Biomarkers of marine pollution observed in species of mullet living in two eastern Mediterranean harbours

F. Telli Karakoc, A. Hewer, D. H. Phillips, A. F. Gaines and G. Yuregir

The activities of enzymes associated with xenobiotic metabolism and/or oxidative processes, and the levels of aromatic-DNA adducts, have been determined in the livers of grey mullet (*Oedalechilus labeo* and *Lisa ramada*) living in two eastern Mediterranean harbours. Glutathione peroxidase (GSH-P) activity was 2.5 times higher (~ 9 IU g^{-1} liver) and glutathione reductase (GSSG-R) activity was twice as high (~ 2.5 IU g^{-1} liver) in fish from the more polluted harbour at Mersin than in the harbour near Erdemli. Superoxide dismutase (SOD) activity was 25% lower (4.3 IU g^{-1} liver) in the more polluted harbour. The concentrations of glutathione and malondialdehyde varied both with species and environment by a factor of 2.5–3. DNA adducts in liver were determined by ^{32}P-postlabelling. In *Oedalechilus labeo* in the more polluted harbour, adduct levels were 258 ± 21 adducts per 10^8 nucleotides (mean ± SE); two groups of *Lisa ramada* were distinguished having 261 ± 48 and 30 ± 6 adducts per 10^8 nucleotides, respectively. The average adduct level in a group of mullet of mixed species in the less polluted harbour was 3.3 ± 2.3 adducts per 10^8 nucleotides. The results illuminate the ability of mullet to live in contaminated marine environments, and show that enzyme activities and liver DNA adduct levels can serve as indicators of marine pollution.

Keywords: *fish, DNA adducts, liver enzyme activity, PAHs, ^{32}P-postlabelling.*

Abbreviations: *G-6-PD, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GSH-P, glutathione peroxidase; GSSG, oxidized glutathione; GSSG-R, glutathione reductase; MDA, malondialdehyde; PAHs, polycyclic aromatic hydrocarbons; SOD, superoxide dismutase.*

Introduction

Many marine environments have a history of chemical pollution from industrial, domestic and agricultural sources. A major scientific task in the study of an ecosystem is to relate the biochemistry of living organisms to their environment, and this requires the use of suitable biomarkers that reliably describe the state of health of an ecosystem and its vulnerability or resilience to pollution.

In studies of the health of fish along pollution gradients there has often been observed a strong positive correlation between concentrations of pollutants such as polycyclic aromatic hydrocarbons (PAHs), the prevalence of liver neoplasms in bottom-dwelling fish (Malins *et al.* 1984, Couch and Harshbarger, 1985, Mix 1986, Zdanowicz *et al.* 1986, Myers *et al.* 1987, 1991, Black and Baumann 1991), cytochrome P450 1A1 activity (Goksøyr *et al.* 1992, 1994) and the levels of aromatic-DNA adducts (Dunn *et al.* 1987, Varanasi *et al.* 1989b, Maccubbin *et al.* 1990). Laboratory experiments having established that PAHs induce both P450 activity (Goksøyr 1995) and adduct formation in fish (Varanasi *et al.* 1989a, Kurelec *et al.* 1991, Potter *et al.* 1994), it may be supposed that the positive correlation between concentrations of pollutants and the numbers of DNA adducts with the observation of lesions to be the consequence of a cause and effect relationship (Myers *et al.* 1991), although there have been reports of adducts in fish that are not pollution related (Kurelec *et al.* 1989). Thus fish which assimilate both water and sediment may provide useful information both on the potential carcinogenicity of the environment they inhabit and on the biochemical responses necessary to remain healthy.

PAHs are metabolized by the hepatic microsomal cytochrome P450 system through the formation of electrophilic intermediates. They may be oxidized to form highly reactive epoxy derivatives which in turn may be converted to dihydroxy derivatives and detoxified by glutathione. The free radicals generated by oxidative processes are quenched by the cellular antioxidant system using superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-P), glutathione reductase (GSSG-R) and eventually the final electron acceptor, NADPH, generated by glucose-6-phosphate dehydrogenase (G-6-PD). Thus, such enzymes present in fish liver as SOD, GSH-P, GSSG-R and G-6-PD, which are involved in the processing of hydrocarbons and other pollutants, and in the regulation of the concentration of free radicals and reactive oxygen species, may be induced (Halliwell and Gutteridge 1989). Measurement of the induced activities will therefore provide information not only about the contamination of the marine environment but also about the success of the fish in living amidst the contamination. Similar information is provided by determination of the concentrations of glutathione (GSH), which forms conjugates with xenobiotic compounds, and of malondialdehyde (MDA), an end product of the biochemical oxidation of unsaturated hydrocarbons and a measure of lipid peroxidation. In fish, as in many mammals including man, PAHs initiate carcinogenesis after becoming covalently bound to cellular DNA and this binding can be monitored by postlabelling techniques (Gupta 1985, Phillips *et al.* 1986, Stein *et al.* 1989).

