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BIOMARKERS FOR REDOX-ACTIVE GENOTOXINS IN CONTAMINATED SEDIMENTS: A MECHANISTIC APPROACH

by
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Abstract

Feral brown bullhead catfish and environmental sediments were collected from three sites in the Niagara River system in northern New York state. The sites were Black Creek, a relatively uncontaminated reference site, the Love Canal - 102nd Street dump site, principally contaminated with chlorinated hydrocarbons (CHs) and chlorinated pesticides, and a site in the Buffalo River, principally contaminated with polycyclic aromatic hydrocarbons (PAHs). A variety of putative biomarkers of genotoxicity and/or oxidative stress was measured in hepatic tissues of these fish. Both brown bullhead and channel catfish were also exposed to the sediments and sediment fractions under laboratory conditions and similar metabolic indices were evaluated in hepatic tissues of these fish. In general, metabolic responses were more pronounced in feral fish than in laboratory exposed fish. The most sensitive biomarkers of exposure to the contaminated sediments, both in situ and in the laboratory, appeared to be the levels of fluorescent aromatic compounds in the bile of the fish and the activity of hepatic ethoxyresorufin-O-deethylase, an indicator of cytochrome P4501A activity. Levels of cytochromes P450 and P4501A and of hydrophobic xenobiotic-DNA adducts also showed marked responses in feral fish. Fish from the site enriched with PAHs (Buffalo River) showed the highest levels of bile FACs and DNA adducts. Fish from the site enriched with CHs (Love Canal) had the highest levels of cytochromes P450 and P4501A and also the highest hepatic EROD activities. There were no essential differences in the responses of channel catfish and brown bullhead to sediment exposure under laboratory conditions. These results indicate that metabolic indices of benthic animals can successfully be used as biomarkers of exposure to environmental contaminants, especially when a suite of responses is evaluated, and that strong associations between specific responses and classes of chemicals suggest that a further refinement of this approach is feasible.

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Summary and Recommendations

Past research documents that inland waterways in the United States are contaminated to varying degrees with organic compounds that are potential carcinogens, especially certain polycyclic aromatic hydrocarbons (PAHs) and chlorinated hydrocarbons (CHs). Extensive epizootics of neoplasia in benthic fish found in contaminated waterways have also been found. The role of these and other compounds in the development of human cancer and the risk that they present to human populations remains unclear. Even a thorough evaluation of contaminant types and levels in diverse aquatic ecosystems is difficult, expensive and time-consuming. The development of sensitive methodologies, economical in terms of both time and money, and accurate in their assessment of the kinds and levels of potential carcinogens, is obviously desirable to address this situation. Such methodology should be based on a thorough understanding of the underlying metabolism and physiology of the animals involved, and ideally should assess various sub-lethal effects of chemical pollution rather than acute symptoms, allowing time for possible remediation. It is with this approach in mind that the research described in this report was undertaken.

The riparian lands of the Niagara River system in northern New York state consist of both relatively unimpacted areas as well as major metropolitan and industrialized regions. The pollution status of many areas in the river system has been thoroughly documented and the Niagara River has been the subject of previous work of this sort. In accordance with ongoing studies conducted by the US Fish and Wildlife Service and with the assistance of personnel from Buffalo State College, we collected samples of aquatic sediments and resident brown bullhead catfish (Ameriurus nebulosus), a demersal species endemic to the area, from three sites. A tributary of the Niagara River known as Black Creek, a slow-moving, relatively uncontaminated stream in Canada, was chosen as the reference site. Two heavily polluted sites were also chosen. A site in the Buffalo River impacted by metropolitan runoff and polluted by effluents from a variety of heavy industries was selected because it was enriched in PAHs. Another site in a bay near the infamous Love Canal-102nd Street dump site was chosen because it is heavily loaded with CHs and chlorinated pesticides. Hepatic tissues from fish taken from these sites were evaluated for a number of metabolic indices associated with genotoxicity and/or oxidative stress from exogenous chemicals. Sediments from these sites and fractions thereof were also used to expose both brown bullhead and channel catfish (Ictalurus punctatus) in a controlled, laboratory setting so similar measurements could be made.

Determination of fluorescent aromatic compounds (FACs) in the bile of the fish served as a direct measure of exposure to hydrophobic xenobiotics. Indices of oxidative stress examined included ethoxyresorufin-O-dethylase activity (EROD) , levels of cytochrome P450 and P4501A, and measurement of NADPH cytochrome P450 reductase, all of which have been previously shown to be induced by xenobiotics that can potentially cause oxidative damage. Indices of genotoxicity included a determination of the percentage of single strand breaks in hepatic DNA as evaluated by an alkaline unwinding assay, determination of the oxidatively damaged nucleotide 8-hydroxy-deoxyguanosine, and direct measurement of xenobiotic-DNA adducts by ^{32}P-postlabeling. Cellular glutathione status, which has also been shown to be affected by exposure to various xenobiotics, was also measured.

The major findings of our study were as follows:

(1) Overall, the most sensitive biomarkers of exposure to the contaminated sediments appeared to be the levels of FACs in the bile of the fish and hepatic EROD activities. Cytochrome P450 and P4501A levels and levels of hydrophobic xenobiotic-DNA adducts, as detected by ^{32}P-postlabeling (PPL), also showed marked responses in feral fish. Fish from the site with the highest concentrations of PAHs (Buffalo River) showed the highest levels of bile FACs and DNA adducts. Fish from the site dominated by CHs exhibited the highest levels of cytochrome P450, P4501A and had the highest hepatic EROD activities.

(2) There were no essential differences in the responses of channel catfish and brown bullhead to exposure to the sediments under laboratory conditions. Difficulties experienced with the laboratory exposures made interpretation of the results more difficult, but in general, responses were less marked than in the feral fish.

The fact that some of the potential biomarkers examined showed differential responses to different suites of contaminants bodes well for this approach to environmental monitoring. The results of this research indicate that the evaluation of a suite of biomarkers, carefully chosen on the basis of potential effects of putative contaminants on the metabolism of the sentinel organism, can accurately assess the type of contamination at a given site, although more studies of this sort are obviously necessary to refine the methodology. These results also do not rule out laboratory exposures of animals to contaminated environmental sediments as an effective means for the appraisal of the pollution status of many different sites in a limited amount of time. These methods should prove still more efficacious when combined with others, such as sediment chemical analysis and tests to determine the toxicity of specific contaminants on selected benthic species.

Introduction

Neoplasia in Fish

It has been well documented that many bodies of water in the United States are contaminated to varying degrees with potentially carcinogenic substances, but the role of this pollution in the etiology of human cancer remains unclear. It has been generally recognized that chemical pollution of aquatic environments is associated with elevated rates of neoplasia in fish, and this is a cause for concern as to possible effects on human health. The goal of our research is the development of sensitive methods, soundly based on knowledge of fundamental biochemical mechanisms, for the assessment of the carcinogenic potential of sediments from polluted aquatic environments.

Elevated rates of neoplasia have been documented for many fish species from chemically polluted waters. Species include English sole (Parophrys vetulus) from the Puget Sound (Malins et al. 1985; Malins and Haimnot 1991), tomcod (Microgadus tomcod) from the Hudson River (Smith et al. 1979), mummichog (Fundulus heteroclitus) (Vogelbein 1991), white suckers, (Catostomus commersoni) from Lake Ontario (Sonstegard 1978) and the Detroit River (Maccubbin and Ersing 1991), who also found tumors in brown bullhead (Ameriurus nebulosus), redhorse suckers (Moxostoma sp.), bowfin (Amia calva) and walleye (Sitzostedion vitreium). Neoplasia have also been found in brown bullhead form the Fox River in Illinois (Brown et al. 1973), the Black River in Ohio (Baumann et al. 1982), and other sites in the midwest (Baumann et al. 1991). In 1983, Black reported the frequency of neoplasia in freshwater drum (Aplodinotes grunniens) from polluted sites to be 8.85%, while fish from a relatively clean site had neoplasia frequencies ranging from 0 - 3.7%. Neoplasia have also been documented in bivalves; Mix (1986) has reviewed these instances.

Aquatic sediments are composed of inorganic and organic particulate matter that eventually settles to the bottom of the water. Hydrophobic compounds introduced into the water can adsorb to the surfaces of these particles and be deposited in the sediments, often leading to much higher concentrations of these compounds in the sediments than in the overlying waters. Contaminants are also more persistent and less mobile in sediments than in the overlying waters. Organic chemical pollutants in sediments include polychlorinated biphenyls (PCBs), chlorinated pesticides, polycyclic aromatic hydrocarbons (PAHs), polycyclic aromatic ketones (PAKs) and quinones, and nitrogen containing compounds such as carbazoles and amines (Malins et al. 1984; Rogerson et al. 1985; West, et al. 1986). Compounds from each of these classes have been found to be genotoxic, redox-active and carcinogenic. Similar xenobiotics have been found in tissues of aquatic animals.

Chlordane and PCBs were found in tissues of channel catfish from the lower Mississippi River (Leiker et al. 1991). Christiansen et al. (1991) found chlordane, PCBs, DDT, dieldrin, heptachlor, and trifluralin in muscle tissue of channel catfish from the Missouri River, adjoining Nebraska. The Niagara River Toxics Committee (NRTC) reported high levels of PCBs, mirex, chlordane and dioxin in spotted shiner (NRTC, 1984). Hickey and co-workers (1990) found high levels of PCBs (530 - 2200 ng/g tissue), chlordane (10-20 ng/g tissue) and metabolites of DDT (30 - 90 ng/g tissue) in brown bullhead taken from the Niagara River in the vicinity of Love Canal, one of the sites from which sediments and fish were sampled for this study.

In 1957, a correlation between aquatic pollution and tumors in fish was first suggested, and it is now generally accepted that such a relationship indeed exists. It has been much more difficult to associate a particular chemical with elevated tumor frequencies observed in situ. Malins and co-workers (1984; 1985) statistically analyzed data on sediment contaminant levels, tissue contaminant levels and frequencies of neoplasia and found significant correlations between the presence of neoplasia and levels of PAHs in tissue and bile of fish. A number of other studies (Varanasi et al. 1982; 1985; Hendricks et al. 1985; Shugart et al. 1987) have also examined potential roles for PAHs in carcinogenisis in aquatic animals. Studies have shown the transformation of benzo(a)pyrene (BaP) to its diol metabolites by channel catfish (Hinton et al. 1981) and by brown bullhead (Swain and Melius 1984; Sikka et al. 1990a; b). There have also been studies showing that microsomal fractions (S9s) from several species of fish can activate PAHs and aromatic amines to mutagenic substances in the Ames test (Balk et al. 1982; Guobatis et al. 1986; Milling and Maddock, 1986; Winston et al. 1988). Unpublished data from our laboratory has also demonstrated that channel catfish S9 can enhance the mutagenicity of fractions of contaminated sediments. Other studies, using mammalian S9 to enhance the mutagenicity of sediment fractions have also been done (Sato et al. 1983; West et al. 1986; Fabacher et al. 1988). These studies have indicated that sediment fractions that have the greatest genotoxic potential are enriched in PAHs, carbazoles, aromatic amines, and various other nitrogen-containing polycyclic aromatics and quinones.

Given the above, it is obviously beneficial to have at hand practical, relatively easy methods to assess aquatic contamination. Chemical analysis of sediments is time-consuming, labor intensive, and costly. Observations of extensive occurrence of neoplasia in fish populations is generally diagnostic of contamination of the aquatic environment with carcinogens, but the surveys necessary to document these epizootics are also expensive and time-consuming to do. Additionally, they only provide evidence that damage has been done; it would be much more desirable to have an earlier warning of potential problems. Ergo, it is desirable to document sub-lethal effects associated with exposure of fish to the compounds of interest and to assess these endpoints as biomarkers of exposure. It is with this approach that our study is concerned.

Justification of Fish Species Utilized

We have used both channel catfish and brown bullhead as model organisms in the study described herein. The rationale for the use of both species is as follows.

Channel catfish are an important species in aquaculture and as such have been well studied. Considerable information on their physiology and metabolism is available. The nutritional requirements of the species is well documented (National Research Council 1983), and several nutritionally adequate diets are commercially available. Modified diets can also be easily obtained. Channel catfish have been used frequently in routine aquatic toxicity studies, so background toxicological data are extant; some of these data have been obtained by our laboratory, and support the appropriateness of our selection of the species. Fish are commercially available in various sizes and ages and are easily adapted to culture in the laboratory.

There are drawbacks to the use of channel catfish as well. They are not present in every contaminated body of water, even though they are widely distributed. We are unaware of any reports of tumors observed in channel catfish although, as referenced above, significant concentrations of potential carcinogens have been found in tissues of feral fish.

Tumors have been frequently reported in another species of catfish, the brown bullhead, (Baumann et al. 1982; Black 1983; Grizzle et al. 1984; Fabacher and Baumann, 1985) which may also be a more truly demersal species than the channel catfish. The species is also widely distributed, and it is abundant in the Niagara River, the freshwater system studied in the project reported on herein, as well as in waters in North Carolina near the location of our laboratory. Brown bullhead are more difficult to adapt to and maintain in the laboratory and are commercially available only on a limited basis. While available data suggest that brown bullhead are more prone to cancer than channel catfish, this is by no means a well documented hypothesis, for there are little comparative data on the two species. Data obtained on both species as biomonitors are therefore valuable.

Rationale for Selection of Biomarkers

Exposure of fish to potentially carcinogenic compounds has been shown to be associated with a number of metabolic effects short of tumors. These include increased mixed function oxidase (MFO) activities, particularly EROD activity, with concomitant increases in cytochrome P450 levels, detection of PAH metabolites in bile, alterations in the levels hepatic glutathione and direct damage to DNA. Each of these is discussed more fully below.

Measurements of MFO activities. Many organic contaminants, including PCBs and PAHs, induce hepatic MFO components in fish, while others, such as mirex, kepone and DDT, do not (Lech et al. 1982). Induction of MFO associated activities by xenobiotics has been reported in rainbow trout (Onchorynchus mykiss) (Vodicinik et al. 1981), carp (Cyprinus carpio) (Melancon et al. 1981), brook trout (Salvelinus fontinalus) (Addison et al. 1982) and scup (Stenotomus chysops) (Stegeman et al. 1981). Field studies showing MFO induction in fish taken from contaminated environments include those of Klotz and co-workers (1983) with scup, Stegeman's group (1987) with winter flounder, Monod et al. (1988) with nase (Chondrostoma nasus), Elskus and Stegeman (1989a,b) with marsh killifish (Fundulus heteroclitus) and VanVeld et al. (1990) with spot (Leistomus xanthurus).

