DETERMINATION OF THE LEVELS OF POLYCYCLIC AROMATIC HYDROCARBONS AND BIOMARKER ENZYME ACTIVITIES IN STRIPED RED MULLET (*MULLUS SURMULETUS*) CAUGHT FROM THE WEST BLACK SEA REGION OF TURKEY

by
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ABSTRACT

Determination of the Levels of Polycyclic Aromatic Hydrocarbons and Biomarker Enzyme Activities in Striped Red Mullet (*Mullus Surmuletus*) Caught from the West Black Sea Region of Turkey

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Induction of CYP1A and associated 7-ethoxyresorufin O-deethylase (EROD) activity are the best characterized and used biomarkers of exposure of fish to organic pollutants such as PCBs, PAHs and dioxins. The presence of CYP1A inducer chemicals in the West Black Sea coast of Turkey have been shown by EROD activity measurements in flathead mullet (*Mugil cephalus*), golden grey mullet (*Liza aurata*) and soiuy mullet (*Mugil soiuy*) in our previous study (Bozcaarmutlu et al., 2009). In this study, our aim was to assess the suitability of striped red mullet (*Mullus surmuletus*) in monitoring of environmental pollution and continue to monitor the pollution present in the West Black Sea coast of Turkey in flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) samples caught from the West Black Sea coast of Turkey. Fish samples were caught from five

different stations having a varying degree of pollution in the West Black Sea coast of Turkey in 2011. Highly elevated EROD activities were measured in all fish species caught from Zonguldak and Ereğli Harbours. The lowest EROD activities were measured in all fish samples caught from Amasra and Kefken. The differences in EROD activities between stations were at comparable values between striped red mullet and the other fish species. This result indicates that striped mullet is a suitable organisms to monitor CYP1A inducer chemicals. In addition to EROD activity measurements, the levels of glutathione S-transferase (GST), catalase and glutathione reductase activities were also measured in striped red mullet samples. The GST and glutathione reductase enzyme activities were insensitive to the pollutants present in different stations in the West Black Sea Region of Turkey. Besides these biochemical parameters, the concentrations of 13 different polycyclic aromatic hydrocarbons were also measured in fish liver tissues to determine the types of PAHs present in fish liver tissues. The concentrations of PAHs measured in striped red mullet were higher than those measured in flathead mullet and golden grey mullet in all sampling stations.

Keywords: Biomonitoring, Biomarkers, 7- Ethoxyresorufin O-deethylase (EROD), Glutathione S-transferase (GST), Glutathione Reductase, Flathead Mullet (*Mugil cephalus*), Black Sea, Catalase, Polycyclic Aromatic Hydrocarbons (PAHs), Golden Grey Mullet (*Liza aurata)*, Cytochrome P4501A (CYP1A), Striped Red Mullet (*Mulus surmuletus*).

ÖZET

Batı Karadeniz Bölgesi'nden Yakalanan Tekir Balıklarında (*Mullus Surmuletus*) Polisiklik Hidrokarbon Düzeylerinin ve Biyobelirteç Enzim Aktivitelerinin Belirlenmesi

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Yüksek Lisans, Kimya Bölümü

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CYP1A'nın indüklenmesi ve bununla ilgili 7-etoksirezorufin O-deetilaz (EROD) aktivitesi, balıkların PCB'ler, PAH'lar ve dioksinler gibi organik kirleticilere maruz kalma düzeyinin belirlenmesinde kullanılan en iyi çalışılmış biyobelirteçtir. Daha önce yaptığımız çalışmamızda Türkiye'nin Batı Karadeniz kıyılarında CYP1A protein düzeyinde artışa sebep olan kimyasalların varlığı has kefal balığı (*Mugil cephalus*), sarıyanak kefal balığı (*Liza aurata*) ve rus kefal balığı (*Mugil soiuy*) örneklerinde gösterilmişti (Bozcaarmutlu ve ark., 2009). Bu çalışmada amacımız tekir balığının (*Mullus surmuletus*) çevre kirliliğinin izlenmesinde uygunluğunu değerlendirmek ve Türkiye'nin Batı Karadeniz kıyısında var olan kirliliği has kefal balığı ve sarıyanak kefal balığı örneklerinde izlemeye devam etmektir. Balık örnekleri Türkiye'nin Batı Karadeniz kıyısında bulunan

farklı düzeyde kirli olan beş istasyondan 2011 yılında yakalanmıştır. Oldukça yüksek EROD aktiviteleri Zonguldak ve Ereğli Liman'larından yakalanan bütün balık türlerinde ölçülmüştür. En düşük EROD aktiviteleri Amasra ve Kefken'den yakalanan bütün balık türlerinde ölçülmüştür. Tekir balıklarında istasyonlar arasında ölçülen EROD aktivitesindeki fark diğer iki balık türü ile karşılaştırılabilecek düzeydedir. Bu sonuç bize tekir balığının CYP1A indükleyicisi kimyasalların izlenmesine uygun bir organizma olduğunu göstermiştir. EROD aktivitesi ölçümlerine ek olarak, glutathione S-transferaz (GST), katalaz ve glutatyon redüktaz enzim aktivitesi düzeyleri de tekir balığı örneklerinde ölçülmüştür. GST ve glutatyon redüktaz enzim aktivitelerinin Türkiye'nin Batı Karadeniz kıyılarında bulunan kirliliğe duyarlı olmadığı bulunmuştur. Biyokimyasal parametrelerin yanında, balık karaciğer dokularında bulunan PAH türlerini belirlemek için 13 farklı polisiklik aromatik hidrokarbon düzeyleri de ölçülmüştür. Bütün örnek toplanan bölgelerde tekir balıklarında ölçülen PAH düzeyleri has kefal ve sarıyanak kefal balıklarında ölçülen PAH düzeylerinden fazladır.

Anahtar Kelimeler: Biyomonitoring, Biyobelirteç, 7-Etoksirezorufin O-deetilaz (EROD), Glutatyon S-Transferaz (GST), Glutatyon Redüktaz, Has Kefal Balığı (*Mugil cephalus*), Karadeniz, Katalaz, Polisiklik Aromatik Hidrokarbonlar (PAH), Sarıyanak Kefal Balığı (*Liza aurata*), Sitokrom P4501A (CYP1A), Tekir Balığı (*Mullus surmuletus*).

DEDICATED TO MY FAMILY

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AChE	Acetylcholineesterase
ε-ACA	ε-Amino Caproic Acid
Arnt	Aromatic Hydrocarbon Receptor Nuclear Transporter
AhR	Aromatic Hydrocarbon Receptor
BSA	Bovine Serum Albumin
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CYP	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
EROD	7-Ethoxyresorufin O-deethylase
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione (Reduced form)
GSSG	Glutathione (Oxidized form)
GST	Glutathione S-Transferase
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic Acid
H_2O_2	Hydrogen Peroxide
HSP90	90 kDA Heat-Shock Protein
MFO	Mixed Function Oxidase
MO	Monooxygenase
NADH	Nicotinamide Adenine Dinucleotide (Reduced form)
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate (Oxidized form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced form)
OCP	Organochlorine Pesticides

PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl
PCDD	Polychlorinated Dibenzodioxins
PCDF	Polychlorinated Dibenzofuran
PMSF	Phenylmethanesulfonyl Fluoride
POP	Persistent Organic Pollutant
SOD	Superoxide Dismutase
Tris	2-amino-2-(hydroxymethyl)- propane-1,3-diol
TÜBİTAK	The Scientific and Technological Research Council of Turkey
UDP	Uridine Diphosphate
UNEP	United Nations Environment Programme
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
XAP2	X-associated Protein 2
XRE	Xenobiotic Response Element

INTRODUCTION

Life began in the marine environment and continues to exist in its diverse habitats. At the present time, marine environments are seriously getting affected by the oil spills, the dumping of toxic chemicals from industrial areas and the dumping of untreated sewage. Marine organisms are exposed to many different chemicals throughout their lives. Some of these chemicals may accumulate in these organisms. Since oceans and seas are the biggest natural sources of water and food, the determination of toxic chemicals and their effects in edible fish is important.

1.1. Persistent Organic Pollutants

Persistent organic pollutants (POPs) are a group of chemicals that have high lipid solubility. They are resistant to the biological, photolytic and chemical degradation processes. They are divided into two groups (Figure 1.1). The first group is intentionally produced persistent organic chemicals. This group covers organochlorine pesticides (OCP) and polychlorinated biphenyls (PCB). The second group is unintentionally produced persistent organic chemicals. The members of this group are polycyclic aromatic hydrocarbons (PAHs), polychlorinated dioxins (PCDDs) and polychlorinated furans (PCDFs).

Pesticides are the chemicals used to destroy uneconomic organisms such as insects. Environmental Protection Agent (EPA) classifies pesticides into two main categories as chemical pesticides and biopesticides. Chemical pesticides include organochlorine pesticides, organophosphate pesticides, carbamate derivatives and pyrethroids pesticides. Biopesticides include microbiological pesticides, biochemical pesticides and preservatives that are located into plants.

Polychlorinated biphenyls (PCBs) are not present in nature. They are synthesized chemically. There are 209 different types of polychlorinated biphenyls. The usage of these chemicals is forbidden by Environmental Protection Agency (EPA) in 1979. The presence of these chemicals has been shown in the recently published studies related with air and sea pollution (Den Besten et al., 2001; Tanabe, 2002; Galindo et al., 2003; Kuzyk et al., 2005; Nesto et al., 2007).

Polychlorinated dioxins (PCDDs) and polychlorinated furans (PCDFs) are chlorinated chemicals that combine two benzene rings and include two or one oxygen atom. These chemicals are produced during manufacturing process of organic chemicals (e.g. pesticides) and plastic materials, burning process of rubbish, coal and forest fire. Because of their stability in the environment, they can easily accumulate in organisms. Dioxins are the most toxic chemicals among the man-made chemicals. Dioxins cause deleterious effect on the reproduction system and immune system. In addition, they are directly related with cancer formation in the living organisms. Among the dioxins, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic dioxin produced by humans in the world.

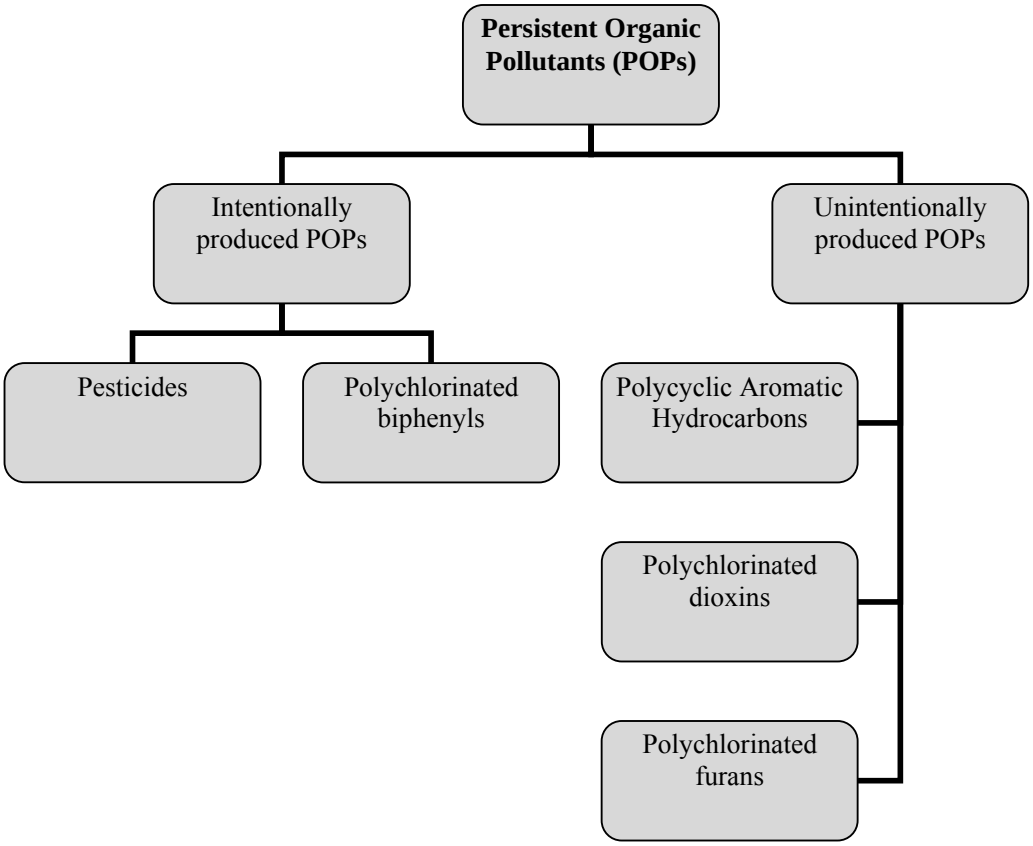


Figure 1.1. Classification of persistent organic pollutants

Polycyclic aromatic hydrocarbons (PAHs) are molecules including carbon and hydrogen atoms. They have two or more benzene rings in their structure. They are lipid-soluble chemicals. There are more than one hundred polycyclic aromatic hydrocarbons. Polycyclic aromatic hydrocarbons are formed during the pyrolysis or combustion reaction of petroleum and its products, coal, wood and tobacco. The list of the sixteen most toxic PAHs indicated by Environmental Protection Agency (EPA) is given in Table 1.1. Polycyclic aromatic hydrocarbons (PAHs) are toxic

chemicals. They are related with a series of harmful effects in organisms including immune and endocrine dysfunction, mutations and cancer (Aas et al., 2000).

1.2. Biomarkers

Chemical analyses give us valuable information about the levels and types of chemicals present in living organisms. However, organisms are exposed to dozens of different chemicals at the same time. It takes up much more time to measure these chemicals. However, biomarkers give us information about the presence of pollutants and the impact of these chemicals on organisms. They are frequently used in field studies for monitoring pollutants.

Metallothioneins, DNA adducts, acethylcholinesterase (AChE), oxidative stress and antioxidant enzymes, and biotransformation enzymes are widely used biomarkers in fish.

1.2.1. Metallothioneins

Metallothioneins are cysteine rich-proteins that are induced by heavy metals. Thiol groups of cysteine residues have a capacity to bound both endogenous (Zn, Cu, Se, etc.) and exogenous (Cd, Hg, Ag, etc.) heavy metals. Metallothioneins are generally used in the monitoring of metal ions in aquatic environments. The level of

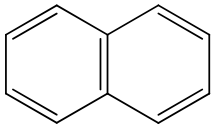
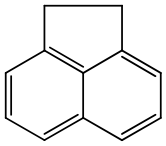
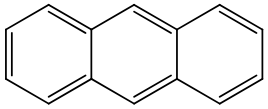
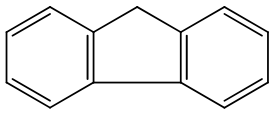
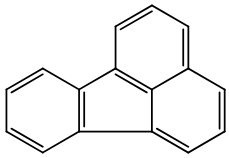
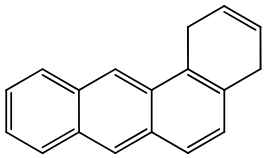
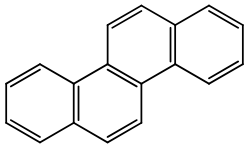
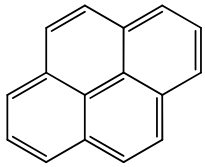
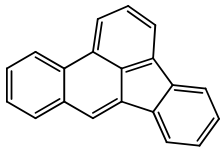
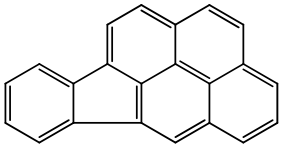
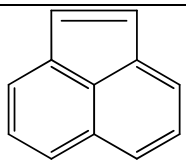
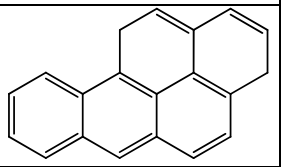
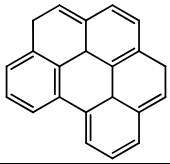
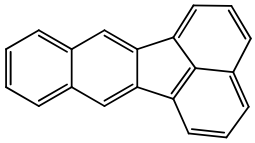
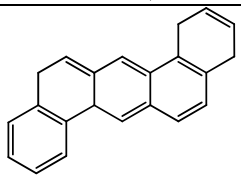
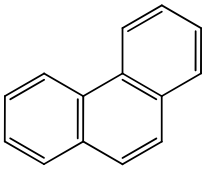
Name	*Chemical Structure*	*Name*	*Chemical Structure*
naphthalene		**acenaphthene**	
anthracene		**fluorene**	
fluoranthene		**benz[a]anthracene**	
chrysene		**pyrene**	
Benzo(b) fluoranthene		**indeno[1,2,3-c,d] pyrene**	
acenaphthylene		**benzo[a]pyrene**	
benzo[g,h,i] perylene		**Benzo(k) fluoranthene**	
dibenz[a,h] anthracene		**phenanthrene**	

Table 1.1. The structures of the proposed PAHs to be followed in environmental pollution studies by EPA.

metallothioneins increases in the presence of metal ions. This property forms the basis of the usage of metallothioneins as a biomarker (Livingstone, 1993).

1.2.2. DNA Adducts

The level of DNA adduct formation is also used as a biomarker of exposure to toxic chemicals in living organisms. Highly reactive molecules interact with DNA and form DNA adducts. Covalent bond formation between DNA and the molecules causes mutations in DNA. Cancer formation may initiate in the cell because of this structural change (Lyons at al., 2004). The amount of DNA adducts gives information about the presence of reactive molecules in the environment and it also acts as early warning signal of more serious effects.

