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BIOMARKERS OF POLYCYCLIC AROMATIC HYDROCARBON (PAH) EXPOSURE IN FISH AND THEIR APPLICATION IN MARINE MONITORING

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1. EXECUTIVE SUMMARY

While the direct measurement of organic pollutants, by chemical analysis can provide detailed information regarding the spatial distribution of contamination, it provides little indication of the biological impact of these compounds. Recent advances in the field of ecotoxicology have provided a number of molecular biomarkers that begin to allow a diagnostic approach to the assessment of effects. The integrated use of these biomarkers has been suggested by the International Council for the Exploration of the Sea (ICES) and the Oslo and Paris Commission (OSPAR) working groups as an effective means of determining the impact of pollution in the aquatic environment. Of the biomarkers currently available for use, the induction of hepatic EROD activity, production of bile metabolites and the formation of DNA adducts have shown potential for elucidating exposure of animals to polycyclic aromatic hydrocarbons (PAH). This study was commissioned to investigate:

- The use of this suite of diagnostic biomarkers as indicators of genotoxic pollution by PAH.
- To deploy them in surveys of estuarine, coastal and offshore biota, to establish the extent of PAH mediated effects in the marine environment.
- To investigate the links between these biomarkers, chemical analysis and indicators of gross effects such as disease.

The key findings were:

- The mixed function oxygenase (MFO) enzyme system, as measured by hepatic EROD activity levels in flounder is induced significantly above baseline levels in fish collected from a number of UK estuaries.
- In certain areas, such as the Tyne estuary, fish are displaying signs of chronic carcinogenic/genotoxin exposure as indicated by high DNA adduct occurrence.
- Coastal and offshore data have proved more difficult to interpret but clear biomarker 'hotspots', particularly off the north east coast and in Liverpool Bay, have been established.

Meaningful correlations with chemical analysis and PAH mediated disease have been difficult to elucidate. However, the study has highlighted the importance of PAH as a marine contaminant and demonstrated the value in the integrated use measurements of EROD, bile metabolites and DNA adducts as a suite of tools for investigating the extent of PAH mediated effects. Other influences on biomarker activity (seasonality, etc.) are discussed and recommendations for restrictions on assay use for monitoring and future work are made.

2. INTRODUCTION

The aquatic environment is a major sink for many potentially hazardous chemical pollutants, emitted from industrial and domestic sources (De Flora *et al*., 1991; Law *et al*., 1997; Woodhead *et al*., 1999). The presence of genotoxins (chemicals capable of interacting and modifying the genetic material of exposed organisms) in these discharges is well documented (Kirsch-Volders, 1984). Additional studies have identified one group of these chemicals (polycyclic aromatic hydrocarbons - PAHs) in both the sediment and water column (Law *et al*., 1997; Woodhead *et al*., 1999), and accumulated in the tissue of aquatic organisms (Kadhim, 1990). A considerable amount of indirect evidence suggests that such contamination can account for a number of adverse effects in exposed species, including increases in direct toxicity (Matthiessen *et al*., 1998), hepatic tumours (Myers *et al*., 1990) and reproductive impairments (Nagler and Cyr, 1997). This concern over the possible genotoxic contamination of the aquatic environment has lead to increased efforts to evaluate the relationship between pollutant concentration and subsequent deleterious effects in exposed organisms.

While the direct measurement of organic pollutants, by chemical analysis, can provide detailed information regarding the spatial distribution of contamination (Law *et al*., 1997; Woodhead *et al*., 1999), it provides little indication of the biological impact of these compounds. The recent advances in the field of ecotoxicology have provided a number of molecular 'biomarkers' (cellular and/or physiological parameters that signify exposure to, or damage incurred by, environmental pollutants) that allow the assessment of the impact of these genotoxic compounds upon exposed aquatic organisms. The integrated use of these biomarkers has been suggested as an effective means of determining the impact of pollution in the aquatic environment (McCarthy and Shugart, 1990). Of the 'biomarkers' currently available for use, the induction of EROD activity, production of bile metabolites and the formation of DNA adducts have shown great potential for identifying levels of exposure to PAHs following contamination of the aquatic environment (Livingstone *et al*., 1997; Burgeot *et al*., 1996; Harvey *et al*., 1997).

EROD activity

The mixed function oxygenase system (MFO) is an important pathway for metabolism of xenobiotics in aquatic vertebrates. The degree of induction of one of its components, the cytochrome P450s, can be readily assessed through the *in vitro* measurement of

7-ethoxyresorufin-O-deethylase (EROD) activity. The levels of these cytochrome P450s are known to be increased by the presence of environmentally significant xenobiotics, such as PAHs and PCBs. Due to this induction, the measurement of EROD activity has been used extensively as a biomarker of exposure following xenobiotic contamination of the aquatic environment (Stegeman *et al*., 1988; Burgeot *et al*., 1996). MFO metabolism is particularly important when dealing with PAH effects as its activity on this group of compounds can create the production of genotoxic intermediates which can damage DNA and lead to neoplasia.

1-hydroxy pyrene bile metabolites
It is not generally practical to measure PAHs in tissues of fish as they are rapidly metabolised and excreted in bile. However, previous studies have indicated the analysis of bile for the presence of PAHs and/or their metabolites to be a useful tool in aquatic biomonitoring (Britvic *et al*., 1993). The exposure of fish to PAHs was investigated by the biliary levels of 1-hydroxy pyrene, which has previously been demonstrated to be a major metabolite in the bile of fish exposed to PAH polluted sediments (Ariese *et al*., 1993).

^{32}P-postlabelling for the detection of DNA adducts
Previous studies have indicated that the levels of genotoxin-DNA complexes (adducts) in selected aquatic bioindicator species could be used as biomarkers of environmental contamination (Harvey *et al*., 1997; Lyons *et al*., 1997). DNA adducts are covalently bound addition products formed when electrophilic chemical species attack the nucleophilic sites in DNA. Extensive experimental data supports their role in the initiation of chemical carcinogenesis (Miller and Miller, 1981). Furthermore, the quantitative analysis of DNA adducts enables the determination of the biologically active levels of exposure to genotoxic chemicals, by taking into account physiological factors such as absorption, metabolism and detoxification involved in genotoxin adduct formation.

The detection of these biomarkers (EROD activity, bile metabolites and DNA adducts) was therefore used in this study as 'early warning' indicators of pollution-related genetic toxicology caused by PAHs. Laboratory calibration studies were also conducted (using flounder, *Platichthys flesus*) to demonstrate the potential for the PAH benzo[*a*]pyrene (a potent carcinogen present in crude oil) to induce the biomarkers used in this report.

3. METHODOLOGY

3.1 Laboratory calibration studies

Flounder used in the tank experiments were collected from the Alde estuary in Suffolk. The Alde was chosen as a clean location with low levels of industrial, agricultural or domestic inputs. The collection and testing were completed in November/December 1997 to minimise the potential of seasonal influences affecting the results obtained. Fish were kept in large aquaria and acclimated to laboratory conditions (15°C ±2, 31 ±2‰ salinity and 7.9 ±0.2 pH) before initiation of the test.

Flounder were exposed to 3 treatments via intraperitoneal (IP) injection using corn oil as the contaminant carrier. Treatments involved a single injection and consisted of two doses of the model PAH carcinogen benzo[*a*]pyrene (B[*a*]P) at levels of 1 and 20 mg kg^{-1} and one group of fish receiving the corn oil only. On days 0, 2, 4, 8, 14 and 21 post injection 4-5 fish were taken from each treatment. Liver samples were taken for the determination of EROD activity and the levels of DNA adducts. Bile samples were also taken for the determination of bile metabolites. The biomarkers were determined as described below and the results are discussed.

3.2 Sample collection from the field

Samples analysed for this contract were obtained from a series of 3 CEFAS offshore research cruises (*Cirolana* 6b/96 (July 1996), *Cirolana* 5b/97 (June 1997) and *Cirolana* 3b/98 (June 1998)) and a series of estuarine sampling locations from Environment Agency research vessels (September-December 1997).

Two fish species were targeted as test organisms: dab (*Limanda limanda*) for near and offshore sites and flounder (*Platichthys flesus*) in estuaries. Fish were caught in Granton trawls at sea and with 2 m beam trawls in estuaries.

Once on deck, target species were separated into tanks containing flowing seawater. Dissections were performed on deck within 1 hour of capture. Sections of liver were removed for EROD and adduct analysis. These were stored in cryovials in liquid nitrogen (~195ºC). Notes were also taken on fish condition, length, sex, gonad length and parasitism. Bile samples were taken when possible, but the gall bladders were frequently found to be empty. All fish over 15 cm were taken as samples.

EROD analysis was carried out for every site and these data were used to inform the more targeted DNA adduct analysis, which is a more complex and expensive procedure.

Samples of surface (0-2 cm depth) sediment were collected for PAH analysis from a 0.1 m^2 Day grab at coastal and offshore sites, or by means of a hand-held Van Veen grab from an inflatable boat when inshore. Samples were transferred to hexane-rinsed glass jars with aluminium foil inserts and kept frozen at -20ºC until analysis (Law *et al.,* 1988).

3.3 EROD activity determination

Liver samples were kept in liquid nitrogen until analysis approximately 3 months after collection. A 200 mg (±10) slice of liver was homogenised with 1 ml of ice cold homogenising buffer (50 mM TRIS pH 7.5, 1 mM EDTA, 1 mM dithiothrietol, 150 mM NaCl) using six strokes of a Potter-Elvehjem automatic homogeniser set at 4000 rpm. The homogenate was then transferred to a lidded polyethylene eppendorf tube and stored on ice. The homogenates were then centrifuged at 10,000 g for 20 minutes in a refrigerated unit at 4°C. After centrifugation homogenates were kept on ice and assayed within 30 minutes.

EROD analysis was performed using a modification of the method described in Stagg *et al*. (1995). A Perkin Elmer LS50B fluorometric spectrometer was used with a cuvette stirring function with settings of 535 nm excitation and 580 nm emission. All assay reagents were kept at 20°C (±1) in a water bath to control the assay temperature. The reaction mixture, final volume 2 ml, contained 1.96 ml assay buffer (100 mM pH 7.5 TRIS, 100 mM NaCl), 20 µl liver homogenate, 10 µl ethoxyresorufin substrate (0.4 mM in dimethyl sulphoxide, DMSO) and 10 µl of resorufin internal standard (25 µM in DMSO). The standard equates to an addition of 250 pM of resorufin against which the assay was calibrated. To ensure quality control on the substrate and standard a batch was made and then 1-2 ml aliquots were frozen and defrosted prior to use in the assay. The reaction was initiated by the addition of 10 µl NADPH (0.25 mM) and emission readings were recorded at 0, 15, 30, 45 and 60 seconds post addition.

EROD activity was normalised to protein content and expressed as pM resorufin/min/mg protein. Protein analyses were carried out on the same liver homogenate as the EROD analyses using a plate reader modification of the method described by Bradford (1976) with a bovine serum albumin standard.

3.4 Bile PAH metabolite analysis

Two methods were employed for the analysis of bile metabolites in this study. All samples of dab bile from environmental monitoring stations were analysed using an enzymatic method in conjunction with high performance liquid chromatography (HPLC). Briefly, 10-100 µl of raw bile was hydrolysed with β-glucuronidase (2-3 hrs at 37°C), which converts the conjugated bile metabolites into free 1-hydroxy pyrene (10 HP). This was then subsequently analysed by HPLC using fluorescence detection (flow rate 1.25 ml min^{-1}; excitation 346 nm; emission 383 nm). A second rapid method consisted of analysing raw bile using synchronous fluorescence spectrometry (SFS) for the glucuronide conjugate (Ariese *et al*., 1993). Samples of bile removed from specimens of flounder used in the laboratory calibration study were analysed by this method.

3.5 Genotoxin-DNA adduct formation

DNA was extracted from hepatic tissue of dab and plaice using a standard high salt method (Miller *et al.,* 1988). All samples were deemed essentially free from RNA and protein contamination and were subsequently adjusted to a final concentration of 1 µg DNA/µl water and stored at -70ºC prior to postlabelling. The DNA from 3 individuals was pooled into one sample for each postlabelling reaction. The pooling of tissue from each site was conducted on the basis of EROD activity.

Samples were analysed for DNA adducts using the ^{32}P-postlabelling assay as described previously (Harvey *et al*., 1997). Briefly, 10 µg samples of pooled DNA were digested to deoxyribonucleoside 3'-monophosphates in a total volume of 30 µl of digestion mix. 29 µl of this mix was then subjected to a butanol enhancement procedure (Gupta *et al*., 1982), evaporated to dryness and then redissolved in 9.5 µl of water. The remaining 1 µl of the DNA digest was diluted and held for labelling of the normal undamaged nucleotides for subsequent quantification (Jones *et al*., 1991). Adducted and normal nucleotides were labelled separately, but simultaneously, for 2 hours using 2 µl of labelling buffer (100 mM bicine, NaOH, pH 7.6, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine), 0.5 µl T4 polynucleotide kinase (10 u μl^{-1}; Boehringer Mannheim) and 8 µl and 2 µl of [γ ^{32}P] ATP (> 6000 Ci mol^{-1}, 10 µCi μl^{-1}; Amersham) respectively.

The adducted deoxyribonucleoside-3'-5'-biphosphates present in the sample were then purified and separated from their normal undamaged counterparts using multidimensional anion exchange thin layer chromatography (TLC), on polyethyleneimine (PEI)-cellulose plates (Machery Nagel) essentially as described previously (Jones *et al*., 1991). The levels of DNA adduct radioactivity were determined using an AMBIS Radioanalytical Scanning System (Lablogic). Upon quantification of both the adducted and normal nucleotides, the relative adduct labelling values were calculated and converted to number of adducted nucleotides per 10^8 undamaged nucleotides. Appropriate negative and positive controls were analysed throughout the studies as described by Harvey and Parry (1998).

