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Adduct Distributions in Piscine DNA: South-Eastern Black Sea

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The south-eastern Black Sea is relatively unpolluted and contains polynuclear aromatic hydrocarbons (PAHs) only of the order of 1 $\mu g\,l^{-1}$. Pelagic and benthic fish were monitored during three successive winters from 1993 to 1996 for PAHs and for DNA adducts. The fish caught (*Mugil* sp., *Mullus barbatus*, *Alosa fallax*, *Merlangius merlangus*, *Sprattus sprattus* and *Platichthys flesus*) varied annually in length, weight and species composition. Although their livers were oily and contained significant concentrations of PAHs (11–56 $\mu g\,g^{-1}$), ^{32}P-postlabelling relatively low levels of hydrophobic DNA adducts (3–20 per 10^8 nucleotides) in either fish livers or blood. A significant fraction of the DNA adducts in the blood samples, unlike those in the livers, were dephosphorylated by nuclease-P1.

Keywords: Black Sea; fish PAHs; DNA adducts; ^{32}P-postlabelling.

The recent decline in the number of fish species due to the impact of the changing Black Sea environment is notorious (Mee, 1992). Nevertheless, there has been surprisingly little study of the physiology and health of the remaining fish. *Sprattus sprattus* and *Merlangius merlangus*, and presumably therefore other fish species in the south-eastern Black Sea, are parasitized by nematodes, occasionally to such an extent that their livers are all but completely consumed (Telli Karakoç and Doran, 1997). Under these circumstances care is needed in distinguishing the effects of pollution on the health of the fish. ^{32}P-postlabelling furnishes a sensitive technique to determine DNA adducts formed by the covalent binding of electrophilic metabolites of potentially genotoxic xenobiotics ingested by the fish (Gupta and Kurelec, 1993). Adduct formation is an early event in the initiation of cancer (Phillips *et al.*, 1986; Maccubbin, 1993) and persistent, high concentrations of DNA adducts, such as those observed in fish in polluted eastern Mediterranean harbours, imply a significant hazard to health. Thus, the magnitude of DNA-adduct formation provides information both about the pollution of the marine environment and about the piscine response to the pollution.

The oceanography of the Black Sea is well described (Degens and Ross, 1974; Oğuz *et al.*, 1996) as are the piscine inhabitants (Fisher, 1979; Hoar *et al.*, 1979). Overfishing and recent changes in the Black Sea ecosystem have reduced both the number of fish species and the population of individual species (Kıdeyş, 1994). Nevertheless, the coastal shelf of the south-eastern Black Sea still houses pelagic, demersal and benthic fish, both herbivores and carnivores, all of which can be sampled. Furthermore, as a result of the sterilization of the former breeding grounds on the north-west Black Sea shelf and the presence of a rim current, such pelagic fish as Sprat (*Sprattus sprattus*) now circumnavigate the Black Sea annually (Avşar, 1993) and, to this extent, sampling of the fish stock in the south-east may provide information, albeit limited, about the extent of pollution throughout the Sea.

Previously, we have shown that whereas the seawater in the north-eastern Mediterranean contains only $\sim 1\,\mu g\,l^{-1}$ of polynuclear aromatic hydrocarbons (PAHs), concentrations in sediments and discharges of waste can be large and, in consequence, grey mullet inhabiting polluted harbours may possess aromatic–DNA adducts at the high level of 1 per million nucleotides (Yilmax *et al.*, 1997). These studies are now extended to the examination of the fish species living in the south-east of the Black Sea sampling during three successive winters from 1993/1994 to 1995/1996.

Methodology

Sampling

Figure 1 shows the region of open coastal water in the south-eastern Black Sea and the locations where the fish stock was sampled. Fish were captured by trawl/purse-seiner by the vessels R/V Sürat Araştırma-1 and R/V Bilim. A 1–2 km trawl occurred in mid-January in 1994; in late January in 1995 short trawls sampled coastal water from Samsun to Trabzon (Fig. 1), and in February 1996 1–2 km were trawled in

