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Sequential expression of biomarkers in Bluegill Sunfish exposed to contaminated sediment

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The temporal expression of various biological rsponses was determined in Bluegill Sunfish *Lepomis macrochirus* exposed under controlled laboratory conditions to sediment containing high concentrations of polynuclear aromatic hydrocarbons, polychlorinated biphenyls and heavy metals. Liver, gill, blood, kidney, brain, spleen and intestine were removed from Sunfish sampled at 1, 2, 4, 8, 16, and 40 weeks post-exposure. Biomarker data were recorded for specific proteins, enzymatic activities, DNA integrity, and histopathology. Biomarkers in the laboratory exposed fish were similar to those of indigenous Sunfish sampled from the site of origin of the contaminated sediment. Several patterns of development of biomarkers over time were also evident. For example, the responses of certain biomarkers are not time-dependent (i.e., intestine and gill ATPase activities) while that of others, such as brain ATPase activity, liver cytochrome P450 and NADPH content, stress proteins, chromatin proteins and DNA strand breaks, fluctuate over time. Still other biomarkers, such as EROD activity, zinc protoporphyrin content of the blood, and DNA adducts, showed marked increases over time. Such patterns need to be considered when comparing laboratory and field results and deciding which biomarkers to use for biomonitoring programs. Implications for natural selection and population/community level responses are also discussed.

Keywords: biomarkers; Bluegill; sediment; pollution; EFPC

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Abbreviations: AchE, acetylcholinesterase; ALAD, δ-aminolevulenic acid dehydratase; BaP, benzo[*a*]pyrene; BaPDE, benzo[*a*]pyrene diol-epoxide; DDW, double distilled water; EFPC, East Fork Poplar Creek; EROD, ethoxyresorufin-*O*-deethylase; PAH, polycyclic aromatic hydrocarbon; PCB, polychlorinated biphenyl; PAS, periodic acid Schiff; ZPP, zinc protoporphyrin.

Introduction

There is a need for more work demonstrating that biological responses to pollutants in the laboratory are comparable with field data. This need arises for several reasons. First, most studies that examine the effects of anthropogenic contaminants on aquatic organisms focus either on laboratory or field research, with only a few investigations directly linking the two. Second, a large proportion of laboratory studies report results obtained from a single time point or from short term exposures, with relatively little information on long term development of biological aberrations in response to toxicants. More detailed information is needed to understand the mechanisms of action of pollutants on aquatic animals, and is necessary to properly interpret laboratory findings with respect to field data and vice versa. Results obtained in the laboratory may be contradictory to field data if the improper length of exposure is chosen (e.g., Shugart, 1988). Third, in the field it is difficult to control environmental variables which may affect or modulate toxicological responses. Therefore, field data must be validated with appropriate laboratory studies in which these environmental correlates can be controlled.

Many studies, both in the laboratory and in the field, focus on only one or, at most, a few endpoints. Although focusing on a small number of endpoints may be adequate for monitoring exposure to a particular compound, it has limited utility in predicting the effect of pollution, which often occurs as a complex mixture in the environment, on the overall health of the organism. Therefore there is a need for more studies examining multiple biological endpoints in response to toxic stress. Currently, there is a trend in ecotoxicology to use toxic responses of organisms as sentinels of environmental contamination. These biological indicators of contamination (referred to as 'biomarkers') can be much more sensitive and ecologically relevant than simply measuring the levels of contaminants in the environment (McCarthy and Shugart, 1990). Thus, information from studies with multiple endpoints would also be useful in determining which organ systems or tissues would be the most sensitive or relevant systems to obtain biomarker data. There is also an effort underway to develop short-term (e.g., biochemical and molecular) predictors of long-term (e.g., population and community) effects on aquatic systems (Adams *et al.*, 1989).

In particular, the Biological Monitoring and Abatement Program (Loar *et al.*, 1988) at the Oak Ridge National Laboratory has been monitoring the biological responses of aquatic orgnanisms in East Fork Poplar Creek (EFPC). EFPC receives industrial effluent from the Y-12 Plant, a US Department of Energy (DOE) nuclear weapons production facility. Redbreast Sunfish *Lepomis auritus* in EFPC have been found to have elevated tissue levels of PCBs and mercury (Kornegay, 1991), as well as biomarkers indicative of contaminant-induced stress (Adams *et al.*, 1989, 1990; Shugart, 1990). These biomarkers have also been correlated with community and ecosystem level responses, such as species diversity and community structure (Adams *et al.*, 1989).

Therefore the principal aims of this study are: 1, to examine the sequential and temporal development of various biomarkers in a fish exposed to a known contaminated sediment over a reasonable period of time; 2, to evaluate and compare a wide suite of biomarkers in a variety of organs and organ systems, and 3, to determine if biological responses observed in the laboratory exposed fish are comparable with those seen in the field. These objectives were addressed by exposing Bluegill Sunfish *Lepomis macrochirus* in the laboratory to EFPC sediment for various periods of time. Endpoints

Table 1. Suite of biomarkers examined in Bluegill Sunfish exposed to EFPC sediment

Biomarker[a]	Tissue[b]	Contaminant/reference[c]
Na^+/K^+ ATPase	B,G,I,K	metals and PCBs/Lakshmi *et al.*, 1991; La Roca and Carlson, 1979
AchE	B	metals and PAHs/Shaw and Panigrahi 1990; Danga and Masurekar, 1989
EROD/cytochrome P450/ microsomal NADH/ NADPH	L	PAHs and PCBs/Adams *et al.*, 1990
Liver somatic index	L	PAHs and PCBs/Adams *et al*, 1990; Fletcher *et al.*, 1982
Spleen somatic index	S	metals and hydrocarbons/Lowe-Jinde and Niimi, 1986; Adams *et al.*, 1990
PAH metabolites	bile	Krahn *et al.*, 1984
DNA: strand breaks	L	Shugart, 1988, 1990
DNA: BaP adducts	L,G,rbc	Shugart, 1987
Hb: BaP adducts	rbc	Shugart, 1985, 1987
DNA content	L	metals and PAHs/Bickham, 1990
ALAD/ZPP/Hct	rbc	metals/Hodson *et al.*, 1980; Cory-Slechta *et al.*, 1989; Cyriac *et al.*, 1989; Flora *et al.*, 1991
Histopathology	G,K,L	metals and PAHs/Nath and Kumor, 1989; Kinubagaran and Joy, 1988
Stress proteins	G	metals/Bradley, 1992
Chromatin proteins	rbc	PAHs/C.W. Theodorakis and S.J. D'Surney, unpublished data

[a]Abbreviations: AchE, acetylcholinestrase activity; ALAD, δ-aminolevulenic acid dehydratase activity; EROD, ethoxyresorufin-*O*-deethylase activity; Hb, haemoglobin; Hct, hemotocrit; NADH, NADH-cytochrome b_5 reductase activity; NADPH, NADPH-cytochrome *c* reductase activity; ZPP, zinc protoporphyrin content of the blood.
[b]Tissue in which biomarker examined (B, brain; G, gill; I, intestine; K, kidney; L, liver; rbc, red blood cell).
[c]Environmental contaminants known to elicit a change in biomarker response.

include a suite of biomolecular, anatomical and histological responses. Particular endpoints were chosen because they are altered: 1, in Redbreast Sunfish in EFPC; 2, in fish or other organisms from areas with similar pollution; or 3, in fish exposed in the laboratory to compounds known to be present in the sediment of EFPC (Table 1). The particular organs examined were chosen because they were primary target organs or were the site of exposure and/or detoxification.

Materials and methods

Chemicals

All reagents and enzymes explicitly mentioned in this paper were purchsed from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Where the reader is referred to another paper for specific methodology, the sources of the reagents used in those assays can be found in their respective references. All organic solvents were purchased from J.T. Baker (Phillipsburg, NJ).

Sediment source

Contaminated sediment was collected by grab sample near the headwaters of EFPC, just downstream from the Y-12 plant. Control sediment was collected from a relatively clean headwater stream on the DOE Reservation.

Experimental design

The collected sediment was sieved through a 2 mm metal sieve. Approximately three litres of wet sediment was placed in the bottom of each of twelve, 76 litre glass aquaria. Six of the aquaria received contaminated sediment and six received control sediment. Thirty-two litres of charcoal-filtered, UV sterilized, dechlorinated tap water was then added to the aquaria and allowed to equilibrate with the sediment for one week before fish were added. In addition, approximately 30 litres of contaminated sediment was added to a 378 litre aquarium. This aquarium was filled as needed with water in order to provide fresh, contaminated water to the contaminated tanks. The water was continously aerated throughout the experiment in all aquaria. Ten hatchery-reared Bluegill Sunfish, measuring 80–100 mm (standard length) and weighing 25–40 g, were then added to each aquarium (five males and five females). The fish were acclimated to laboratory conditions in clean water for one week prior to the start of the experiments, and fed *ad libitum* four times per week with approximately 17 g of live tubificid worms (*Tubifex* spp.) apportioned to each tank at each feeding throughout the experiment. Fish in the contaminated and control tanks were fed worms which were kept for one week in the contaminated and control sediment, respectively. Every day approximately one quarter of the water from each tank was removed and replaced with fresh contaminated or uncontaminated water, depending on the treatment. Water from the 378 litre aquarium was allowed to equilibrate for at least three days with the contaminated sediment before being used to fill the contaminated aquaria.