Here, we report such measurements on species of grey mullet living in two eastern Mediterranean harbours ~ 50 km apart, one the comparatively small and shallow harbour at the Institute of Marine Sciences near Erdemli, and the other, the harbour of the port of Mersin.

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MATERIALS AND METHODS

Water samples

Surface and bottom water samples were collected from within the harbour near Erdemli (36.60°N, 34.30°E), and a sample of sediment was also taken for analysis. Surface water samples were collected within the harbour at Mersin (36.80°N, 34.75°E) and also close to a site of discharge of city waste just outside the harbour; sediment samples were also taken at both sites.

Fish

Grey mullet (11 *Oedalechilus labeo* and 15 *Lisa ramada*) were captured from Mersin harbour in October 1995. A mixed collection ($n = 16$) of grey mullet consisting of *O. labeo, L. ramada* and *Mugil auratus* were captured from Erdemli in June, 1995. None of the mullet from Mersin or Erdemli were spawning. Grey mullet spawn throughout the year in the eastern Mediterranean and in Mersin and Erdemli the difference between fish caught in June and in October is minimal. Blood from an incision in the tail was collected into EDTA and the fish were then sacrificed. The weight, sex and species of the mullet were noted. The fish were examined for evidence of illness or disease and their livers were excised.

Fresh fish liver (1–2 g) was homogenized in ice-cold 1.15% KCl in a Teflon homogenizer. Protein determinations were carried out by the procedure using the Folin phenol reagent originally described by Lowry *et al.* (1951). Aliquots of the crude homogenate were centrifuged at 19000 *g* at 4 °C. The supernatants were retained for the following assays.

Glutathione peroxidase (GSH-P)

GSH-P catalyses the oxidation of GSH to GSSG by peroxides, a suitable substrate for assays being *t*-butyl hydroperoxide. The rate of formation of GSSG, and hence the enzyme activity, was measured by monitoring the subsequent reduction of GSSG by GSSG-R spectrophotometrically at 340 nm as described below. An assay medium was prepared by mixing 100 µl of 1 M Tris–HCl, 5 mM EDTA buffer (pH 8), 20 µl of 0.1 M GSH, 100 µl of 10 U ml^{-1} GSSG-R, 100 µl of NADPH, 10 µl of centrifuged supernatant and 670 ml of distilled water and incubating this medium for 10 min at 37 °C. After the addition of 10 µl of freshly prepared 7 mM *t*-butyl hydroperoxide the optical density at 340 nm was compared with that of the appropriate blank every 10 min. The enzyme activity was calculated (in µmol per g of liver and per g of protein) from the rate of change of optical density, the dilution factor being 100 (Beutler 1984).

Glutathione reductase (GSSG-R)

GSSG-R catalyses the reduction of oxidized glutathione (GSSG) by NADPH or NADH to GSH. Activities may be measured by following the concentration of NADPH spectrophotometrically at 340 nm (molar extinction coefficient 6220). An assay medium was prepared containing 100 mM sodium phosphate buffer (pH 6.8), 05 mM EDTA, 1 mM GSSG and 0.12 mM NADPH. Medium (0.9 ml) and supernatant (0.1 ml) were mixed at 37 °C and the optical density of the mixture was recorded every 10 min. From the rate of change of optical density, the enzyme activity was calculated in µmol per g of liver and per g of protein, the dilution factor being 10 for Erdemli fish and 100 for Mersin fish (Beutler 1984).

Glucose-6-phosphate dehydrogenase (G-6-PD)

G-6-PD catalyses the oxidation of glucose-6-phosphate (G-6-P) to 6-phosphogluconolactone which rapidly and spontaneously hydrolyses to 6-phosphogluconate (6-PGA).