MFO induction has been implicated in increased risks of cancer and genetic damage because biotransformation of xenobiotics by the MFO system can produce intermediates far more mutagenic than the parent compound. BaP, a known carcinogen, is metabolically converted to its proximate carcinogen, the 7,8-dihydrodiol, by the hepatic P450 system of brown bullhead (Steward, et al, 1990a,b), English sole and starry flounder (Platichthys stellatus) (Varanasi, et al. 1989). Such reactive metabolites can bind directly to DNA, forming mutagenic adducts (McCarthy, et al. 1989, Sikka, et al, 1990b).

The MFO system is composed of multiple isozymes, the activities of which are associated with cytochrome P450. Cytochrome P450 has been found in mammals, birds, fish, amphibians and various invertebrates, including mollusks, crustaceans and insects (Bend and James 1978; Livingstone 1990). The MFO system consists of the cytochrome P450 isozymes and NADPH-cytochrome P450 reductase. Electrons flow from NADPH through the reductase flavoprotein to cytochrome P450, which inserts one atom of dioxygen into the substrate being oxygenated and reduces the other oxygen atom to water. (Guengrich and Leibler 1985) Cytochrome P450 isozymes have different catalytic activities. Induction causes de novo synthesis of cytochrome P450 protein; specific isozyme induction depends on the particular operators targeted by each inducer. Delineation of the induction of various isozymes has been proposed as a biomarker for environmental pollution (Payne et al. 1987).

Induction of the MFO system can be measured in several ways. Total P450 content can be determined spectrophotometrically. Activities of various isozymes can be directly assayed by measurement of the transformation of specific substrates. P450 isozymes belonging to immunologically cross-reactive groups can be assayed with antibodies. Measurement of NADPH-cytochrome P450 reductase is also possible.

Direct spectrophotometric determination of total cytochrome P450 measures both constitutive and inducible proteins and hence does not accurately reflect specific catalytic function. However, degradation of cytochrome P450 to the inactive p420 form can be accurately assessed by this method. The extent of this degradation is variable and is heavily influenced by sample handling and preparation (Stegeman and Kloepper-Sams, 1987).

Specific isozyme activities that have been measured include aryl hydrocarbon hydroxylase (Ahh), which uses BaP as a substrate, ethoxycoumarin-o-deethylase activity and EROD activity. EROD activity has been shown to be a sensitive indicator of contaminant exposure (Masfaraud et al. 1990). Increased EROD activity is sometimes seen in fish inhabiting PAH contaminated waters (Elskus and Stegeman 1989b; VanVeld et al. 1990; Di Giulio et al. in press) and also in laboratory studies in which fish were exposed to specific environmental contaminants (Melanchon et al. 1987; Gooch et al.1989). However, there have been instances when EROD activity was not a reliable indicator of exposure. Induction of EROD was not observed in tumorous brown bullhead from the Black River in Ohio (Fabacher and Baumann 1985). These fish had enlarged livers, as measured by an increase in the somatic-liver index, causing the authors to speculate that the fish had adapted to the polluted environment and increased the total activity of the hepatic MFO system by this means. Other studies have also

reported liver enlargement associated with exposure to environmental contaminants (Fletcher et al. 1982; Sloof et al. 1983). Gallagher and Di Giulio (1989) also reported a lack of EROD induction in brown bullhead collected from a stream contaminated with organic compounds and trace metals. Lack of induction in this case was attributed to the presence of the trace metals, which have been shown to inhibit MFO activity in mammals (Alvares et al. 1972) and fish (George and Young 1986).

A knowledge of specific P450 isozymes involved in particular reactions is necessary to understand catalytic function and allow for interspecies comparisons of metabolic activity (Stegeman and Kloepper-Sams, 1987). Determination of specific P450 isozymes is accomplished by immunoblotting with monoclonal or polyclonal antibodies to identify individual enzyme forms or immunologically cross-reactive groups. The P450 isozyme most commonly identified in fish is the PAH-inducible form known as P450e in scup, LM_4 in rainbow trout and P450c in cod (Kloepper-Sams and Stegeman, 1989). These isozymes are all representatives of the P4501A gene family. Measurement of isozymes with polyclonal antibodies is semi-quantitative, but has been shown to correlate well with contaminant exposure in several species. An indirect, enzyme-linked immunoabsorbant assay (ELISA), using rabbit anti-cod P450c as the primary antibody has been used to reliably measure cytochrome P4501A in field collected and laboratory exposed fish across a wide range of species (Collier et al. 1991; Goksoyr 1991; Ronis et al. 1991).

MFO activity can also be assessed by measurement of NADPH - cytochrome P450 reductase. This enzyme transfers electrons from NADPH to cytochrome P450 and may also transfer electrons to phase 1 metabolic intermediates (Cohen and d'Arcy Doherty 1987). This latter process can generate free radicals, which are potentially damaging to cellular macromolecules, including DNA. Since the enzyme does not only transfer electron to cytochrome P450, its activity may not be an accurate measure of MFO induction (Stegeman and Kloepper-Sams 1987). Carlson, working in our laboratory, observed no concomitant increase in NADPH - cytochrome P450 reductase activity with increased EROD activity induced by BNF exposure in either channel catfish or brown bullhead (unpublished). However, Winston, et al. (1988) found increased activity in channel catfish taken from a site contaminated with PAHs and PCBs, with concomitant increases in cytochromes P450 and b_5.

Other correlations between various MFO indices have also been observed. A correlation between induced P4501A levels and EROD activity was reported in fish induced with either BNF or environmental PAHs (Goksoyr et al. 1987; Elskus and Stegeman 1989). A similar correlation was seen in fish taken from contaminated sites (Stegeman et al. 1987). Collier and co-workers (1992) have published results showing general concordance between

cytochrome P4501A levels and Ahh and EROD activities. Kloepper-Sams and Stegeman (1989) have shown a close correlation between P450 and EROD activity over a 20 day period in BNF induced killifish. However, studies cited earlier have shown that exposure to contaminants is not always indicated by increased EROD levels. Additionally, Stegeman and Kloepper-Sams argue that induction of other P450 isozymes may occur independently of EROD induction. Gooch et al. (1989) reported depressed EROD activity in scup treated with a PCB congener, concomitant with P4501A induction. Thus, the best method for assessing overall MFO induction appears to be the use of several indices of activity, and that is the approach we have selected in the studies reported on herein.

Bile Metabolites. Early approaches in the direction of assessment of exposure of fish to chemical contamination involved direct analysis of tissues for contaminant residues, but only trace amounts of PAHs were found in fish taken from heavily polluted sites (Baumann et al. 1982; Malins, et al. 1982). Such and approach is clearly not feasible for appraising lower levels of contamination. Malins and Hodgins (1981) showed that fish assimilate and metabolize PAHs, and Krahn and associates (1984) developed an analytical technique to measure these metabolites in bile, in the hope that this would serve as a more sensitive index of exposure. This technique has since been widely adopted. Krahn's group (1986) discovered correlations between PAH metabolites in the bile of English sole taken from polluted sites and increased frequencies of neoplasia and liver lesions. However, they also noted that intersite variability existed and that a direct correlation between the concentrations of sediment PAHs and bile metabolites could not be established, a fact that they attributed to the migratory habits of the English sole. Maccubbin et al. (1988) found correlations between high concentrations of BaP and phenanthrene equivalents in the bile of brown bullhead and sediment PAH concentrations. They also noted that the concentration of BaP equivalents in brown bullhead was 10-fold higher than the value determined for English sole taken from sites with similar contaminant loads, suggesting a more efficient metabolism of PAHs by brown bullhead. Collier and Varanasi (1991) found a correlation between FACs in the bile of English sole injected with BaP or sediment extract and increased Ahh activity. FACs in bile remained elevated over controls even after the decline of Ahh activity, suggesting that this parameter might be a more sensitive indicator of exposure than MFO induction. Meyers and co-workers (1991) found that FACs in bile, while sometimes correlated with increased MFO activity and neoplasia frequencies, are not directly predictive of their occurrence. However, it is apodictic that the presence of FACs in bile does show the assimilation and metabolism of PAHs by fish, whatever the source of the compounds.

Glutathione status. Glutathione is a tripeptide composed of glycine, cysteine and glutamic acid. It is virtually ubiquitous in living systems, having been found in tissues in millimolar concentrations (Kretzschmar and Klinger 1990). Glutathione can exist as a tripeptide with the sulfhydryl group of cysteine reduced (GSH), or this group may be oxidized to form a disulfide linkage between two glutathione molecules (GSSG). Most hepatic glutathione exists as GSH. The ultimate source of electrons for the maintenance of GSH in the reduced state is NADPH (Reed and Beatty 1986).

Glutathione is thought to serve in a number of in vivo detoxication processes. Many xenobiotics can be conjugated to GSH by the action of the glutathione-S-transferases (GSTs) (Chasseaud 1979). Glutathione peroxidase, an enzyme that catalyzes the oxidation of GSH with the concomitant reduction of H_2O_2 to water is thought to be an important protective mechanism against H_2O_2 toxicity in mammals. Glutathione peroxidase has also been shown to reduce organic peroxides, possibly diminishing the potentially deleterious effects of lipid peroxidation (Di Giulio et al. 1989). Xenobiotics can also alter the cellular glutathione status by affecting the activity of γ-glutamyl transpeptidase (GCT), an enzyme that functions in GSH catabolism by cleaving the glutamyl residue from the tripeptide (Kretzschmar and Klinger 1990). Until recently, cellular glutathione status and the activities of the various enzymes active in the transformations of the molecule have received relatively little attention as biomarkers for exposure to xenobiotics. However several recent studies have focused on these parameters. A study in Finland (Lindstrom-Seppa and Oikari 1990,) documented increases in total hepatic glutathione in feral fish exposed to bleached pulp mill effluent: no changes in GST activity in exposed fish were found, however. Stein and his associates (1992) observed increases in total hepatic glutathione levels in several species of fish taken from contaminated sites in Puget Sound. Such increases were not observed in English sole exposed to contaminated sediment in the laboratory in an earlier study, however. (Stein et al 1987) Di Giulio, et al. (in press) observed slight increases in GST activity in channel catfish exposed to contaminated sediment and marked increases in total hepatic glutathione.

DNA single-strand breakage. Many genotoxic, mutagenic and/or carcinogenic compounds are presumed to cause their pernicious effects by binding directly to nucleotides in the DNA. One study has shown that the carcinogenic potential of a compound is better correlated with binding to DNA than with mutagenicity in the Ames test. (Parodi et al. 1982) This study also found that DNA fragmentation, as measured by an alkaline elution assay, was well correlated with the affinity a compound exhibited for DNA binding. The extent of DNA strand breakage has also been positively correlated with the mutagenicity that a compound demonstrates in the Ames test. (Daniel et al. 1985) The relationship between DNA adducts and strand

breakage could be due to the formation of alkali-labile apurinic or apyridinic sites due to DNA alkylation (Lindahl and Nyberg 1972). Other, more unstable adducts can cause strand breakage directly (Shugart 1988). Kohn (1983) suggested that quantitation of DNA damage could be a useful indicator of the genotoxicity of environmental pollutants.

Shugart (1988) adapted the alkaline unwinding assay for use with fish DNA. The procedure takes advantage of the fact that bisbenzamide (Hoechst dye 33258) forms a fluorescent product upon binding to DNA, the intensity of which is greatly reduced when the dye is bound to single-stranded rather than double-stranded DNA. Shugart used the assay to demonstrate increased strand breakage in the livers of bluegill sunfish (Lepomis macrochirus) exposed to 1 ppm BaP. He also used the technique to quantitate DNA strand breakage in fish taken from polluted sites (Shugart 1990). Nacci and his associates did similar work with bivalves (Nacci et al. 1992). Di Giulio and co-workers used the alkaline unwinding assay to assess strand breakage in channel catfish exposed to contaminated environmental sediments under laboratory conditions (Di Giulio et al. in press).

Formation of 8-hydroxydeoxyguanosine (8-OHdG). When 2'-deoxyguanosine is hydroxylated by a hydroxyl radical, the product formed is 8-OHdG. Floyd and his associates (1986) developed an extremely sensitive HPLC technique, using electrochemical detection, to assay for this species. The technique has been successfully employed to measure 8-OHdG in plants and Cundy and his group (1988) have used it in animals. Malins and Haimnot (1990) found higher levels of 8-OHdG in fish with hepatic tumors, as compared to healthy fish. They also determined that 8-OHdG levels in a population of English sole with a low (3%) frequency of liver neoplasia were intermediate between those found in cancerous fish and those in healthy fish (Malins and Haimnot, 1991). Given these results, it can be concluded that this technique shows considerable promise as an index of genotoxicity.

Hepatic-xenobiotic DNA adducts. As has been stated previously, the carcinogenicity and mutagenicity of a number of chemicals have been directly correlated with the binding of those compounds to DNA. Covalent binding of exogenous compounds occurs soon after exposure (Shugart, 1992). The fraction of the compound present exogenously that actually forms DNA adducts is minuscule, making quantitation of the adducts difficult at best. The ^{32}P-postlabeling technique is extremely sensitive and can detect 1 adduct in 10^9-10^{10} nucleotides and has become well accepted as an indicator of DNA damage. It has been employed successfully in a number of field studies (Dunn et al. 1987; Stein et al. 1989; Varanasi et al. 1989b; Maccubbin, et al. 1990). Varanasi (1989b) found good agreement between adduct levels and FACs in bile, but Maccubbin and his co-workers did not. Stein's group has observed elevated levels of DNA adducts in fish from

polluted sites in the Puget Sound, but correlation with other biochemical indices of exposure is mixed. These workers concluded that a broad range of biochemical indices is desirable to better assess exposure to xenobiotics (Stein et al. 1992). That is the approach we have taken in the study described herein.

The Niagara River system

The Niagara River flows northward and connects Lake Erie to Lake Ontario (Fig 1). The river is 58 kilometers long and is divided into lower and upper reaches by Niagara Falls. The flow rate is approximately 5700 m^3/sec (Hickey et al. 1990). The river divides into two channels approximately five miles north of its source at Lake Erie. The Chippewa channel circles Grand Island to the west and carries 57% of the river's flow. The Towanda channel flows on the eastern side of Grand Island, through the highly industrialized region comprised by the greater metropolitan area of Buffalo, NY. Industries present include petrochemical, chemical manufacture, steel production and cloth dyeing. Pollution problems are worsened by general urban runoff. Both Canadian and American populations draw drinking water from the river. The river also supports an excellent sport fishery and provides considerable waterfowl habitat (Hickey et al. 1990). In contrast, riparian lands along the Chippewa channel are relatively undeveloped. Few heavy industries exist; slight leaching from landfills and the Fort Erie Water Pollution Control Plant are the only point sources of organic chemical pollution on this side of the river (NRTC 1984). The diverse nature of the Niagara River system has caused significant environmental degradation in the Towanda channel, and potential impacts on human health are cause for concern. The Niagara River Toxics Committee (NRTC), comprised of environmental agencies from both the U. S. and Canada, was established to identify sources of pollution in the watershed, recommend control programs and to develop programs for long term monitoring of the pollution status of the river. The NRTC published a report in 1984 which includes water analyses, a survey of extant biota and identification of major polution sources. This information facilitates the selection of sites with specific contaminant profiles. Our reference site was Black Creek, a slow-moving creek in Ontario, Canada, adjacent to the Chippewa channel. It flows through residential areas, undeveloped forests and wetlands, and lacks significant point sources of pollution. Non-point sources are recreational motorboats, runoff from residential areas and a highway that passes over the creek. Black Creek is not pristine (total PAHs = 2800 ng/g: PCBs = 55 ng/g) but the NRTC noted that none of the chemical levels exceeded ambient sediment criteria. It has been used in the past as an uncontaminated reference site for similar studies.

