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**ASSESSMENT OF POLLUTION IN THE WEST BLACK SEA
COAST OF TURKEY USING BIOMARKER
RESPONSES IN FISH**

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A part of this study was presented in poster format at the 14th International Symposium on Pollutant Responses in Marine Organisms (Primo 14) (May 6-9, 2007).

ABSTRACT

Aim of this study was to determine the extent of pollution in the West Black Sea Coast of Turkey by measuring CYP1A associated EROD activity, phase II enzyme, glutathione S-transferase and antioxidant enzymes, catalase and glutathione reductase activities and immunochemical detection of CYP1A protein level in the liver of mullet. The fish samples were caught from six locations having a varying degree of pollution in the West Black Sea Region of Turkey in August 2005, 2006 and 2007. Mulletts caught from Zonguldak Harbour, Ereğli Harbour and Güllüç Stream's Mouth displayed 6-9 fold higher EROD, 2-4 fold higher glutathione S-transferase and 2-3 fold higher catalase activities than the reference site, Amasra. Total polyaromatic hydrocarbon levels in mullets caught from these locations were also significantly higher (2-4 fold) than Amasra. The results of this study indicate that Zonguldak Harbour, Ereğli Harbour and Güllüç Stream are highly polluted by polycyclic aromatic hydrocarbons and related contaminants.

Keywords: Black Sea, Biomonitoring, CYP1A, Catalase, Chemical pollution, EROD, Glutathione S-transferase, Mullet.

1. INTRODUCTION

The Black Sea has been increasingly threatened by pollutants over the past decades as a result of accidental crude oil spills, dumping of toxic industrial wastes, discharge of domestic wastes from coastal settlements and industrial and domestic pollutants carried by rivers. Some industrial areas, rivers and coastal cities in the Black Sea have tendency to create local pollution (Mee, 1992). The 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). Industrial areas found in Düzce and Adapazarı drain their discharges into Efteni Lake. The Melen Stream originates from this lake and flows through many industrial and agricultural areas. The Güllüç Stream is very close to the Ereğli Harbour and Ereğli Iron and Steel Factory. This small stream was highly polluted primarily by domestic waste discharges from Ereğli and villages around the stream. Zonguldak and Ereğli, with increasing population, shipping activities, uncontrolled discharges from industries and coastal settlements, are among the most polluted regions in the Black Sea. Zonguldak is also an important coal mining area in Turkey. It suffers from particulate discharges from the thermal power plant and coal processing wastes, resulting in permanently elevated turbidity levels along the coast of the city (Tuncer et al., 1998).

Chemical analyses of the sea water and sediment give valuable information about the levels of chemical pollutants. However, organisms are often exposed to complex mixtures of pollutants, including polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polyaromatic hydrocarbons (PAHs), alkyltin compounds, and metals. Chemical analyses do not reveal the impact of these pollutants on organisms. The use of biochemical markers fulfills this purpose. Induction of CYP1A (P4501A) and associated EROD activity are the best characterized and used biochemical markers of exposure of fish to organic pollutants such as PCBs, PAHs and dioxins. CYP1A induction provides a useful “tool” for the assessment of exposure to these types of organic pollutants and acts as an early warning sign for potential harmful effects of many organic molecules (Payne et al., 1987; Goksøyr and Förlin, 1992; Bucheli and Fent, 1995; Stegeman, 1995; Addison, 1996; Arinç and Sen, 1999; Miller et al., 2003; Arinç et al., 2000, 2001). Glutathione-S-transferases (GSTs) catalyse the conjugation of electrophilic compounds (or phase I metabolites) with reduced glutathione (GSH) and are one of the main enzymes involved in xenobiotic phase II metabolism. 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. Benzo[*a*]pyrene as well as other PAHs and PCBs have been shown to cause significant alterations in the activities of antioxidant enzymes in laboratory experiments with molluscs and fish (Otto and Moon, 1995, Van der Oost et al., 2003). Changes in the levels of phase II enzymes, glutathione S-transferase and antioxidant enzymes have also been proposed as biomarkers of contaminants in a variety of marine organisms, including invertebrates, mussels, and fish (Otto and Moon, 1995, Livingstone, 2001; Orbea et al., 2002; Sen and Kirikbakan, 2004; Ferreira et al., 2005; Martínez- Gómez et al., 2006).