The rate of reaction and hence the enzyme activity is again followed by monitoring the optical density at 340 nm due to the accompanying formation of NADPH. The assay medium comprised 0.3 ml of pH 8 buffer (1 M Tris–HCl, 5 mM EDTA), 0.3 ml of 0.1 M $MgCl_2$, 0.3 ml of 2 mM NADP, 0.3 ml G-6-P and 1.7 ml of distilled water. Liver supernatant (0.1 ml) was added to this and the optical density at 340 nm was compared every 19 min with that of the appropriate blank. The enzyme activity was calculated in µmol per g of liver and per g of protein from the rate of change of optical density, the dilution factor being 100 for Erdemli fish and 150 for Mersin fish (Beutler 1984).

Superoxide dismutase (SOD)

Superoxide radicals generated from a mixture of xanthine and xanthine oxidase react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) to yield a red formazan dye absorbing at 505 nm. The formation of the dye is inhibited by the presence of SOD which catalyses the dissociation of the superoxide radical.

Assays utilized a commercial kit from the Randox laboratories (Crumlin, Co. Antrim, UK). Measurement of optical densities on the mixing of preformed reagents and again 30 s later, gave the rate of formazan dye formation. This was compared with the rate in the presence of liver supernatant and in the presence of blanks. The inhibition by supernatant and hence its SOD activity was calculated by comparison of the change in optical density with the change caused by the presence of known concentrations of standard solutions of SOD according to the manufacturer's instructions.

Reduced glutathione (GSH)

Virtually all of the non-protein sulphydryl of red cells is in the form of GSH which readily reduces 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a disulphide, to form bright yellow anions having a molar extinction coefficient of 13600 at 412 nm. Accordingly, 3 ml of the precipitating solutions (100 ml of which contained 1.67 g of glacial metaphosphoric acid, 0.2 g of disodium EDTA and 30 g of NACl) were added to 2 ml of the liver supernatant. After standing for 5 min the mixture was filtered through medium or coarse filter paper. An aliquot (1 ml) of filtrate was added to 4 ml of 0.3 M Na_2HPO_4 solution. The absorption at 412 nm was recorded against a blank prepared under the same conditions but containing distilled water instead of sample. The absorption at 412 nm was recorded again after DTNB reagent (1 ml containing 0.2 mg DTNB in 1% sodium citrate solution) was added to the filtered solution. From the difference in the optical densities the GSH activity was calculated in µmol per g of liver and per g of protein, the appropriate dilution factor being 2.5 (Beutler 1984).

Malondialdehyde (MDA)

Lipid peroxidation of the liver supernatant was measured by the thiobarbituric acid method using 1,1,3,3-tetramethoxypropane as standard. Results were expressed as µmol MDA formed per g of protein calculated by comparison with the absorbance of standards at 532 nm (Bus and Gibson 1979, Ohkawa *et al.* 1979).

Determination of aromatic-DNA adducts

DNA isolation

The procedures of Gupta (1984) and Gill *et al.* (1985) were adapted for the isolation of DNA from fish liver and blood. Blood (500 µl) was incubated overnight in 0.1 M NaCl, 0.01 M Tris–HCl, 0.01 M EDTA, pH 8.0 (1.5 ml) containing 2% SDS and 100 µl proteinase K (10 mg ml^{-1}). Similarly, liver (0.3–0.5 g) was incubated in 2 ml of this solution. The mixtures were then extracted with an equal volume of redistilled phenol saturated with 20 mM Tris (pH 8.0) and centrifuged to separate the layers. The aqueous phase was further extracted with an equal volume of phenol/sevag (chloroform:isoamyl alcohol; 24:1) and centrifuged. DNA was then precipitated by the addition of ethanol, washed with 70% ethanol and dried. The DNA was dissolved in sterile, distilled water (500 µl) and the volume was made up to 1.5 ml with 1 mM EDTA. The solution was cen

at 4 °C to separate glycogen (liver samples only). The partially purified DNA was incubated at 37 °C for 30 min with 30 µl RNase A (10 mg ml^{-1}), 30 µl RNase T1 (50000 U ml^{-1}) and 240 µl of 50 mM Tris (pH 7.4). After extraction twice with equal volumes of sevag, 5.0 M NaCl (20 µl) and ethanol (2 vol.) were added. The DNA precipitated overnight at -20 °C. The DNA was dissolved without drying in 1/100 SSC (2 ml). The concentrations of DNA were confirmed from measurements of the optical densities at 230, 260 and 280 nm. The DNA solutions were frozen and sent from Cukurova University by air to the Institute of Cancer Research.