Fish were sampled from the tanks at 1, 2, 4, 8, 16, and 40 weeks after the exposure was begun. Although there were six aquaria total for each tretment, only three of the aquaria were sampled at each sampling period, with the other half being sampled at the next sampling period. In order to minimize tank effects, the six control and six exposed tanks were each randomly designated to be sampled at week 1 or week 2 (three tanks total for each week for each treatment). This randomization procedure was repetaed for weeks 4 and 8 and then for weeks 16 and 40. Although both male and female fish were exposed to the sediment, only the analysis for females is reported here. All fish were anaesthetized in a solution of 50 ml diethyl ether per litre water, and blood was collected in EDTA or heparinized vacuum tubes via caudal vein puncture. The fish were then killed by overdose of anaesthetic followed by spinal scission. Organ samples were frozen in liquid nitrogen and stored under liquid nitrogen until analysis. Samples of skeletal muscle were frozen for polychlorinated biphenyl (PCB) and heavy metal analysis. Organ tissues were not used for chemical analyses because they were used in biomarker analyses.

Sediment analysis

Both control and exposed sediments were analyzed for heavy metals (EPA method 200.7; US EPA, 1983), hexane-extracted (EPA method 3550; US EPA, 1986) and analyzed for PCBs and PAHs (EPA methods 8080 and 8270, respectively; US EPA,

1986). PCBs were quantified by Aroclor number (EPA method 8081; US EPA, 1986) and by congeners. Individual congeners were identified according to retention time, and the external standard method using peak area for quantification. Eleven dichloro- to hexachlorobiphenyl congeners were identified. Congener standards were obtained from ULTRA Scientific (Kingston, RI).

Water analysis

Physical and chemical parameters. At the time of sampling of the fish, water samples were also collected, filtered through a 0.45 μm filter and centrifuged at 10000 rpm for 15 min to remove suspended particles. The temperature and pH were measured immediately after collection. The pH was measured on an Orion pH meter (Orion Research, Boston, MA). The remainder of the water was acidified with Ultrex nitric acid (J.T. Baker, Phillipsburg, NJ; 1 ml acid per l water) and stored at 4 °C until analysis (no more than 24 h storage for organic extraction). The amount of organic carbon dissolved in the water was also monitored throughout the experiment. Total dissolved organic and inorganic carbon was determined on an O.I. model 700 total organic carbon analyzer (Q.I. Corporation, College Station, TX). Metal concentrations in the water were determined by inductively coupled plasma (ICP) spectroscopy (EPA method 200.7; US EPA, 1983).

PCB analysis. Fifty ml of water were extracted as described by Dillon and Burton (1991); the extracts were dried and the residue was resuspended in 1 ml hexane. All glassware was acetone-rinsed before use. One μl hexane extract was injected into a Perkin-Elmer model 8500 gas chromatograph equiped with a DB5 fused-silica capillary column (30 m × 0.25 mm ID) and an electron capture detector. Temperature programming followed established EPA procedures (EPA method 8080; US EPA, 1986). Congeners were identified as above.

PAH analysis. Ten ml of water were passed through a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA). The PAHs were then eluted with 3 ml methanol, concentrated to 100 μl, dissolved in acetonitrile + water (1:1) and analyzed by HPLC with fluorescence detection (Maccubin *et al.*, 1984). Quantitation was performed as with the PCBs. PAH standards were obtained from ULTRA Scienfitic (Hope, RI).

Analysis of biological materials

PCB analysis. Fillets were ground with Na_2SO_4 and Soxhlet extracted (EPA method number 3540; US EPA 1986) with acetone as the solvent. The extract was concentrated on a rotary evaporator, and the residue was redissolved in hexane with 3 × 3 ml washes of the evaporator flask. The extract ws taken to dryness and redissolved in 5 ml hexane. Aliquots of 1 ml were removed for lipid determination by gravimetric methods. The remainder was cleaned on a Florosil column and analysed by gas chromatography as before. PCBs were again identified according to congener and Aroclor number.

Heavy metal analysis. Fillets were ashed (Stahr *et al.*, 1977), dissolved in 1 N HCl, filtered, and diluted to 10 ml with double distilled water (DDW). Metal determinations were performed on an ICP spectrophotometer as with the water. All glass- and porcelainware

was acid washed and DDW rinsed before use. The same preparation for metal and organic chemical analyses was performed on the tubificid worms after 1 week of exposure to the contaminated sediment, with the exception that the PAHs and PCBs were extracted wtih sonication (EPA method 3550; US EPA, 1986).

Biomarker analysis

Protein determinations. Protein concentrations were determined for all assays using the method of Bradford (1976) with Bio-Rad reagents (Bio-Rad Corporation, Richmond, VA).

Na^+/K^+ ATPase activity. Frozen brain, gill and kidneys were homogenized with a glass homogenizer in a buffer consisting of 300 mM sucrose, 20 mM EDTA, 100 mM imidazole and 0.1% sodium deoxycholate (Zaugg, 1982). ATPase activity of the homogenate was measured as the cleavage of ATP to form inorganic phosphate. Inorganic phosphate was measured spectrophotometrically (Ames and Dubin, 1960) before and after incubation of tissues at 37 °C for 10 min. Appearance of additional phosphate was assumed to be attributable to ATPase activity. Na^+/K^+ ATPase was discriminated from all other ATPase or phosphorylase activities by comparing the results of the assay with and without ouabain (Zaugg, 1982).

Acetylcholinesterase activity. Brains were homogenized with 0.25–0.5 ml 150 mM NaCl, 6 mM Tris buffer (pH 7.4), 1 mM $MgCl_2$, and 0.5 mM EDTA. Then 0.1 ml of this homogenate was mixed with 0.45 ml of Na^+–K^+ phosphate buffer (pH 8.0) and the activity was determined spectrophotometrically (Halbrook *et al.*, 1992).

Liver and spleen parameters. Liver microsomes were prepared by homogenizing tissues in sodium phosphate buffer using a motor-driven Potter-Elvehjem glass and Teflon homogenizer (Talboy's Instrument Co., Emerson, NJ; Jimenez *et al.*, 1988) and subsequently isolating the microsomes by differential centrifugation (McKee *et al.*, 1983). Liver microsomal ethoxyresorufin-*O*-deethylase (EROD) activity was determined fluorometrically by an automated procedure, while cytochromes P450 and b_5 concentrations were determined spectrophotometrically (Jimenez *et al.*, 1988). Microsomal NADPH cytochrome *c* reductase activity was measured photometrically by the methods of Phillips and Langdon (1962), with a modified reaction mixture consisting of 50 mM Tris (pH 7.43), 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1.1 mg ml^{-1} horse heart cytochrome *c*, 0.175 mM NADPH, and 2–10 μg microsomal protein (Loar *et al.*, 1988). NADH-cytochrome b_5 reductase activity was measured by methods identical to that for NADPH cytochrome *c* reductase, except that 0.25 mM NADH was used in place of NADPH (Loar *et al.*, 1988). Cytochromes P450 and b_5 concentrations were each measured using their characteristic oxidized and reduced spectra (Johannsen and DePierre, 1978). Liver and spleen somatic indices were measured as a percentage of total body mass.

Bile metabolites. The bile was diluted to 0.5% in DDW and analyzed by HPLC with relative quantitation using BaP as the standard (Krahn *et al.*, 1986).

DNA damage. DNA strand breaks were determined by an alkaline unwinding assay and expressed as 'F values' or 'n values' (Shugart, 1988). The F value is inversely

proportional to the number of strand breaks. The n value is a measure of the relative amount of strand breaks in exposed fish compared to controls. For example, an n value of 1.5 indicates that exposed fish have 150% more strand breaks than controls. In addition, an alternative method of determining relative strand breaks was performed. This was done using acrylamide gel electrophorsis under alkaline conditions (Freeman and Thompson, 1990) with the same DNA as the unwinding assays.

DNA adducts of BaP were determined for gill, liver, and blood DNA. DNA from livers and gills was isolated by homogenization in a buffer solution (250 mM NaCl, 100 mM Tris-HCl, 100 mM EDTA and 1% Sarcosyl detergent), extraction with phenol + chloroform and chloroform, and digestion for 1 h successively with RNaseA and proteinase K (100 $\mu g\ ml^{-1}$ for both) at 50 °C. The digestate was extracted again with phenol + chloroform and chloroform. The DNA in the extract was precipitated with ethanol, dried and resuspended in 1 ml DDW. Aliquots of 100 µl were removed for DNA determination by fluorescence methods (Shugart, 1988). BaP adducts in the remaining portion were acid hydrolyzed and quantitated by HPLC with fluorescence detection (Shugart, 1987).

