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9 10	A zra Pozoa rmutlu ^{1*} Conon Sanmaz ¹ Zulavha Ayaun ¹ Emal Arina ²
10	Azra Bozcaarmutu , Canan Sapinaz , Zuleyna Aygun , Emer Armç
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14	¹ Department of Chemistry Abant Izzet Baysal University Bolu Turkey
15	² Biochemistry Graduate Programme and Department of Biological Sciences. Middle East
16	Technical University. Ankara, Turkey
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21	* Corresponding author: <u>bozcaarmutlu_a@ibu.edu.tr</u> (A. Bozcaarmutlu).
22	Tel: +90-374-2541247 and Fax: +90-374-2534964
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29	Tonutant Responses in Marine Organisms (Trino 14) (May 0-9, 2007).
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50 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. Mullets caught from Zonguldak Harbour, Ereğli Harbour and Gülüç 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 Gülüç Stream are highly polluted by polycyclic aromatic hydrocarbons and related contaminants. Keywords: Black Sea, Biomonitoring, CYP1A, Catalase, Chemical pollution, EROD, Glutathione S-transferase, Mullet.

100 **1. INTRODUCTION**

101

102 The Black Sea has been increasingly threatened by pollutants over the past decades 103 as a result of accidental crude oil spills, dumping of toxic industrial wastes, discharge of 104 domestic wastes from coastal settlements and industrial and domestic pollutants carried by 105 rivers. Some industrial areas, rivers and coastal cities in the Black Sea have tendency to 106 create local pollution (Mee, 1992). The Sakarya River, from its source in the West 107 Anatolia, flows through many industrial and agricultural areas and drains into the Black 108 Sea. It carries many pollutants to the Black Sea from inner regions of Turkey (Tuncer et 109 al., 1998; Barlas, 1999). Industrial areas found in Düzce and Adapazarı drain their 110 discharges into Efteni Lake. The Melen Stream originates from this lake and flows through many industrial and agricultural areas. The Gülüç Stream is very close to the Ereğli 111 112 Harbour and Ereğli Iron and Steel Factory. This small stream was highly polluted primarily 113 by domestic waste discharges from Ereğli and villages around the stream. Zonguldak and Ereğli, with increasing population, shipping activities, uncontrolled discharges from 114 industries and coastal settlements, are among the most polluted regions in the Black Sea. 115 116 Zonguldak is also an important coal mining area in Turkey. It suffers from particulate 117 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). 118

119

120 Chemical analyses of the sea water and sediment give valuable information about the levels of chemical pollutants. However, organisms are often exposed to complex 121 122 mixtures of pollutants, including polychlorinated biphenyls (PCBs), polychlorinated 123 dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polyaromatic 124 hydrocarbons (PAHs), alkyltin compounds, and metals. Chemical analyses do not reveal 125 the impact of these pollutants on organisms. The use of biochemical markers fulfills this 126 purpose. Induction of CYP1A (P4501A) and associated EROD activity are the best 127 characterized and used biochemical markers of exposure of fish to organic pollutants such 128 as PCBs, PAHs and dioxins. CYP1A induction provides a useful "tool" for the assessment 129 of exposure to these types of organic pollutants and acts as an early warning sign for 130 potential harmful effects of many organic molecules (Payne et al., 1987; Goksøyr and Förlin, 1992; Bucheli and Fent, 1995; Stegeman, 1995; Addison, 1996; Arinc and Sen, 131 132 1999; Miller et al., 2003; Arinc et al., 2000, 2001). Glutathione-S-transferases (GSTs) 133 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 134 135 metabolism. GSTs detoxify a number of environmental carcinogens and epoxide intermediates (Gallagher et al., 1996). Glutathione S-transferase activity measurement is 136 137 also used as a marker of oxidative stress (Rodriguez-Ariza et al., 1993; Martínez- Gómez 138 et al., 2006). The induction of antioxidant enzyme activities represents a cellular defense 139 mechanism to neutralize toxic effects of reactive oxygen species. Benzo[a]pyrene as well 140 as other PAHs and PCBs have been shown to cause significant alterations in the activities 141 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-142 transferase and antioxidant enzymes have also been proposed as biomarkers of 143 144 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; 145 146 Ferreira et al., 2005; Martínez- Gómez et al., 2006).