^{32}P-Postlabelling

DNA was digested enzymatically and subjected to ^{32}P-postlabelling analysis using the butanol extraction procedure to enhance the sensitivity of the assay, essentially as described (Gupta 1985, Stein *et al.* 1989). Labelled digests were resolved by thin-layer chromatography on polyethyleneimine(PEI)–cellulose sheets using the multi-directional solvent systems described previously (Phillips *et al.* 1986, 1987), DNA adducts were visualized by autoradiography and quantitated by means of their ^{32}P-decay.

PAHs in liver

Portions of livers dried under reduced pressure (0.2 g) at 40 °C were weighed into round-bottomed flasks and refluxed with 0.75 g of potassium hydroxide and 20 ml of ethanol for 90–120 min. PAH-free hexane (20 ml) was added during cooling and when the flasks attained room temperature sufficient distilled water was added to cause two layers to separate. The lower, aqueous layer was extracted with two further 20 ml aliquots of PAH-free hexane. The volume and fluorescence of the combined extracts was noted before spiking with 0.85 µg of chrysene. Fluorescence was measured at excitation wavelengths of 267 and 310 nm, and emission wavelengths of 360, 380 and 400 nm. The 'chrysene equivalent' of extractable PAHs in each liver was calculated from fluorescence emission before and after spiking and from the weight of the dried sample (UNEP 1986).

Statistics

Data were analysed by Student's *t*-test. One-tailed *p* values are reported.

Results

Harbours

Both harbours from which grey mullet were captured contain saline Mediterranean water and enjoy the same seasonal climate and approximately the same daily weather. The characteristics of the surface waters and the sediments of both harbours are listed in Table 1. Values obtained from monitoring eastern Mediterranean coastal waters over the last 10 years are shown for comparison. The Erdemli harbour is small (~200 m diameter) and shallow (~2 m deep). The sediment is yellow grey and sandy, and although the water appears to be clean, the harbour does contain PAHs. Mersin is a fair-sized port whose harbour facilities occupy ~2 km by 0.5 km. Although some water appears clean, much of the surface is covered by a film of oil and there is a discharge of domestic waste at the entrance to the harbour. The highly polluted sediment consists of a thin, black ooze which is difficult to sample. The waters of both harbours are well oxygenated; they are rich in inorganic nutrients compared with the concentrations usually found in surface water around the eastern Mediterranean coast. The salinity of all harbour water samples analysed was between 29 and 32‰.

Sample	$NO_3 + NO_2$ (µM)	o-PO_4 (µM)	Dissolved oxygen (DO) (µM)	PAH (µg l^{-1})	PAH (sed.[a]) (µg g^{-1})
Erdemli surface water	25.0	1.37	254	1.04	
Erdemli bottom water	11.1	0.16	242	1.04	8.0 ± 2.2
Mersin harbour water[b]	36.3	5.1	235	5.4	219 ± 10[d]
Mersin harbour discharge[c]	80.3	71	0	267	148[d]
Coastal surface water	< 0.2	< 0.05	~225	0.5	

Table 1. Analysis of harbour waters and sediments.

[a] sed, sediment.
[b] Surface water sampled within harbour.
[c] Surface water sampled at site of city waste discharge immediately outside harbour.
[d] Sediment samples taken at same site as surface water sampling.

Mullet

Although they had been living in polluted waters all the captured fish were apparently healthy. No macroscopic lesions or deformities were observed and none of the internal organs was obviously diseased. All the fish were similar in appearance and morphology to grey mullet. The major species present in Mersin harbour were *Lisa ramada* and *Oedalechilus labeo*, neither of which showed any signs of spawning. The former species were more numerous and decidedly larger, the mean weight of those captured being 514 ± 30 g (n = 16) and 345 ± 20 g (n = 11), respectively (mean ± standard error). The difference was significant, $p < 0.0005$.

The mixed species of mullet observed at Erdemli had a mean weight of 301 ± 35 g (n = 16) which was significantly different from the mean weight, 453 ± 25 g of all species in Mersin harbour ($p < 0.005$).

