REVIEW

DNA adducts in marine and freshwater fish as biomarkers of environmental contamination

Wolfgang Pfau

The analysis of DNA modifications in aquatic animals may serve as a sensitive marker of exposure to genotoxic contaminants. This is of importance in assessing water quality regarding pollution with genotoxic compounds, food safety analysing edible aquatic animals and in terms of ecotoxicology. Covalent modification of DNA is considered a crucial event in chemical carcinogenesis and thus may be considered a biomarker of an early genotoxic effect. Measuring DNA adducts is unique in that these lesions may be considered a biomarker of both exposure and effect. A number of studies have described the analysis of the DNA isolated from the liver of both freshwater and marine fish. Considerable levels of DNA adducts have been observed in some animals from contaminated lakes or rivers. Low levels were observed in DNA from the liver of marine fish. The background levels of DNA adducts have to be determined in animals from appropriate uncontaminated control sites that are matched for species, gender, age and season of the year. It is of crucial importance to consider the species analysed since there have been reports of the non-responsiveness of some species.

Keywords: *DNA adducts, ^{32}P-postlabelling, fish, environmental toxicology.*

Introduction

The occurrence of tumorigenic lesions in fish has been employed as a biomarker for the assessment of water pollution (Mix 1986, Malins *et al.* 1988). However, this biological endpoint has proved to be a rather crude parameter, detecting only very heavy contamination of the environment with carcinogenic pollutants (deRaat *et al.* 1990). Furthermore, the detection of neoplastic diseases in fish is a late biomarker of effect which provides no information on the contaminants that led to this biological response.

DNA adducts

Covalent modifications of cellular DNA (DNA adducts) are generally regarded as initiating lesions in chemically-induced neoplasms (Kriek *et al.* 1984). Quantitative relationships have been established between dose, DNA adduct level and biological endpoints in a number of mutagenesis tests, transformation assays and animal carcinogenicity tests *in vitro* and *in vivo* in rodents (Autrup 1991). The determination of DNA adducts in experimental animals and humans exposed to genotoxic compounds is a useful tool in cancer research. A number of experimental studies with laboratory fish (see below) have led to the assumption that these results can be transferred from mammalian species to teleosts: most of the chemicals classified as carcinogens require metabolic activation in order to exert their biological effect. This activation is mediated by cytochrome P450 oxidases or prostaglandin synthetases (phase I) and may require further activation by conjugating enzymes (phase II). The active metabolites are electrophilic, and will react with cellular DNA to form covalent adducts. Guanine is generally the major target for the binding of alkylating or bulky polycyclic aromatic agents to DNA. It has been demonstrated that fish have the enzymatic capability to perform these metabolic transformations (reviewed by Stegeman 1993). The induction of hepatic enzymes as a result of environmental pollution or experimental dosing with PAH, polychlorinated biphenyls or dioxines has been applied as a useful biomarker in freshwater and marine organisms including fish (Goksoyr and Förlin 1992, Collier *et al.* 1995).

In experimental studies (Varanasi *et al.* 1986, 1989a, Stein *et al.* 1993) it has been shown that adducts formed by benzo[*a*]pyrene in the liver DNA of fish accumulate over time. Therefore DNA adducts appear to be especially suited for the determination of chronic exposure to genotoxic compounds. It has been shown that covalent DNA modifications are persistent for longer periods of time (up to several months), similar to or even longer than DNA adducts in mammalian tissues (Varanasi *et al.* 1989a). Although several studies have addressed the repair of DNA adducts in fish (Sikka *et al.* 1990, Kurelec *et al.* 1991), the recent insights into the mechanisms of DNA repair in mammalian cells have not as yet been investigated in detail (Bohr 1995).