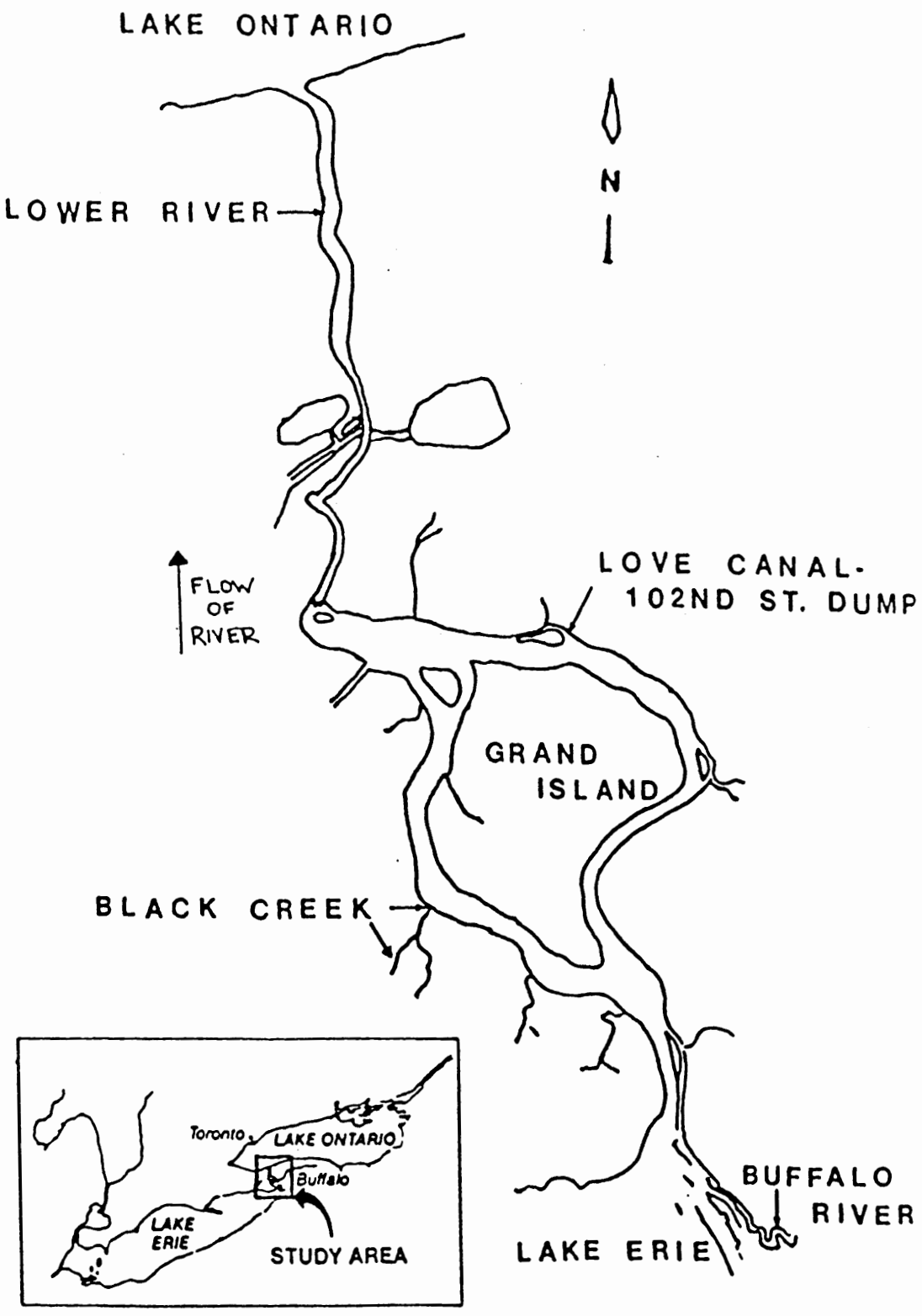

Fig. 1. Map of the Niagara River System

Love Canal is an abandoned channel adjacent to the confluence of the Little Niagara River and the Towanda channel of the Niagara river. From 1942-1953, the Hooker Electrochemical Company dumped nearly 21,800 tons of chemical waste generated by its pesticide and plasticizer production facility into the abandoned channel at 102nd street. Love Canal gained national notoriety in 1978 when chemical odors and oily leachate began to invade basements of a nearby residential area. President Jimmy Carter declared the area to be a human health hazard and families were displaced; the dump site was subsequently covered with a clay cap. The hazardous waste sites drain directly into the Niagara River in the area referred to as the 102nd street bay; it is here that our sampling site was located. The soil, leachate, biota and nearby creeks have been previously shown to contain high levels of PCBs, dioxins, furans, PAHs and pesticides (Hickey et al. 1990).

The Love Canal area has been the site of numerous studies. Of particular relevance to our work are studies on feral fish conducted by the US Fish and Wildlife Service (USFWS). Brown bullhead from the vicinity of Love Canal have demonstrated a 5% frequency of non-parasitic lesions as compared with a frequency of less than 1% at the Black Creek reference site. Filets showed elevated concentrations of PCBs (530-2200 ng/g), chlordane (10-20 ng/g) and DDT metabolites (30-90 ng/g). The only detectable chlorinated organic compounds in filets of fish taken from the reference site was DDE (10-20 ng/g) (Hickey, et al. 1990). Studies by the USFWS are ongoing at the Love Canal site.

The Buffalo River is formed by the confluence of the Cayuga, Buffalo and Cazenovia creeks and flows through the city of Buffalo, NY, draining into Lake Erie near the head of the Niagara River. Extensive dredging has resulted in decreased flow and an amplification of seasonal temperature fluctuations and variations in water level. The riparian land has considerable heavy industry which includes grain mills, chemical manufacture, coke and steel production, oil refining and sewage treatment. Lake vessels gain access to these industries via the river.
The Buffalo River has been subjected to intensive scrutiny by the New York State Department of Environmental Conservation (NYSDEC). The Buffalo River Remedial Action plan (RAP) defines pollution sources and recommends remedial action and plans for long-term monitoring. Water quality has improved greatly in the past 20 years but pollution levels still exceed New York State standards. Data collected by Environment Canada in 1981 show high levels of PCBs (480 ng/g) and heavy metals (Cd, 2 ppm; Pb, 109 ppm; Zn, 179 ppm) while data on sediment contamination indicated high levels of PAHs (BaP, 103 ng/g; phenanthrene, 1665 ng/g; chrysene, 653 ng/g) in 1989 (NYSDEC 1989). Fish consumption has been restricted due to unacceptable levels of PCBs and chlordane in carp. In sum, the Buffalo River is one of the more highly polluted areas in the Niagara system.

Materials and Methods

Site Selection

The sampling sites were selected in accordance with ongoing studies conducted by the US. Fish and Wildlife Service and co-ordinated by Dr. John Hickey, Cortland, NY. Sites were selected based on information in the NRTC report (1984). Goals for site selection included, (1) to select sites highly polluted with PAH's or other compounds of interest, (2) to have sediments with similar physical characteristics and (3) to select a reference site ecologically similar to the polluted sites. Three sites, a reference site and two polluted site, were chosen for sampling. The reference site was near the mouth of Black Creek (BC), a small tributary of the Chipewa channel about 4 miles downstream from the bifurcation (Fig. 1). This site has been used previously as a reference for studies on sediment toxicity (Hickey et al. 1990). One of the polluted sites was approximately one mile upstream from the mouth of the Buffalo River (Buffalo, NY). A second polluted site was chosen near the Love Canal-102nd Street dump site (Wheatfield, NY).

Sediment Collection, Handling and Analysis

Sediment samples were collected at the sites of feral fish collection in July 1991 by personnel from Buffalo State College. Samples from each site were pooled and mixed with a mortar mixer to assure uniformity of subsamples. Subsamples were sent to the Northwest Fisheries Center (Seattle, WA) for chemical analysis. Subsamples used to prepare sediment extract and for the laboratory sediment exposures were stored in plastic pails in the cold room at 4°C before use. Total organic content of sediments was determined by the weight differential between samples dried at 90°C and samples ignited in a muffle furnace.

Fish

Juvenile channel catfish used in the sediment extract injection experiment and the laboratory sediment exposure were obtained from Aquaculture Advisory Service, Garner, NC. These fish were 13 - 18 cm in length and weighed between 50 g and 93 g. Juvenile brown bullhead for the laboratory MFO induction experiment were obtained from Zett's fish hatchery, Drifting, PA. Juvenile brown bullhead for the laboratory sediment exposure were obtained from the Pamlico Aquaculture Facility, Aurora, NC. These fish ranged from 10 - 25 cm in length and weighed between 35 g and 270 g.

Laboratory Sediment Extract Injection Experiment

An extract of sediment collected at the Buffalo River site was prepared according to Krone et al. (1986). A total of 1.934 g of extract was made from 1.8 kg of sediment. Briefly, 100 g aliquots of sediment, to which 300 g of sodium sulfate had been added to absorb excess water, were extracted twice with 500 ml of dichloromethane. Extractions were carried out on a rotary shaker at 100 rpm, the first for 18 hours and the second for 6 hours. The extracts were pooled and evaporated to about 100 ml under warm air, then to dryness under warm N_2. The extract was stored in the dark under N_2 until it was prepared for injection into fish. This was done by dissolving 48.9 ml of extract in 3.26 ml of acetone and blending this mixture with an equal volume of Alkamuls EL-620.

Twelve fish were injected interperitoneally (i.p.) with sediment extract at a dose of 7.5 mg/kg body weight and 12 fish were injected with a sham injection containing equal volumes of acetone and Alkamuls EL-620. Six uninjected fish were maintained with the others to serve as absolute controls. Fish were maintained in 100 l polyethylene aquaria under flow through conditions. Water temperatures over the course of the exposure were stable at 22 +/- 1°C. Six fish from each group were sacrificed by cervical dislocation on days 2 and 7; the uninjected controls were sacrificed on day 2 of the exposure. Livers were excised and divided into five parts before freezing at -70°C for later assays. Assays done were for EROD activity, DNA single strand breaks, 8-OHdG and total and oxidized glutathione.

Laboratory Sediment Exposure Experiments

Preparation of aquaria Sediment was bedded in 100 l polyethylene aquaria to a depth of 3 cm. Each sediment was randomly assigned a position in each of 4 identical blocks of aquaria. A plastic grid with a 1.27 cm square mesh, 1.27 cm thick was pressed into the top of each sediment bed to prevent loss of sediment due to the fish swimming above it and to prevent their digging into it. Control tanks contained plastic grids, but no sediment.

Exposure of channel catfish Prior to the introduction of fish, flow-through conditions at a rate of 125 ml per minute (approximately 2.4 turnovers per day) were established and maintained for seven days. Ten juvenile channel catfish were introduced into each tank at the start of the exposure, which was to last for 24 days. Flow rate was held constant over the course of the exposure and water temperature was stable at 15 +/- 1°C. Fish were fed every 3 to 5 days with Purina floating catfish chow. On sample days, two fish per aquarium were killed by cervical dislocation. Bile was removed from the gall bladders with a hypodermic syringe. Livers were excised and

washed in cold 0.15M KCl. Livers were cut into 3 parts, which were later used for assays of total and oxidized glutathione, DNA single strand breaks and EROD activity. Liver and bile samples were maintained on ice for no more than 3 hours before freezing at -70°C.

Exposure of brown bullhead. Prior to the introduction of fish, a flow rate of 100 ml per minute was established. Seven fish were added to each tank; each tank contained one extra fish in case of mortality during the experiment. Water temperature over the course of the exposure was stable at 18°C - 19°C and pH ranged between 6.7 and 7.2. Fish were fed floating Purina Catfish Chow ad libitum for two hours every day; uneaten food was removed at the end of this period. Six fish were killed on days 4, 12 and 24 by cervical dislocation. Bile was removed from the gall bladders with a hypodermic syringe. Livers were excised and immediately removed to ice-cold 0.15M KCL. Livers were later divided into 3 parts and frozen at -70°C for later analysis of total and oxidized glutathione, DNA single strand breaks and EROD activity.

Laboratory MFO Induction Study

Juvenile brown bullhead catfish were held for 1 week prior to use under static conditions in 100 l polyethylene aquaria. Water was changed every four days to keep ammonia concentrations less than 1 ppm. Fish were fed Westco (Charlotte, NC) floating catfish food to satiation every other day. Fifteen fish were injected i.p. with β-napthoflavone (BNF) at a dose of 10 mg/kg body weight (low dose) and 17 fish were injected with a dose of 100 mg/kg body weight (high dose). Five fish were injected with an equivalent amount of corn oil carrier. BNF was prepared for injection by suspension of 13 mg in 1 ml of corn oil. Three uninjected fish were used as absolute controls. One group of 5-6 fish from each treatment was sacrificed at 48 hours, 72 hours and 7 days post-injection. Livers were excised and microsomes prepared immediately. Microsomes were assayed for EROD activity, cytochrome P450 content and P4501A content. Absolute controls were sacrificed at 7 days, not at each time point, so no statistical comparisons among the treatments were made.

Field Studies

Adult brown bullhead, weighing between 72 g and 647 g, were collected from the three sites in June and September 1991 with nets and electroshock apparatus. In June, live fish were placed on ice for up to 4 hours while being taken to Buffalo State College for sacrifice and dissection. In September, fish were sacrificed immediately upon removal from the river. In either case, bile was removed from the gall bladder with a hypodermic syringe and frozen in

liquid N_2, livers were excised, washed in ice-cold 0.15M KCl, pH 7.4, cut into pieces of approximately 250 mg each and frozen in liquid N_2. Samples were kept in liquid N_2 until transported to the Duke University Ecotoxicology Laboratory, where they were transferred to a -70°C freezer. Some liver samples were thawed and used to prepare microsomes. A 10 µl aliquot was removed from each microsome preparation, diluted to a concentration of 10 µg protein/ml in 50mM $NaHCO_3$, frozen and shipped on dry ice to the Northwest Fisheries Center for measurement of P4501A content. The remaining microsomes were refrozen at -70°C before use in assays for EROD activity, cytochrome P450 content and NADPH-cytochrome P450 reductase activity determinations.