Blood cell nuclei were isolated by the methods of Hewish and Burgoyne (1973), except that phosphate-buffered saline plus 10mM EDTA was used as the buffer. The cells were lysed with Nonidet P-40 detergent and the nuclei were collected by centrifugation. The nuceli were lysed with 1% Sarcosyl detergent and the DNA was purified and the adducts were quantitated as above.

Haemoglobin adducts. After the blood cells were lysed and the nuclei pelleted, the supernatant containing the haemoglobin was collected. The globin was separated from the heme and precipitated in acidified acetone (Shugart, 1985). The globin was then dried, weighed, and resuspended with sonication into 1 ml DDW. The BaP-globin adducts were quantitated as above.

DNA content and variability. Frozen liver samples were transported in liquid nitrogen to the Center for Biosystematics and Biodiversity at Texas A & M University, and stored at −63 °C until analysis. Monodispersed nuclear suspensions for flow cytometry (FCM) were made form 2–3 mm blocks of the tissue samples. Tissue suspensions were prepared following the detergent–trypsin method for the preparation of nuclei for FCM analysis by Vindelov *et al.* (1983). The intercalative binding fluorochrome propidium iodide (PI) was used to form fluorescent complexes with DNA in the tissue suspensions. RNA in the suspensions was destroyed by pretreatment with RNase. The stained tissue suspensions of both control and exposed samples were assigned numbers and blindly analysed for relative DNA content on a Coulter EPICS Profile II flow cytometer. The Profile II was equipped with a 488 nm argon laser to excite the DNA-PI fluorescent complexes.

Blood parameters. δ-Aminolevulinic acid dehydratase activity (ALAD) was determined colourometrically (Mitchell *et al.*, 1977). Initially, EDTA and citrate, both efficient chelators of metal ions (Klassen *et al.*, 1987), were used in the assay, but this may have affected enzyme activity. Therefore a second assay replaced EDTA and citric acid with heparin and HCl.

Zinc protoporphyrin (ZPP) content of fresh blood was measured on an automated zinc protoporphyrin meter (Aviv Corp., Lakewood, NJ). Hematocrits were measured by

drawing up fresh blood into a microcapillary tube and spinning it for 20 min in an EIC MB micro hematocrit centrifuge (Needham Heights, MA).

Histopathology. Once tissues were removed from the specimens, they were immediately immersed in Karnovski's fixative. Fixed tissues were embedded in paraffin following routine procedures, sectioned at 5 μm, and stained with hematoxylin and eosin for general observations or with periodic acid–Schiff (PAS) reagent for demonstration of complex carbohydrates (Bancroft, 1977). After staining, slides were numbered and shipped to the Gulf Coast Research Laboratory for blind analysis.

Stress and chromosomal protein analysis. For stress protein determinations, whole frozen tissues were shipped in liquid nitrogen to the University of Maryland (Baltimore County Campus) for analysis. Upon arrival, gill homogenates were prepared and electrophoresed on a polyacrylamide gel according to established procedures (Bradley and Ward, 1989). Gels were then stained with Coomassie Blue (Higgins and Dahmus, 1979) or Western blotted (Burnette, 1981) with polyclonal anti-HSP_{70}, which crossreacts with HSP_{90} in daphnids (Bradley, 1992). The stress proteins were visualized with peroxidase-linked anti-IgG (Winston, 1989).

For analysis of red blood cell chromatin proteins, blood cell nuclei were prepared from 50 μl blood using the techniques of Hewish and Burgoyne (1973). The chromatin proteins were prepared and electrophoresed on a 13% polyacrylamide gel (Laemmli, 1970) and stained as above. The relatve amounts of proteins in each band were compared qualitatively using a Scanning Laser Densitometer (LKB, Bromma, Sweden).

Statistics

Because the numerical distributions of many of the samples seemed skewed, differences between control and exposed fish were determined using a nonparametric (Wilcoxon rank-sum) test (Hollander and Wolfe, 1973). The authors acknowledge that a rigorous statistical study of the data was not possible because of the small sample size ($n = 3$, unless otherwise noted), however, the experimental design was predicated by the logistical constraints on available laboratory space, personnel, and the long duration of the exposure. Moreover, the objectives of this study are more concerned with qualitative trends with time of exposure rather than an estimation of precise quantitative differences between fish.

Results and discussion

Chemical analysis

Physical and chemical parameters for water samples are listed in Table 2. These values did not differ significantly between aquaria within treatments :$p > 0.50$) and were also similar between treatments (i.e., contaminated and uncontaminated aquaria). Organic carbon content of the water did not vary between aquaria or treatments or over time, except for the slightly elevated levels the first week (Table 2).

Determination of the concentrations of the various xenobiotics in the sediment, water and fish tissues was necessary in order to show a causal relationship between exposure and response. PCB analysis included both congener-specific and Aroclor specific analyses. This was because previous studies determined PCB loads using Aroclor specific

Table 2. Water quality parameters for water equilibrated with EFPC sediment

Parameter	Week	Sediment	
		Control	EFPC
Temperature (°C)	1	21	21
	2	20	21
	4	21	21
	8	21	21
pH	1	8.1	8.2
	2	8.5	8.3
	4	8.2	8.0
	8	8.0	8.1
Total inorganic carbon ($mg\ l^{-1}$)	1	7.59	8.36
	2	7.89	7.98
	4	6.99	7.89
	8	7.45	8.01
Calcium ($mg\ l^{-1}$)	1	54.0	34.0
	2	55.0	44.0
	4	60.0	47.0
	8	52.0	39.0
Total organic carbon ($mg\ l^{-1}$)	1	8.65	8.40
	2	6.82	5.49
	4	6.13	5.01
	8	6.84	5.23

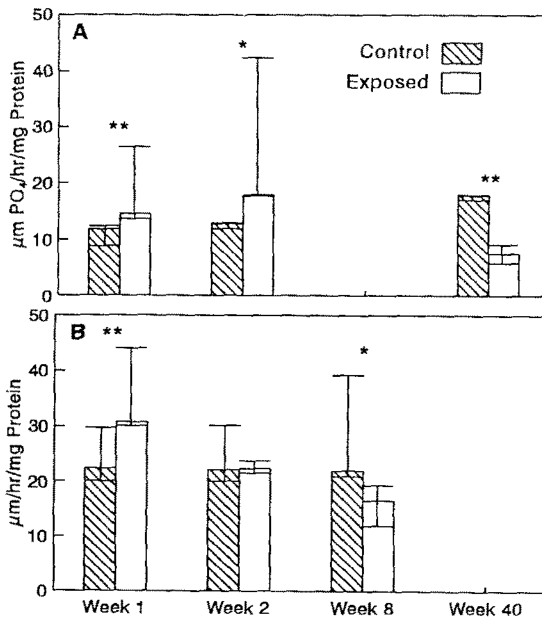

Fig. 1. ATPase (a) and acetylcholinesterase (b) activities from the brains of Bluegill Sunfish exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars marked * represent the range $p < 0.10$, and those marked ** represent $p < 0.05$.

analyses. Significant levels of PCBs, PAHs and heavy metals were present in contaminated sediment (Table 3). No PAHs were detected in the control sediment. PAHs, PCBs and metals were also present in the water equilibrated with EFPC sediment (Table 4). There were no differences within treatments ($p > 0.50$) and there were no consistent trends over time.

Table 3. Representative PCBs, PAHs and heavy metals found in EFPC sediment

Compound	Sediment Control	Sediment EFPC	Worms
PCBs[b] (μg per g wet weight)			
22′ DBC	–[c]	205.24	7.98
24 DCB	–	31.98	7.97
44 DCB	–	9.48	2.62
246 TCB	–	22.06	0.36
22′4 TCB	–	78.12	0.67
22′5 TCB	–	100.47	1.99
22′55′ TtCB	–	13.28	20.2
22′455′ PCB	–	5.56	2.67
22′466′ PCB	–	5.49	0.86
22′33′44′ HCB	–	4.08	2.15
22′44′55′ HCB	–	2.19	1.38
22″44′66′ HCB	–	11.04	1.21
Total Aroclors[d]	0.49	7.78	–
PAHs (μg per g wet weight)			
Fluorene	ND	9.10	1.48
Phenanthrene	ND	22.98	4.40
Anthracene	ND	22.90	4.43
Benzanthracene	ND	2.97	1.61
Chrysene	ND	98.01	11.89
Pyrene	ND	120.80	10.93
Benzo[*k*]fluoranthrene	ND	2.17	0.98
Benzo[*a*]pyrene	ND	66.56	7.72
Dibenz[*a*,*h*]anthracene	ND	14.33	2.48
Heavy metals (ng per g wet weight)			
Al	370	530	208
As	6.1	85	1.26
Cu	3.6	140	0.71
Ni	8.6	64	8.86
Pb	6.1	75	1.26
Zn	83.0	890	6.42

[a]Not detected.
[b]DCB, dichlorobiphenyl; TCB, trichlorobiphenyl; TtCB, tetrachlorobiphenyl; PCB, pentachlorobiphenyl; HCB, hexachlorobiphenyl.
[c]This particular assay was not performed.
[d]PCBs identified as Aroclors rather than individual congeners.

fed to control fish. Also, the stomach contents in a subsample (n = 12) of fish fed just before death contained an average of 0.04 g worms per g body weight.