147

148 The studies concerning the effects of pollutants on the Black Sea ecosystem are 149 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).

200 2. MATERIALS AND METHODS

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202 **2.1 Chemicals**

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204 Acrylamide, anti-rabbit IgG alkaline phosphatase antibody, ε-amino caproic acid 205 7-ethoxyresorufin, N-2-hydroxyethylpiperazine-N'-2-ethansulphonic (e-ACA), acid 206 (HEPES), β -mercaptoethanol, N,N'-methylene bisacrylamide, phenylmethanesulfonyl 207 fluoride (PMSF), resorufin sodium salt were purchased from Sigma-Aldrich, Saint Louis, 208 Missouri, USA. Bromophenol blue, N,N-dimethylformamide and nitro blue tetrazolium 209 chloride were purchased from Merck, Darmstadt, Germany. 5-bromo-4-chloro-3-210 indolylphosphate disodium salt (BCIP), glucose-6-phosphate dehydrogenase, α -D-glucose-211 6-phosphate monosodium salt, polyethylene sorbitan monolaurate (Tween 20), sodium 212 dodecyl sulfate (SDS) were purchased from Calbiochem, California, USA. Ammonium 213 persulfate, N, N, N', N' tetrametylethylene diamine (TEMED), trans blot tranfer medium 214 (pure nitrocellulose membrane) were purchased from Bio-Rad Laboratories, California, 215 USA. β -Nicotinamide adenine dinucleotide phosphate disodium salt (NADP⁺), β -216 nicotinamide adenine dinucleotide, reduced form (NADPH), phenazine methosulfate 217 (PMS) were purchased from Applichem, Darmstadt, Germany. All other chemicals were 218 of analytical grade and were obtained from commercial sources at the highest grade of 219 purity available.

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221 2.2 Sampling Sites and Fish

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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 soiuy*), flathead mullet (*Mugil cephalus*) and golden grey mullet (*Liza aurata*), each weighing 600-750 g, were captured by fish net.

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231

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Figure 1

232 **2.3 Preparation of Tissue Samples**

234 Fish were killed by decapitation and livers were removed immediately by avoiding 235 the gall bladders and flash frozen in liquid nitrogen. Tissues were taken from liquid 236 nitrogen and cut into two pieces without thawing. One of the pieces was wrapped by 237 freezing bags and covered by aluminum foil and then put back into liquid nitrogen. This 238 piece was later used in PAH analysis. The other piece of the liver tissue was used in the 239 preparation of microsomes and thawed on ice. Liver microsomes were prepared as 240 described before (Adali and Arinç, 1990; Arinç and Sen, 1993) except that homogenization 241 solution contained 10 mM EDTA, 0.25 mM PMSF and 0.25 mM &-ACA. The homogenate was centrifuged at 13300×g for 40 minutes. The microsomes were sedimented from the 242 243 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 244 245 1.15% KCl solution containing 10 mM EDTA and resedimented by ultracentrifugation at 246 70000xg for 50 minutes. The washed microsomes were resuspended in 10% glycerol 247 containing 10 mM EDTA. Aliquots of microsomes were gassed with nitrogen and stored in 248 liquid nitrogen until used. All the procedures involved in the preparation of microsomes 249 and cytosols were performed at 0-4°C.

250 **2.4 Biochemical Methods**

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252 Protein concentrations of microsomes and cytosols were determined by the method 253 of Lowry et al. (1951). Bovine serum albumin was used as a standard.