Microsome Preparation

Microsomes were prepared from frozen (Payne et al. 1988) or fresh liver tissue according to the method of Erikkson et al. (1978) and stored at -70°C until use.

Bile Analysis

Bile samples were analyzed for xenobiotic metabolites by a modification of the method of Krahn, et al. (1984). HPLC analysis was done with a Perkin-Elmer Series 400 liquid chromatograph using a reverse phase C_{18} analytical column (Rainin Instrument Co.), protected by a 3 cm reverse phase C_{18} guard column (Keystone Scientific). An Upchurch A-318 precolumn filter was placed before all of the columns for all injections. The fluorescence detector was a Perkin Elmer model LS-3 fluorescence spectrometer. Samples were measured at excitation/emission wavelength pairs of 286nm/380nm to detect phenanthrene metabolites and metabolites of other 3-ring PAH's, and at 380nm/430nm to detect BaP metabolites, and metabolites of other 4 or 5-ring PAH's. Chromatography conditions consisted of a linear gradient of 100% 5 ppm acetic acid to 100% methanol over 10 minutes, followed by 20 minutes of 100% methanol, followed by a linear gradient back to 100% 5 ppm acetic acid. Conditions of 100% 5 ppm acetic acid were maintained for 10 minutes between injections. The flow rate of the mobile phase was 1.0 ml per minute. Chromatograms were recorded and analyzed on a microcomputer equipped with the EZ Chrom Chromatography data system, version 4.0 (Scientific Software, Inc., San Ramon, CA)

For the field samples, five samples per month per site were randomly selected for analysis. For the laboratory exposures, bile from fish in each treatment group was pooled by mixing 20 µl aliquots from each sample, then diluted 10 fold with water before injection.

EROD Activity

EROD activity was measured using a modification of the procedure of Burke and Mayer (1974). Briefly, a reaction mixture containing 25 µl of microsomes and 5 ul of 400 µM ethoxyresorufin in 1.962 ml of 0.1 M Tris-HCl (pH 7.8) was preincubated for 2 minutes before 7.5 µl of 50 mM NADPH was added. The change in fluorescence at 586 nm , with an excitation wavelength of 530 nm, was recorded for 3 minutes. Endogenous rates of ethoxyresorufin deethylation were subtracted from the rates for all samples. Specific activities were calculated from a standard curve produced by measurement of known concentrations of resorufin.

Cytochrome P450

The reduced carbon monoxide spectrum of cytochrome P450 was measured according to the method of Matsubara, et al. (1976). After recording a baseline spectrum, samples were reduced with a few milligrams of Na_2SO_4, then CO gas was bubbled through the sample and difference spectrum recorded against a sample that had not been reduced with Na_2SO_4. Total cytochrome P450 was calculated using an extinction coefficient of 106 $mM^{-1}cm^{-1}$.

Cytochrome P4501A

Cytochrome p4501A was measured with the semi-quantitative ELISA assay of Goksoyr (1991). Rabbit anti-cod P450c was used as the primary antibody. Measurements were made by Dr. Tracy Collier of the Northwest Fisheries Center, Seattle, WA.

NADPH Cytochrome P450 Reductase

A method adapted from Guengerich (1985) was used to assay this activity. Briefly, the reaction mixture consisted of 700 ul of 10 mM KCN/0.3 M KPO_4, 100 µl of 0.4mM cytochrome c in 10 mM KPO_4, 100 µl 1 mM NADPH and 100 µl microsomes. Absorbance at 550 nm was measured for 3 minutes. Specific activities were determined using an extinction coefficient of 0.21 mM^{-1} cm^{-1}.

DNA Single Strand Breaks

The alkaline unwinding procedure described by Shugart (1988) was used to assess single strand breaks in hepatic DNA. DNA was prepared from thawed liver samples weighing between 100 - 350 mg. Samples were homogenized in 1 ml of 0.1 M NH_4OH with a dounce homogenizer and teflon pestle. The homogenate was emulsified with 5 ml of chloroform/isoamyl alcohol/phenol (24/1/25 v/v/v) and allowed to stand on ice for 30 min. Phases were separated by centrifugation at 4°C at 8500 x g for 30 min. The aqueous phase was removed and the DNA purified by gel filtration on Sephadex G-50. Sephadex G-50 was equilibrated in buffer containing 150 mM NaCl, 1 mM MgCl, 0.5 mM EDTA and 10 mM Tris, pH 7.4, and packed to approximately 3 ml in columns made from 5 ml disposable hypodermic syringes. DNA purity was assessed spectrophotometrically. A_{260}/A_{280} ratios for the samples ranged from 1.7 to 1.9.

Strand breakage was quantitated by measurement of the binding of Hoechst dye 33258 to double-stranded, single-stranded, and partially unwound DNA. For the double-stranded samples, Hoescht dye in 50 µl each of 0.1 N NaOH and 0.1 N HCl and 3 ml of 0.2 M KPO_4 were mixed with 100 µl of DNA while vortexing. Samples were incubated for at least 15 minutes before fluorescence was measured on a Perkin Elmer LS-3 fluorometer at the wavelength pair of 360_{ex}:450_{em}. Single-stranded DNA samples were prepared by mixing 100 µl DNA with 50 µl of 0.1 N NaOH while vortexing, then placing the solution at 90C for 90 min. After incubation, 50 µl of 0.1 N HCl was added to the sample while vortexing to neutralize the NaOH. Immediately thereafter, 10 ul of 1mM EDTA/0.1% SDS was added and the sample was aspirated five times with a 100 µl Hamilton syringe with a size 22S needle. Hoescht dye in 3 ml of KPO_4 buffer was added, and the sample allowed to incubate for 30 minutes before fluorescence measurement. The single-stranded sample exhibited 46% of the double-stranded sample for DNA from channel catfish. Partially unwound samples of DNA were prepared by combining DNA and 0.1 N NaOH as above and incubating at 37C for 30 minutes. Samples with more single strand breakage should unwind to a greater degree under these conditions. Samples were treated as described for totally unwound samples before fluorescence measurement.

F values were calculated as

$$F = (X_{sample} - X_{ss}DNA)/(X_{ds}DNA - X_{ss}DNA)$$

as discussed by Kanter and Schwartz, 1982, where X is the observed fluorescence.

8-OHdG Assay

Hepatic DNA for use in this assay was isolated by the method of Gupta (1984). An aliquot containing 400 μg of DNA was incubated with 20 μg nuclease P1 for 45 minutes at 37C; 1.3 units of alkaline phosphatase were added and incubation continued 90 minutes longer. 8-OHdG present in the samples was measured with an HPLC method adapted from Park, et al. (1989). The mobile phase was 5% methanol in 50 mM KPO_4 pH 5.0. Nucleosides were separated with a reverse phase column (250 mm x 4.6 mm) packed with 5μm particles. A Perkin Elmer LC-95 UV/Vis spectrometer was used to detect 2'-deoxyguanosine (2'dG) by measuring absorbance at 260nm. An EG&G Princeton Applied Research Model 400 Electrochemical detector equipped with a glass carbon electrode, operated at an applied voltage of 600 mV and adjusted for a full-scale output of 2 nA was used to detect 8-OHdG.

Hepatic Xenobiotic-DNA Adducts

Dr. John Stein of the Northwest Fisheries Center determined the concentration of hydrophobic xenobiotic-DNA adducts on 5-7 randomly selected samples from each site and time point. This was done by using the nuclease P1 version of the ^{32}P postlabeling method described by Randerath, et al. (1981) as modified by Varanasi, et al. (1989).

Glutathione Analysis

Total and oxidized glutathione were measured by a procedure adapted from Griffith (1980). Briefly, liver samples were homogenized 5 times(w/v) of 5% 5-sulfosalicylic acid (SSA), yielding 20% homogenates. Homogenates were centrifuged at 5000 x g at 4°C and supernatants used for the analysis.

For total glutathione, 100 μl of supernatant was diluted with 900 μl deionized water and kept on ice. A 100 μl aliquot of the diluted supernatant was mixed with 750 μl of 0.24 mM NADPH in 125 mM Na-phosphate/6.3 mM EDTA and 100 μl DTNB, and allowed to incubate for a minute or two, after which 50 μl of glutathione reductase were added and absorbance at 412 nm was measured.

Samples in which oxidized glutathione was measured were treated differently. An aliquot of 500 μl of undiluted supernatant from liver homogenate was combined with 10 μl of 2-vinylpyridine (2-VP) and 30 μl of triethanolamine (TEA). This mixture was incubated for 45 minutes before absorbance at 412 nm was measured as for total glutathione. The pH of the samples was measured before spectrophotometric analysis to be sure that it

did not exceed 7.0. Samples in which the pH was too high were discarded and prepared again, with less TEA used to neutralize them, and the pH rechecked.

Concentrations of total and oxidized glutathione were determined by comparison to standard curves. Total glutathione standards in a range of 0 - 20 μM were prepared in 0.412% SSA and oxidized glutathione standards in a range of 0 - 10 μM were prepared in 4.12% SSA. Oxidized glutathione standards were treated with 2-VP and TEA.

Protein Analysis

Microsomal protein concentrations were determined by the bicinchoninic acid protein analysis kit marketed by Sigma Chemical Co., St, Louis, MO. Bovine serum albumin was used as a standard.

Chemicals

The 7-ethoxyresorufin (resorufin ethyl ether) was obtained from Molecular Probes, Inc., Junction City, OR. The 8-OHdG standard was a kind gift from Dr. R.A. Floyd, Oklahoma Medical Research Foundation, Molecular Toxicology Research Group. CO gas was obtained from National Welders, Inc., Durham NC. Liquid nitrogen was obtained from the Duke University Cryogenics Laboratory. Alkamuls-EL 620 was obtained from Rhone-Poulenc, Cranbury, NJ. All other chemicals were of the highest grade commercially available and were obtained from Sigma Chemical Co., St. Louis, MO.

Statistical Methods

Laboratory sediment extract injection and sediment exposure experiments. These data were evaluated as to whether they were normally distributed using normal probability plots in Statgraphics, a software package for personal computers marketed by STSC, Inc., Rockville, MD. Both data sets were found to be normally distributed. Comparisons between treatments were made by ANOVA at $\alpha = 0.05$ by the Neuman-Keuls method of multiple comparisons using Kwikstat, a software package marketed by TexaSoft/Mission Technologies, Cedar Hill, TX.

Field data. These data were analyzed using the SAS statistical analysis package for personal computer, version 6.03, marketed by SAS Institute, Cary, NC. Data were tested to see whether they were normally distributed with the Shapiro-Wilkes Test ($p < 0.05$) Since data were determined not to be normally distributed, non-parametric methods were used for analysis. The NPARWAY1 procedure in the SAS package, a non-parametric, one-way

ANOVA based on rank, with multiple comparisons, was used to determine relationships among collection site and biochemical responses. Intrasite sex and season comparisons were made with the Mann-Whitney U test. The Spearman Rank Correlation Coefficient was used to examine correlations among various indicies. All analyses were done at a significance level of $p < 0.05$.

No significant differences were observed between sexes at any of the sites, except for EROD activities and NADPH-cytochrome P450 reductase activities at Love Canal in June. Since these were the only exceptions, sexes were pooled for subsequent analyses. Seasonal differences were significant for some assays and not others, so sampling times were not pooled in subsequent analyses.

Graphics were generated with the Statview SE + Graphics statistical graphics system, a software package marketed by Abacus concepts, Berkeley, CA. The asymmetry of confidence intervals around the median is typical of data that are not normally distributed and that have been analyzed by non-parametric methods. The methods employed here reflect data based on rank rather than on absolute value.

RESULTS

Analysis of Xenobiotics present in Sediments

Data showing the concentrations of selected compounds in sediments collected from the three sites are shown in Table 1. In general, the analyses revealed high concentrations of PAH's at the Buffalo River site, high concentrations of PCB's at the Love Canal site and lower but measurable concentrations of both PAH's and PCB's at the Black Creek (reference) site. Total PAH concentrations (wet weight) were approximately 5500 ng/g at Buffalo River, 3300 ng/g at Love Canal and 440 ng/g at the reference site. Total organic content of the sediments, as a percentage of initial weight were 4.6 for Buffalo River and 6.1 for the reference site. This value was not determined for sediment from Love Canal.

Sediment Extract Injection Experiment

Bile analysis. Fig. 2 shows phenanthrene and BaP equivalents found in bile samples from channel catfish injected with extract prepared from the Buffalo River sediment, compared with sham injected and uninjected fish. Phenanthrene equivalents increased nicely over the time course to a value about three times that in sham injected and control fish. BaP equivalents showed a similar increase.

Data for the other biochemical indicies measured in this experiment are shown in Table 2.

DNA single strand breaks. Data showing the number of single strand breaks in DNA from uninjected, sham injected and extract injected fish are presented graphically in Fig. 3. The number of single strand breaks in extract injected fish was not significantly different from either of the controls.

EROD activity. Fig. 4 is a histogram showing EROD activity in control and treated fish. Injected fish showed a significant ($p < 0.05$), ten-fold increase over the uninjected control after two days, which had declined to basal levels after seven days. A three to four fold, statistically non-significant increase over the uninjected control was observed in the sham injected fish after two days; this difference also was not apparent in the samples taken at seven days.

Table 1. Sediment chemical concentrations of selected contaminants (ng/g dry wt) from the sampling sites in the Niagara River system.

Compound	Black Creek (Reference site)	Buffalo River	Love Canal
Total PAHs	<442	<5,527	<3,338
naphthalenes[a]	<28	<208	<112
phenanthrene	12	440	240
anthracene	<2	180	73
chrysene	22	540	250
fluoranthenes	42	590	460
benzo(e)pyrene	16	190	210
benzo(a)pyrene	13	350	230
Total PCBs	22.1	258.6	1160
mirex	<0.2	<1	130
lindane(γBHC)	0.3	<0.6	24
aldrin	<0.1	<0.7	11
dieldrin	<0.1	<0.6	1
DDT+DDE+DDD	<2.5	<12.9	35

[a] - includes naphthalene, 2-methylnaphthalene, 1-methylnaphthalene, 2,6-dimethylnaphthalene, acenaphthalene and 2,3,5-trimethylnaphthalene

Table 2. Sediment extract injection experiment.
Data for the biochemical assays.
Values are means +/- standard errors.