In the fillets, PCB concentrations were high (Table 5) but heavy metal concentrations were low (Table 6). While heavy metal concentrations fluctuated over time with no consistent trends (Table 6), PCBs showed a general trend of increasing concentration over time (Table 5). The lower chlorinated congeners had lower bioconcentration factors than did the higher chlorinated congeners (Table 5). The total PCB concentration, determined by Aroclor analysis, of week 16 exposed fish was 5.55 $\mu g\ g^{-1}$. Recovery of

The worms used to feed the fish exposed to EFPC sediment had high levels of PAHs, PCBs and heavy metals (Table 3). These contaminants were not detected in the worms

Table 4. PCB, PAH and heavy metal concentrations (μgl) of water equilibrated with EFPC sediment

Compound	Week 1	Week 2	Week 4	Week 8
PCB congeners[a]				
22′ DCB	220.6	62.0	52.2	57.8
24 DCB	55.0	13.0	18.2	15.4
44 DCB	42.5	8.2	7.6	7.5
246 TCB	5.6	1.4	0.9	0.8
22′4 TCB	2.6	0.8	1.0	0.7
22′5 TCB	3.0	3.0	0.7	1.3
22′55′ TtCB	20.0	2.8	2.8	2.3
22′455′ PCB	7.4	0.9	0.7	0.8
22′466′ PCB	10.7	0.7	1.2	1.0
22′33′44′ HCB	17.2	0.4	0.3	0.3
22′44′55′ HCB	11.5	4.4	0.3	0.4
22′44′66′ HCB	5.2	0.6	0.7	0.6
PAH congeners				
Fluorene	0.5	0.5	0.4	0.3
Phenanthrene	2.1	2.6	2.3	2.2
Anthracene	1.2	1.1	1.1	1.2
Banzanthracene	0.1	0.1	0.04	0.04
Chrysene	6.8	5.4	3.3	2.8
Pyrene	4.1	3.8	3.0	5.5
Benzo[*k*]fluoranthrene	0.04	0.04	0.04	0.2
Benzo[*a*]pyrene	0.6	0.6	0.2	0.5
Dibenz[*a,h*]anthracene	0.1	0.1	0.1	0.2
Heavy metal				
Al	60	50	57	110
As	< 50[b]	<50[b]	50	< 50[b]
Cd	< 7[b]	< 7[b]	7	< 7[b]
Cu	16	7	8	< 5[b]
Ni	12	4	7	6
Pb	50	30	32	34
Zn	54	24	22	18

[a]DCB, dichlorobiphenyl; TCB, trichlorobiphenyl; TtCB, tetrachlorobiphenyl; PCB, pentachlorobiphenyl; HCB, hexachlorobiphenyl.
[b]Detection limit.

spiked samples ranged from 85–94% for various PCB congeners and 70–95% for metals. Precision ranged from ± 5–8% for PCBs and ± 8–12% for heavy metals.

These analyses indicated that PAHs, PCBs and heavy metals were accumulated by the fish, giving credence to the idea that it was these chemicals that affected changes in biomarker responses. In addition, analyses also revealed congener specific patterns of PCB bioaccumulation which are similar to findings from the literature (see Shaw and Connell (1986)). These results provide strong evidence that the source of the PCBs is the sediment. Aroclor-specific analyses were included in this study to compare results with reported results from EFPC. However, we feel that Aroclor analyses may underestimate the actual levels of PCBs as reflected by the results of this study, especially for less chlorinated congeners. This may be due to the different congeners in pure industrial mixtures having different uptake, depuration, sediment adsorption/desorption and degradation rates and different aqueous solubilities. Reported values of PCB body burdens for EFPC fish were much less than those in the present study. This may be due to the fact that congener-specific analysis was used in the laboratory but Aroclor-specific analysis was used for EFPC fish. However, even the subsample of laboratory-exposed fish in which the PCBs were identified as Aroclors were found to have higher concentrations than EFPC fish. This could be due to exposure concentrations in the field differing from those in the laboratory, or to the fact that a different species was used in the lab (Bluegill) than in the field (Redbreast Sunfish). Differences in bioaccumulation or biomarker response between individuals from different tanks and time points were apparently not due to fluctuations in water concentrations of contaminants, which remained relatively constant. It is interesting to note that the heavy metals present seemed to equilibrate rapidly with the body tissues of the fish, while the PCB concentrations increased over time. These contrasting ptterns of accumulation may have had an effect on the temporal patterns of biomarker expression described below.

Biomarker analyses

All probability (alpha) values refer to the Wilcoxon rank-sum test. Data for some weeks are missing due to insufficient tissue to perform all assays on all samples. Although all fish had well developed gonads, reproductive activity was not apparent during this study. The results will be discussed according to organ system and/or the trend of response.

Brain enzyme activity. Both AchE and brain Na^+/K^+ ATPase activities were higher in exposed fish relative to control after 1 week, but decreased over time (Fig. 1). Because the brain enzyme activities are affected by xenobiotics, there may also be implications for behavioural toxicology studies. Most behavioural studies involve short-term exposures, but the results from this study indicate that the effects on brain enzyme activities from long-term exposures are different from short-term exposures, and alterations of behavioural patterns may follow the same trends. In laboratory studies, short-term exposure to some xenobiotics produces behavioural hyperactivity in fish (Steele, 1985; Drummond and Russom, 1990) which is consistent with increased enzyme activities in the present study. But, the results from this study imply that long-term behavioural alterations might be different from the short-term.

Gill and intestine parameters. The Na^+/K^+ ATPase activities of the gill and intestine were decreased in exposed fish compared to controls (Fig. 2). Histopathological examination of the gills of exposed specimens indicated erosion of the epithelium and damage

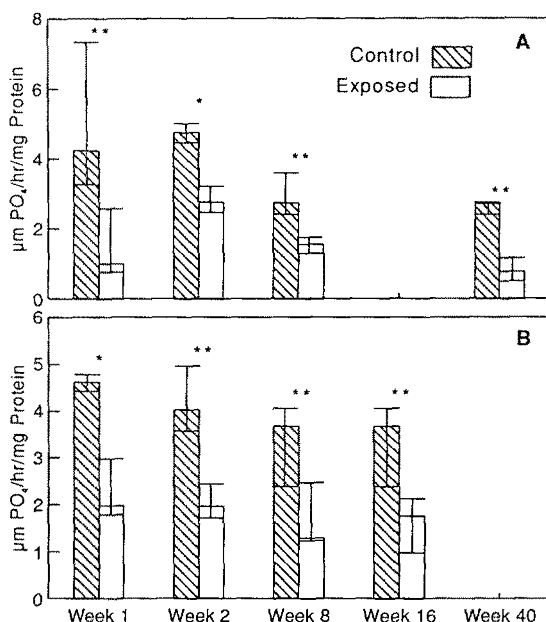

Fig. 2. Na^+/K^+ ATPase activities from the gills (A) and intestines (B) of Bluegill Sunfish exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars marked * represent the range $p < 0.10$, and those marked ** represent $p < 0.05$.

to the secondary lamellae. With PAS staining there appeared to be a reduced number of mucous cells in many exposed fish. None of the changes, however, showed temporal trends.

Liver parameters. In exposed fish EROD activity increased with time and was greater than controls (Fig. 3). The liver somatic indices seemed not to be affected until the fourth week, but thereafter showed a trend similar to EROD. Microsomal concentrations of cytochromes P450 and b_5 showed fluctuations in response but no consistent trends over time. The same was true for liver microsomal NADH-cytochrome b_5 and NADPH-cytochrome *c* reductase activities. In general, the activities in exposed fish for these parameters were higher than in controls, especially for the longer exposures (Fig. 4). Histopathological examination of liver tissue showed focal necrosis in specimens at 1 and 4 weeks of exposure. In several 8 and 16 week specimens, scattered individual hepatocytes appeared necrotic. PAS staining indicated glycogen depletion in exposed specimens at 1 and 4 weeks.

Bile metabolites. The bile contained many fluorescent compounds found to chromatograph with retention times similar to PAH metabolites (Krahn *et al.*, 1984). Relative amounts of these chemicals in the bile of exposed fish were found to be over 300-fold greater than those in control fish (Table 7), but did not vary with time.