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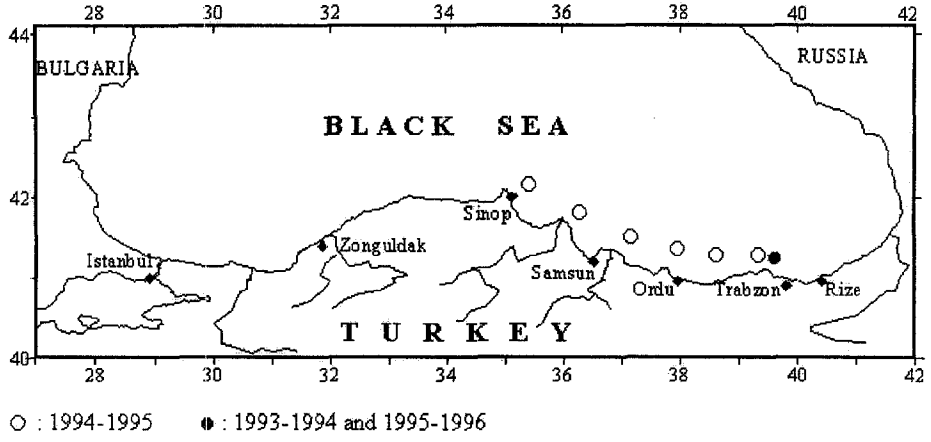

Fig. 1 South-eastern Black Sea showing sampling locations.

the same region as in 1994. Each year's objective was to sample the entire available fish stock. Table 1 reports the average properties of the euphotic zone during these winters and it provides information as to the concentrations of PAHs in the Black Sea. Essentially all the fish, pelagic, demersal and benthic, that were observed at the sampling locations were captured; it was assumed that these fish were characteristic of the entire stock. Anchovy were ignored; the sex, length, weight and apparent species of all other fish were noted. Blood was withdrawn from the caudal fin vein and livers were excised; these samples were stored frozen on board ship and transported forzen to the laboratory for anlaysis.

Determination of DNA adducts

DNA was isolated from blood and livers by phenol-chloroform extraction and freed from RNA and protein by digestion with RNase and proteinase K. The purity of the DNA samples was confirmed by their ultraviolet absorption at 230, 260 and 280 nm (Gupta, 1984). Cold DNA samples were transported to the Institute for Cancer Research where DNA adduct concentrations were determined. DNA samples were digested so that they were totally hydrolysed to their constituent nucleotides. The concentration of adducted nucleoside-3′-monophosphates was enhanced either by butanol extraction or by digestion with nuclease P1. Adducted nucleotides were converted to ^{32}P-labelled 3′,5′-bisphosphates and thereafter separated by thin layer chromatography. The concentrations of adducted material, and hence the concentrations of adducts in the original DNA, were derived from autoradiographs. The entire procedure is essentially that given by Gupta (1982, 1993) and described in detail in Telli Karakoç *et al.* (1997).

PAH concentrations of liver samples

Samples (0.2–0.5 g) of freeze-dried liver were refluxed with 0.7 g of KOH in 20 ml of alcohol for 90–120 min. Essentially all the liver dissolved. Hexane (20 ml) was added to the saopnified liver as it cooled and, after the solution had stood overnight, sufficient distilled water was added to form a two-phase system. The upper, hexane phase was removed and the lower aqueous alcohol phase was extracted with two further 20 ml portions of hexane. (A few samples gave foamy, saponified solutions which were difficult to separate into two phases and were discarded.) The hexane extracts were bulked and partially purified by chromatography on a silica gel column (the silica was dried for 4 h at 25°C and partially purified by chromatography on a silica gel column (the silica was dried for 4 h at 25°C and partially deactivated for 24 h with 3% water) eluted with hexane and dichloromethane/hexane. The fluorescence of the eluates (excitation at 310 nm and emission at 360 nm) was compared with that of chrysene (UNEP, 1986). The procedure was checked by a limited number of gas chromatography and gas chromatography/mass spectrometry analyses of the eluates through these techniques are generally less sensitive than the determination of fluorescence.

TABLE 1

Winter coastal water in the southern Black Sea (averages, 0–75 m)[a]

Temperature (°C)	Salinity[b]	NO_3+NO_2 (μmol)	PO_4 (μmol)	DO[c] (μmol)	PAH[d] (μg l^{-1})
7.1–19.5	17.9–18.2	0.15–0.54	0.05–0.5	275–350	0.036–1.06

[a]Average depth.
[b](ppt).
[c]DO: dissolved oxygen.
[d]Analyses of sea water, 1993–95.

Statistics

Single-tailed Student's *t*-tests established whether differences between means were significant.