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255 EROD activity of the mullet liver microsomes was determined by the 256 spectrofluorometric method of Burke and Mayer (1974), with some modifications. Assay 257 conditions optimized for gilthead seabream (Sparus aurata) liver microsomes by Arinç and 258 Sen (1994) were also used in this study. The reaction mixture contained 0.1 M potassium 259 phosphate buffer, pH 7.8, 0.1 M NaCl, 2.4 mg bovine serum albumin (BSA), 1.5 µM 7-260 ethoxyresorufin, 50-100 µg of fish liver microsomal protein, and 0.5 mM NADPH 261 generating system in a final volume of 2.0 mL. The reaction was initiated by the addition 262 of substrate and followed for 2 minutes in a spectrofluorometer. Finally, a known amount 263 of resorufin was added as an internal standard to the reaction mixture and the increase in 264 fluorescence was recorded. EROD activity was calculated using the fluorescence increase 265 caused by the addition of resorufin.

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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 Stransferase activity was calculated as nmol CDNB conjugate formed min⁻¹ mg⁻¹ protein at 25°C, using 9.6 mM⁻¹ cm⁻¹ as an extinction coefficient (ϵ_{340}).

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275 Catalase (CAT) activity of mullet liver cytosols was determined according to the 276 method of Aebi (1984). The reaction mixture contained 50 mM potassium phosphate 277 buffer, pH 7.5, 10 mM H₂O₂ and fish liver cytosols. The rate of reaction was followed by 278 measuring the consumption of H₂O₂ spectrophotometrically at 240 nm for 1 minute. 279 Specific catalase activity was calculated as nmol H₂O₂ consumed min⁻¹ mg⁻¹ protein at 280 25°C, using 36.4 mM⁻¹ cm⁻¹ as an extinction coefficient (ϵ_{240}).

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282 Glutathione reductase (GR) activity of mullet liver cytosols was determined 283 according to the method of Carlberg and Mannervick (1985). The reaction mixture 284 contained 100 mM potassium phosphate buffer, pH 7.6, 0.5 mM EDTA, pH 7.6, 0.1 mM 285 NADPH and fish liver cytosols in a final volume of 2.0 mL. The reaction was started by 286 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 287 288 unit of glutathione reductase activity was calculated as nmol NADPH oxidized min⁻¹ mg⁻¹ protein at 25°C, using 6.22 mM⁻¹ cm⁻¹ as an extinction coefficient (ε_{340}). 289

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291 **2.5 Western Blot Analysis**

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293 Cytochrome P4501A (CYP1A) protein levels were determined using the polyclonal 294 antibodies produced against purified leaping mullet (*Liza saliens*) liver cytochrome 295 P4501A (Arinç and Sen, 1999). Western blot analysis of fish liver microsomes was carried 296 out essentially as described by Towbin et al. (1979). Sodium dodecyl sulfate– 297 polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli 298 (1970) and proteins were transferred from slab gels to nitrocellulose membrane by use of a 299 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.

308 2.6 PAH Analysis

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310 Spectrofluorometric measurements of the total PAH concentration in fish liver 311 tissues were performed according to the method described by UNEP, 1992. The principle 312 of the method is based on the extraction of PAHs by suitable pure solvent. Mullet livers 313 were dried in an oven at 40 °C overnight. Dried tissue (0.2 g) was ground and refluxed 314 with 20 mL of ethanol containing 0.75 g of KOH for 120 minutes by constant heating. 315 Then 20 mL of PAH-free hexane was added to the flask and mixed very well and waited 316 until the flask reached to room temperature. When the flask was attained to room 317 temperature, it was poured into a separatory funnel and sufficient amount of distilled water 318 was added. The lower aqueous phase was extracted further two times with 20 mL of PAH-319 free hexane. The fluorescence intensities of the pooled samples were measured at 310 nm 320 excitation and 360 nm emission wavelengths. The fluorescence intensity of the pooled 321 samples was compared with the fluorescence intensity of known concentrations of 322 chrysene standard.

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324 2.7 Statistical Analysis

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Results were expressed as means ± 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.

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350 3. RESULTS

Table 1

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352 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 353 354 and glutathione reductase activities were measured. Table 1 illustrates biomarker enzyme 355 activity results. The lowest biomarker activities were measured in fish samples (so-iuy 356 mullet, flathead mullet and golden grey mullet) caught from Amasra. The biomarker 357 enzyme activities of different mullet species were compared with the corresponding mullet 358 species caught from Amasra. This relatively clean site was considered as a reference site to 359 demonstrate the extent of pollution.

