH	Catalytic activity

1.7. CYP1A1 Regulation

The mechanism of enzyme induction has been studied most extensively for cytochrome P450 isozymes, especially CYP1A which is the best studied biomarker for environmental contamination in aquatic ecosystems. Knutson and Poland identified a high affinity receptor molecule namely aromatic hydrocarbon receptor (AhR) in the cytosol and proposed a model to describe the mechanism by which TCDD and related compounds regulate gene expression through this receptor (AhR) (Poland and Knutson, 1982).

The Ah-receptor is a ligand-activated transcription factor residing in the cell cytoplasm as a complex with heat-shock protein (HSP90) molecule whose function is to maintain unliganded AhR in a configuration that facilitates ligand binding. Enzyme induction is initiated by the binding of a specific xenobiotic, such as PCDDs, PCBs, PCDFs and PAHs (Poland and Knutson, 1982; Whitlock et al., 1996; Denison and Heath-Pagliuso, 1998; Whitlock, 1999; Denison et al., 2002; Van der Oost et al., 2003).

The regulation of CYP1A by the cytosolic aromatic hydrocarbon receptor (AhR), when the organism is exposed to a toxicant, is shown in Figure 1.8. The hydrophobic inducer (ligand) passively diffuses into the cell and binds to the AhR which is present in the cytosolic compartment as a multiprotein complex containing two molecules of HSP90 (a heat shock protein of 90 kDa), XAP2 (X-associated protein 2 (Meyer et al., 1998)) (also referred to as AIP or ara9 (Carver and Bradfield, 1997; Ma and Whitlock, 1997)) and p23 (a co-chaperone protein of 23 kDa)

(Kazlauskas et al., 1999). Following ligand binding, the HSP90 molecule, XAP2, and p23 are released, and the ligand-AhR complex translocates into the nucleus of the cell. Within the nucleus, the liganded AhR dimerizes with a nuclear protein, Arnt (Ah receptor nuclear translocator), thereby generating the DNA-binding AhR:Arnt heteromer (Henry and Gasciewicz, 1993; Hord and Perdew, 1994; Pollenz et al., 1994; Hankinson, 1995; Whitlock et al., 1996; Denison et al., 2002). Formation of the AhR:Arnt heterodimer converts the complex into its high affinity DNA binding form (Probst et al., 1993; Hankinson, 1995; Denison et al., 2002) and binding of this complex to its specific DNA recognition site, known as the xenobiotic regulatory element (XRE), initiates synthesis of the messenger RNA (mRNA) responsible for transcribing the appropriate sequence for CYP1A1 synthesis. The cytochrome P450 molecule is then incorporated into the membrane of the endoplasmic reticulum (Denison et al., 1988; Stegeman and Hahn, 1994; Whitlock et al., 1996; Whitlock, 1999; Denison et al., 2002).

The transcriptional regulation of CYP1A in fish through a transcription factor, aromatic hydrocarbon receptor (AhR), is similar to that in mammals (Stegeman and Hahn, 1994; Hahn and Karchner, 1995; Hahn, 1998; Miller et al., 2003; Van der Oost et al., 2003). The AhR has been characterized in several species of fish (Heilmann et al., 1988; Hahn et al., 1994; Willett et al., 1995; Abnet et al., 1999a,b; Karchner et al., 1999; Tanguay et al., 1999) and in several fish cell lines (Lorenzen and Okey, 1990; Swanson and Perdew, 1991; Hahn et al., 1993; Pollenz and Necela, 1998; Hestermann et al., 2000; Billiard et al., 2002).

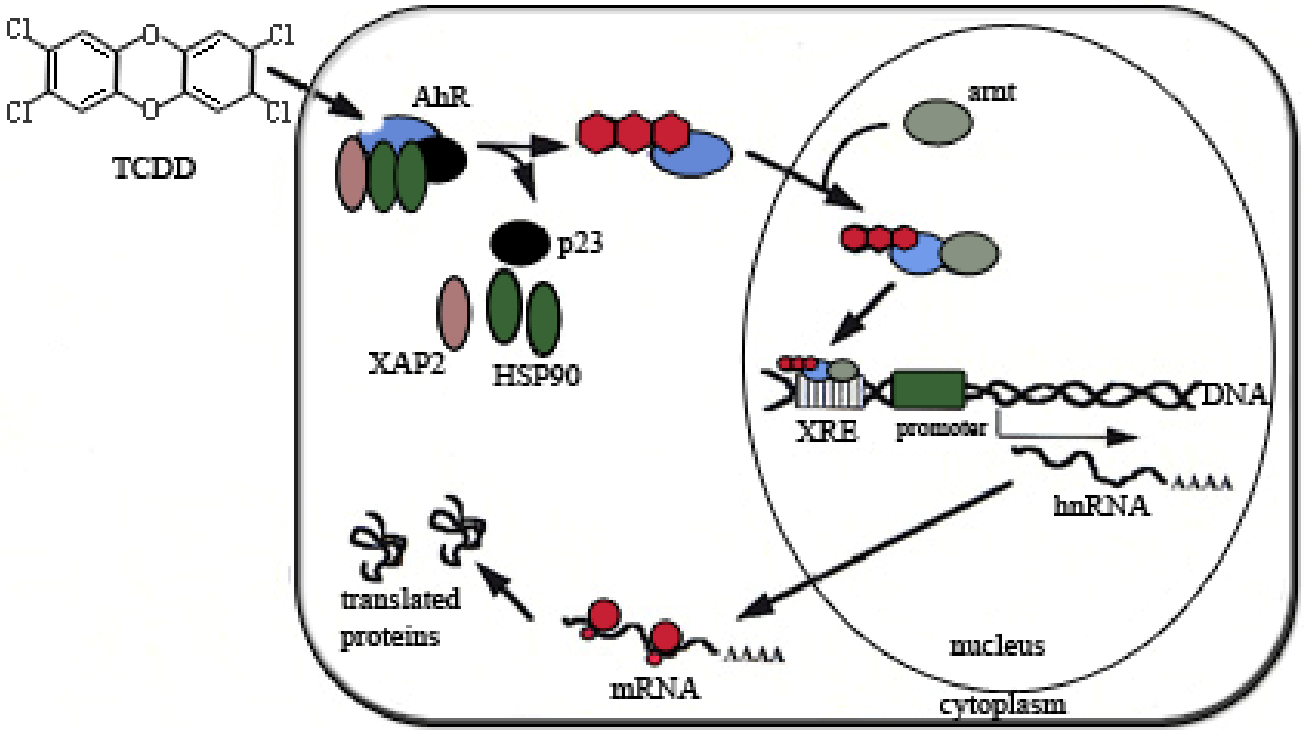


Figure 1.8: The regulation of CYP1A by the cytosolic aromatic hydrocarbon (Ah) receptor (taken from Whitlock, 1986 and modified according to Denison et al., 2002).

1.8. The Black Sea

The Black Sea is the world's largest enclosed sea and covers an area of 423,000 km^2 with depth of 2212 m, respectively. Its only connection with the World's Oceans is via the Turkish Bosphorus Strait, which links it with the Mediterranean (via the Sea of Marmara). The Black Sea is also connected to the Sea of Azov by the strait of Kerch. The Sea has six coastal countries: Turkey, Bulgaria, Romania, Ukraine, the Russian Federation and Georgia.

These countries organized a convention for the protection of the Black Sea against pollution in 1992 in Bucharest (known as the Bucharest Convention). In order to provide a practical program of actions for cleaning up and protecting the Black Sea, the Black Sea Environmental Program (BSEP) was founded in 1993. The Black Sea Environmental Program formed the base of the Black Sea Action Plan. The overall aim of the Black Sea Action Plan is to "enable the population of the Black Sea region to enjoy a healthy living environment in both urban and rural areas, and to attain a biologically diverse Black Sea ecosystem with viable natural populations of higher organisms, including marine mammals and sturgeons, and which will support livelihoods based on sustainable activities such as fishing, aquaculture and tourism in all Black Sea countries" (Strategic Action Plan for the Rehabilitation and Protection of the Black Sea, 1996).

Today, the most important pollution caused problem in the Black Sea is the massive over-fertilization of the sea by compounds of nitrogen and phosphorus, largely as a result of agricultural, domestic and industrial sources. This over-

fertilization, produces a phenomenon called eutrophication, has changed the structure of the Black Sea ecosystem. The nitrogen and phosphorus compounds (termed as *nutrients*) enter the Black Sea from the 17 countries in its drainage basin, particularly through rivers. Another problem is the lack of proper sewage and wastewater treatment systems in most cities in the Black Sea Region. This insufficiently treated sewage and wastewater have been discharging either through the rivers or directly to the Black Sea, cause microbiological contamination. Oil pollution is the other problem threatens Black Sea coastal ecosystems. Currently levels of oil pollution are not high in the open Black Sea but are unacceptably high in many coastal areas and river mouths. Oil enters the environment as a result of accidental and operational discharges from vessels, as well as through land based sources (Strategic Action Plan for the Rehabilitation and Protection of the Black Sea, 1996). According to Black Sea scientists, every year about 30000 tons of oil enters the sea from domestic sewage plants, 15500 tons from industry (including the oil industry), and 53000 tons flows down the Danube River. The total annual discharges around 98500 tons.

Heavy metals, such as cadmium, copper, chromium and lead, are usually associated with waste from industry and the ash remaining from burning coal for generating electricity. Pesticides are mostly introduced through rivers and streams discharging from agriculture. Radioactive substances have been introduced to the Black Sea in small quantities from nuclear power generation and in more significant amounts as a result of the Chernobyl accident in 1986. Reservoirs in the Dnieper River however, still contain huge amounts of radioactive material locked up in their sediments.

The final class of problematic pollutants is solid waste, dumped into the sea from ships and some coastal towns. As an enclosed sea, the Black Sea is particularly vulnerable to this form of pollution (Strategic Action Plan for the Rehabilitation and Protection of the Black Sea, 1996).

The studies on the Black Sea Coasts of Turkey mainly focused on the chemical analyses. According to these studies, the Turkish Coasts of the Black Sea is polluted with heavy metals (Tuncer et al., 1998; Barlas, 1999; Tüzen, 2003). The Kızılırmak, Yeşilırmak, Sakarya, Filyos and Gülüç Rivers are important sources for Cd, Cu, Pb and Zn (Tuncer et al., 1998; Barlas, 1999). In addition to inorganic pollutants, the presence of pesticides and PCBs, including lindane, heptachlor, heptachlor epoxy, aldrin, dieldrin, endrin, DDE, DDD and DDT have been shown in different studies (Tanabe et al., 1997; Tuncer et al., 1998; Bakan and Büyükgüngör, 2000; Bakan and Ariman; 2004; Kurt and Ozkoc, 2004). The presence of polyaromatic hydrocarbons have been also shown in the edge of Bosphorus Strait and Trabzon, Yomra (Telli-Karakoç et al., 2001; Readman et al., 2002). In another study, polyaromatic hydrocarbon level has been measured in the surface sediment taken from east-west transect in the southern half of the Black Sea. In this study, the sediments have been collected from the open sea. It has been reported that the level of polyaromatic hydrocarbon in the west part of the Black Sea was higher than that in the east part (Wakeham, 1996).

1.9. Test Species

In this study, three kinds of mullet species used, namely so-iuy mullet (*Mugil soiuy*), golden grey mullet (*Liza aurata*), and flathead mullet (*Mugil cephalus*). The mullets are economically important marine fish belong to the family of Mugilidae. They live predominantly inshore in the protected waters of whole Mediterranean Sea, Black Sea, Sea of Azov and along the Atlantic coast. They are resistant and tolerant to pollutants and to various environmental conditions. Mullets are herbivorous scavengers, feeding primarly on algae and detrital (dead) plant material.

So-iuy mullet also called Russian grey mullet live predominantly in Russia, Korea, China, Black Sea and Mediterranean Sea. Length of so-iuy mullet in the Black Sea is usually 20-30 cm. Golden grey mullet live in Eastern Atlantic, Mediterranean and Black Sea. The flathead mullet live in California, USA, Nova Scotia, Canada to Brazil, Cape Cod to southern Gulf of Mexico, most of West Indies and Caribbean, Mediterranean Sea and Black Sea.

Mullets are very important for our economy due to the marketing of their meat and eggs. There is a large commercial and sport fishing demand for this organism and consumed in large quantities in Turkey, which increase the importance of this organism from the ecological and toxicological standpoints.

1.10. The Aim of the Study

Marine organisms are most often exposed to complex mixtures of pollutants, including PAHs, PCBs, PCDFs, PCDDs, alkyltin compounds and metals. Even though chemical analysis in the sea water and sediment give valuable information about the levels of chemical pollutants, they do not reveal the impact of pollutants on organisms. Induction of CYP1A and associated 7-ethoxyresorufin O-deethylase (EROD) activity is the best characterized and used biochemical marker of exposure of fish to organic pollutants such as PCBs, PAHs and dioxins. CYP1A induction is used as a useful tool for the assessment of exposure to these types of organic pollutants and early warning sign for potential harmful effects of many organic molecules.

In this study, our aim was to determine and monitor the degree of PAHs, PCBs and dioxins type pollution in the West Black Sea Coast of Turkey by measuring cytochrome CYP1A associated 7-ethoxyresorufin O-deethylase (EROD) activity and immunochemical detection of CYP1A protein level in mullet liver microsomes and to find the places of areas that create local pollution in the West Black Sea Region of Turkey. In addition, total polyaromatic hydrocarbon levels were measured in the mullet liver samples to show the presence and extent of one of the CYP1A inducer organic pollutants in sampling stations. Finally, the relation between the results of monitoring study and ecological consequences was discussed in this study.

MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Bromophenol blue ($C_{19}H_{10}BrO_5S$; 108122), chrysene (841690), diethanolamine (DEA; $C_4H_{11}NO_2$; 803116), N,N-dimethylformamide ($HCON(CH_3)_2$; 102937), dimethyl sulfoxide (DMSO; $(CH_3)_2SO$; 116743), ethanol (C_2H_5OH; 100983), glycerol ($C_3H_8O_3$; 104092), n-hexane ($CH_3(CH_2)_4CH_3$; 104368), hydrochloric acid (HCl; 100314), magnesium chloride hexahydrate ($MgCl_2.6H_2O$; 105833), methanol (CH_3OH; 106007), nitro blue tetrazolium chloride (NBT; $C_{40}H_{30}Cl_2N_{10}O_6$ 124823), potassium chloride (KCl; 104936), potassium dihydrogen phosphate (KH_2PO_4; 104871), dipotassium hydrogen phosphate (K_2HPO_4:105101), potassium hydroxide (KOH; 105033), sodium carbonate (Na_2CO_3; 106392), sodium chloride (NaCl; 106404), sodium hydroxide (NaOH; 106462), zinc chloride ($ZnCl_2$; 108816) were purchased from Merck KGaA, Darmstadt, Germany.

Acrylamide (C_3H_5NO; A8887), anti-rabbit IgG alkaline phosphatase antibody (A3687), ε-amino caproic acid (ε-ACA; $C_6H_{13}NO_2$ A2504), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris, T1378), bovine serum albumin (BSA; A7511 or A7888), copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$; C7631), resorufin ethyl ether ($C_{14}H_{11}NO_3$; E3763), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; $C_{10}H_{14}N_2Na_2O_8.2H_2O$; E5134), folin-ciocalteu's phenol reagent (F9252), N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES; $C_8H_{18}N_2O_4S$; H3375), glycine ($C_2H_5NO_2$; G7126), β-mercaptoethanol (M6250), N,N'-methylene bisacrylamide (BIS; $C_7H_{10}N_2O_2$; M7256), phenylmethanesulfonyl fluoride (PMSF; $C_7H_7FO_2S$; P7626), resorufin sodium salt (23,015-4) were purchased from Sigma-Aldrich, Saint Louis, Missouri, USA.

5-bromo-4-chloro-3-indolylphosphate disodium salt (BCIP; 203788), glucose-6-phosphate dehydrogenase (346774), α-D-glucose-6-phosphate monosodium salt (346764), polyxyethylene sorbitan monolaurate (Tween 20; 655204), sodium dodecyl sulfate (SDS; 428023) were purchased from Calbiochem, San Diego, California, USA.

Ammonium persulfate (161–0700), N, N, N', N' tetrametylethylene diamine (TEMED; 161–0801), trans blot tranfer medium (pure nitrocellulose membrane; 162-0115) were purchased from Bio-Rad Laboratories, Richmond, California, USA.

β-nicotinamide adenine dinucleotide phosphate disodium salt ($NADP^+$; $C_{21}H_{27}N_7NaO_{17}P_3$; A1394), phenazine methosulfate (PMS;$C_{14}H_{14}N_2O_4S$; A2212) were purchased from Applichem GmbH, Darmstadt, Germany.

The primary antibody (polyclonal antibodies produced against purified leaping mullet liver cytochrome P4501A in rabbits) was prepared by Dr. Alaattin Şen in the Department of Biology in Middle East Technical University, Ankara.

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 samples were collected in August 2005, August 2006 and August 2007 from the different stations of the West Black Sea Region of Turkey. Three different species of mullet were captured by fish net. These are soiuy mullet (*Mugil soiuy*), flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*). Each mullet was weighted as about 600-700g. The fish sampling stations were shown in Figure 2.1.

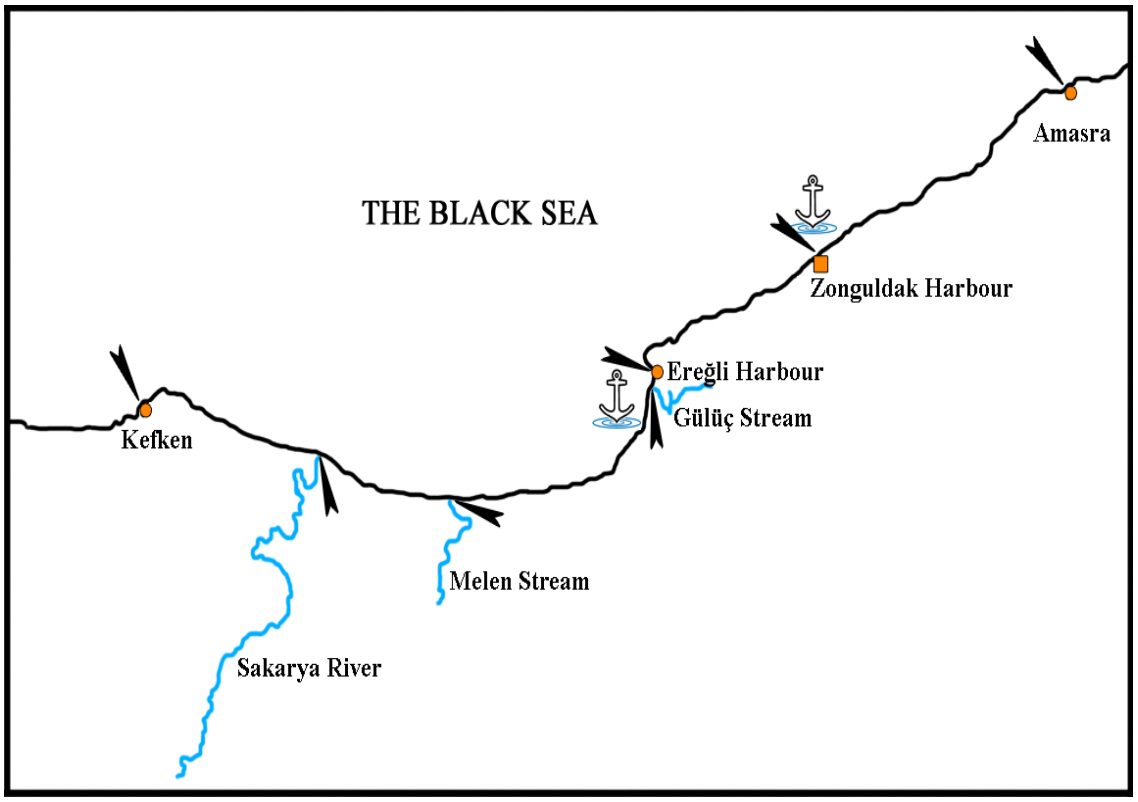


Figure 2.1. The sampling stations in the West Black Sea Region of Turkey.

2.2. Methods

2.2.1. Preparation of Fish Liver Microsomes

The liver microsomes were prepared by the method described by Arınç and Şen (1993). Fish were killed by decapitation and the livers, weighing approximately 9-12 grams, were removed immediately. In order to prevent the spillage of the contents of the gall bladders that are known to be inhibitory to monooxygenase activities, the gall bladders were removed carefully with scissors. 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 to our university laboratory in Bolu prior to preparation of microsomes and PAH analyses.

Microsomes were prepared in Middle East Technical University, Biology Department and Gülhane Military Medical Academy, Biochemistry Department in Ankara. The liver samples were transported from our university laboratory in Bolu to laboratory in Ankara in liquid nitrogen.

Microsomes were prepared from one fish liver at a time separately. In the laboratory, the livers were taken from liquid nitrogen and cut into two pieces without thawing. One of the pieces was wrapped by freezing bags and covered by aluminum foil and then put back into liquid nitrogen. These pieces were used in PAH analyses.

The other piece was used in microsome preparation and thawed on ice and 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 pieces with scissors. The resulting tissue mince was homogenized in 1.15% KCl solution containing 10 mM 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,with twelve passes.

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 mullet 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.

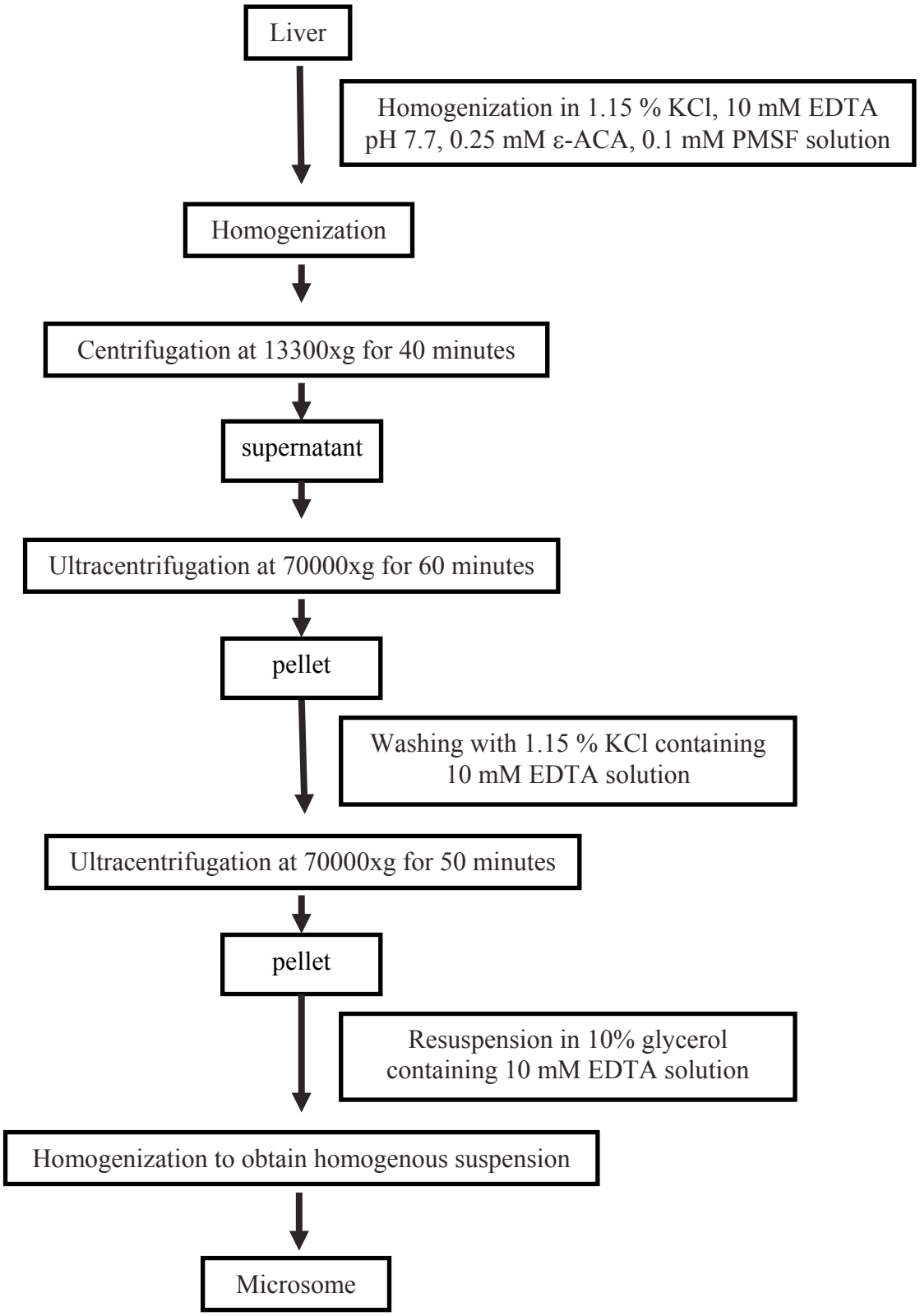


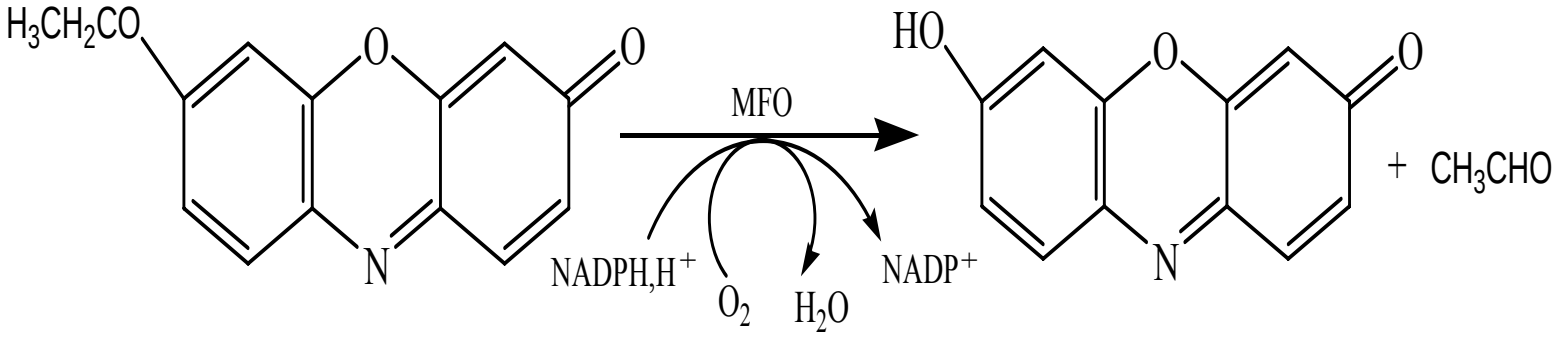
Figure 2.2. Major steps of the preparation of microsomes from fish liver.

2.2.2. Protein Determination

Concentrations of microsomal proteins were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as standard. The mullet liver microsomes were 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-ciocalteu's 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 200 μg/mL was plotted and used for the determination of protein concentration of samples.

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

Cytochrome P4501A (CYP1A) associated 7-Ethoxyresorufin-O-deethylase activity of fish liver microsomes were determined by the method described by Burke and Mayer (1974) with some modifications. Assay conditions optimized for gilthead seabream liver microsomes by Arınç and Şen (1994) was also used for fish species in this study. Figure 2.3 shows the O-dealkylation reaction of ethoxyresorufin catalyzed by monooxygenases in the presence of molecular oxygen and NADPH.



7- ethoxyresorufin

7- hydroxyresorufin

Figure 2.3. 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 2.1, 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, 25, 50 or 100 μg microsomal protein, 0.5 mM NADPH generating system (constituents of the generating system were given in Table 2.2) 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 F-4500, Hitachi High-Tecnologies Corporation, 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. 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.	25, 50 and 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	12 mg/mL
Ethoxyresorufin	10 µM	0.3	1.5 µM
NADPH Generating System*		0.3	0.5 mM
Distilled Water		Completed to 2.0 mL	

*NADPH generating system was prepared as described in Table 2.2.