Treatment	**F-value**	**EROD act.** pmol/mg/ min	**TGSH** umol/g	**GSSG** umol/g	**%GSSG**
control	0.663 ± 0.047	2.34 ± 0.50	1.19 ± 0.115	0.0187± 0.0015	3.22 ± 0.33
sham inj. day 2	0.669 ± 0.043	4.96 ± 2.30	1.09 ± 0.074	0.0156 ± 0.0020	2.90 ± 0.37
extract inj. day 2	0.761 ± 0.073	26.11 ± 9.55	1.02 ± 0.123	0.0179 ± 0.0025	3.62 ± 0.51
sham inj. day 7	0.376 ± 0.072	9.00 ± 1.62	0.096 ± 0.068	0.0175 ± 0.0032	3.60 ± 0.54
extract inj. day 7	0.608 ± 0.107	5.68 ± 2.31	0.93 ± 0.165	0.0157 ± 0.0032	3.81 +/- 1.03

F-value - Fraction of hepatic DNA present as double stranded after alkaline unwinding

TGSH - Total hepatic glutathione

GSSG - oxidized glutathione

% GSSG - percent of total hepatic glutathione present as GSSG

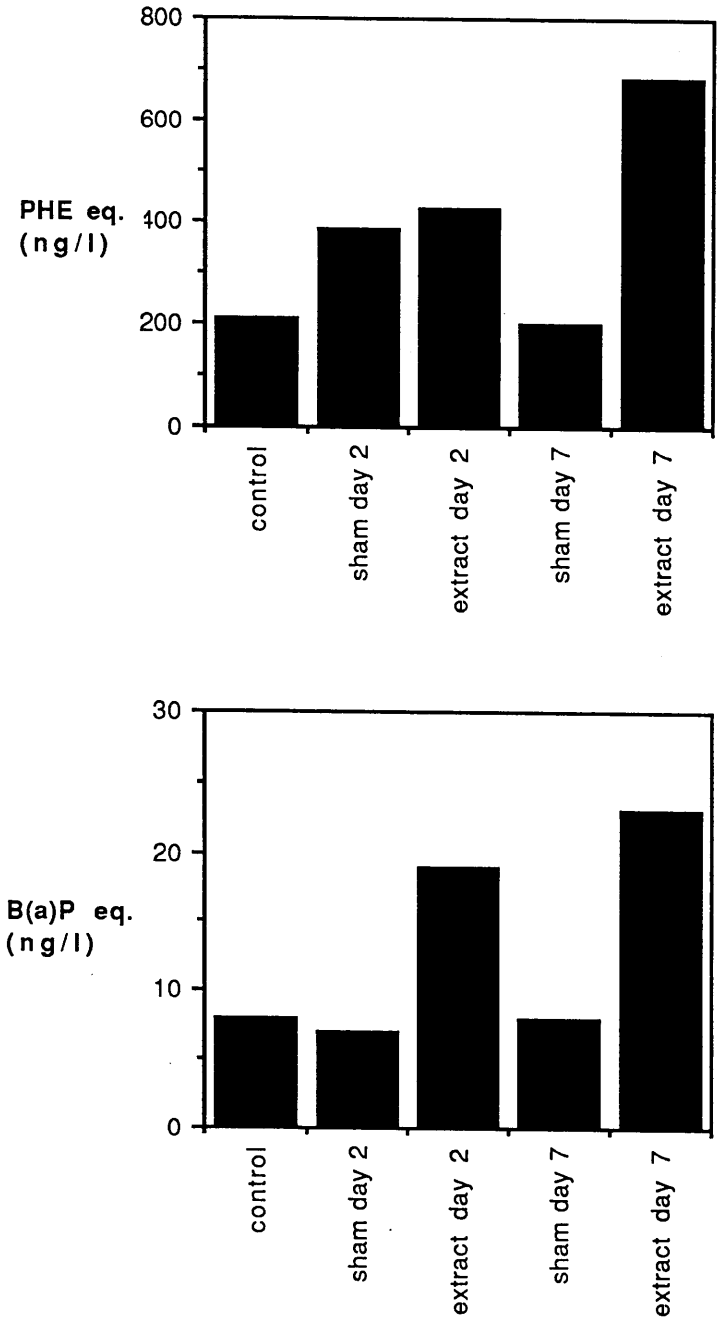

Fig. 2 PAH equivalents in bile of catfish from the sediment extract injection experiment

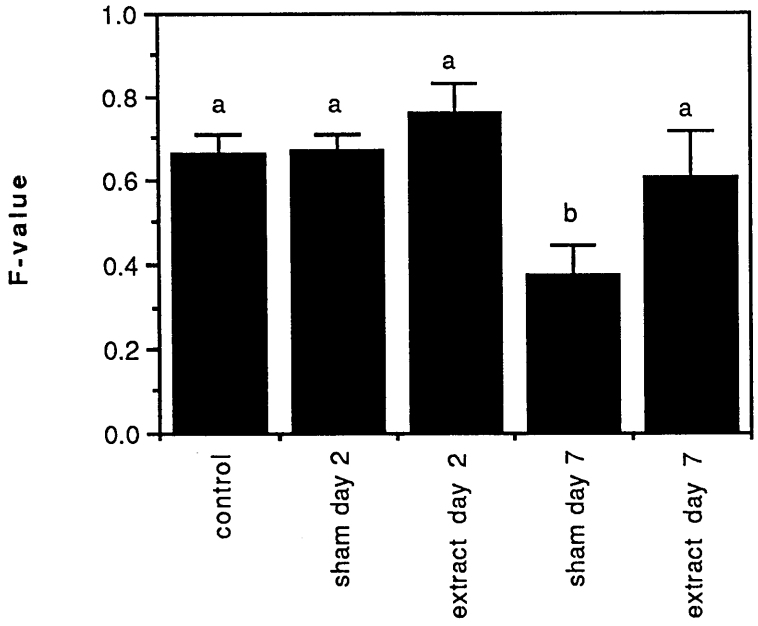

Fig. 3. Fraction of DNA present as double-stranded after alkaline unwinding for the sediment extract injection experiment.

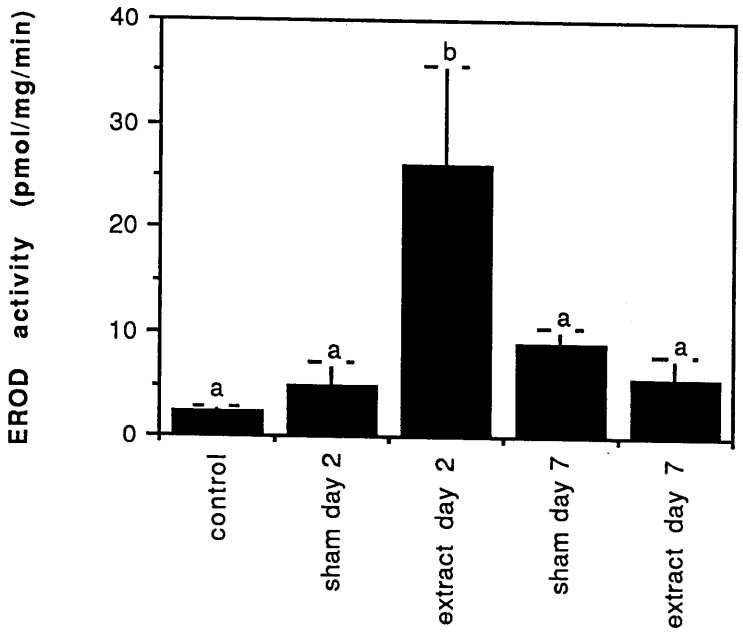

Fig. 4. EROD activity in hepatic tissues of catfish from the sediment extract injection experiment

Glutathione These data are shown graphically in Fig. 5. No significant differences between the injected fish and either of the controls in total or oxidized glutathione, or in the percent of the total glutathione present in the oxidized form, were found.

Laboratory Sediment Exposure Experiments

Bile analysis. Fig. 6 shows phenanthrene and BaP equivalents present in the bile of channel catfish exposed to the three sediments. Control fish were exposed to no sediment. A definite increase in the level of phenanthrene metabolite in the controls was noted over the time course. Levels of these metabolites also increased in fish exposed to Buffalo River and Love Canal sediments and were consistently higher than the controls at all time points, and higher than fish exposed to sediment from the reference site on days 12 and 24. Measurable levels of BaP equivalents were also present in the bile of the control fish at all time points. No great differences between control fish and sediment exposed fish was seen on days 4 or 12. Bile from fish exposed to the Buffalo River sediment showed approximately a two fold increase in BaP equivalents over the others at day 24.

Levels of phenanthrene and BaP equivalents present in the bile of brown bullhead exposed to the three sediments are shown in Fig. 7. Control fish were exposed to no sediment. Levels of both phenanthrene and BaP equivalents were highest in fish exposed to sediment from the Buffalo River site, followed by those exposed to sediment from Love Canal. Only the Buffalo River results were significantly different ($p< 0.05$) from the others. Significant levels ($p<0.05$) of both BaP and phenanthrene equivalents were also present in the bile of control fish sampled at 4 days. Fish exposed to Buffalo River sediment showed an approximate three-to fourfold increase in BaP equivalents and an approximate twofold increase in phenanthrene equivalents over average control values present on days 12 and 24. Approximate twofold increases in both BaP and phenanthrene equivalents over average levels present on days 12 and 24 in controls were observed in bile from fish exposed to Love Canal sediment.

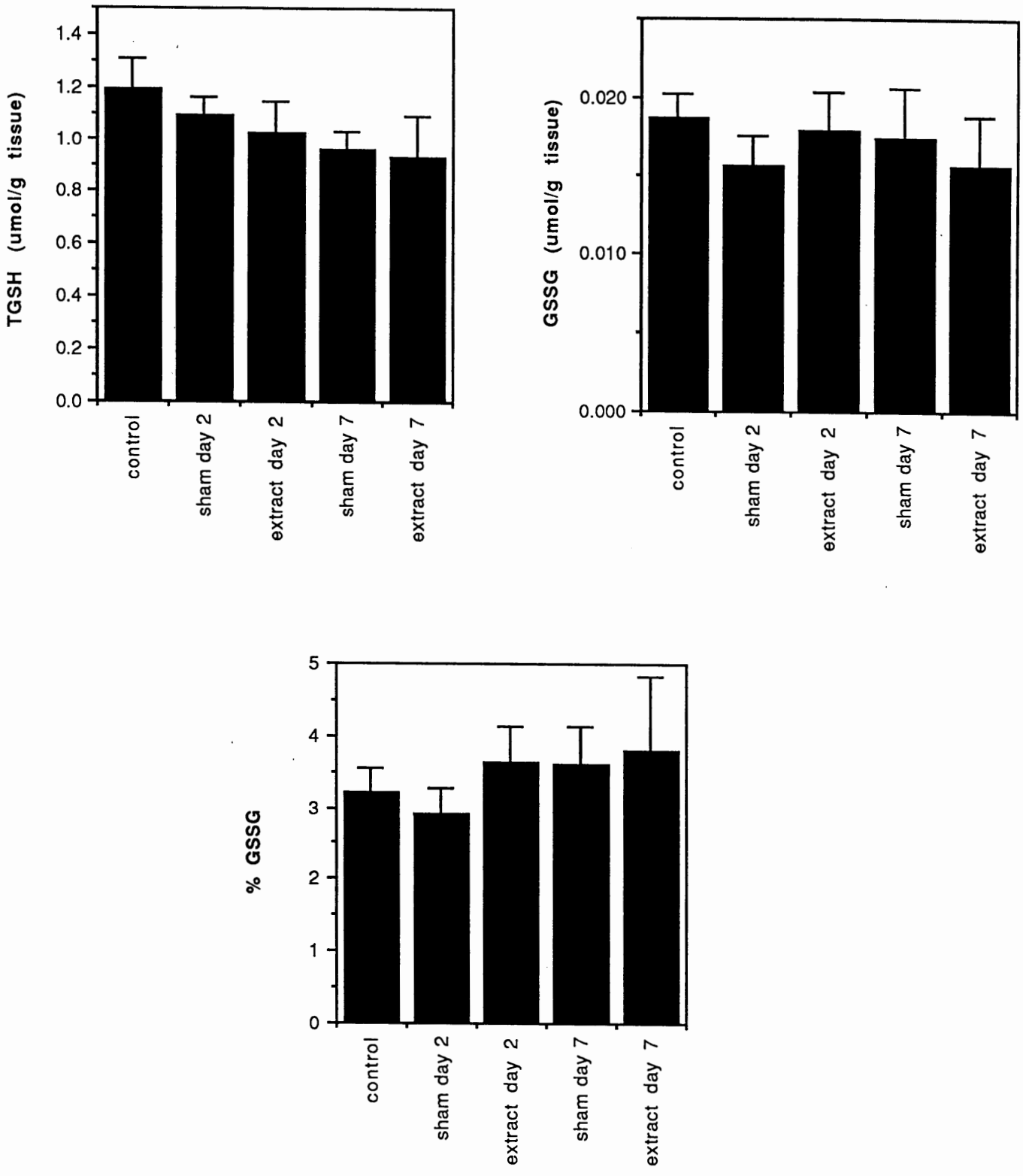

Fig. 5. Hepatic glutathione levels in catfish from the sediment extract injection experiment