The studies concerning the effects of pollutants on the Black Sea ecosystem are relatively few (Telli-Karakoç et al., 2001; Bozcaarmutlu et al., 2006, 2008). Monitoring

biological effects by using biomarkers and their implementation into environmental monitoring programmes in the Black Sea fall behind the advanced studies taking place in the USA, Canada and most of the Western and Southern Europe. In this study, our aim was to determine and monitor the extent of pollution in the West Black Sea Coast of Turkey by measuring CYP1A associated EROD activity, phase II enzyme, glutathione S-transferase and antioxidant enzymes, catalase and glutathione reductase activities and immunochemical detection of CYP1A protein level in mullet liver. Besides these biochemical parameters, total PAH concentrations were determined in fish liver tissues to show the presence of one of the inducer organic pollutants in fish samples. Mullet is chosen as a test organism. It is an economically important marine fish due to the marketing of their meats and eggs. It is a pelagic fish 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. Since mullet is resistant to pollutants, it is widely used in biomonitoring programs of contaminated waters (Arinç and Sen, 1999; Arinç et al., 2000; Ferreira et al., 2004). Biomarker enzyme activities such as components of cytochrome P450 system, glutathione S-transferase and antioxidant enzymes have been well characterized in mullet (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).

2. MATERIALS AND METHODS

2.1 Chemicals

Acrylamide, anti-rabbit IgG alkaline phosphatase antibody, ϵ -amino caproic acid (ϵ -ACA), 7-ethoxyresorufin, N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid (HEPES), β -mercaptoethanol, N,N'-methylene bisacrylamide, phenylmethanesulfonyl fluoride (PMSF), resorufin sodium salt were purchased from Sigma-Aldrich, Saint Louis, Missouri, USA. Bromophenol blue, N,N-dimethylformamide and nitro blue tetrazolium chloride were purchased from Merck, Darmstadt, Germany. 5-bromo-4-chloro-3-indolylphosphate disodium salt (BCIP), glucose-6-phosphate dehydrogenase, α -D-glucose-6-phosphate monosodium salt, polyethylene sorbitan monolaurate (Tween 20), sodium dodecyl sulfate (SDS) were purchased from Calbiochem, California, USA. Ammonium persulfate, N, N, N', N' tetramethylethylenediamine (TEMED), trans blot transfer medium (pure nitrocellulose membrane) were purchased from Bio-Rad Laboratories, California, USA. β -Nicotinamide adenine dinucleotide phosphate disodium salt (NADP^+), β -nicotinamide adenine dinucleotide, reduced form (NADPH), phenazine methosulfate (PMS) were purchased from AppliChem, Darmstadt, Germany. All other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2 Sampling Sites and Fish

The fish samples were collected in August 2005, August 2006 and August 2007 from six different locations of the West Black Sea Region of Turkey. The fish sampling stations were shown in Figure 1. As indicated in this figure, Amasra was used as the reference site. Three different species of mullet, namely so-iuy mullet (*Mugil soiyu*), flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*), each weighing 600-750 g, were captured by fish net.

Figure 1

2.3 Preparation of Tissue Samples

Fish were killed by decapitation and livers were removed immediately by avoiding the gall bladders and flash frozen in liquid nitrogen. Tissues were taken from liquid nitrogen and cut into two pieces without thawing. One of the pieces was wrapped by freezing bags and covered by aluminum foil and then put back into liquid nitrogen. This piece was later used in PAH analysis. The other piece of the liver tissue was used in the preparation of microsomes and thawed on ice. Liver microsomes were prepared as described before (Adali and Arinç, 1990; Arinç and Sen, 1993) except that homogenization solution contained 10 mM EDTA, 0.25 mM PMSF and 0.25 mM ϵ -ACA. The homogenate was centrifuged at 13300xg for 40 minutes. The microsomes were sedimented from the supernatant by centrifugation at 70000xg for 60 minutes. The supernatant fraction (cytosol) was removed and stored at -80°C until use. The microsomal pellet was resuspended in 1.15% KCl solution containing 10 mM EDTA and resedimented by ultracentrifugation at 70000xg for 50 minutes. The washed microsomes were resuspended in 10% glycerol containing 10 mM EDTA. Aliquots of microsomes were gassed with nitrogen and stored in liquid nitrogen until used. All the procedures involved in the preparation of microsomes and cytosols were performed at 0-4°C.