METHODS

The detection and quantitation of DNA adducts present a challenging analytical problem. Since these lesions are formed at very low levels the sensitivity of an assay must be very high. Several new methods have been developed to detect carcinogen–DNA adducts in mammalian (including human) tissues and blood cells (Table 1), including immunochemical, biochemical and physical techniques (Phillips 1989). Experimental studies often make use of radioactively labelled compounds which are applied to the animals (Sikka *et al.* 1991, Zhang *et al.* 1992) (Table 2). However in field studies this approach is not feasible. The immunological methods use antibodies raised against specific DNA adducts, thus the chemical structure of the DNA adduct to be analysed has to be known (Nakatsuru *et al.* 1990, Poirier 1993, Pfohl-Leszkowicz *et al.* 1996). Typically, antibodies are raised against DNA modified with benzo[*a*]pyrene diol epoxide. It has been shown that these antibodies cross-react with similar DNA adducts formed by other PAH diol epoxides, data derived from these immunochemical assays are considered to reflect the total of PAH–DNA adducts (Poirier 1993). In order to increase the specificity and sensitivity of the biochemical and the physical methods they have been combined with an immunoaffinity purification step (Autrup 1991).

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Method	Sensitivity[a]	Cost	Comments	Reference
^{3}H-label/HPLC	10	High	Labelled carcinogens required, not suitable for biomonitoring feral fish	Phillips 1989, Varanasi *et al.* 1986
^{14}C-label/HPLC	100			Sikka *et al.* 1991
HPLC/fluorescence	100	Low	Applicable only to fluorescent compounds	Shugart *et al.* 1987
GC/MS	10	High	Derivatization required	Talaska *et al.* 1992, Friesen *et al.* 1996
Immunoassay	1	Low	Structure of adduct must be known and antibody available	Poirier 1993
^{32}P-Postlabelling	0.1	Low	Preferably bulky, aromatic adducts	Randerath *et al.* 1981, Phillips 1989

Table 1. Methods for DNA adduct analysis that have been applied to the study of DNA adduct formation in marine or freshwater fish.

[a] Approximative sensitivity in adducts per 10^{8} nucleotides.

Species		Carcinogen[a]	Method[b]	Result[c]	Reference
English sole	*Parophrys vetulus*	BaP	^{3}H-label	Species-dependent adduct levels	Varanasi *et al.* 1986
Starry flounder	*Platichthys stellatus*				
English sole	*Parophrys vetulus*	Sediment extract	PL/Butex	Diagonal zone	Varanasi *et al.* 1989b
English sole	*Parophrys vetulus*	BaP	PL	BPDE-dG	Varanasi *et al.* 1989a
Rainbow trout	*Oncorhynchus mykiss*	AFB	^{3}H and ELISA	Species-dependent adduct levels	Nakatsuru *et al.* 1990
Coho salmon	*Oncorhynchus kisutch*				
Southern flounder	*Paralychthys lethostigma*	BaP and metabolites	^{3}H-label		James *et al.* 1991
Brown bullhead	*Ictalurus nebulosus*	BaP and metabolites	^{14}C-label	BPDE-dG, persistence of adducts	Sikka *et al.* 1991
Carp	*Cyprinus carpio*	BaP	PL, Butex	BPDE-dG	Kurelec *et al.* 1991
Carp	*Cyprinus carpio*	Diesel-2 oil slick	PL	Persistence and repair of adducts	Kurelec *et al.* 1992
English sole	*Parophrys vetulus*	BaP	^{3}H-label	BPDE-dG	Nishimoto *et al.* 1992
English sole	*Parophrys vetulus*	BaP, DBC	PL/P1	Cumulative adduct formation, persistence	Stein *et al.* 1993
Rainbow trout	*Oncorhynchus mykiss*	AFB	^{3}H-label	Temperature dependent adduct levels	Zhang *et al.* 1992
Rainbow trout	*Oncorhynchus mykiss*	BaP	PL	Adduct levels dependent on route of administration	Potter *et al.* 1994
Killifish	*Fundulus grandis*	BaP	PL/P1		Willet *et al.* 1995
	Fundulus similis				
Rainbow trout	*Oncorhynchus mykiss*	Effluent discharges (aquarium control)	PL/Butex	DNA adducts in gill and liver	Sagelsdorff 1995

Table 2. Experimental studies on DNA adduct formation in aquarium fish.

