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DNA adducts as a biomarker of polycyclic aromatic hydrocarbon exposure in aquatic organisms: relationship to carcinogenicity

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The use of DNA adduct measurement as a biomarker of exposure to polycyclic aromatic hydrocarbons (PAHs) is now well established in ecotoxicology. In particular, DNA adduct levels in aquatic organisms has been found to produce a better correlation with PAH exposure than PAH concentrations in organisms. DNA adducts levels are most commonly determined using the ^{32}P-postlabelling assay which measures total aromatic adducts. The relationship between relative DNA adduct formation and carcinogenicity has been investigated for a number of carcinogenic and non-carcinogenic PAHs using an *in vitro* system. Our results demonstrate that relatively high levels of DNA adducts can be produced by some non-carcinogenic PAHs, while other non-carcinogenic compounds do not produce detectable adducts. In addition, it has been shown that all carcinogenic PAHs investigated produce DNAadducts and that a relationship exists between relative adduct formation and carcinogenic potency. An investigation of adduct levels in fish liver and crustacean hepatopancreas in Oxley Ck, Brisbane has shown that higher than expected DNA adduct levels were correlated with the presence of carcinogenic and non-carcinogenic PAHs with high relative adduct forming potential.

Keywords: DNA, adducts, biomarkers, PAHs, aquatic, carcinogenic.

Abbreviations: 2AB, 2-aminobiphenyl; 3MC, 3-methylcholanthrene; 9,10DMA, 9,10-dimethylanthracene; Ant, anthracene; BaA, benz[*a*]anthracene; BaP, benzo[*a*]pyrene; BbF, benzo[*b*]fluoranthene; Benz, benzidine; BeP, benzo[*e*]pyrene; BghiP, benzo[*g,h,i*]perylene; BkF, benzo[*k*]fluoranthene; Chr, chrysene; Cor, coronene; DbacA, dibenz[*a,c*]anthracene; DbaeP, dibenz[*a,e*]pyrene; DbahA, dibenz[*a,h*]anthracene; DbaiP, dibenz[*a,i*]pyrene; DbF, dibenzofluoranthene; DMBA, dimethylbenzanthracene; DMSO, dimethyl sulphoxide; Flu, fluoranthene; Nap, naphthacene; PAHs, polycyclic aromatic hydrocarbons; Per, perylene; Phe, phenanthrene; Pic, picene; Pyr, pyrene; RAF, relative adduct formation; Tri, triphenylene.

Introduction

Ecotoxicological investigations of environmental contamination now commonly use determination of aromatic DNA adducts in aquatic organisms as a biomarker of exposure to various genotoxins, especially polycyclic aromatic hydrocarbons (PAHs) (Van Schooten *et al.* 1995, French *et al.* 1996, Karakoc 1997, 1998, Willett *et al.* 1997). In contrast, the concentrations of PAHs in biota often do not relate to the level of environmental contamination (Van der Oost *et al.* 1994). These findings may be explained by induction of metabolizing enzymes (mixed function oxidases) in areas of higher contamination. The mixed function oxidases of the Cytochrome P450 family however are also responsible for metabolic activation of PAHs to electrophilic forms capable of binding to cellular macromolecules including DNA

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(Hall and Grover 1990, Mallet *et al*. 1991, Van der Oost *et al*. 1994). Thus it would be expected that DNA adducts should be a biomarker of exposure to PAHs.

Many studies of DNA adducts in aquatic organisms use the ^{32}P-postlabelling assay for determination of total aromatic adducts (Baumann *et al*. 1998, Ericson *et al*. 1998, Wirgin and Waldman 1998). It is however not clear whether levels of adducts measurable by this technique relate to the carcinogenicity of the PAHs to which the organism was exposed. Indeed it has been shown that genotoxic PAHs that are not carcinogenic may also be capable of forming DNA adducts (ECETOC 1989, Au 1991). It has been shown that DNA adduct formation is only one stage in the multi-stage process of chemical carcinogenesis and that mis-repair of adducts to form mutations is necessary for carcinogenesis to proceed (Hruszkewycz *et al*. 1992, Ross *et al*. 1994).

Methodology

Biota samples were analysed for lipid content gravimetrically after extraction with acetone/hexane (50/50). The extract was used for determination of PAHs after clean-up. This involved application of the extract to Florisil columns and elution with 6% and 10% diethyl ether in hexane. The fractions were concentrated, transferred to dichloromethane and passed through a gel permeation column. This procedure is described in detail in Mueller *et al*. (1999). Analysis of cleaned-up extracts from all samples was performed using gas chromatography–mass spectrometry operating in full ion scan mode as described in Mueller *et al*. (1999).