The average wet weights of livers in the mullet were also very different, being 8.4 ± 0.6, 5.9 ± 0.4 and 3.4 ± 0.6 g for species *Lisa ramada, Oedalechilus labeo* and Erdemli mixed species, respectively. Again, the differences were significant ($p < 0.025$). The weights of the livers were proportional to the weights of the fish, the constant of proportionally being essentially the same for species *Oedalechilus labeo* and *Lisa ramada* in Mersin harbour but less for the mullet sampled at Erdemli. Thus 24 (out of 27; excluding outliers) mullet livers sampled from Mersin harbour gave an excellent correlation with the weights of the fish (r^2 = 0.91). Similarly, 14 (out of 16; excluding outliers) mullet livers from Erdemli also correlated with the weight of the fish (r^2 = 0.95), the slopes of the two graphs being 0.018 and 0.013, respectively, the difference being significant ($p < 0.025$). Thus, whilst there was no difference between the ratios of liver to weight in species *O. labeo* and *L. ramada,* the mullet in the cleaner harbour at Erdemli possessed less liver per gram of fish than the mullet in the more polluted harbour at Mersin

Enzyme or compound	*Lisa ramada* (Mersin) mean ± SE		*Oedalechilus labeo* (Mersin) mean ± SE		Erdemli 'mixed' mean ± SE	
	IU g^{-1} liver	μmol g^{-1} protein	IU g^{-1} liver	μmol g^{-1} protein	IU g^{-1} liver	μmol g^{-1} protein
GSH-P	9.4 ± 0.5 (n = 15)	26.6 ± 1.0 (n = 14)	8.7 ± 0.7 (n = 11)	22.7 ± 2.3 (n = 9)	3.7 ± 0.4 (n = 14)	19 ± 2 (n = 14)
GSSG-R	2.3 ± 0.1 (n = 15)	7.9 ± 0.7 (n = 14)	2.8 ± 0.1 (n = 11)	8.7 ± 1.0 (n = 10)	1.4 ± 0.1 (n = 15)	6.0 ± 0.4 (n = 14)
G-6-PD	14.2 ± 1.7 (n = 15)	42.4 ± 4.9 (n = 15)	10.8 ± 2.8 (n = 11)	29.1 ± 6.6 (n = 11)	11.7 ± 0.8 (n = 15)	54 ± 6 (n = 16)
SOD	4.2 ± 0.4 (n = 13)	14 ± 2 (n = 13)	4.4 ± 0.5 (n = 11)	16 ± 3 (n = 10)	6.1 ± 0.25 (n = 15)	28 ± 2 (n = 15)
GSH	0.2 ± 0.02 (n = 15)	0.5 ± 0.05 (n = 15)	0.06 ± 0.01 (n = 11)	0.21 ± 0.04 (n = 11)	0.1 ± 0.01 (n = 16)	0.46 ± 0.07 (n = 16)
MDA	0.45 ± 0.07 (n = 12)	1.4 ± 0.2 (n = 12)	0.43 ± 0.05 (n = 10)	2.2 ± 0.4 (n = 10)	0.2 ± 0.015 (n = 14)	1.0 ± 0.1 (n = 14)

Table 2. Comparison of enzyme activities and concentrations in fish livers.
Erdemli 'Mixed': mullet in Erdemli harbour.

Whereas the concentrations of protein in the livers of species *O. labeo* and *L. ramada* were the same, 350 ± 30 mg g^{-1}, they were significantly higher than the concentrations of protein in the livers of Erdemli mullet, 230 ± 15 mg g^{-1} ($p < 0.001$).

Consistent with the degree of pollution of the two harbours, mullet livers contained significant concentrations of extractable PAHs; mullet livers from Erdemli contained 9.3 ± 1.2 μg g^{-1} dry wt (n = 13) and mullet livers from the more polluted Mersin harbour contained 34.3 ± 7.2 μg g^{-1} (n = 11), the mean concentrations being significantly different ($p < 0.005$).

Enzyme activities

Table 2 compares the activities of GSH-P and GSSG-R, of G-6-PD and of SOD and the concentrations of GSH and MDA observed in the livers of species *Lisa ramada* and *Oedalechilus labeo* from Mersin, and the mixed species from Erdemli.

The difference in GSH-P activity, expressed as per g of protein, between *L. ramada* taken from Mersin and Erdemli fish was significant ($p < 0.01$). Differences in activities per g of liver in samples taken from Mersin and Erdemli were highly significant ($p < 0.0005$).

The difference in GSSG-R activity per g of protein between the two Mersin species was not significant but the difference in activity between samples taken from Mersin and Erdemli was ($p < 0.025$). When activities are expressed per g of liver the differences between species is significant (p ~0.001).

G-6-PD activity, when expressed per g of protein, was lower in *O. labeo* than samples in the other species ($p < 0.0025$). However, differences in activities were not significant when expressed per g of liver.