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362 363

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

364 The highest EROD activities were found in mullets (so-iuy mullets, flathead 365 366 mullets, and golden grey mullets) caught from Zonguldak Harbour in all sampling years 367 (Table 1). Mullets caught from Zonguldak Harbour had about 6-9 fold higher EROD activities than those obtained from Amasra. The highly elevated EROD activities were also 368 369 measured in mullets captured from Ereğli Harbour which were about 7-9 fold higher with 370 respect to the values obtained from Amasra. Gülüc Stream's Mouth was the other highly 371 polluted station in this study. EROD activities of mullets samples caught from this station 372 were about 6 fold higher than those obtained from Amasra. In Figure 2, the data were 373 presented separately for each species. Statistically significant differences were indicated in 374 this figure with different letters (p<0.05). EROD activities of soluy mullets caught from 375 Zonguldak Harbour and Gülüç Stream's Mouth were significantly different from the souiy 376 mullets caught from Amasra, Melen Stream's and Sakarya River's Mouths. EROD 377 activities of flathead mullets caught from Zonguldak Harbour were significantly different 378 from Amasra. EROD activities of golden grey mullets caught from Zonguldak and Ereğli 379 Harbours were significantly different from Amasra. EROD activities of mullets caught 380 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 381 382 EROD activities of fish caught from all sampling stations except Sakarya River's Mouth.

- 383 384
- Figure 2
- 385

386 CYP1A protein level was also determined in mullet liver microsomes using the polyclonal antibodies produced against leaping mullet cytochrome P4501A. Figure 3 387 388 illustrates the western blot analysis results of mullets caught from six different stations in 389 three sampling years. Polyclonal antibody anti-leaping mullet cytochrome P4501A showed strong cross-reactivity with liver microsomes of so-iuy mullets, flathead mullets and 390 391 golden grey mullets caught from different stations of the West Black Sea. The CYP1A 392 bands of mullets caught from Zonguldak and Ereğli Harbours and Gülüc Stream's Mouth 393 were stained more intensely than those obtained from Amasra. Liver microsomes of 394 mullets caught from Melen Stream's Mouth and Sakarya River's Mouth showed moderate 395 cross reactivity with polyclonal anti-mullet P4501A. Liver microsomes of mullets caught 396 from Amasra showed low cross-reactivity with polyclonal antibody anti-mullet P4501A. 397 The results clearly indicated that mullets caught from the polluted sites had both highly 398 induced EROD activity and elevated CYP1A protein content. The relationship between 399 CYP1A protein levels and EROD activities was also examined with correlation analysis

400 (Figure 3). CYP1A protein levels were highly correlated with the EROD activity results in 401 the mullet samples caught from different stations of the West Black Sea Coast of Turkey 402 (R^2 ranged between 0.92-0.96).

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When EROD activities of different mullet species were analyzed using ANOVA, species differences were not significant (p=0.714). When the species specific crossreactivity 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.

- 413
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- 415
- 416 3.2 Glutathione S-transferase Activity

Figure 4

Figure 3

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418 The highest total glutathione S-transferase activities were measured in mullets 419 caught from Zonguldak Harbour in all sampling years which were about 4 fold higher than those obtained from Amasra (Table 1). Mullets caught from Gülüc and Melen Streams' 420 421 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 422 423 separately for each species. Glutathione S-transferase activities of soluy mullets caught 424 from Zonguldak Harbour, Melen and Gülüç Streams' Mouths were not significantly 425 different from each other. However, they were significantly different from Amasra. Glutathione S-transferase activities of soluy mullets caught from Sakarya River's Mouth 426 427 (represented by "ab") were not statistically different from Zonguldak Harbour, Gülüç and 428 Melen Stream's Mouths and Amasra. Glutathione S-transferase activities of golden grey 429 mullets caught from Ereğli Harbour (represented by "ab") were 2-3 fold higher than those 430 obtained from Amasra, however these differences were not significantly different from Amasra and Zonguldak Harbour. Glutathione S-transferase activities of mullets caught 431 432 from Ereğli Harbour showed significant difference between years. The highest GST 433 activities were measured in the fish samples caught in 2005.