3.6 Analysis of PAH in sediments

After thawing, wet sediments were well mixed and sub-samples of 1 to 15 g were placed in Teflon centrifuge tubes with anhydrous sodium sulphate and acetone. A known concentration of internal standard (anthracene-d_{10}) was added directly to the sample prior to extraction. Samples were extracted using a MSE Soniprep 150 for 15 minutes with the probe operated sequentially at maximum power for 55 s and then at zero power for 5 s.

Samples were then centrifuged and the acetone extract decanted, this was repeated twice further and the extracts combined and adjusted to 50 ml with acetone. An aliquot of the acetone extract was then filtered through a 0.5 µm filter (millex LCR4), to remove particulates, using a syringe into an autosampler vial which was refrigerated until analysis.

Analyses were conducted using a commercially available automated PAH analyser (Chrompack UK) based on HPLC with fluorescence detection (see Law *et al.,* 1997). Briefly, a 50 µl aliquot of the extract was transferred to a ChromSpher PI SPE column and rinsed with 50% methanol/water (v/v). PAH are strongly retained on the column and mono-aromatics and aliphatics are rinsed off. PAH are then desorbed from the column in back-flush mode by the analytical eluent (acetonitrile/water) and transferred to the analytical column (ChromSpher 5 PAH) via a guard column. The various PAH components to be quantified were separated by gradient elution and detected using ultra-violet fluorescence. Further technical detail of the technique is given in Woodhead *et al.,* 1999.

4. RESULTS

4.1 Laboratory calibration studies

4.1.1 EROD induction

The EROD induction levels resulting from the exposure of flounder to B[*a*]P and corn oil alone are displayed in Figure 1(b) and Table 1. The first observation is that the expected large induction did not occur with mean EROD induction levels never exceeding 100 pmol/min/mg protein), even in the high doses. Similar studies have demonstrated large EROD response to B[*a*]P exposure via intramuscular and dietary routes using acetone as the solvent carrier (Goksøyr *et al*., 1996). The study used intraperitoneal injection and corn oil, which is a routinely used method and has been shown to induce EROD readily in previous studies (Murphy and Gooch, 1997). Although the induction levels were relatively low the results do indicate elevated EROD levels from Day 8 occurring in specimens dosed with 20 mg kg^{-1} B[*a*]P. The elevated EROD levels were found to be statistically significant ($p<0.05$) for 20 mg kg^{-1} B[*a*]P on Day 14 only. This indicates a clear lag in the induction response in this experiment that was more prolonged than expected.

4.1.2 Detection of bile metabolites

The conjugated 1-hydroxypyrene-equivalent glucuronide concentrations measured in bile following exposure of flounder to B[*a*]P and corn oil alone are displayed in Figure 1(c) and Table 1. Treatment with 20 mg kg^{-1} B[*a*]P led to clear increases in the levels of conjugated glucuronide detected, with concentrations up to an order of magnitude higher than controls. Treatment

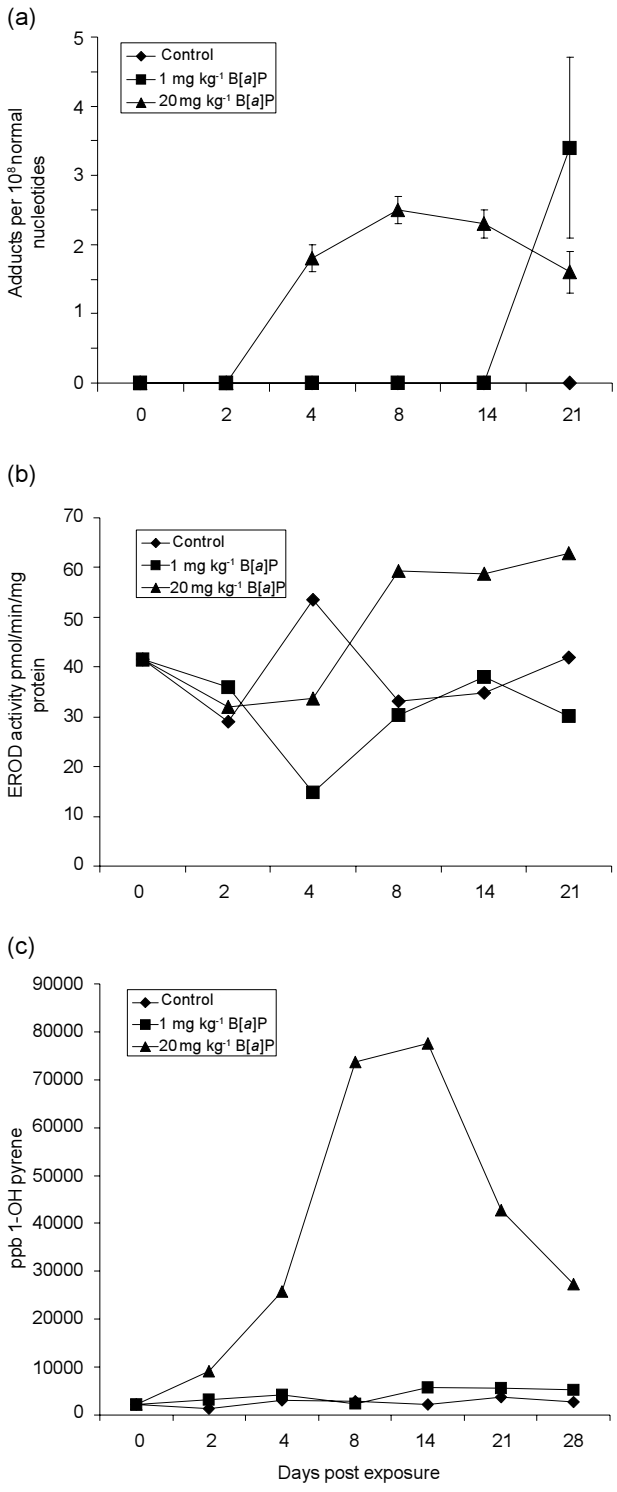

Figure 1. (a) Levels of DNA adducts following ^{32}P-postlabelling of hepatic DNA from flounder, (Platichthys flesus) exposed to corn oil (control), 20 mg kg^{-1} and 1 mg kg^{-1} benzo[a]pyrene (B[a]P) by a single IP injection. (b) Quantitative levels of hepatic EROD activity (pmol/min/mg protein) following IP injection of corn oil (control), 1 mg kg^{-1} benzo[a]pyrene and 20 mg kg^{-1} benzo[a]pyrene. (c) Quantitative levels of bile metabolites (ppb 1-OH pyrene equivalents) following IP injection of corn oil (control), 1 mg kg^{-1} benzo[a]pyrene and 20 mg kg^{-1} benzo[a]pyrene

Table 1. Levels of hepatic DNA adducts, hepatic EROD activity and bile metabolites for a time course study, flounder (Platichthys flesus) exposed to 1 mg kg^{-1} and 20 mg kg^{-1} benzo[a]pyrene (B[a]P)

Exposure group	Levels of DNA adducts per 10^8 nucleotides	EROD activity (pmol/mg/min protein)	Bile metabolites (ppb 1-OH pyrene equivalents)
Untreated control	0.0	41.6 ± 4.5[b]	2266.4 ± 450.2[b]
Corn oil Control			
D2	0.0	29.0 ± 10.5	1336 ± 340.0
D4	0.0	53.5 ± 14.6	2980 ± 566.4
D8	0.0	33.3 ± 4.7	2848 ± 710.4
D14	0.0	34.9 ± 2.8	2160 ± 246.2
D21	0.0	41.9 ± 10.2	3756.2 ± 885.9
1 mg kg^{-1} B[*a*]P			
D2	0.0	36.0 ± 6.6	3173.2 ± 408
D4	0.0	14.8 ± 12.0	4219 ± 600.9
D8	0.0	30.3 ± 5.8	2405 ± 1481
D14	0.0	38.1 ± 7.3	5722.2 ± 1329.7
D21	3.4 ± 1.3[a]	30.2 ± 7.3	5685 ± 1750
20 mg kg^{-1} B[*a*]P			
D2	0.0	32.0 ± 3.9	9140.2 ± 1948
D4	1.8 ± 0.2	33.7 ± 5.0	25821.4 ± 7456.1
D8	2.5 ± 0.2	59.4 ± 11.0	73757.8 ± 13563.5
D14	2.3 ± 0.2	58.8 ± 7.8	77678.4 ± 36086.1
D21	1.6 ± 0.3	62.8 ± 9.7	42726 ± 24005.4

[a] *(M±SE, n = 2). 4-5 fish pooled per sample.*
[b] *M±SE n = 4-5.*

with 1 mg kg^{-1} B[*a*]P was less conclusive. Statistical analysis of the mean level of conjugated glucuronide detected revealed that significantly higher levels of conjugate were only detected in the 1 mg kg^{-1} treated samples on Day 2 and Day 14. A wide range of PAH compounds have previously been detected in fish food and corn oil, and so it is likely that the relatively low levels of PAH metabolites found in the control specimens may have been due to the presence of PAH in their food or in the corn oil carrier (C. Kelly, pers. comm.).

4.1.3 Detection of DNA adducts

The results obtained following the exposure of *P. flesus* to B[*a*]P and corn oil alone are displayed in Figure 1(a) and Table 1. Treatment with 20 mg kg^{-1} B[*a*]P induced a single adduct per 10^8 nucloetides, which was detectable after 4 days and persisted through until Day 21 (see Figure 2(a-c) and Annex 1). Administration of a single IP injection of 1 mg kg^{-1} B[*a*]P also induced a qualitatively similar adduct in hepatic tissue of *P. flesus*. Though the adduct was only

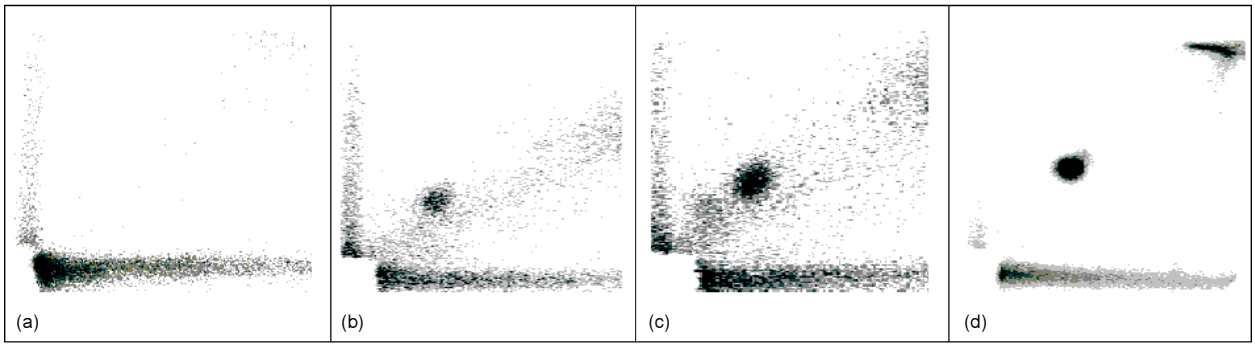

Figure 2. Typical DNA adduct profiles produced following the ^{32}P-postlabelling of hepatic DNA from:
(a) P. flesus administered corn oil only via I.P. injection,
(b) P. flesus administered 1 mg kg^{-1} B[a]P in corn oil via I.P. injection,
(c) P. flesus administered 20 mg kg^{-1} B[a]P in corn oil via I.P. injection,
(d) Positive control consisting of P. flesus hepatic DNA treated in vitro with 1.5 mM B[a]P diol epoxide

detectable 21 days after treatment. This lag in the formation of DNA adducts has been documented in previous studies using aquatic species (Shugart *et al*., 1990), and is thought to reflect the slow absorption of the genotoxin and its biotransformation into DNA reactive metabolites.

4.1.4 Calibration study discussion

The tank calibration studies clearly showed that a representative PAH, benzo[*a*]pyrene, has the capacity to induce EROD activity, bile metabolites and DNA adducts in flounder. The three biomarkers showed broad agreement in terms of peak response timing and level. Whilst adducts and bile metabolites were clearly induced after a short lag period the relatively low induction of EROD activity was unexpected. Similar studies have demonstrated large EROD response to B[*a*]P exposure via intramuscular and dietary routes using acetone as the solvent carrier (Goksøyr *et al.,* 1996). The study used intraperitoneal injection and corn oil which is a routinely used method and has been shown to induce EROD readily in previous studies (Murphy and Gooch, 1997). Although the induction levels were relatively low, the results do indicate elevated EROD levels occurring in specimens dosed with 20 mg kg^{-1} B[*a*]P from Day 8. The elevated EROD levels were found to be statistically significant ($p<0.05$) for 20 mg kg^{-1} B[*a*]P on Day 14 only. This indicates a clear lag in the induction response in this experiment that appeared to be more prolonged than expected. Nevertheless a clear inter-biomarker link was established as a result of PAH exposure.

4.2 EROD induction in wild dab (1996-1998)

4.2.1 Results

It is known that EROD activity in flatfish varies temporally on a seasonal basis connected to the spawning season. The results from summer cruises also suggest that EROD activity levels in dab (*Limanda limanda*) vary spatially around the UK.