DNA isolation and DNA adduct determination in organisms

The method for DNA isolation and adduct determination is described in Shaw *et al*. (1995). Briefly, DNA was isolated after deproteinization using proteinase-K and salt precipitation. The DNA was then precipitated with ethanol and RNA was removed enzymatically with RNase T1 and RNase A. DNA adduct levels were determined using the ^{32}P-postlabelling assay based on the procedure of Gupta (1993). The DNA was enzymatically hydrolysed into individual nucleotides using micrococcal nuclease and spleen phosphodiesterase. Nuclease P1 was used to degrade normal nucleotides and thereby enhance the relative concentration of adducted nucleotides. Nucleotides were then radiolabelled using [^{32}P] ATP with polynucleotide kinase as catalyst. The labelled nucleotides were separated using two dimensional thin layer chromatography and adducts were visualized by autoradiography and quantified using a Packard Autoimager multiwire detector.

Relative adduct formation experiments

Individual PAHs were added to an incubation mixture consisting of the following and incubated according to the method of Shaw *et al*. (1995): PAH–5 µl of 500 µg ml^{-1} of each compound in DMSO; microsomal protein–500 µg; NADPH–400 µg; calf thymus DNA–200 µg; 0.1 M TRIS at pH 7.4–500 µl.

(Rat liver microsomes were obtained from rats pretreated for 48 h with 50 mg kg^{-1} of 3-methylcholanthrene dosed intraperitoneally in corn oil.)

Determination of relative adduct formation (RAF) by PAHs

The determination of DNA adduct concentrations was based on the calculation of total radioactivity corresponding to all spots on the chromatogram. Adducts were calculated in femtomole of adducts per microgram of DNA and in adducts per normal nucleotides. The RAF values were calculated on a molar basis for all PAHs and are expressed relative to benzo[*a*]pyrene as unity. The equation for calculation of RAF is shown below:

$$RAF = \frac{[PAH\ adduct]}{[BaP\ adduct]} \times \frac{PAH\ Mwt}{Bap\ Mwt}$$

Results

DNA adduct levels in biota

The results of DNA adduct determinations in samples from Brisbane River and Moreton Bay are presented in table 1. The corresponding adduct results from

Table 1. Results of DNA adduct determination in organisms from Moreton Bay/Brisbane River.

Organism	Location	Organ	DNA adduct conc. in adducts/normal nucleotides	DNA adduct conc. in fmol adducts μg^{-1} DNA
Whiting	Luggage Pt	Liver	1 in 10^9	0.003
Flathead	Coffee Pots	Liver	<5 in 10^{10}	<0.002
Silver bream	Long Island	Liver	<5 in 10^{10}	<0.002
Catfish	Luggage Pt	Liver	<5 in 10^{10}	<0.002
Flat nose	Bramble Bay	Liver	<5 in 10^{10}	<0.002
Bream	–	Liver	<5 in 10^{10}	<0.002
Flathead	Pumistone Passage	Liver	<5 in 10^{10}	<0.002
Flounder	Waterloo Bay	Liver	<5 in 10^{10}	<0.002
Flat nose	Nudgee Bch	Liver	4 in 10^9	0.01
Silver bream	Eastern Bay	Liver	<5 in 10^{10}	<0.002
Catfish	Refinery	Liver	<5 in 10^{10}	<0.002
Ray	Pumistone Pass	Liver	<5 in 10^{10}	<0.002
Flathead	Pumistone Pass	Liver	5 in 10^{10}	0.002
Ray	–	Liver	<5 in 10^{10}	<0.002
Flounder	Pumistone Pass	Liver	5 in 10^9	0.015
Silver bream	Nudgee Bch	Liver	<5 in 10^{10}	<0.002
–	–	Liver	<5 in 10^{10}	<0.002
Flathead	Eastern Bay	Liver	<5 in 10^{10}	<0.002
Catfish	City	Liver	9 in 10^9	0.03
Silver bream	Nudgee Bch	Liver	4 in 10^9	0.015

Oxley Ck are presented in table 2. It can be readily observed that the adduct levels in Moreton Bay (from <0.002 to 0.015 fmol adducts μg^{-1} DNA) are approximately two orders of magnitude lower than those from Oxley Ck (from 0.2 to 2.3 fmol. adducts μg^{-1} DNA). Adduct levels in the city reach of the Brisbane R. were intermediate in concentration between Oxley Ck and Moreton Bay (0.03 fmol adducts μg^{-1} DNA). The biotic concentrations of PAHs in various organisms from Brisbane R., Moreton Bay and Oxley Ck are presented in figure 1 as maximum concentration ranges due to variations in detection limit between individual samples.