[a] Fish were treated with carcinogenic compounds (BaP: benzo[*a*]pyrene, AFB: aflatoxin B1, DBC: dibenzocarbazole) or complex mixtures.

[b] Methods employed for DNA adduct analysis were PL: ^{32}P-postlabelling with enhancement procedures butanol extraction (Butex, Gupta 1985) or nuclease P1 enhancement (P1, Reddy and Randerath 1986); or ^{3}H or ^{14}C-labelled compounds were administered and radioactivity bound to hepatic DNA was determined.

[c] Specific results of the study, i.e. identification of the major DNA adduct formed by BaP, the adduct that is formed by direct reaction of *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene with deoxyguanosine (BPDE-dG) or dependance of adduct formation on species, water temperature, route of administration or organ examined.

The fluorescence-based techniques rely heavily on the intrinsic fluorescence properties of the carcinogen. Thus, synchronous fluorescence scanning or HPLC analysis with fluorescence detection have been restricted to the analysis of benzo[*a*]pyrene or aflatoxin-derived adducts (Shugart *et al.* 1987, Phillips 1989, Wang and O'Laughlin 1992). Analysis of DNA adducts by GC/MS techniques has the advantages of a relatively high sensitivity with additional structural information on the carcinogen. It has been shown that PAH-modified DNA quantitatively releases the tetrol moieties following acid hydrolysis. Polycyclic aromatic amines bound to DNA are released by mild alkaline hydrolysis (Friesen *et al.* 1996). The necessary derivatization techniques have been developed and GC/MS analysis has been applied in experimental studies with rodents or in human biomonitoring experiments (Talaska *et al.* 1992, Friesen *et al.* 1996). The application of these very recent developments to studies on DNA adducts in fish appears to be very promising.

^{32}P-Postlabelling

The most extensively applied method in the study of DNA adducts is the ^{32}P-postlabelling assay developed by Randerath and coworkers (Randerath *et al.* 1981). The advantages of this method are the versatility with regard to adduct structure, the extremely low limit of detection, the applicability to DNA adducts of unknown structure and the relatively low cost of the assay.

DNA adducts of the 'bulky' types formed by carcinogens containing a multi-ring aromatic moiety are most easily detected, but adducts formed by other groups of compounds can be analysed by modifications of the chromatographic conditions (Phillips 1989). Briefly this method consists of DNA isolation from the tissue and enzymatic hydrolysis of DNA to 3′-phospho-nucleotides. These monophosphates are then enzymatically labelled with radioactive [^{32}P]-phosphate at the 5′-position using polynucleotide kinase and [γ-^{32}P]-ATP. This step is often preceded by an