The results from *Cirolana* 6b/96 (July 1996) are shown in Figure 3 and Table 2. These reveal an unusually high result (2549 pmol/min/mg protein) in those dab taken 30 miles off Milford Haven. It is tempting to suggest that this was a residual effect of the *Sea Empress* oil spill but this was not borne out by other observations from nearer the spill (Kirby *et al.,*1999). This result appears to be more associated with the fact that these fish had by far the smallest gonado-somatic index (GSI). It is known that EROD activity in gonadally mature females is significantly lower than in immature or spent females

Table 2. Hepatic EROD Activity in dab - 3 year trend

Site	Mean hepatic EROD (pmol/min/mg protein)								
	Cirolana 6b/96 (July)			Cirolana 5b/97 (June)			Cirolana 3b/98 (June)		
	n	Mean	SD	n	Mean	SD	n	Mean	SD
Morecambe Bay	20	1040	606	20	2083	1139	19	1176	841
Liverpool Bay	20	983	707	20	1782	858	20	1456	955
Burbo Bight	20	1146	960	20	1397	789	-	-	-
S.E. Isle of Man	20	601	368	20	869	837	-	-	-
Red Wharf Bay	20	1156	907	20	757	599	20	602	389
Dundrum Bay	-	-	-	16	1286	603	20	880	578
Inner Cardigan Bay	-	-	-	20	792	470	21	520	467
Outer Cardigan Bay	12	864	479	20	808	587	-	-	-
30m SW of Milford	20	2549	1400	-	-	-	-	-	-
St. Brides Bay	20	1035	619	-	-	-	-	-	-
Carmarthen Bay - East	22	1655	1156	20	691	663	-	-	-
Carmarthen Bay - Mid	6	1429	948	20	1081	945	-	-	-
Inner Carmarthen Bay	24	1604	1046	20	1323	813	-	-	-
Carmarthen Bay - West	20	1592	739	20	993	543	-	-	-
Celtic Deep	-	-	-	20	562	287	-	-	-
Lyme Bay	-	-	-	-	-	-	20	245	165
Rye Bay	-	-	-	-	-	-	20	385	251
Off Tyne/Amble	20	1828	1167	20	1520	1350	20	1400	789
Off Tees	20	1424	820	20	1454	710	-	-	-
Off Flamborough	-	-	-	-	-	-	20	1569	752
Off Humber	20	449	234	20	580	335	20	998	685
The Wash	10	830	653	-	-	-	20	218	259
Dogger Bank	20	1343	902	20	668	479	20	1261	1125
West Dogger Bank	-	-	-	-	-	-	20	873	569
Bremerhaven 1	-	-	-	-	-	-	20	1010	870
Bremerhaven 7	-	-	-	-	-	-	20	1135	1007

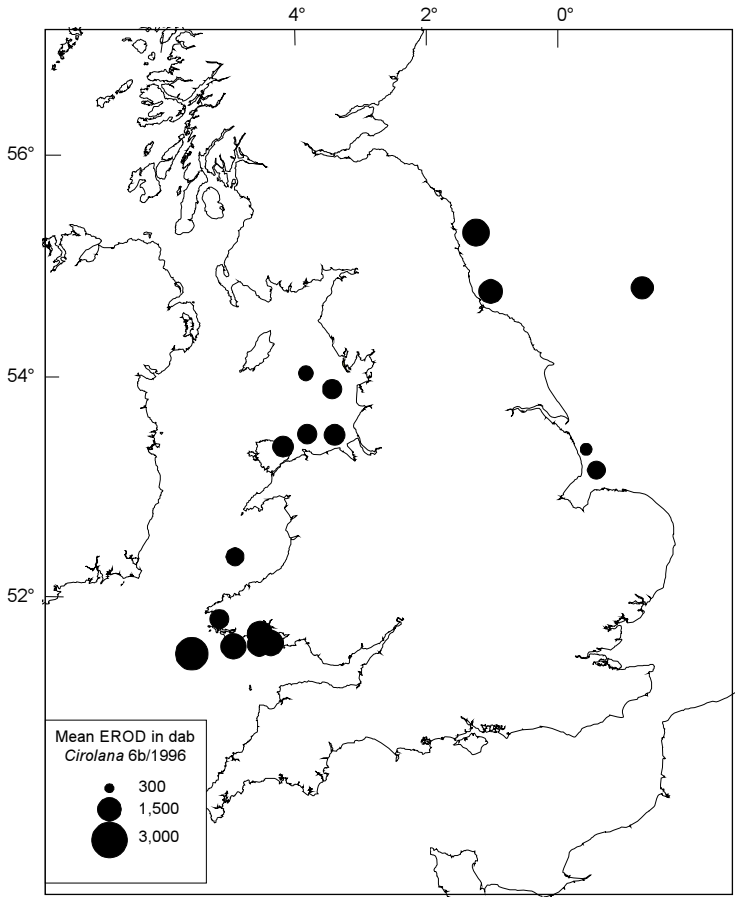

Figure 3. Hepatic EROD (pM/min/mg protein) activity in dab from Cirolana 6b/96 (July)

(Elskus *et al.,* 1992) in winter flounder and that this phenomenon is linked to the elevated levels of the sex hormone oestradiol and its ability to act as a P450/ EROD suppressant. However, the high EROD activity levels in those fish sampled off the Tyne and Tees tended to coincide with relatively large GSI figures. It is clear that the interactions between the MFO system and circulating sex hormones requires further elucidation. These data have proved difficult to interpret and the fact that there was a strong correlation of female GSI with latitude (0.83) would suggest that spatially disparate populations can be at different stages in their reproductive cycle. It appears advisable to compare fish which are at the same stage of their reproductive cycle.

The results from the *Cirolana* 5b/97 (June 1997) cruise are shown in Figure 4 and Table 2. Contrastingly, these appeared to offer a relatively clear pattern of EROD induction. Of the 13 stations sampled there was a clear split between those showing relatively high levels of induction (>1280 pmol/min/mg protein) and those with relatively low levels (< 870 pmol/min/mg protein). The interpretation is slightly confused by the fact that female GSI and EROD induction are quite well correlated in fish on the east of the country whereas those on the west show no such relationship. In general, these correlations are not apparent across all sites, but those fish showing high EROD levels were all taken in the vicinities of contaminated estuaries and those showing low levels were from offshore sites or relatively uncontaminated bays.

The results from the *Cirolana* 3b/98 (June/July 1998) cruise are shown in Figure 5 and Table 2. Once again the induction shows the pattern observed in previous years with all sites off the north east coast and in Liverpool Bay showing levels of hepatic EROD activity in excess of 1000 pmol/min/mg protein Fish collected from sites in the English Channel, Cardigan Bay and Wash area all showed low levels of EROD induction.

4.2.2 Discussion

The cruise data have been very difficult to interpret and suggests that much more needs to be known about the environmental influences on MFO activity before the monitoring data can be more confidentally used to interpret biological effects of contaminants. However, general, but clear, patterns of induction have emerged over the three annual datasets. Sites in Liverpool Bay and off the north east coast routinely have significantly elevated EROD activity whilst those in areas such as Cardigan Bay have been consistently low. These general trends were very important in targeting areas for the analysis of genetic damage by means of DNA adducts.

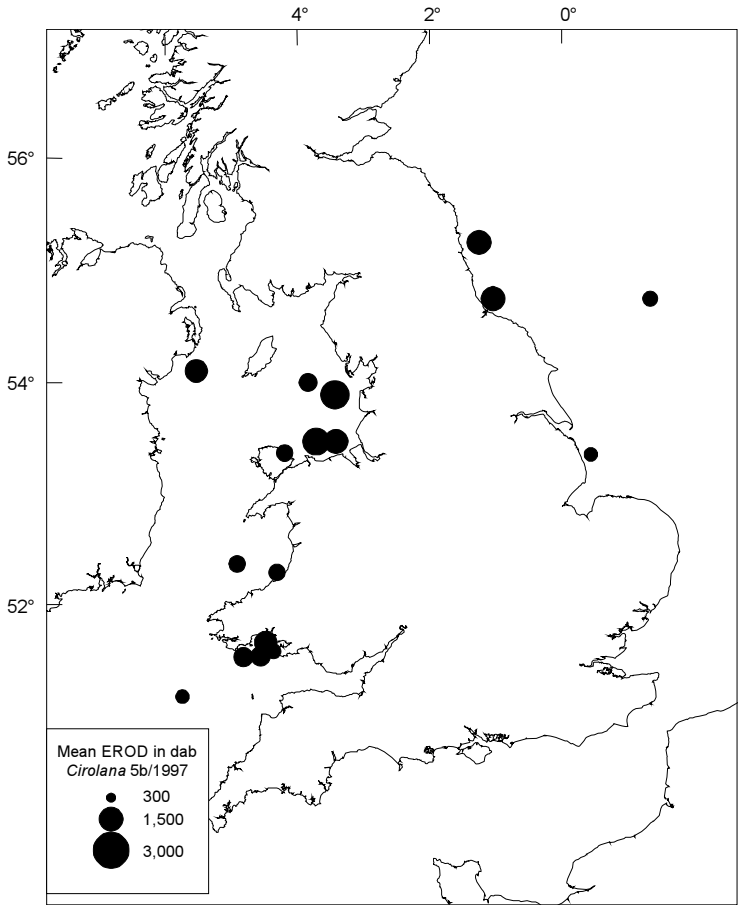

Figure 4. Hepatic EROD (pmol/min/mg protein) activity in dab from Cirolana 5b/97 (June)

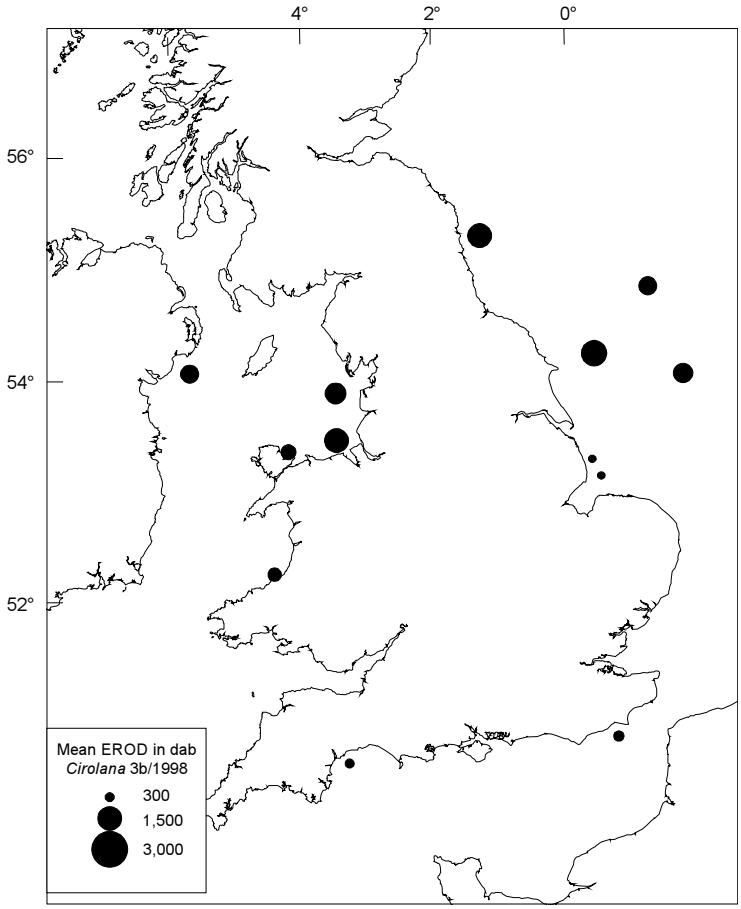

Figure 5. Hepatic EROD (pmol/min/mg protein) activity in dab from Cirolana 3b/98 (June)

4.3 DNA adducts in wild dab (1996)

4.3.1 Results

The mean hepatic DNA adduct levels for dab sampled during the *Cirolana* 6b/96 (June 1996) research cruise are displayed in Table 3 and Figure 6. No DNA adducts were observed in pooled samples collected from outer Cardigan Bay during 1996, indicating that this location was indeed relatively free of anthropogenic pollution and therefore suitable as a control site (see Figure 7(a) - also see Annex 1). PAH data from this site supports the lack of DNA adduct detection, with ∑PAH values for the sediment documented at the low value of 26 μg kg^{-1} dry weight (Woodhead *et al*., 1999). The analysis of hepatic DNA adducts in pooled specimens of dab collected from other UK offshore sites (off Milford Haven 26.8 ±2.0; Liverpool Bay 16.2 ±9.4; off Tyne 4.0 ±2.6; off Humber 7.6 ±1.0; Dogger Bank 5.6 ±2.3) all contained some samples displaying a detectable level of adducts (see Table 3 and Figure 6). In the majority of cases the DNA adduct profiles detected consisted of diagonal radioactive zones (DRZs) of genotoxin-DNA adducts (see Figure 7(b-c) - also see Annex 1).

4.3.2 Discussion

The samples obtained from off Milford Haven in 1996 all contained elevated DNA adduct levels, with profiles consisting of DRZs, indicating exposure to complex mixtures of PAH (see Figure 7(b)). It is considered that the detection of DNA adducts in these samples was as a direct consequence of the *Sea Empress* oil spill, which occurred off the West Wales coast in February of that year. Surprisingly, no elevations in DNA adduct levels were detected in fish from two other sites sampled from around the *Sea Empress* affected area, mid and west Carmarthen Bay (Harvey *et al*., 1999). Indeed the levels of adducts detected at these two sites (mid Carmarthen Bay 1.2 ±0.4 and west Carmarthen Bay 0.0 ±0.0 adducts per 10^8 nucleotides) are similar to levels detected at outer Cardigan Bay and are representative of levels detected in dab collected from uncontaminated sites during a previous study (Harvey *et al*., 1997). This inconsistency in the detection of genotoxin induced DNA adducts from the *Sea Empress* affected area is thought to be due to factors such as the influence of fish migration and the patchy distribution of hydrocarbon contamination following the oil spill.