434 435

Figure 5

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437 *3.3 Catalase Activity*

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439 The highest catalase activities were detected in mullets caught from Sakarya 440 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 Gülüç Stream's 441 442 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 443 444 species. Catalase activities of soiuy mullets caught from Zonguldak Harbour, Melen 445 Stream's and Sakarya River's Mouths were not statistically different from each other. However, they were significantly different from Amasra. Catalase activities of soiuy 446 447 mullets caught from Gülüc Stream's Mouth (represented by "ab") were not statistically 448 different from Zonguldak Harbour, Melen Stream's and Sakarya River's Mouths and 449 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.

455 456

Figure 6

457 458

3.4 Glutathione Reductase Activity

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460 Highly elevated glutathione reductase activities were measured in mullets caught from Zonguldak and Ereğli Harbours, Gülüc and Melen Streams' Mouths (Table1). In 461 462 2005, mullets caught from Melen Stream's Mouth had the highest glutathione reductase 463 activities among the other sampling sites which were 1.7 fold higher than those obtained from Amasra. Glutathione reductase activities of mullets caught from Gülüc Stream's 464 Mouth were 1.6 fold higher than those obtained from Amasra in 2005 and 2006. 465 466 Glutathione reductase activities of mullets captured from Ereğli Harbour were 1.6 fold 467 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 468 higher than those obtained from Amasra, respectively. In Figure 7, the data were presented 469 470 separately for each species. Glutathione reductase activities of soluy mullets caught from 471 Melen and Gülüç Streams' Mouths were significantly different from Amasra and Sakarya 472 River's Mouth. Glutathione reductase activities of soluy mullets caught from Zonguldak 473 Harbour (represented by "ab") were not statistically different from Gülüc, Melen Streams', 474 Sakarya River's Mouths and Amasra. Glutathione reductase activities of golden grey 475 mullets caught from Zonguldak and Ereğli Harbours were significantly different from 476 Amasra. Significant differences between years were not found in glutathione reductase 477 activities of fish caught from all sampling stations except Sakarya River's Mouth.

478 479

Figure 7

480 481

482 *3.5 PAH Analysis*

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484 Besides biomarker enzyme activity measurements, total PAH concentrations were 485 determined in mullets caught in 2006 to show the presence and extent of one of the organic 486 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 487 488 from Zonguldak Harbour. Total PAH concentrations of fish caught from Zonguldak and 489 Ereğli Harbours and Gülüç Stream's Mouth were significantly different from Amasra. 490 Total PAH concentrations of fish caught from Zonguldak Harbour were significantly 491 different from fish caught from Ereğli Harbour. However, total PAH concentrations of fish 492 caught from Ereğli Harbour and Gülüç Stream's Mouth were not significantly different from each other. The lowest total PAH concentrations were measured in mullets caught 493 494 from Amasra. Total PAH concentrations of fish caught from Sakarya River's and Melen 495 Stream's Mouths (represented as "bc") were not significantly different from Amasra, 496 Ereğli Harbour and Gülüç Stream's Mouth.

497

498 Table 2

499

500 501 502 503 504 505 506	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).
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550 **4. DISCUSSION**

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552 In this study, cytochrome P4501A (CYP1A) associated EROD activity, phase II 553 enzyme, glutathione S-transferase and antioxidant enzymes, catalase and glutathione 554 reductase activities were measured in mullet liver fractions to determine and monitor the 555 extent of pollution in the West Black Sea Coast of Turkey. Highly elevated biomarker 556 enzyme activities were found in the mullets caught from Zonguldak and Ereğli Harbours, 557 and Gülüç Stream's Mouth. Zonguldak is an important coal mining area in Turkey. The 558 harbour of this city is polluted especially with coal processing wastes and discharges of 559 industrial and domestic wastes. Cohen and coworkers (1994) have shown that coal by-560 products such as coal-tar significantly induce EROD activity in medika liver. In addition, 561 Vignier and coworkers (1994) have demonstrated that EROD activity significantly 562 increases in winter flounder in coal-tar contaminated estuary. Creosote is a dark liquid 563 made from coal tar. It is a complex mixture of organic compounds, containing about 85% 564 PAHs. Hyötyläinen and Oikari (1999) and Whyte and coworkers (2000) have demonstrated in different studies that the extracts of creosote-contaminated sediments 565 566 highly induce EROD activities in rainbow trout. In this study, the lowest biomarker 567 enzyme activities were measured in mullets caught from Amasra. The biomarker enzyme 568 activities obtained from the other stations were compared with those obtained from 569 Amasra. 6-9 fold increase in EROD activities and elevated CYP1A protein levels were 570 found in mullets caught from Zonguldak Harbour with respect to Amasra. This result 571 clearly indicates that coal mining activities create pollution in Zonguldak Harbour.