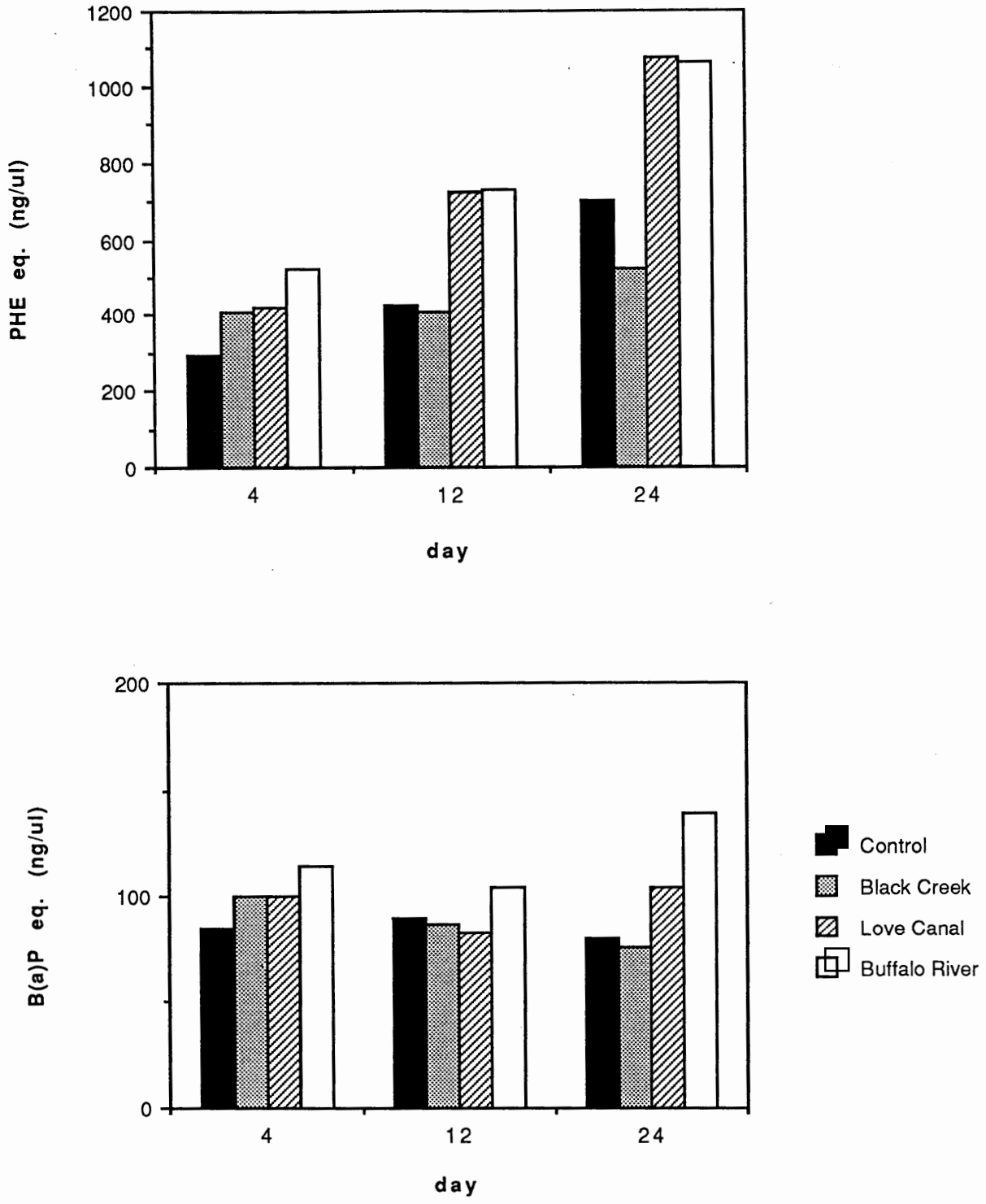

Fig. 6. PAH equivalents in bile of catfish exposed to sediments

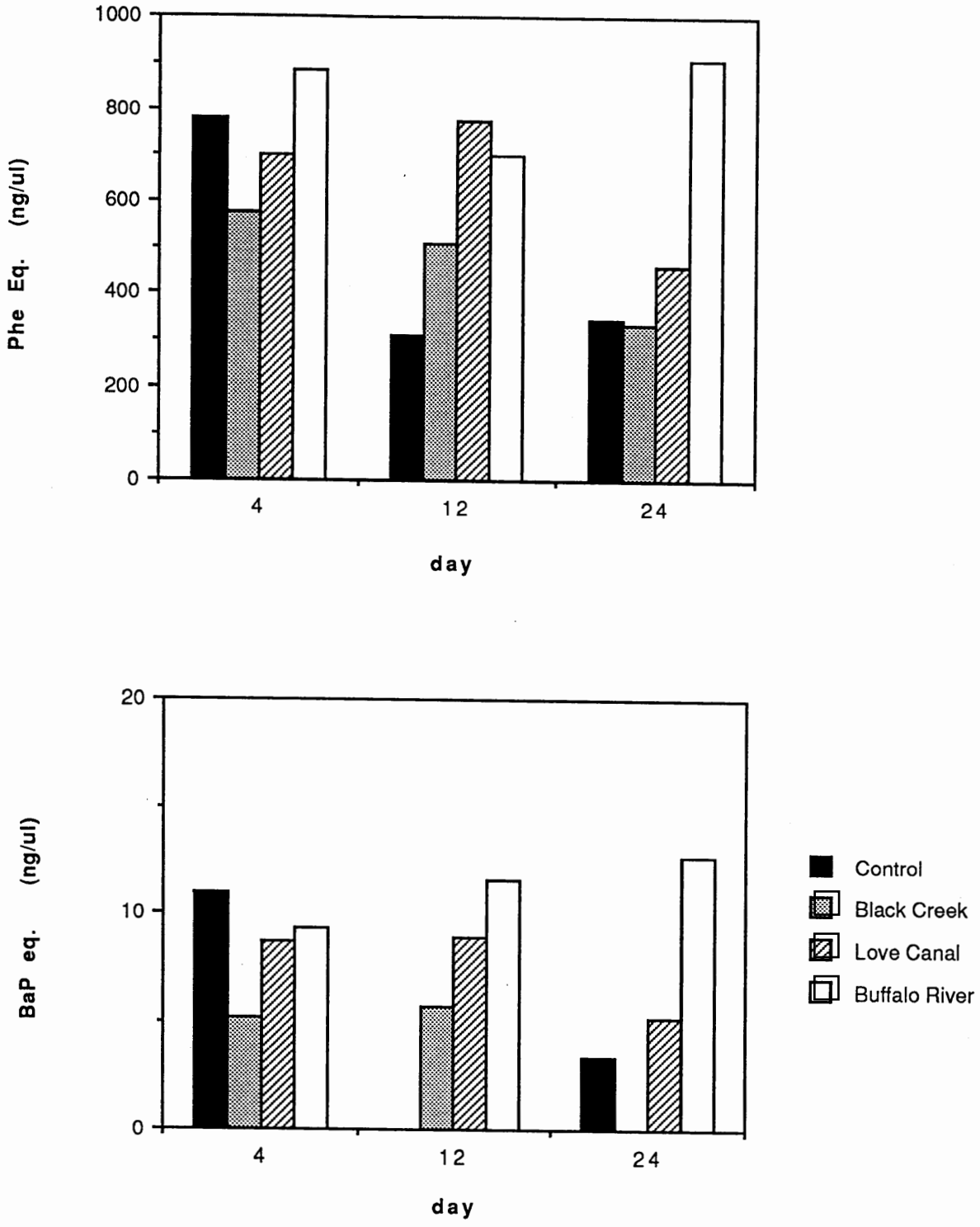

Fig. 7. PAH equivalents in bile of brown bullhead exposed to sediments

DNA single strand breaks F-values for DNA from control and exposed channel catfish are shown in Table 3 and in Fig. 8. No significant differences were noted between any of the treatments at any of the time points.

F-values from control and exposed brown bullhead are shown in Table 4 and Fig. 9. F-values calculated for control fish on day 4 were significantly higher ($p < 0.01$) than those for any other time in any other treatment group. The value was significantly less ($p < 0.01$) for control fish on day 12 than the value for any other time in any other treatment group. F-values calculated for fish exposed to sediment from the Buffalo River site was significantly greater ($p < 0.05$) on day 12 than those from fish exposed to sediment from the same site sampled on days 4 and 24.

EROD activity. EROD activities in control and exposed channel catfish are shown in Table 5 and in a histogram in Fig.10. The only statistically significant differences between any of the treatments occurred on day 4, between the control and the reference site, and the two polluted sites. EROD activities in fish from the polluted sites were depressed by an order of magnitude at this time point.

EROD activities in control and exposed brown bullhead are shown in Table 6 and are presented graphically in Fig. 11. EROD activities were greatest on day 4 for all treatments. Significant increases ($p< 0.05$) of approximately twofold were observed between fish exposed to sediment from the reference site those exposed to sediment from the Buffalo River site. High levels of EROD activities were also present in control fish on days 4 and 12.

Glutathione. Values for hepatic concentrations of total and oxidized glutathione, and the percent of total glutathione present in the oxidized form in exposed and unexposed channel catfish are shown in Table 7 and presented graphically in Fig. 12. On day 4, the control fish had higher levels of total hepatic glutathione than those exposed to the Love Canal sediment. No other significant differences in any of the other glutathione indicies were seen at this time. On day 12, the concentrations of total hepatic and oxidized glutathione were significantly ($p< 0.05$) elevated in the exposed fish, although intrasite differences were not present. The percent of the total glutathione present in the oxidized form was also elevated in the exposed fish; the only significant difference in this index was between fish exposed to the sediment from Love Canal and the others. This index also showed a significant index over the values from day 4 in all groups, due to the increase in the oxidized glutathione levels. On day 24, levels of total hepatic glutathione in the exposed fish remained higher than the controls; again, intrasite differences were not present. Levels of oxidized glutathione

Table 3. Channel catfish sediment exposure.
Fraction of DNA (F-value) present in the double stranded form in hepatic tissue after alkaline unwinding.
Values are means ± standard errors.

Treatment	**day 4**	**day 12**	**day 24**
control	0.228 ± 0.043	0.345 ± 0.060	0.331 ± 0.061
Black Creek	0.137 ± 0.019	0.180 ± 0.035	0.359 ± 0.062
Love Canal	0.221 ± 0.074	0.278 ± 0.068	0.226 ± 0.024
Buffalo River	0.194 ± 0.041	0.351 ± 0.100	0.252 ± 0.056

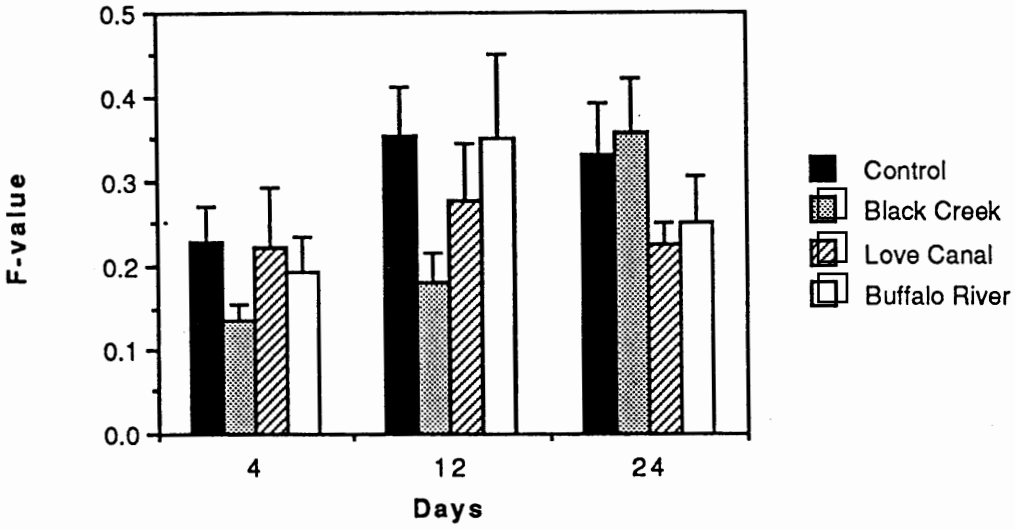

Fig 8. Fraction of DNA present as double-stranded in hepatic tissues of catfish exposed to sediments.

Table 4. Brown bullhead sediment exposure.
Fraction of DNA (F) existing as double strand after alkaline treatment.
Mean values.

Treatment	**day 4**	**day 12**	**day 24**
control	0.741	0.074	0.303
Black Creek	0.325	0.287	0.245
Love Canal	0.258	0.279	0.339
Buffalo River	0.171	0.434	0.240

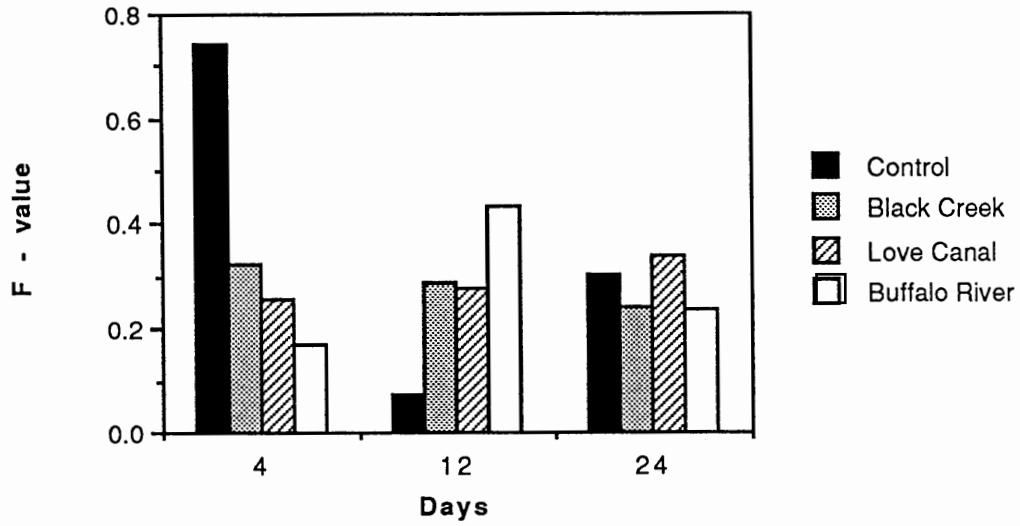

Fig 9. Fraction of DNA present as double-stranded in hepatic tissues of brown bullhead exposed to sediments.

Table 5. Channel catfish sediment exposure.
EROD activity (pmol/mg/min)
Values are means ± standard errors.

Treatment	**day 4**	**day 12**	**day 24**
control	8.70 ± 1.79^{a}	0.307 ± 0.261	0.076 ± 0.027
Black Creek	12.89 ± 3.46^{b}	0.224 ± 0.200	0.088 ± 0.024
Love Canal	0.171 ± 0.095^{b}	0.013 ± 0.006	0.038 ± 0.011
Buffalo River	0.093 ± 0.040^{b}	0.070 ± 0.012	0.059 ± 0.024

a,b - values with different superscripts are significantly different at the $p<0.05$ level. No differences were found between unmarked values.

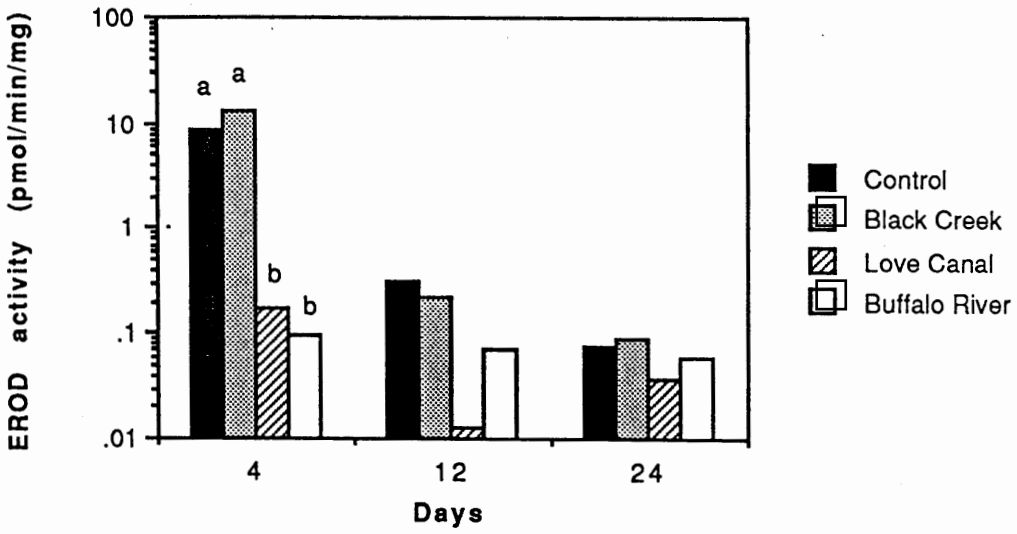

Fig 10. EROD activities of hepatic tissues of catfish exposed to sediments.