DNA damage. At 1 week there were more strand breaks in the exposed than control fish. After 2 weeks there was no difference between treated and control fish, while after 8 weeks the exposed fish seemed to have fewer strand breaks than controls. After 40 weeks

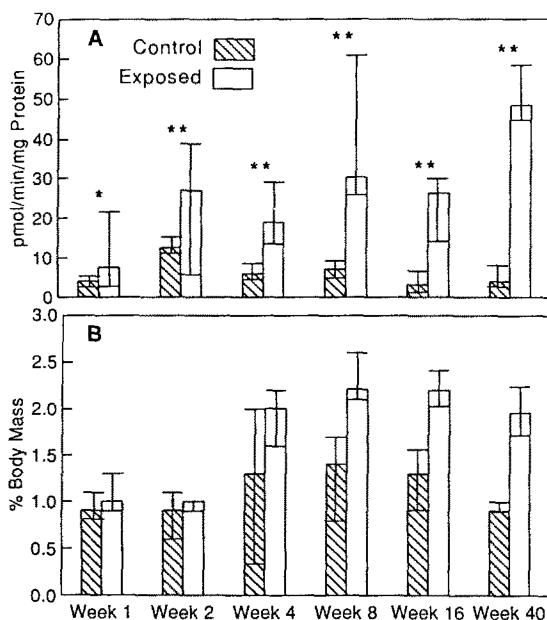

Fig. 3. Ethoxyresorufin-*O*-deethylase (EROD) activity (A) and liver somatic index (B) from the livers of Bluegill Sunfish exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Error bars marked * represent the range $p < 0.1$, and those marked ** represent $p < 0.05$.

Table 5. Median (range) PCB concentrations in Bluegill Sunfish exposed to water equilibrated with EFPC sediment

Congener[a]	PCB concentration (μg PCB per g wet weight) week 1	week 2	week 4	BCF[b]
22′ DCB	2.93 (1.89–3.11)	7.09 (4.46–9.71)	37.90 (37.06–38.81)	726
24 DCB	0.36 (0.24–0.48)	1.64 (1.36–1.92)	7.03 (6.19–8.78)	386
44 DCB	1.69 (0.89–1.89)	3.59 (3.11–5.06)	22.77 (16.13–30.65)	2996
22′4 TCB	1.42 (0.91–1.67)	0.63 (0.29–0.86)	3.40 (1.92–3.96)	3400
22′5 TCB	2.14 (0.96–2.98)	0.88 (0.62–1.14)	1.42 (1.01–1.52)	2029
22′55′ TtCB	0.28 (0.09–0.56)	1.15 (1.17–1.23)	7.99 (3.42–9.73)	2854
22′455′ PCB	0.99 (0.30–1.09)	1.17 (1.07–1.19)	2.46 (2.97–2.95)	3514
22′466′ PCB	1.02 (0.60–1.34)	1.13 (1.09–1.16)	3.73 (3.61–11.01)	3108
22′33′44′ HCB	0.32 (0.05–0.45)	2.15 (2.10–2.19)	6.13 (6.09–6.39)	20 433
22′44′55′ HCB	0.36 (0.09–0.43)	1.30 (1.24–1.36)	3.38 (3.24–3.47)	11 267
22′44′66′ HCB	0.29 (0.08–0.46)	0.63 (0.52–0.74)	1.43 (1.31–3.37)	2024
Σ PCB	12.00	20.12	97.64	

[a]DCB, dichlorobiphenyl; TCB, trichlorobiphenyl; TtCB, tetrachlorobiphenyl; PCB, pentachlorobiphenyl; HCB, hexachlorobiphenyl.
[b]Bioconcentration factor: concentration in the fish ($\mu g\ kg^{-1}$) ÷ concentration in the water ($\mu g\ l^{-1}$). The bioconcentration factor was calculated for the highest concentration (i.e., week 4) only.

Table 6. Median (range) concentration of heavy metals in Bluegill Sunfish tissue exposed to water equilibrated with EFPC sediment

Metal	Metal concentration (ng per g wet weight)				
	Week 1	Week 2	Week 4	Week 8	Week 16
Al	43 (30–56)	46 (32–110)	42 (30–53)	49 (126–52)	41 (85–97)
As	76 (49–80)	60 (57–91)	50 (41–59)	95 (49–140)	80 (23–56)
Cd	13 (7–17)	20 (8–93)	18 (10–26)	17 (5–28)	9 (3–14)
Cu	37 (24–157)	26 (19–106)	20 (7–32)	19 (14–23)	16 (6–26)
Ni	49 (10–92)	32 (13–45)	34 (27–40)	55 (39–70)	29 (24–33)
Pb	65 (38–88)	86 (35–163)	71 (33–109)	88 (25–150)	95 (50–140)
Zn	99 (43–54)	109 (89–128)	79 (42–115)	89 (5–73)	96 (49–142)

of exposure the contaminated fish again appeared to have more strand breaks than controls (Table 7). Short-term results in this study are comparable with other laboratory studies (Shugart, 1988), but different from results from the field (Shugart, 1990). Temporal fluctuations in the level of strand breakage could be due to changes in activity of DNA-damage compensatory mechanisms.

Results from the gel electrophoresis assay indicated that samples with lower F values (i.e., more strand breaks) had a greater electrophoretic mobility (i.e., lower molecular weight) than samples with higher F values (Fig. 5). This indicates that gel electrophoresis could be used as an alternative method for examination of DNA strand breakage. Quantitation could be accomplished with laser densitometry (Freeman and Thompson, 1990). An advantage of this technique is that it requires much less tissue than the alkaline unwinding assay.

BaP-DNA adducts in the gill were apparent after the first week of exposure, with increasing concentration over time (Table 7). In contrast, adducts to liver DNA were detected only in the longest exposures. Blood DNA adducts were apparent after only 1 week and increased more rapidly than in the gill (Table 7). The chromatograms also suggested fluorescent adducts other than BaPDE were also present.

Adducts to the haemoglobin were detected after 1 week of exposure, increased after 2 weeks, then remained relatively constant over time (Table 7). No DNA or hemoglobin adducts were detected in control fish. Measurement of hemoglobin adducts, although less sensitive than DNA adducts, could be used as a surrogate to assess exposure to genotoxins. Other studies show a correlation between hemoglobin adducts, genotoxicity and carcinogenesis in mice *Mus musculus* (Shugart, 1986).

DNA content. DNA content, as determined by flow cytometry, of cells in the G_1 phase of the cell cycle exhibited a normal distribution about the mean. The relative DNA content for each fish was measured as the mean content for G_1 cells, and cell-to-cell variation in DNA content was measured as the % coefficient of variation about this mean. The average amount of DNA was found to be greater in control than in exposed liver cells, but this pattern is significant only at the 0.05 level at 8 and 40 weeks of exposure (Table 7). The cell-to-cell variation in DNA content was slightly greater in exposed than in control fish (Table 7). An exception to this pattern is the variation in DNA content in exposed fish at 8 weeks, which is less than that of control fish. There were no differences

Table 7. Biomarkers indicative of exposure to genotoxic agents in tissues of Bluegill Sunfish exposed to EFPC sediment

Tissue	Week	Sediment[d] Control	EFPC
PAH bile metabolites (μg per ml bile)			
Bile	1	0.20 (0.19–0.21)	67.1 (62.1–86.8)**
	4	0.19 (0.17–0.19)	61.9 (47.1–63.2)**
	8	0.20 (0.19–0.23)	63.1 (57.0–69.3)**
	16	0.17 (0.16–0.19)	61.9 (55.3–64.4)**
DNA strand breaks (F values)			
Liver	1	0.71 (0.59–0.76)	0.46 (0.33–0.50)**
	2	0.58 (0.49–0.59)	0.56 (0.55–0.58)
	8	0.65 (0.47–0.69)	0.84 (0.74–0.94)**
	16	0.68 (0.55–0.70)	0.35 (0.28–0.42)**
			DNA content per cell[b]
Liver	1	65.0 (60.8–69.1)	60.9 (58.8–64.2)
	2	66.9 (66.4–69.0)	65.8 (65.7–65.9)
	8	66.7 (60.8–69.1)[c]	61.1 (60.7–61.3)**
	40	66.8 (61.0–68.3)[c]	62.5 (61.9–64.6)**
DNA cell-to-cell variability[d]			
Liver	1	4.5 (4.3–4.6)	5.6 (4.6–6.1)*
	2	4.8 (4.8–5.7)	5.8 (5.4–6.3)*
	8	4.7 (3.5–5.7)	3.9 (3.8–4.4)
	40	4.7 (4.5–4.8)	5.3 (4.8–5.7)*
DNA–BaP adducts (ng adduct per g DNA)			
Liver	1	ND[e]	ND
	2	ND	ND
	40	ND	2.60 (1.42–2.95)
Gill	1	ND	1.78 (1.62–1.80)
	8	ND	5.58 (1.62–5.74)
	16	ND	22.28 (20.8–24.0)
	40	ND	18.34 :8.69–28.0)
Blood	1	ND	3.41 (3.34–3.48)
	2	ND	20.23 (19.4–21.1)
Haemoglobin–BaP adducts (ng adduct per g haemoglobin)			
Blood	1	ND	0.02 (0.01–0.03)
	2	ND	0.11 (0.10–0.12)
	40	ND	0.12 (0.05–0.18)

[a]Values indicate medians with ranges in parentheses: those marked * are $p < 0.10$ and those marked ** are $p < 0.05$ using the Wilcoxon rank-sum test.
[b]Determined by flow cytometry as mean amount of DNA per cells in G_1; units are relative fluorescence units.
[c]Samples for which n (number of samples) = 6, in all other cases n = 3.
[d]Determined by flow cytometry as coefficient of variation of the mean amount of DNA per cell; units are relative fluorescence units.
[e]Not detected.