Table 2.2. Preparation of NADPH generating system.

Constituents	Stock Solutions	Volume to be taken (ml)	Final concentration in 0.5 mL reaction mixture
Glucose-6-phospate	100 mM	0.025	2.5 mM
$MgCl_2$	100 mM	0.025	2.5 mM
HEPES, pH:7.8	200 mM	0.074	14.6 mM
$NADP^+$	20 mM	0.0235	0.5 mM
Glucose-6-phosphate dehydrogenase	200 U/mL	0.0025	0.5 Units
Final Volume		0.150	

2.2.4. Western Blot Analysis – Protein Blotting

Cytochrome P4501A protein level was determined by using the polyclonal antibodies produced against purified leaping mullet liver cytochrome P4501A1.

Firstly, 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 2.3 in a given order.

Table 2.3. 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 (29.2% Acrylamide containing 0.8% N,N'-methylene bisacrylamide solution)	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 (mL)	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 2 minutes exactly.

After application of the samples, gel set up was placed for separation of proteins in an electrical field in Scie-Plas Mini Electro-Blotting Unit. The Cell was connected to the power supply (Elite 200, Wealtec Corp., Sparks, NV, USA) and electrophoresis was run at 10 mA constant current and corresponding voltage. When electrophoresis was completed, gel was removed from the cell for western blot analysis which was carried out as described by Towbin et al. (1979) with some modifications. Gel was 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 2.4. 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 Scie-Plas Mini Electro-Blotting Unit and the cell was filled with cold transfer buffer. Voltage was set to constant 90V and transfer process was carried out in the refrigerator at 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; 20 mM 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 40 minutes in order to fill the empty spaces between bound proteins in order to prevent the non-specific binding of antibodies on the membrane.

The blot was incubated with primary antibody for 2 hours. As primary antibody, polyclonal antibodies produced against purified leaping mullet liver cytochrome P4501A1 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, 1:10000 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 2.4 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. The final images of the blots were photographed using computer based gel imaging instrument (Infinity 3000-CN-3000 darkroom, Vilber Lourmat, Marre-la-Vallee Cedex 1, France), and densities of bands was analzed using The Scion Image software for windows (Version 4.0.2, Scion Corporation, Maryland, USA) as a quantitative tool to determine cytochrome P4501A protein levels.

Table 2.4. Preparation of substrate solution for immunodetection.

Solution A: 2.67 mL of 1.5 M Tris-HCl, pH 8.8 4.0 mL of 1 M 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 40.0 mL (pH of the solution was adjusted to 9.55 with saturated Tris before completing to final volume)
Solution B: 2.0 mg/mL phenazine methosulfate in distilled water.
Solution C: 5.44 mg BCIP in 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.

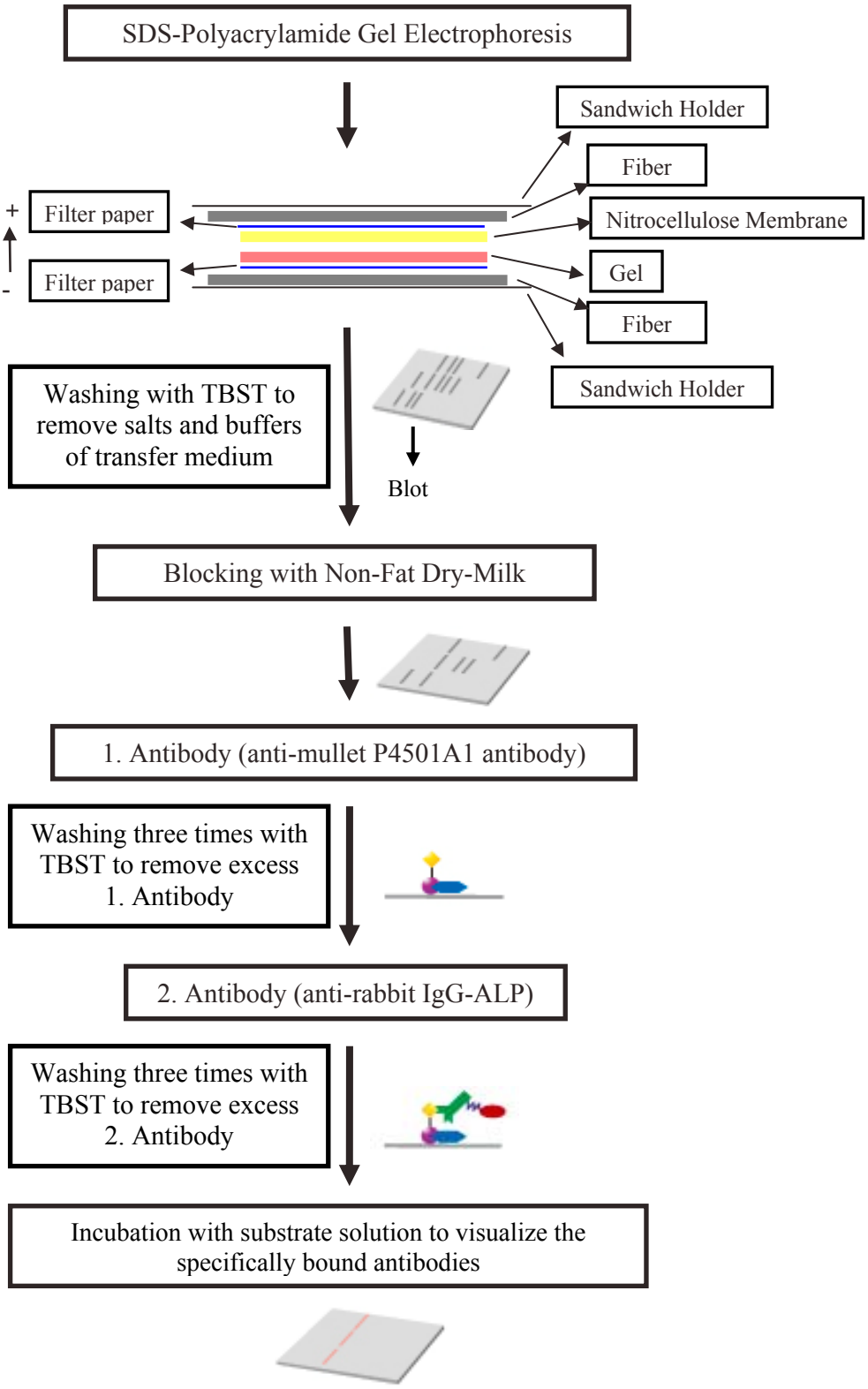


Figure 2.4. Major steps of western blot analysis.

2.2.5. Spectrofluorometric Measurement of Total Polyaromatic Hydrocarbon in Fish Liver Tissue

Spectrofluorometric measurements of the total PAHs concentration in fish liver tissues were performed according to the method described by UNEP, 1992. 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. They cannot be dissolved in water or other polar solvents. Therefore highly hydrophobic and nonpolar solvents such as benzene or hexane can be used for this purpose. Extraction of desired compounds from a biological tissue generally requires a pretreatment step to get ride 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 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 grinded in a mortar. 0.2 g of dried and grinded tissue put into a round bottomed flask and refluxed with 20 mL of ethanol containing 0.75 g of KOH for 120 minutes by constant heating (for saponification process). Then 20 mL of PAH-free hexane was added to flask (to extract PAHs from saponification solution) and

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 samples was compared with the fluorescence of series of reference solutions, 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 and 2 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 and 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 (g).

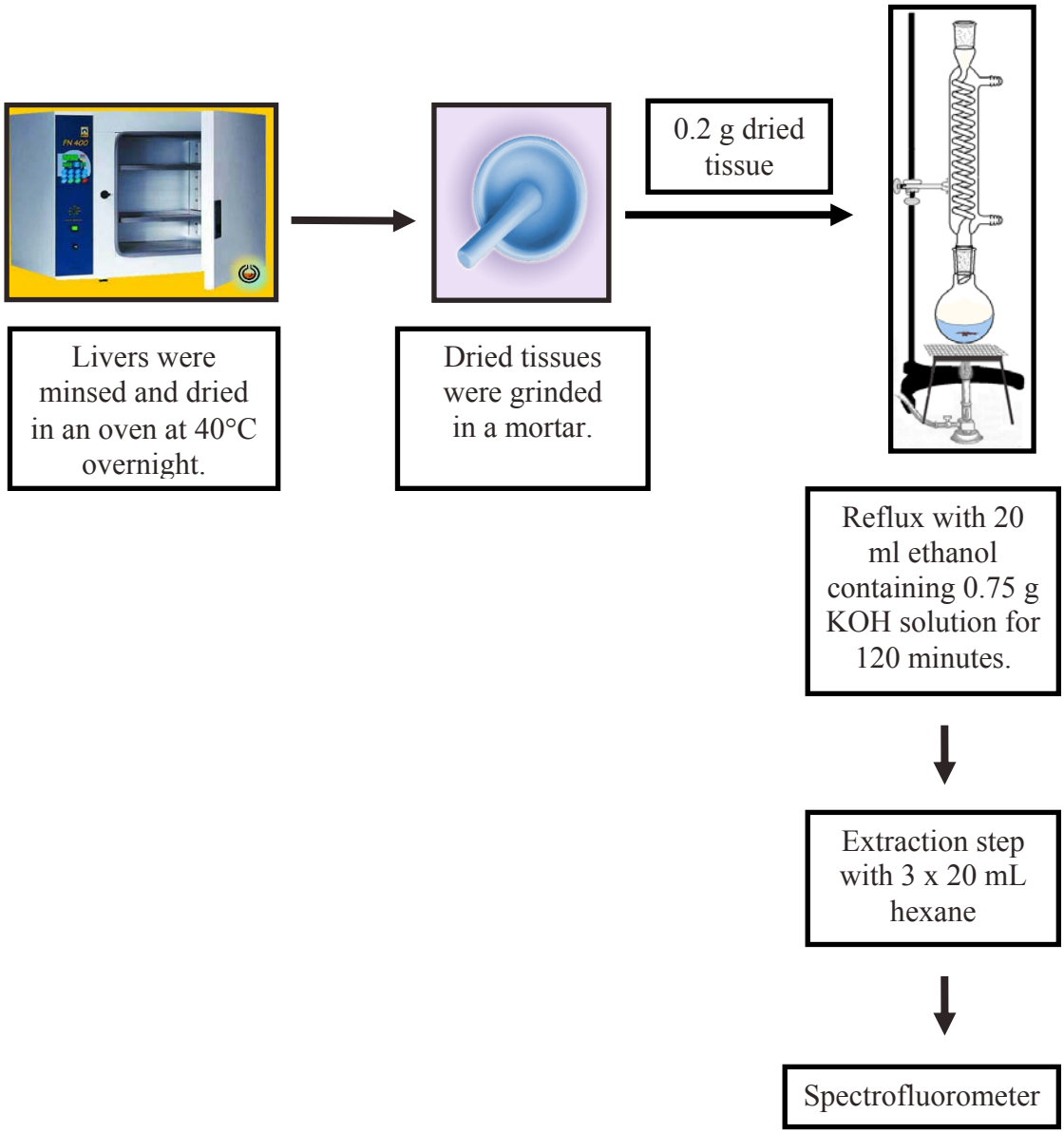


Figure 2.5. Major steps of the total polyaromatic hydrocarbon determination method in fish liver tissue.

2.2.6. Statistical Analysis

The results were expressed as means ± standard error of mean (SEM). To test differences among the mean values, one-way ANOVA were applied with Dunnett's Multiple Comparison Post Hoc Test. The analyses were carried out using the SPSS statistical package (SPSS 15.0 for Windows Evaluation Version).

RESULTS

In this study 150 fish samples were collected in August 2005, August 2006 and August 2007 from different stations of the West Black Sea Region of Turkey to determine the extent of PAHs, PCBs, and dioxin type pollutions by measuring CYP1A associated 7-ethoxyresorufin O-deethylase (EROD) activity and immunochemical detection of CYP1A protein level in mullet liver microsomes. Besides these biochemical parameters, total PAH concentrations were determined in fish liver tissues collected in August 2006 to show the presence and extent of one of the CYP1A inducer organic pollutants in sampling stations. Three different mullet species were used throughout this study, namely soiuy mullet *(Mugil soiuy)*, flathead mullet (*Mugil cephalus*), and golden grey mullet (*Liza aurata*).

3.1. Liver Microsomal 7-Ethoxyresorufin O-Deethylase Activity of Mullets Caught from Different Stations of West Black Sea Region of Turkey in August 2005.

Microsomes were prepared from one fish liver at a time separately and a sample number was given to each of the microsome preparation throughout this study. Cytochrome P4501A dependent 7-ethoxyresorufin O-deethylase (EROD) activity was determined in all microsome preparations. The principle of the method is based on the conversion of 7-ethoxyresorufin into resorufin which is measured

spectrofluorometrically at 535 nm (excitation) and 585 nm (emission) wavelengths (Burke and Mayer et al., 1974).

In 2005, 60 mullets were caught from six different stations (Zonguldak Harbour, Ereğli Harbour, Gülüç Stream Mouth, Melen Stream Mouth, Sakarya River Mouth, and Kefken) of the West Black Sea Coast of Turkey. Table 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, and 3.7 show microsomal EROD activity results obtained from the livers of each fish caught from the six different stations along the West Black Sea Coast of Turkey. All activities were measured in duplicates and sometimes in triplicates.

The average EROD activities of liver microsomes of mullets caught from six different stations of the West Black Sea Region of Turkey were summarized in Table 3.8 and presented in Figure 3.1 as a graph. EROD activities were represented as mean ± standard error of mean.