Table 6. Brown bullhead sediment exposure.
EROD activity(pmoles $mg^{-1}min^{-1}$). Mean values.

Treatment	**day 4**	**day 12**	**day 24**
control	5.433	6.443	3.564
Black Creek	3.884	3.143	2.615
Love Canal	6.138	4.463	6.117
Buffalo River	7.947	7.353	3.039

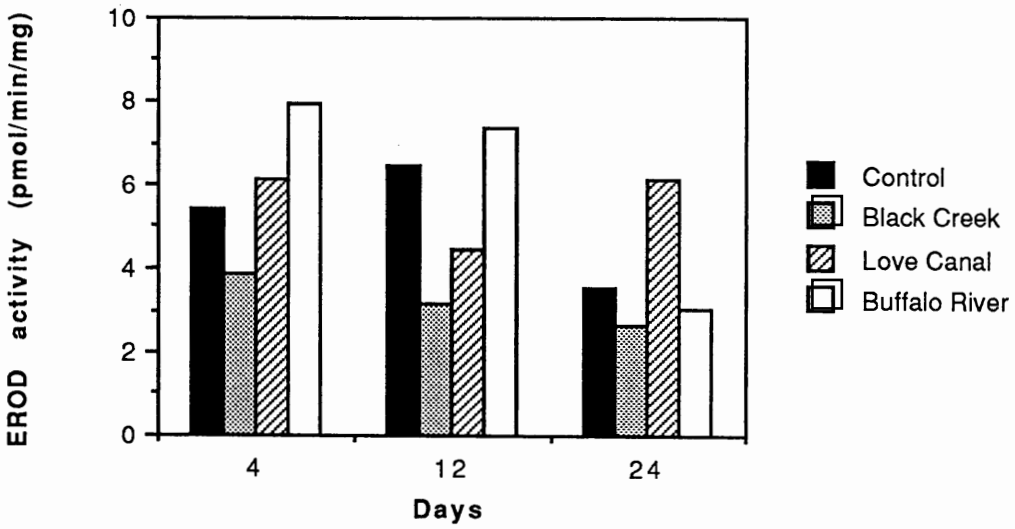

Fig. 11 EROD activities of hepatic tissues of brown bullhead exposed to sediments

Table 7. Channel catfish sediment exposure
Hepatic glutathione levels.
Values are means +/- standard errors.

Total glutathione (umol/g tissue)

	day 4	day 12	day 24
control	1.548 ± 0.148	0.660 ± 0.141	1.006 ± 0.193
Black Creek	1.228 ± 0.113	1.641 ± 0.055*	1.579 ± 0.185
Love Canal	1.038 ± 0.097	1.482 ± 0.071*	1.591 ± 0.315
Buffalo River	1.232 ± 0.013	1.700 ± 0.086*	1.510 ± 0.147

Oxidized glutathione (umol/g tissue)

	day 4	day 12	day 24
control	0.010 ± 0.0015	0.017 ± 0.0110	0.029 ± 0.0046
Black Creek	0.012 ± 0.0022	0.061 ± 0.0060*	0.021 ± 0.0031
Love Canal	0.012 ± 0.0026	0.087 ± 0.0084*	0.018 ± 0.0020
Buffalo River	0.013 ± 0.0024	0.085 ± 0.120*	0.018 ± 0.0020

% oxidized glutathione

	day 4	day 12	day 24
control	0.7 ± 0.12	2.6 ± 1.5	2.8 ± 0.92
Black Creek	1.0 ± 0.23	3.7 ± 0.31	1.4 +/- 0.21*
Love Canal	1.3 ± 0.41	5.9 ± 0.69*	1.4 ± 0.34*
Buffalo River	1.1 ± 0.20	5.0 ± 0.62	1.3 ± 0.17*

*statistically different from control ($p < 0.05$)

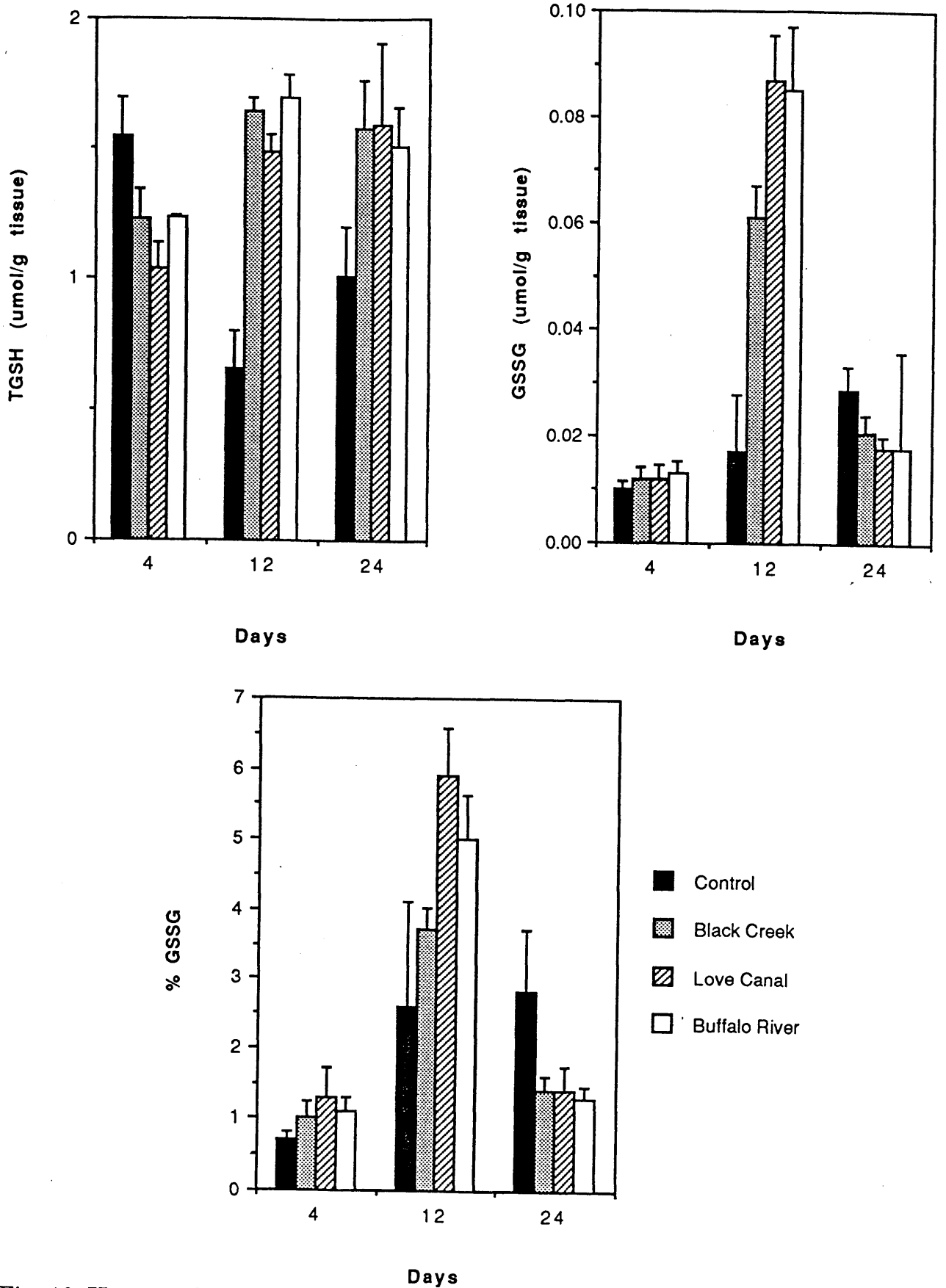

Fig. 12 Hepatic glutathione levels in catfish exposed to sediments

declined significantly ($p < 0.05$) in the exposed fish compared to the levels present at day 12, while levels in the control fish increased, although not significantly. There were no significant difference between the exposed and control fish at day 24. There was a significant decrease in the percent of total glutathione present in the oxidized form in the exposed fish at this time.

Results for brown bullhead are shown in Table 8 and Fig. 13. No differences in the percentage of oxidized glutathione were evident. The mean level of total glutathione was significantly lower ($p < 0.01$) on day 24 than on day 4 for all treatments. No other differences were noted.

Field Studies

Since the data from the field studies were found to be non-normally distributed, a non-parametric format, the box and whisker plot, has been chosen to display them in Figs. 14 - 19. From top to bottom of each box, 90%, 75% 50% (median), 25% and 10% of the values are represented. Notches in the box depict the 95% confidence band around the median; if the notches do not overlap vertically, the medians may be considered significantly different at the $p < 0.05$ level.

Bile Analyses. Chromatograms of bile from brown bullhead catfish taken from the polluted sites showed a complex mixture of metabolites whether phenanthrene or BaP metabolites were measured. Fluorescent aromatic compounds measured using phenanthrene wavelength pairs were significantly (ten-to fortyfold) greater in fish from the Buffalo River site than in those from the other sites in both June and September (Table 9). Significantly more phenanthrene equivalents were found in fish taken from Love Canal as compared with the reference site in June, but no differences between these two sites was seen in September (Fig. 14). Median values for the concentration of BaP equivalents in the bile were significantly (nine-to twelvefold) higher in fish from the Buffalo River site than both of the other sites at both sampling times. No difference between fish from Love Canal and the reference site was seen (Fig. 15). Median values for phenanthrene equivalents were significantly (six-to twentyfold) higher than BaP equivalents for fish from all three sites.

Median, maximum and minimum values for hepatic mono-oxygenase activities for the laboratory exposure of brown bullhead catfish to BNF are shown in Table 10. We did this experiment to have some basis for comparison for the data from the field samples, which are shown in Table 11.

Table 8. Brown bullhead sediment exposure.
Hepatic Glutathione levels.
Mean Values.

Total glutathione (umol/g tissue)	**day 4**	**day 12**	**day 24**
control	1.01	0.822	0.657
Black Creek	0.877	0.829	0.890
Love Canal	0.965	0.741	0.771
Buffalo River	0.976	0.842	0.722
% oxidized glutathione			
control	2.398	2.117	2.304
Black Creek	1.846	2.660	2.809
Love Canal	1.835	2.660	2.809
Buffalo River	2.583	2.324	2.565

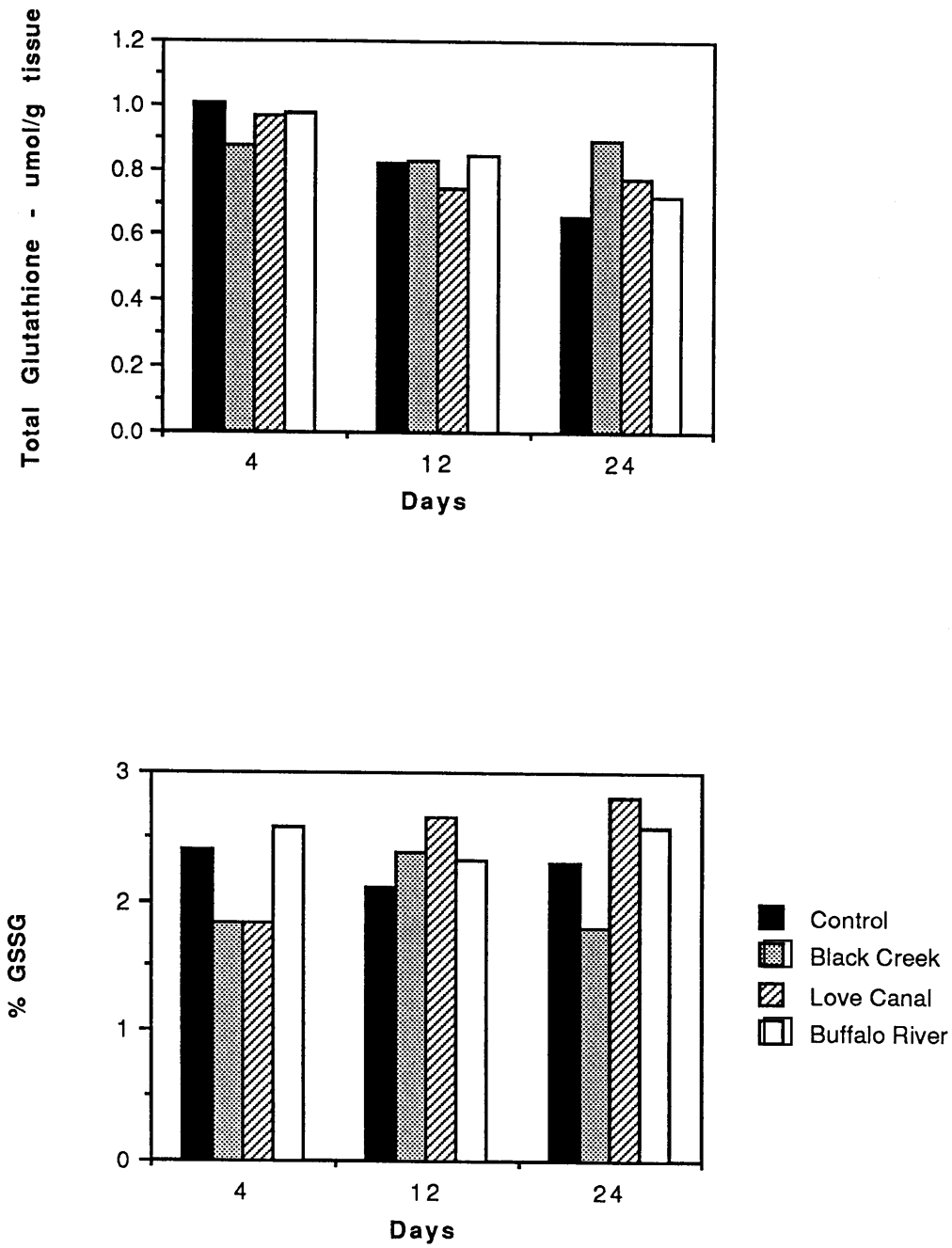

Fig. 13 Hepatic glutathione levels in brown bullhead exposed to sediments

Table 9. Data for biochemical assays - Field studies (June & Sept.,1991)
Median*, minimum and maximum values**, sample sizes***