Results

The south-eastern Black Sea

The average analyses of winter coastal water in the south-eastern Black Sea (Table 1) confirm that the surface water becomes comparatively rich by mixing with deeper regions during the winter. Primary production, limited by the low intensity of the winter light, blooms during the early spring. Despite the concern for the Black Sea there is as yet little pollution of the southern Black Sea by PAHs, the concentrations shown in Table 1 being similar to those in unpolluted regions of the Antarctic (Weber and Bicego, 1990).

Unfortunately, we know little about PAH concentrations in sediments of the south-eastern Black Sea.

Fish

Fish were sampled from 1993/1994 to 1995/1996; anchovy were ignored but all other species captured in significant numbers were analysed. The characteristics of each year's catch are given in Table 2. The table shows that over the 3-year period a range of pelagic, demersal and benthic fish were captured, including herbivores, omnivores and carnivores. However, each year's catch differed markedly both in species' distribution and in the sizes of the individual fish, the variation in the composition of the catch probably being due to variation in fishing activity. In these years there were marked differences in the recorded total catch of *Mugil* sp. and of *Mullus barbatus* in the eastern Black Sea (Fisheries Statistics, 1993, Fisheries Statistics, 1994). The significance of these fluctuations in enhanced by the diminished biodiversity and fish stock in the Black Sea.

DNA adducts

Figure 2 shows autoradiographs formed from the ^{32}P radioactivity emitted by typical thin layer chromatograms (TLCs) of the post-labelled, adducted nucleotides derived from DNA in the livers and blood of the sampled fish. The TLCs exhibit a 'diagonal zone' characteristic of overlapping DNA adducts obtained from PAHs — and other hydrophobic carcinogens — previously observed in tissues from humans, mice, fish and other organisms (e.g. Phillips *et al.*, 1993, and references therein). In addition, Fig. 2 displays a limited number of clearly resolved TLC spots in the neighbourhood of the characteristic diagonal zone.

TABLE 2

Characteristics of fish samples.

Common–Turkish and Latin names[a]	Habitat	Type of food	Sex[b]	Length[c] (cm)	Weight[d] (g)
1993/1994					
Mullet–Kefal	Pelagic	Omnivore	M 4	21.1±1.4	82±1.5
Mugil sp.			F 1	24.3	118
Red mullet–Barbunya	Demersal	Carnivore	M&F	10±0.4	5±1
Mullus barbatus			3		
Shad–Tirsi	Pelagic	Planktivore	M2	24.8±2.8	62±15
Alosa fallax			F 3	24.7±1.5	104±16
Whiting–Mezgit	Demersal	Carnivore	M&F	13.5±0.4	16.7±1.2
Merlangius merlangus			3		
Sprat–Çaça	Pelagic	Planktivore	M&F	10±0.8	5.1±0.9
Sprattus sprattus			10		
1994/1995					
Flounder–Pisi	Benthic	Carnivore	M 1	19.5	66.0
Platichthys flesus			F 10	20.5±0.5	154±11
Whiting–Mezgit	Demersal	Carnivore	M 9	19.2±0.4	54±3
Merlangius merlangus			F 11	20.2±0.3	57±3
1995/1996					
Mullet–Kefal	Pelagic	Omnivore	M 4	25.1±1.4	143±22
			F 2	29.1±0.6	225±7
Mugil sp.			J 5	23.5±1.1	114±18
Red mullet–Barbunya	Demersal	Carnivore	M 2	15.5±0.5	45±3
Mullus barbatus			F 16	17.0±0.3	58±3
Whiting–Mezgit	Demersal	Carnivore	F 15	25.5±0.5	146±13
Merlangius merlangus					
Sprat–Çaça	Pelagic	Planktivore	F 15	11.9±0.1	10±0.3
Sprattus sprattus					
Flounder–Pisi	Benthic	Carnivore	M 6	21.5±1.3	92±21
Platichthys flesus			F 14	27±1	209±16

[a]Classification and common Turkish and English nomenclature from Fisher, 1979.
[b]M = male; F = female; J = juvenile; M&F = no distinction made between male and female.
[c]Total length in cm (mean ± standard error).
[d]Weight of fresh fish free of surface water (mean ± standard error). Where no standard error is shown there is only a single result.