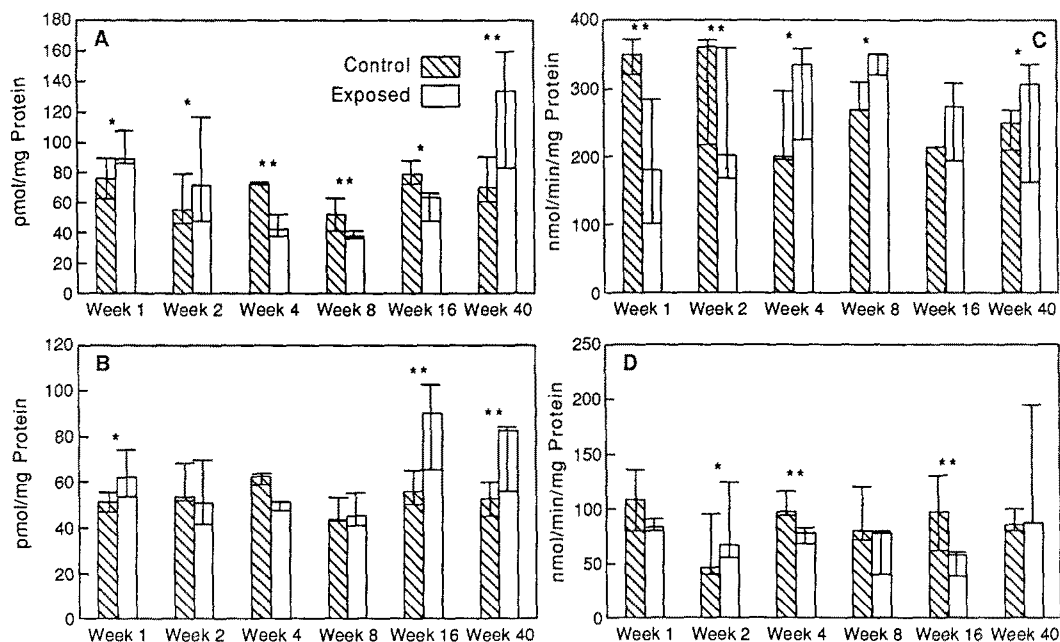

Fig. 4. Microsomal cytochromes P450 (A) and b_5 (B) concentrations, NADH-cytochrome b_5 reductase activity (C) and NADPH-cytochrome *c* reductase activity (D) from the livers of Bluegill Sunfish exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars marked * represent the range $p < 0.10$, and those marked ** represent $p < 0.05$.

between control and exposed fish in the proportion of cells in G1, S or G2 phases of the cell cylce ($p > 0.03$, data not shown). This indicates that flow cytometry can be used to detect genotoxic responses in fish. What is less clear, however, is the molecular basis for these patterns. Because the relative proportions of cells in each phase of the cell cycle are the same in control and exposed fish, it is unlikely that these patterns are due to different rates of mitosis. Increased cellular variability, as a result of mutagen exposure, was observed in small mammals (McBee and Bickham, 1988) and Slider Turtles *Trachemys scripta* (Bickham *et al.*, 1988; Lamb *et al.*, 1991) exposed to environmental genotoxins, and in laboratory rats exposed to the clastogenic drug triethylenemelamine (Bickham *et al.*, 1992). Although the molecular basis for the alteration of mean DNA content is unclear, increased cell-to-cell variability is likely due to choromosomal breakage (Bickham, 1990; Bickham *et al.*, 1992).

Blood, kidney and spleen. ALAD activity, when EDTA and citric acid were used in the assay, was greater in exposed than in control fish (Table 8). This pattern was the opposite of that found in other studies of fish exposed environmentally to lead (Hodson *et al.*, 1980). When EDTA and citrate were replaced with heparin and HCl, the activity of the exposed fish was found to be lower than that of control fish (Table 8). It should be noted that the control fish did not seem to be affected by the assay protocol. ALAD inhibition

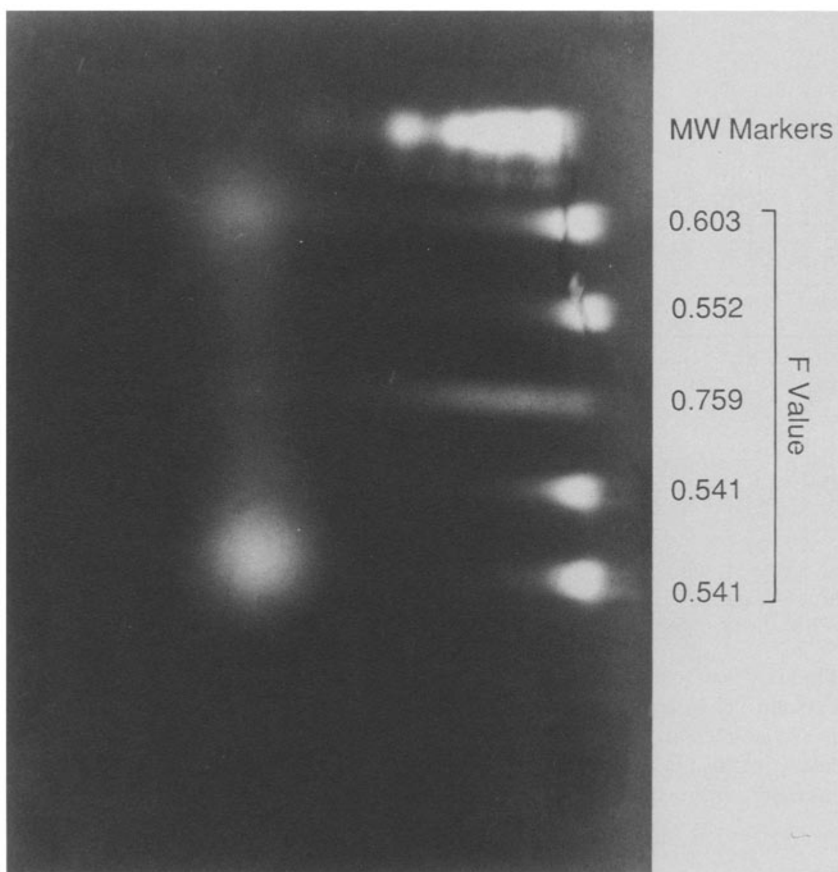

Fig. 5. Photograph of an agarose gel in which DNA from the livers of Bluegill Sunfish have been electrophoresed. The electrophoresis was run under denaturing conditions to differentiate between DNA samples with different numbers of strand breaks. The DNA was stained with ethidium bromide and photographed under UV light. 'F values' indicated above the lanes were obtained using the alkaline unwinding assay, and are inversely proportional to the number of DNA stand breaks.

is due to noncovalent binding of the metal to the protein (Finnelli *et al.*, 1975), so the presence of chelators in the assay may promote dissociation of metals from the ALAD molecules. If so, higher ALAD activity in exposed fish with chelators suggests higher concentrations of enzyme than in control fish, which may be a compensatory response to low enzyme activity. Further work is needed to clarify these results.

Haematocrits were higher in exposed fish after one week, but fell below controls thereafter (Fig. 6). One should be aware that hematocrit is also affected by physiological and reproductive states (Blaxhall, 1972); thus, field results should be interpreted with caution.

ZPP content of the blood was consistently higher in treated fish, with the values increasing over time (Fig. 6). It has been shown that zinc can substitute for iron in heme