1.2.3. Acetylcholinesterase

Acetylcholinesterase (AChE) is a serine protease that hydrolyze the neurotransmitter acetylcholine. The first effect of organophosphates and carbamates is upon acetylcholinesterase activitiy. Acetylcholinesterase activity is inhibited in the presence of these chemicals (Pena-Llopis et al., 2003). Acetylcholinesterase activity is usually used in the biomonitoring studies of neurotoxic chemicals in aquatic pollution studies (Galgani et al., 1992, Burgeot et al., 1996; Fossi at al., 1996; Stien et al., 1998; Bresler et al., 1999; Sturm et al., 1999; Corsi et al., 2003; Lionetto et al., 2004).

1.2.4. Oxidative Stress and Antioxidant Enzymes

Oxidative stress can be described as the production and accumulation of reactive oxygen intermediates like superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals (Lesser, 2006). Reactive oxygen species (ROS) were produced by endogenous reactions (these are the intermediary metabolites of energy generation reactions of mitochondria or reactions in liver achieved by cytochrome P450 dependent system) or exogenous sources (smoking, environmental pollutants exposure, excess alcohol intake, radiation exposure, bacterial, fungal or viral infections).

Reactive oxygen species can damage organism when they react with DNA or important cellular component like cell membrane. Some of the reactive oxygen species (ROS) such as superoxide radical anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH^-$) cause lipid peroxidation, enzyme inactivation and DNA adduct formation (Davies, 1995; Jifa et al., 2006). Organisms have antioxidant protection system to prevent harmful effects of free radicals. Antioxidant protection system contains antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) and many low molecular weight antioxidants such as glutathione, β-carotene (vitamin A), ascorbic acid (vitamin C) and tocopherol (vitamin E) (Halliwell and Gutteridge, 1999).

Fish are sensitive to the effect of reactive oxygen species as the other aerobic organisms. It has been shown that PAH, PCB, OCP and PCDD type pollutants alter

the levels of antioxidants in fish (Di Giulio et al., 1989; Rudneva-Titova and Zherko, 1994; Otto and Moon, 1995; Chen et al., 1998; Bello et al., 2001; van der Oost et al., 2003; Stephensen et al., 2005).

1.2.4.1. Catalase

Catalase (CAT) is a tetrameric enzyme that catalyze the metabolism of hydrogen peroxide (H_2O_2) into molecular oxygen (O_2) and water. All mammalian and non-mammalian aerobic cells and many anaerobic organisms are protected against the effect of oxidative damage by catalase enzyme (Halliwell and Gutteridge, 1999; Dama´sio et al., 2007).

The reaction catalyzed by catalase takes place in two steps. Catalase contains four porphyrin heme groups. During reaction, the heme is oxidized into an oxyferryl species (compound I) with the first hydrogen peroxide molecule. During this redox reaction, one oxidation equivalent is removed from the iron and one from the porphyrin ring to generate a porphyrin cation radical. Then, compound I is oxidized by second hydrogen peroxide to regenerate the resting state enzyme, water and oxygen (Switala and Loewen, 2002).

Catalase has been used as a biomarker in several fish species living in polluted areas (Burgeot et al., 1996; Di Giulio et al., 1989; Monteiro et al., 2006; Peixoto et al., 2006; Regoli et al., 2002; Rudneva-Titova and Zherko, 1994; van der Oost et al., 2003).

1.2.4.2. Glutathione Reductase

Glutathione (L-γ-glutamyl- cysteinyl-glycine) is a tripeptide, produced through two consecutive ATP-dependent enzymatic reactions in cytosol. First of all, glutamate reacts with cysteine by glutamate-cysteine ligase (GCL) and γ-glutamylcysteine is formed. After that, reduced glutathione is produced from glutamylcysteine and glycine by GSH synthetase catalyzed reaction (Lu, 1999). Reduced glutathione has a role in the maintenance of thiol disulfide status (acting as a redox buffer), free radical scavenging, detoxification of electrophiles and harmful xenobiotics (Ritola et al., 2000; Peña-Llopis et al., 2001). Antioxidant enzymes have a key role in glutathione redox cycle (Figure 1.2).

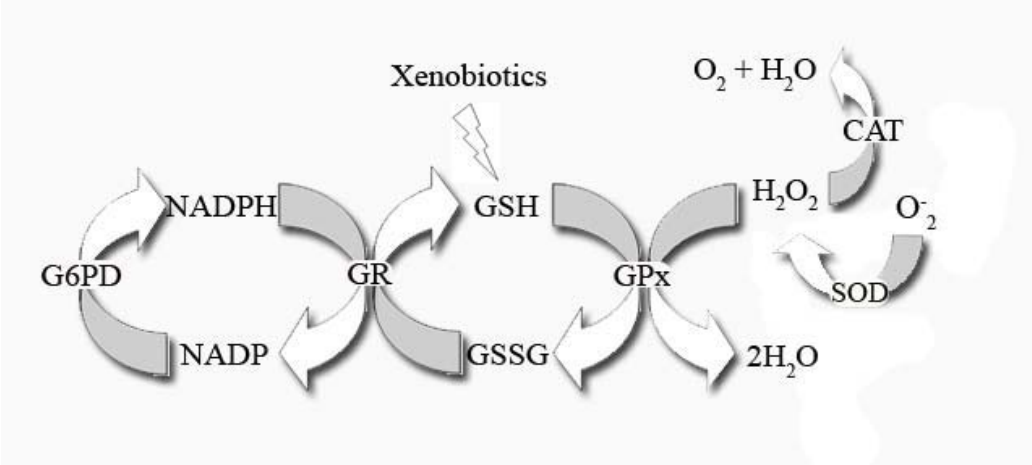


Figure 1.2. Glutathione Redox Cycle (taken and modified from Grosicka-Maciag et al., 2007)

The homeostasis of reduced (GSH) and oxidized (GSSG) glutathione under oxidative stress conditions are maintained by glutathione reductase (GR) activity (Winston and Di Giulio, 1991). Glutathione reductase (GR) is an important flavoprotein in response to oxidative stress, heavy metals and herbicides in both plants and animals. It reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) at the expense of NADPH.

Reduction of oxidized glutathione is achieved by a multistep reaction. Glutathione reductase is initially reduced by NADPH. The reduced glutathione reductase (GRred) reacts with two molecules of oxidized glutathione (GSSG). The products of this reaction are two molecules of glutathione (GSH) and the oxidized glutathione reductase.

Glutathione reductase has been used as a biomarker in several fish species in environmental pollution monitoring studies (Borković et al., 2008; Martínez- Gómez et al., 2006; Regoli et al., 2002; Trenzado et al., 2006)

1.2.5. Biotransformation Enzymes

Biotransformation is described as the conversion of xenobiotics to the water soluble chemicals by enzyme catalyzed reactions (Lech and Vodicnik, 1985; Van der Oost et al., 2003). Biotransformation reactions can be classified into two major reactions as phase I and phase II. In general, the first step in biotransformation of lipid soluble chemicals is achieved by phase I reactions such as oxidation, reduction or hydrolysis reaction. At the end of these reactions, one of the proper group for

conjugation reaction such as carboxyl, hydroxyl, amino or sulfhydryl groups is added on the molecule. At the end of the phase I reaction, molecule can lose its activity, be converted to another molecule having same activity or be converted to more toxic molecule. Phase II reactions convert chemicals or metabolites to less lipophilic form by conjugation reactions with sugar derivatives or amino acids. At the end of phase II reactions, the molecules are converted into less lipid soluble form and excreted from the body.

Oxidative biotransformation of xenobiotics is mainly catalyzed by cytochrome P450-dependent monooxygenase system in mammalian and some nonmammalian organisms. The cytochrome P450 dependent monooxygenase system is a coupled membrane-bound electron transport system present in endoplasmic reticulum and mitochondria. The cytochrome P450-dependent monooxygenase system catalyzes monooxygenation reactions of exogenous molecules (xenobiotics) such as organochlorines, dioxins, pesticides, antioxidants organic solvents, dyes and environmental pollutants and endogenous molecules such as prostaglandins, steroids, biogenic amines, fatty acids, bile acids, vitamin D_3 and retinoids (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, and N-acetyl transferases are the important phase II enzymes present in the living organisms. Conjugation reactions are addition type of reactions in which polar chemical groups (sugar and amino acids) are covalently added to xenobiotics.

Glutathione S-transferase enzymes are dimeric proteins that have a key role in the detoxification of endogenous and foreign electrophilic compounds (Mainwaring et al., 1996). They are present in cytosol, mitochondria and endoplasmic reticulum. Detoxification process is achieved by conjugating the reactive molecules to the nucleophile scavenging tripeptide glutathione (GSH, γ-glu-cys-gly) (Jemth and Mannervik et al., 1999). All GST isozymes use reduced GSH as an acceptor species, but they differ in the specificity with which different substrates are transferred to the cysteine thiol of GSH. GSTs have been classified as alpha (α), mu (μ), pi (π), sigma (σ), theta (θ) and zeta (δ) according to primary amino acid sequences.

Glutathione S-transferase requires an electrophilic center for conjugation reaction. This center can be provided by a carbon, a nitrogen, or a sulfur atom that are present in arene oxides, aliphatic and aromatic halides, α-, β- unsaturated carbonyls, organic nitrate esters, organic thiocyanates, olefins, organic peroxides, quinines and sulfate esters (Mannervik et al., 1985; Mannervik and Danielson, 1988). Many pesticides are detoxified by glutathione S-transferase such as alachlor, atrazine, dichloro-diphenyltrichloroethane (DDT), lindane, and methyl parathion.

Polycyclic aromatic hydrocarbons and halogenated xenobiotics induce glutathione S-transferase activity in fish (Vigano et al., 1995; Van der Oost et al., 2003; Martínez- Gómez et al., 2006). Total glutathione S-transferase activity measurements have been used in many different field studies to monitor the pollution present in different fish species (Regoli et al., 2002; Rodriguez-Ariza et al., 1993; Şen and Kirikbakan, 2004; Moreira and Guilhermino, 2005; Sureda et al., 2006;

Martínez- Gómez et al., 2006; Rocher et al., 2006; Tim-Tim et al., 2009; Vidal-Liñàn et al., 2010)

1.3. Cytochrome P450-Dependent Monooxygenases

Cytochrome P450-dependent monooxygenases (MO) or mixed function oxidases (MFO) are a multi-gene family of enzyme containing more than 11290 different cytochrome P450s (CYPs) (Nelson, 2009). Cytochrome P450 enzymes are predominantly found in liver. In addition they are also present in intestine, brain, skin, prostate gland, and placenta (Arınç and Philpot, 1976; Adalı and Arınç, 1990; Lester et al. 1993; Lewis, 2001; Anzenbacherova and Anzenbacher, 2001; Arukwe, 2002; Ortiz-Delgado et al., 2002; Siroka and Drastichova, 2004; Arınç et al., 2005; Arınç et al., 2007).

Nomenclature of all cytochrome P450 genes and proteins were proposed by Nebert and coworkers in 1987. This system based on the presence of common amino acid sequence. Cytochrome P450 genes are named as CYP according to the rule of nomenclature (or cyp in the case of mouse genes). The CYP designation is followed by an Arabic numeral. It denotes the gene family which share more than 40% amino acid sequence identity (Figure 1.3). A capital letter which is written after arabic numeral is used for subfamily. 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. The last number in the nomenclature is used for one gene or one protein.

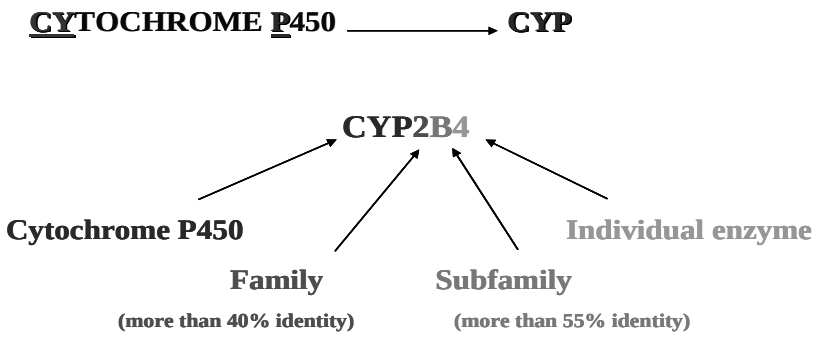


Figure 1.3. Nomenclature of cytochrome P450s

General reactions catalyzed by cytochrome P450 are monooxygenation reactions. In these reactions, one atom of the atmospheric oxygen is introduced into the structure of organic molecule (RH) and the other atom is reduced into the water in the presence of cytochrome P450, cytochrome P450 reductase, NADPH and lipid (Figure 1.4). Cytochrome P450s (CYPs) are used as a catalyst in many oxidation reactions including hydroxylation, O-, N-, and S-dealkylation reactions, N-, S- and P-oxidation, dehalogenation, epoxidation and deamination reactions.

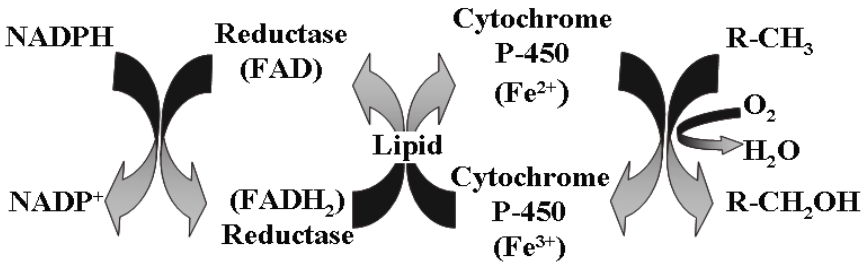


Figure 1.4. General reaction catalyzed by the cytochrome P450-dependent mixed function oxidase system.

Cytochrome P450s are heme containing proteins. The characteristic CO-difference spectrum gives maximum absorbance at wavelengths near 450 nm when the heme iron is reduced with sodium dithionate and combined with carbon monoxide. The name of these proteins is directly related with the absorbance at 450 nm (Omura and Sato, 1964 a, b).

During the cytochrome P450 dependent monooxygenase reaction, conformational changes occur in the structure of cytochrome P450. Cytochrome P450 can transfer electrons to heme from NADPH via a nearby NADPH-cytochrome P450 reductase flavoprotein. Then, reduced cytochrome-substrate complex is formed by NADPH-cytochrome P450 reductase. The reduction of the heme iron from its normal ferric state to the ferrous state allows one molecule of oxygen (O–O) to bind. The substrate, cytochrome and oxygen complex receive another electron by two different pathways. The first pathway is through the same flavoprotein as before. The second pathway is through different flavoprotein in which the electron is first passed through cytochrome *b5* (another cytochrome present in endoplasmic reticulum). The

second pathway for the second electron can also use NADPH as electron donor. With the addition of second electron to the complex, one oxygen atom inserts into the substrate to yield the product while the other atom of oxygen is reduced to water. Figure 1.5 shows the general reactions catalyzed by the cytochrome P450-dependent mixed function oxidase system in endoplasmic reticulum (Rose and Hodgson, 2004).

1. 4. Fish Cytochrome P450 Mixed Function Oxidase System

Fish also have cytochrome P450 enzymes. CYP1A, CYP3A, CYP2K, CYP2M, CYP2N, CYP2P, CYP2V, CYP2X, CYP3C, CYP4T and CYP2B-like are the cytochrome P450 subfamilies that have been purified or cloned from different fish species (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; Uno et al, 2012). Fish enzymes have a role in the metabolism of endogenous and exogenous substances similar to mammalian enzymes. CYP1A are mainly studied because of its role in carcinogen activation and its role as a biomarker in environmental monitoring studies (Gelboin, 1980; Conney, 1982; Payne et al., 1987; Stegeman, 1995; Bucheli and Fent, 1995; Addison, 1996; Şen and Arınç, 1998; Arınç et al., 2000).

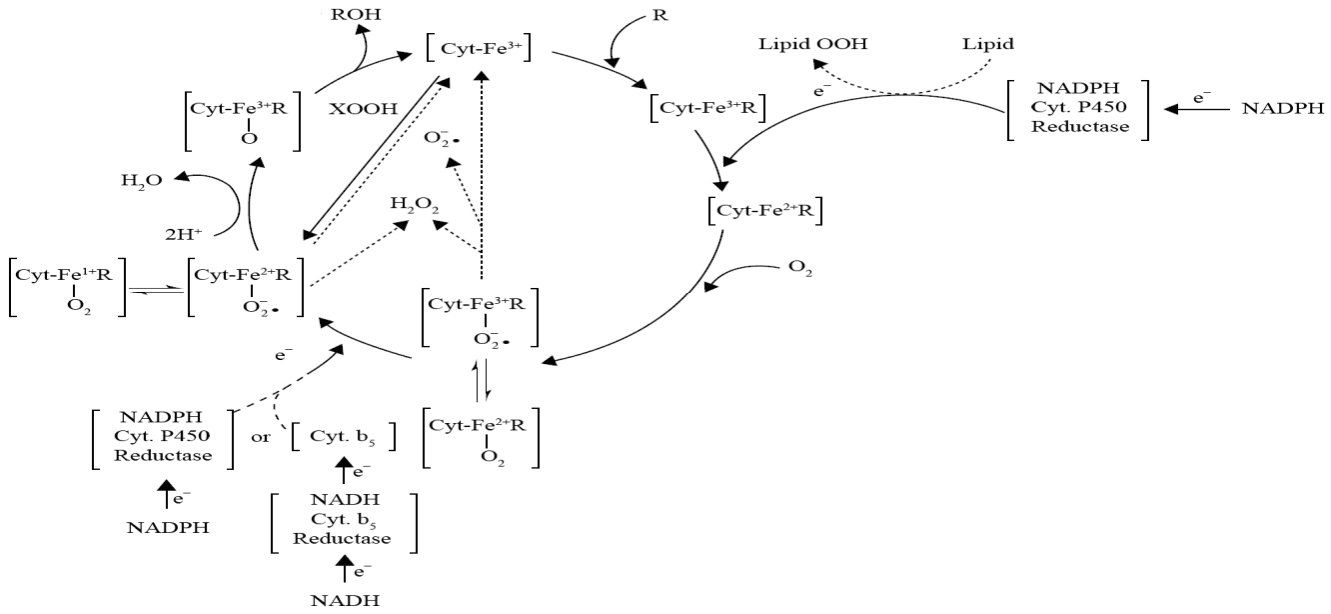


Figure 1.5. General reaction catalyzed by cytochrome P450-dependent monooxygenase system in endoplasmic reticulum (taken from Rose and Hodgson, 2004)

1.5. Cytochrome P4501A (CYP1A) in Fish Species

CYP1A1 and CYP1A2 are the isoenzymes of CYP1A present in mammals. The gene product of CYP1A1 and CYP1A2 metabolize polyaromatic hydrocarbons and arylamines into toxic and/or reactive intermediates (Dey et al., 1989). The occurrence of cytochrome P4501A1 (CYP1A1) has been demonstrated in different fish species. Cytochrome P4501A1 has been purified from rainbow trout, perch, marine species such as scup, cod and leaping mullet (Williams and Buhler, 1982; Williams and Buhler, 1984; Miranda et al., 1989; Miranda et al., 1990; Andersson, 1992; Zhang et al., 1991; Klotz et al., 1983; Klotz et al., 1986; Stegeman et al., 1990; Goksøyr, 1985; Goksøyr et al., 1986; Şen and Arınç, 1998; 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).