Species		Location		Method[a]	Adduct levels[b] RAL (10^8)	Correlation of DNA adduct levels with contamination[c]	Reference
Brown bullhead	*Ictalurus nebulosus*	Buffalo River	Great Lake estuaries	P1	7.0	+	Dunn *et al.* 1987
		Detroit River		HPLC	5.4		
		(aquarium control)[d]			(1.5)[d]		
Chub	*Leuciscus cephalus*	Sava River	New Zagreb, Croatia	Butex	0.5	–	Kurelec *et al.* 1989a
Barbel	*Barbus barbus*		Karlovac, Croatia		1.9		
Bream	*Abramis brama*				7.5		
Carp	*Cyprinus carpio*	(Korana River)[d]			(0.5)[d]		
Brown bullhead	*Ictalurus nebulosus*	Cuyahoga River	Ohio		56.6	+	Dunn *et al.* 1990
		Black River	Ohio		42.2		
		Hamilton Harbour	Lake Ontario		21.7		
		Bay of Quinte	Lake Ontario		7.9		
		(Lake Haddie)[d]			(6.6)[d]		
Oyster toadfish	Opsanus tau	Elizabeth River	Virginia		4.7–14.1	+	Collier *et al.* 1993
		(Chesapeake)[d]			(0.5)[d]		
Bream	*Abramis brama*	River Elbe	Several stations,	Butex	21.9–45.7	+	Pfau *et al.* 1994
		(Lake Belau)[d]	Germany		(0.5)[d]		
Roach	*Rutilus rutilus*	Nieuwe Meer Lake	Amsterdam	P1	2.9	–	van der Oost *et al.* 1994a
		(Gasperplan Lake)[d]			(2.8)[d]		
Eel	*Anguilla anguilla*	Amerika Harbour	Amsterdam,	P1	34.3	+	van Schooten *et al.* 1995, van der
		Volgermeerpolder	The Netherlands		23.2		Oost *et al.* 1994b
		(Diemerzeedijl)[d]			(4.7)[d]		
White sucker	*Catostomus commersoni*	St Lawrence River	Montreal Canada		12.9	+	El Adlouni *et al.* 1995
		(St Francois River)[d]	Windsor, Canada		(5.7)[d]		

Table 3. Field studies on DNA adducts in various species of freshwater fish from a number of contaminated rivers or lakes. DNA adduct levels are compared with fish of the same species from local reference sites. Studies were performed on DNA from fish liver employing the ^{32}P-postlabelling assay with different enrichment procedures.

[a] Variations of the ^{32}P-postlabelling assay: Butex: butanol extraction (Gupta 1985), P1: nuclease P1 enrichment (Reddy and Randerath 1986), HPLC: fractionation of adducts by reversed-phase HPLC (Dunn *et al.* 1987).

[b] RAL = relative adduct labelling of DNA modifications detectable by ^{32}P-postlabelling expressed as DNA adducts per 10^8 nucleotides (interval or mean levels from several animals per station).

[c] A significantly elevated level of DNA adducts was observed (+) or was not observed (–) in the livers of fish from a contaminated environment compared with the reference site.

[d] Reference sites and DNA adduct background levels observed in animals from these sites in parenthesis.

enrichment of the modified nucleotides, extracting these into *n*-butanol in the presence of a phase-transfer agent, HPLC fractionation or differential hydrolysis of non-modified nucleotides with nuclease P1 (Gupta 1985, Reddy and Randerath 1986, Dunn *et al.* 1987). Following the labelling reaction the labelled material is separated by chromatographic methods. Conventionally, this is done by multidirectional thin layer chromatography on ion exchange polyethylene imide cellulose plates that yield patterns of spots or areas of radioactivity upon autoradiography (Randerath *et al.* 1981, Phillips 1989). The quantitation is achieved by Cerencov counting of the excised spots or more conveniently with a phospho-imager or similar apparatus. Alternatively, reversed-phase HPLC methods have been developed that allow on-line detection and quantitation of DNA adducts with improved chromatographic resolution of labelled adducts (Pfau and Phillips 1991, Zeisig and Möller 1995). Some problems exist with the interlaboratory reproducibility of these ^{32}P-postlabelling DNA adduct analyses. While in several trials the qualitative reproducibility was satisfactory, the quantitative results differed considerably between laboratories. Efforts to standardize the existing protocols are under way (Phillips and Castegnaro 1993). The high sensitivity of the ^{32}P-postlabelling assay is also its weakness. Many different spots or diffuse areas of radioactivity are observed in this assay but the structure of these and the biological consequences remain unknown. To date it has been impossible to identify most of the ^{32}P-labelled adducts since none of the spectroscopic methods available reaches into these femtomole levels of sensitivity.