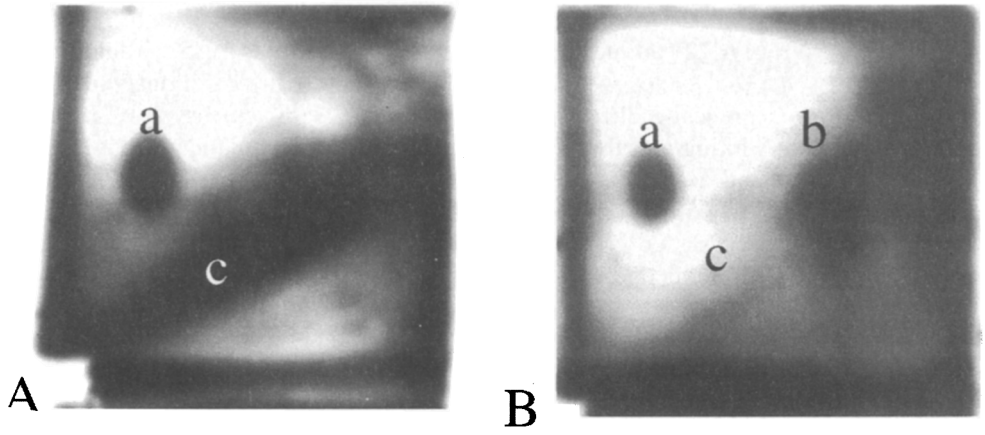

Fig. 2 Representative autoradiographs of thin-layer chromatograms of ^{32}P-postlabelled digests of DNA from Black Sea fish. **A**, liver DNA; **B**, blood DNA. The patterns show discrete adduct spots (a and b) and a diffuse diagonal radioactive zone (c).

Such spots generated only a small percentage of the observed radioactivity; their position and intensity varied from year to year but, in any given year, appeared to be independent of the fish species. These spots probably arose from adducts formed by the covalent binding to DNA of unidentified adventitious or endogeneous compounds. Thus, in 1993/1994 two spots were observed, one of which, observable only from blood samples and occupying a similar position to an RNA adduct on the autoradiograph (Beach and Gupta, 1992) corresponded to 1.0 ± 0.3 adducts per 10^8 nucleotides ($n = 23$) in all fish species. Similarly, the second spot corresponded to 1.3 ± 0.5 ($n = 20$) and 1.3 ± 0.2 ($n = 22$) adducts per 10^8 nucleotides in liver and blood samples, respectively, from all fish species.

Average DNA adduct concentrations

Table 3 summarizes the concentrations of DNA adducts found in piscine liver and blood during the three periods of sampling. The results are calculated from the radioactivities displayed by the diagonal zones of the autoradiographs. When there were sufficient results from both sexes, no significant difference could be observed between adduct concentrations from males and females. Table 3 shows that DNA adduct concentrations frequently differed between species but adduct concentrations were low and sometimes rather similar. Considering Table 3 winter by winter: In 1993/1994 there were no significant difference between DNA adduct concentrations in red mullet, whiting or shad livers, the mean for these species being 5.5 ± 0.6 ($n = 11$) per 10^8 nucleotides. The larger concentrations observed in (grey) mullet (9.5 per 10^8 nucleotides) and sprat (19.7 per 10^8 nucleotides) were significantly different ($p < 0.025$). Although statistics reveal some significant differences between the adduct concentrations in blood from different species, all the concentrations were similar and relatively few samples were

TABLE 3

Average adduct concentrations per 10^8 nucleotides (mean $\pm$ standard error). Number of samples given in parentheses

1993/1994	Sprat	Mullet	Whiting	Red mullet	Shad
Liver	19.7 ± 3.8 (11)	9.5 ± 0.7 (12)	6.5 ± 1.1 (4)	6.3 ± 2.0 (3)	4.0 ± 0.5 (4)
Blood	3.7 ± 0.5 (7)	5.5 ± 0.5 (4)	5.8 ± 1.6 (2)	3.4 ± 0.6 (3)	4.5 ± 0.5 (6)
1994/1995	**Whiting**	**Flounder**			
Liver	3.1 ± 0.4 (18)	3.3 ± 0.6 (10)			
Blood	4.4 ± 1.8 (14)	3.5 ± 0/9 (12)			
1995/1996	**Sprat**	**Mullet**	**Whiting**	**Red mullet**	**Flounder**
Liver	11.6 ± 1.1 (16)	11 ± 2 (13)	6.8 ± 0/8 (12)	16.2 ± 2 (8)	9.3 ± 1.4 (15)
Blood	–	12 ± 2 (12)	5.3 ± 1.5 (14)	8.1 ± 1.4 (8)	13 ± 2.5 (15)

examined; it seems realistic to bulk all the determinations to give a mean of 4.4 ± 0.3 ($n = 22$) adducts per 10^8 nucleotides.