Fish CYP1A and mammalian CYP1A have similarities in biocatalytic and immunological properties (Stegeman, 1995). Induction of CYP1A1 in fish has been observed with various PAHs, PCBs, 2,3,7,8-TCDDs, PCDFs, PCDDs, and other halogenated compounds, sediments extracts, bleached kraft mill effluents and crude oils (Arınç et al., 1978; Law and Addison, 1981; Spies et al., 1982; Förlin et al., 1985; Andersson et al., 1987; Vandermeulen, 1990; Collier and Varanasi, 1991; Goksøyr and Förlin, 1992; Arınç and Şen, 1994; Addison, 1996).

Induction of cytochrome P4501A proteins and its associated enzyme activities aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin O-deethylase

(EROD) in fish have been extensively used in many different environmental pollution monitoring studies (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). At first, the aryl hydrocarbon hydroxylase activity was proposed to be used as a biomarker (Payne, 1976). However, 7- ethoxyresorufin O-deethylase (EROD) has been used instead of aryl hydrocarbon hydroxylase (AHH) in recent environmental pollution studies. The reason of this is related with the substrate of aryl hydrocarbon hydroxylase reaction. The substrate of this reaction is very toxic chemical and gives cross reaction with other CYP isoenzymes. The level of cytochrome P450 protein in fish can also be identified by immunochemical methods such as western blot or ELISA (enzyme-linked immunosorbent assay) using mono- or polyclonal antibodies produced against CYP1A protein (Stegeman et al., 1986; Goksøyr and Solberg, 1987; Goksøyr et al. 1991; Goksøyr and Förlin, 1992; Bucheli and Fent, 1995; Goksøyr and Husøy, 1998). Induction of cytochrome P4501A can also be determined by measuring the mRNA level of cytochrome P4501A.

1.6. Induction of Cytochrome P4501A (CYP1A)

Many exhaustive studies have been carried out to identify the induction mechanism of CYP1A. Poland and Knutson (1982) showed the role of aromatic hydrocarbon receptor (AhR) in the induction of CYP1A. They proposed a model for this receptor (AhR) to define regulatory mechanism of gene expression of 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. The Ah-receptor is a ligand-activated transcription factor present in the cell cytoplasm together with heat-

shock protein 90 (HSP90), XAP_2 and p23 protein molecule whose function is to maintain unliganded AhR in a configuration that facilitates ligand binding. Enzyme induction is initiated by the binding of specific xenobiotic such as PCDD, PCB, PAH and PCDF to Ah-receptor (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). Inducer chemical passes into the cell by passive diffusion and binds to Ah-receptor complex (Figure 1.6). After the binding of ligand to AhR receptor, HSP90 molecules are cleavaged from XAP_2 and p23 complex. Ligand-AhR complex passes into cell nucleus and forms AhR:Arnt heteromer with aryl hydrocarbon nuclear translocation proteins (Arnt) (Henry and Gasciewicz, 1993; Hord and Perdew, 1994; Pollenz et al., 1994; Hankinson, 1995; Whitlock et al., 1996; Denison et al., 2002). This complex binds xenobiotic regulatory element (XRE) in DNA and initiates the transcription of messenger RNA (mRNA) encoding CYP1A1 protein (Probst et al., 1993; Hankinson, 1995; Denison et al., 2002). Synthesized CYP1A1 protein translocates in the membrane of endoplasmic reticulum (Denison et al., 1988; Stegeman and Hahn, 1994; Whitlock et al., 1996; Whitlock, 1999; Denison et al., 2002).

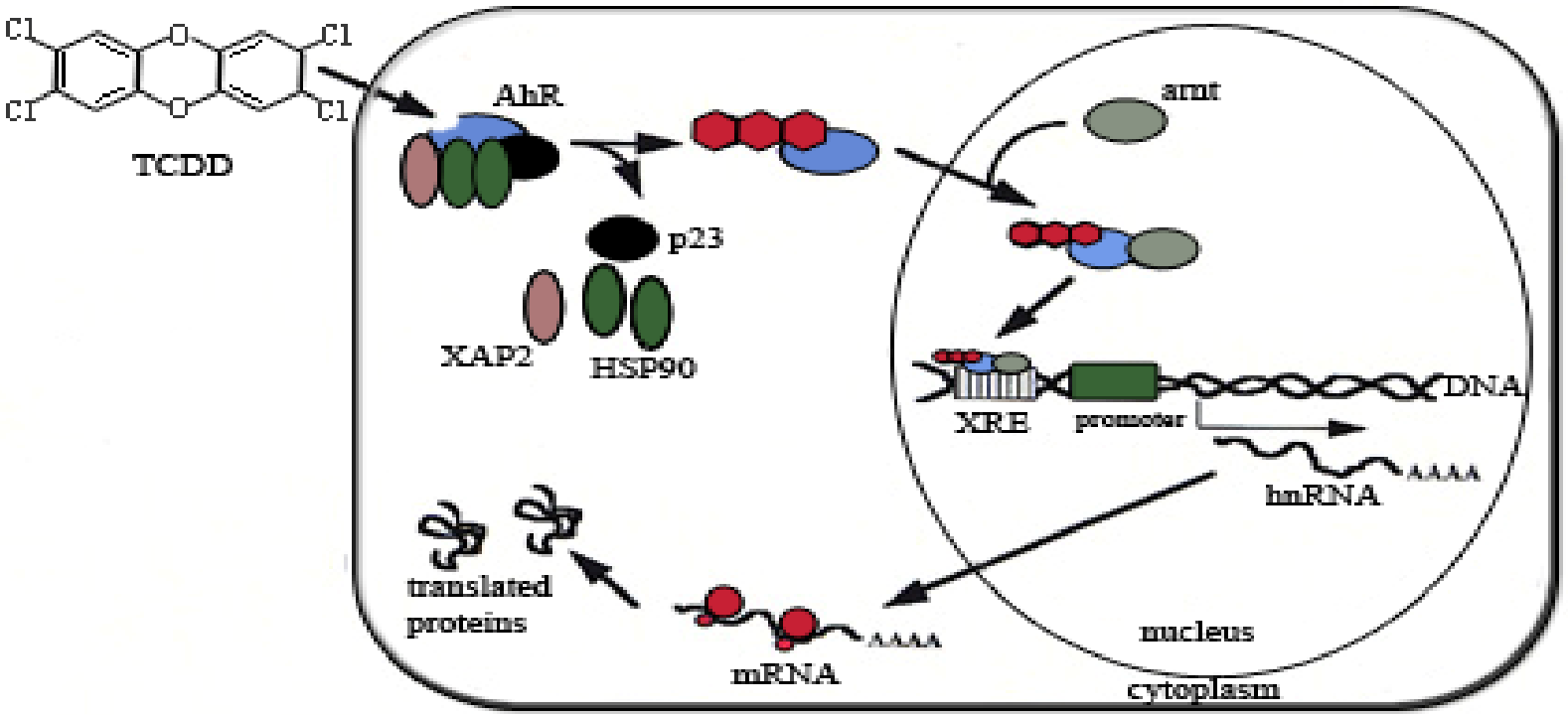


Figure 1.6. The mechanism of CYP1A induction by TCDD (taken from Whitlock, 1986 and modified according to Denison et al., 2002).

1.7. The Black Sea

The Black Sea is the largest sea in the world with 2200 m depth and 423000 km^2 area. There are six coastal countries in the Black Sea: Turkey, Bulgaria, Romania, Ukraine, the Russian Federation and Georgia. The Black Sea is under threat of pollution as a result of industrial, anthropogenic and agricultural activities. Many cities around the Black Sea have no treatment system for canalization and effluent water. This canalization and effluent water is spilled into Black Sea directly or by streams. The other problem threatening the Black Sea ecosystem is petroleum pollution. The levels of petroleum related pollutants are not so high in offshore, however the levels are inadmissibly high in coastlines and estuaries. Oil enters into the environment as a result of accidental and operational discharges and ship spills and discharges from land based sources (Strategic Action Plan for the Rehabilitation and Protection of the Black Sea, 1996).

The coastal countries organized a meeting in 1992 in Bucharest to protect the Black Sea ecosystem from pollution. They signed Bucharest Convention. After this convention, studies about the environmental pollution in Black Sea increased gradually. The studies on the Black Sea coast of Turkey mainly focused on the chemical analysis. According to these studies, heavy metal pollution has been reported in the Black Sea coast of Turkey (Tuncer et al., 1998; Barlas, 1999; Tüzen, 2003). The Kızılırmak, Yeşilırmak, Sakarya, Filyos and Gülüç Rivers carry Cd, Cu, Pb and Zn metals into the Black Sea (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, Boke-Ozkoc et al., 2007). Bakan and Ariman (2004) measured aldrin, heptachlor, DDT and HCH isomers in sea water and surface sediment. OCPs have been measured in sediment and mussel samples that were taken from Bosphorus Strait (Okay et al., 2011). The presence of DDT has been reported in rivers, streams and canalization in Black Sea (Tuncer et al., 1998). In another study, the presence of endosulfan I, endosulfan II and DDE have been shown in sardines and anchovy (Çakıroğulları and Secer, 2011). The presence of polycyclic aromatic hydrocarbons have been reported in the edge of Bosphorus Strait and Trabzon, Yomra (Telli-Karakoç et al., 2001; Readman et al., 2002). In addition, the levels of the polycyclic aromatic hydrocarbon have been measured in surface sediment samples taken from the middle of the Black Sea. The levels of polycyclic aromatic hydrocarbons in the west part of the Black Sea were higher than the east part of the Black Sea (Wakeham, 1996). The studies concerning the fish were limited. The presence of polycyclic aromatic hydrocarbons have been shown by total PAH measurements in our laboratory in three mullet species soiuy mullet (*Mugil soiuy*), flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) caught from the West Black Sea Coast of Turkey (Bozcaarmutlu et al., 2009). Highly elevated EROD activities have been reported in the fish samples caught from Zonguldak and Ereğli Harbours and Gülüç Stream Mouth (Bozcaarmutlu et al., 2009).

1.8. Test Species

Striped red mullet (*Mullus surmuletus*) was used as model organism in this study. Striped red mullet is a member of *Mullidae* family. It has red colored spots along the upper portion of head, backbone and fins. Striped red mullet has stripes on its two dorsal fins. It is found in the Mediterranean Sea, Black Sea and Atlantic Ocean. It is a bottom-feeder fish. Striped red mullet lives in shallow waters with sandy bottoms. It feeds with crustaceans and mollusks (Lobropoulou et al., 1997). Striped red mullet is an economically important fish and widely consumed in Turkey. There is only one environmental pollution study in striped red mullet. It is restricted with metallothionein and metal ions measurements (Filipovic and Raspor, 2003).

The other organisms used in this study were flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*). They are pelagic fishes inhabiting usually inshore regions, enters lagoons and estuaries along the Atlantic coasts to the north of Bay of Biscay, also the whole of the Mediterranean, Black Sea and Sea of Azov. Mullets are herbivorous organism, feeding primarily on algae and dead plant materials. Mullet species are widely used in biomonitoring programs of environmental pollution (Arinç and Sen, 1999; Arinç et al., 2000; Ferreira et al., 2004, Bozcaarmutlu et al., 2009). Biomarker enzyme activities such as components of cytochrome P450 system, glutathione S-transferase and antioxidant enzymes have been well characterized in different mullet species (Sen and Arinç, 1998a, b; Bozcaarmutlu and Arinç, 2004, 2007, 2008; Ferreira et al., 2004; Sen and Kirikbakan, 2004; Bozcaarmutlu, 2007; Sen and Semiz, 2007).

1.9. The Aim of the Study

The Black Sea has been increasingly threatened by pollutants over the past decades. The presence of polycyclic aromatic hydrocarbons has been shown in our laboratory by total PAH measurements in the liver of flathead mullet (*Mugil cephalus*), so-iuy mullet (*Mugil soiuy*) and golden grey mullet (*Liza aurata*) caught from the West Black Sea Coast of Turkey in 2005, 2006 and 2007 (Bozcaarmutlu et al., 2009). The presence of organochlorine pesticides have been shown in the liver of flathead mullet (*Mugil cephalus*) caught from the West Black Sea Coast of Turkey in 2009 (Bozcaarmutlu et al., 2012). Biomarker enzyme activities have also been measured in the liver of flathead mullet (*Mugil cephalus*), so-iuy mullet (*Mugil soiuy*) and golden grey mullet (*Liza aurata*) caught from the West Black Sea Region of Turkey in 2005, 2006 and 2007 (Bozcaarmutlu et al., 2009). Highly elevated EROD activities have been measured in the mullet caught from Zonguldak Harbour, Ereğli Harbour and Gülüç Stream's Mouth. It has been well documented in many studies that interspecies differences have been reported in biomarker enzyme activities against pollutants. In this study, striped mullet samples were caught from the same locations in the West Black Sea Coast of Turkey.

The objectives of this study were

1- to assess suitability of striped red mullet (*Mullus surmuletus*) in monitoring of environmental pollution by using cytochrome P4501A related 7-ethoxyresorufin O-deethylase activity, glutathione S-transferase, catalase and glutathione reductase activities.

2- to determine the concentrations of 13 different polycyclic aromatic hydrocarbons in the liver tissue of striped red mullet caught from the West Black Sea Region of Turkey.

3- to continue monitoring the impact of pollution present in the West Black Sea Region of Turkey on flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*).

4- to determine the concentrations of 13 different polycyclic aromatic hydrocarbons in the liver tissue of flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*).

5- to compare EROD activities measured in striped red mullet with the EROD activities measured in flathead mullet and golden grey mullet.

6- to compare the concentrations of 13 different polycyclic aromatic hydrocarbons measured in the liver tissue of striped red mullet with the concentrations of polycyclic aromatic hydrocarbons in the liver tissue of flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*).

MATERIALS AND METHODS

2.1. Materials

Aluminium oxide (Al_2O_3; 101077), dipotassium hydrogen phosphate (K_2HPO_4; 105101), ethanol (C_2H_5OH; 100983), florisil (112518), glycerol ($C_3H_8O_3$; 104092), n-hexane ($CH_3(CH_2)_4CH_3$; 104368), methanol (CH_3OH; 106007), potassium chloride (KCl; 104936), potassium dihydrogen phosphate (KH_2PO_4; 104871), sodium chloride (NaCl; 106404) and sodium hydroxide (NaOH; 106462) were purchased from Merck KGaA, Darmstadt, Germany.

ε-Amino caproic acid (ε-ACA; $C_6H_{13}NO_2$ A2504), bovine serum albumin (BSA; A7511 or A7888), ethylene diamine tetraacetic acid (EDTA; $C_{10}H_{14}N_2Na_2O_8.2H_2O$; E5134), Folin-Ciocalteu reagent (F9252), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; $C_8H_{18}N_2O_4S$; H3375), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH; $C_{21}H_{26}N_7Na_4O_{17}P_3$; A1395), and phenylmethane sulfonyl fluoride (PMSF; $C_7H_7FO_2S$; P7626) were purchased from Sigma-Aldrich, (Saint Louis, Missouri, USA).

Glucose-6-phosphate dehydrogenase (346774) and α-D-glucose-6-phosphate monosodium salt (346764) were purchased from Calbiochem (San Diego, California, USA).

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

Contract Laboratory Program-PAH standard (CLP-PAH) (2000 ng/µl concentration in methylene chloride) were purchased from Absolute Standards.

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

2.2. Methods

2.2.1. Fish Collection

Striped red mullet (*Mullus surmuletus*), flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) were caught from different locations of the West Black Sea coast of Turkey in 2011. Figure 2.1 shows the fish sampling stations in the West Black Sea coast of Turkey. Fish samples were killed by decapitation. Livers were removed from fish by scissors immediately to prevent the spillage of the contents of gall bladders. Then, striped red mullet livers were packaged with freezing

bags and aluminum foil. They were frozen by putting into liquid nitrogen. Freshly frozen fish livers were transported to our laboratory in Bolu.