2.4 Biochemical Methods

Protein concentrations of microsomes and cytosols were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

EROD activity of the mullet liver microsomes was determined by the spectrofluorometric method of Burke and Mayer (1974), with some modifications. Assay conditions optimized for gilthead seabream (*Sparus aurata*) liver microsomes by Arinç and Sen (1994) were also used in this study. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.8, 0.1 M NaCl, 2.4 mg bovine serum albumin (BSA), 1.5 μ M 7-ethoxyresorufin, 50-100 μ g of fish liver microsomal protein, and 0.5 mM NADPH generating system in a final volume of 2.0 mL. The reaction was initiated by the addition of substrate and followed for 2 minutes in a spectrofluorometer. Finally, a known amount of resorufin was added as an internal standard to the reaction mixture and the increase in fluorescence was recorded. EROD activity was calculated using the fluorescence increase caused by the addition of resorufin.

Total glutathione S-transferase (GST) activity of mullet liver cytosols was determined according to the method of Habig et al. (1974). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 1.3 mM GSH and fish liver cytosols. The reaction was initiated by the addition of 1 mM CDNB in a final volume of 3.0 mL. The thioether formation was followed at 340 nm for 2 minutes. Specific total glutathione S-transferase activity was calculated as nmol CDNB conjugate formed $\text{min}^{-1} \text{mg}^{-1}$ protein at 25°C, using 9.6 $\text{mM}^{-1} \text{cm}^{-1}$ as an extinction coefficient (ϵ_{340}).

Catalase (CAT) activity of mullet liver cytosols was determined according to the method of Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 10 mM H_2O_2 and fish liver cytosols. The rate of reaction was followed by measuring the consumption of H_2O_2 spectrophotometrically at 240 nm for 1 minute. Specific catalase activity was calculated as nmol H_2O_2 consumed $\text{min}^{-1} \text{mg}^{-1}$ protein at 25°C, using 36.4 $\text{mM}^{-1} \text{cm}^{-1}$ as an extinction coefficient (ϵ_{240}).

Glutathione reductase (GR) activity of mullet liver cytosols was determined according to the method of Carlberg and Mannervick (1985). The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.6, 0.5 mM EDTA, pH 7.6, 0.1 mM NADPH and fish liver cytosols in a final volume of 2.0 mL. The reaction was started by adding 1 mM oxidized glutathione (GSSG). Then the GSH formation was followed by measuring the NADPH utilization spectrophotometrically at 340 nm for 5 minutes. One unit of glutathione reductase activity was calculated as nmol NADPH oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein at 25°C, using 6.22 $\text{mM}^{-1} \text{cm}^{-1}$ as an extinction coefficient (ϵ_{340}).

2.5 Western Blot Analysis

Cytochrome P4501A (CYP1A) protein levels were determined using the polyclonal antibodies produced against purified leaping mullet (*Liza saliens*) liver cytochrome P4501A (Arinç and Sen, 1999). Western blot analysis of fish liver microsomes was carried out essentially as described by Towbin et al. (1979). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) and proteins were transferred from slab gels to nitrocellulose membrane by use of a Bio-Rad Trans-Blot electrophoretic transfer apparatus. The proteins on membranes were

first reacted with 1:5000 diluted leaping mullet CYP1A antibodies and then with the enzyme-linked secondary antibody, and alkaline phosphatase activity was detected by the method of Ey and Ashman (1986). The final images of the blots were photographed using a computer-based gel imaging instrument (Infinity 3000-CN-3000 darkroom, Vilber Lourmat, Marne-la-Vallee Cedex 1, France), and relative peak area (R.P.A.) of the blots was analyzed using the Scion Image software for Windows (Version 4.0.2, Scion Corporation, Maryland, USA) as a quantitative tool to determine CYP1A protein levels.

2.6 PAH Analysis

Spectrofluorometric measurements of the total PAH concentration in fish liver tissues were performed according to the method described by UNEP, 1992. The principle of the method is based on the extraction of PAHs by suitable pure solvent. Mullet livers were dried in an oven at 40 °C overnight. Dried tissue (0.2 g) was ground and refluxed with 20 mL of ethanol containing 0.75 g of KOH for 120 minutes by constant heating. Then 20 mL of PAH-free hexane was added to the flask and mixed very well and waited until the flask reached to room temperature. When the flask was attained to room temperature, it was poured into a separatory funnel and sufficient amount of distilled water was added. The lower aqueous phase was extracted further two times with 20 mL of PAH-free hexane. The fluorescence intensities of the pooled samples were measured at 310 nm excitation and 360 nm emission wavelengths. The fluorescence intensity of the pooled samples was compared with the fluorescence intensity of known concentrations of chrysene standard.