It can be readily observed that the PAH profile in Oxley Ck biota is dominated by higher molecular weight compounds such as BghiP, DbahA and BaP. The PAH profile in organisms from Brisbane R. and Moreton Bay are however dominated by lower molecular weight compounds such as Phe, Flu and Pyr.

Table 3 gives the RAF values for a number of PAHs with BaP as unity. It can be observed that some PAHs do not form detectable DNA adducts while others form adducts to varying degrees up to a maximum of three times that formed by BaP. Values for the product of RAF and maximum concentration ranges for PAHs in Oxley Ck biota are presented in figure 2. Thus, using this approach, the data predict that DNA adduct formation in biota from Oxley Ck is dominated by several higher molecular weight PAHs; BaA, Chr, Tri, BbF, BkF, BeP, BaP, DbahA, BghiP. In contrast, the majority of adduct formed in organisms from Brisbane R. and Moreton Bay are predicted to occur from the following PAHs: Pyr, Chr, Tri, BbF, BkF.

The relationship between RAF and carcinogenicity can be investigated with the use of carcinogenicity indices such as that of Richard and Woo (1990) and the Gene-Tox index (Nesnow *et al.* 1986). Figure 3 shows the relationship between RAF and

Table 2. Results of DNA adduct determination on crab hepatopancreas and fish liver from Oxley Ck.

Organism	Location	Organ	DNA adduct conc. in adducts/normal nucleotides	DNA adduct conc. in fmol adducts μg^{-1} DNA
Crab	Downstream	Hepatopancreas	4 in 10^7	1.3
Crab	Upstream	Hepatopancreas	7 in 10^7	2.3
Crab	Downstream	Hepatopancreas	5 in 10^7	1.7
Crab	Upstream	Hepatopancreas	<5 in 10^8	<0.2
Crab	Downstream	Hepatopancreas	7 in 10^7	2.3
Crab	Upstream	Hepatopancreas	<5 in 10^8	<0.2
Crab	Downstream	Hepatopancreas	<5 in 10^8	<0.2
Crab	Upstream	Hepatopancreas	5 in 10^7	1.7
Bony bream	Downstream	Liver	9 in 10^8	0.3
Bony bream	Downstream	Liver	6 in 10^7	2
Bony bream	Downstream	Liver	4 in 10^7	1.2
Catfish	Downstream	Liver	6 in 10^7	2
Catfish	Downstream	Liver	4 in 10^7	1.4
Catfish	Downstream	Liver	5 in 10^7	1.6

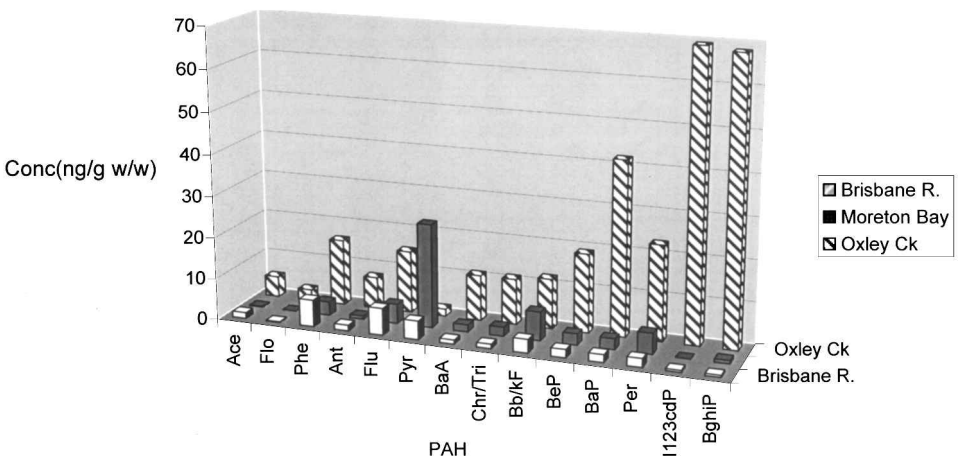

Figure 1. Maximum concentration ranges for PAHs in organisms from Brisbane R., Moreton Bay and Oxley Ck.