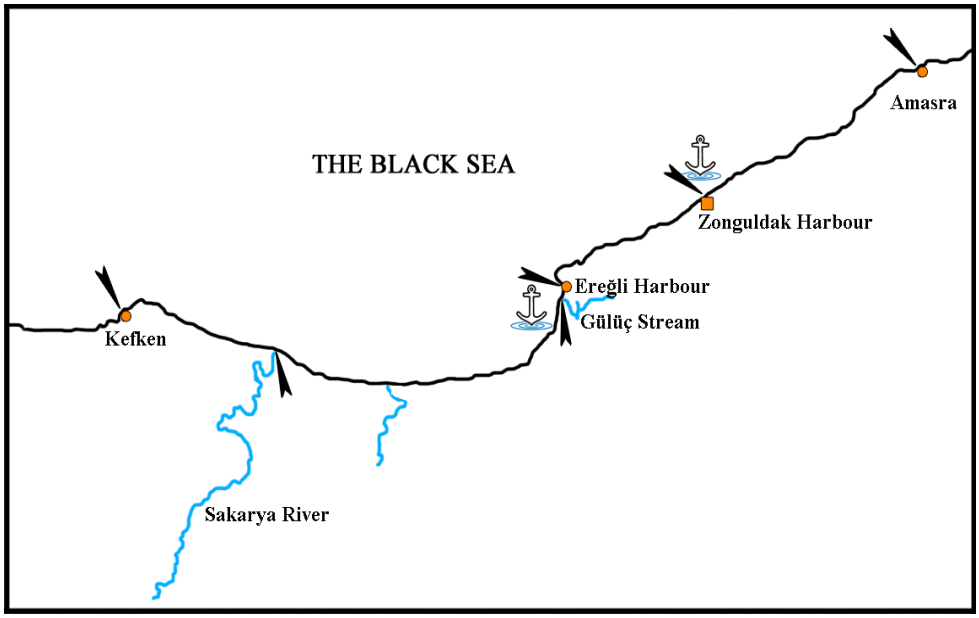


Figure 2.1. The sampling stations in the West Black Sea coast of Turkey

2.2.2. Preparation of Fish Liver Microsomes

Liver microsomes were prepared by the method described by Arınç and Şen (1993). Livers were taken from liquid nitrogen and thawed on ice. All processes for microsome preparation were carried in 0-4 $^{\circ}$C ice bath. Livers were firstly washed with cold distilled water. Then, they were washed with cold 1.15% KCl solution to remove blood as much as possible. Then the tissues were drained by a paper towel. They were weighed and minced with scissors.

The resulting tissue mince was homogenized in 2.5 mL of homogenization solution containing 1.15% KCl, 10mM EDTA pH 7.7, 0.25 mM ε-ACA, and 0.1 mM PMSF for each gram of mullet liver tissue. Homogenization process were performed by using Potter-Elvehjem glass homogenizer packed in crushed ice, coupled motor (Black & Decker, V850 multispeed drill) driven Teflon pestle at 2400 rpm. Fifteen passes were made for homogenization. Then, the homogenate was centrifuged at 13300xg by Sigma 3K30 Refrigerated Centrifuge (Saint Louis, Missouri, USA) with 12156 rotor at 13300×g 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 supernatant fraction was taken and recentrifuged at 45000 rpm (105000×g) for 60 minutes using Beckman Optima L-100K ultracentrifuge (Beckman Coulter Inc., Fullerton, California, USA) with 70.1 Ti rotor. The supernatant fraction (cytosol) was taken and put into Eppendorf and stored in deep freeze at - 80 $^{\circ}$C until used. The pellet was suspended in 1.15% KCl solution containing 10 mM EDTA and resedimented by ultracentrifugation at 45000 rpm (105000×g) for 50 minutes. The supernatant was discarded. Then the 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 Teflon-glass homogenizer in order to obtain homogenous suspension. Microsomes were transferred into Eppendorf tubes and stored in liquid nitrogen after gassing with nitrogen.

2.2.3. Protein Determination

Concentrations of microsomal and cytosolic proteins were determined as described by Lowry et al (1951). Bovine serum albumin (BSA) was used as a standard. The mullet liver microsome and cytosols were diluted 200 times to bring the concentration of proteins to the linear range. 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 the test tubes. Five different concentrations of standard were prepared from 1 mg/mL stock standard solution. All of the tubes including standards 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. The tubes were 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. The tubes were incubated at room temperature for 30 minutes. At the end of this period, the color intensity of the tubes was measured at 660 nm. The standard calibration curve of BSA from 20 to 200 µg/mL was plotted and used for the determination of protein concentrations of microsomes and cytosols.

2.2.4. Determination of 7-Ethoxyresorufin-O-deethylase Activity in Fish Liver Microsomes

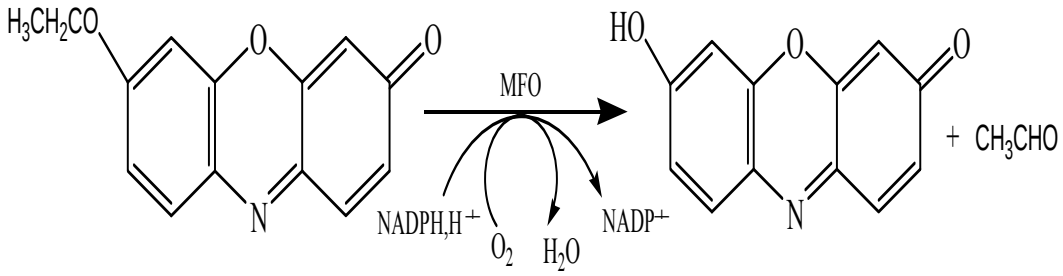
Cytochrome P4501A (CYP1A) associated 7-ethoxyresorufin-O-deethylase (EROD) activity of fish liver microsomes were determined as described by Burke and Mayer (1974) with some modifications. Assay conditions optimized for gilthead seabream liver microsomes by Arınç and Şen (1994) were also used in this study.

According to the method, 0.5 mM of substrate solution was prepared by dissolving 1 mg of 7-ethoxyresorufin in 8.32 mL DMSO. Then 10 μM daily solution was prepared in 1:50 dilution with 0.2 M potassium phosphate buffer (pH 7.8) containing 0.2 M NaCl from 0.5 mM stock solution. 1 mM stock resorufin standard was prepared by dissolving 2.35 mg of resorufin in 10 mL of DMSO. Then 5 μM daily solution was prepared in 1:200 dilution with 0.2 M potassium phosphate buffer (pH 7.8) containing 0.2 M NaCl from 1 mM stock solution.

A typical reaction medium included 0.1 M potassium phosphate buffer (pH 7.8), 0.1 M NaCl, 2.4 mg BSA, 1.5 μM 7-ethoxyresorufin, 25-100 μg microsomal protein, 0.5 mM NADPH generating system in a final volume of 2.0 mL in a fluorometer cuvette (Table 2.1). NADPH generating system was obtained by incubation of 2.5 mM magnesium chloride, 2.5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase, 14.6 mM HEPES buffer, pH 7.8 and 0.5 mM $NADP^+$ at 37 $^{\circ}C$ about 5 minutes. The reaction was started by the addition of substrate and followed for three minutes in spectrofluorometer (Hitachi F-4500, Hitachi High-Technologies 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. The increase in flourescence in a minute via enzyme activity was compared with the fluorescence of the resorufin standard. Enzyme activity was expressed as the formation of resorufin (pmol) for milligram of protein per minute.

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 0.4M NaCl	400 mM	0.5	100 mM
BSA	12 mg/mL	0.2	1.2 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	

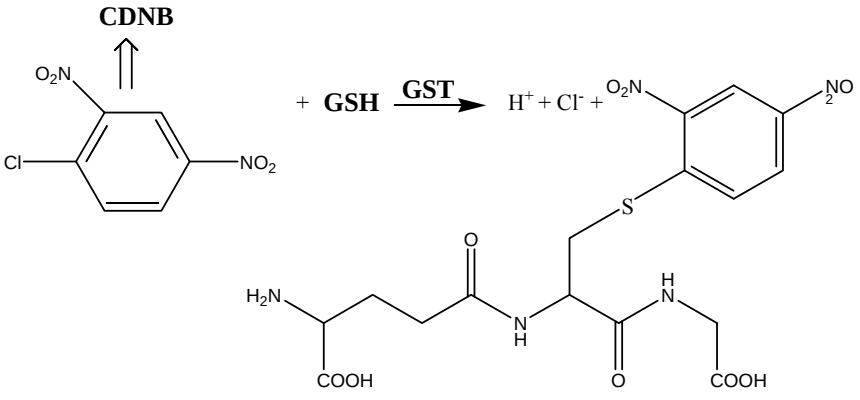


7- ethoxyresorufin 7-hydroxyresorufin

Figure 2.2. Ethoxyresorufin O-deethylase reaction.

2.2.5. Glutathione S-Transferase Activity in Fish Liver Cytosols

Glutathione S-transferase activity measurements were determined according to the method described by Habig et al (1974). This method is based on conjugation reaction between 1-chlorine-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) by glutathione S-transferases (GSTs). 2,4-dinitrophenyl glutathione complex is formed at the end of the reaction (Figure 2.3). 1-chlorine-2,4-dinitrobenzene (CDNB) is the common substrate of all GSTs. Total glutathione S-transferase activity is measured by this method. According to this method, a typical reaction medium contained 2.5 mL of 50 mM potassium phosphate buffer, pH 7.5, 0.2 mL of 20 mM GSH, 0.150 mL of cytosol (diluted 1/100-1/250 times). The reaction was started with the addition of 0.150 mL of 20 mM CDNB. The formation of 2,4-dinitrophenyl glutathione complex was monitored at 340 nm about 2 minutes. Enzyme activity were calculated as nmole 2,4-dinitrophenyl glutathione conjugate formed by per milligram protein in one minute at 25°C, using 9.6 $mM^{-1}cm^{-1}$ as an extinction coefficient (ε_{340}**).**



1-glutathione-2,4-dinitrobenzene (DNB_SG)

Figure 2.3. Metabolism of 1-chlorine-2,4-dinitrobenzene (CDNB) by glutathione S-transferases.

2.2.6. Catalase Activity in Cytosol

Catalase activity of fish samples was determined according to method described by Aebi (1984). The principle of the method depends on the decomposition of H_2O_2 by catalase (Figure 2.4). In the UV range, H_2O_2 has an absorbance at 240 nm. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. According to this method, mullet cytosols were pretreated with 1% Triton X-100 for 10 minutes. Then, the mixture was diluted 100-400 times with 50 mM phosphate buffer pH 7.5. The total dilution of sample was 1000-4000. 2 mL diluted sample was put into two quartz cuvettes. 1 mL of hydrogen peroxide was added into the reaction cuvette and 1 mL potassium phosphate buffer solution was added into the reference cuvette. The activity was measured by following consumption of hydrogen peroxide at 240 nm for 1 minute. Catalase activity was calculated as nmole hydrogen peroxide consumed per milligram protein in one minute at 25°C using 36.4 $mM^{-1}cm^{-1}$ as an extinction coefficient (ε_{240}**).**

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Figure 2.4. General reaction catalyzed by catalase.

2.2.7. Glutathione Reductase Activity in Fish Liver Cytosols

Glutathione reductase activity was measured according to method of Carlberg and Mannervick (1985). Glutathione reductase catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to glutathione (GSH) (Figure 2.5). The reduction of GSSG is determined indirectly by measuring the consumption of NADPH. In this method, typical reaction medium contained 100mM of potassium phosphate buffer pH 7.6, 0.5 mM of EDTA pH 7.6, 0.1 mM of NADPH and fish liver cytosol in a final volume of 2 mL. The reaction was started by adding 1 mM oxidized glutathione (GSSG). The decrease in NADPH was monitored at 340 nm for 5 minutes. Enzyme activities were calculated as nmole NADPH oxidized per milligram protein in one minute at 25 °C, using 6.22 $mM^{-1}cm^{-1}$ as an extinction coefficient (ε_{340}**).**

$$ROH + 2GSSG + NADPH + H^{+} \xrightarrow{GR} ROOH + 2GSH + NADP^{+}$$

Figure.2.5. General reaction catalyzed by glutathione reductase.

2.2.8. Measurement of Concentrations of Polycyclic Aromatic Hydrocarbons (PAHs) in Fish Samples

2.2.8.1. Extraction and Clean-up Procedure

Polycyclic aromatic hydrocarbons were determined in striped red mullet caught in 2009-2011 and in flathead mullet and golden grey mullet caught in 2011 in this study. Polycyclic aromatic hydrocarbons were extracted from the liver tissues according to method described by UNEP (1992). Fish liver tissues were dried in an oven at 40 °C overnight. Dried tissue was (0.1 g) ground and refluxed with 20 mL ethanol containing 0.75 g KOH for 120 minutes at constant heating. Then, 20 mL of PAH-free hexane was added into the flask mixed well and waited until the flask reached to room temperature. The mixture was taken into a separatory funnel and sufficient amount of distilled water was added. Water was added to see the phase difference between ethanol and hexane phases. The upper hexane phase was taken with glass pipette into conical evaporator flask. The lower aqueous phase was extracted further two times with 20 mL of the PAH-free hexane. The hexane phases were collected into the same conical evaporator flask. The solvent part of the extract was evaporated until dryness in a vacuum rotary evaporator at 40 °C. Then the flask was washed with 1 mL hexane to dissolve all of PAHs. PAHs were taken from the flask and put into a vial. The flask was further washed with 1mL hexane three times more. All PAHs were collected into the same vial. For clean-up, glass column (1 cm i.d. x 15 cm length) containing a pieces of glass wool on a glass frit was used. The column was filled with 1 g activated florisil, anhydrous sodium sulphate and alumina, respectively. The column was wetted and equilibrated with 10 mL hexane

prior to use and then this solvent was discarded. 1 mL of hexane was added into the sample containing vial. The vial was mixed on the vortex at maximum speed. The dissolved sample was applied into the preequilibrated column. The vial was washed four times more to eliminate sample losses and collected hexane was applied into the column. After the application of all the sample into the column, PAHs were eluted from the column with 40 mL of ethyl acetate:hexane (1:1) into a conical evaporatory flask. The eluate was evaporated until dryness in a vacuum rotary evaporator at 40 °C. The flask was washed with 1 mL hexane to dissolve all PAHs. Then the eluate was transferred to a vial. The flask was washed four times more with hexane and eluate were transferred to the same vial. The analyte was evaporated by gentle stream of nitrogen gas after the addition of each hexane phase until dryness. At the end of the last evaporation, the analyte was dissolved in 1mL acetonitrile.

2.2.10. Analyses of Sample

Analysis of polycyclic aromatic hydrocarbons (PAHs) were made by Agilent 1100 series high performance liquid chromatography (HPLC). HPLC's program was modified from Pekey and coworkers (2007). Table 2.2 shows operation parameters for HPLC.

Table 2.2. Operation parameters of HPLC for PAHs

Operation Parameters	**PAHs**
HPLC column	30 m x 250 µm x 0.25 µm nominal film column 5% phenyl methyl siloxane ,TRB-5MS capillary column
Detector	Diode array detector (DAD)
Wavelength	270nm
Injection volume	20 µL
Column temperature	20 °C
Mobile phase	Water : Acetonitrile (Gradient)
Flow rate	0-8.5 min.:40:60 flow : 1.2 mL min-1 10-20 min.:10:90 flow : 1.0 mL min-1 27-40 min.: 0:100 flow: 1.2 mL min-1

2.2.11. Method Validation

Extraction efficiency (percent recovery) of the method was determined by analyzing spiked fish samples. The recoveries of PAHs were determined in three separate fish liver samples. Fish liver samples were spiked with the working solution containing 13 PAHs, then extracted and analyzed in the same way as the real samples. Mean recoveries of PAHs were given in Table 2.3. The recoveries of PAHs ranged from 41% to 98% for fish samples and the results were not been corrected for these recoveries.

Calibration curves were obtained from matrix-matching calibration solutions. Matrix-matching calibration solutions were prepared from the fish extracts (n=20) spiked with PAHs to the final concentration 0.2-1.0 ng/mL. The squared correlation coefficients, r^2, were greater than 0.989 (Table 2.3). The concentrations of PAHs were determined by comparing the peak height of the samples and the slope of calibration curve.

The limits of detection (LOD) were calculated by using ten replicate analyses of 0.2 ng mL^{-1} standard. LOD values were 3 times of the standard deviation of ten replicates. The limits of detection (LOD) were given in Table 2.3. The LODs of PAHs were in the range of 0.032 -0.640 ng/g dry weight. Limits of quantification (LOQ) values were defined as ten times the standard deviation of ten replicates (Table 2.3).

2.2.12. Statistical Analysis

Data were analyzed by one-way ANOVA. When significant differences were observed, Tukey HSD Post Hoc Test was applied for a pair-wise comparison. The analyses were carried out using the SPSS statistical package (SPSS 15.0 for Windows Evaluation Version).

Table 2.3. Validation parameters. Recoveries, r^2, LOD and LOQ values for the method that were optimized for PAH measurement in the fish liver.