2.7 Statistical Analysis

Results were expressed as means $\pm$ standard error of the mean (S.E.M.). Data were analyzed by two-way ANOVA using years and sampling sites as independent variables. 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). No significant sex dependent differences were observed in the fish samples. Therefore the data were pooled.

3. RESULTS

In this study 147 fish samples were collected from six different locations of the West Black Sea Coast of Turkey. In all samples, EROD, glutathione S-transferase, catalase and glutathione reductase activities were measured. Table 1 illustrates biomarker enzyme activity results. The lowest biomarker activities were measured in fish samples (so-iuy mullet, flathead mullet and golden grey mullet) caught from Amasra. The biomarker enzyme activities of different mullet species were compared with the corresponding mullet species caught from Amasra. This relatively clean site was considered as a reference site to demonstrate the extent of pollution.

Table 1

3.1 7-Ethoxyresorufin O-deethylase (EROD) Activity and CYP1A Protein Level

The highest EROD activities were found in mullets (so-iuy mullets, flathead mullets, and golden grey mullets) caught from Zonguldak Harbour in all sampling years (Table 1). Mulletts caught from Zonguldak Harbour had about 6-9 fold higher EROD activities than those obtained from Amasra. The highly elevated EROD activities were also measured in mullets captured from Ereğli Harbour which were about 7-9 fold higher with respect to the values obtained from Amasra. Güllüç Stream's Mouth was the other highly polluted station in this study. EROD activities of mullets samples caught from this station were about 6 fold higher than those obtained from Amasra. In Figure 2, the data were presented separately for each species. Statistically significant differences were indicated in this figure with different letters ($p < 0.05$). EROD activities of so-iuy mullets caught from Zonguldak Harbour and Güllüç Stream's Mouth were significantly different from the so-iuy mullets caught from Amasra, Melen Stream's and Sakarya River's Mouths. EROD activities of flathead mullets caught from Zonguldak Harbour were significantly different from Amasra. EROD activities of golden grey mullets caught from Zonguldak and Ereğli Harbours were significantly different from Amasra. EROD activities of mullets caught from Melen Stream's Mouth were 2 fold higher than Amasra, however these differences were not statistically significant. Significant differences between years were not found in EROD activities of fish caught from all sampling stations except Sakarya River's Mouth.

Figure 2

CYP1A protein level was also determined in mullet liver microsomes using the polyclonal antibodies produced against leaping mullet cytochrome P4501A. Figure 3 illustrates the western blot analysis results of mullets caught from six different stations in three sampling years. Polyclonal antibody anti-leaping mullet cytochrome P4501A showed strong cross-reactivity with liver microsomes of so-iuy mullets, flathead mullets and golden grey mullets caught from different stations of the West Black Sea. The CYP1A bands of mullets caught from Zonguldak and Ereğli Harbours and Güllüç Stream's Mouth were stained more intensely than those obtained from Amasra. Liver microsomes of mullets caught from Melen Stream's Mouth and Sakarya River's Mouth showed moderate cross reactivity with polyclonal anti-mullet P4501A. Liver microsomes of mullets caught from Amasra showed low cross-reactivity with polyclonal antibody anti-mullet P4501A. The results clearly indicated that mullets caught from the polluted sites had both highly induced EROD activity and elevated CYP1A protein content. The relationship between CYP1A protein levels and EROD activities was also examined with correlation analysis

(Figure 3). CYP1A protein levels were highly correlated with the EROD activity results in the mullet samples caught from different stations of the West Black Sea Coast of Turkey (R^2 ranged between 0.92-0.96).

Figure 3

When EROD activities of different mullet species were analyzed using ANOVA, species differences were not significant ($p=0.714$). When the species specific cross-reactivity pattern of anti-leaping mullet CYP1A protein was compared, different mullet species having similar EROD activity showed similar degree of staining and gave almost the same EROD activity/CYP1A protein level (R.P.A.) ratio (Figure 4). The induction responses to organic pollutants, both with respect to EROD activities and CYP1A protein levels, were not different between these mullet species.