The activities of SOD in Mersin *L. ramada* and *O. labeo* were the same but were lower than those of Erdemli harbour ($p < 0.0025$).

GSH concentrations, expressed per g of liver, showed significant differences ($p < 0.025$); expressed in terms of protein the concentration of species *O. labeo* was significantly low (p ~0.005).

Concentrations of malondialdehyde, expressed per g of liver, were the same in Mersin *L. ramada* and *O. labeo*, but were significantly higher than the concentration of Erdemli species ($p < 0.001$); expressed in terms of protein the differences in activity between species *O. labeo* and *L. ramada* from Mersin and the Erdemli species were barely significant ($p < 0.05$).

DNA adducts

Postlabelling analysis revealed that all the liver DNA samples possessed aromatic and/or hydrophobic DNA adducts. This was manifested in the appearance of characteristic zones of radioactive material on TLC plates on which the labelled DNA digests had been subjected to multi-directional chromatography (Figure 1).

Quantitation of the levels of DNA adducts in each sample was achieved by assaying the radioactive zones on the chromatograms and the results are shown in Table 3. The two major species from Mersin harbour, *L. ramada* and *O. labeo*,

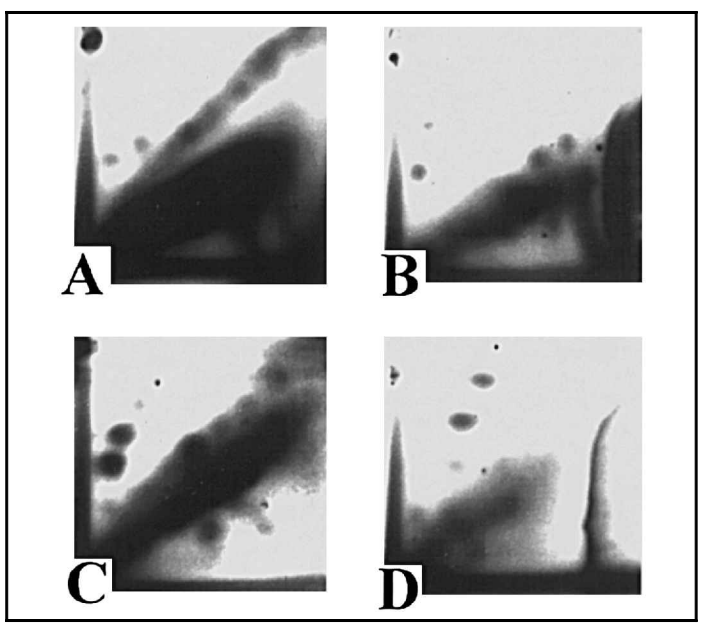

Figure 1. Autoradiographs of ^{32}P-postlabelled digests of mullet liver and blood DNA chromatographed on polyethyleneimine–cellulose TLC. The DNA adducts were resolved by multi-directional chromatography using urea-containing solvents. The origins, located in the lower left corner of each chromatogram, were excised before autoradiography. Autoradiography was for 60 h at -75 °C. A, Liver DNA from the highly polluted harbour at Mersin; B, liver DNA from the less polluted harbour near Erdemli; C, blood DNA from Mersin; D, blood DNA from Erdemli.

Site and species	Liver, adducts per 10^8 nucleotides (mean ± SE)	Blood, adducts per 10^8 nucleotides (mean ± SE)
Mersin		
L. ramada	130 ± 37 (*n* = 14)	14.3 ± 2.8 (*n* = 8)
L. ramada group 1	30 ± 6 (*n* = 8)	
L. ramada group 2	261 ± 48 (*n* = 6)	
O. labeo	258 ± 21 (*n* = 6)	
Erdemli		
Mixed species	3.3 ± 2.3 (*n* = 10)	

Table 3. Aromatic/hydrophobic DNA adducts in mullet.

both showed very high levels of DNA adducts compared with the fish in the less polluted harbour at Erdemli. In the case of *L. ramada*, the fish fell into two distinct groups, one of which had adduct levels comparable to those found in *O. labeo*, and the other considerably lower. Thus there was an apparent bimodal distribution of adducts in this species. It is not known why there was this large difference between otherwise indistinguishable fish. Although it is straightforward to isolate DNA from fish blood, there has been little previous investigation of DNA adducts in fish blood. Postlabelling analysis of blood DNA from eight fish from both groups also revealed a diagonal band of radioactivity on thin-layer chromatography similar to that seen with liver DNA (Figure 1). The levels at which these adducts were present were much lower than the adduct levels in liver (Table 3).