the Gene-Tox index. It can be observed that the three PAHs that were not found to produce adducts in this work; Ant, Phe and Per are classified as 'insufficient evidence for carcinogenicity'. The compounds 2AB, Pyr and BeP are classified as 'insufficient evidence for carcinogenicity' but did form detectable levels of adducts. All of the compounds classified as 'positive'-evidence of carcinogenicity were shown to form adducts to varying degrees. Figure 4 shows the relationship between RAF and the index of Richard and Woo (1990). Again it can be observed that a number of compounds, Cor, Flu, Per, Phe and Pic, regarded as either 'low evidence for carcinogenicity' or 'low-low/moderate evidence for carcinogenicity' did not form detectable adducts. On the other hand, Tri, classified as 'low' formed substantial levels of adducts. All compounds classified as 'low-moderate' to 'high' formed detectable levels of adducts.

Table 3. Relative adduct formation by various polycyclic aromatic compounds.

PAH	Adduct formation relative to benzo[a]pyrene as unity
Fluorene (Flo)	0
Phenanthrene (Phe)	0
Anthracene (Ant)	0
Fluoranthene (Flu)	0.05
Pyrene (Pyr)	0.15
Benzo[*a*]fluorene (BaF)	0
Benzo[*b*]fluorene (BbF)	0
Benz[*a*]anthracene (BaA)	0.8
9,10-Dimethylanthracene (9,10DMA)	3.0
7,12-Dimethylbenz[*a*]anthracene (7,12DMBA)	0.9
Triphenylene (Tri)	3.0
Chrysene (Chr)	2.0
Benzo[*a*]pyrene (BaP)	1.0
Benzo[*e*]pyrene (BeP)	0.35
Benzo[*b*]fluoranthene (BbF)	0.80
Benzo[*k*]fluoranthene (BkF)	0.75
Perylene (Per)	0
Dibenz[*a*,*c*]anthracene (DbacA)	0.65
Picene (Pic)	0
Dibenz[*a*,*h*]anthracene (DbahA)	0.74
Benzo[*g*,*h*,*i*]perylene (BghiP)	0.32
3-Methylcholanthrene (3MC)	1.5
Naphthacene (Nap)	0
2-Aminobiphenyl	0.1
Benzidine	0.05
Coronene (Cor)	0
Dibenz[*a*,*e*]pyrene (DbaeP)	0.2

Source: Shaw et al. (1995).

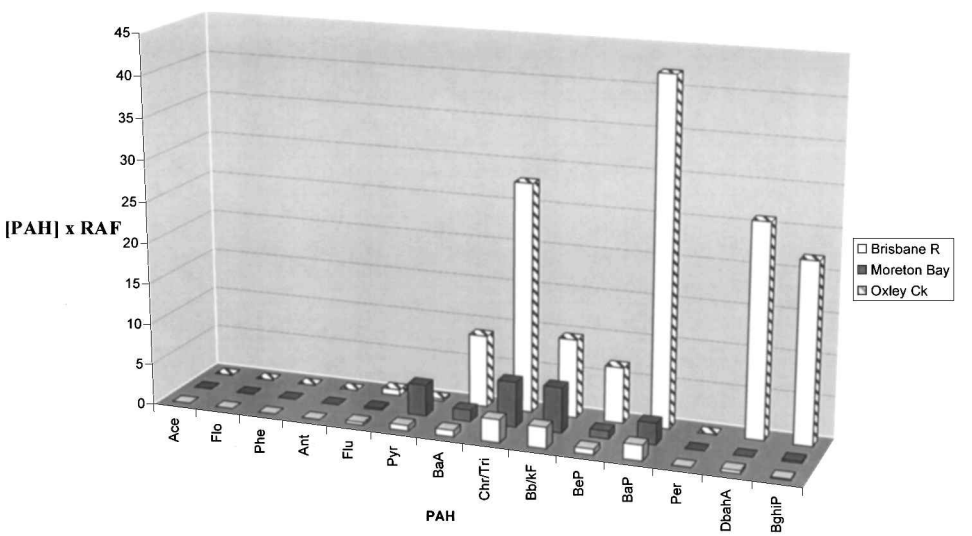

Figure 2. Product of maximum PAH concentration and RAF.