Figure 4

3.2 Glutathione S-transferase Activity

The highest total glutathione S-transferase activities were measured in mullets caught from Zonguldak Harbour in all sampling years which were about 4 fold higher than those obtained from Amasra (Table 1). Mulletts caught from Güllüç and Melen Streams' Mouths also displayed highly elevated total glutathione S-transferase activities which were 3-4 fold higher than those obtained from Amasra. In Figure 5, the data were presented separately for each species. Glutathione S-transferase activities of soiyu mullets caught from Zonguldak Harbour, Melen and Güllüç Streams' Mouths were not significantly different from each other. However, they were significantly different from Amasra. Glutathione S-transferase activities of soiyu mullets caught from Sakarya River's Mouth (represented by "ab") were not statistically different from Zonguldak Harbour, Güllüç and Melen Stream's Mouths and Amasra. Glutathione S-transferase activities of golden grey mullets caught from Ereğli Harbour (represented by "ab") were 2-3 fold higher than those obtained from Amasra, however these differences were not significantly different from Amasra and Zonguldak Harbour. Glutathione S-transferase activities of mullets caught from Ereğli Harbour showed significant difference between years. The highest GST activities were measured in the fish samples caught in 2005.

Figure 5

3.3 Catalase Activity

The highest catalase activities were detected in mullets caught from Sakarya River's and Melen Stream's Mouths and Zonguldak Harbour in 2005, which were about 3 fold higher than those obtained from Amasra (Table 1). Fish sampled from Güllüç Stream's Mouth also displayed highly elevated catalase activities which were 2 fold higher than those obtained from Amasra. In Figure 6, the data were presented separately for each species. Catalase activities of soiyu mullets caught from Zonguldak Harbour, Melen Stream's and Sakarya River's Mouths were not statistically different from each other. However, they were significantly different from Amasra. Catalase activities of soiyu mullets caught from Güllüç Stream's Mouth (represented by "ab") were not statistically different from Zonguldak Harbour, Melen Stream's and Sakarya River's Mouths and Amasra. Catalase activities of flathead mullets caught from Zonguldak Harbour were

significantly different from Amasra. Catalase activities of golden grey mullets caught from Zonguldak and Ereğli Harbours were significantly different from Amasra. Although highly elevated catalase activities were measured in mullets caught in 2005, this enzyme activity significantly decreased in the mullets caught from Melen Stream's and Sakarya River's Mouths and Ereğli Harbour in the following sampling years.

Figure 6

3.4 Glutathione Reductase Activity

Highly elevated glutathione reductase activities were measured in mullets caught from Zonguldak and Ereğli Harbours, Güllüç and Melen Streams' Mouths (Table1). In 2005, mullets caught from Melen Stream's Mouth had the highest glutathione reductase activities among the other sampling sites which were 1.7 fold higher than those obtained from Amasra. Glutathione reductase activities of mullets caught from Güllüç Stream's Mouth were 1.6 fold higher than those obtained from Amasra in 2005 and 2006. Glutathione reductase activities of mullets captured from Ereğli Harbour were 1.6 fold higher than those obtained from Amasra. In 2006, glutathione reductase activities of so-iuy mullets and golden grey mullets captured from Zonguldak Harbour were 1.5 and 1.4 fold higher than those obtained from Amasra, respectively. In Figure 7, the data were presented separately for each species. Glutathione reductase activities of so-iuy mullets caught from Melen and Güllüç Streams' Mouths were significantly different from Amasra and Sakarya River's Mouth. Glutathione reductase activities of so-iuy mullets caught from Zonguldak Harbour (represented by "ab") were not statistically different from Güllüç, Melen Streams', Sakarya River's Mouths and Amasra. Glutathione reductase activities of golden grey mullets caught from Zonguldak and Ereğli Harbours were significantly different from Amasra. Significant differences between years were not found in glutathione reductase activities of fish caught from all sampling stations except Sakarya River's Mouth.

Figure 7

3.5 PAH Analysis

Besides biomarker enzyme activity measurements, total PAH concentrations were determined in mullets caught in 2006 to show the presence and extent of one of the organic pollutants in the sampling stations. Table 2 illustrates the total PAH concentrations in mullet liver tissues. The highest total PAH concentrations were measured in mullets caught from Zonguldak Harbour. Total PAH concentrations of fish caught from Zonguldak and Ereğli Harbours and Güllüç Stream's Mouth were significantly different from Amasra. Total PAH concentrations of fish caught from Zonguldak Harbour were significantly different from fish caught from Ereğli Harbour. However, total PAH concentrations of fish caught from Ereğli Harbour and Güllüç Stream's Mouth were not significantly different from each other. The lowest total PAH concentrations were measured in mullets caught from Amasra. Total PAH concentrations of fish caught from Sakarya River's and Melen Stream's Mouths (represented as "bc") were not significantly different from Amasra, Ereğli Harbour and Güllüç Stream's Mouth.