Species

Experimental studies have established differences of hepatic DNA adduct formation, e.g. between two species of salmonid fish, rainbow trout and coho salmon, upon treatment with aflatoxin B1 (Nakatsuru *et al.* 1990). The studies published so far on DNA adducts in feral fish have been pilot studies with the aim of establishing the suitability of this biomarker in a number of different species (Tables 3 and 4).

Fish species with a small migration tendency and a high abundance in the investigated aquatic environment are considered most suitable (Jedamski-Grymlas *et al.* 1994). It has been suggested that benthic species living close to the sediment and feeding on the sediment or invertebrates living therein are ideal for monitoring the contamination of the sediment. A correlation between adduct

Species		Location		Method[a]	Adduct levels[b] RAL (10^8)	Correlation of DNA adduct levels with contamination[c]	Reference
English sole	*Parophrys vetulus*	Duwamish Waterway	Seattle, Washington	Butex	2.6	+	Varanasi *et al.* 1989b
		Eagle Harbor			1.7		
		(Useless Bay)[d]			(< 0.03)[d]		
Winter flounder	*Pseudopleuronectes americanus*	Boston Harbor	Boston, MA	Butex	0.9	+	Varanasi *et al.* 1989b
Mugil	*Mugil auratus*	Mediterranean	Croatian coast	P1/Butex	0.9	–	Kurelec *et al.* 1989b
English sole	*Parophrys vetulus*	North Sea and Irish Sea			–	+	Poginsky *et al.* 1990
Dab	*Limanda limanda*						
Winter flounder	*Pseudopleuronectes americanus*	Long Island Sound	Norwalk, New Haven Niantic, Connecticut	–	–	+	Gronlund *et al.* 1991
English sole	*Parophrys vetulus*	Pudget Sound:	Seattle, Washington		14–18	+	Stein *et al.* 1992
Rock sole	*Lepidopsetta bilineata*	Duwamish waterway					
Starry flounder	*Platichthys stellatus*	(Polnell point)[d]			(2.0–6.7)[d]		
Dab	*Limanda limanda*	North Sea	Several stations in the German Bight	Butex	No adducts	–	Chipman *et al.* 1992
Dab	*Limanda limanda*	North Sea	Several stations in the German Bight	P1	0.1–8.4	+	Poginsky *et al.* 1990
Red mullet	*Mullus barbatus*	Mediterranean	Several sites: Spanish, French, Italian Coast	P1/Butex ELISA (PAH)	Up to 5	+	Pfohl-Leszkowicz *et al.* 1996
Hardhead sea catfish	*Arius felis*	Gulf of Mexico	Galveston, Texas		–	–	McDonald *et al.* 1996

Table 4. Field studies on DNA adducts in marine fish. Hepatic DNA adduct levels were determined using variations of the ^{32}P-postlabelling assay or immunochemical analysis (ELISA) employing antibodies generated against DNA adducts of benzo[*a*]pyrene diol epoxide. See legend to Table 3.

formation in fish liver DNA and sedimental contamination has been shown for flounder fish (Collier *et al.* 1993) and eel (van der Oost *et al.* 1994b). Aromatic contaminants of high molecular weight tend to bind to the sediment and DNA adducts formed by these polycyclic compounds are most easily detected by ^{32}P-postlabelling analysis of liver DNA. However, studies of rainbow trout exposed to industrial effluents have shown that they develop DNA adducts both in the liver and to a considerable extent in the gill (Sagelsdorff 1995). The variation of DNA adduct levels depending on the route of administration (feed, water or intraperitoneal injection) has also been shown in experimental studies (Varanasi *et al.* 1986, Potter *et al.* 1994).