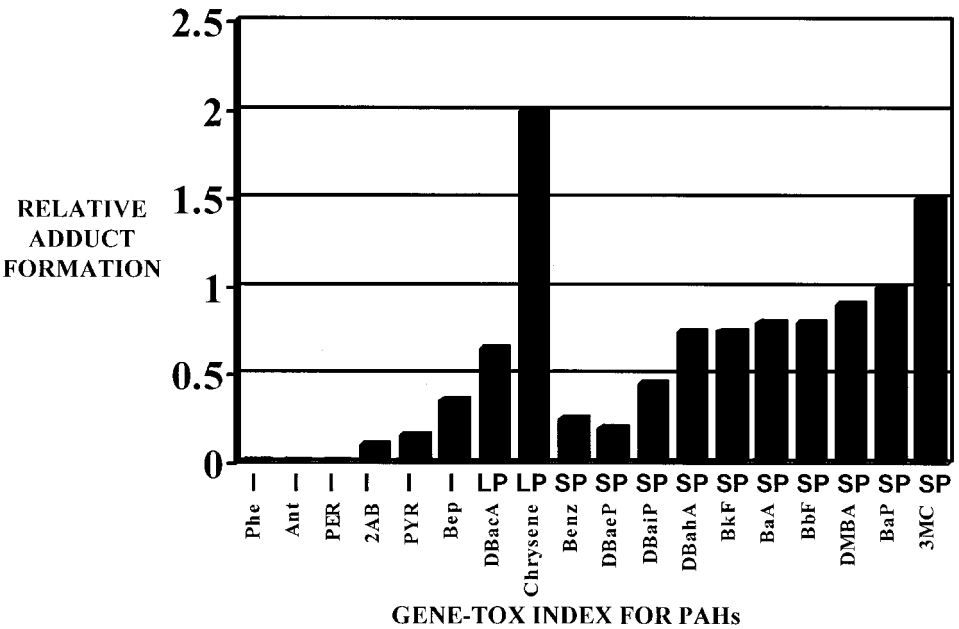

Figure 3. Relationship between RAF and Gene-Tox index.

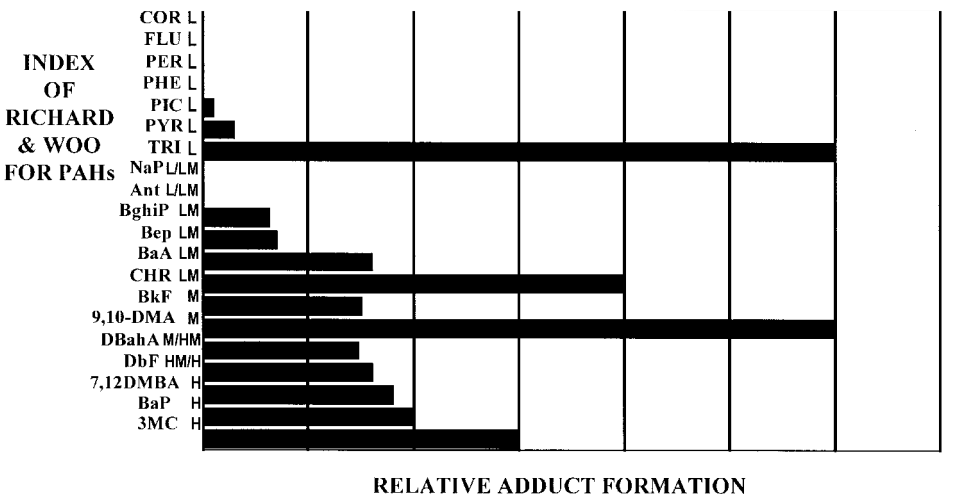

Figure 4. Relationship between RAF and Index of Richard and Woo.