Table 2

The relationship between biomarker activities and total PAH concentrations was also examined with correlation analysis (Pearson). The highest positive correlation was found between EROD activities and total PAH concentrations ($r=0.491$, $p<0.01$). Glutathione reductase and glutathione S-transferase activities were slightly correlated with total PAH concentrations ($r=0.392$, $p<0.01$) and ($r=0.283$, $p<0.05$), respectively. Correlation between total PAH concentrations and catalase activities was positive but not significant ($r=0.210$, $p=0.083$).

4. DISCUSSION

In this study, cytochrome P4501A (CYP1A) associated EROD activity, phase II enzyme, glutathione S-transferase and antioxidant enzymes, catalase and glutathione reductase activities were measured in mullet liver fractions to determine and monitor the extent of pollution in the West Black Sea Coast of Turkey. Highly elevated biomarker enzyme activities were found in the mullets caught from Zonguldak and Ereğli Harbours, and Güllüç Stream's Mouth. Zonguldak is an important coal mining area in Turkey. The harbour of this city is polluted especially with coal processing wastes and discharges of industrial and domestic wastes. Cohen and coworkers (1994) have shown that coal by-products such as coal-tar significantly induce EROD activity in medaka liver. In addition, Vignier and coworkers (1994) have demonstrated that EROD activity significantly increases in winter flounder in coal-tar contaminated estuary. Creosote is a dark liquid made from coal tar. It is a complex mixture of organic compounds, containing about 85% PAHs. Hyötyläinen and Oikari (1999) and Whyte and coworkers (2000) have demonstrated in different studies that the extracts of creosote-contaminated sediments highly induce EROD activities in rainbow trout. In this study, the lowest biomarker enzyme activities were measured in mullets caught from Amasra. The biomarker enzyme activities obtained from the other stations were compared with those obtained from Amasra. 6-9 fold increase in EROD activities and elevated CYP1A protein levels were found in mullets caught from Zonguldak Harbour with respect to Amasra. This result clearly indicates that coal mining activities create pollution in Zonguldak Harbour.

İzmir Bay is one of the most polluted sites in Turkey. Highly elevated EROD activities have been reported in mullets (*Liza saliens* and *Mugil cephalus*) caught from the most polluted site of İzmir Bay. In these studies, EROD activities of *Liza saliens* were reported as 1293 ± 292 pmol min⁻¹ mg⁻¹ protein in 1995 and 1028 ± 287 pmol min⁻¹ mg⁻¹ protein in 1999 (Arinç and Sen, 1999; Arinç et al., 2000). EROD activities of *Mugil cephalus* caught from the same site were 1398 ± 410 pmol min⁻¹ mg⁻¹ protein in 1999 (Arinç et al., 2000). In our study, highly elevated EROD activities were found in mullets caught from Zonguldak and Ereğli Harbours, and Güllüç Stream's Mouth, suggesting the presence of organic pollutants such as PAHs and PCBs in these sampling stations in the West Black Sea Coast of Turkey.

Glutathione S-transferases are involved in the detoxification reactions of xenobiotics. Induction of glutathione S-transferase have been reported in a number of field studies in fish exposed to organic contaminants (Lenartova et al., 1997; Pandey et al., 2003; Martínez- Gómez et al., 2006; Sen and Kirikbakan, 2004). In our study, highly elevated total glutathione S-transferase activities measured in mullets caught from Zonguldak Harbour ($950-1147$ nmol min⁻¹ mg⁻¹ protein), Ereğli Harbour (932 ± 131 nmol min⁻¹ mg⁻¹ protein), Güllüç Stream's Mouth (1352 ± 154 nmol min⁻¹ mg⁻¹ protein) and Melen Stream's Mouth (1192 ± 140 nmol min⁻¹ mg⁻¹ protein) are at the same order of magnitude of that reported by Sen and Kirikbakan (2004) in mullets (*Liza saliens*) caught from highly polluted site of İzmir Bay in Turkey (1183 ± 35.9 nmol min⁻¹ mg⁻¹ protein).