Endogenous adducts

In order to obtain meaningful results with any biomarker the selection of an appropriate reference site is crucial. While aquarium-raised fish appear to be ideal with respect to minimized pollution, the financial effort is high and the artificial situation may be misleading. Chemical analysis of the water and/or sediment from the reference site would be required but in a number of studies DNA adduct levels in fish liver DNA have been reported and assessed against another biomarker. It has been pointed out that ^{32}P-postlabelling analysis may detect considerable levels of DNA adducts unrelated to anthropogenic water pollution (Kurelec *et al.* 1989a, Ray *et al.* 1995). The nature of these adducts is not clear and they might be due to natural genotoxic components in the diet or be of endogenous origin. Endogenously-formed DNA modifications have also been detected in laboratory mammals (Randerath *et al.* 1986, Nath *et al.* 1996) and are apparently dependent on diet, age and, in the case of wildliving fish, are season dependent. Although described for a number of fish species as being considerable the levels reported by Kurelec *et al.* (1989b) are relatively low compared with other studies (Table 4). Furthermore, in this study of DNA adduct formation in the Mediterranean sea, the contamination status of the polluted site was not based on chemical analyses.

However, fish from polluted and reference sites should be matched for species, gender, age and caught at the same time of the year. Determination of age should be done by scale analysis since size or body weight of animals may be reduced in heavily polluted areas.

The age of the animals appears to be of crucial importance: in experimental studies Varanasi *et al.* (1989) showed that in juvenile English sole higher adduct levels were induced compared with adult animals. The accumulation of adducts has been mentioned above, thus animals living in a contaminated environment for years show higher levels of covalent DNA modification.

Identification of DNA adducts

Whereas in laboratory experiments the exposure to a defined chemical leads to a typical pattern of covalent modification the exposure of wild animals typically occurs to complex mixtures. The ^{32}P-postlabelling analysis is especially suited for the high number of DNA adducts resulting from these exposures, resulting in a typical diagonal zone of radioactivity upon ion-exchange TLC analysis (Figure 1). Even with a few discrete adduct spots detectable, only a small amount of information can be gained with regard to the chemical structure of the adducts measured. Applying different variations of the assay employing a nuclease P1 hydrolysis (Reddy and Randerath 1986) or enrichment of hydrophobic adducts prior to the labelling (butanol extraction (Gupta 1985, Gupta and Earley 1988) or chromatographic techniques (Dunn *et al.* 1987) give some information about the nature of adducts. The nuclease P1 enrichment procedure is generally considered to enhance DNA adducts derived from polycyclic aromatic hydrocarbons or their activated metabolites, the diol epoxides, especially in the case of benzo[*a*]pyrene, the model compound