Table 3. Levels of hepatic DNA adducts (number of adducted nucleotides per 10^8 normal nucleotides) detected in pooled L. limanda samples collected from British Coastal waters in July 1996

Sample Site	Position	July-1996[d]	∑PAH[e]
Outer Cardigan Bay	52°23.34N 04°53.50W	0.0 ± 0.0[a] (4)[b]	26
Mid Carmarthen Bay	51°33.32N 04°32.50W	1.2 ± 0.4 (4) [0.0-2.0][c]	N. A.[f]
West Carmarthen Bay	51°23.05N 04°51.55W	0.0 ± 0.0 (4)	N. A.
Offshore Milford Haven	51°29.77N 04°51.55W	26.8 ± 2.0 (4) [24.3-32.7]	N.A
Liverpool Bay (Burbo Bight)	53°28.29N 03°15.58W	16.2 ± 9.4 (4) [0.0-33.8]	714 – 1091
Off Tyne	55°00.50N 01°08.00W	4.0 ± 2.6 (4) [0.0-11.6]	1495
Off Humber	54°00.00N 02°00.00E	7.6 ± 1.0 (4) [5.2-9.5]	132 – 212
Dogger Bank	54°48.28N 01°15.84 E	5.6 ± 2.3 (4) [0.78-10.0]	N. A.

[a] *Mean adduct levels ± SE.*
[b] *Numbers in parentheses represent number of pooled samples analysed (3 fish per pooled sampled).*
[c] *Numbers in brackets represent range of DNA adduct levels detected.*
[d] *Sample date.*
[e] *Concentrations of ∑PAH (15) in surface sediments μg kg^{-1} dry weight (Woodhead et al., 1999).*
[f] *N. A. not available.*

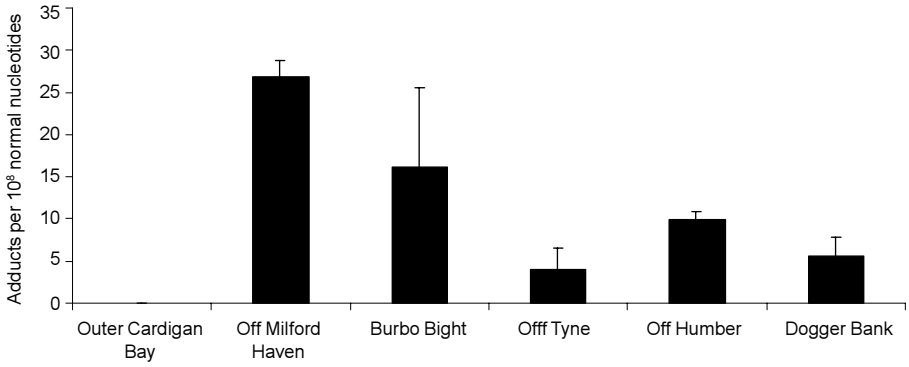

Figure 6. Bar chart showing the levels of DNA adducts (adducted nucleotides per 10^8 normal nucleotides) detected in hepatic L. limanda samples collected from sites around UK coastal waters (Cirolana 6b/96 - July 1996). Values are expressed as mean ± SE, n = 4, pools of three fish

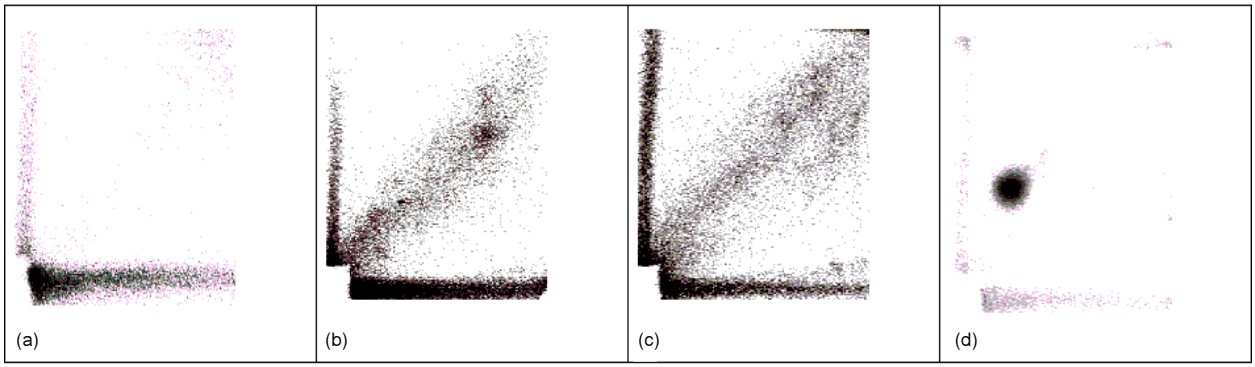

Figure 7. Typical DNA adduct profiles obtained from ^{32}P-postlabelling:
(a) L. limanda displaying no DNA adducts from a pooled sample of hepatic DNA from individuals collected from Outer Cardigan Bay,
(b) Diagonal radioactive zone (DRZ) of radiolabelled L. limanda hepatic DNA adducts from a pooled sample from off Milford Haven. The detection of a DRZ indicates the presence of a number of overlapping bulky aromatic adducts, characteristic of PAH exposure,
(c) Representative DNA adduct profile from a pooled sample of L. limanda hepatic DNA from individuals collected from Burbo Bight, again a DRZ is detectable, indicating exposure to a complex mixture of genotoxins,
(d) Positive control consisting of L. limanda hepatic DNA treated in vivo with 1.5 mM B[a]P diol epoxide

Samples of dab collected from Liverpool Bay, off Humber, Dogger Bank and off Tyne all displayed DNA adduct profiles characteristic of PAH exposure (Table 3 and Figure 7(c)). In the majority of cases the DNA adduct profiles detected consisted of diagonal radioactive zones (DRZs) of genotoxin-DNA adducts (see Figure 7(b-c)). While such DNA adduct profiles are characteristic of exposure to complex mixtures of genotoxins it is difficult to relate the levels of adducts to specific groups of environmental contaminants. However, the DRZs detected in this report share similarities in chromatographic characteristics with adduct profiles detected in wild fish collected from PAH contaminated environments. Significantly, identical adduct profiles have been shown to be induced following exposure of English sole (*Pleuronectes vetulus*) and winter flounder (*Pseudopleuronectes americanus*) to PAH contaminated sediment (Varanasi *et al*., 1989; French *et al*., 1996). Indeed the laboratory calibration studies detailed in this report demonstrate that exposure to the PAH, B[*a*]P, results in the detection of DNA adducts migrating to positions on the chromatographic plate as occupied by the DRZs (see Figure 2(b-c) and Figure 7(b-c)).

Chemical analysis of sediments at these sites (except Dogger Bank where no data was available) indicate that the ∑PAH levels are elevated with respect to the control site, Outer Cardigan Bay. However, the ranking of sites (see Table 3) based on ∑PAH data does not correspond to a ranking based on DNA adduct levels (∑PAH = Off Tyne > Liverpool Bay > Off Humber > Outer Cardigan Bay; DNA adduct levels = Liverpool Bay > Off Humber > Off Tyne > Outer Cardigan Bay). Such results indicate the problems encountered when trying to extrapolate from the presence of contaminants at a particular location to detectable biological responses in exposed organisms, and as such reflect the plethora of factors influencing contamination effect. For instance, dab are known to be highly migratory and can move considerable distances between various areas and hence no simple relationship exists between the levels of DNA adducts in such a species and the levels of contamination at their site of capture.

4.4 DNA adducts and bile metabolites in wild dab (1998)

4.4.1 Results

Following the detection of DNA adducts in dab liver at certain near shore sites in 1996 (see Section 4.3) dab were again collected on the *Cirolana* 3b/98 (July 1998) research cruise. The mean hepatic DNA adduct levels for selected sites for this cruise are displayed in Table 4. While DNA adducts representative of exposure were detected at Liverpool Bay those samples from Off Tyne were devoid of detectable levels DNA adducts. Again levels of DNA adducts were undetectable at the Outer Cardigan Bay site, consistent with previous findings (see Section 4.3). The observation of elevated levels of bile metabolites in fish from Liverpool Bay (315.9 ±16.2) supports the hypothesis that PAH exposure was responsible for the formation of DNA adducts (see Table 4). However, although bile metabolite analysis of dab sampled from Off Tyne (199.3 ±17.3) in 1998 indicated that levels were similar to those from Cardigan Bay (186.3 ±16.2) (Table 4).

4.4.2 Discussion

Previous studies of DNA adduct prevalence in dab from Liverpool Bay (Lyons, 1998) demonstrated that temporal variations in DNA adduct levels can be detected when sampling over successive years from

Table 4. Levels of pooled hepatic DNA adducts (number of adducted nucleotides per 10^8 normal nucleotides) and conjugated bile metabolites (ppb 1-OH pyrene equivalents) detected in pooled L. limanda samples collected from around the British Isles during the Cirolana 3b/1998 (June 1998) cruise

Sample Site	Position	DNA adducts	Bile metabolites[d]
Outer Cardigan Bay	52°23.34N 04°53.50W	0.0 ± 0.0[a] (4)[b]	186.3 ± 16.2 (18)
Liverpool Bay (Burbo Bight)	53°28.29N 03°15.58W	5.6 ± 2.2 (4) [0.0-9.5][c]	315.9 ± 23.9 (11)
Off Tyne	55°00.50N 01°08.00W	0.0 ± 0.0 (4)	199.3 ± 17.3 (5)

[a] *Mean adduct levels ± SE.*

[b] *Numbers in parentheses represent number of pooled samples analysed (3 fish per pooled sampled).*

[c] *Numbers in brackets represent range of DNA adduct levels detected.*

[d] *SFS measurements of conjugated glucoronides, expressed as ppb 1-OH pyrene equivalents.*

Table 5. Mean EROD and other variables in flounder from English estuaries caught in September-December 1997

Location	Samples (n)	EROD (pmol/min/mg protein)	Length (cm)	Weight (g)	GSI Female	GSI Male	HSI	CF
Alde (Reference)								
North of Orford	24	38.7	25.4	156.8	0.72	0.76	1.03	0.95
Mersey								
Speke	16	117.9 *	25.0	185.3	0.82	-	1.35	0.96
Bromborough	11	161.6 *	28.1	238.2 *	0.98	-	1.58 *	0.98
Tees								
Tees Dock	4	99.8 *	23.9	171.4	1.47	0.17	1.77 *	1.26 *
Redcar Terminal	4	95.6	22.0	125.3	1.04	0.08	1.66 *	1.17 *
Humber								
Whitton	3	17.4	22.4	106.0	1.40	-	1.27	0.94
Foul Holme Channel	9	77.0	21.9	117.8	1.11	-	1.79 *	0.98
Spurn Head	13	84.6 *	25.4	181.6	1.27	0.86	0.92	1.07 *
Southampton Water								
Bird Pile Beacon	12	62.1	27.2	218.5	2.52 *	1.36	1.42 *	0.95
Wear								
Wearmouth Bridge	4	84.6	20.4	99.6	0.69	0.18	1.44	1.08 *
Tyne								
Scotswood Bridge	26	70.4 *	29.7 *	288.1 *	2.36 *	1.35	1.70 *	1.05 *
Redheugh	41	90.0 *	27.2	237.2 *	1.72 *	1.37	1.78 *	1.05 *
Newcastle	22	95.8 *	25.4	183.7	1.53	0.56	1.68 *	1.02
Hebburn	28	54.3	24.2	176.3	1.94 *	0.80	1.59 *	1.08 *
Jarrow	30	94.8 *	20.9 *	105.5	1.51	0.16	1.74 *	1.04 *
Lloyds Hailing Station	3	57.6	28.2	271.6	2.54	0.27	2.03 *	1.19 *

** Significantly different ($p<0.05$) from the Alde reference site*

the same site. The lack of detectable DNA adducts in dab collected from Off Tyne in 1998, is again thought to reflect the difficulties of using a migratory species in biomonitoring studies (implications of which are discussed further in Section 5). The DNA adduct and bile metabolite data appears to reflect the presence of elevated levels of PAH in the sediment from Liverpool Bay, in samples collected in 1998. This is in contrast to the DNA adduct, bile metabolite and sediment PAH data from Off Tyne and Outer Cardigan Bay in 1998. No differences could be seen in the levels of DNA adducts and bile metabolites from dab sampled from these two sites. However, the sediment PAH data for Off Tyne ($\sum$PAH = 1495 µg kg^{-1} dry weight) was significantly elevated over Cardigan Bay ($\sum$PAH = 26 µg kg^{-1} dry weight) collected in 1996. This discrepancy in biomarker data again highlights the problems associated with linking biomarker responses in a migratory species to point sources of contaminants and is thought to reflect the time prior to capture that populations have resided in a polluted area.

4.5 EROD activity in flounder from English estuaries (September-December 1997)

4.5.1 Results

Mean EROD activities from each estuarine site are displayed in Table 5. Significantly ($p<0.05$) elevated activity, compared to the Alde reference site, was evident in nine out of the 16 stations sampled. The sites from the Mersey and Tees showed the greatest induction, reaching a maximum of 161.6 pmol/min/mg protein, a four-fold increase in induction over the reference level, at the Bromborough site in the Mersey. The span of EROD activities from all the sites sampled is represented in Figure 8.