Chronic exposure to organic pollutants increases reactive oxygen species (ROS) formation (Livingstone 2001). The antioxidant enzyme catalase is one of the most responsive enzymes to ROS (Halliwell and Gutteridge, 1999). Elevated catalase enzyme activities have been reported in several fish species in various laboratory and field studies (Otto and Moon, 1995; Orbea et al., 2002; Ferreira et al., 2005). Significantly high catalase

activities were measured in mullets caught from Zonguldak and Ereğli Harbours and Melen Stream's and Sakarya River's Mouths, indicating the presence of ROS generating pollutants in these regions. The other biomarker enzyme activity measured in this study was glutathione reductase. Glutathione reductase maintains GSH/GSSG homeostasis under oxidative stress conditions. Induction of glutathione reductase activity have been reported in various field studies in fish exposed to organic pollutants such as PAHs, PCBs and halogenated xenobiotics (Rodriguez-Ariza et al., 1993; Van der Oost et al., 2003; Martínez- Gómez et al., 2006). Significantly high glutathione reductase activities have been found in mullets caught from Zonguldak and Ereğli Harbours, Güllüç and Melen Streams' Mouths, suggesting the presence of PAHs, PCBs and halogenated xenobiotics in these sampling sites.

Biomarker enzyme activities of fish caught from Sakarya River's Mouth displayed significant differences between sampling years. Sakarya River is the third longest river of Turkey (824 km). It rises from the center of Anatolia and joins with Porsuk, Göksu, Kirmir, Mudurnu and Çark Streams. The river runs through many industrial and agricultural areas before reaching to the Black Sea. The results of this study suggested the existence of qualitative and quantitative differences of contaminants in the sampling station between years. However, it is difficult to determine which chemicals specifically affected the biomarker activities in the environmental monitoring studies. Biomarkers reveal the interactive effects of chemical contaminants on organisms.

In the Black Sea, total PAH concentrations in fish samples have been determined by Telli-Karakoç and coworkers (2001) in mullet samples caught from Trabzon, Yomra. In our study, the highest total PAH concentrations were measured in so-iuy mullets and golden grey mullets caught from Zonguldak Harbour ($54 \pm 5 \mu\text{g g}^{-1}$ dried liver weight, $n=13$ and $47 \pm 6 \mu\text{g g}^{-1}$ dried liver weight, $n=9$, respectively) are at the same order of magnitude of that reported by Telli-Karakoç and coworkers for mullets (*Mugil saliens*) ($55.7 \pm 16.6 \mu\text{g g}^{-1}$ dried liver weight) caught from Trabzon, Yomra. In our study, significantly high total PAH concentrations were also measured in mullets caught from Ereğli Harbour and Güllüç Stream's Mouth. The sampling areas arranged in decreasing order of total PAH concentrations are Zonguldak Harbour>Güllüç Stream Mouth>Ereğli Harbour>Melen Stream Mouth>Sakarya River Mouth>Amasra.

The relationship between biomarker activities and total PAH concentrations was also analyzed. Biomarker activities of fish were moderately related with total PAH concentrations, suggesting the presence of the other organic contaminants such as PCBs and/or dioxins in these regions besides polycyclic aromatic hydrocarbons.

CONCLUSION

In this study, the contribution of Turkish rivers and harbours present in the West Black Sea Region of Turkey to the pollution of the Black Sea was assessed using biomarker responses of fish. The elevated biomarker responses found in this study indicate that Zonguldak Harbour, Ereğli Harbour and Güllüç Stream are highly polluted by polycyclic aromatic hydrocarbons and related contaminants. In addition, Melen Stream and Sakarya River also bring some PAHs, PCBs and other type of pollutants from the inner parts of Anatolia and Black Sea Region of Turkey. The results reported here also supported the conclusion of previous field studies that mullet is a suitable fish species for biomonitoring studies.

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Table 1. Mean values of biomarker enzyme activities and Standard Error of Mean (SEM)