Discussion

Compared with open coastal waters in the eastern Mediterranean, the two harbours studied here, though well oxygenated, are unusually rich in inorganic nutrients and are polluted with PAHs. Nevertheless, the mullet appear to thrive under these conditions, the dominant species in the more polluted harbour at Mersin being much larger than the mullet living in the cleaner harbour at Erdemli. This suggests that more food is available in Mersin than in Erdemli harbour and this would be consistent with the richness of Mersin water in inorganic nutrients. Mullet possessed livers in accordance with their size, the more polluted environment in Mersin harbour inducing proportionately slightly larger livers that are richer in protein, consistent with the observations of previous workers (Bagnasco *et al.* 1991). In order to thrive in Mersin harbour the body must metabolize and rid itself of its load of pollutants: the magnitudes of the activities and concentrations recorded in Table 2 are a direct consequence of the concentrations noted in Table 1. This is emphasized by the significant concentrations of PAHs observed in both harbours and also in the mullet livers, particularly in the livers of mullet from the more polluted Mersin harbour. In fish, as in other animals, xenobiotics are generally metabolized in the endoplasmic reticulum, organic compounds being oxidized and excreted either through the gall bladder as water-soluble products or as such metabolites as GSH conjugates. We have yet to measure the activity of the cytochrome P450 1A1 system responsible for the initial oxidation of PAHs. However, MDA concentrations (Table 2), a measure of overall lipid peroxidation activity (Winston and Di Giulio 1991), indicate that more lipid peroxidation occurred in the mullet livers in the more polluted Mersin harbour. GSH concentrations in the livers were of the order of 0.05 to 0.2 $\mu g\ g^{-1}$ of wet liver and, for reasons which are still unclear, varied with species (Table 2). Thus the GSH concentrations were clearly greater than the concentrations of PAHs in mullet livers from Erdemli harbour (accepting an average molecular mass of 200–300 for PAHs) but only of the same order of magnitude as the concentration of PAHs in livers of Mersin fish. In these field experiments one may regard the metabolism of xenobiotics as a steady state flow process and the fact that GSH concentrations are similar whether the mullet livers came from Mersin or Erdemli suggests that these concentrations were sufficient to conjugate all the unoxidized, water-insoluble PAHs passing through the livers. Presumably PAHs are not the only xenobiotics present in the harbours and it would be of interest to determine the total concentration of material forming GSH conjugates as it passes through the mullet livers. The GSH balance is maintained by the levels of the GSH-P, GSSG-R and G-6-PD activities. GSH-P decomposes the hydroperoxides present in the liver whose reactivity might furnish radicals and endanger the health of the liver. GSSG-R replaces the GSH which is used up and the NAD(P)H which is consumed in replacing the GSH is regenerated through the G-6-PD system. Higher activities of GSH-P and GSSG-R are induced in mullet livers from Mersin than in mullet livers from Erdemli harbour. This is most clearly seen when the activities are expressed per g of liver (Table 2). *In vitro* experiments have shown that the activities of enzymes such as peroxidase and reductase activities decrease and subsequently increase on further insult, the latter effect often being due to the induction of specific isoenzymes (van Caneghem 1984, Washburn and Di Giulio 1991, Pedradjas *et al.* 1993). In field experiments the steady state conditions maintain the induced activity. Variances were rather large and G-6-PD activities showed no significant differences either between species or between the two harbours. The activities were higher than those of the other enzyme systems studied here and may be presumed to be sufficient to regenerate all the required NAD(P)H even in Mersin harbour. The SOD activities of *L. ramada* and *O. labeo* species in Mersin harbour were the same but both were significantly lower than the SOD activities of grey mullet in Erdemli harbour. SOD cleaves superoxide anions formed, for example, by the reduction of oxygen by the flow of electrons through microsomes, and induction results in isoenzymes with increased activity (Winston and Di Guilio 1991, Pedrajas *et al.* 1993). The anions can be involved in both the formation and the decomposition of peroxides (Winston and Di Giulio 1991). Reduced SOD activity has been associated with the presence of several (van Caneghem 1984, Bagnasco *et al.* 1991, Babo and Vasseur 1992) though not all (Washburn and Di Guilio 1988) xenobiotics and carcinomas (Bize *et al.* 1980) and is consistent with increased organic pollution of the environment (Bagnasco *et al.* 1991).