In 1994/1995 there were no significant difference between adduct concentrations in whiting and flounder either in their livers (which possessed a mean concentration of 3.2 ± 0.4 ($n = 28$) adducts per 10^8 nucleotides) or in their blood (which possessed a mean concentration of 4.0 ± 1.0 ($n = 26$) adducts per 10^8 nucleotides).

In 1995/1996 there was no significant difference between the adduct concentrations either in the livers of whiting and flounder or in the livers of mullet and sprat. Adduct concentrations per 10^8 nucleotides in livers are best described as being 16.2 ± 2 ($n = 8$) in red mullet, 11.3 ± 1.1 ($n = 29$) in mullet+sprat and 8.2 ± 0.8 ($n = 27$) in whiting+flounder. Similarly, adduct concentrations per 10^8 nucleotides in blood samples are best described as being 12.6 ± 1.6 ($n = 27$) in mullet+flounder and 6.3 ± 1.1 ($n = 22$) in whiting+red mullet. Expressed in this way, the differences between species and groups of species are significant ($p < 0.025$).

Table 4, taken from the data summarized in Table 3, shows the low, rather constant level of DNA adduct formationin whiting (*Merlangius merlangus*) which persisted throughout the three winters. The consistency of these concentrations, measured at 12-monthly intervals, gives confidence in the reality of the differences between species recorded in Table 3.

Butanol and nuclease-P1 enhancement

All the DNA adduct concentrations encapsulated in Table 3 were obtained after the concentrations of adducted nucleotides had been enhanced by butanol extraction (Gupta, 1985). Table 5 compares some of these adduct concentrations with those obtained from the same samples by the alternative nuclease-P1 enhancement technique (Reddy and Randerath, 1986). With the exception of the DNA adduct concentrations in grey mullet livers in 1995/1996, when nuclease-P1 enhancement gave high results, both enhancement techniques found similar adduct concentrations in liver samples, the agreement being as good as that established in the recent intercalibration exercise (Phillips and Castegnaro, 1993). However, nuclease-P1 enhancement gave him results, both enhancement techniques found similar adduct concentrations in liver samples, the agreement being as good as that established in the recent intercalibration exercise (Phillips and Castegnaro, 1993). However, nuclease-P1 enhancement consistently furnished apparently low adduct concentrations in DNA extracted from blood. Water-soluble aromatic amines and alkylating agents would behave in this way (Gupta, 1993; Hemminki *et al.*, 1993). Inspection of the raw data summarized in Table 5 suggests that the nuclease-P1 technique missed a significant but limited number of blood samples possessing relatively high DNA adduct concentrations.

TABLE 4

Adduct levels (per 10^8 nucleotides) in whiting (*Merlangius merlangus*).

	1993/1994	1994/1995	1995/1996
Liver	6.5 ± 1.1	3.1 ± 0.4	6.8 ± 0.8
Blood	5.8 ± 1.6	4.4 ± 1.8	5.3 ± 1.5
Average wt (g)	17	56	146

PAH concentrations in fish livers

Table 6 shows the PAH concentrations observed in fish livers sampled from the south-eastern Black Sea in 1995/1996. Though the Black Sea water and sediment generally contain low concentrations of PAH, PAH concentrations in livers sampled from the south-eastern Black Sea were higher than those in fish livers from the eastern Mediterranean, even from those in polluted sites (Telli Karakoç *et al.*, 1997). This may partly due to the presence of polyunsaturated alkenes (squalene) which GC/MS showed to be present in hexane eluates of Black Sea livers and which would have contributed to the measured fluorescence attributed to PAHs. However, Table 6, and especially Table 7 which compares grey mullet (*Mugil* sp.), suggests that the high PAH concentrations in livers from fish in the relatively cold winter Black Sea water may have been

TABLE 5

DNA adduct concentrations per 10^8 nucleotides obtained via the butanol, B and the nuclease-P1, N, enhancement techniques.