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573 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 574 most polluted site of İzmir Bay. In these studies, EROD activities of Liza saliens were 575 reported as 1293±292 pmol min⁻¹ mg⁻¹ protein in 1995 and 1028±287 pmol min⁻¹ mg⁻¹ 576 577 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 (Arinc 578 579 et al., 2000). In our study, highly elevated EROD activities were found in mullets caught 580 from Zonguldak and Ereğli Harbours, and Gülüç Stream's Mouth, suggesting the presence 581 of organic pollutants such as PAHs and PCBs in these sampling stations in the West Black 582 Sea Coast of Turkey.

583

Glutathione S-transferases are involved in the detoxification reactions of 584 585 xenobiotics. Induction of glutathione S-transferase have been reported in a number of field 586 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 587 588 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 589 min⁻¹ mg⁻¹ protein), Gülüç Stream's Mouth (1352±154 nmol min⁻¹ mg⁻¹ protein) and Melen 590 591 Stream's Mouth (1192±140 nmol min⁻¹ mg⁻¹ protein) are at the same order of magnitude of 592 that reported by Sen and Kirikbakan (2004) in mullets (*Liza saliens*) caught from highly 593 polluted site of İzmir Bay in Turkey (1183±35.9 nmol min⁻¹ mg⁻¹ protein).

594

595 Chronic exposure to organic pollutants increases reactive oxygen species (ROS) 596 formation (Livingstone 2001). The antioxidant enzyme catalase is one of the most 597 responsive enzymes to ROS (Halliwell and Gutteridge, 1999). Elevated catalase enzyme 598 activities have been reported in several fish species in various laboratory and field studies 599 (Otto and Moon, 1995, Orbea et al., 2002; Ferreira et al., 2005). Significantly high catalase

600 activities were measured in mullets caught from Zonguldak and Ereğli Harbours and 601 Melen Stream's and Sakarya River's Mouths, indicating the presence of ROS generating 602 pollutants in these regions. The other biomarker enzyme activity measured in this study 603 was glutathione reductase. Glutathione reductase maintains GSH/GSSG homeostasis under 604 oxidative stress conditions. Induction of glutathione reductase activity have been reported 605 in various field studies in fish exposed to organic pollutants such as PAHs, PCBs and 606 halogenated xenobiotics (Rodriguez-Ariza et al., 1993; Van der Oost et al., 2003; 607 Martínez- Gómez et al., 2006). Significantly high glutathione reductase activities have 608 been found in mullets caught from Zonguldak and Ereğli Harbours, Gülüç and Melen 609 Streams' Mouths, suggesting the presence of PAHs, PCBs and halogenated xenobiotics in 610 these sampling sites.

611

612 Biomarker enzyme activities of fish caught from Sakarya River's Mouth displayed 613 significant differences between sampling years. Sakarya River is the third longest river of 614 Turkey (824 km). It rises from the center of Anatolia and joins with Porsuk, Göksu, Kirmir, Mudurnu and Cark Streams. The river runs through many industrial and 615 616 agricultural areas before reaching to the Black Sea. The results of this study suggested the 617 existence of qualitative and quantitative differences of contaminants in the sampling 618 station between years. However, it is difficult to determine which chemicals specifically 619 affected the biomarker activities in the environmental monitoring studies. Biomarkers 620 reveal the interactive effects of chemical contaminants on organisms.