Discussion

The use of the determined RAF coefficients and PAH concentrations in fish and crabs has predicted that adduct formation would be dominated by several individual PAHs. While the use of DNA adduct determinations in aquatic organisms as a biomarker of PAH exposure has been shown to correlate well with

environmental contamination by these compounds (Van Schooten *et al.* 1995, French *et al.* 1996, Willett *et al.* 1997, Karakoc *et al.* 1998), the relationship between DNA adducts and exposure to carcinogenic PAHs is not generally known. Examination of figures 3 and 4 show that the predicted adduct formation in Oxley Ck (see figure 2) is dominated by several carcinogenic PAHs. A considerable proportion of the adduct formation is however attributable to the several lesser carcinogenic PAHs. In Brisbane R. and Moreton Bay the predicted adduct formation is dominated by the non- or low carcinogenic PAHs, Tri, Chr and Pyr, while the carcinogenic BaP, BbF and BkF are also predicted to contribute to the adduct formation at these localities. It can thus be expected that a mixture of both carcinogenic and non-carcinogenic PAHs would be responsible for DNA adduct formation by PAHs in contaminated areas. In the process of multistage chemical carcinogenesis, measurement of DNA adducts in organisms lies between the stages of activation and genetic change to produce the initiated cell (Harris 1985). The fact that DNA adducts are produced by some PAHs therefore does not guarantee that mutation and carcinogenesis will result (Ross *et al.* 1994). DNA adduct measurement can be regarded as a biochemical effect monitor for humans (ECETOC 1989). It is thus considered that for ecological risk assessment purposes, DNA adduct measurement is more relevant for aquatic organisms than environmental monitoring or biological monitoring. In addition, the levels of DNA adducts in exposed aquatic organisms has been found to correlate well with other biomarkers including induction of Cytochrome P450 measured as EROD activity, glutathione S-transferase activity, biliary PAH metabolites, glutathione peroxidase activity (Karakoc *et al.* 1997, Willett *et al.* 1997, Camatini *et al.* 1998). Epidemiological evidence and laboratory studies have produced relationships between DNA adduct levels in a number of fish species from contaminated locations and both hepatic and skin neoplasms (Baumann, 1998, Myers *et al.* 1998, Reichert *et al.* 1998). It has been found however that levels of aromatic adducts were not always predictive of the vulnerability to neoplasia in populations and species from contaminated areas (Wirgin and Waldman 1998).

This study has demonstrated the commonly found association between levels of aromatic DNA adducts and environmental exposure to PAHs by aquatic organisms. In addition it has been shown that relative adduct formation potential varies substantially with the individual PAH. In particular, it was found that all carcinogenic PAHs investigated form detectable quantities of DNA adducts, but that some of the non-carcinogenic PAHs also form relatively high levels of adducts. In aquatic organisms it was predicted that adducts found are due to both carcinogenic and non-carcinogenic PAHs. The relevance of DNA adduct levels to neoplasms in aquatic organisms will thus depend on the PAH composition of contaminated environments.

References

Au, W. 1991, Monitoring human populations for effects of radiation and chemical exposures using cytogenetic techniques. *Occupational Medicine: State of the Art Previews*, **6**, 597–611.

Baumann, P. 1998, Epizootics of cancer in fish associated with genotoxins in sediment and water. *Mutation Research–Reviews in Mutation Research*, **411**, 227–233.

Camatini, M., Bonfanti, P., Colombo, A. and Urani, C. 1998, Molecular approaches to evaluate pollutants. *Chemosphere*, **37**, 2717–2738.

ECETOC 1989, *DNA and Protein Adducts: Evaluation of their use in Exposure Monitoring and Risk Assessment* (Brussels: ECETOC), Monograph No 13.

ERICSON, G., LINDESJOO, E. and BALK, L. 1998, DNA adducts and histopathological lesions in perch (*Perca fluviatilis*) and northern pike (*Esox lucius*) along a polycyclic aromatic hydrocarbon gradient on the Swedish coastline of the Baltic Sea. *Canadian Journal of Fisheries and Aquatic Sciences*, **55**, 815–824.

FRENCH, B., REICHERT, W., NISHIMOTO, M., SANBORN, H. and STEIN, J. 1996, Accumulation and dose–response of hepatic DNA adducts in English sole (*Pleuronectes vetulus*) exposed to a gradient of contaminated sediments. *Aquatic Toxicology*, **36**, 1–16.

GUPTA, R. 1993, 32P-postlabelling analysis of bulky aromatic adducts. In *Postlabelling Methods for Detection of DNA Adducts*. D. Phillips, M. Castegnaro and H. Bartsch, eds (Lyon, France: IARC Scientific Publications), pp. 11–23.

HALL, M. and GROVER, P. 1990, Polycyclic aromatic hydrocarbons; metabolism, activation and tumour initiation. In *Handbook of Experimental Pharmacology*, Volume 94, C. Cooper and P. E. Grover, eds (London: Springer-Verlag), pp. 327–372.