1994/1995	Flounder	Whiting	
Liver B	3.3 ± 0.6 ($n = 10$)	3.1 ± 0.4 ($n = 18$)	
Liver N	5.2 ± 1.5 ($n = 8$)	3.0 ± 0.7 ($n = 20$)	
Blood B	3.5 ± 0.9 ($n = 12$)	4.4 ± 1.8 ($n = 14$)	
Blood N	0.75 ± 0.2 ($n = 12$)	1.0 ± 0.2 ($n = 14$)	
1995/1996	**Flounder**	**Mullet**	**Red mullet**
Liver B	9.3 ± 1.4 ($n = 15$)	11 ± 2 ($n = 13$)	16.2 ± 2 ($n = 8$)
Liver N	–	25.4 ± 2.2 ($n = 6$)	17.8 ± 4.9 ($n = 5$)
Blood B	13 ± 2.5 ($n = 15$)	12 ± 2 ($n = 12$)	8.1 ± 1.4 ($n = 8$)
Blood N	6.8 ± 1.3 ($n = 19$)	4.6 ± 0.7 ($n = 12$)	4.2 ± 1 ($n = 8$)

TABLE 6

PAH concentrations in liver (μg chrysene equivalent per g dry wt; mean ± standard error) south-eastern Black Sea, 1995/1996.

Flounder	Red mullet	Whiting	Sprat	Mullet
11.5 ± 1.5 (n = 12)	18 ± 2.5 (n = 18)	26.5 ± 4 (n = 15)	35 ± 9 (n = 6)	56 ± 16.5 (n = 7)

TABLE 8

Piscine DNA adducts: liver/blood ratios.

1993/1994	1994/1995	1995/1996
Mullet–others	Flounder–Whiting	Mullet–Red mullet–Whiting
1.7–1.2	0.9–0.7	1.0–1.8–1.0

due to the high proportion of lipids in the livers. The livers sampled from the Black Sea were obviously more oily than the livers which we have previously sampled from the warmer eastern Mediterranean. In fact, many Black Sea livers were covered in a yellow oil, which gave a yellow lacquer on freeze-drying. This material, which was not observed in eastern Mediterranean livers, was removed from hexane extracts by column chromatography.

Discussion

The PAH concentrations summarized in Table 1 and elsewhere (IAEA, 1996) indicate the south east Blck Sea to be little polluted, the concentrations of PAH in the water column being similar to those in unpolluted Antarctic waters (Weber and Bicego, 1990) and few of the fish we have examined possessed visible xternal tumours. Consistent with this, Table 3 shows the concentrations of piscine DNA adducts observed in the south-eastern Black Sea to be of a similar order of magnitude to the concentrations, less than or equal to 10 per 10^8 nucleotides, observed in livers of grey mullet (*Mugil* sp.) living in open eastern Mediterranean coastal water (Yilmax *et al.*, 1997) and to be much less than the concentrations of adducts observed in fish inhabiting polluted Mediterranean or North American waters (Dunn *et al.*, 1987; Malins *et al.*, 1984; Varanassi *et al.*, 1989; Maccubbin *et al.*, 1990; Yilmax *et al.*, 1997). These observations permeate this discussion.

Whereas for individual species in certain years there appear to be correlations between the length and weight of the fish and the concentration of DNA adducts in the liver, statistical analysis reveals no persistent correlations between these parameters and certainly none which apply for all species. Although our experience with grey mullet (*Mugil* sp.) (Yelli Karakoç *et al.*, 1997; Yilmax *et al.*, 1997) suggests that pollution can generate high concentrations of DNA adducts in these species, comparison of Table 2 and Table 3 shows no simple relationships between DNA adduct concentrations and the feeding habits of the species. In contrast to published investigations of fish inhabiting polluted waters in North America (Malins *et al.*, 1984; Myers *et al.*, 1987; Varanasi *et al.*, 1989; Stein *et al.*, 1990), we possess no evidence that in the south-eastern Black Sea benthic organisms suffer from undue pollution. Both flounder (*Patichthys flexus*), a benthic carnivore, and whiting (*Merlangius merlangus*), a demersal carnivore, generally possessed low concentrations of adducts (Table 3) whereas sprat (*Sprattus sprattus*), a pelagic planktivore, and red mullet (*Mullus barbatus*), a demersal carnivore, sometimes possessed somewhat higher adduct concentrations. Table 8 recalls that concentrations of DNA adducts observed in fish wintering in the south-eastern Black Sea were similar in liver and in blood samples. When fish live in an entirely uncontaminated marine environment, the ratio of (background) DNA adduct concentrations in liver and blood samples will be close to unity as, in fact, is generally observed in Table 8. When fish inhabit a polluted environment, particularly when the fish ingest sediment, the ratio of DNA adducts in liver and blood samples may exceed 1 significantly. For example the grey mullet species, *Lisa ramada*, inhabiting a polluted eastern Mediterranean harbour, provided DNA adduct concentrations in liver and blood samples in the ratio of 9.1:1 (Telli Karakoç *et al.*, 1997). On this basis, the ratio of their adduct concentrations in liver and blood being close to 2, there is reason to expect that grey and red mullet in the south-eastern Black Sea suffered from some pollution in 1993/1994 and 1995/1996 respectively. Table 5 establishes that whereas the levels of adducts in blood and livers were similar, DNA adducts in blood, unlike those in liver, were cleaved by nuclease-P1. The structures of the DNA adducts in blood and liver were different. It may be that a significant proportion of the adducts inb lood were derived from such xenobiotics as arylamines (Delclos *et al.*, 1993). Further work is needed to establish whether and how this happens.