Soiuy mullets and flathead mullets that caught from Zonguldak Harbour showed the highest EROD activities (3237±539 pmole/min/mg protein, n=7 and 3600±368 pmole/min/mg protein, n=2, respectively) which were followed by soiuy mullets that caught from Gülüç Stream Mouth (2402±261 pmole/min/mg protein, n=13) and golden grey mullets that caught from Ereğli Harbour (1871±391 pmole/min/mg protein, n=16) (Table 3.1, 3.2, 3.3 and 3.4). The lowest EROD activity was found in flathead mullet caught from Kefken (484 pmole/min/mg protein, n=1) (Table 3.7). The EROD activities of soiuy mullets sampled from Melen Stream Mouth and Sakarya River Mouth were similar to each other (1065±176 pmole/min/mg protein, n=11 and 904±216 pmole/min/mg protein, n=10,

respectively) (Table 3.5 and 3.6). The fish samples captured from these stations had lower microsomal EROD activities than the fish samples captured from Zonguldak and Ereğli Harbours, and Gülüç Stream Mouth.

Table 3.1. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Zonguldak Harbour (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	5280-5241	5261
2	4947-4970	4959
3	2375-2479	2427
4	2550-2419	2485
5	2048-2149	2099
6	1638-1753	1696
7	3850-3619	3735
Average Activity ± Standard Error of Mean n= 7		3237±539

Table 3.2. EROD activities of flathead mullets (*Mugil cephalus*) caught from Zonguldak Harbour (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	3316–3148	3232
2	3943–3991	3967
Average Activity ± Standard Error of Mean n= 2		3600±368

Table 3.3. EROD activities of golden grey mullets (*Liza aurata*) caught from Ereğli Harbour (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	1856-1693	1775
2	466-443	455
3	1769-1694	1732
4	1412-1225	1319
5	1135-1048	1092
6	1979-1871	1925
7	1743-1716	1730
8	1554-1509	1532
9	1226-1226	1226
10	2932-2817	2875
11	6993-6986	6990
12	3194-3315	3255
13	392-363	378
14	1429-1452	1441
15	560-635	598
16	1649-1586	1618
Average Activity ± Standard Error of Mean n= 16		1871±391

Table 3.4. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Gülüç Stream Mouth (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	3964-4034	3999
2	1716-1754	1735
3	1792-1779	1786
4	2960-3130	3045
5	2371-2437	2404
6	834-844	839
7	3029-2884	2957
8	2554-2525	2540
9	2091-2025	2058
10	2628-2588	2608
11	3942-4115	4029
12	1770-1795	1783
13	1495-1397	1446
Average Activity ± Standard Error of Mean n= 13		2402±261

Table 3.5. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Melen Stream Mouth (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	143-136	140
2	1830-1990	1910
3	1911-1998	1955
4	1502-1591	1547
5	1162-1249	1206
6	765-771	768
7	1018-982	1000
8	820-832	826
9	382-369	376
10	1290-1211-1290	1264
11	730-706	718
Average Activity ± Standard Error of Mean n= 11		1065±176

Table 3.6. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Sakarya River Mouth (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	1378-1380	1379
2	1228-1236-1246	1237
3	2585-2530	2558
4	367-296	332
5	695-655	675
6	987-1032-905	975
7	444-464	454
8	441-433	437
9	470-456	463
10	522-533	528
Average Activity ± Standard Error of Mean n= 10		904±216

Table 3.7. EROD activity of flathead mullet (*Mugil cephalus*) caught from Kefken (August 2005).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	478-489	484

Table 3.8. The average EROD activities of mullets caught from different stations of the West Black Sea Region of Turkey (August 2005).

Sampling Stations	Fish Species	Number of Fish Liver	Average EROD Activity ± Standard Error of Mean
Zonguldak Harbour	*Mugil soiuy*	7	3237±539
Zonguldak Harbour	*Mugil cephalus*	2	3600±368
Ereğli Harbour	*Liza aurata*	16	1871±391
Gülüç Stream Mouth	*Mugil soiuy*	13	2402±261
Melen Stream Mouth	*Mugil soiuy*	11	1065±176
Sakarya River Mouth	*Mugil soiuy*	10	904±216
Kefken	*Mugil cephalus*	1	484

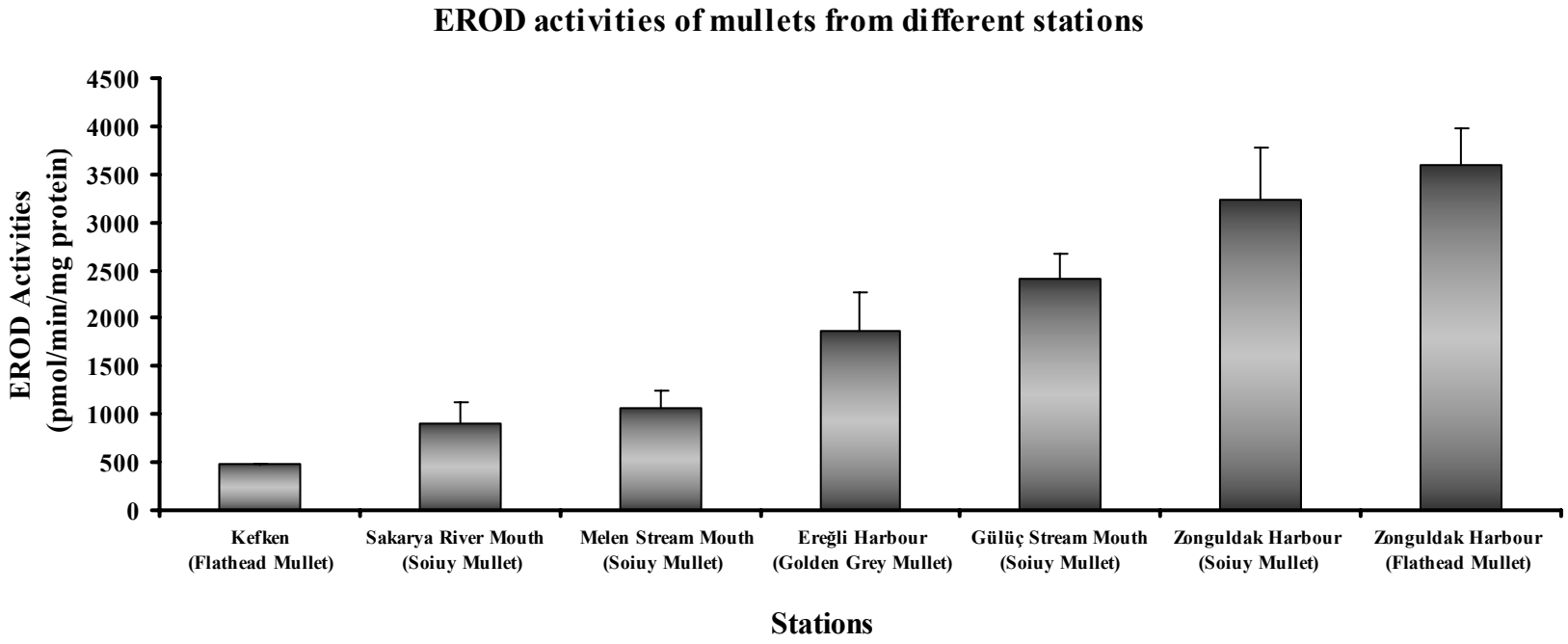


Figure 3.1. EROD activities of mullets caught from different stations of the West Black Sea Coast of Turkey (August 2005).

3.2. Liver Microsomal 7-Ethoxyresorufin O-Deethylase Activity of Mullets Caught from Different Stations of West Black Sea Region of Turkey in August 2006.

In 2006, 71 mullets were caught from six different stations (Zonguldak Harbour, Ereğli Harbour, Gülüç Stream Mouth, Melen Stream Mouth, Sakarya River Mouth, Amasra) of West Black Sea Region of Turkey. Table 3.9, 3.10, 3.11, 3.12, 3.13, 3.14, 3.15 and 3.16 show microsomal EROD activities obtained from the livers of each fish caught from the six different stations along the West Black Sea Region of Turkey. All activities were measured in duplicates.

The average EROD activities of liver microsomes of mullets caught from six different stations of the West Black Sea Region of Turkey were summarized in Table 3.17 and presented in Figure 3.2 as a graph. EROD activities were represented as mean ± standard error of mean.

The highest EROD activities were measured in the fish samples caught from Zonguldak Harbour and Gülüç Stream Mouth (2559±315 pmole/min/mg protein, n=13; 2415±426 pmole/min/mg protein, n=11, respectively) in 2006. Table 3.9 and 3.12 show the EROD activities of liver microsomes of each soiuy mullets caught from Zonguldak Harbour and Gülüç Stream Mouth, respectively. Golden grey mullets sampled from Ereğli Harbour had also high microsomal EROD activities (2037±280 pmole/min/mg protein, n=12) (Table 3.11.). Table 3.13 shows the EROD activities of liver microsomes of soiuy mullets caught from Melen Stream Mouth. Soiuy mullets that caught from Melen Stream Mouth had lower EROD activities

(818±217 pmole/min/mg protein, n=9) than that of EROD activities from Zonguldak Harbour, Gülüç Stream Mouth and Ereğli Harbour. However this value was higher than that of EROD activities from Amasra. In 2006, the lowest EROD activities were measured in soiuy mullets caught from Amasra (Table 3.15).

Table 3.9. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Zonguldak Harbour (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	2454-2414	2434
2	3505-3598	3552
3	3588-3517	3553
4	1652-1739	1696
5	2617-2582	2600
6	1214-1171	1193
7	632-571	602
8	3455-3397	3426
9	2516-2548	2532
10	1180-1188	1184
11	2605-2662	2634
12	3376-3316	3346
13	4470-4552	4511
Average Activity ± Standard Error of Mean n= 13		2559±315

Table 3.10. EROD activities of golden grey mullets (*Liza aurata*) caught from Zonguldak Harbour (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	3114-3286	3200
2	1887-2034	1961
3	3197-3163	3180
4	2873-2937	2905
5	1901-1959	1930
6	2206-2199	2203
7	2637-2497	2567
8	1078-1089	1084
9	1735-1633	1684
Average Activity ± Standard Error of Mean n= 9		2302±240

Table 3.11. EROD activities of golden grey mullets (*Liza aurata*) caught from Ereğli Harbour (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	1933-2115	2024
2	2094-2169	2132
3	3264-3424	3344
4	4065-4061	4063
5	1450-1598	1524
6	2167-2009	2088
7	1000-968	984
8	1570-1428	1499
9	1508-1570	1539
10	1852-1669	1761
11	661-660	661
12	2847-2798	2823
Average Activity ± Standard Error of Mean n= 12		2037±280

Table 3.12. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Gülüç Stream Mouth (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	4734-4701	4718
2	3271-3172	3222
3	3963-3789	3876
4	458-431	445
5	1491-1438	1465
6	2223-2044	2134
7	3256-3139	3198
8	3648-3491	3570
9	2229-2342	2286
10	777-789	783
11	870-870	870
Average Activity ± Standard Error of Mean n= 11		2415±426

Table 3.13. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Melen Stream Mouth (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	278-278	278
2	456-442	449
3	1471-1460	1466
4	441-477	459
5	850-875	863
6	564-571	568
7	866-899	883
8	2246-2169	2208
9	192-182	187
Average Activity ± Standard Error of Mean n= 9		818±217

Table 3.14. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Sakarya River Mouth (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	187-189	188
2	531-554	543
3	459-441	450
4	457-464	461
5	779-788	784
6	113-115	114
7	426-423	425
8	485-474	480
9	596-644	620
10	313-306	310
Average Activity ± Standard Error of Mean n= 10		438±62

Table 3.15. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Amasra (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	416-410	413
2	476-444	460
3	408-395	402
Average Activity ± Standard Error of Mean n= 3		425±18

Table 3.16. EROD activities of flathead mullets (*Mugil cephalus*) caught from Amasra (August 2006).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	474-511	493
2	547-556	552
3	541-501	521
4	577-618	598
Average Activity ± Standard Error of Mean n= 4		541±22

Table 3.17. The average EROD activities of mullets caught from different stations of the West Black Sea Region of Turkey (August 2006).

Sampling Stations	Fish Species	Number of Fish Liver	Average EROD Activity ± Standard Error of Mean
Zonguldak Harbour	*Mugil soiuy*	13	2559±315
Zonguldak Harbour	*Liza aurata*	9	2302±240
Ereğli Harbour	*Liza aurata*	12	2037±280
Gülüç Stream Mouth	*Mugil soiuy*	11	2415±426
Melen Stream Mouth	*Mugil soiuy*	9	818±217
Sakarya River Mouth	*Mugil soiuy*	10	438±62
Amasra	*Mugil soiuy*	3	425±18
Amasra	*Mugil cephalus*	4	541±22

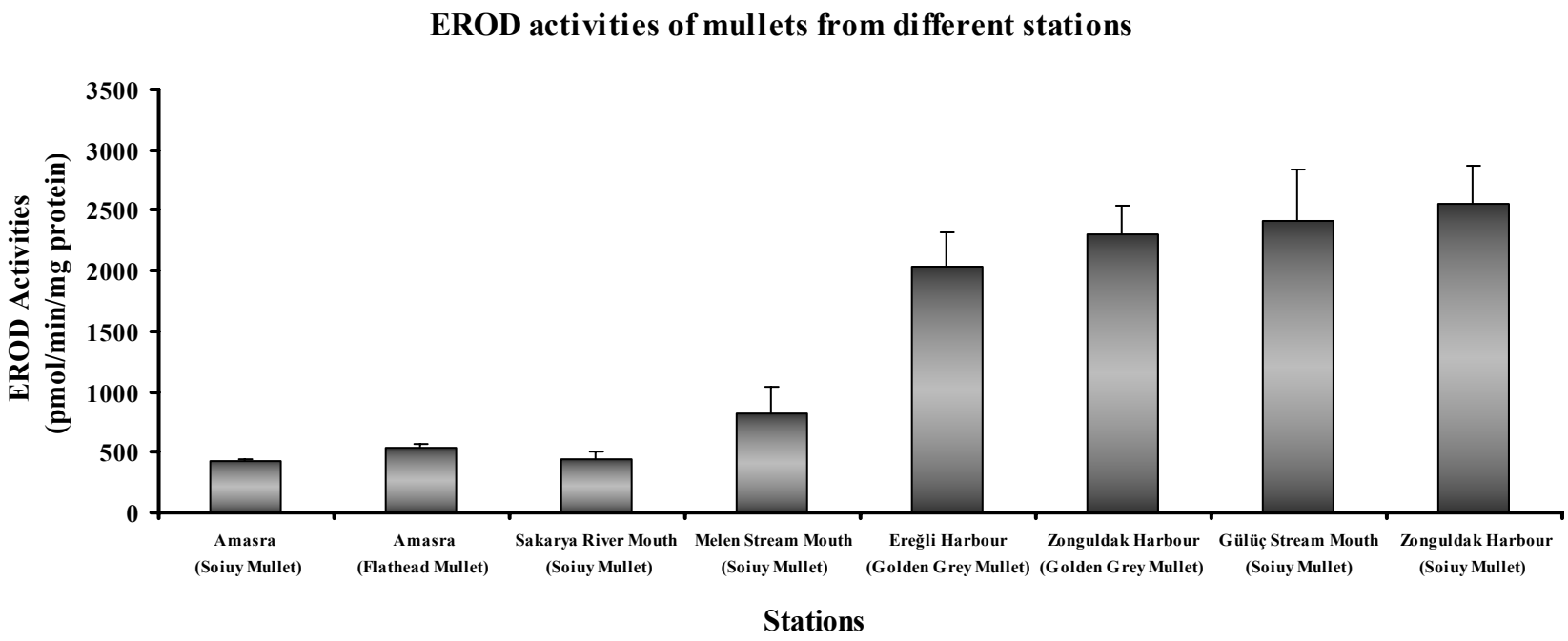


Figure 3.2. EROD activities of mullets collected from different stations of the West Black Sea Region of Turkey (August 2006).