HARRIS, C. 1985, Future directions in the use of DNA adducts as internal dosimeters for monitoring human exposure to environmental mutagens and carcinogens. *Environmental Health Perspectives*, **62**, 185–191.

HRUSZKEWYCZ, A., CANELLA, K., PELTENEN, K., KOTRAPPA, L. and DIPPLE, A. 1992, DNA polymerase action on benzo[*a*]pyrene–DNA adducts. *Carcinogenesis*, **13**, 2347–2352.

KARAKOC, F., HEWER, A., PHILLIPS, D., GAINES, A. and YUREGIR, G. 1997, Biomarkers of marine pollution observed in species of mullet living in two eastern Mediterranean harbours. *Biomarkers*, **2**, 303–309.

KARAKOC, F., TULI, A., HEWER, A., GAINES, A., PHILLIPS, D. and UNSAL, M. 1998, Adduct distributions in piscine DNA: south-eastern Black Sea. *Marine Pollution Bulletin*, **36**, 696–704.

MALLET, W., MOSEBROOK, D. and TRUSH, M. 1991, Activation of *trans* 7,8-dihydroxy-7,8dihydrobenzo[*a*]pyrene to diolepoxides by human polymorphonuclear leukocytes on myeloperoxidase. *Carcinogenesis*, **12**, 521–524.

MUELLER, J., CONNELL, D., SADLER, R., SHAW, G. and MORTIMER, M. 1999, *Bioaccumulation of Toxicants–Final Report* (Brisbane: Brisbane River/Moreton Bay Wastewater Study).

MYERS, M., JOHNSON, L., HOM, T., COLLIER, T., STEIN, J. and VARANASI, U. 1998, Toxicopathic hepatic lesion in subadult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: relationships with other biomarkers of contaminant exposure. *Marine Environmental Research*, **45**, 47–67.

NESNOW, S., ARGUS, M., BERGMAN, H., CHU, K., FRITH, C., HELMES, T., MCGAUGHTY, R., RAY, R., SLAGA, V., TENNANT, R. *et al*. 1986, Chemical carcinogens. A review and analysis of the literature of selected chemicals and the establishment of the Gene-Tox carcinogen data base. *Mutation Research*, **185**, 1–195.

REICHERT, W., MYERS, M., PECK-MILLER, K., FRENCH, B., ANULACION, B., COLLIER, T., STEIN, J. and VARANISI, U. 1998, Molecular epizootiology of genotoxic events in marine fish: Linking contaminant exposure, DNA damage and tissue-level alteration. *Mutation Research–Reviews in Mutation Research*, **411**, 215–225.

RICHARD, A. and WOO, Y. 1990, A CASE-SAR analysis of polycyclic aromatic hydrocarbon carcinogenicity. *Mutation Research*, **242**, 285–303.

ROSS, H., LEE, H., HARVEY, R. and DIPPLE, A. 1994, Comparison of the sites of reaction of three polycyclic aromatic hydrocarbons in the *sup*F gene. *Mutation Research*, **306**, 91–101.

SHAW, G., BARRON, W. and CONNELL, D. 1995, The use of in vitro DNA adduct formation to estimate the genotoxicity of residues at contaminated sites. *Chemosphere*, **30**, 1957–1968.

VAN DER OOST, R.-J., VAN SCHOOTEN, F., ARIESE, H., HEIDA, K., SATUMALAY, K. and VERMEULEN, N. 1994, Bioaccumulation, biotransformation and DNA binding of PAHs on feral eel (*Anguilla anguilla*) exposed to polluted sediments. A field survey. *Environmental Toxicology and Chemistry*, **13**, 859–870.

VAN SCHOOTEN, F., MAAS, L., MOONEN, E., KLEINJANS, J. and VAN DER OOST, R. 1995, DNA dosimetry in biological indicator species living on PAH-contaminated soils and sediments. *Ecotoxicology and Environmental Safety*, **30**, 171–179.

WILLETT, K., MCDONALD, S., STEINBERG, M., BEATTY, K., KENNICUTT, M. and SAFE, S. 1997, Biomarker sensitivity for polynuclear aromatic hydrocarbon contamination in two marine fish species collected in Galveston Bay, Texas. *Environmental Toxicology and Chemistry*, **16**, 1472–1479.

WIRGIN, I. and WALDMAN, J. 1998, Altered gene expression and genetic damage in North American fish populations. *Mutation Research–Fundamental and Molecular Mechanics of Mutagenesis*, **399**, 193–219.