Inspection of the standard errors displayed in Table 3 shows that in some species the total variance in DNA adduct concentrations was approximately equal to the mean, indicating that sampling gave a Poisson

TABLE 7

PAH concentrations in livers of grey mullet (*Mugil* sp.) (μg chrysene per g dry wt; mean ± standard error).

	Black Sea	Eastern Mediterranean[a]	Eastern Mediterranean[b]
PAH	56 ± 16.5 (n = 7)	9.3 ± 1.2 (n = 13)	34.3 ± 7.2 (n = 11)
DNA adducts/10^8 nucleotides	11.3	3.3	155

[a]Reference harbour (Telli Karakoç *et al.*, 1997).
[b]Polluted harbour (Telli Karakoç *et al.*, 1997).

distribution, but in the majority of the instances recorded in Table 3, the variance exceeds the mean DNA adduct concentration markedly. Thus the adduct concentrations were clustered about a selection of values. A straightforward example of clustering occurred in 1993/1994 when DNA adduct concentrations in livers of sprat averaged 19.7 per 10^8 nucleotides in 1993/1994 with a total variance of 158.8 (Table 3) and these samples formed two groups about mean adduct concentrations of 38 ± 8.5 ($n = 5$) and the more normal value of 4.3 ± 1.0 ($n = 6$) per 10^8 nucleotides (the two groups are significantly different, $p \approx 0.001$). Clustering (separation into groups) of adduct concentrations, both in liver and blood samples, often occurred in Table 3. It implies that different fish of the same species and apparently inhabiting the same environment developed significantly different concentrations of adducts. At the moment we know of no clear explanation for this. Previously, two groups of *Lisa ramada* (*Mugil* sp.) living in a polluted eastern Mediterranean harbour were distinguished by possessing DNA adduct concentrations in their livers of 261 ± 48 and 30 ± 6 per 10^8 nucleotides (Telli Karakoç *et al.*, 1997). These two groups of *Lisa ramada* had a rational distribution. The larger fish possessed somewhat larger livers with somewhat higher concentrations of protein. Accordingly, they metabolized xenobiotics more actively and displayed lower concentrations of DNA adducts (Telli Karakoç *et al.*, 1997). The south-eastern Black Sea shows very much less pollution than the eastern Mediterranean harbour and one finds no consistent correlations between the size of the fish (or their sex or their length) and their concentration of adducts. The reasons why certain fish — superficially, apparently by chance — in the south-eastern Black Sea develop higher adduct concentrations than others, apparently identical and living in the same environment, may be important and should be explored. Such exploration might provide insight as to why certain individual organisms — even humans — living in a reasonably unpolluted ecosystem are nevertheless susceptible to carcinogenesis.

Table 6 shows the PAH concentrations observed in fish livers sampled from the south-eastern Black Sea in 1995/1996. Supposing the PAH concentrations in fish livers to be in a steady state, then one may approximate