Zone	Year	N	Fish Species	EROD ^a	GST ^b	CAT ^b	GR ^b
Zonguldak Harbour	2005	7	<i>Mugil soiuy</i>	3237±539	1260±207	322±34	14.9±1.9
	2006	13	<i>Mugil soiuy</i>	2559±315	950±135	283±46	20.4±0.8
	2006	9	<i>Liza aurata</i>	2302±240	1067±208	163±16	16.2±1.6
	2007	5	<i>Mugil cephalus</i>	3017±565	1017±231	171±12	11.5±2.1
Ereğli Harbour	2005	16	<i>Liza aurata</i>	1871±391	932±131	195±18	17.4±0.7
	2006	12	<i>Liza aurata</i>	2037±280	585±64	138±12	18.1±0.8
	2007	5	<i>Liza aurata</i>	2233±464	406±29	121±12	17.9±1.3
Gülüç Stream Mouth	2005	13	<i>Mugil soiuy</i>	2402±261	1352±154	281±32	20.8±0.9
	2006	11	<i>Mugil soiuy</i>	2415±426	830±154	224±30	20.7±1.3
Melen Stream Mouth	2005	11	<i>Mugil soiuy</i>	1065±176	851±129	349±32	23.1±1.9
	2006	9	<i>Mugil soiuy</i>	818±217	1192±140	200±20	18.7±1.8
Sakarya River Mouth	2005	10	<i>Mugil soiuy</i>	904±216	810±159	352±17	19.7±1.7
	2006	10	<i>Mugil soiuy</i>	438±62	735±134	257±32	9.6±0.7
	2007	4	<i>Mugil soiuy</i>	1642±470	1016±349	187±19	11.3±0.8
Amasra	2006	3	<i>Mugil soiuy</i>	425±18	288±165	123±20	13.4±0.8
	2006	4	<i>Mugil cephalus</i>	541±22	263±62	117±21	11.3±0.5
	2007	5	<i>Liza aurata</i>	259±72	285±37	100±11	11.2±1.7

^a pmol min⁻¹ mg⁻¹ protein^b nmol min⁻¹ mg⁻¹ protein

Table 2 Total PAH concentrations in the livers of mullets collected from six sampling stations of the West Black Sea Coast of Turkey in 2006. Homogenous subsets (groups do not differ significantly) were indicated by the same letters (p<0.05).

Zone	N	Fish Species	PAH Concentration *
Zonguldak Harbour	13	<i>Mugil soiuy</i>	54 ± 5 (a)
	9	<i>Liza aurata</i>	47 ± 6 (a)
Ereğli Harbour	12	<i>Liza aurata</i>	28 ± 3 (b)
Gülüç Stream Mouth	11	<i>Mugil soiuy</i>	35 ± 3 (ab)
Melen Stream Mouth	9	<i>Mugil soiuy</i>	25 ± 3 (bc)
Sakarya River Mouth	8	<i>Mugil soiuy</i>	21 ± 3 (bc)
Amasra	3	<i>Mugil soiuy</i>	12 ± 2 (c)
	4	<i>Mugil cephalus</i>	11 ± 2 (c)

* $\mu\text{g g}^{-1}$ dry weight

FIGURE LEGENDS

Figure 1 Fish sampling stations in the West Black Sea Coast of Turkey. * Reference site.

Figure 2 Ethoxyresorufin O-deethylase (EROD) activities of liver microsomes of mullets caught from six sampling stations of the West Black Sea Coast of Turkey in 2005, 2006, and 2007. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p < 0.05$).

Figure 3 Western blot analysis of CYP1A protein contents and correlation between microsomal EROD activities and CYP1A protein levels of mullets caught from different stations of the West Black Sea Coast of Turkey in 2005 (A), 2006 (B), and 2007 (C). 80 μ g microsomal protein was applied to each slot. (A) Line 1-9 (*Mugil so-iuy*) and Line 10-12 (*Liza aurata*); (B) Line 1-7 (*Mugil so-iuy*) and Line 8-12 (*Liza aurata*); (C) Line 1-4 (*Liza aurata*), Line 5-8 (*Mugil so-iuy*), Line 9-12 (*Mugil cephalus*).

* Sampled in 2006

** Sampled in 2007

Figure 4 Comparison of anti-leaping mullet CYP1A cross reactivity pattern of different mullet species. The intensities of CYP1A protein bands of different mullet species present in the same western blot result (Figure 3C) were compared together with corresponding EROD activities and EROD activity/CYP1A protein level (R.P.A.) ratios. Different mullet species having similar EROD activity showed similar degree of staining and gave almost the same EROD activity/CYP1A protein level (R.P.A.) ratio.

Figure 5 Glutathione S-transferase (GST) activities of mullets caught from six sampling stations of the West Black Sea Coast of Turkey in 2005, 2006, and 2007. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p < 0.05$).

Figure 6 Catalase activities of mullets caught from six sampling stations of the West Black Sea Coast of Turkey in 2005, 2006, and 2007. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p < 0.05$).

Figure 7 Glutathione reductase activities of mullets caught from six sampling stations of the West Black Sea Coast of Turkey in 2005, 2006, and 2007. Homogenous subsets (groups do not differ significantly) were indicated by the same letters ($p < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7