Polycyclic Aromatic Hydrocarbons	Recovery (%) (n=4)	R^2	LOD* (ng g^{-1} dried weight)	LOQ** (ng g^{-1} dried weight)
Acenaphthylene	86	0.997	0.263	0.876
Acenaphthene	41	0.999	0.032	0.108
Phenanthrene	75	1.000	0.037	0.122
Anthracene	59	0.980	0.453	1.509
Fluoranthene	69	0.995	0.051	0.171
Pyrene	86	0.999	0.491	1.635
Benz[a]anthracene	85	0.999	0.112	0.373
Benzo(b)fluoranthene	98	0.999	0.155	0.518
Benzo(k)fluoranthene	85	0.997	0.279	0.929
Benzo(a)pyrene	73	0.998	0.118	0.394
Indeno(1,2,3,c,d pyrene)	82	0.996	0.640	2.132
Dibenzo(a,h) anthracene	74	0.996	0.256	0.854
Benzo(g,h,i)perylene	78	0.989	0.173	0.577

* LOD: Limit of Detection
** LOQ: Limit of Quantitation

RESULTS

The biomarker enzyme activities were measured in striped red mullet (*Mullus surmuletus*) caught from the West Black Sea coast of Turkey in 2011. CYP1A associated 7-ethoxyresorufin O-deethylase (EROD) activity was measured in microsomes of fish livers. Glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activities were measured in cytosols of fish livers. Besides these biochemical parameters, the levels of 13 different polycyclic aromatic hydrocarbons were determined in striped red mullet liver caught from the West Black Sea coast of Turkey in 2009-2011 by HPLC analysis. In addition, EROD activities and the levels of 13 PAHs were also measured in flathead mullet (*Mugil cephalus*) and golden grey mullet (Liza aurata) samples caught from the West Black Sea coast of Turkey in 2011. These fish species were compared with respect to EROD activities and the levels of polycylic aromatic hydrocarbons.

3.1. Biomarker Enzyme Activities Measured in Striped Red Mullet (*Mullus surmuletus*)

3.1.1. 7-Ethoxyresorufin O-Deethylase Activity of Striped Red Mullets (*Mullus surmuletus*)

Striped red mullet samples were caught from different stations of West Black Sea coast of Turkey in 2011. Liver samples obtained from striped red mullet were very small. It was impossible to obtain sufficient microsomes and cytosols for the biomarker enzyme activity measurement. Therefore, each microsomes were prepared from the combination of four different striped red mullet livers. Cytochrome P4501A dependent 7-ethoxyresorufin O-deethylase (EROD) activity was determined in all microsome preparations.

Striped red mullets were caught from Zonguldak Harbour, Ereğli Harbour, Sakarya River Mouth, Amasra, and Kefken. Tables 3.1-3.8 show microsomal EROD activity results obtained from the livers caught from five different stations along the West Black Sea coast of Turkey. All activities were measured in duplicates and sometimes in triplicates. The highest average EROD acitivity was found for striped red mullet samples caught from Zonguldak Harbour. 7-Ethoxyresorufin O-deethylase activity was 308±56 pmole/min/mg protein (Average activity± SEM, N=20) (Table 3.1). Striped red mullet samples were caught from two different locations within Ereğli Harbour. The average EROD activity of striped red mullet caught in the center of Ereğli Harbour was 180±30 pmole/min/mg protein (Average activity± SEM, N=20) (Table 3.2). Beside this location, striped red mullet samples were

caught in front of the Erdemir Iron and Steel Works to measure the effects of wastes of this plant on organisms living in this region. EROD activity of striped red mullet samples caught in front of Erdemir Iron and Steel Works was 130±9 pmole/min/mg protein (Average activity± SEM, N=11) (Table 3.3). The average EROD activity of striped red mullet samples caught from Amasra was 52±6 pmole/min/mg protein (Average activity± SEM, N=24) (Table 3.5). Table 3.4 shows the average EROD activity of striped red mullet samples caught from Sakarya River Mouth. EROD activity of striped red mullet was 31±7 pmole/min/mg protein (Average activity± SEM, N=16) in this region. In 2011, the fish samples were collected from three different locations in Kefken. These stations were named as outside part of Kovanağzı, inside part of Kovanağzı and outside part of Kefken Harbour. The average EROD activities of striped red mullet samples caught from these stations were 21±4 pmole/min/mg protein, (N=20), 72±9 pmole/min/mg protein (N=20) and 30±8 pmole/min/mg protein (N=20), respectively (Table 3.6, 3.7, 3.8). Figure 3.1 illustrates the graphical representation of EROD activities with standard error of the mean.

Table 3.1. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Zonguldak Harbour

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	16	280
2	Female	13	239
3	Female	14	154
4	Female	12	389
5	Female	10	476
Average activity ± SEM N= 20*			308±56

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

Table 3.2. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Ereğli Harbour

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	15	258
2	Female	13	138
3	Female	12	131
4	Female	18	246
5	Female	18	125
Average activity ± SEM N= 20*			180±30

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

Table 3.3. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught in front of Erdemir Iron and Steel Works in Ereğli Harbour

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	13	122
2	Female	15	148
3	Female	14	119
Average activity ± SEM N= 11*			130±9

* Each microsomal fraction was prepared from four liver samples except sample 1 (prepared from three liver samples). N= represent total striped red mullet liver number.

Table 3.4. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Sakarya River Mouth

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	13	33
2	Female	12	11
3	Female	15	35
4	Female	14	46
Average activity ± SEM N= 16*			31±7

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

Table 3.5. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Amasra

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	11	53
2	Female	17	62
3	Female	14	40
4	Female	19	61
5	Female	13	69
6	Female	13	29
Average activity ± SEM N= 24*			52±6

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

Table 3.6. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from outside part of Kovanağzı in Kefken

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	12	15
2	Female	14	22
3	Female	18	26
4	Female	8	11
5	Female	15	30
Average activity± SEM N= 20*			21±4

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

Table 3.7. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from inside part of Kovanağzı in Kefken

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	22	81
2	Female	13	93
3	Female	16	46
4	Female	16	53
5	Female	17	85
Average activity ± SEM N= 20*			72±9

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

Table 3.8. EROD activities and microsomal protein concentrations of striped red mullet *(Mullus surmuletus)* caught from outside part of Kefken Harbour

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	11	42
2	Female	14	54
3	Female	10	22
4	Female	17	20
5	Female	16	13
Average activity ± SEM N= 20*			30±8

* Each microsomal fraction was prepared from four liver samples. N= represent total striped red mullet liver number.

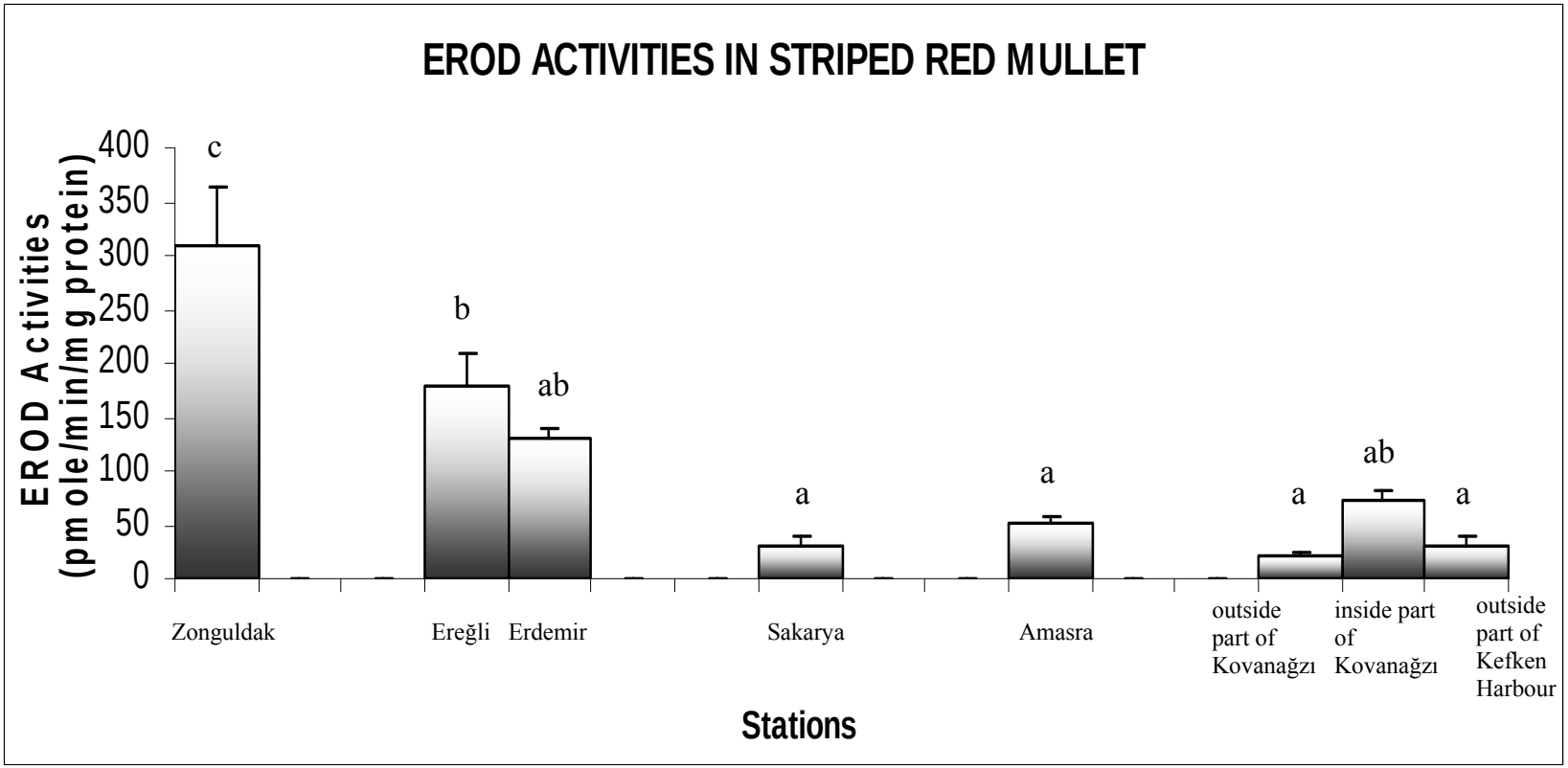


Figure 3.1. EROD activities of striped red mullet (*Mullus surmuletus*) caught from different stations: Zonguldak Harbour, Ereğli Harbour, Erdemir Iron and Steel Works, Sakarya River Mouth, Amasra, outside part of Kovanağzı, inside part of Kovanağzı and outside part of Kefken Harbour. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p<0.05$).

3.1.2. Glutathione S-Transferase Activity of Striped Red Mullets (*Mullus surmuletus*)

During microsome preparation, cytosols were also prepared for glutathione S-transferase (GST), catalase (CAT) and glutathione reductase (GR) activity measurements. Tables 3.9- 3.16 show cytosolic GST activity results obtained from striped red mullet samples caught from five different stations along the West Black Sea Coast of Turkey. All activities were measured in duplicates and sometimes in triplicates. Table 3.9 shows cytosolic protein and GST activities of striped red mullets samples caught from Zonguldak Harbour. The average GST activity was 852 ± 39 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.10 shows cytosolic protein and GST activities of striped red mullet samples caught from Ereğli Harbour. The average GST activity was 718 ± 35 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.11 shows cytosolic protein and GST activities of striped red mullet samples caught in front of Erdemir Iron and Steel Works in Ereğli Harbour. The average GST activity was 672 ± 10 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=11) in this region. Table 3.12 shows cytosolic protein and GST activities of striped red mullet samples caught from Sakarya River Mouth. The average GST activity was 851 ± 111 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=16) in this region. Table 3.13 shows cytosolic protein and GST activities of striped red mullet samples caught from Amasra. The average GST activity was found 779 ± 57 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=24) in this region. Table 3.14 shows cytosolic protein and GST activities of striped red mullet samples caught outside part of Kovanağzı in Kefken. The average GST activity was

1035 ± 136 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=20) in this region. 3.15 shows cytosolic protein and GST activities of striped red mullet samples caught inside part of Kovanağzı in Kefken. The average GST activity was 831 ± 33 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=20) in this region. 3.16 shows cytosolic protein and GST activities of striped red mullet samples caught outside part of Kefken Harbour. The average GST activity was 1056 ± 45 nmole CDNB-GSH/min/mg protein (Average activity± SEM, N=20) in this region. Figure 3.2 illustrates the graphical representation of GST activities with standard error of the mean.

Table 3.9. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Zonguldak Harbour

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	12	975
2	Female	10	853
3	Female	10	894
4	Female	13	770
5	Female	12	767
Average activity ± SEM N= 20			852 ± 39

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.10. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Ereğli Harbour

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	11	730
2	Female	10	741
3	Female	13	833
4	Female	9	646
5	Female	13	641
Average activity ± SEM N= 20			718±35

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.11. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught in front of the Erdemir Iron and Steel Works in Ereğli Harbour

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	9	690
2	Female	9	669
3	Female	7	658
Average activity ± SEM N= 11			672±10

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.12. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Sakarya River Mouth

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	5	1060
2	Female	7	1015
3	Female	10	727
4	Female	7	602
Average activity ± SEM N= 16			851±111

*Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.13. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught from Amasra

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	6	928
2	Female	12	598
3	Female	9	914
4	Female	7	716
5	Female	10	662
6	Female	6	856
Average activity± SEM N= 24			779±57

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.14. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught outside part of Kovanağzı in Kefken

Sample Number	Sex	CytosolicProtein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	5	1546
2	Female	7	803
3	Female	11	854
4	Female	5	890
5	Female	5	1082
Average activity ± SEM N= 20			1035±136

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.15. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught inside the Kovanağzı in Kefken

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	9	856
2	Female	11	819
3	Female	9	908
4	Female	8	859
5	Female	12	711
Average activity ± SEM N= 20			831±33

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.16. GST activities and cytosolic protein concentrations of striped red mullet *(Mullus surmuletus)* caught outside the Kefken Harbour

Sample Number	Sex	Cytosolic Protein (mg/ml)	GST Activity (nmole CDNB-GSH/min/mg protein)
1	Female	4	955
2	Female	4	1089
3	Female	4	1012
4	Female	6	1214
5	Female	5	1010
Average activity ± SEM N= 20			1056 ± 45

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

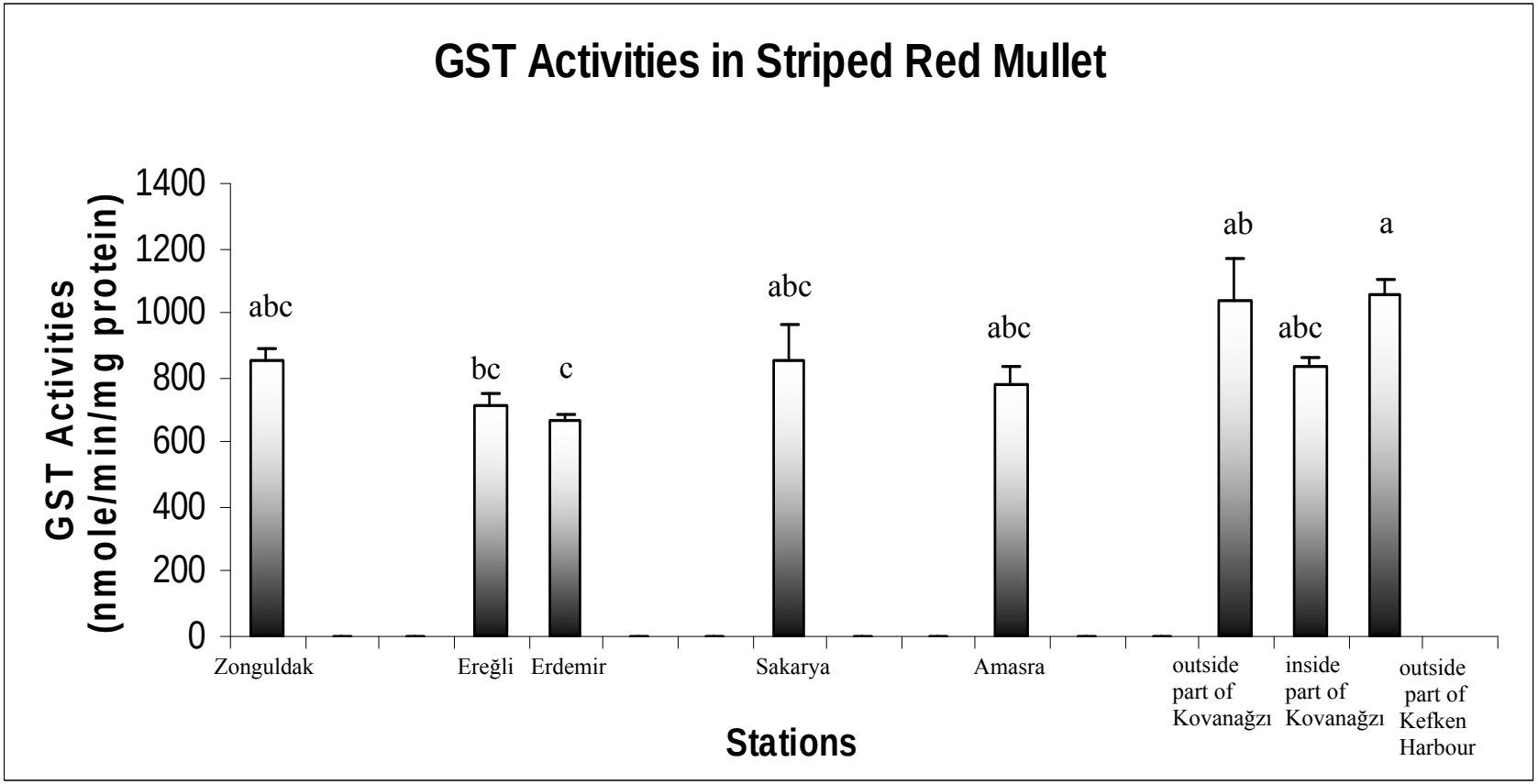


Figure 3.2. GST activities of striped red mullet (*Mullus surmuletus*) caught from different stations: Zonguldak Harbour, Ereğli Harbour, Erdemir Iron and Steel Works, Sakarya River Mouth, Amasra, outside part of Kovanağzı, inside part of Kovanağzı and outside part of Kefken Harbour. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p<0.05$).