3.3. Liver Microsomal 7-Ethoxyresorufin O-Deethylase Activity of Mullets Caught from Different Stations of West Black Sea Region of Turkey in August 2007.

For this study, 19 mullets were caught from four different stations (Zonguldak Harbour, Ereğli Harbour, Sakarya River Mouth, Amasra) of the West Black Sea Region of Turkey. Table 3.18, 3.19, 3.20 and 3.21 show microsomal EROD activities obtained from the livers of each fish caught from the four different stations along the West Black Sea Region of Turkey. All activities were measured in duplicates.

The average EROD activities of liver microsomes of mullets caught from four different stations of the West Black Sea Region of Turkey were summarized in Table 3.22 and presented in Figure 3.3 as a graph. EROD activities were represented as mean ± standard error of mean.

Flathead mullets that caught from Zonguldak Harbour showed the highest EROD activities (3017±565 pmole/min/mg protein, n=5) which were followed by the EROD activities of golden grey mullets caught from Ereğli Harbour (2233±464 pmole/min/mg protein, n=5) and soiuy mullet caught from Sakarya River Mouth (1642±470, n=4). Table 3.18, 3.19 and 3.20 show the EROD activities of liver microsomes of each mullets caught from Zonguldak Harbour, Ereğli Harbour and Sakarya River Mouth, respectively. The lowest microsomal EROD activities were measured in golden grey mullets from Amasra (259±72 pmole/min/mg protein, n=5) in 2007 (Table 3.21).

Table 3.18. EROD activities of flathead mullets (*Mugil cephalus*) caught from Zonguldak Harbour (August 2007).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	929-844	887
2	3208-3081	3145
3	4279-4176	4228
4	3574-3545	3560
5	3175-3351	3263
Average Activity ± Standard Error of Mean n= 5		3017±565

Table 3.19. EROD activities of golden grey mullets (*Liza aurata*) caught from Ereğli Harbour (August 2007).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	3058-3121	3090
2	1619-1466	1543
3	1498-1486	1492
4	1424-1434	1429
5	3670-3548	3609
Average Activity ± Standard Error of Mean n= 5		2233±464

Table 3.20. EROD activities of soiuy mullets *(Mugil soiuy)* caught from Sakarya River Mouth (August 2007).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	1407-1408	1408
2	2057-2057	2057
3	2624-2670	2647
4	452-455	454
Average Activity ± Standard Error of Mean n= 4		1642±470

Table 3.21. EROD activities of golden grey mullets (*Liza aurata*) caught from Amasra (August 2007).

Sample Number	Activity (pmol/min/mg protein)	Average Activity (pmol/min/mg protein)
1	460-446	453
2	152-156	154
3	412-421	417
4	143-152	148
5	124-120	122
Average Activity ± Standard Error of Mean n= 5		259±72

Table 3.22. The average EROD activities of mullets caught from different stations of the West Black Sea Region of Turkey (August 2007).

Sampling Stations	Fish Species	Number of Fish Liver	Average EROD Activity ± Standard Error of Mean
Zonguldak Harbour	*Mugil cephalus*	5	3017±565
Ereğli Harbour	*Liza aurata*	5	2233±464
Sakarya River Mouth	*Mugil soiuy*	4	1642±470
Amasra	*Liza aurata*	5	259±72

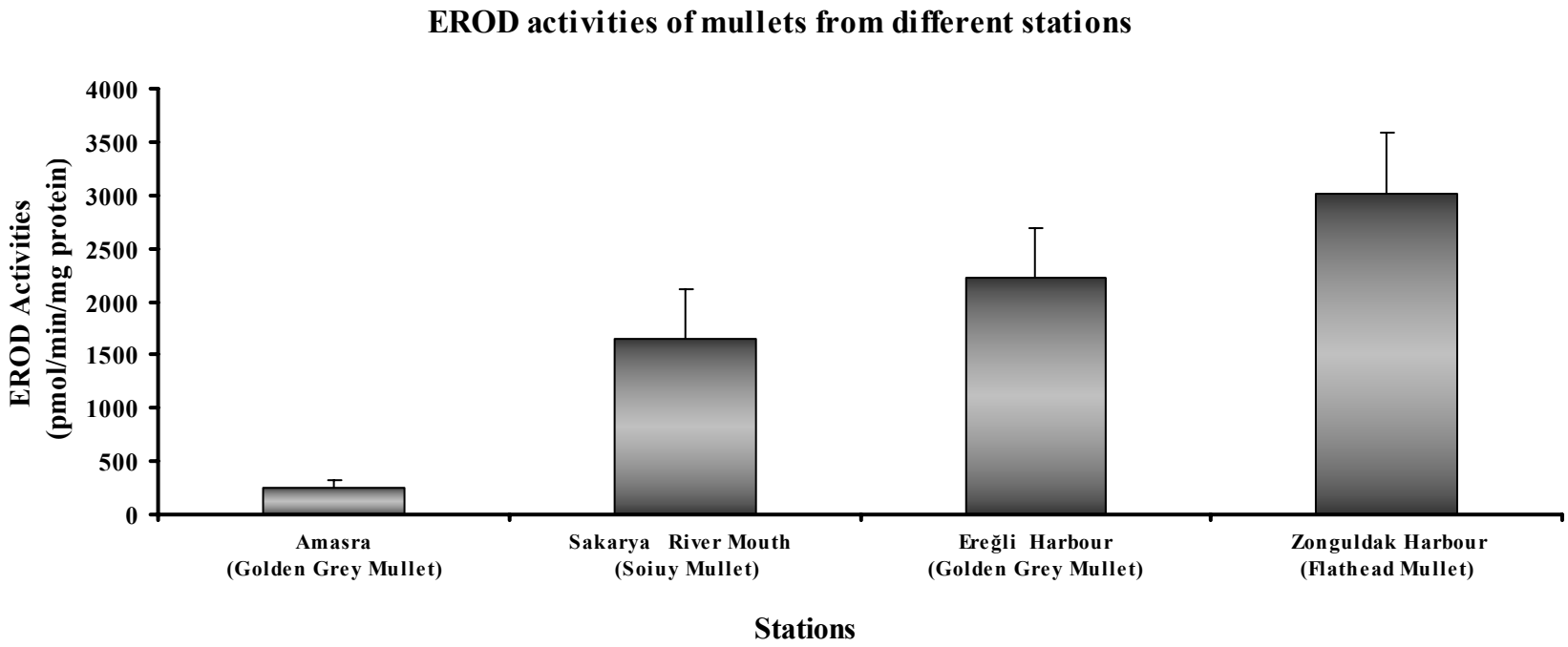


Figure 3.3. EROD activities of mullets collected from different stations of the West Black Sea Region of Turkey (August 2007).

3.4. Western Blot Analysis of Fish

Cytochrome P4501A protein level was determined by western blot analysis using a polyclonal anti-mullet P4501A1 IgG and anti-rabbit IgG-ALP as primary and secondary antibodies, respectively. Polyclonal anti–mullet P4501A1, was prepared by Dr. Alaattin Şen in the Department of Biology in Middle East Technical University, showed strong cross-reaction with liver microsomes of three fish mullet species, soiuy mullet *(Mugil soiuy)*, flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*).

The western blot analyses were performed for some mullet samples caught in each sampling years. Microsomal CYP1A protein levels were quantified by densitometry and the intensity of each band was quantified as an arbitrary unit, relative peak area (R.P.A) by Scion Image software program.

Cross-reactivities of cytochrome P4501A in liver microsomes of mullets caught in 2005 by western blot analysis were given in Figure 3.4. In table 3.23, CYP1A protein levels and respective EROD activities were presented.

In 2005, polyclonal anti-mullet P4501A1 showed strong cross-reactivity with liver microsomes of the mullet caught from Zonguldak Harbour (lane 7, 8), Ereğli Harbour (lane 10, 11) and Gülüç Stream Mouth (lane 5, 6). In comparison with these sampling stations, liver microsomes of the mullets caught from Melen Stream Mouth (lane 3, 4) and Sakarya River Mouth (lane 1, 2) showed moderate cross reactivity

with polyclonal anti-mullet P4501A1. Polyclonal anti-mullet P4501A1 showed very low cross-reactivity with the liver microsomes of mullets caught from Amasra (lane 9, 12).

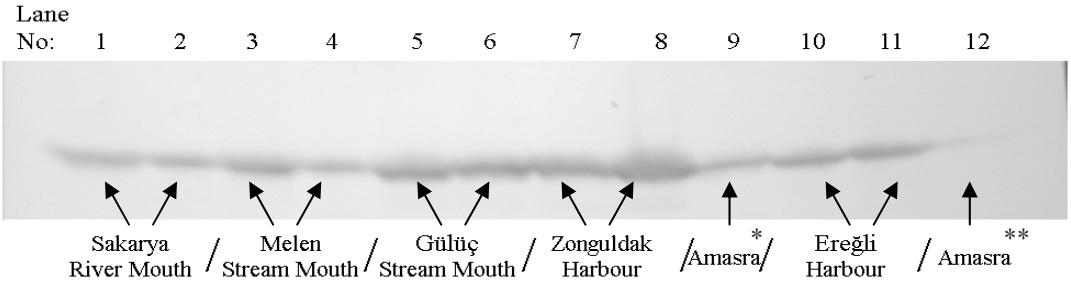


Figure 3.4. Immunochemical detection of liver microsomal cytochrome P4501A of mullets caught from the different stations of the West Black Sea Region of Turkey (August 2005). An 80 µg microsomal protein was applied to each slot.

* Sampled in 2006

** Sampled in 2007

Table 3.23. EROD activities and band intensities of each sample in the western blot analysis of mullet samples caught in 2005.

Lane No	EROD Activity (pmol/min/mg protein)	Relative Peak Area (R.P.A)
1	1379	1874
2	975	1520
3	1955	2247
4	1000	1357
5	3045	2664
6	2608	2603
7	2485	2674
8	3735	4210
9	413	976
10	1925	2430
11	2875	2659
12	148	152

In Figure 3.5, microsomal CYP1A protein levels and respective EROD activities from the fish samples caught in 2005 were given together. Figure 3.6 shows the correlation between CYP1A protein levels and microsomal EROD activities.

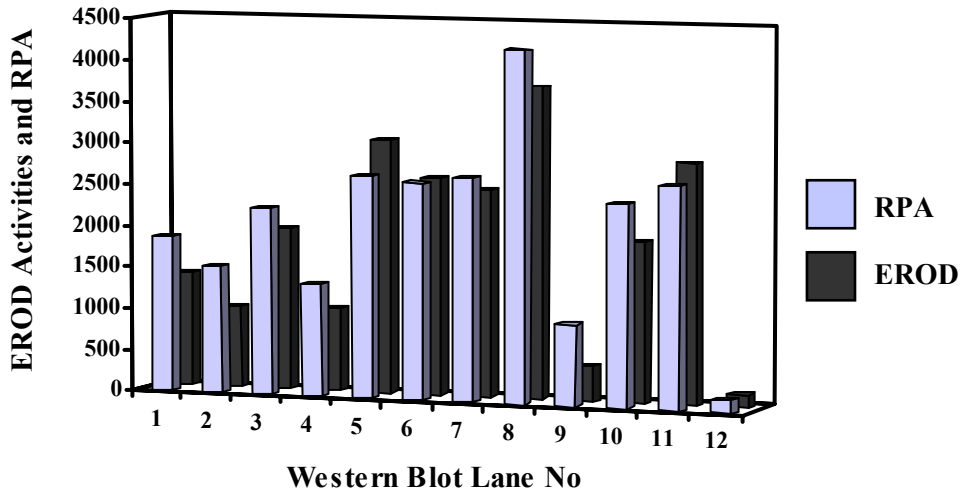


Figure 3.5. Microsomal CYP1A protein levels of mullets caught in 2005 and respective EROD activities.