The mean length, weight, gonado-somatic index (GSI), hepato-somatic index (HSI) and condition factor (CF) for each site are shown in Table 5. The range of each of these variables is also shown in Figure 9. There was a wide span of mean length (20-30 cm) in the data set but only two sites produced fish that were significantly ($p<0.05$) different from the Alde, the Scotswood Bridge and Jarrow fish on the Tyne which were longer and shorter respectively. This broad size range was generally mirrored in the mean weights with no clear pattern of size distribution between or within estuaries.

The mean GSIs for each site were calculated individually for male and female specimens. Female fish from the reference site demonstrated relatively low GSIs whilst those from the other estuaries were generally higher, some significantly so (Southampton Water and Tyne). Notably, the female fish from the Mersey had GSIs similar to those of the reference site. GSI in males from the reference site were higher than from the Tees, Wear and lower Tyne, though not significantly so, but lower than in the Humber, Southampton Water and the upper Tyne.

The mean HSIs were significantly elevated compared to the Alde reference site at all but two of the Humber sites and the Wear site, with particularly high levels apparent in the Tees and Tyne estuaries.

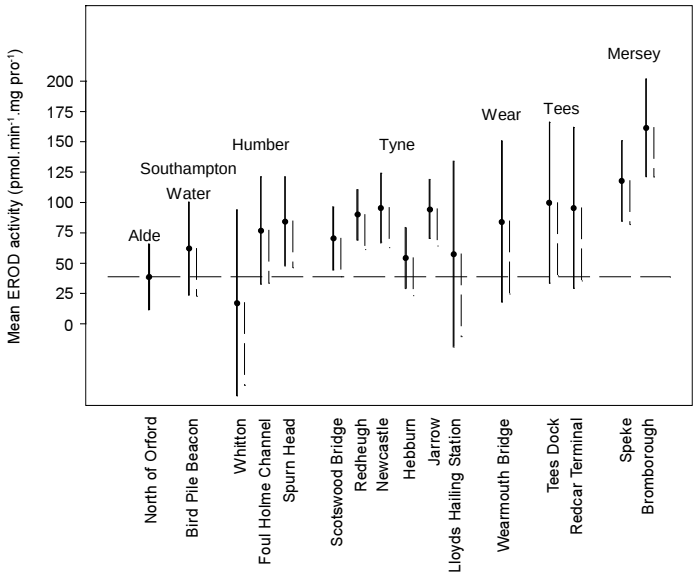

Figure 8. Mean hepatic EROD activity in flounder from English estuarine sites (• is the mean EROD level; —— the 95% confidence interval for mean; horizontal --- the mean EROD level at reference (Alde) site; vertical --- the graphical representation of one-sided t-test against reference site)

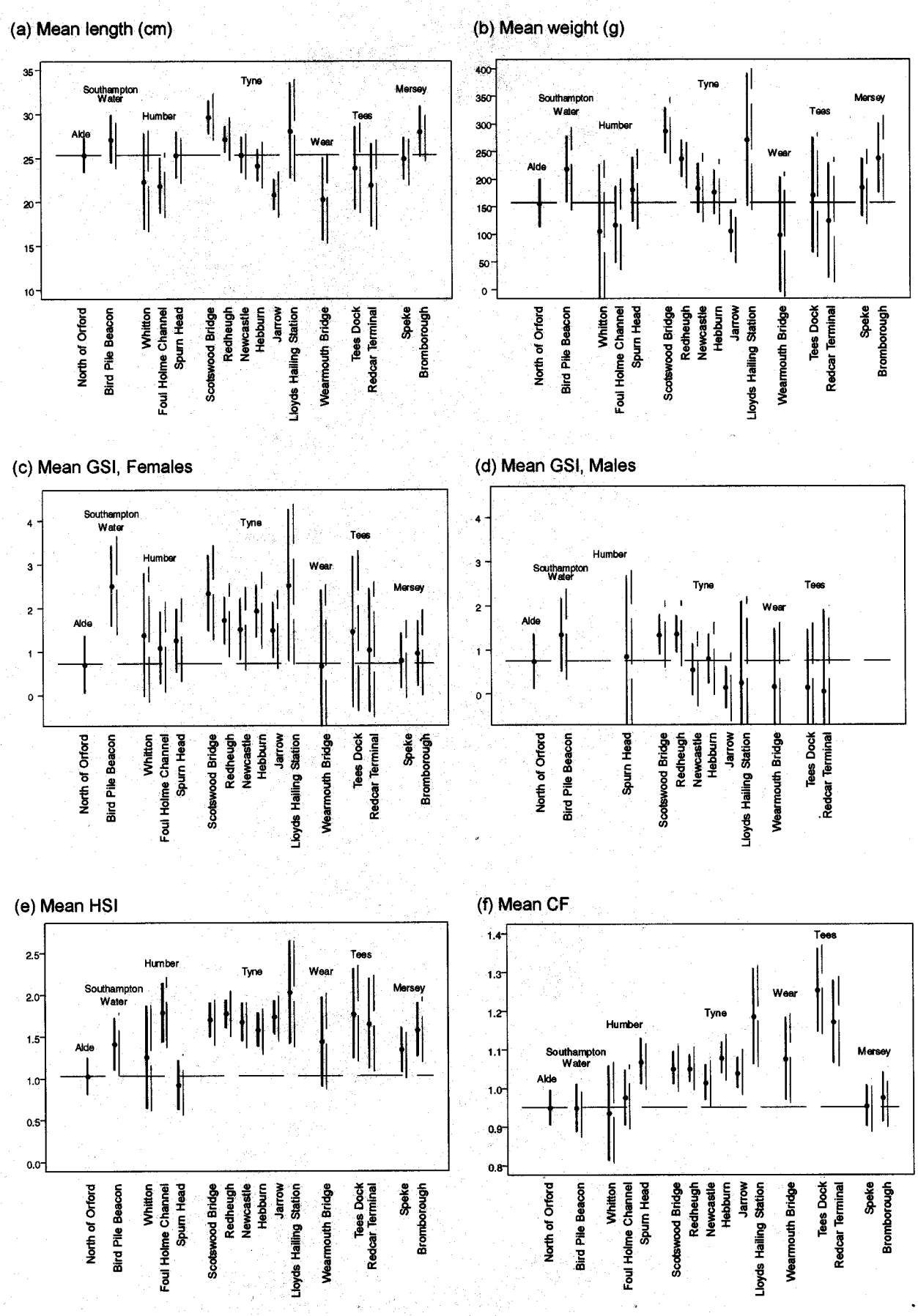

Figure 9. Mean values for other measured variables (length, weight, GSI, HSI, CF) in estuarine caught flounder (• is the mean level; —— the 95% confidence interval for mean; horizontal --- the mean level at reference (Alde) site; vertical --- the graphical representation of one-sided t-test against reference site)

Mean CFs were calculated for each station as an indication of the nutritional state/health of the animals. Significantly elevated CFs were apparent at all of the Tyne sites, in the Tees, the Wear and at Spurn Head in the outer Humber. The fish from the Mersey, Southampton Water and the remaining Humber sites had mean CFs comparable to the reference site.

Table 6 shows inter-gender comparisons within each site for a range of mean measurements. This analysis could only be done for samples from 6 sites, in addition to the reference, as these were the only sites from which sufficient numbers of males and females were captured. When mean values of various variables for each sex are compared to the reference site, in general, they closely mirror the results gained for the pooled gender data. No significant inter-gender differences were demonstrated for any variable at any site suggesting that sex was not a significant factor in intra- or inter-site differences.

Linear correlation coefficients were calculated between mean EROD activity and mean length, weight, GSI, HSI and CF for pooled gender data, males and females across all sampled sites. Also, due to the extensive range of sites sampled on the Tyne a comparable intra-estuary correlation analysis for EROD against the same variables from this estuary was carried out. These analyses are summarised in Table 7.

When all sites are included in the correlation analysis only weak correlations can be demonstrated between mean EROD activity and any other measured variable for pooled or single gender datasets. However, when the correlation analysis is performed on the dataset from the Tyne sites alone, some stronger relationships begin to emerge, which are more apparent in the females than in the males. In particular female mean EROD activity is quite strongly negatively correlated to both GSI ($r = -0.84$) and CF ($r = -0.80$).

Total PAH and PCB concentrations in flounder livers from each of the four estuaries are shown in Table 8. The reported figures are means of all the pooled samples and therefore represent an integrated value for the estuary.

4.5.2 Discussion

It is clear from this survey that the mixed function oxygenase (MFO) enzyme system is significantly induced in flounder captured in a number of English estuaries. These results presumably reflect the anthropogenic contamination of estuaries such as the Mersey, Tees and Tyne, and suggest that the flounder (and probably other fish) populations in these areas are subject to sub-lethal contaminant stress. The hepatic concentrations of PAH and PCBs (Table 8) suggest that the relatively high levels of these contaminants in fish from the rivers Mersey and (for PAH) Tees may be a significant factor in the EROD induction observed. The survey allows a ranking of contamination in the estuaries in terms of their EROD inducing capabilities in flounder (in ascending order): Alde, Southampton Water, Humber, Tyne, Wear, Tees and Mersey, which to some extent reflects the hepatic accumulation of MFO inducing compounds (Table 8) in these areas. Kirby *et al*. (1998) ranked a number of English estuarine waters on the basis of the toxicity of hexane extracts of seawater to the harpacticoid copepod, *Tisbe battagliai* and the estuaries sampled, common to both studies, were ranked: Southampton Water < Tyne < Mersey < Wear < Tees. It can be seen that the ranking of estuaries was similar in the two studies. This agreement between these disparate studies is not surprising bearing in mind the correlation found by Kirby *et al*. (1998) between biological water quality, as measured by the *T. battagliai* assay, and the PAH concentrations in water.

If the EROD results obtained from the River Tyne are observed in isolation it is evident that in this estuary there are inter-site differences in EROD activity with the mean activity at Hebburn (54.3 pmol/min/mg protein) being significantly less than mean EROD activity at three other Tyne stations: Redheugh, Newcastle and Jarrow. A recently published survey of sediment toxicity in the River Tyne (Matthiessen *et al*., 1998) suggested that sediments taken from the Jarrow area in 1992 were relatively non-toxic compared to other areas in the estuary, and that the most toxic sediments were sampled from the Redheugh/Newcastle area. The same study also recorded the highest sedimentary PCB and hydrocarbon concentrations at Redheugh/Newcastle, which paralleled the elevated EROD activity expressed in samples from these sites.

Gonado-somatic index is a measure of gonadal maturity and has been shown to be well correlated with EROD activity in dab during the spawning season (Kirby *et al*., 1999). It has been shown that GSIs in North Sea flounder (*Platichthys flesus*) start to increase in September for both males and females in preparation for winter spawning, dropping back to baseline levels in February and March for females and males respectively (Janssen *et al*., 1995; 1996(a)). In the present study the mean female GSI at the reference site was 0.72 in October 1997, which suggests that these fish were in their resting/pre-spawning phase. Those females sampled at the two Mersey sites 2 months earlier had low mean GSI values of 0.82 and 0.98, which were not significantly different from those seen at the reference site. However, at all other sites (which were sampled at approximately the same time as the Alde), except the Wear, mean female GSIs were elevated with respect to the reference site, significantly so in Southampton Water and three sites on the Tyne. No clear explanation exists as to why significantly enlarged female gonads were present at these sites. One possibility is that excessive

Table 6. Inter-gender differences in EROD activity and other variables in flounder caught in September - December 1997

Location	Sample Size		EROD		Fish Length(cm)		Fish Weight(g)		HSI		C.F.	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Alde (Reference)												
North of Orford	14	10	36.1	42.5	25.3	25.5	157.4	155.9	1.06	0.99	0.96	0.93
Southampton Water												
Bird Pile Beacon	7	5	78	39.9	26.5	28.2	204.9	237.7	1.59	1.18	0.95	0.96
Tyne												
Scotswood Bridge	9	17	55.7	78.2	29.6 *	29.8 *	293.2 *	285.5 *	1.88 *	1.61 *	1.05	1.06 *
Redheugh	21	20	98.5 *	81	27.3	26.6	231.7	242.9	1.67 *	1.89 *	1.02	1.08 *
Newcastle	12	10	91.2 *	101.3 *	27	23.4	213.7	147.7	1.80 *	1.54 *	1.01	1.02
Hebburn	17	11	60.5	44.8	23.5	25.4	162	198.3	1.60 *	1.57 *	1.09 *	1.07 *
Jarrow	15	15	85.7 *	103.8 *	22.1	19.6 *	127.4	83.6	1.81 *	1.67 *	1.03	1.05 *

** Signficantly different ($p<0.05$) from the Alde reference site.*

Table 7. Linear correlation coefficients (r) for mean EROD with other variables in estuarine flounder (September-December 1997)

	Number of Stations	Length	Weight	GSI	HSI	CF
All Estuaries						
All	16	-0.09	0.05	-	0.21	0.22
Female	13	0.09	0.14	-0.33	0.31	0.24
Male	7	-0.54	-0.37	-0.44	0.11	0.52
Tyne Only						
All	6	-0.55	-0.60	-	-0.17	-0.67
Female	5	0.01	-0.24	-0.84	-0.07	-0.80
Male	5	-0.51	-0.51	-0.43	0.09	-0.63

Table 8. Mean total PAH and PCB in bulked samples of flounder liver from selected estuaries

Location	PAH ($\mu g\ kg^{-1}$ wet weight)	PCB ($\mu g\ kg^{-1}$ wet weight)
River Mersey	363	1082
River Tees	365	n/a
River Humber	71.2-100.6	424
River Alde	109.7	32

n/a = not analysed

exposure to environmental oestrogens in these estuaries could have led to precocious female sexual maturation. However, it seems more likely that these differences are genetic or climatic in nature. The high female mean GSI (2.52) in the Southampton Water samples would suggest that these fish had entered the phase of gonadal maturation, as expected in December.