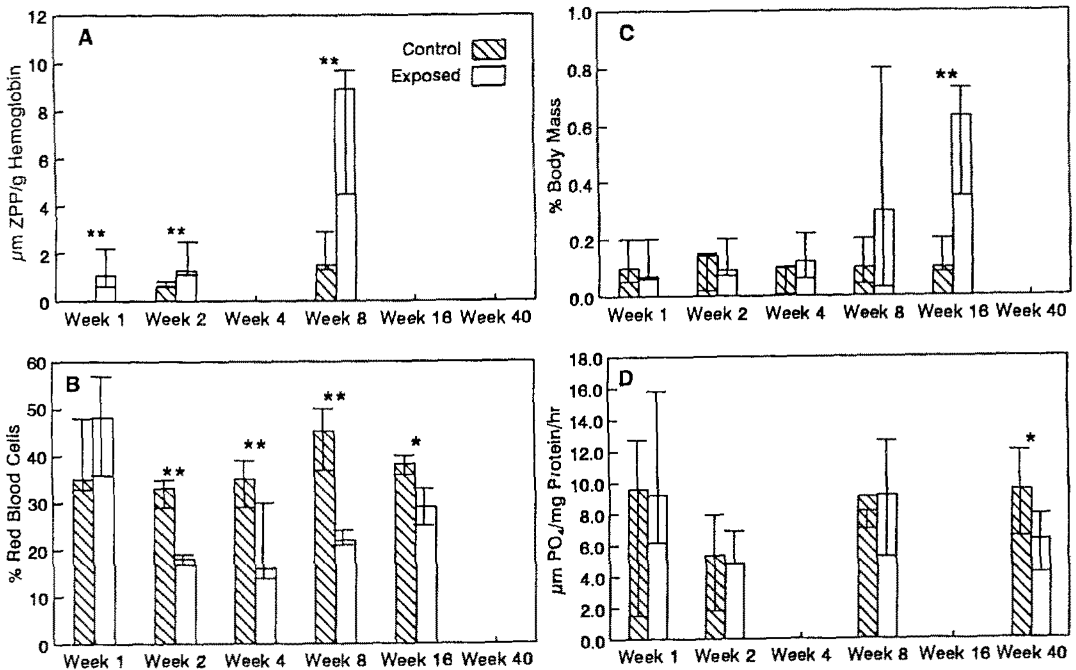

Fig. 6. ZPP content of the blood (A), hematocrits (B), spleen somatic indices (C), and kidney ATPase activities (D) of Bluegill Sunfish exposed to water contaminated with PAHs, PCBs and heavy metals over several weeks. Both control and exposed fish are indicated. Error bars marked * represent the range $p < 0.10$ and those marked ** represent $p < 0.05$.

molecules if porphyrin synthesis is disturbed by lead (Cory-Slechta *et al.*, 1989; Labbe and Rettmer, 1989) or other metals (Missenar *et al.*, 1989; Flora *et al.*, 1991). Thus, an elevated level of ZPP in the blood can be used as a biomarker of pollutant exposure; however, to our knowledge, there are no studies that have used the levels of ZPP in fish blood as a biomarker. It has been found, interestingly, that Great Blue Herons *Ardea herodias* roosting along Poplar Creek have ZPP levels very similar to the exposed fish at week 8 (unpublished data). The trend of increasing response over time can be seen with the spleen somatic index, but the only significant difference between control and exposed fish was week 16 (Fig. 6). These markers indicate a general disruption in hematopoietic function.

Kidney ATPase activity in exposed fish was not depressed until week 16 (Fig. 6). Indications of renal damage, tubular degeneration and the presence of eosinophilic inclusion bodies in tubular epithelial cells, occurred in two of the three fish at week 16. No histopathological lesions were found in kidneys of control specimens from that time period. Thus, tubular damage is indicated at both the tissue and biochemical levels after chronic exposure to toxicants.

Stress protein analysis. Western blots of HSP_{70} from the gills indicated that this stress protein is present at higher concentrations in exposed fish at weeks 1 and 2. However,

Table 8. δ-Aminolevulinic acid dehydratase activity (nmol per ml red blood cells per h) for Bluegill Sunfish exposed to EFPC sediment

Treatment	Week	Sediment[a] Control	EFPC	Alpha value[b]
With chelators[c]	2	888 (789–974)	985 (931–2273)	0.10
	4	753 (699–753)	1238 (769–1486)	0.05
	8	750 (626–874)[d]	990 (877–1047)	0.10
	16	801[e]	943[e]	–
Without chelators	1	867 (863–914)	917 (823–1081)	0.35
	2	852 (652–922)	372 (265–578)	0.05
	8	899 (737–1046)	712 (688–753)	0.10

[a]Values indicate medians with the ranges in parentheses. $n = 3$ for all samples unless otherwise indicated.
[b]Probability levels for the Wilcoxon rank-sum test.
[c]Metal chelators used in the assay were EDTA and citrate. Chelation of metals could affect inhibition of ALAD by lead.
[d]Sample with $n = 2$.
[e]Sample with $n = 1$.

there was no difference in the amount of HSP_{70} from weeks 4 and 8, and by week 16 the amount of this stress protein in exposed fish was less than that from controls. Total staining of gill proteins indicated that proteins of 55 and 90 kDa were expressed more strongly in exposed fish from 1, 2, 4, and 8 weeks of exposure. However, the 90 kDa protein did not cross-react with anti-HSP_{70}, as it does in Daphnids (Bradley, 1992) and other species (C. Gonzalez and B.P. Bradley, unpublished data). Both Western blot and total protein analysis revealed that some exposed fish responded to a greater degree than others, while some did not respond at all.

Chromosomal protein analysis. Total staining of red blood cell chromatin proteins revealed that a wide suite of proteins, ranging in size from 14 kDa to greater than 100 kDa, showed striking changes in temporal patterns of expression in exposed fish. These data are summarized in Table 9. After 1 week of exposure, there was a decrease in the concentration of most chromatin proteins of exposed fish compared with controls. By the second week, and continuing through week 4, there seemed to be a general increase in expression of chromatin proteins in exposed fish. Proteins in exposed fish were once again depressed at week 8. The data from weeks 16 and 40 indicate a general induction of proteins, except for those > 100 kDa, which showed a decrease in exposed fish. In addition, the proteins tentatively identified as histones H1 and H5 showed a shift from three bands in control fish to two in the exposed, resulting from loss of putative H5 histone. This may indicate a change in the state of red cell differentiation in exposed fish. In vertebrates with nucleated red cells, there is an increase in the portion of H5 histone relative to the related H1 histone as erythrocytes age and the nuclei become pycnotic (Palyga, 1990; Greenaway and Murray, 1971). Therefore, in exposed fish a loss of older mature erythrocytes may occur concurrent with increased recruitment from precursor cells in exposed fish. At week 40, the protein bands tentatively identified as the histone H2a–H2b–H3–H4 nucleosome complex showed a change from the two intense bands observed in control fish to three in the exposed. The histones were identified by their

Table 9. Comparison of the relative intensity of chromosomal protein bands[a] from Bluegill Sunfish exposed to EFPC sediment, examined in a polyacrylamide electrophoretic gel

	Week					
Protein	1	2	4	8	16	40
H2–H3[b]	ND[c]	ND	ND	ND	ND	2→3[d]
20 kDa[e]	C>E	ND	ND	C>E	ND	E≫C
H1–H5	3→2[f]	ND	ND	3→2	3→2	3→2
36–42 kDa[g]	C>E	ND	C>E	C>E	ND	C>E
43 kDa	C>E	ND	ND	E>C	E>C	E≫C
50 kDa	C>E	E≫C	ND	C>E	C>E	E≫C
60 kDa	C>E	ND	ND	C>E	ND	E≫C
70 kDa	C>E	E≫C	E≫C	C>E	C>E	E≫C
78 kDa	C>E	E>C	E≫C	C>E	E≫C	E≫C
48–97 kDa[g]	C>E	E>C	C>E	C>E	E>C	E>C
>100[g]	C>E	E≫C	C>E	C>E	C>E	C>E

[a]E > C signifies that this band is more intense in exposed fish; E ≫ C signifies that this protein band is much more intense in exposed fish; C > E signifies that this protein band is more intense in control fish.
[b]Indicates a histone complex.
[c]No difference between control and exposed fish.
[d]Indicates a condition where there are two protein bands in this complex for control fish and three protein bands for exposed fish.
[e]Identifies an unknown protein by approximate molecular weight (kDa).
[f]Indicates a condition where there are three protein bands in this complex for control fish and two protein bands for exposed fish.
[g]A suite of protein bands not previously identified, but showing similar patterns.

migration in SDS gels relative to chicken erythrocyte standards, and by the fact that these protein bands comprise greater than 50% of chromosomal protein in fish erythrocyte nuclei. The nucleosome histone densitometric profiles were invariant for both control and exposed fish except for week 40. The alterations seen at week 40 may be the result of histone modification and changes in chromatin packaging or structure, possibly as a result of a change in the composition of red cell maturational stages or toxicological effects on erythrocytes.

Interestingly, the gill stress proteins and blood chromatin proteins exhibit temporal responses similar to each other and to the trends of DNA damage. At present, however, the precise relationship between stress proteins, chromatin proteins and strand breakage is not known, but the chromatin proteins of molecular weights 43, 50, 60, 70 and 78 kDa (Table 9) were highly expressed in exposed fish relative to controls at week 40. A number of stress proteins are homogolous in molecular weight to those (70 kDa) induced in this study. Specific antibody probes to stress/heat shock proteins may be used to identify the class of proteins induced in fish exposed to anthropogenic agents. To our knowledge, there is no other study which has used fish blood chromatin proteins as a biomarker of exposure.

Comparison between biomarkers

A significant aspect of this study is that patterns of biochemical and molecular responses are also seen at the organ or tissue level. For example, both EROD activity and liver

somatic indices (LSI) show a trend of increasing magnitude of the response with time (Fig. 3). Both factors seem to be reliable indicators of liver damage, since focal or single cell necrosis also occurred in fish with elevated EROD and LSI values. Furthermore, skeletal muscle concentrations of PCBs also increase with time. Although liver concentrations of PCBs were not determined, it may be assumed that they too increased over time, and this may be a partial explanation for increasing values for EROD and LSI over time.