There is comparatively little difference in the induced enzyme activities between species *L.*

Mersin harbour. This is consistent with the supposition that species *L. ramada* is more numerous – and larger – not because it is more successful in ridding itself of pollutants but because it is assimilating more food.

Besides variation in enzyme activity due to differences in species and to induction by the environment, one might expect to see variation resulting from differences in the vitality of the fish. In other words, within a given species and a selected harbour one might expect to observe enzyme activity varying according to the metabolic activity of individual fish. Unfortunately all we can say is that we have found no correlation between induced enzyme activities in species *L. ramada* and *O. labeo* and the weights of the fish.

All the concentrations of aromatic DNA adducts displayed in Table 3 are high; the concentrations observed in Mersin *L. ramada* and *O. labeo* are among the highest recorded in fish livers (Dunn *et al.* 1987, Kurelec *et al.* 1989, Varanasi *et al.* 1989b, Maccubbin *et al.* 1990, Dunn 1991). Even the concentrations found in mullet in the Erdemli harbour are high, consistent with pollution of the harbour by PAHs and the concentrations of PAHs in the livers, however, the higher concentrations of adducts found in mullet from Mersin harbour are indicative of gross pollution.

The formation of aromatic-DNA adducts by covalent binding between oxidized PAHs and DNA is, in a sense, a side reaction competing with the smooth removal of urine-soluble oxidized PAH and the smooth excretion of GSH conjugates. This, indeed, is what Tables 2 and 3 demonstrate. As we have seen, testifying to their successful metabolism of xenobiotics, the mullet in the grossly polluted Mersin harbour possessed higher MDA concentrations, significantly higher GSH-P and GSSG-R activities and lower SOD activities in their livers than the mullet inhabiting the Erdemli harbour.

From their adduct concentrations species *L. ramada* may be subdivided into subspecies *L. ramada* group 1 and *L. ramada* group 2, the latter having similar adduct levels to *O. labeo* but the former possessing adduct concentrations of an order of magnitude lower (although still higher than the concentrations found in Erdemli harbour). Whereas there is no significant difference between the adduct concentrations in *O. labeo* and *L. ramada* group 2, the adduct levels in groups 1 and 2 and in Erdemli 'mixed' are significantly different ($p < 0.0005$). The reasons for the distinction between the two groups of *L. ramada* are not clear. The average weight of the low adduct subspecies, *L. ramada* group 1, 567 ± 40 g, was significantly higher than the average weight of subspecies *L. ramada* group 2, 440 ± 40 g ($p < 0.01$). In fact, the eight specimens of *L. ramada* group 1 include the four largest mullet analysed (all weighing more than 600 g). *L. ramada* group 1 possessed higher average GSH concentrations than group 2 (0.19 ± 0.02 μmol g^{-1} liver compared with 0.13 ± 0.025) and lower average GSH-P and SOD activities (24 ± 2.5 and 10 ± 2.0 IU g^{-1} of protein, respectively, compared with 31 ± 2 and 17 ± 3). The relationship between the subgroups of *L. ramada* appears analogous to the relationship between livers from Erdemli and Mersin harbours but the significance of the differences in enzyme activities is weak and requires further investigation.

We have yet to determine cytochrome P450 1A1 activities in mullet and our results are not always consistent with observations with previous workers on other species of fish inhabiting other seas (Bagnasco *et al.* 1991). Nevertheless it is worth remarking that the description of the assimilation and metabolism of PAHs which has been developed primarily for mammals – including man (Schoor and Couch 1979, Sims and Grover 1981, Ashurst *et al.* 1983, Phillips *et al.* 1987, Varanasi *et al.* 1989a, Stein *et al.* 1990) – is entirely consistent with the observations we have reported on mullet living in waters polluted by PAHs. Fish biochemistry responds rapidly to the marine environment (Couch and Harshbarger 1985, Kurelec *et al.* 1991) and observations on mullet provide general insight into the efficient biochemistry necessary to maintain a healthy physiology.

Conclusions

The results presented demonstrate that although the waters of the eastern Mediterranean are relatively clean, the two harbours examined here are polluted. Several species of mullet inhabit these harbours without obvious ill-effect but exhibit pollution-related alterations in several liver enzyme activities; they also have elevated levels of aromatic/hydrophobic DNA adducts characteristic of exposure to PAHs. They are thus useful indicator organisms for monitoring the effect of pollution on the aquatic environment.

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