Mean male GSI values were calculated for fewer sites (Table 5) and only included in statistical analyses when sample numbers exceeded three. The GSI value of 0.76 from the Alde reference site in October was relatively high and suggests that the males at this site may have begun to enter their gonadal maturation phase as expected in September-December (Janssen *et al.*, 1996(a)). This also explains the high figure (1.36) for the December sampled fish from Southampton Water. However, of the sites where mean male GSI was statistically compared to the reference site none were found to be significantly ($p<0.05$) different. These GSI figures correspond well to those gained in a study by Allen *et al.* (1997) in 1996.

Previously EROD activity in flounder has been shown to exhibit high levels of seasonal fluctuation but these influences appear to be at a minimum during the period June to October (Eggens *et al*., 1996). The influence of reproduction may significantly obscure any biomarker response to contaminants (Goksøyr *et al*., 1996) and therefore the samples taken in this study were timed to coincide with a period when reproductive influences were thought to be minimal. In general this appears to be the case, and so we are confident that the inter-estuary differences shown are contaminant induced. However, correlation analysis (Table 7) of mean GSI with mean EROD does suggest that even in the optimal sampling period there may be a residual effect of the reproductive cycle on the MFO system in some estuaries. For example, the female samples from the Tyne sites showed a high negative correlation (r = -0.84) of EROD with GSI which reflects a trend that is more evident during the reproductive season of several flatfish species (Elskus *et al*., 1992; Eggens *et al*., 1996; Kirby *et al*., 1999).

Reference to Table 5 shows that mean hepato-somatic indices were significantly increased with respect to the reference site at all the Tyne and Tees sites as well as other sites on the Mersey, Humber and Southampton Water and, whereas Lye *et al*. (1997) only noted this effect in males, Table 6 demonstrates that this increase in liver size is evident in both sexes. An increase in HSI in fish exposed to contaminated sediments has been observed in previous field studies (Everaarts *et al*., 1993; Theodorakis *et al*., 1992) and the latter authors showed this increase to mirror a concurrent trend in elevated EROD activity in bluegill sunfish. Janssen *et al*., (1996(b)) have shown that HSI increases in female flounder at the onset of, and during, vitellogenesis, and Allen *et al*. (1997) have demonstrated that where HSI is high, plasma vitellogenin levels in flounder are elevated. It seems likely therefore that the observed increase in HSI in this study, in both sexes, is caused by a combination of contaminant effects on the hepatic system.

Condition factor is a measure of the nutritive state of the fish (Saborowski and Buckholz, 1996) and in flounder shows a strong seasonal fluctuation (Eggens *et al*., 1996). CF in flounder reaches a maximum just prior to the onset of vitellogenesis, after the fish have been feeding voraciously during the summer months, and begins to decrease thereafter as a result of sporadic feeding and mobilisation of body energy stores associated with migration and spawning (Janssen *et al*., 1995). In this study significantly ($p<0.05$) higher condition factors were apparent at all but one of the Tyne sites, all the Tees sites, the Wear site and at Spurn Head in the Humber compared to the reference site (Table 5). When the Tyne Estuary data is examined in isolation (Table 6) it can be seen that the high CF levels compared with the reference site fish are more associated with males rather than females, with all sites showing a significantly higher CF for males as opposed to only one site for females. This finding is consistent with the fact that female but not male GSIs were in general higher in the Tyne compared to the Alde, and would suggest that the females were investing energy in gonad maturation at this time. These differences in CF are most probably due to local and seasonal factors influencing the condition of the fish. Condition factor is strongly negatively correlated (r = -0.80) to EROD

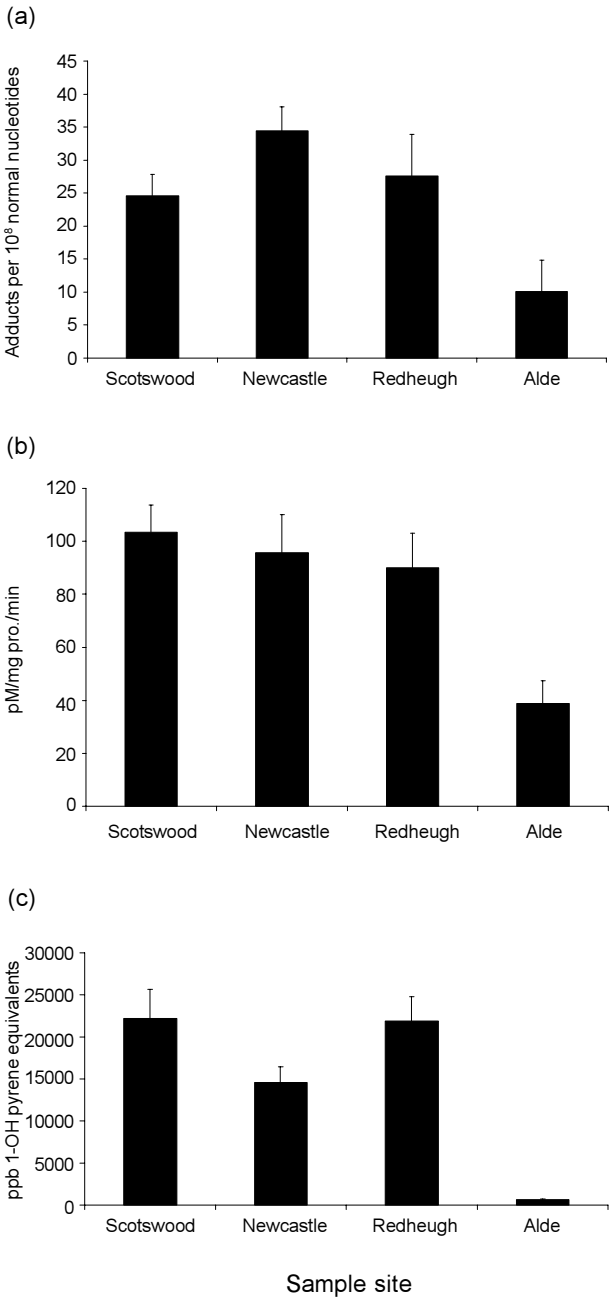

Figure 10. Levels of (a) DNA adducts, (b) EROD activity and (c) bile metabolites (mean ± SE) detected in samples of flounder (P. flesus). Fish were collected from polluted sites along the Tyne Estuary (Scotswood, Newcastle and Redheugh) and from the Alde, an estuary deemed to be essentially free from anthropogenic contamination in September-December 1997

activity in Tyne female flounder (Table 7) which is almost certainly directly linked to the correlation of GSI with EROD in these fish.

4.6 Tyne Estuary - DNA adducts and bile metabolites as biomarkers of genetic damage (October 1997)

Studies detailing flounder EROD activity in this report (see Section 4.5) have shown that the MFO enzyme system is significantly induced in a number of English estuaries. It has been postulated that these results reflect the anthropogenic contamination of estuaries such as the Tyne, Mersey and Tees. While the MFO system is essential for the detoxification of a diverse array of xenobiotics (including PAH), its induction may also produce deleterious side effects. For example while the majority of xenobiotics are rendered less harmful, others (particularly 4-5 ringed PAH) may form genotoxic metabolites with potentially mutagenic and/or carcinogenic properties. The River Tyne Estuary is known to receive considerable amounts of anthropogenic inputs, including municipal sewage discharges (268,652 m^3 day^{-1}) and industrial effluents (1,162,931 m^3 day^{-1}). This is thought to contribute significantly to the high levels of PAH contamination in the Tyne (12,548-43,470 µg kg^{-1} dry weight, ∑PAH), including carcinogens such as benzo[*a*]pyrene and benzo[*a*]anthracene at sediment concentrations of 952-3310 and 945-4130 µg kg^{-1} dry weight respectively (Woodhead *et al*., 1999).

In view of the association between PAH contamination, EROD induction, genetic damage (genotoxicity) and neoplasia observed in experimental animal studies, we conducted further surveys determining the potential for DNA adduct formation and the presence of PAH-bile metabolites to act as biomarkers of PAH exposure in flounder collected from the Tyne. For purposes of this study DNA adducts and bile metabolites were also analysed from flounder collected from the Alde Estuary, an area known to receive minimal municipal wastes (145 m^3 day^{-1}) and no recorded industrial discharges.

Samples of flounder liver and bile were obtained from sample cruises as described in Section 3.2. Liver tissue and bile were excised, placed in vials and immediately transferred to liquid nitrogen. DNA adduct frequencies and bile metabolites were determined as described previously (Section 3.5).

4.6.1 Results

Clear differences were observed in flounder captured from the contaminated Tyne Estuary and the reference Alde Estuary for both hepatic DNA adducts and bile metabolites (Table 9 and Figure 10). Results show that bile metabolites were significantly ($p<0.05$) higher in flounder sampled from the Tyne (Scotswood = 22246.9 ±3407.6; Newcastle = 14572.4 ±1887.6; Redheugh = 21872.4 ±2935.5) compared to those captured from the reference Alde Estuary (632.21 ±55.9). A similar result was observed for DNA adducts with samples analysed from the Tyne containing elevated adduct levels (Scotswood = 24.6 ±3.2; Newcastle = 34.4 ±3.7; Redheugh = 27.6 ±6.3) compared to fish collected from the reference Alde Estuary (10.1 ±4.8).

The DNA adduct profiles detected in this study are displayed in Figure 11 (also refer to Annex 1). Typically DNA adduct profiles observed in fish captured in the Tyne consisted of a diagonal radioactive zone (DRZ) of ^{32}P-labelled adducts (see Figure 11(b-c)). Previous studies have demonstrated such profiles to be representative of exposure to complex mixtures of aromatic and/or hydrophobic genotoxins, such as those formed by PAH. For example, identical DNA adduct profiles were observed in laboratory studies following exposure of English sole (*Pleuronectes vetulus*) to sediment contaminated with PAH (French *et al*., 1996). Furthermore, field investigations have also detected DNA adduct profiles consisting of DRZs when fish are sampled from PAH contaminated waterways (Dunn *et*

Table 9. Levels of DNA adducts per 10^8 nucleotides and EROD activity (pmol/mg/min protein) in pooled liver tissue in Platichthys flesus samples from a reference estuary (Alde) and an estuary known to contain elevated levels of contamination (Tyne)

Sample Location	Levels of DNA adducts per 10^8 nucleotides	EROD activity (pmol/mg/min protein)	Bile metabolites (glucuronide conjugates[f])
Tyne Scotswood	24.6 ± 3.2[a] (5)[b] [16.6-30.5][c]	103.4 ± 10.3 (26)[e]	22246.9 ± 3407.6 (16) [2618.9-47213.6]
Tyne Newcastle	34.4 ± 3.7 (5) [22.6-43.4]	95.8 ± 14.2 (22)	14572.4 ± 1887.6 (13) [5685.5-29265.9]
Tyne Redheugh	27.6 ± 6.3 (5) [16.5-51.7]	90.0 ± 12.9 (41)	21872.4 ± 2935.5 (27) [2049.8-72386.9]
Alde	10.1 ± 4.8 (7) [0.0-35.8]	38.7 ± 8.8 (24)	632.21 ± 55.9 (14) [338.5-1028.9]

[a] *Mean adduct levels ± SE.*

[b] *Numbers in parentheses represent number of pooled samples analysed (5 fish per pooled sample).*

[c] *Numbers in square brackets represent range of DNA adduct levels detected.*

[e] *Number if individuals sampled for EROD activity*

[f] *SFS measurements of conjugated glucoronides, expressed as ppb 1-OH pyrene equivalents.*

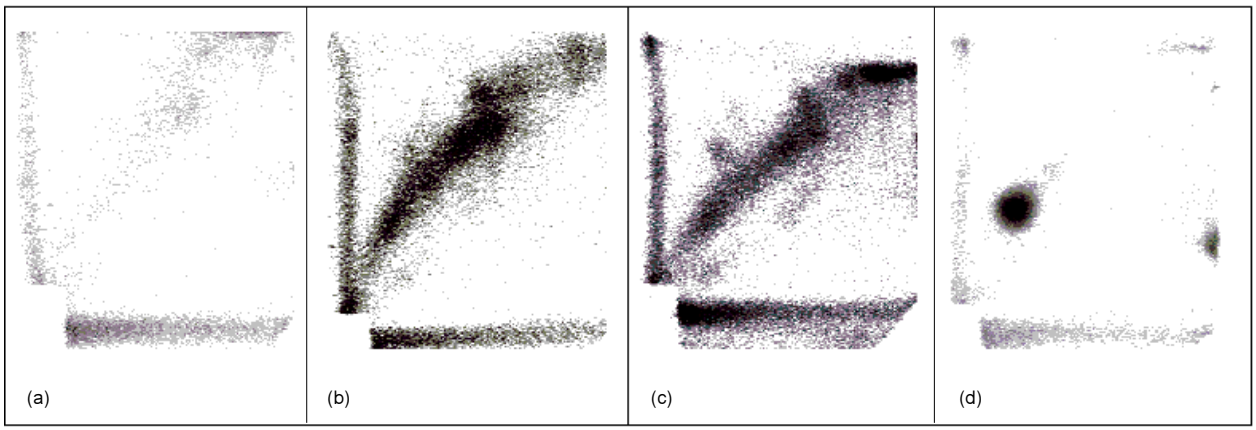

Figure 11. ***Typical DNA adduct profiles obtained from ^{32}P-postlabelling:***
(a) Pooled P. flesus hepatic DNA sample displaying a low level of DNA adducts from individuals collected from the Alde Estuary,
(b) Diagonal radioactive zone (DRZ) of radiolabelled P. flesus hepatic DNA adducts from a pooled sample from Scotswood off the Tyne Estuary. The detection of a DRZ indicates the presence of a number of overlapping bulky aromatic adducts, characteristic of PAH exposure,
(c) Representative DNA adduct profile from a pooled sample of P. flesus hepatic DNA from individuals collected from Newcastle Bridge on the Tyne Estuary, again a DRZ is detectable, indicating exposure to a complex mixture of genotoxins,
(d) Positive control consisting of L. limanda hepatic DNA treated in vivo with 1.5 mM B[a]P diol epoxide

al., 1987; Ericson *et al*., 1995; Harvey *et al*., 1997; Lyons *et al*., 1997).