Also, ATPase activity seems to be a good correlate of tissue damage. For example, depression of ATPase activity of the gill was apparent after only 1 week, and the relative amount of inhibition remained stable over time (Fig. 2). The same was true of tissue damage to the gill. In the kidney, on the other hand, both ATPase depression (Fig. 6) and tubular damage were apparent only after long-term exposures.

Molecular responses at the DNA level also paralleled the cytological responses. In comparing the results from flow cytometry and the alkaline unwinding assays, it can be seen that the amount of cell-to-cell variation in DNA content is lowest at 8 weeks, and that the number of DNA strand breaks in exposed fish is also lowest at 8 weeks (Table 7). This suggests some relationship between these two parameters, the common factor probably being DNA damage and/or repair, but the molecular mechanism behind this relationship remains to be elucidated.

It is also interesting to note that different biochemical markers from the same organ show similar patterns. For instance, both brain ATPase and AchE activities are initially induced in exposed fish relative to controls, but are later inhibited (Fig. 1). This is not surprising, since both enzymes are membrane-bound proteins found in high concentrations in neuronal tissue (Alberts *et al.*, 1983).

Also, organs which share related functions show similar trends of response. In this respect, both the kidney and the spleen are involved in haematopoiesis in fish (Gromman, 1982). Both organs also show delayed responses to toxicants, indicating a general disruption of haematopoietic function after chronic exposure. ZPP levels also increased dramatically after chronic exposure (Fig. 6), and this may be a correlate of haematopoietic function. Two other correlates of haematopoiesis, ALAD activity (without chelators) and hematocrit, show parallel temporal patterns of response. Both biomarkers in exposed fish are equal to or slightly greater than those of control fish after 1 week, but fall below controls thereafter (Table 8, Fig. 6). This is understandable, since ALAD is the rate-limiting step in the synthesis of haeme (Labbe and Rettmer, 1989), and is an essential component of blood formation.

The results from some organs, on the other hand, show different patterns of response from others. DNA adducts in the gill and blood appear earlier and at larger concentrations than in the liver (Table 7). The rapid accumulation of DNA adducts in blood compared with gill may reflect the fact that mature red blood cells lose their ability to synthesize DNA (Mahajan and Dheer, 1980), so they may have reduced DNA repair capabilities as well.

Generally, the fact that biomarker responses follow similar trends suggests that information from one marker or organ can imply information about other markers or organs. One example is that depression of ATPase activity seems to be closely correlated with tissue damage. As another exmaple, DNA–BaP adducts in the blood seem to be an early indication that these adducts will show up in the liver at a later time point.

Biomarkers in the gill, intestine, and blood seem to respond quickly to contaminants. In general, responses remain relatively stable over time, with the exceptions of stress and

chromatin proteins. Biomarkers in organs which are internal to the site of exposure (brain, kidney, liver, spleen) show increasing responses over time. Several factors could play a role in these phenomena, for example: 1, the gill and intestine are directly exposed to the toxins; 2, there may be tissue-specific differences in the amount of resistance to or recovery from the action of toxicants; 3, the response of internal organ alterations over time may be due to accumulated injury or to the action of compensatory systems which can cope with short- but not long-term insult; 4, xenobiotics may equilibrate within the various tissues at different rates. Heavy metals reached equilibration relatively quickly, but PCB concentrations increased over time. This may be an important consideration in fatty tissues, such as the liver and brain; 5, physiological acclimation to the toxic environment may result in shifting patterns of responses.

Comparisons with EFPC studies

In general, results from the longer laboratory exposures more closely resemble those found in EFPC fish. This is particularly true for DNA strand breakage and liver mixed-function oxidase. Although these responses in the laboratory are qualitatively similar to those in Redbreast Sunfish in EFPC, the magnitude of the response is much greater in EFPC fish than in the laboratory (Table 10). This could be due to the fact that the EFPC fish are subjected to lifetime exposures, or to differences between Bluegill and Redbreast Sunfish. In addition, it had been found that certain biomarkers (e.g., DNA strand breaks) show a much more marked response to toxicants when the animals are stressed

Table 10. Summary of values for biomarkers from Redbreast Sunfish living in East Fork Poplar Creek

	Field data[a]		Laboratory data[b]	
Marker (units)	Reference stream	EFPC	Control sediment	EFPC sediment
Microsomal cytochrome P450 content (pmol per mg protein)	400	80	80	120
Microsomal cytochrome b_5 content (pmol per mg protein)	40	100	275	325
Microsomal NADH-cytochrome b_5 reductase aactivity (nmol per mg protein per min)	230	380	60	90
Microsomal NADPH-cytochrome c reductase activity (nmol per mg protein per min)	90	170	110	120
EROD activity (pmol per mg protein per min)	2	120	5	50
Liver somatic index (% body mass)	1.0	1.6	1.0	2.0
Strand breaks (N value)	–	4.0	–	1.7

[a]Sources: Loar *et al.* (1988) Jimenez *et al.* (1990) and Shugart (1990).
[b]Source: this study at longest exposure period.

with temperature or organic solvents (Shugart, 1988). In the laboratory, the fish are kept at a constant temperature, fed *ad libitum*, and supplied with water that is fully saturated with air. Thus, for fish in the laboratory the number of stressors other than xenobiotic stress is kept to a minimum, whereas this is not true for EFPC fish. Natural selection, poulation and community interactions, abiotic mediating factors in the field, or any combination thereof, could also affect these responses.

In addition, the variability of the response between individuals may reflect genetic differences in the ability to resist or adapt to toxicological stress. In many cases, the variability in unexposed animals is somewhat less than in exposed animals (e.g., see Figs 3 and 4). In fact, increased variability in responses has been proposed as a biomarker in itself (M.S. Adams, Environmental Sciences Division, ORNL, personal communication). Obviously, inherent differences in the resistance to stressors will not become apparent until the fish are stressed. Individuals which are more adept at compensating for any physiological perturbation, however slight, would have a selective advantage over others in the population.

Comparisons with other studies

This study is also significant in that it is an important link between single-contaminant laboratory studies and field studies, which often examine a wide suite of organic and inorganic chemicals. Note that biomarkers seen with exposure to single toxicants are also seen when the fish are exposed to EFPC sediment. For example, BaP adducts are a specific marker for BaP exposure (Shugart, 1987). In this study, the fish were exposed to BaP as well as numerous other PAHs, organic, and inorganic compounds, but still displayed responses similar to single-exposure experiments.

Another significant aspect of this study is that the results for some Bluegill biomarkers show the same trends as results from other fishes or even other vertebrates, for example, rodents (Shugart, 1987; McBee and Bickham, 1988), turtles (Bickham et al., 1988) or herons (see above). This is an important component of linking biomarker data with community and ecosystem level responses. Also, the ecological relevance of single-species tests is strengthened by the fact that results from one species are applicable to other species. In particular, this implies that there is no need to sample every species in an ecosystem in order to demonstrate an effect of anthropogenic contamination on that ecosystem.

As a final note, we would like to point out that there are water-associated factors (e.g., Ca^{2+} concentration, water hardness, pH, and dissolved organic carbon) which may potentiate the availability and toxicity of xenobiotics in the sediment. These factors may modulate xenobiotic interactions between the sediment and water (e.g., partitioning, adsorption/desorption, etc.). In this study, as in field studies, these interactions were allowed to take place during exposure of the fish.

Conclusion

The essence of our findings may be summarized as follows. 1, Laboratory results using contaminated sediment parallel those found in fish collected from the site of the sediment's origin. This illustrates that laboratory and field studies supplement each other. The responses of fish exposed to the sediment in the laboratory are similar to but

lesser in magnitude than EFPC fish, perhaps due to species-specific differences, lifetime toxicant exposures, natural selection, population and community interactions, or abiotic mediating factors in the field. 2, The duration of a laboratory exposure requires consideration, since different biomarkers show different trends of response over time. Choosing a biomarker appropriate for the time course of the study is an important consideration. Certain biomarkers are affected by xenobiotics but remain stable over time, so they should provide comparable field and short-term laboratory data. Some biomarker responses fluctuate over time, while others show steady time-dependent increases. For these markers only long-term laboratory data may be comparable with the field. 3, Variation in biomarker response can be quite large. This may have a genetic basis and could have evolutionary and ecological implications. 4, Certain biomarkers show very similar trends over time, while others are quite different. This has to be taken into account when deciding which organ or organ system to use as biomarkers. Overall, the blood and the organs at the site of exposure seem to respond quicker and with greater magnitude than internal organs. Internal organs seem to be more indicative of chronic exposures. Generally, the blood seems to be a good indicator of stress, and has the advantages that it is easy to collect and data can be recorded from live specimens. Thus, data can be collected from one fish over several points in time, which may reduce individual variability in time-course experiments. Also, for field studies, animals do not need to be permanently removed from the ecosystem. Finally, non-lethal biomonitoring may seem more acceptable to certain aspects of society.

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