621

622 In the Black Sea, total PAH concentrations in fish samples have been determined 623 by Telli-Karakoc and coworkers (2001) in mullet samples caught from Trabzon, Yomra. In 624 our study, the highest total PAH concentrations were measured in so-iuy mullets and golden grey mullets caught from Zonguldak Harbour (54 \pm 5 µg g⁻¹ dried liver weight, n=13 625 and 47±6 μ g g⁻¹ dried liver weight, n=9, respectively) are at the same order of magnitude 626 627 of that reported by Telli-Karakoç and coworkers for mullets (Mugil saliens) (55.7±16.6 µg 628 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 Gülüc 629 630 Stream's Mouth. The sampling areas arranged in decreasing order of total PAH 631 concentrations are Zonguldak Harbour>Gülüç Stream Mouth>Ereğli Harbour>Melen 632 Stream Mouth>Sakarya River Mouth>Amasra.

633

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.

638

639 CONCLUSION

640

641 In this study, the contribution of Turkish rivers and harbours present in the West 642 Black Sea Region of Turkey to the pollution of the Black Sea was assessed using 643 biomarker responses of fish. The elevated biomarker responses found in this study indicate 644 that Zonguldak Harbour, Ereğli Harbour and Gülüç Stream are highly polluted by 645 polycyclic aromatic hydrocarbons and related contaminants. In addition, Melen Stream and 646 Sakarya River also bring some PAHs, PCBs and other type of pollutants from the inner 647 parts of Anatolia and Black Sea Region of Turkey. The results reported here also supported 648 the conclusion of previous field studies that mullet is a suitable fish species for 649 biomonitoring studies.

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658

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ACCEPTERMANUSCRIP

Zone	Vear	N	Fish Species	EROD ^a	GST ^b	CAT ^b	GR ^b
Zonguldak Harbour	2005	7	Mugil soiuv	3237+539	1260+207	322+34	14 9+1 9
Zongulaun Halooul	2006	13	Mugil soiuy Mugil soiuy	2559+315	950+135	283+46	204+08
	2006	9	Liza aurata	2302+240	1067+208	163+16	16.2 ± 1.6
	2000	5	Mugil conhalus	2017 ± 565	1007 ± 200 1017 ± 221	103 ± 10	10.2 ± 1.0 11 5 ± 2 1
	2007	5	mugii cephaius	3017±303	1017±231	1/1±12	11.3±2.1
Ereğli Harbour	2005	16	Liza aurata	1871±391	932±131	195±18	17.4±0.7
C C	2006	12	Liza aurata	2037±280	585±64	138±12	18.1±0.8
	2007	5	Liza aurata	2233+464	406+29	121+12	17.9+1.3
		-					
Gülüç Stream Mouth	2005	13	Mugil soiuy	2402±261	1352±154	281±32	20.8±0.9
,	2006	11	Mugil soiuv	2415 ± 426	830±154	224±30	20.7 ± 1.3
Melen Stream Mouth	2005	11	Mugil soiuy	1065±176	851±129	349±32	23.1±1.9
	2006	9	Mugil soiuy	818±217	1192 ± 140	200±20	18.7 ± 1.8
			0 ,				
Sakarya River Mouth	2005	10	Mugil soiuy	904±216	810±159	352±17	19.7±1.7
-	2006	10	Mugil soiuy	438±62	735±134	257±32	9.6±0.7
	2007	4	Mugil soiuy	1642±470	1016±349	187±19	11.3±0.8
Amasra	2006	3	Mugil soiuy	425±18	288±165	123±20	13.4±0.8
	2006	4	Mugil cephalus	541±22	263±62	117±21	11.3±0.5
	2007	5	Liza aurata	259±72	285±37	100 ± 11	11.2 ± 1.7

 \checkmark

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

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

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

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).

* μg 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 µg 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





EROD Activities of Mullets

Figure 2







EROD Activity (pmol min⁻¹ mg⁻¹ protein)

CYP1A protein level (R.P.A.)

EROD Activity/CYP1A protein level



Total Glutathione S-Transferase Activies of Mullets









Glutathione Reductase Activities of Mullets