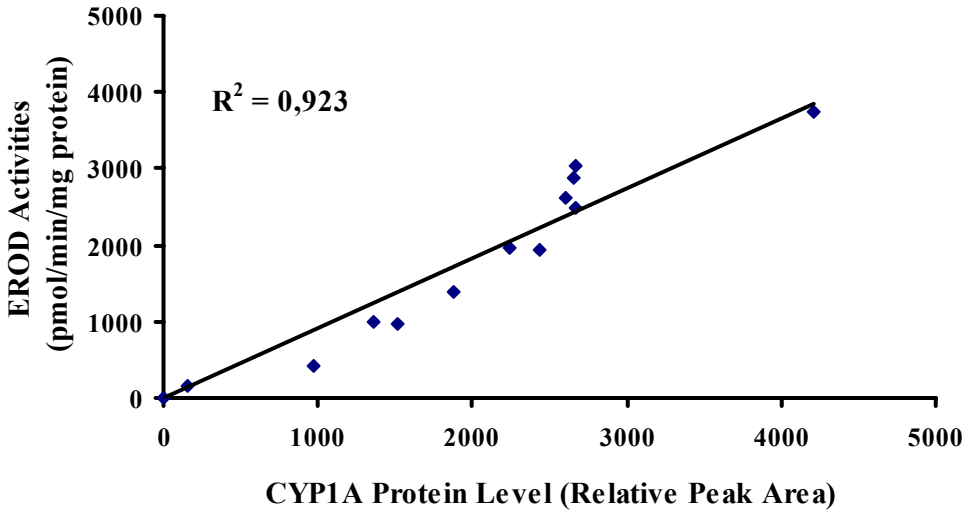


Figure 3.6. Correlation between CYP1A protein levels and microsomal EROD activities.

Cross-reactivities of cytochrome P4501A in liver microsomes of mullets caught in 2006 were given in Figure 3.7 and results were represented for each lane with respective EROD activities in Table 3.24. In 2006, polyclonal anti-mullet P4501A1 showed strong cross-reactivity with liver microsomes of the mullet caught from Zonguldak Harbour (lane 6, 7, 8 and 9), Gülüç Stream Mouth (lane 4, 5) and Ereğli Harbour (lane 9,10), respectively. Compare to these sampling stations, liver microsomes of the mullets caught from Melen Stream Mouth (lane 3) and Sakarya River Mouth (lane 1) showed moderate cross reactivity with polyclonal anti-mullet P4501A1. Mullets caught from Amasra, showed very low microsomal cross reactivity with polyclonal anti-mullet P4501A1.

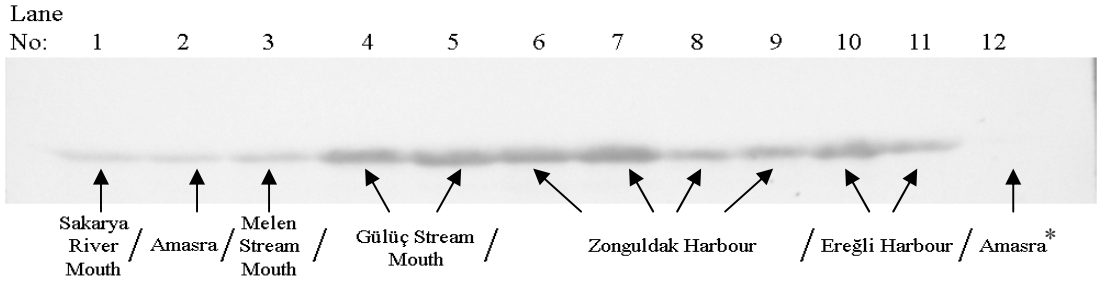


Figure 3.7. Immunochemical detection of liver microsomal cytochrome P4501A of mullets caught from the different stations of the West Black Sea Region of Turkey (August 2006). An 80 µg microsomal protein was applied to each slot.

* Sampled in 2007

Table 3.24. EROD activities and band intensities of each sample in the western blot analysis of mullet samples caught in 2006.

Lane No	EROD Activity (pmol/min/mg protein)	Relative Peak Area (R.P.A)
1	784	450
2	402	273
3	863	554
4	3570	1384
5	3876	1596
6	3346	1511
7	4511	1894
8	2203	930
9	2567	1079
10	4063	1543
11	2823	882
12	148	47

In Figure 3.8, microsomal CYP1A protein levels and respective EROD activities from the fish samples caught in 2006 were given together. Figure 3.9 shows the correlation between CYP1A protein levels and microsomal EROD activities.

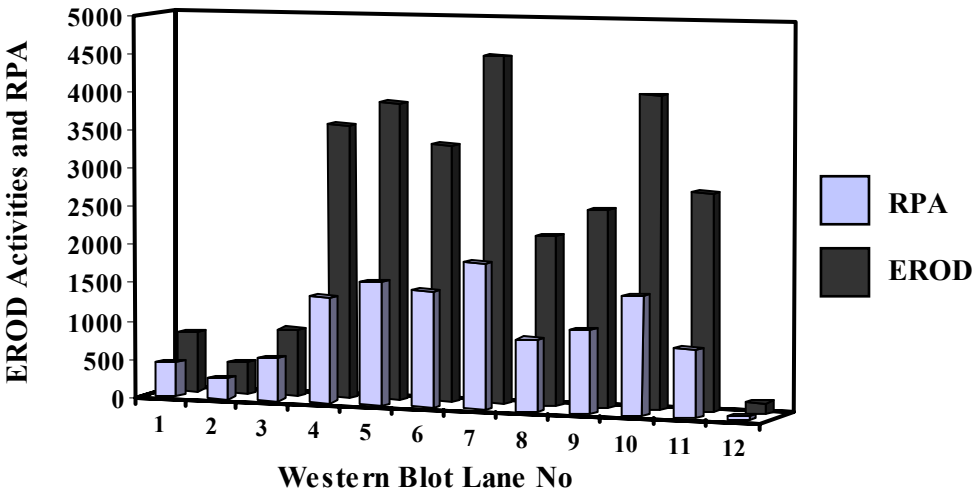


Figure 3.8. Microsomal CYP1A protein levels of mullets caught in 2006 and respective EROD activities.

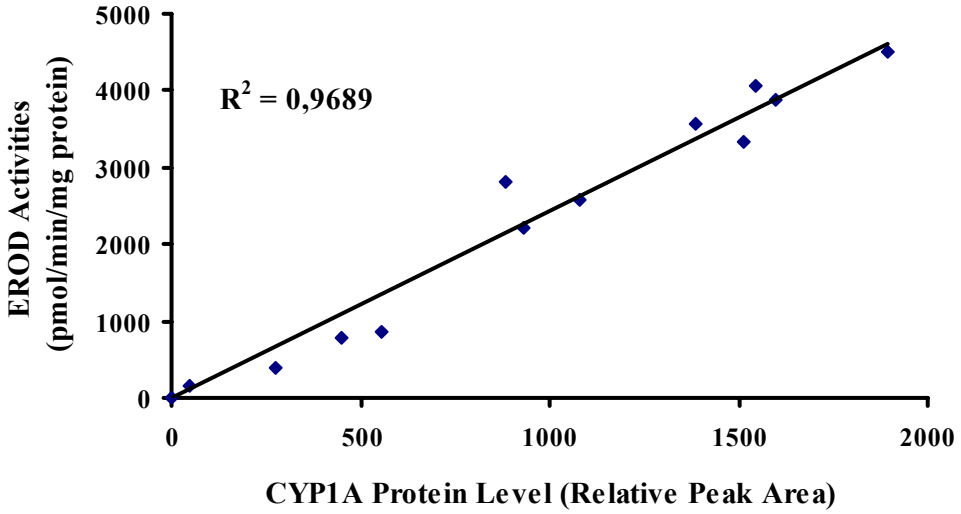


Figure 3.9. Correlation between CYP1A protein levels and microsomal EROD activities.

Cross-reactivities of cytochrome P4501A in liver microsomes of mullets caught in 2007 were given in Figure 3.10 and results were represented for each lane with respective EROD activities in Table 3.25. In this year, polyclonal anti-mullet P4501A1 showed strong cross-reactivity with liver microsomes of the mullet caught from Zonguldak Harbour (lane 10, 11) and Ereğli Harbour (lane 3, 4). Liver microsomes of the mullets caught from Sakarya River Mouth (lane 7, 8) showed moderate cross reactivity with polyclonal anti-mullet P4501A1 compare to Zonguldak and Ereğli. The liver microsomes of the mullets caught from Amasra (lane 1, 2, 5, 6, 9, and12) showed very low cross reactivity with polyclonal anti-mullet P4501A1.

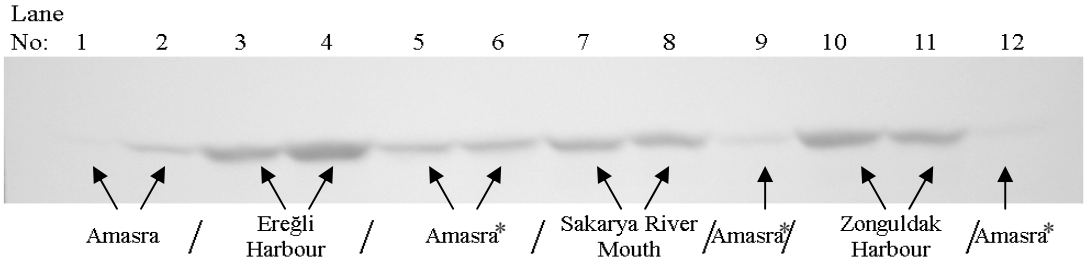


Figure 3.10. Immunochemical detection of liver microsomal cytochrome P4501A of mullets caught from the different stations of the West Black Sea Region of Turkey (August 2007). An 80 µg microsomal protein was applied to each slot.

* Sampled in 2006

Table 3.25. EROD activities and band intensities of each sample in the western blot analysis of mullet samples caught in 2007.

Lane No	EROD Activity (pmol/min/mg protein)	Relative Peak Area (R.P.A)
1	148	75
2	453	379
3	3090	1836
4	3609	2449
5	402	669
6	413	741
7	2057	1232
8	2647	1250
9	521	319
10	3263	2155
11	3145	1875
12	493	230

In Figure 3.11, microsomal CYP1A protein levels and respective EROD activities from the fish samples caught in 2007 were given together. Figure 3.12 shows the correlation between CYP1A protein levels and microsomal EROD activities.

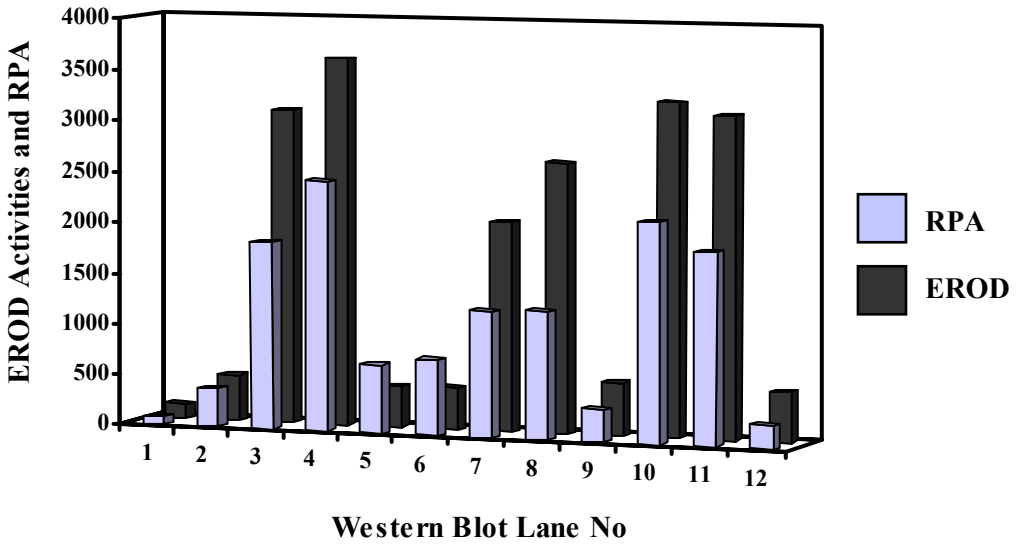


Figure 3.11. Microsomal CYP1A protein levels of mullets caught in 2007 and respective EROD activities.

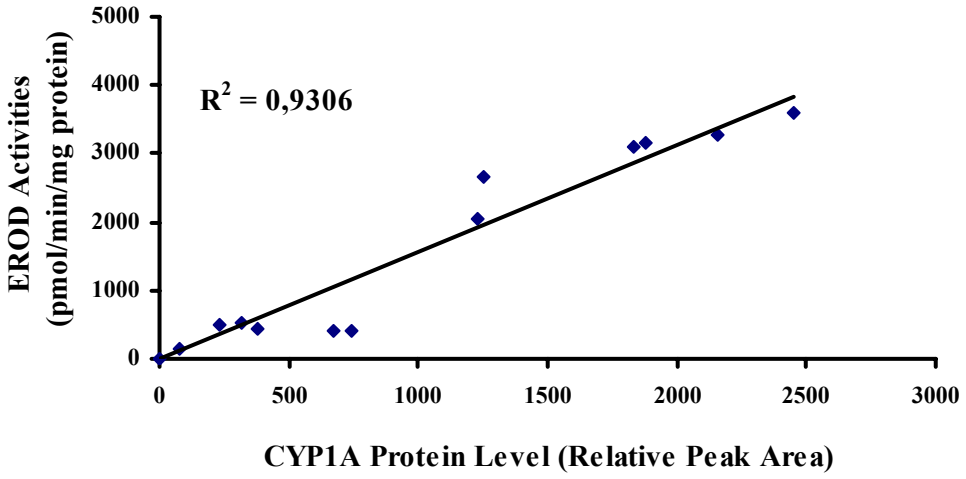


Figure 3.12. Correlation between CYP1A protein levels and microsomal EROD activities.

3.5. Total Polyaromatic Hydrocarbon Concentrations of Fish Liver Tissues

Total polyaromatic hydrocarbon (PAH) concentrations in the liver tissues of fish caught in 2006 were determined to show the presence of one of CYP1A inducer chemicals in sampling stations by using a spectrofluorometric method described by UNEP, 1992. Total PAH concentrations were calculated by comparing the fluorescence of the "chrysene equivalent". Total PAH concentrations were determined in individual fish liver and expressed as μg/g dried liver weight.

Total PAH concentrations were determined in 69 mullets that were caught from six different stations (Zonguldak Harbour, Ereğli Harbour, Gülüç Stream Mouth, Melen Stream Mouth, Sakarya River Mouth, Amasra) of the West Black Sea Region of Turkey.