$$k_m(\mathrm{PAH})_m = k_l K(\mathrm{PAH}) \qquad (1)$$

where $(\mathrm{PAH})_m$ is the observed concentration of PAH in the marine environment and the left-hand side of eqn (1) gives the rate at which PAH enters the liver. This may be the rate at which food enters the fish or it may be the rate at which PAH diffuses into the liver, whichever is the slower. $(\mathrm{PAH})_l$ is the measured concentration of PAH in the liver, most of which will be dissolved in lipids. $K(\mathrm{PAH})_l$ gives the concentration of PAH in the aqueous phase of the liver (and in equilibrium with the PAH in the lipids) and the right-hand side of eqn (1) gives the rate (of the slow stage) of the oxidative metabolism and excretion of the PAH from the liver; k_l can be expressed straightforwardly in terms of Michaelis–Menten parameters assuming the concentration of PAH in the aqueous phase of the liver to be a tenth to a hundredth of the Michaelis constant. eqn (1) expresses the dependence of the observed concentration of PAHs in fish livers on the eating habits and the rate of ingestion of food by the fish, by the rate of metabolism of the xenobiotic and the proportion of lipids in the livers. The oily texture of the Black Sea livers has already been remarked upon. As eqn (1) leads one to expect, the PAH concentrations displayed in Table 6 vary with species (concentrations in *Platichthys flesus*, *Mullus* sp., *Merlangius merlangus* and *Mugil* sp. being significantly different, $p < 0.05$). Variances in Table 6 are large and concentrations were grouped (clustered). There was no intraspecies correlation between the concentrations of PAH and the lengths and weights of the fish or the concentrations of DNA adducts in the livers. Again, the lack of correlation between the mean concentrations of PAH shown in Table 6 and the corresponding average concentrations of DNA shown in Table 3 must be emphasized. *Platichthys flesus*, *Mugil* sp. and *Sprattus sprattus* all had the same low levels of DNA adducts in their livers though they possessed very different concentrations of PAH. Table 7 confirms the subtlety of the relationship between PAH and DNA adduct concentrations; mullet in the Black Sea possessed rather high PAH concentrations but relatively low adduct concentrations in their livers. DNA adduct formation is a side reaction competing with the conjugation and excretion of metabolized xenobiotics (PAH!). Tables 3 and 6 demonstrate the ratio of the rates of these two competing reactions (and perhaps the rate of repair of the DNA) to be more important in determining the levels of adducts in different fish species living in the unpolluted Black Sea environment than the concentrations of PAHs in the livers.

The concentrations in Table 6 are entirely different to those observed in livers from *Parophrys vetelus* (English sole) in polluted North American waters (Varanasi *et al.*, 1985). Gas chromatography/mass spectrometry revealed no PAHs in the *Parophrys vetelus* livers though the livers fluoresced. The absence of PAHs was confirmed by elegant experiments in which fish assimilating sediments dosed with radioactive PAH showed no radioactivity in their livers. It was concluded that nearly all the PAHs in the livers were present in metabolized forms (Varanasi *et al.*, 1985). In these North American samples k_l appears to have been larger than any of the other parameters in eqn (1) whereas Table 7 suggests that in the south-eastern Black Sea it was $k_m/k_l K$ that was large. The discussion we have given in the previous paragraphs stems from the investigations of Sunay (1982) who determined

PAH concentrations in eggs, gills, muscle and livers of eastern Mediterranean fish. Gas chromatography revealed the presence of benzo(a)pyrene, pyrene, chrysene and fluorene in hexane extracts of the fish livers. The fluorescence spectra of these extracts were observed to be similar to the spectra of hexane extracts of eastern Mediterranean sediments (Sunay, 1982) and this provides a sound experimental basis for eqn (1) though further work is needed.

Conclusions

The south-eastern Black Sea shows little evidence of pollution by PAHs and fish sampled during the winters of 1993–1996, though infested by nematodes, showed few signs of being affected by pollution. Thus, hydrophobic DNA adduct concentrations were generally low both in livers and blood, ranging from 3 to 20 adducts per 10^8 nucleotides and there were no consistent correlations between adduct concentrations and the sex, length, weight or feeding habits of the fish. Whereas concentrations of adducts showed significant differences between species of fish, benthic fish, unlike those in polluted North American waters, did not possess markedly high concentrations of adducts. The similar, low adduct concentrations in liver and blood appear to be characteristic of a clean marine environment; in our observations pollution causes not merely higher concentrations of hydrophobic adducts but also an increase in the ratio of adduct concentrations in liver and blood. Nevertheless, in the south-eastern Black Sea the structures of DNA adducts in liver and blood were different, those in blood possessing a fraction which could be cleaved by nuclease-P1 and the reasons for this should be elucidated.

Fish livers were oily and apparently contained relatively high concentrations of PAHs. The level of hydrophobic DNA adducts in the livers appeared to depend on the relative rate of adduct formation compared to the rate of conjugation and excretion of xenobiotics. Certain fish developed higher concentrations of hydrophobic DNA adducts than others, apparently identical and living in the same habitat. Further work is required to elucidate how this happens.

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