3.1.3. Catalase Activity of Striped Red Mullets (*Mullus surmuletus*)

Catalase activities (CATs) were also measured in the cytosols of striped red mullet liver. Tables 3.17-3.24 show cytosolic catalase activity results obtained from the livers caught from five different stations along the West Black Sea coast of Turkey. All activities were measured in duplicates and sometimes in triplicates. Table 3.17 shows catalase activities of striped red mullet samples caught from Zonguldak Harbour. The average catalase activity was 120 ± 13 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.18 shows catalase activities of striped red mullet samples caught from Ereğli Harbour. The average CAT activity was 136 ± 14 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.19 shows catalase activities of striped red mullet samples caught in front of Erdemir Iron and Steel Works in Ereğli Harbour. The average catalase activity was 173 ± 18 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=11) in this region. Table 3.20 shows catalase activities of striped red mullet samples caught from Sakarya River Mouth. The average catalase activity was 89 ± 10 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=16) in this region. Table 3.21 shows catalase activities of striped red mullet samples caught from Amasra. The average catalase activity was 92 ± 5 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=24) in this region. Table 3.22 shows catalase activities of striped red mullet samples caught outside part of Kovanağzı in Kefken. The average catalase activity was 126 ± 2 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.23 shows catalase activities of striped red mullet samples caught inside part of Kovanağzı in Kefken. The average catalase activity was 96 ± 13

nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.24 shows catalase activities of striped red mullet samples caught inside part of Kovanağzı in Kefken. The average catalase activity was 130 ± 10 nmole H_2O_2 consumed/min/mg protein (Average activity± SEM, N=20) in this region. Figure 3.3 illustrates the graphical representation of catalase activities with standard error of the mean.

Table 3.17. Catalase activities of striped red mullet *(Mullus surmuletus)* caught from Zonguldak Harbour

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	136
2	Female	150
3	Female	133
4	Female	98
5	Female	81
Average activity± SEM n= 20		120±13

***** Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.18. Catalase activities of striped red mullet *(Mullus surmuletus)* caught from Ereğli Harbour

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	142
2	Female	178
3	Female	140
4	Female	126
5	Female	92
Average activity± SEM N= 20		136±14

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.19. Catalase activities of striped red mullet *(Mullus surmuletus)* caught from Erdemir region in Ereğli Harbour

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	208
2	Female	161
3	Female	152
Average activity± SEM n= 11		173±18

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.20. Catalase activities of striped red mullet *(Mullus surmuletus)* caught from Sakarya River Mouth

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	97
2	Female	110
3	Female	84
4	Female	64
Average activity± SEM N= 16		89±10

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.21. Catalase activities of striped red mullet *(Mullus surmuletus)* caught from Amasra

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	96
2	Female	80
3	Female	109
4	Female	86
5	Female	81
6	Female	101
Average activity± SEM N= 24		92±5

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.22. Catalase activities of striped red mullet *(Mullus surmuletus)* caught outside part of Kovanağzı in Kefken

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	126
2	Female	130
3	Female	118
4	Female	126
5	Female	128
Average activity± SEM N= 20		126±2

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.23. Catalase activities of striped red mullet *(Mullus surmuletus)* caught inside part of Kovanağzı in Kefken

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	126
2	Female	72
3	Female	125
4	Female	95
5	Female	64
Average activity± SEM N= 20		96±13

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.24. Catalase activities of striped red mullet *(Mullus surmuletus)* caught outside part of Kefken Harbour

Sample Number	Sex	Catalase Activity (nmole H_2O_2 consumed/min/mg protein)
1	Female	104
2	Female	122
3	Female	118
4	Female	157
5	Female	149
Average activity± SEM N= 20		130±10

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

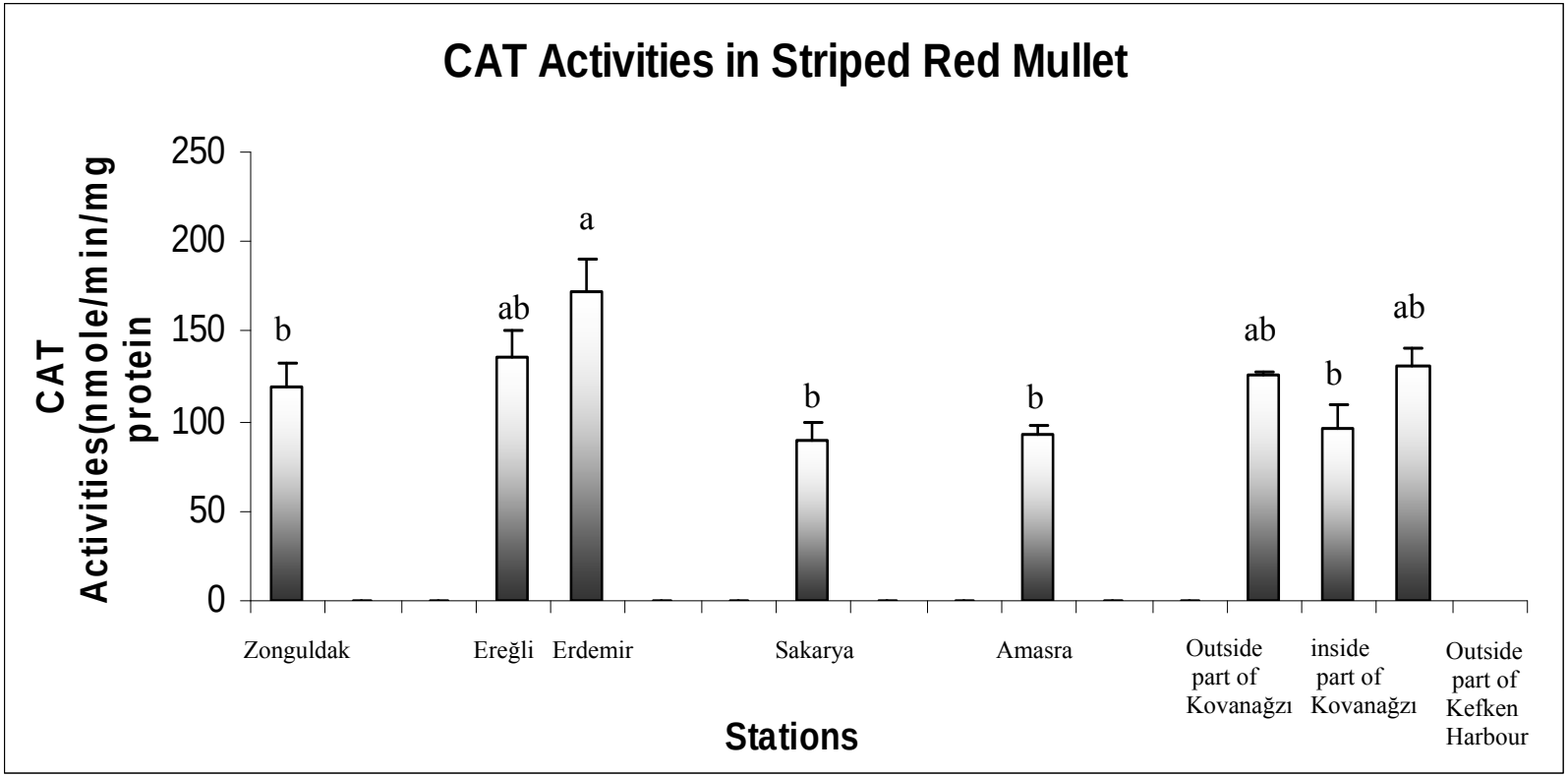


Figure 3.3. CAT activities of striped red mullet (*Mullus surmuletus*) caught from different stations: Zonguldak Harbour, Ereğli Harbour, Erdemir Iron and Steel Works, Sakarya River Mouth, Amasra, outside part of Kovanağzı, inside part of Kovanağzı and outside part of Kefken Harbour. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p<0.05$).

3.1.4. Glutathione Reductase Enzyme Activity of Striped Red Mullets (*Mullus surmuletus*)

Glutathione reductase is another antioxidant enzyme measured in striped red mullet. Tables 3.25-3.32 show glutathione reductase activity results obtained from the livers caught from five different stations along the West Black Sea coast of Turkey. All activities were measured in duplicates and sometimes in triplicates. Table 3.25 shows glutathione reductase activities of striped red mullet samples caught from Zonguldak Harbour. The average glutathione reductase activity was 7.6 ± 1.1 nmole/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.26 shows glutathione reductase activities of striped red mullet samples caught from Ereğli Harbour. The average glutathione reductase activity was 8.2 ± 0.8 nmole/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.27 shows glutathione reductase activities of striped red mullet samples caught in front of Erdemir Iron and Steel Works in Ereğli Harbour. The average glutathione reductase activity was 7.8 ± 0.5 nmole/min/mg protein (Average activity± SEM, N=11) in this region. Table 3.28 shows glutathione reductase activities of striped red mullet samples caught from Sakarya River Mouth. The average glutathione reductase activity was 8.1 ± 1.0 nmole/min/mg protein (Average activity± SEM, N=16) in this region. Table 3.29 shows glutathione reductase activities of striped red mullet samples caught from Amasra. The average glutathione reductase activity was 7.7 ± 0.9 nmole/min/mg protein (Average activity± SEM, N=24) in this region. Table 3.30 shows glutathione reductase activities of striped red mullet samples caught outside part of Kovanağzı in Kefken. The average GR activity was 8.8 ± 0.7 nmole/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.31 shows glutathione

reductase activities of striped red mullet samples caught inside part of Kovanağzı in Kefken. The average glutathione reductase activity was 7.9 ± 1.0 nmole/min/mg protein (Average activity± SEM, N=20) in this region. Table 3.32 shows glutathione reductase activities of striped red mullet samples caught outside part of Kefken Harbour. The average glutathione reductase activity was 8.2 ± 0.4 nmole/min/mg protein (Average activity± SEM, N=20) in this region. Figure 3.4 illustrates the graphical representation of glutathione reductase activities with standard error of the mean.

Biomarker enzyme activity results measured in striped red mullet caught from the West Black Sea coast of Turkey were summarized in Table 3.33. EROD activities of striped red mullet caught from Zonguldak Harbour was significantly different from the other sampling stations ($p<0.05$). There was no significant difference in EROD activities of striped red mullet caught from two different stations in Ereğli Harbour. However, EROD activities of striped red mullet caught from the center of Ereğli Harbour was significantly different from Zonguldak Harbour, Sakarya, Amasra and Kefken. There was no significant difference in EROD activities between Amasra, Sakarya, outside part of Kovanağzı in Kefken and outside part of Kefken Harbour. There were no meaningful differences in glutathione S-transferase and glutathione reductase activities measured in striped red mullet samples between stations. However, the catalase activity of striped red mullets caught in front of Erdemir Iron and Steel Works was significantly different from Amasra and inside part of Kovanağzı.

Table 3.25. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught from Zonguldak Harbour

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	7.4
2	Female	7.6
3	Female	11.5
4	Female	4.8
5	Female	6.5
Average activity± SEM N= 20		7.6± 1.1

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.26. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught from Ereğli Harbour

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	7.6
2	Female	6.8
3	Female	11.0
4	Female	9.3
5	Female	6.5
Average activity± SEM N= 20		8.2±0.8

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.27. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught in front of the Erdemir Iron and Steel Works in Ereğli Harbour

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	7.0
2	Female	8.7
3	Female	7.8
Average activity± SEM N= 11		7.8±0.5

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.28. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught from Sakarya River Mouth

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	7.3
2	Female	10.9
3	Female	8.1
4	Female	6.2
Average activity± SEM N= 16		8.1±1.0

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.29. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught from Amasra

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	7.8
2	Female	11.2
3	Female	9.2
4	Female	5.9
5	Female	5.4
6	Female	6.8
Average activity± SEM N= 24		7.7±0.9

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.30. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught outside part of Kovanağzı in Kefken

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	10.0
2	Female	8.3
3	Female	7.2
4	Female	7.7
5	Female	11.0
Average activity± SEM N= 20		8.8±0.7

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.31. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught inside part of Kovanağzı in Kefken

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	6.6
2	Female	6.8
3	Female	9.2
4	Female	11.4
5	Female	5.6
Average activity± SEM N= 20		7.9±1.0

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

Table 3.32. Glutathione reductase activities of striped red mullet *(Mullus surmuletus)* caught outside Kefken Harbour

Sample Number	Sex	Glutathione Reductase Activity (nmole/min/mg protein)
1	Female	8.4
2	Female	6.6
3	Female	8.7
4	Female	9.3
5	Female	8.0
Average activity± SEM N= 20		8.2±0.4

* Each microsomal fraction was prepared from four liver samples. N= represent total mullet liver number.

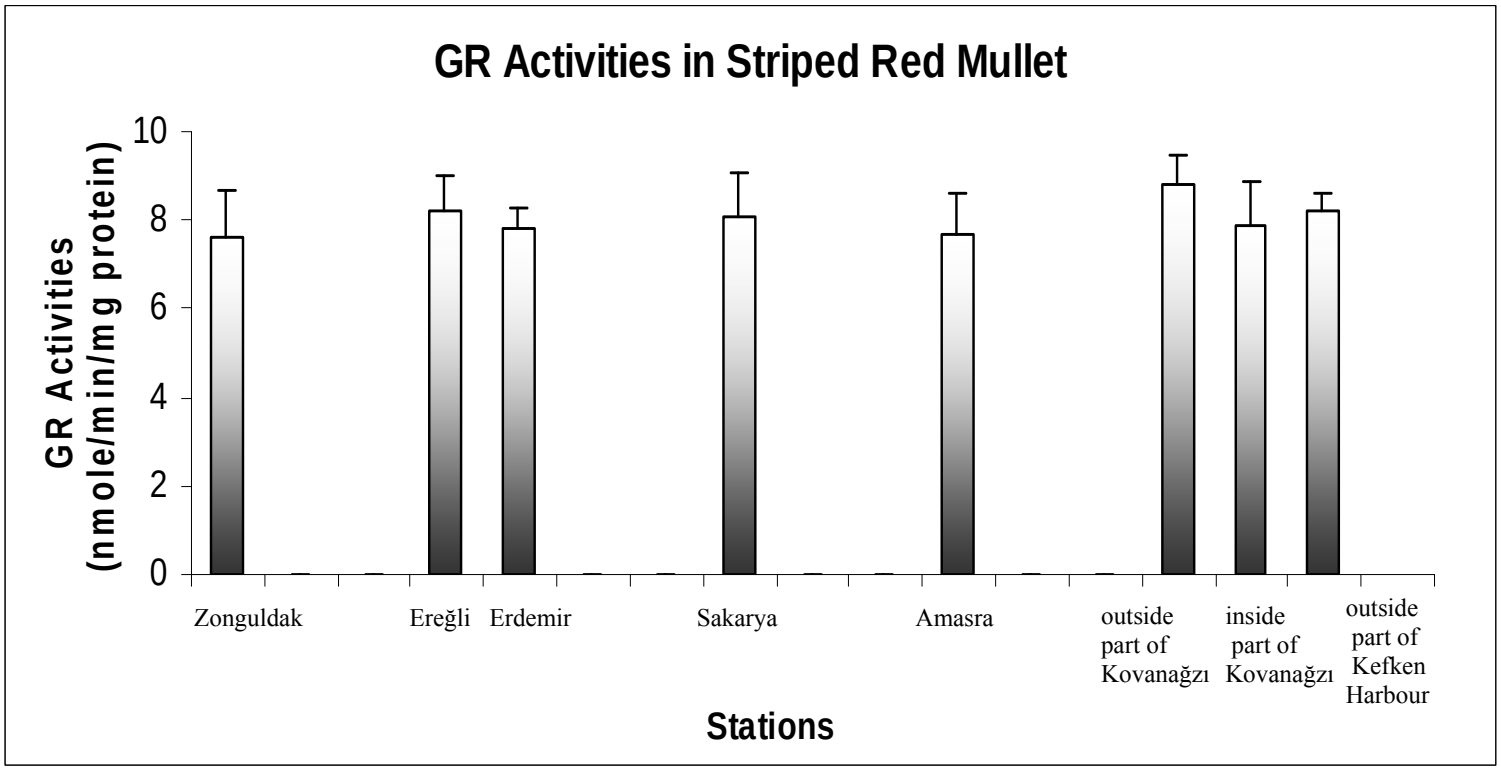


Figure 3.4. GR activities of striped red mullet (*Mullus surmuletus*) caught from different stations: Zonguldak Harbour, Ereğli Harbour, Erdemir Iron and Steel Works, Sakarya River Mouth, Amasra, outside part of Kovanağzı, inside part of Kovanağzı and outside part of Kefken Harbour.