The average concentrations of the total PAH results obtained from the livers of each fish caught from six different stations along the West Black Sea Region of Turkey were shown in Table 3.26 and presented in Figure 3.13 as a graph. The average concentrations of the total PAH were represented as mean ± standard error of mean. Soiuy mullets and golden grey mullets caught from Zonguldak Harbour displayed highly elevated total PAH concentrations which were 54±5 μg/g dried liver weight (n=13) and 47±6 μg/g dried liver weight (n=9), respectively. Soiuy mullets caught from Gülüç Stream Mouth had also high total PAH concentrations (35±3 μg/g dried liver weight, n=11) which were followed by golden grey mullets caught from Ereğli Harbour (28±3 μg/g dried liver weight, n=12), soiuy mullets caught from Melen Stream Mouth (25±3 μg/g dried liver weight, n=9) and soiuy mullets caught

from Sakarya River Mouth (21±3 μg/g dried liver weight, n=8). The lowest total PAH concentrations were measured in soiuy mullets and flathead mullets caught from Amasra which were 12±2 μg /g dried liver weight (n=3) and 11±2 μg/g dried liver weight (n=4), respectively.

Table 3.26. The average total PAH concentrations in the livers of the mullets caught from different stations of the West Black Sea Region of Turkey in 2006.

Sampling Stations	Fish Species	Number of Fish Liver	Average PAH conc. μg/g dried weight ± Standard Error of Mean
Zonguldak Harbour	Soiuy Mullet (*Mugil soiuy*)	13	54±5
Zonguldak Harbour	Golden Grey Mullet (*Liza aurata*)	9	47±6
Ereğli Harbour	Golden Grey Mullet (*Liza aurata*)	12	28±3
Gülüç Stream Mouth	Soiuy Mullet (*Mugil soiuy*)	11	35±3
Melen Stream Mouth	Soiuy Mullet (*Mugil soiuy*)	9	25±3
Sakarya River Mouth	Soiuy Mullet (*Mugil soiuy*)	8	21±3
Amasra	Soiuy Mullet (*Mugil soiuy*)	3	12±2
Amasra	Flathead Mullet (*Mugil cephalus*)	4	11±2

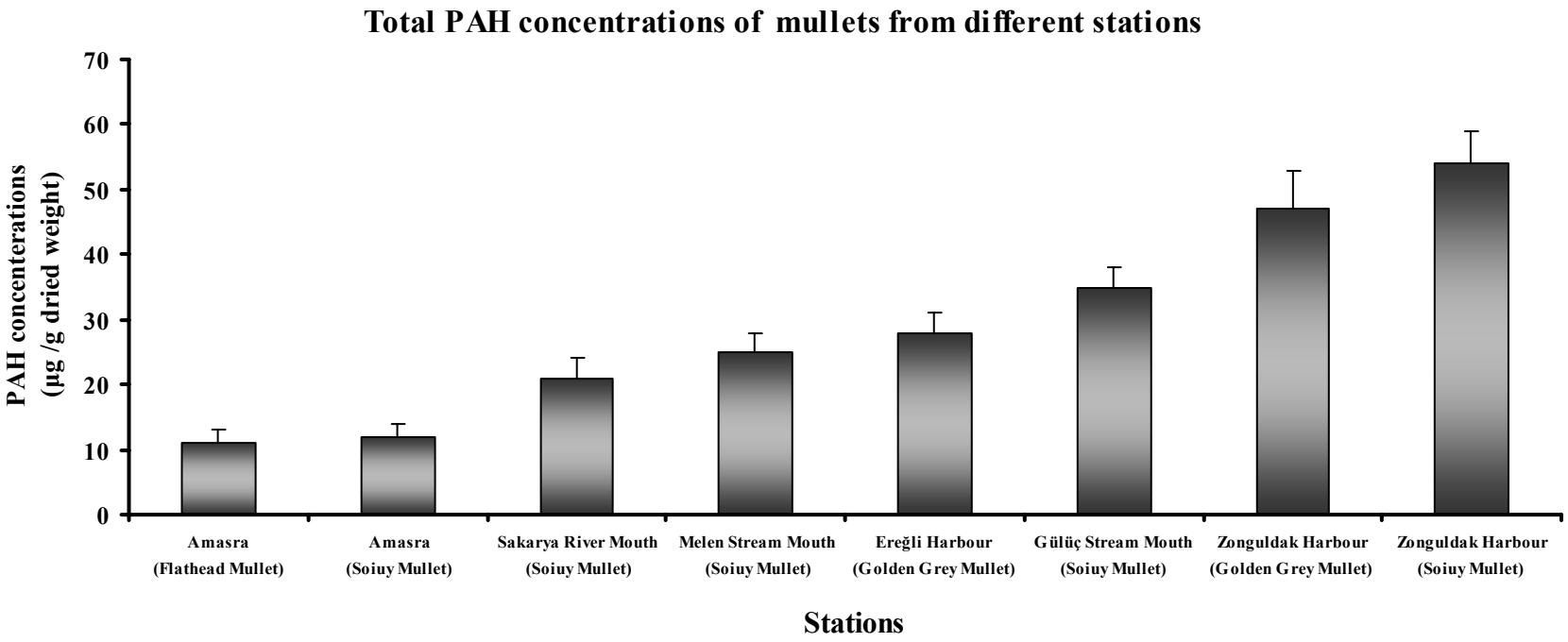


Figure 3.13. Total PAH concentrations in the livers of mullets collected from different stations of the West Black Sea Region of Turkey (August 2006).

DISCUSSION

The Black Sea has been increasingly threatened by pollutants over the past decades due to several factors such as uncontrolled shipping, unmanaged fishing, accidental crude oil spills and dumping of toxic industrial wastes and discharge of domestic wastes from coastal settlements (Mee, 1992). The importance of sea pollution and the effects of pollutants on the ecosystem of the Black Sea have been recognized by coastal countries. They became more co-operative in taking joint actions to protect the Black Sea against pollution. The first joint action was held in Bucharest in 1992 that formed the basis for future actions to protect the Black Sea. Another meeting concerning such actions was held in Oddesa in 1993, where the ministers of the participating countries signed a declaration. In order to provide a practical program of actions for cleaning up and protecting the Black Sea, the Black Sea Environmental Program (BSEP) was founded in 1993. The BSEP assisted the countries to prepare the Black Sea Action Plan. A Strategic Action Plan was developed based on the recommendations in the Bucharest Convention and Odessa Declaration and was signed by the countries surrounding the Black Sea in İstanbul in 1996.

After the Bucharest Convention and Odesa Declaration, numbers of studies on the environmental pollution have increased in the participant countries. Concentrations of inorganic pollutants, polyaromatic hydrocarbons, pesticides and

PCBs have been measured in sea water and sediment in different sites of the Black Sea. The results of these studies were presented in Black Sea Transboundary Diagnostic Analysis Report (2007). According to the results of chemical analysis, oil pollution threats Black Sea coastal ecosystems. Other toxic substances such as pesticides and heavy metals do not appear to pollute the entire Black Sea but appear as "hot spots" (places where pollution levels are unacceptably high) near well-identified sources. Among these pollutants, pesticides are mostly introduced through rivers and streams discharging from agriculture.

The studies on the Turkish Coasts of the Black Sea have demonstrated that the Kızılırmak, Yesilırmak, Sakarya, Filyos and Gülüç Rivers were important sources for Cd, Cu, Pb and Zn (Tuncer et al., 1998; Barlas, 1999). In addition to inorganic pollutants, the presence of pesticides and PCBs, including lindane, heptachlor, heptachlor epoxy, aldrin, dieldrin, endrin, DDE, DDD and DDT have been shown in different studies in the Black Sea (Tuncer et al., 1998; Bakan and Ariman, 2004). The use of DDT in Turkey has been banned for the last 30 years. Despite this restriction, Tuncer and coworkers have shown that DDT is present in Turkish rivers, streams and domestic and industrial discharges, which indicates their illegal use. Readman and coworkers have shown the presence of polyaromatic hydrocarbons in the sediment of Bosphorus Strait (2002). In another study, polyaromatic hydrocarbon level has been measured in the surface sediment taken from east-west transect in the southern half of the Black Sea. According to the results of this study, the level of polyaromatic hydrocarbon in the west part of the Black Sea is higher than that in the east part (Wakeham, 1996).

Organisms are often exposed to complex mixtures of pollutants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polyaromatic hydrocarbons (PAHs), alkyltin compounds, and metals. Even though chemical analysis in the sea water and sediment give valuable information about the levels of chemical pollutants, they do not reveal the impact of pollutants on organisms. Induction of CYP1A and associated 7-ethoxyresorufin O-deethylase activity (EROD) is the best characterized and used biochemical marker of exposure of fish to organic pollutants such as PCBs, PAHs and dioxins. CYP1A induction is used as a useful tool for the assessment of exposure to these types of organic pollutants and early warning sign for potential harmful effects of many organic molecules. The CYP1A response measured as EROD activity has been incorporated into some major monitoring programs, e.g., the National Status and Trends Program in the United States, the North Sea Task Force Monitoring Master Plan of the North Sea Nations in Europe, and the MED POL program for the study of pollutant effects in the Mediterranean Sea (Goksøyr and Förlin, 1992; Collier et al., 1992; Viarengo et al., 2000).

Some industrial areas, rivers and coastal cities in the Black Sea have tendency to create local pollution (Mee, 1992; Tuncer et al., 1998). Sakarya River, from its source in the West Anatolia, flows through many industrial and agricultural areas including Eskişehir and Adapazarı (Sakarya) and drains to the Black Sea. It has been well known that the Sakarya River carries many pollutants to the Black Sea from inner regions of Turkey (Tuncer et al., 1998; Barlas, 1999). Industrial areas found in Düzce and Adapazarı drain their discharges into Efteni Lake. The Melen Stream originates from this lake and flows through many industrial and agricultural areas.

Gülüç Stream is very close to Ereğli Harbour and Ereğli Iron and Steel Factory. It has been reported that this small stream was highly polluted primarily by domestic waste discharges from Ereğli and villages around the stream. It also flows through many agricultural areas. Tuncer and coworkers (1998) have reported for Gülüç Stream and the other small streams that drain to the Black Sea that if no action is taken to stop domestic waste discharges into these small streams, they will soon become open sewer. Zonguldak and Ereğli, with increasing population, shipping activities, uncontrolled discharges from industries and coastal settlements, are among these polluted local regions. Zonguldak is an important coal mining area in Turkey. Zonguldak suffers from particulate discharges from the thermal power plant and coal processing wastes, resulting in permanently elevated turbidity levels along the coast of the city (Tuncer et al., 1998).

In this study, our aim was to determine and monitor the degree of PAHs, PCBs and dioxins type pollutants in several locations in the West Black Sea Coast of Turkey by measuring cytochrome CYP1A associated 7-ethoxyresorufin O-deethylase (EROD) activity and immunochemical detection of CYP1A protein level in mullet liver microsomes and to find the places of areas that create local pollution in the West Black Sea Region of Turkey. In addition, total polyaromatic hydrocarbon levels were measured in the mullet liver samples to show the presence of one of CYP1A inducer chemicals in sampling stations and to find the extent of PAH contaminants in the caught mullet tissues.

Mullet is chosen as a test organism. Because it is resistant and tolerant to pollutants and various environmental conditions. It can live in polluted areas (Balık

et al., 1992; Arınç and Şen, 1999). It is also consumed in large quantities in Turkey. It is commonly used for monitoring environmental exposure of fish to contaminants (Arınç and Şen, 1999; Arınç et al., 2000; Ferreira et al., 2004). All the measurements were performed in the liver tissues. Liver plays an important role in vital functions of basic metabolism and it is the major organ of accumulation, biotransformation, and excretion of contaminants in fish.

In this study 150 fish samples were collected from the different stations of the West Black Sea Coast of Turkey. In all samples, EROD activities were measured. Figure 4.1 illustrates average EROD activity results of mullets caught from different stations of the West Black Sea Coast of Turkey in 2005, 2006 and 2007. The lowest EROD activities were measured in fish samples (soiuy mullet, flathead mullet and golden grey mullet) caught from Amasra. Amasra is the touristic place in the West Black Sea Region of Turkey. Since there were no industrial settlements and agricultural activities in this small town, this place appeared to be good reference site. Kefken is another touristic place in this region of Turkey. Kefken was considered as reference site at the beginning of this study. However, only one fish sample could be caught in this station and its EROD activity was not considered in the statistical analysis.