4.6.2 Discussion

The high concentration of conjugated bile metabolites, as detected by synchronised fluorescence spectrometry (SFS) in this study demonstrates that PAH contaminants present in the Tyne were bioavailable to the flounder and that the enhanced EROD response (see Section 4.5) was in part due to the uptake and subsequent detoxification of these xenobiotics. Furthermore, the observation of elevated levels of DNA adducts, characteristic of PAH exposure signifies that a proportion of the bioavailable PAH (most probably the carcinogenic 4-5 ringed PAH, such as benzo[*a*]pyrene) were being metabolised by the MFO system to metabolites capable of reacting with the DNA in exposed flounders. Previous studies, including those presented in this report (see Section 4.1) have demonstrated marine flatfish species to be capable of metabolising benzo[*a*]pyrene to carcinogenic metabolites, such as diol-epoxides and phenol-oxides (for an overview see Stein *et al*., 1990). This assumption is further supported by the observation that the positive control (Figure 11(d)) consisting of the mutagenic diol-epoxide metabolite of benzo[*a*]pyrene, migrates to a position on the chromatogram that is covered by the DRZs detected in Tyne flounder.

As mentioned previously, DNA adducts are one of the initial stages in the progression of chemical carcinogenesis and their detection demonstrates the interaction of genotoxic chemicals with DNA and therefore provides us with an estimation of carcinogenic exposure for flounder. However, DNA adducts are only biomarkers of genotoxic exposure. As structural modifications to the genetic material, they are transient in nature, with cellular DNA repair systems reverting adducted nucleotides back to their original conformation before cell replication. It is when DNA adducts are miss-repaired or persist through to the DNA replication stage of the cell cycle that manifestations of genotoxicity occur. The replication of DNA containing adducted or miss-repaired nucleotides has the potential to introduce DNA mutations into the genetic material of the daughter cells, so 'fixing' the genetic damage into the genome. This may result in various manifestations of genotoxicity, including impairments in immunoresponse, decreased reproductive capacity and the induction of neoplasia, which collectively comprise a condition termed 'the genotoxic disease syndrome' (Kurelec, 1993).

Previous studies have associated the presence of DNA adducts, indicative of exposure to complex PAH mixtures with increases in the incidents of neoplastic lesions in marine flatfish (Maccubin *et al*., 1990; Stein *et al*., 1990). To date no studies have been conducted to establish frequencies of pre-neoplastic and neoplastic lesions in flounder inhabiting the Tyne or other equally contaminated English waterways. However, data from other European estuaries suggest levels of contamination similar to that found in the Tyne may lead to the onset of neoplastic diseases (Koehler, 1990). The detection in this study of elevated EROD, bile metabolites and DNA adducts demonstrates that the PAH contamination of the Tyne is having a sub-lethal genotoxic effect in the resident flounder, which may potentially lead to the induction of neoplastic disease.

Prolonged chronic exposure to PAH contaminated sediments may also lead to reproductive impairments as PAH have been shown to have endocrine disrupting ability. Previous *in vitro* studies have demonstrated the anti-oestrogenic action of PAH including acenaphthene, benz[*a*]anthracene, benz[*a*]pyrene, dibenz[*a*,*h*]anthracene (Tran *et al*., 1996; Chaloupka *et al*., 1992). Furthermore, studies have shown that a 5 month pre-spawning exposure of maturing male American plaice to ∑PAH sediment concentrations of 39,800 μg kg^{-1} dry weight affects sperm viability and leads to a 48% reduction in the hatching success of larvae from eggs of unexposed females (Nagler and Cyr, 1997). The European flounder confines itself to its home estuary for up to 8 months of the year, only migrating offshore to spawn. The levels of ∑PAH detected in the Tyne (12,548-43,470 μg kg^{-1} dry weight) indicate that similar reproductive impairments may be occurring in other fish species inhabiting this, and other equally polluted estuaries. Furthermore, we previously have demonstrated genetic damage, as detected by elevated levels of DNA adducts, in the reproductive organs of the inter-tidal teleost *Lipophrys pholis* following PAH exposure (Lyons *et al*., 1997 and Harvey *et al*., 1999). Therefore, it is not inconceivable to suggest that the PAH concentrations present in contaminated English estuaries may be exerting both an anti-oestrogenic and mutagenic affect in the reproductive organs of exposed fish.

In summary it has been demonstrated that the use of suite of biochemical indices, along with selected chemical analyses enhances the ability to detect genotoxin exposure and provides not only an estimate of the bioavailability of a group of compounds, but also a measure of subsequent sub-lethal genotoxic effects. Viewing the data as a whole it seems probable that the flounder populations in the Tyne (and potentially other industrialised estuaries) are facing a significant threat to their long-term health and reproductive capabilities. More research is urgently required to establish the significance of these findings and to determine in full the potential ecosystem effects of chronic contaminant exposure at present levels.

5. OVERALL DISCUSSION

Livingstone *et al*. (1997) mention several instances where increased EROD activity in fish has been linked to higher order effects in areas such as disease and reproduction. This study has shown that the MFO system of flounder is significantly induced above that expected at particular states of the reproductive cycle in several English estuaries. Whilst the MFO system is essential for detoxification of certain xenobiotics and the metabolism of some endogenous compounds (for example steroid hormones and vitamins), its induction may also produce deleterious side effects. First, whilst many xenobiotics are rendered less harmful, others form carcinogenic or genotoxic compounds after transformation by the MFO system (e.g. formation of benzo[*a*]pyrene diol-epoxide from the parent PAH compound). Secondly, since essential endogenous substances such as steroid hormones are metabolised by the MFO system, its elevated activity could have serious repercussions for an organism's normal reproductive development. However, it is also possible that exposure to exogenous oestrogenic hormones and their mimics is actually causing some of the differences in EROD induction observed. On balance, it seems probable that flounder populations (and probably other species) in industrialised English estuaries are facing a significant threat to their long-term health and viability from anthropogenic contamination. More research is required to establish the significance of these findings in terms of estuarine ecosystems and fisheries.

The data for the three biomarkers in question has proven difficult to interpret. Whilst, in general, the results for bile metabolites of PAH, EROD and DNA adducts show global elevations at the same sites, no unambiguous link to known PAH levels at near-/offshore sites has been manifest. Figure 12 shows PAH concentrations in sediment from around the UK and whilst it is clear that elevated levels occur off the NE coast which correspond well to the high induction of biomarkers observed in this area there is not a concomitantly high PAH signal in Liverpool Bay which also showed relatively high biomarker responses. However, this apparent inconsistency is explained by the fact that both EROD and DNA adducts also respond to many other planar compounds (e.g. PCBs) and these could be of greater significance in areas of lower PAH contamination. For the estuarine work with flounder, however, a stronger biomarker- PAH link was evident and this may indicate that the use of a less migratory species allows more confident interpretation of results. It is also likely that seasonal influences contribute significantly to the spatial and temporal variance (especially for EROD) that has been shown.

Figure 12 shows general trends in sediment PAH contamination but 'snapshot' levels taken from discreet samples, whilst informative, do not necessarily represent the wider local area. Therefore, great care needs to be taken in the extrapolation of these data to estimate the exposure of benthic fish such as dab and flounder. Perhaps of greater use is the analysis of the constituent PAH data in an effort to identify the likely sources of contamination. One approach is to produce concentration ratios of various PAH compounds of the same molecular mass (isomers) as these can differ depending on the source of contamination. Woodhead *et al.* (1999) applied two such ratios to the data used to generate Figure 12. The first of these was the ratio of benzo[*k*]fluoranthene (BkF) to BkF plus benzo[*e*]pyrene (BeP) and the second was benzo[*a*]pyrene (BaP) to BeP plus BaP. Theoretically a high ratio is indicative of PAH from anthropogenic combustion processes (pyrolytic) and low ratios are more associated with PAH derived

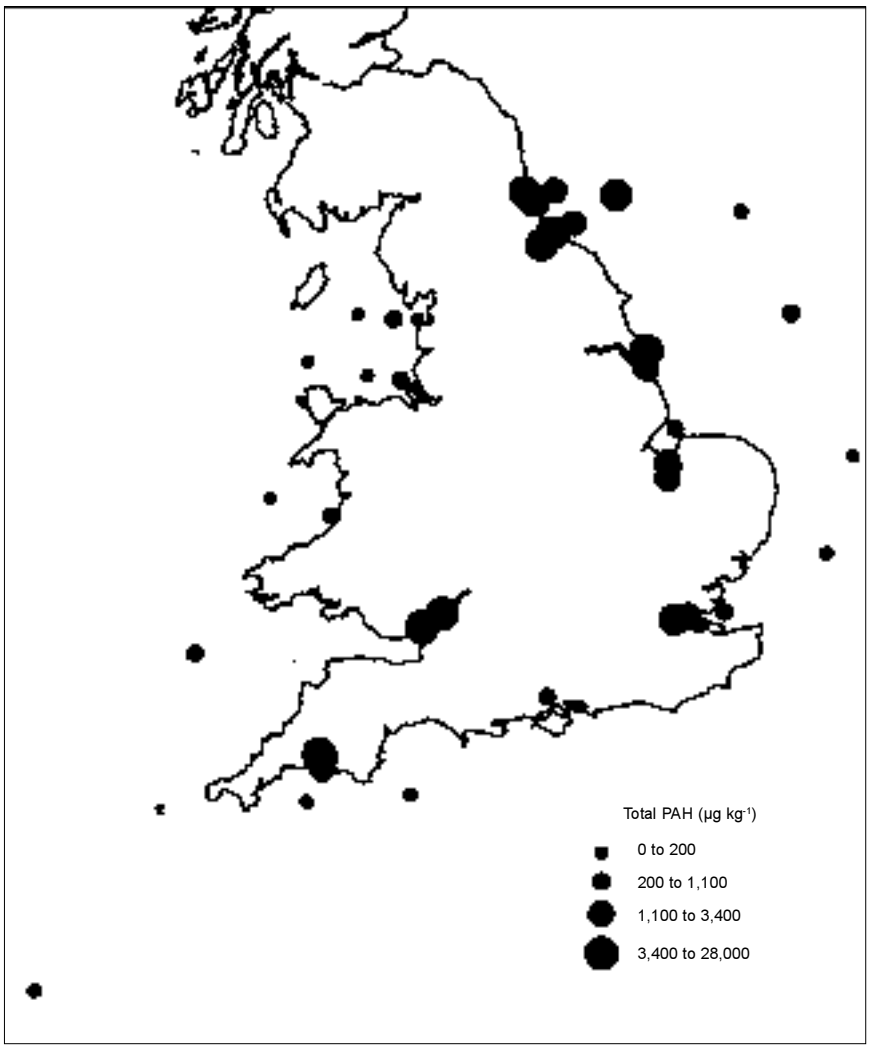

Figure 12. Concentrations (mg kg^{-1}) of PAH (sum of 10 individual PAH compounds) in sediments from around the UK (1993-1996)

from oil and its refined products (petrogenic). Values for these ratios of >0.5 were noted in a number of areas including Inner Cardigan Bay and in the Tees river. Levels of <0.3 were recorded at areas offshore of the Tees and Tyne, Morecambe Bay, S.E. Isle of Man and St. Brides Bay and Woodhead *et al.* (1999) also noted low ratios from samples taken in Milford Haven. The conclusion that can be drawn from the data is that PAH derived from combustion appears to dominate especially in certain industrialised estuaries such as the Tees and Tyne but that in specific areas associated with the petroleum industry, e.g. Milford Haven, petrogenic sources of PAH can make a significant contribution to the overall load.

The most likely gross consequence of genotoxic poisoning would be the occurrence of neoplastic growths. Liver neoplasias denoted 'nodules' are routinely monitored for in dab liver by the CEFAS Weymouth Laboratory. Figure 13 shows the occurrence of gross liver nodules in dab caught around the UK coast from 1993 to 1996. Once again no clear link emerges between nodule occurrence and the induction of the PAH biomarker suite although the NE coast stands out as a region of elevated prevalence. Migration effects are potentially of great significance in this respect. The development of a neoplastic lesion is a relatively slow process, with the progression from genotoxic exposure to the detection of observable changes in tissue architecture taking months if not years. DNA adducts, while directly implicated in chemical carcinogenesis, are relatively early stage alterations in the progression from genotoxin exposure to disease manifestation. Therefore, in highly migratory species it is difficult to link early stage carcinogenesis biomarkers with latent endpoints such as alterations in liver pathology.