Table 3.33. Mean values of biomarker enzyme activities and standard error of mean (SEM) for striped red mullet (*Mullus surmuletus*). Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p<0.05$)

Locations	N	EROD*	GST**	CAT**	GR**
Zonguldak Harbour	20	308 ± 56^{c}	852 ± 39^{abc}	120 ± 13^{b}	7.6 ± 1.1
Ereğli Harbour	20	180 ± 30^{b}	718 ± 35^{bc}	136 ± 14^{ab}	8.2 ± 0.8
Ereğli H. (Erdemir)	11	130 ± 9^{ab}	672 ± 10^{c}	173 ± 18^{a}	7.8 ± 0.5
Sakarya River Mouth	16	31 ± 7^{a}	851 ± 111^{abc}	89 ± 10^{b}	8.1 ± 1.0
Amasra	24	52 ± 6^{a}	779 ± 57^{abc}	92 ± 5^{b}	7.7 ± 0.9
Outside part of Kovanağzı	20	21 ± 4^{a}	1035 ± 136^{ab}	126 ± 2^{ab}	8.8 ± 0.7
Inside part of Kovanağzı	20	72 ± 9^{ab}	831 ± 33^{abc}	96 ± 13^{b}	7.9 ± 1.0
Outside part of Kefken Harbour	20	30 ± 8^{a}	1056 ± 45^{a}	130 ± 10^{ab}	8.2 ± 0.4
	151				

* pmole min^{-1} mg $protein^{-1}$

** nmole min^{-1} mg $protein^{-1}$

3.2. Liver Microsomal 7-Ethoxyresorufin O-Deethylase Activity of Flathead Mullets (*Mugil cephalus*) and Golden Grey Mullet (*Liza aurata*)

In this study, 46 mullet samples were also caught from Zonguldak Harbour, Ereğli Harbour, Gülüç Stream Mouth, Sakarya River Mouth, Amasra, and Kefken in 2011. Tables 3.34-3.41 show microsomal EROD activity results obtained from the livers of each fish caught from six different stations along the West Black Sea coast of Turkey. All activities were measured in duplicates and sometimes in triplicates. Table 3.34 shows microsomal protein and EROD activities of flathead mullet (*Mugil cephalus*) caught from Zonguldak Harbour. The average EROD activity was 4744 ± 390 pmole/min/mg protein (Average activity± SEM, N=11) in this region. Table 3.35 shows microsomal protein and EROD activities of golden grey mullet (*Liza aurata*) caught from Ereğli Harbour. The average EROD activity was 4243 ± 1037 pmole/min/mg protein (Average activity± SEM, N=5) in this region. Table 3.36 shows microsomal protein and EROD activities of flathead mullets (*Mugil cephalus*) caught from Gülüç Stream Mouth. The average EROD activity was 1936 ± 223 pmole/min/mg protein (Average activity± SEM, N=10) in this region. Table 3.37 shows microsomal protein and EROD activities of flathead mullets (*Mugil cephalus*) caught from Sakarya River Mouth. The average EROD activity was 584 ± 137 pmole/min/mg protein (Average activity± SEM, N=7) in this region. Table 3.38 shows microsomal protein and EROD activities of flathead mullets (*Mugil cephalus*) caught from Amasra. The average EROD activity was 546 ± 85 pmole/min/mg protein (Average activity± SEM, N=4) in this region. Table 3.39 shows microsomal protein and EROD activities of golden grey mullets (*Liza aurata*) caught from

Amasra. The average EROD activity was 316 ± 190 pmole/min/mg protein (Average activity± SEM, N=2) in this region. Table 3.40 shows microsomal protein and EROD activities of flathead mullets (*Mugil cephalus*) caught from Kefken. The average EROD activity was 406 ± 100 pmole/min/mg protein (Average activity± SEM, N=3) in this region. Table 3.41 shows microsomal protein and EROD activities of golden grey mullets (*Liza aurata*) caught from Kefken. The average EROD activity was 339 ± 17 pmole/min/mg protein (Average activity± SEM, N=4) in this region. Figure 3.5 illustrates the graphical representation of EROD activities with standard error of the mean.

Table 3.34. EROD activities and microsomal protein concentrations of flathead mullet *(Mugil cephalus)* caught from Zonguldak Harbour

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	21	5750
2	Male	27	6756
3	Male	28	5103
4	Female	30	4068
5	Female	23	3272
6	Female	30	4058
7	Female	22	3779
8	Female	34	4022
9	Female	27	5606
10	Female	29	6646
11	Female	24	3122
Average Activity ± SEM N= 11*			4744 ± 390

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.35. EROD activities and microsomal protein concentrations of golden grey mullet *(Mugil cephalus)* caught from Ereğli Harbour

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	25	1144
2	Female	18	6885
3	Female	15	5189
4	Female	14	5393
5	Female	25	2604
Average activity ± SEM N= 5*			4243 ± 1037

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.36. EROD activities and microsomal protein concentrations of flathead mullet *(Mugil cephalus)* caught from Gülüç Stream Mouth

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	28	1927
2	Female	25	1283
3	Female	19	1849
4	Female	27	2381
5	Female	16	1287
6	Female	28	1603
7	Female	20	1678
8	Female	18	1706
9	Male	18	1922
10	Female	12	3724
Average activity ± SEM N= 10*			1936 ± 223

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.37. EROD activities and microsomal protein concentrations of flathead mullet *(Mugil cephalus)* caught from Sakarya River Mouth

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmole/min/mg protein)
1	Female	24	1152
2	Female	11	288
3	Female	20	935
4	Female	12	391
5	Female	10	116
6	Female	12	640
7	Female	14	564
Average activity ± SEM N= 7*			584 ± 137

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.38. EROD activities and microsomal protein concentrations of flathead mullet *(Mugil cephalus)* caught from Amasra

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmol/dk./mg protein)
1	Female	14	346
2	Female	16	754
3	Female	17	500
4	Male	20	584
Average Activity ± SEM N= 4*			546 ± 85

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.39. EROD activities and microsomal protein concentrations of golden grey mullet *(Liza aurata)* caught from Amasra

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmol/dk./mg protein)
1	Male	20	506
2	Female	27	126
Average Activity ± SEM N= 2*			316 ± 190

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.40. EROD activities and microsomal protein concentrations of flathead mullet *(Mugil cephalus)* caught from Kefken

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmol/dk./mg protein)
1	Female	19	606
2	Female	15	299
3	Female	9	313
Average Activity ± SEM N= 3*			406 ± 100

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

Table 3.41. EROD activities and microsomal protein concentrations of golden grey mullet *(Liza aurata)* caught from Kefken

Sample Number	Sex	Microsomal Protein (mg/ml)	EROD Activity (pmol/dk./mg protein)
1	Female	23	312
2	Female	6	374
3	Male	17	308
4	Female	9	362
Average Activity ± SEM N= 4*			339 ± 17

* Each microsomal fraction was prepared from one liver. N= represent total mullet liver number.

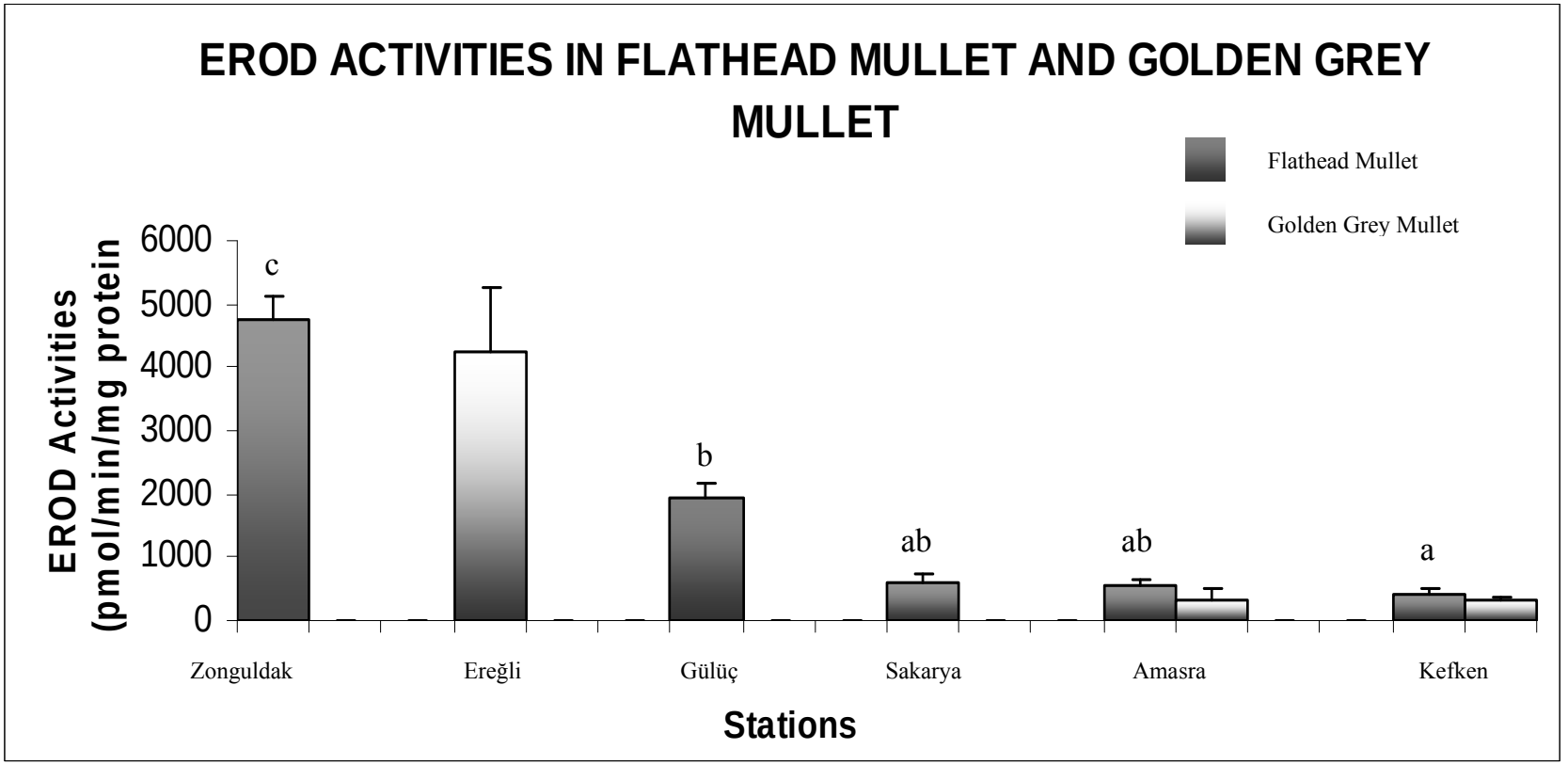


Figure 3.5. EROD activities of flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) caught from different stations: Zonguldak Harbour, Ereğli Harbour, Gülüç Stream Mouth, Sakarya River Mouth, Amasra and Kefken. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p<0.05$).

Table 3.42 shows the summary of the EROD activities measured in flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) caught from the West Black Sea coast of Turkey. The highest EROD activities were found in the mullet samples caught from Zonguldak Harbour. The lowest EROD activities were measured in fish samples (flathead mullet and golden grey mullet) caught from Amasra and Kefken. The EROD activities were compared with the corresponding mullet species caught from these stations. EROD activities of flathead mullet were significantly different from the EROD activities of Amasra and Kefken. EROD activities of flathead mullets caught from Gülüç Stream Mouth were significantly different from Zonguldak Harbour and Kefken.

Table 3.42. Mean values of EROD activities for flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*). Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p<0.05$)

Locations	Year	N	Fish	EROD*
Zonguldak Harbour	2011	11	*Mugil cephalus*	4744 ± 390^{c}
Ereğli Harbour	2011	5	*Liza aurata*	4243±1037
Gülüç Stream Mouth	2011	10	*Mugil cephalus*	1936 ± 223^{b}
Sakarya River Mouth	2011	7	*Mugil cephalus*	584 ± 137^{ab}
Amasra	2011	4	*Mugil cephalus*	546 ± 85^{ab}
	2011	2	*Liza aurata*	316±190
Kefken	2011	3	*Mugil cephalus*	406 ± 100^{a}
	2011	4	*Liza aurata*	339±17

* pmole min^{-1} mg $protein^{-1}$

3.3. Polycyclic Aromatic Hydrocarbon Analyses in Fish Liver Tissues

Polycyclic aromatic hydrocarbon residues were determined in striped red mullet caught in 2009-2011 and in flathead mullet and golden grey mullet caught in 2011 in this study. Table 3.44 and Table 3.45 show the PAHs measured in these fish samples. Acenapththylene, acenaphthene, phenanthrene, anthracene, fluoranthene and benzo[b]fluoranthene were measured in most of the fish collection stations. Benzo[a]anthracene, benzo[k]fluoranthene, benzo[a]pyrene, indeno(1,2,3,-cd)pyrene, dibenz(a,h)anthracene and benzo[g,h,i]perylene were below detection limits in most of the fish samples in all stations.

The concentrations of PAHs measured in striped red mullet were higher than those measured in flathead mullet and golden grey mullet. Acenaphthene, fluoranthene, pyrene, benzo [a] anthracene, benzo [b] fluoranthene, benzo [k] fluoranthene, benzo [a] pyrene, indeno (1,2,3,-cd) pyrene, dibenz (a,h) anthracene and benzo [g,h,i] perylene were below detection limits in striped red mullet caught from Amasra in 2009. Benzo[b]fluoranthene was the highest PAH measured in all sampling stations except for striped red mullet caught from Amasra in 2009. Its concentration ranged between 2.99 and 3.48 µg g^{-1} dry weight. Anthracene was also present in all sampling stations. The concentration of anthracene changed between 1.36 and 5.71 µg g^{-1} dry weight. Phenanthrene was present in all sampling stations and all samples caught in 2009-2011. The concentration of phenanthrene changed between 0.44 and 2.68 µg g^{-1} dry weight. Acenaphthylene was present in all sampling stations except for striped red mullet caught from Amasra in 2010. It ranged between 0.87 and 3.38 µg g^{-1} dry weight.

Benzo[b]fluoranthene measured in flathead mullet and golden grey mullet was not as high as measured in striped red mullet. Anthracene was the highest PAH measured in all sampling stations in flathead mullet and golden grey mullet caught in 2011. Some of the high molecular weight PAHs such as dibenzo (a,h) anthracene and benzo(g,h,i)perylene were also measured in flathead mullet and golden grey mullet samples. But, the incidence of occurrence of these PAHs in fish samples was restricted with one or two samples.

Table 3.43. Thirteen different PAH concentrations in the livers of the striped red mullet (*Mullus surmuletus*) caught from different stations of the West Black Sea coast of Turkey (µg/g dried tissue).

	Years	Fish	Acenaphthylene	Acenaphthene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benz[a]anthracene
Amasra	2009	*Mullus surmuletus*	1.451(2/3)	BDL (3/3)	2.184(3/3)	5.711(1/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)
Amasra	2010	*Mullus surmuletus*	BDL (4/4)	1.135(4/4)	2.431(4/4)	1.372(3/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Amasra	2011	*Mullus surmuletus*	3.376(1/3)	0.228(3/3)	0.437(3/3)	1.733(1/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)
Kefken	2009	*Mullus surmuletus*	2.107(3/3)	0.41(3/3)	2.47(3/3)	1.927(1/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)
Kefken	2010	*Mullus surmuletus*	1.275(6/6)	0.595(6/6)	2.526(6/6)	1.933(5/6)	0.232(1/6)	BDL (6/6)	BDL (6/6)
Kefken	2011	*Mullus surmuletus*	3.041(2/3)	0.25(3/3)	1.216(3/3)	BDL (3/3)	0.196(2/3)	BDL (3/3)	BDL (3/3)
Ereğli Harbour	2009	*Mullus surmuletus*	0.871(3/4)	0.508(4/4)	2.342(4/4)	2.699(4/4)	0.223(1/4)	BDL (4/4)	BDL (4/4)
Ereğli Harbour	2010	*Mullus surmuletus*	1.999(3/4)	0.524(4/4)	2.454(4/4)	1.563(4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Ereğli Harbour	2011	*Mullus surmuletus*	3.233(3/3)	0.245(3/3)	0.453(3/3)	1.744(2/3)	BDL(3/3)	BDL(3/3)	BDL (3/3)
Sakarya River Mouth	2010	*Mullus surmuletus*	1.762(3/4)	0.568(4/4)	2.653(4/4)	1.413(4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Sakarya River Mouth	2011	*Mullus surmuletus*	1.205(2/4)	0.354(3/4)	1.531(4/4)	2.869(4/4)	0.106(1/4)	BDL (4/4)	BDL (4/4)
Zonguldak Harbour	2009	*Mullus surmuletus*	0.890(3/3)	0.583(3/3)	2.387(3/3)	1.636(2/3)	0.198(1/3)	BDL (3/3)	BDL (3/3)
Zonguldak Harbour	2010	*Mullus surmuletus*	1.431(4/4)	0.661(4/4)	2.675(4/4)	2.048(4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Zonguldak Harbour	2011	*Mullus surmuletus*	2.906(4/4)	0.387(4/4)	1.447(4/4)	1.355(3/4)	0.173(2/4)	BDL (4/4)	BDL (4/4)

*BDL= Below Detection Limits

The numbers within the paranthesis indicates the number of samples above detection limit/the total number of samples

Table 3.43. (Continued) Thirteen different PAH concentrations in the livers of the striped red mullets (*Mullus surmuletus*) caught from different stations of the West Black Sea coast of Turkey (µg/g dried tissue).