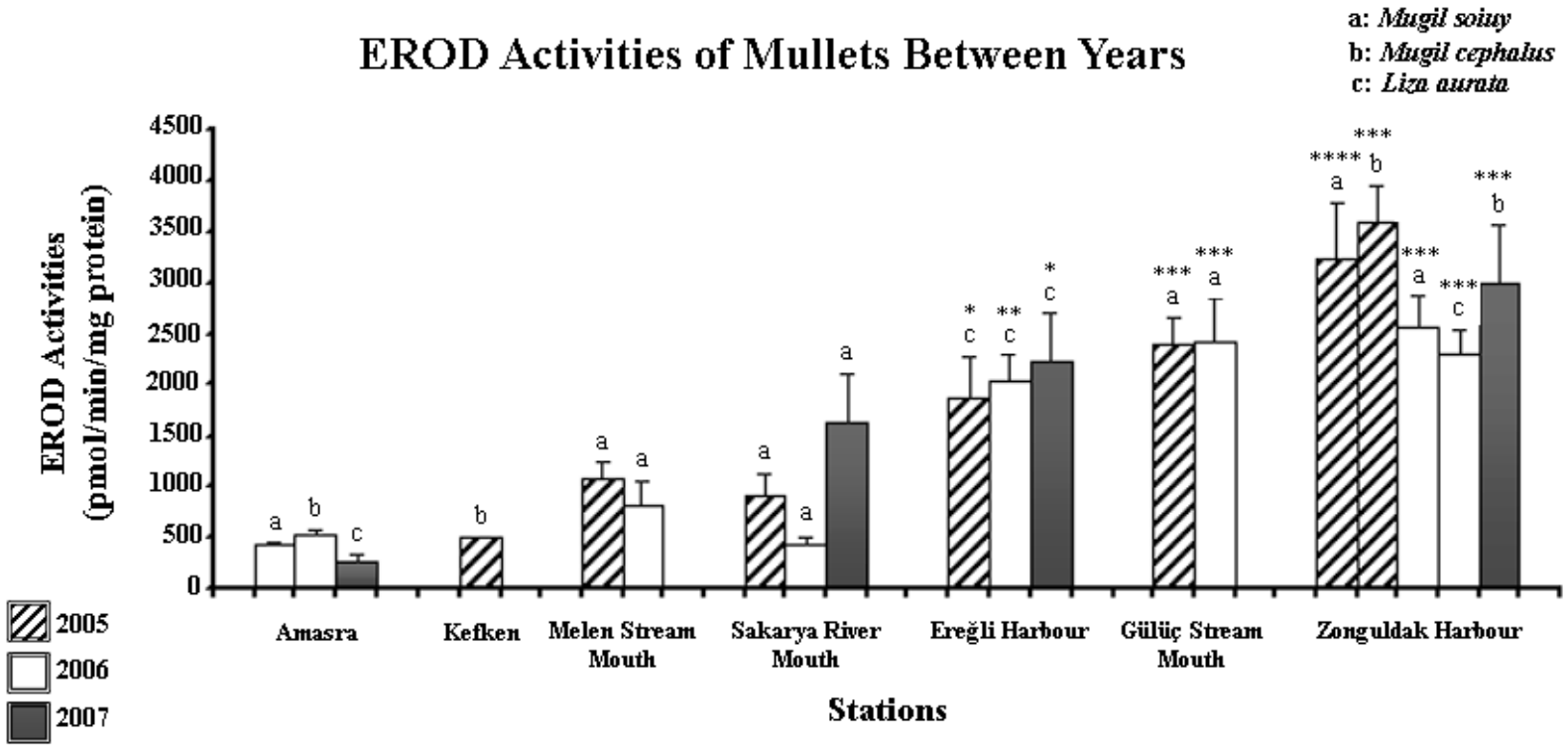


Figure 4.1. EROD activities of mullets sampled from different stations of the West Black Sea Region of Turkey in 2005, 2006 and 2007.

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

*** Significantly different from the lowest values (reference site) with P<0.005

** Significantly different from the lowest values (reference site) with P<0.01

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

The highest liver microsomal EROD activities of soiuy mullets, flathead mullets, and golden grey mullets were recorded in Zonguldak Harbour in all sampling years. EROD activities of soiuy mullets caught from Zonguldak Harbour were 3237±539 pmole/min/mg protein, n=7 in 2005 which were about 8 times higher than the value of soiuy mullets sampled from reference station, Amasra (425±18 pmole/min/mg protein, n=3). In the same year, flathead mullets had also high EROD activities (3600±368 pmole/min/mg protein, n=2) in Zonguldak Harbour which were about 7 times higher than the value of flathead mullets sampled from Amasra (541±22 pmole/min/mg protein, n=4). In 2006, EROD activities of soiuy mullets caught from Zonguldak Harbour were 2559±315 pmole/min/mg protein, n=13 which were about 6 times higher compared with the value of soiuy mullets sampled from reference station, Amasra (425±18 pmole/min/mg protein, n=3). In the same year, golden grey mullets had also high EROD activities (2302±240 pmole/min/mg protein, n=9) in Zonguldak Harbour which were about 9 times higher than the value of golden grey mullets sampled from Amasra (259±72 pmole/min/mg protein, n=5). In 2007, flathead mullets caught from Zonguldak Harbour had high EROD activities (3017±565 pmole/min/mg protein, n=5), which were 6 times higher than flathead mullets caught from Amasra (541±22 pmole/min/mg protein, n=4). This high EROD activity results clearly indicated that Zonguldak Harbour is highly contaminated with PAHs/PCBs and dioxins type pollutants.

Golden grey mullets sampled from Ereğli Harbour, which was another Harbour in this study, also displayed high microsomal EROD activities in all sampling years. In 2005, high EROD activities were measured in golden grey mullets sampled from Ereğli Harbour (1871±391 pmole/min/mg protein, n=16), which were

7 times higher than golden grey mullets caught from Amasra (259±72 pmole/min/mg protein, n=5). In 2006 and 2007, golden grey mullets continue to exhibit high EROD activities (2037±280 pmole/min/mg protein, n=12 and 2233±464 pmole/min/mg protein, n=5, respectively) which were 7.9-8.6 times higher than golden grey mullets caught from Amasra.

Gülüç Stream Mouth is the other highly polluted station in this study. In 2005 and 2006, soiuy mullets caught from the mouth of this stream exhibited high EROD activities (2402±261 pmole/min/mg protein, n=13 and 2415±426 pmole/min/mg protein, n=11, respectively) which were 6 times higher than the value of soiuy mullets sampled from reference station (425±18 pmole/min/mg protein, n=3).

Similar to Gülüç Stream Mouth, soiuy mullets caught from Melen Stream Mouth in 2005 and 2006. EROD activities of soiuy mullets caught from this stream were 1065±176 pmole/min/mg protein, n=11 and 818±217 pmole/min/mg protein, n=9, respectively. These values were 2-2.5 times higher than the EROD activities measured in soiuy mullets from Amasra.

The most interesting EROD activity results were obtained in the mullet samples caught from Sakarya River Mouth between the sampling years. In 2005, EROD activities of soiuy mullets caught from Sakarya River Mouth were 904±216 pmole/min/mg protein, n=10, which were 2 times higher than the value of soiuy mullets sampled from Amasra (425±18 pmole/min/mg protein, n=3). In 2006, EROD activities decreased (438±62 pmole/min/mg protein, n=10). However, EROD activities of the soiuy mullets were 1642±470 pmole/min/mg protein, n=4, in 2007,

which were 4 times higher than the value of soiuy mullets sampled from Amasra. It is well known that Sakarya River brings many pollutants from the inner sites of Turkey. The reason of this fluctuation in the EROD activities between the years is most probably related with the individual extent of CYP1A inducer chemicals. There are several rehabilitation studies in some cities that Sakarya River passes through. As a result of rehabilitation studies, the levels of some pollutants may change between years.

CYP1A protein level was also determined in mullet liver microsomes using the polyclonal antibodies produced against leaping mullet cytochrome P4501A. Polyclonal antibody anti-mullet P4501A showed strong cross-reactivity with liver microsomes of soiuy mullets, flathead mullets and golden grey mullets caught from different stations of the West Black Sea. The CYP1A band in microsomal preparations of mullets from Zonguldak and Ereğli Harbours and Gülüç Stream Mouth was stained more intensely than the reference station, Amasra. Compare to these sampling stations, liver microsomes of the mullets caught from Melen Stream Mouth and Sakarya River Mouth showed moderate cross reactivity with polyclonal anti-mullet P4501A. Liver microsomes of mullets from the reference station (Amasra) show low cross-reactivity with polyclonal antibody anti-mullet P4501A. The relationship between CYP1A protein levels and EROD activities was also examined with correlation analysis. CYP1A protein levels are highly correlated with the EROD activity results in the mullet samples caught from different stations of the West Black Sea (R^2 ranged between 0.92-0.97).

Thus, two biochemical indices, highly induced EROD activities and elevated CYP1A protein levels in the livers of mullets caught from Zonguldak and Ereğli Harbours, and Gülüç Stream Mouth, indicated that these sites are highly contaminated with PAHs and/or PCBs and possibly other toxic compounds. The induction of CYP1A protein level and its associated enzyme activity, EROD, have been reported in a number of field studies in fish exposed to PAHs, PCBs and/or dioxins type contaminants (Ronis et al., 1992; Addison et al., 1994; Addison and Fraser, 1996; Arınç and Şen, 1999; Arınç et al., 2000; Miller et al., 2003; Martínez-Gómez et al., 2006). İzmir Bay is one of the most polluted sites in Turkey. Highly elevated EROD activities have been reported in mullets (*Liza saliens* and *Mugil cephalus*) caught from highly polluted site of İzmir Bay. EROD activities of *Liza saliens* were about 62 times higher than the value at the reference site (Arınç and Şen, 1999). EROD activities of *Mugil cephalus* caught from the same site were about 56 times higher than the value at the reference site (Arınç et al., 2000). Pollution response similar to those recorded in Zonguldak and Ereğli Harbours and Gülüç Stream have been reported in winter flounder collected from Sydney, Nova Scotia, Canada (X6 EROD), in English sole collected from British Columbia, near coastal pulp mills, Canada (X8 EROD), in channel catfish collected from Buyou Meto, Arkansas, USA (X6 EROD), in English sole collected from Vancouver Harbour (X4 EROD) and in two flat fish, *Lepidorhombus boscii* and *Callionymus lyra*, caught from northern Iberian Shelf (X2 and X4 EROD, respectively) (Ronis et al., 1992; Addison et al., 1994; Addison and Fraser, 1996; Arınç et al., 2000; Miller et al., 2003; Martínez-Gómez et al., 2006).

Cohen and coworkers have shown that coal by products such as coal-tar (a tar formed from distillation of coal and coal-tar can be further distilled to give various aromatic compounds) significantly induce EROD activity in medika liver (1994). In addition, Vignier and coworkers have demonstrated that EROD activity significantly increases in winter flounder caught from coal-tar contaminated estuary (1994). Creosote is a dark liquid made from coal-tar. It is a complex mixture of organic compounds, containing about 85% PAHs. Hyötyläinen and Oikari (1999) and Whyte and coworkers (2000) have demonstrated in different studies that the extract of creosote-contaminated sediments highly induce EROD activities in rainbow trout. Highly induced EROD activity results obtained in this study clearly indicates that coal mining activities create sea pollution in Zonguldak Harbour. Recently, the surface sediment of Zonguldak Harbour has been collected and the excavated sediment has been dumped into the open sea in the Black Sea. It is clear that this sediment will create pollution in the open sea in the years to come.

Besides these biochemical parameters, total PAH concentrations were determined in fish caught in 2006 to show the presence and extent of one of the CYP1A inducer organic pollutants in the sampling stations. The lowest total PAH concentration was measured in fish samples (soiuy mullet and flathead mullet) caught from Amasra (12±2 μg/g dried liver weight, n=3 and 11±2 μg/g dried liver weight, respectively). This region was considered as reference site.

The highest total PAH concentrations of soiuy mullets and golden grey mullets were recorded in Zonguldak Harbour. Total PAH concentrations of soiuy mullets caught from Zonguldak Harbour were 54±5 μg/g dried liver weight, n=13

which were 4.5 times higher compared with the value of soiuy mullets sampled from reference station. Golden grey mullets caught from Zonguldak Harbour had also high total PAH concentrations which were 47±6 μg/g dried liver weight, n=9. Golden grey mullets sampled from Ereğli Harbour also displayed high total PAH concentrations which were 28±3 μg/g dried liver weight, n=12.

Total PAH concentrations were also measured in the mullet samples caught from river and stream mouths. Total PAH concentrations of soiuy mullets caught from Gülüç Stream Mouth were also higher than the value obtained from reference station. Total PAH concentrations were measured as 35±3 μg/g dried liver weight, n=11, which were 3 times higher than soiuy mullets caught from Amasra. Total PAH concentrations of soiuy mullets caught from Melen Stream Mouth were measured as 25±3 μg/g dried liver weight, n=9, which were 2 times higher than the reference station. Total PAH concentrations of soiuy mullets caught from Sakarya River Mouth were 21±3 μg/g dried liver weight, n=8, which were 1.75 times higher than soiuy mullets caught from Amasra.

The results of the total PAH analyses clearly indicated the presence of one of the CYP1A inducer chemicals in Zonguldak Harbour, Ereğli Harbour, Sakarya River, Gülüç and Melen Streams. In previously reported studies, Readman and coworkers have been shown the presence of polyaromatic hydrocarbons (Σ 17 isomers) in the surface sediments taken from several stations in the Black Sea. Highest concentrations have been reported at sites influenced by the Danube (30.5-608 ng/g dry weight), Odessa (66.9-635 ng/g dry weight), the Port of Sochi (61.9-368 ng/g dry weight) and Bosphorus (13.8-531 ng/g dry weight) (Readman et al.,

2002). Comparable distributions have been inferred by Wakeham working with "off-shore" sediments (1996). Total PAH concentrations in fish samples have been performed by Telli-Karakoç and coworkers in mullet samples caught from Trabzon, Yomra (2001). In our study, the highest total PAH concentrations measured in soiuy mullets and golden grey mullets caught from Zonguldak Harbour (54±5 μg/g dried liver weight, n=13 and 47±6 μg/g dried liver weight, n=9, respectively) are at the same order of magnitude of that reported by Telli-Karakoç and coworkers (2001) for mullets (*Mugil saliens*) (55.7±16.6 μg/g dried liver weight) caught from Trabzon, Yomra, indicating the presence of comparable amount of PAH contaminants in both of the coastline of these cities.

The results of this study clearly indicated that Zonguldak Harbour, Ereğli Harbour, Gülüç Stream, Sakarya River and Melen Stream have tendency to create local pollution in the West Black Sea Region of Turkey. It is well known that millions of liters of untreated sewage run into the rivers and harbours in the West Black Sea Region of Turkey. In Black Sea Transboundary Report, Zonguldak has been reported as "hot spots" (places where pollution levels are unacceptably high) with municipal pollution source type (2007). According to the result of our study, the other "hot spots" may be Ereğli Harbour and Gülüç Stream in the Black Sea with high PAHs, PCBs and other type pollutants. In addition, the results of this study clearly indicated that Melen Stream and Sakarya River also bring some PAHs, PCBs and other type pollutants from the inner parts of Anatolia and the Black Sea Region of Turkey.

CYP1A mostly activates certain classes of PAH pro-carcinogens and other chemicals by forming oxygenated compounds (Parke et al., 1991). Oxygenation of benzo[*a*]pyrene by CYP1A1 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 DNA damage (Stegeman, 1995). Induction of CYP1A1 has been correlated with the development of PAH-associated cancers and other disorders in mammals (Nebert, 1989). Fish is an important food source in many part of the world. Pollutants in fish can be harmful not only by their direct effects on the organisms, but also indirectly producing some diseases and developmental abnormalities in humans.

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