Migrational influences affecting biomarker responses in dab

There are many problems arising from using a migratory species in biomonitoring programmes, not least the fact that determining previous exposure history is difficult. There is a paucity of data detailing the migrational movements of *L. limanda*; the few studies conducted have suggested that populations of dab tend to be a temporary aggregation of fish originating from a larger area (Rijnsdorp *et al*., 1992). Therefore the results of a biological effects monitoring programme using this and

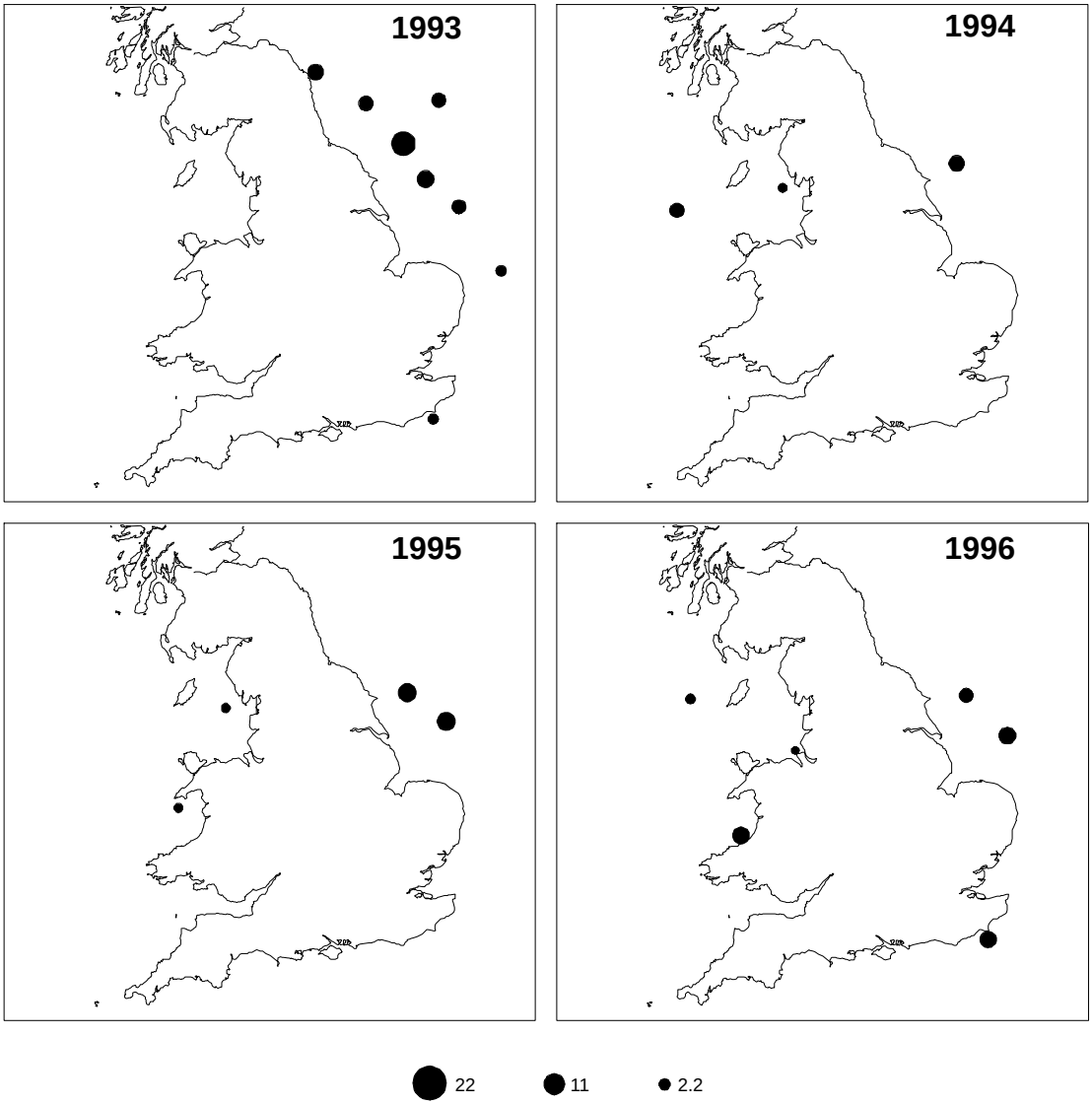

Figure 13. Percentage prevalence levels of gross liver nodules in dab 1993-1996

similar species can only be interpreted in general terms, especially when the endpoints being measured are slowly induced or persistent once induced. The tank exposures detailed in this report (see Section 4.1) indicate that the rate of DNA adduct accumulation in teleosts following exposure to injected PAH is a relatively slow process, reflecting the ability of the organism to remove and repair damaged nucleotides. Indeed Shugart *et al*. (1990) demonstrated that following the continuous exposure of blue gill sunfish to B[*a*]P at 1 µg l^{-1} in the ambient water, DNA adducts were only detectable after 30 days. However, these adducts persisted at detectable levels for several weeks following the cessation of exposure. Therefore it is possible that the migratory movements of sentinel species such as dab may confuse the results of monitoring studies such as those presented in this report.

Migration over an area of 'patchy' contamination levels would decrease the net bioaccumulation of contaminants, enabling the organism to remove the contaminants via the various detoxification pathways, or if adducted to the DNA, removing them via the organism's DNA repair systems. Consequently, the detection of DNA adducts in a migratory species may well depend on the time elapsed between exposure and capture. If the populations of fish at monitoring sites are transient in nature, then movement between sites of heterogeneous contamination may result in fish being collected at a supposed polluted site when in reality they have only just arrived at that location. This factor may well play a critical role in the continued utilisation of *L. limanda* as a biomonitoring species, and further work will be required detailing the migratory movements of *L. limanda* in and around the areas used as monitoring stations.

The problem of migration by monitoring species has been highlighted in previous studies. Cooreman *et al.* (1993) suggested that large variations in EROD activity between individuals at a single site could be attributed to local migrations and Kirby *et al.* (1999) also

suggested that EROD activity distribution patterns after the *Sea Empress* oil spill may have been blurred by migratory activity.

The rationale behind the use of flounder in this study was therefore twofold. First as a means of investigating biomarker relationships in more contaminated areas (i.e. estuaries) and secondly in an effort to target a less migratory species. However, whilst flounder are relatively sedentary, staying in estuaries for most of the spring/summer period, they do migrate offshore in the winter to spawn (Vethaak and Jol, 1996). At certain times of the year (approximately July to October in the UK) flounder can, therefore, be assumed to reflect local conditions as they may have been present for several months but their migratory behaviour leads to some difficulties with data interpretation for the period immediately after migration. Furthermore, the fact that flounder spawn offshore means that for the most sensitive developmental stages (e.g. eggs and larvae) the fish are away from the estuaries where the highest levels of genotoxic chemicals are observed. To allow greater confidence that any biomarker effects detected in fish populations relate to the area of capture, species that breed and reside in an estuary for their entire life cycle would be preferable. The sand goby (*Pomatoschistus minutus*) is a good candidate species for future studies.

Why adducts are relevant biomarkers of environmental contamination

In an organism with active metabolic systems for a given group of contaminants, adducts are generally much longer lived than the mutagens and carcinogens that form them. Therefore the detection of DNA adducts in aquatic organisms can act as an indicator of prior carcinogenic exposure. Indeed, fish collected from even highly contaminated environments are usually characterised by high levels of carcinogen metabolism, low body burdens of carcinogens and high levels of DNA adducts. The presence of carcinogenic DNA adducts would, in addition, suggest a mutagenic/ carcinogenic risk to the aquatic organism itself (Dunn, 1991). Consistent with the high metabolic capability of fish, body burdens of hydrocarbons in finfish collected from around the British Isles are generally very low or undetectable. For instance, following the *Sea Empress* oil spill, total hydrocarbon content (THC) levels in finfish remained relatively low (generally <10 mg kg^{-1}) or not detectable (Law *et al*., 1997). In contrast, studies conducted following the *Sea Empress* oil spill detected elevated levels of DNA adducts in fish collected from oil-affected areas, suggesting that the same process that is eliminating the parent carcinogen from the organism is resulting in the formation of potentially carcinogenic DNA adducts (Lyons *et al*., 1997; Harvey *et al*., 1999). Therefore the detection of DNA adducts provides a reliable marker that exposure to carcinogenic compounds has taken place. Such conclusions would not have been possible if risk assessment had been based purely on body burden analysis.

6. CONCLUSIONS

- The activity of the mixed function oxygenase (MFO) enzyme system, as measured by hepatic EROD activity levels in flounder, is significantly induced in a number of UK estuaries. Some PAH are potent inducers of the MFO system and are also genotoxic, and this study has provided further evidence that PAH are involved in the estuarine responses.

- Flounder residing in the Tyne Estuary are displaying signs of chronic carcinogenic exposure, which is assumed be partly a result of the high levels of sediment-associated PAH. The integrated use of EROD, bile metabolites and DNA adducts has provided a tool for investigating the extent of exposure and has highlighted the need for an in-depth study of industrialised English estuaries to ascertain the ultimate effect of such long-term exposure on the health, growth and reproductive success of the resident biota.

- MFO induction, as measured by hepatic EROD activity levels in dab, at near and offshore sites has been difficult to interpret and no unambiguous link to sediment PAH levels has been established. However, there are clear biomarker 'hotspots', particularly off the north east coast and in Liverpool Bay where the studied biomarkers have been regularly elevated compared to other areas.

- Seasonal influences have contributed significantly to the inter-site differences and has made interpretation more problematical. Migratory behaviour, especially in dab but also in flounder, has also made it difficult to link measured biomarkers to exposure in the area of capture. More research is required to establish the significance of these findings and to determine in full the potential ecosystem effects of chronic contaminant exposure at present levels.

7. RECOMMENDATIONS

- In heavily contaminated estuaries (e.g. Tyne) this study has demonstrated a clear link between the three biomarkers (EROD, bile PAH metabolites and DNA adducts) of PAH contamination although other planar molecules are probably also involved in the EROD and adduct responses. Further research is required to establish the significance of these findings and to determine in full the potential ecosystem effects of chronic contaminant exposure at present levels. The data presented here strongly suggest that carcinogenic disease and impairment of reproduction may be occurring in flounder residing in the Tyne. However, because no disease data was obtained for these samples the potential for

linking the responses to higher order effects (i.e. cancer prevalence) has not yet been realised. A more in-depth biomarker/fish-disease study could help to establish the links between early warning markers and the later incidence of disease. Recent research has provided several new molecular tools for investigating chemically induced carcinogenesis in genes known to be involved with cancer. These, together with more traditional disease monitoring would determine whether the level of exposure in the Tyne is having effects at the population level.

- When using fish as sentinels of pollution effects we need to establish that any biomarker responses/ disease occurence/reproductive impairment discovered is related to the location of capture. The use of migratory fish does not fully allow this. For future studies the use of more sedentary fish species that not only breed but spend their entire life cycle in one area (e.g. an estuary) would create more confidence in pin-pointing areas of effect. A good candidate species for use in estuaries would be the sand goby (*Pomatoschistus minutus*).

- More laboratory calibration studies in which PAH exposure can be controlled and by which biomarker – higher order effects can be unequivocally linked would help the interpretation of field data. Effects on reproduction, for example, are particularly difficult to measure in the environment. The use of small marine fish such as the sand goby (*Pomatoschistus minutus*) would allow effects of reproduction to be studied in the laboratory. Not only is this species relatively easy to keep in captivity but it has a short life-span (12-18 months maximum) so full life-cycle effects can be examined.

- This study has underpinned the current knowledge with respect to the deployment of these biomarkers in environmental monitoring and supports their continued use. In particular, it is recommended that for monitoring purposes (e.g. National Monitoring Plan) EROD can be used as a procedure to 'screen' for effects that can be further investigated by other techniques (e.g. DNA adducts, disease occurence etc.). For results that reflect contaminant effects and minimises the influence of other conditions (e.g. depth, temperature, sediment type etc.) sampling sites should be selected so as to standardise these factors wherever possible. Furthermore, sampling during times of active gonadal maturation or spawning should be avoided as the elevated MFO response during this period can smother the contaminant response. Fish should be sampled during their resting phase or immature specimens selected.

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ANNEX 1. Explanation of Figures 2, 7 and 11

Figures 2, 7 and 11 are provided to allow a qualitative view of types of DNA adduct profiles detected following the various laboratory and field studies undertaken. Each figure (Figure 2(a), 2(b), etc) represents a autoradiograph of a chromatography plate onto which ^{32}P-labelled DNA from the particular sample under investigation has been spotted. DNA adducts (DNA modified by a specific genotoxic metabolite) will migrate to sections of the chromatography plate (this is dependent on both the chemical structure of the particular genotoxin and the chromatography conditions used). The adduct is then visualised by autoradiography and analytical imaging technologies due to the ^{32}P tags on the DNA. As observed in Figure 2(b-d), B[*a*]P adducts migrate to a distinct spot approximately 45 degrees from the origin at the bottom left corner of the plate. As explained in the text, other PAH type adducts tend to migrate to similar positions along this 45 degree line. Therefore, when fish are exposed to complex mixtures of PAH, numerous PAH-DNA adducts are formed. This results in the broad band of radioactivity (termed diagonal radioactive zones, DRZ) detected in fish collected from polluted environments (Figure 7(b-c) and 11(b-c)). Figures 2(a), 7(a) and 11(a) represent samples where no DNA adducts were detected. The radioactivity detectable along the left hand side and bottom edge of the plate represents unincorporated radioactive label and are not included in any analysis.

The reference to proprietary products in this report should not be construed as an endorsement of those products, nor is any criticism implied of similar products which have not been mentioned.