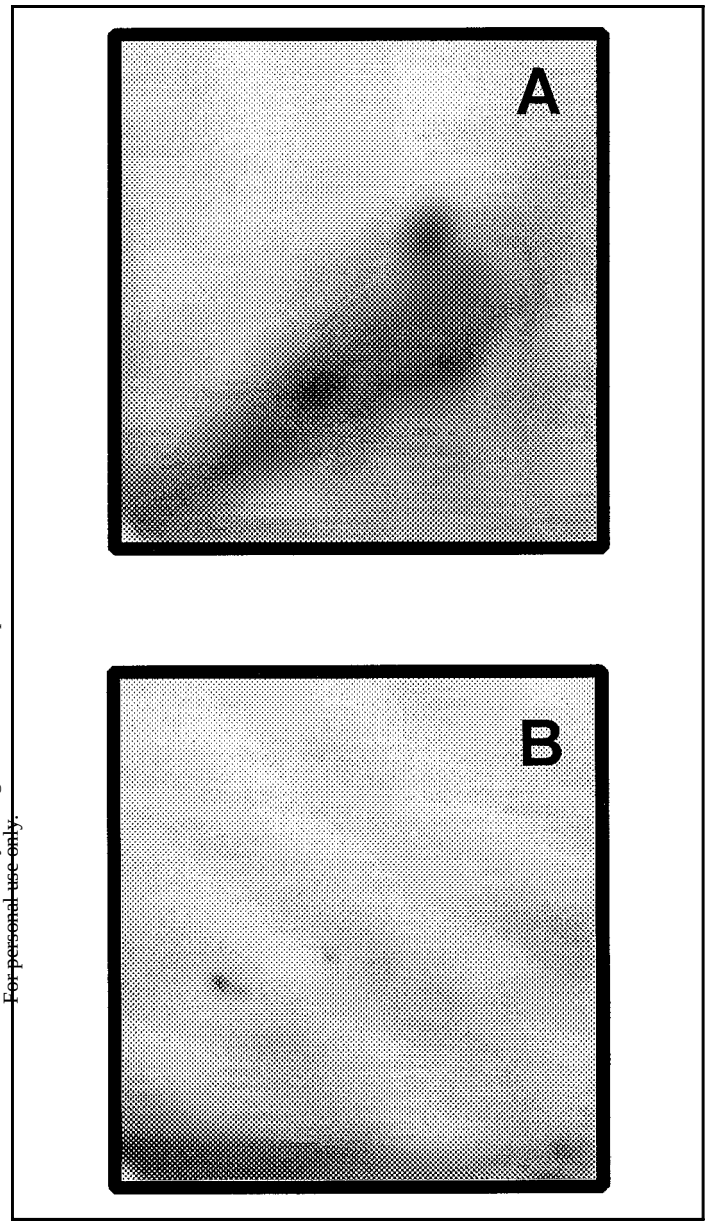

Figure 1. Typical autoradiographic representations of a ^{32}P-postlabelling analysis using multidirectional ion-exchange chromatography of DNA from the liver of a bream from the river Elbe (A) and a reference lake (B, Belauer See). Experimental details are described elsewhere (Pfau *et al.* 1994).

of this class of chemical carcinogens. However, DNA adducts derived from other classes of genotoxic chemicals such as unsaturated aldehydes, quinoid structures, polyhalogenated or heterocyclic compounds from both natural and industrial origin have been shown to be easily detectable by this enrichment procedure (Reddy and Randerath 1986, Gupta and Early 1988, Reddy *et al.* 1990).

Recent developments using high resolution chromatographic techniques (Pfau and Phillips 1991, Zeisig and Möller 1995) will improve the accuracy of identification of DNA adducts from field studies by means of chromatographic comparison with synthetic standard adducts. Using low resolution ion-exchange chromatography, adducts detected in fish liver DNA have been assigned to the major adduct formed by benzo[*a*]pyrene diol epoxide (van Schooten *et al.* 1995, Pfohl-Leszkowicz *et al.* 1996).

The development of dedicated protocols for mass spectrometric analysis of DNA adducts at a very high sensitivity will further this field in the near future. It will thus be possible to measure individual adducts and to identify adducts detected in feral fish, thus allowing retrospective characterization of the contaminant and possibly the polluter responsible.

Conclusions

It has been shown both in the laboratory and in field studies that the analysis of DNA adducts is a promising marker of exposure of fish to certain genotoxic pollutants and complex mixtures. With the exception of a few studies, there has generally been a correlation between pollution status and DNA adduct levels in both freshwater and marine fish. Adduct levels observed in hepatic DNA from fish caught in polluted rivers were higher (up to 56.6 adducts per 10^8 nucleotides) than those from marine fish (up to 18 adducts per 10^8 nucleotides). Compared with the determination of neoplasia in fish the earlier endpoints of DNA damage appear to be far more sensitive both in terms of progression of the disease and of contamination levels. Other endpoints of genotoxicity have been put forward, such as the determination of micronuclei or DNA strand breaks (Shugart *et al.* 1992). The latter has been shown to be confounded by factors such as water oxygen levels (Liepelt *et al.* 1995). Furthermore, these biomarkers of genotoxic effect lack the information about the contaminant that is obtained with DNA adduct studies. With the application of sophisticated methodology DNA adduct determination in feral fish serves as a biomarker of both exposure to genotoxicants and a marker of a very early genotoxic effect in the environment.

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