	Years	Fish	Benzo(b) fluoranthene	Benzo (k) fluoranthene	Benzo (a)pyrene	Indeno(1,3,c,d pyrene)	Dibenzo (a.h) anthracene	Benzo(g,h,i)perylene
Amasra	2009	*Mullus surmuletus*	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)
Amasra	2010	*Mullus surmuletus*	3.438(1/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	0.186(1/4)
Amasra	2011	*Mullus surmuletus*	3.341(3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	0.633 (2/3)	BDL (3/3)
Kefken	2009	*Mullus surmuletus*	3.479(3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)
Kefken	2010	*Mullus surmuletus*	3.440(6/6)	BDL (6/6)	BDL (6/6)	BDL (6/6)	0.260 (1/6)	BDL (6/6)
Kefken	2011	*Mullus surmuletus*	3.318(2/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)
Ereğli Harbour	2009	*Mullus surmuletus*	3.276(4/4)	BDL (4/4)	BDL (4/4)	BDL(4/4)	BDL (4/4)	BDL (4/4)
Ereğli Harbour	2010	*Mullus surmuletus*	3.108(4/4)	BDL (4/4)	BDL (3/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Ereğli Harbour	2011	*Mullus surmuletus*	3.003(3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL (3/3)	BDL(3/3)
Sakarya River Mouth	2010	*Mullus surmuletus*	3.273(4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Sakarya River Mouth	2011	*Mullus surmuletus*	0.182(1/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	0.264(1/4)
Zonguldak Harbour	2009	*Mullus surmuletus*	3.341(3/3)	BDL (3/3)	BDL(3/3)	BDL (3/3)	BDL (3/3)	0.003(1/3)
Zonguldak Harbour	2010	*Mullus surmuletus*	3.475(4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)
Zonguldak Harbour	2011	*Mullus surmuletus*	2.992(4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)	BDL (4/4)

*BDL= Below Detection Limits

The numbers within the paranthesis indicates the number of samples above detection limit/the total number of samples

Table 3.44. Thirteen different PAH concentrations in the livers of the flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) caught from different stations of the West Black Sea coast of Turkey (µg/g dried tissue).

	Years	Fish	Acenaphthylene	Acenaphthene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benz[a]anthracene
Amasra	2011	*Mugil cephalus*	BDL(4/4)	0.606(3/4)	0.932(2/4)	13.976(2/4)	0.143(1/4)	0.868(2/4)	BDL(4/4)
Amasra	2011	*Liza aurata*	1.745(2/2)	0.423(1/2)	BDL(2/2)	8.004(2/2)	BDL(2/2)	BDL(2/2)	BDL(2/2)
Kefken	2011	*Mugil cephalus*	0.388(1/3)	0.934(2/3)	0.491(2/3)	0.849(1/3)	0.068(1/3)	BDL(3/3)	BDL(3/3)
Kefken	2011	*Liza aurata*	0.629(1/3)	0.713(1/3)	0.648(2/3)	2.384(3/3)	0.065(1/3)	BDL(3/3)	BDL(3/3)
Ereğli Harbour	2011	*Liza aurata*	0.961(3/4)	0.509(4/4)	1.18(3/4)	4.621(4/4)	0.153(1/4)	BDL(4/4)	BDL(4/4)
Sakarya River Mouth	2011	*Mugil cephalus*	0.628(4/7)	0.483(4/7)	1.177(6/7)	4.566(5/7)	0.320(1/7)	0.546(4/7)	BDL(7/7)
Gülüç Stream Mouth	2011	*Mugil cephalus*	0.734(6/10)	0.596(8/10)	1.283(10/10)	3.259(8/10)	0.291(3/10)	0.535(1/10)	BDL(10/10)
Zonguldak Harbour	2011	*Mugil cephalus*	1.283(3/10)	0.438(10/10)	0.827(8/10)	3.675(6/10)	0.145(2/10)	BDL(10/10)	BDL(10/10)

*BDL= Below Detection Limits

The numbers within the paranthesis indicates the number of samples above detection limit/the total number of samples

Table 3.44. (Continued) Thirteen different PAH concentrations in the livers of the flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) caught from different stations of the West Black Sea coast of Turkey (µg/g dried tissue).

	Years	Fish	Benzo(b) fluoranthene	Benzo (k) fluoranthene	Benzo (a)pyrene	Indeno(1,2,3,cd) pyrene	Dibenzo (a,h) anthracene	Benzo(g,h,i)perylene
Amasra	2011	*Mugil cephalus*	0.439(4/4)	BDL(4/4)	BDL(4/4)	BDL(4/4)	0.268(1/4)	BDL(4/4)
Amasra	2011	*Liza aurata*	0.510(2/2)	BDL(2/2)	BDL(2/2)	BDL(2/2)	1.241(1/2)	BDL(2/2)
Kefken	2011	*Mugil cephalus*	0.423(1/3)	BDL(3/3)	BDL(3/3)	BDL(3/3)	BDL(3/3)	BDL(3/3)
Kefken	2011	*Liza aurata*	0.475(2/3)	BDL(3/3)	BDL(3/3)	BDL(3/3)	BDL(3/3)	BDL(3/3)
Ereğli Harbour	2011	*Liza aurata*	0.465(1/4)	BDL(4/4)	BDL(4/4)	BDL(4/4)	BDL(4/4)	BDL(4/4)
Sakarya River Mouth	2011	*Mugil cephalus*	0.478(2/7)	BDL(7/7)	BDL(7/7)	BDL(7/7)	0.573(3/7)	BDL(7/7)
Gülüç Stream Mouth	2011	*Mugil cephalus*	0.221(3/10)	BDL(10/10)	BDL(10/10)	BDL(10/10)	0.575(1/10)	BDL(10/10)
Zonguldak Harbour	2011	*Mugil cephalus*	0.327(2/10)	BDL(10/10)	BDL(10/10)	BDL(10/10)	2.128(3/10)	1.640(2/10)

*BDL= Below Detection Limits

The numbers within the paranthesis indicates the number of samples above detection limit/the total number of samples

DISCUSSION

Industrial areas, rivers, coastal cities have tendency to create local pollution in the Black Sea. One of the coastal cities in the West Black Sea Region of Turkey is Zonguldak. Zonguldak is an important coal mining area in Turkey. The harbour of this city is especially polluted with coal processing wastes and discharges of industrial and domestic wastes. Ereğli is a large district of Zonguldak. The harbours of Zonguldak and Ereğli are among the most polluted regions in the Black Sea. Increasing population, shipping activities, uncontrolled discharges from industries and coastal settlements are among the reasons of pollution in these harbours. Another location under the risk of pollution in the West Black Sea Region of Turkey is Sakarya River. Sakarya River, from its source in the West Anatolia, flows through many industrial and agricultural areas and drains into the Black Sea. It carries many pollutants to the Black Sea from inner regions of Turkey (Tuncer et al., 1998; Barlas, 1999). The presence of pollutants has been shown in our previous study in fish samples caught from the West Black Sea coast of Turkey by biomarker enzyme activities. Highly elevated EROD activities have been found in flathead mullet (*Mugil cephalus*), so-iuy mullet (*Mugil soiuy*) and golden grey mullet (*Liza aurata*) samples caught from Zonguldak and Ereğli Harbours and Gülüç Stream Mouth in 2005, 2006 and 2007 (Bozcaarmutlu el al., 2009). In this study, our aim was to assess the suitability of striped red mullet (Mullus surmuletus) in monitoring of environmental pollution by using biomarker enzyme activities and continue to

monitor the pollution in flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) samples caught from the West Black Sea coast of Turkey.

Striped red mullet (*Mullus surmuletus*), flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) were caught from different stations having a varying degree of pollution in the West Black Sea Region of Turkey in 2011. Fish were killed by decapitation and livers were removed immediately by avoiding the gall bladders and flash frozen in liquid nitrogen. The contents of the gall bladder are the inhibitors of cytochrome P450 dependent system. Liver tissues were used in the biomarker enzyme activity measurements and chemical analyses. Liver is the primary organ in the metabolism of xenobiotics. The absorbed molecules especially through the gastrointestinal route are directed toward the liver. Therefore, the concentrations of recently exposed chemicals are higher in the liver than the other organs. The first metabolic reactions on the molecules are achieved by the biotransformation enzymes present in the liver.

Biotransformation reactions can be classified as phase I and phase II. Most of the biotransformation reactions begin with cytochrome P450 dependent phase I reactions. Cytochrome P450 catalyzed reactions are monooxygenase reactions. Both exogenous and endogenous molecules are metabolized by cytochrome P450 dependent monooxygenase reactions. Induction of CYP1A and associated EROD activity are the best characterized and used biomarker of exposure of fish to organic pollutants such as PCBs, PAHs and dioxins. CYP1A induction also acts as an early warning sign for potential harmful effects of many organic molecules. Glutathione-S-transferases (GSTs) are the main enzymes involved in xenobiotic phase II

metabolism. They catalyse the conjugation of electrophilic compounds (or phase I metabolites) with reduced glutathione (GSH). GSTs detoxify a number of environmental carcinogens and epoxide intermediates (Gallagher et al., 1996). Glutathione S-transferase activity measurement is also used as a marker of oxidative stress (Rodriguez-Ariza et al., 1993; Martínez- Gómez et al., 2006). The induction of antioxidant enzyme activities represents a cellular defense mechanism to neutralize toxic effects of reactive oxygen species and metals. It has been shown that the pollutants such as PAHs and PCBs cause significant alterations in the activities of antioxidant enzymes in fish (Otto and Moon, 1995, Van der Oost et al., 2003; Ferreira et al., 2005).

In this study biomarker enzyme activities were measured in striped red mullet samples caught from the West Black Sea coast of Turkey in 2011. All the fish samples were female. They were captured during pre-spawning period. It has been well known that the biomarker enzyme activities decrease after spawning period (Lange et al., 1999). Before spawning period, striped red mullet migrates to shallow waters. It comes near the coasts of Black Sea at the end of April and stays there until the end of June. In this study, the sample collection was carried out at the end of May. CYP1A related EROD activity, glutathione S-transferase, catalase and glutathione reductase activities were measured in the liver of striped red mullet samples. Highly elevated EROD activities were found in striped red mullet samples caught from Zonguldak and Ereğli Harbours. The lowest EROD activities were found in striped red mullet samples caught from Amasra and Kefken. The EROD activities of striped red mullet caught from inside part of Kovanağzı were slightly higher than the outside part of Kovanağzı and Kefken Harbour. However, this

difference was not significant. Striped red mullets caught from Zonguldak Harbour had 6 fold higher EROD activities than those obtained from Amasra. Striped red mullets caught from Zonguldak Harbour had 10 fold higher EROD activities than those obtained from outside part of Kefken Harbour and 14 fold higher EROD activities than those obtained from outside part of Kovanağzı. The EROD activities of striped red mullet caught from Zonguldak Harbour was significantly different from all the other sampling stations ($p<0.05$). Striped red mullet samples were caught from two different locations in Ereğli. High EROD activities were also measured in striped red mullet samples caught from the center of Ereğli Harbour. The other sampling station is around Erdemir Iron and Steel Works. EROD activities of fish caught in front of Erdemir Iron and Steel Works was slightly lower than those obtained from Ereğli Harbour. However, this difference was not significant. This difference may result from the presence of chemicals such as metals around Erdemir Iron and Steel Works. However, the EROD activities of striped red mullet caught from the center of Ereğli Harbour was significantly different from Sakarya River Mouth, Amasra and Kefken. There were no significant differences in EROD activities between Amasra, Sakarya River Mouth, outside part of Kovanağzı in Kefken and outside part of Kefken Harbour. Sakarya River is the third longest river of Turkey. It runs through many industrial and agricultural areas in Anatolia before reaching to the Black Sea. In our study, the EROD activities and the other biomarker enzyme activities of striped red mullet caught from Sakarya River Mouth were not significantly different from relatively clean sites Amasra and Kefken. Many rehabilitation studies have been carried out in Sakarya River especially around Eskişehir in recent years. The amount of rainfall is high in this part of the Turkey in

recent years. As a result of these factors, the amount of pollutants brought by Sakarya River may have decreased in recent years.

In addition to 7-ethoxyresorufin O-deethylase (EROD) activity measurements, the levels of glutathione S-transferase, catalase and glutathione reductase activities were also measured in striped red mullet samples. There were no meaningful differences in glutathione S-transferase and glutathione reductase activities measured in striped red mullet samples between stations. However, the catalase activity of striped red mullets caught in front of Erdemir Iron and Steel Works was significantly different from Amasra and inside part of Kovanağzı. Catalase enzyme activity is induced by heavy metals. Low EROD and GST activities and slightly high catalase activity measured in striped red mullet can be an indicator of the presence of heavy metals around Erdemir Iron and Steel Works. Further studies are needed to show the presence of metals in this area.

There are interspecies differences in response to pollutants. It has been shown in many different biomonitoring studies that CYP1A protein and its related EROD activity induce in all fish species examined in the presence of PAH, PCB, dioxin type pollutants in the environment. However there are conflicting results in GST and antioxidant enzyme activities. There are many studies in which these activities are induced by pollutants present in contaminated waters (Otto and Moon, 1995, Livingstone, 2001; Orbea et al., 2002; Sen and Kirikbakan, 2004; Ferreira et al., 2005; Martínez- Gómez et al., 2006). However, it has been shown in several studies that GST and some antioxidant enzyme activities have lower sensitivity to pollutants in several fish species (Canesi et al., 1999; Gowland et al., 2000; Bebianno et al.,

2007; Tsangaris et al., 2010). For example, the GST activity is not sensitive to PAHs in red mullet (*Mullus barbatus*) (Torre et al., 2010). Striped red mullet (*Mullus surmuletus*) is closely related with red mullet (*Mullus barbatus)*. In our study, the GST activity was insensitive to the pollutants present in different stations in the West Black Sea Region of Turkey. The result obtained from red mullet supports our results obtained in this study.

Flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*) samples were also caught from the West Black Sea coast of Turkey in 2011. EROD activities were measured in the liver tissues. The results obtained in these fish species were compared with the results of striped mullet. Highly elevated EROD activities were measured in mullet samples caught from Zonguldak and Ereğli Harbour. The highest EROD activity was found in flathead mullet samples caught from Zonguldak Harbour. Flathead mullets caught from Zonguldak Harbour had 9 fold higher EROD activities than those obtained from Amasra and had 12 fold higher EROD activities than those obtained from Kefken. The lowest EROD activities were measured in fish samples (flathead mullet and golden grey mullet) caught from Amasra and Kefken. The EROD activities measured in fish samples caught from the other stations were compared with the corresponding mullet species caught from Amasra and Kefken. The EROD activities of flathead mullet caught from Zonguldak Harbour was significantly different from the EROD activities of Amasra and Kefken ($p<0.05$). Highly elevated EROD activities were also measured in golden grey mullets caught from Ereğli Harbour. The EROD activities of fish caught from Ereğli Harbour were significantly different from Kefken ($p<0.05$). Gülüç Stream is another polluted station in the West Black Sea coast of Turkey. The Gülüç Stream is very close to the

Ereğli Harbour and Erdemir Iron and Steel Works. This small stream was highly polluted primarily by domestic waste discharges from Ereğli and villages around the stream. High EROD activities were also measured in flathead mullet samples caught from Gülüç Stream Mouth. EROD activities of flathead mullets caught from Gülüç Stream Mouth was significantly different from Zonguldak Harbour and Kefken ($p<0.05$). The EROD activities of flathead mullet samples caught from Sakarya River Mouth were not different from Amasra and Kefken. This result supports the results obtained in striped mullet caught from the same station. The differences in EROD activities between stations were at comparable values with striped red mullet and the other two fish species. This result indicates that striped red mullet is a suitable organism to monitor CYP1A inducer chemicals.

In our previous study, high EROD activities have been reported in mullet samples caught from Zonguldak and Ereğli Harbours and Gülüç Stream Mouth (Bozcaarmutlu et al., 2009). In 2011, high EROD activities were also measured in the same mullet species caught from the same parts of Turkey. It clearly indicates that the pollutants continue to be present in these parts of Turkey. The average EROD activity of flathead mullet samples caught from Zonguldak Harbour was 3017±565 pmol min^{-1} mg^{-1} protein in 2007. The average EROD activity of flathead mullet samples caught from Zonguldak Harbour was 4744 ± 390 pmol min^{-1} mg^{-1} protein in 2011. This result indicates that the levels of CYP1A inducer chemicals increase in Zonguldak Harbour with time.

Besides these biochemical parameters, the concentrations of 13 polycyclic aromatic hydrocarbons were measured in fish liver tissues to determine the types of

PAHs present in fish liver tissues. The presence of PAHs has been shown previously in our laboratory in flathead mullet (*Mugil cephalus*) by total PAH analyses (Bozcaarmutlu et al., 2009). In this study, the concentrations of PAHs were determined in striped red mullet samples caught from the West Black Sea coast of Turkey in 2009, 2010 and 2011. The concentrations of PAHs were also determined in flathead mullet and golden grey mullets caught from the same stations in 2011. Acenapthlene, acenaphthene, phenanthrene, anthracene and benzo [b] fluoranthene were measured in most of the fish samples. Benzo [a] anthracene, benzo [k] fluoranthene, benzo [a] pyrene, indeno (1,2,3,-cd) pyrene, dibenz (a,h) anthracene and benzo [g,h,i] perylene were below detection limits in most of the fish samples. The concentrations of PAHs measured in striped red mullet were higher than those measured in flathead mullet and golden grey mullet. Most of the measured PAHs had 3-4 rings except benzo[b]fluoranthene. The low molecular weight PAHs cause acute toxicity in living organisms, whereas some of the higher molecular weight PAHs such as benzo[a]pyrene are carcinogenic. The results of this study show that most of the carcinogenic PAHs are not present in all fish samples caught from the West Black Sea coast of Turkey.

In conclusion, the results showed that striped red mullet can be considered as a suitable species to be used in monitoring of environmental pollution. Highly elevated EROD activities measured in flathead mullet and golden grey mullet indicate that the pollutants continue to be present in the West Black Sea coast of Turkey. Striped red mullet is another organism affected from the pollution in this region. Considering its commercial value and abundance, very little is known about striped red mullet. The environmental pollution study using striped red mullet is

restricted with the study related with the determination of metallothionein and metal levels (Filipovic and Raspor, 2003). To our knowledge, this is the first report about biomarker enzyme activities on striped red mullet. In addition, polycyclic aromatic hydrocarbons were determined for the first time in this fish species in this study.

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