Location	**TH-GSH** μmol/g tissue	**8OHdG/ 2'dG** (x 10^5)	**DNA adducts** nmol/mol DNA	**Phe equivalents** ng/g bile	**BaP equivalents** ng/g bile
June					
Buffalo R.	1.57* a 1.17 - 1.88** n = 12***	5.6 3 - 20.1 n = 25	94 a 78 - 214 n = 5	6406 a 2208 - 10855 n = 5	344 a 283 - 1591 n = 5
Love Canal	1.27 a,b 0.87 - 2.64 n = 12	5.7 3.2 - 28.9 n = 27	61 b 22 - 87 n = 7	343 b 198 - 791 n = 5	31 b 19 - 90 n = 5
Black Creek	1.14 b 1.01 - 1.48 n = 6	3.8 1.5 - 11.3 n = 15	20 c 15 - 31 n = 6	151 c 70 - 184 n = 5	19 b 10 - 240 n = 5
September					
Buffalo R.	1.15 0.53 - 1.82 n = 13	8.2 4.5 - 26.5 n = 12	65 a 37 - 250 n = 7	1488 a 1262 - 8019 n = 5	189 a 110 - 2127 n = 5
Love Canal	1.07 0.45 - 1.71 n = 11	10.6 6.8 - 44.9 n = 9	11.5 b 5.0 - 21 n = 6	112 b 8.0 - 755 n = 5	23 b 8.0 - 69 n = 5
Black Creek	1.02 0.60 - 2.01 n = 13	7.7 0.50 - 19.9 n = 9	4.5 c 2.0 - 8.0 n = 6	128 b 52 - 440 n = 5	20 b 10 - 546 n = 5

a,b,c - Medians with the same letter are not significantly different between sites

Table 10. Monooxygenase measurements for laboratory time course study
Brown bullhead exposed to BNF at 10 mg/kg and 100 mg/kg
Median*, minimum and maximum values**, sample sizes***

Exposure group	**Cytochrome P450** nmol/mg protein	**Cyt. P4501A** equiv./ mg protein	**EROD activity** pmol/min/mg prot.
Untreated control	0.27* NR** n = 1***	not detectable NR n = 3	not detectable NR n = 1
Corn oil control	0.042 0.018 - 0.078 n = 3	0 0 - 582.5 n = 3	not detectable NR n = 3
BNF - 10 mg/kg			
48 hours	0.35 0.32 - 0.39 n = 3	580.25 456.2 - 943.5 n = 5	54.6 34.5 - 128.1 n = 5
96 hours	0.19 0.043 - 0.49 n = 3	326.1 0 - 462.2 n = 5	11.9 0 - 28.5 n = 5
168 hours	0.55 0.51 - 0.58 n = 3	504.6 169.77 - 777.3 n = 5	17.9 10 - 30.1 n = 5
BNF - 100 mg/kg			
48 hours	0.23 NR n = 1	857.5 0 - 2125.6 n = 4	4.74 0 - 7.9 n = 4
96 hours	0.25 0.21 - 0.34 n = 3	581.7 502.2 - 863.1 n = 4	21.6 12.2 - 21.9 n = 4
168 hours	0.13 0.052 - 0.18 n = 3	646 486.8 - 1163.1 n = 4	26.5 0 - 34 n = 4

NR - minimum/maximums not reported due to small sample sizes, or all below detection limit

Table 11. Hepatic monooxygenase measurements in field-collected brown bullhead (June & Sept., 1991)
Median*, minimum and maximum values**, and sample sizes***

Location	Cytochrome P450 nmol/mg prot.	Cytochrome P4501A eqiv./mg prot.	EROD activity pmol/min/mg prot.	NADPH-cytochrome P450 reductase nmol/min/mg prot.
June				
Buffalo R.	0.27* a,b 0.11 - 0.8** n = 10***	253.23 a,b 145.1 - 373.45 n = 17	7.77 b 1.82 - 32.51 n = 30	29.65 6.15 - 42.5 n = 30
Love Canal	0.43 a 0.17 - 0.79 n = 10	293.59 a n = 27	24.61 a n = 30	30.8 n = 30
Black Creek	0.19 b 0.125 - 0.79 n = 9	187.37 b 85.8 - 329.5 n = 13	3.76 b 0 - 21.1 n = 19	30.89 0 - 60.94 n = 19
September				
Buffalo R.	0.47 a,b 0.13 - 0.92 n = 13	114.18 a 38.1 - 864.15 n = 14	9.23 b 0 - 30.3 n = 15	ND
Love Canal	0.63 a 0.09 - 0.77 n = 13	241.87 a 36.1 - 434.9 n = 13	25.55 a 4.51 - 85.1 n = 14	ND
Black Creek	0.32 b 0.09 - 0.72 n = 13	84.78 b 0 - 208.2 n = 11	4.19 b 0 - 9.81 n = 14	ND

a,b,c - medians with the same letter are not significantly different between sites ($p < 0.05$).
ND - not determined

EROD activity. In the lab exposure study, the maximum median EROD activity was highest in fish dosed with 10 mg BNF/kg body weight (low dose) at 48 hours post injection. The value was 54.6 pmol/min/mg protein. Maximum median EROD activity in fish dosed with 100 mg BNF/kg body weight (high dose) was 26.5 pmol/min/mg protein, and occurred at 168 hours post-injection.

Median hepatic EROD activities were significantly higher in fish from Love Canal, than in those from the other sites; three-to sixfold higher in June and three-to fivefold higher in September. Significant differences between Buffalo River and the reference site were not present. A box and whisker plot of these data is shown in Fig. 16. EROD activities in Love Canal fish were similar to those in fish induced with the high dose of BNF (Love Canal: 24.6 pmol/min/mg protein in June; 25.5 pmol/min/mg protein in September; high dose BNF 26.5 pmol/min/mg protein.) Values for Buffalo River fish (7.7 pmol/min/mg protein in June; 9.2 pmol/min/mg protein in September) and for fish from the reference site (3.7 pmol/min/mg protein in June; 4.19 pmol/min/mg protein in September) were lower than those for lab induced fish but higher than in unexposed or corn oil injected fish (<1 pmol/min/mg protein).

Cytochrome P450. Median total hepatic cytochrome P450 levels (Table 11) were significantly greater in Love Canal fish (0.43 & 0.634 nmol/mg protein) than in fish from the reference site (0.19 & 0.33 nmol/mg protein) for the June and September samples, respectively. The values for fish from the Buffalo River site fell between those from the other two sites (0.28 & 0.47) nmol/mg protein). These data are shown graphically in Fig. 17. These values correspond well with those measured in laboratory induced fish. Values measured in fish exposed to the low dose of BNF ranged from 0.13 to 0.27 nmol/mg protein over the time course; for fish exposed to the high dose of BNF, the values ranged from 0.19 to 0.54 nmol/mg protein (Table 10). Despite precautions taken during sampling and with sample storage, spectral analyses of cytochrome P450 suggested that some sample degradation had occurred, since peaks at 420 nm were observed in spectra of most samples from all of the sites.

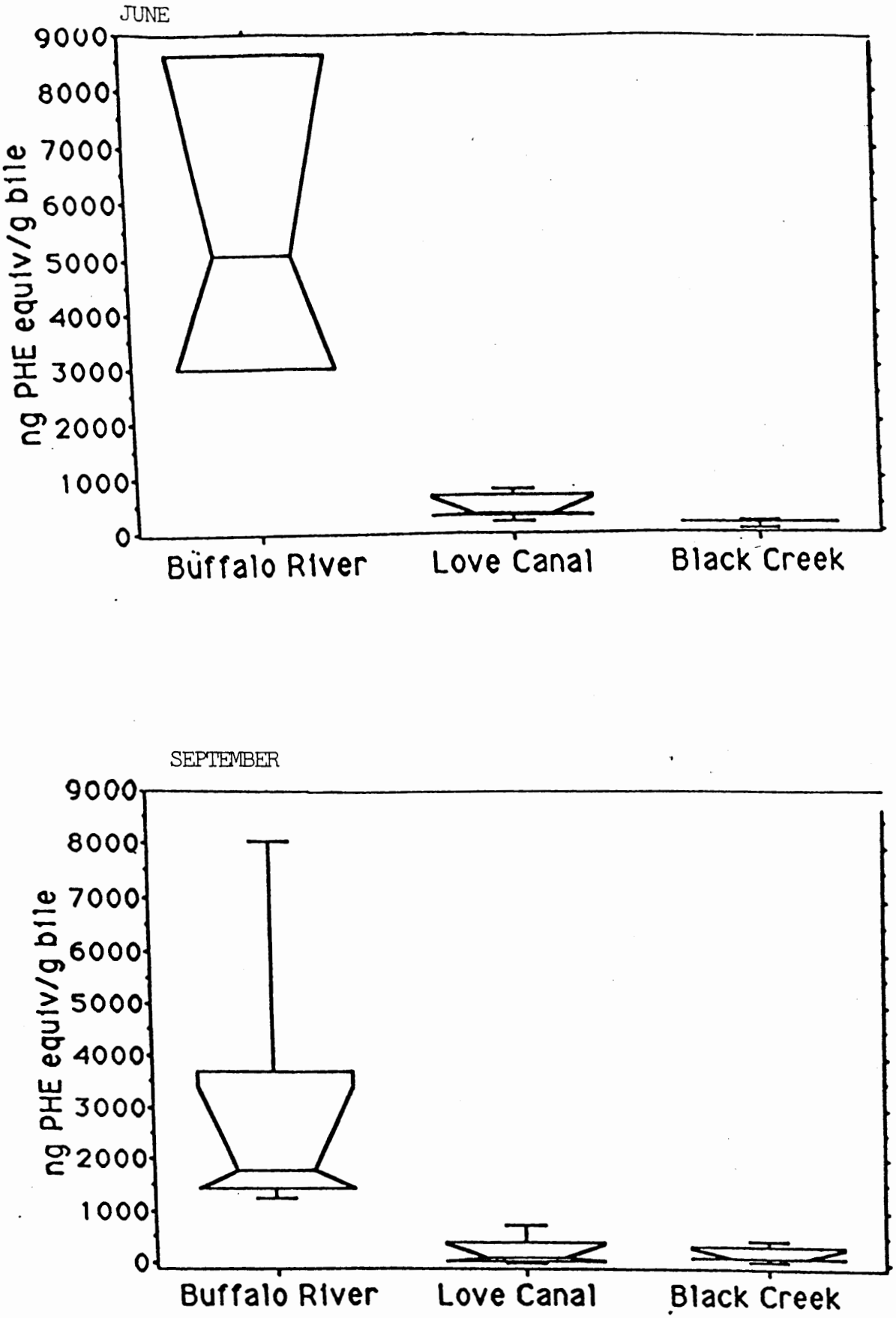

Fig. 14 Fluorescent aromatic metabolite equivalents of phenanthrene by site for June and September samplings

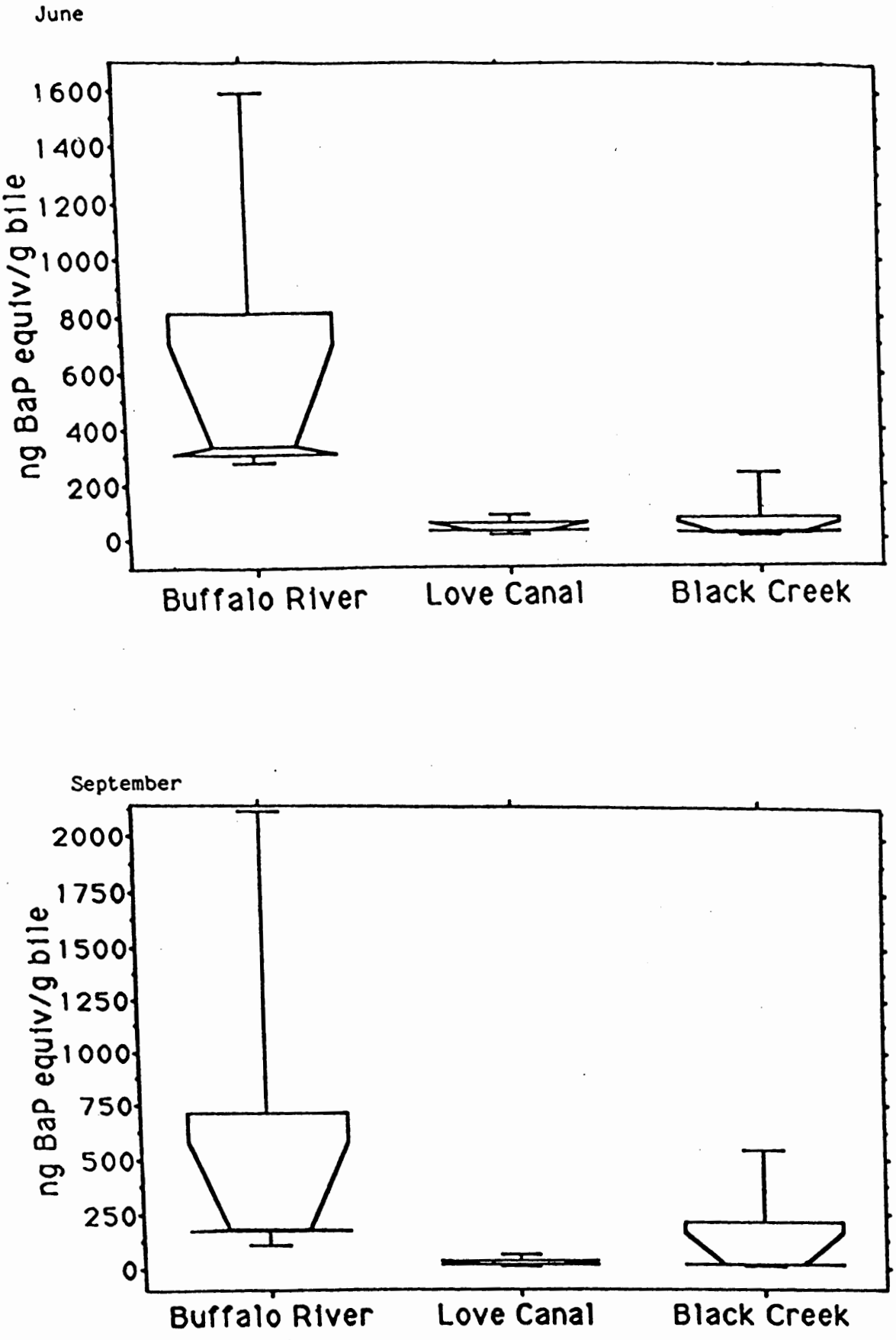

Fig. 15 Fluorescent aromatic metabolite equivalents of benzo(a)pyrene by site for June and September samplings

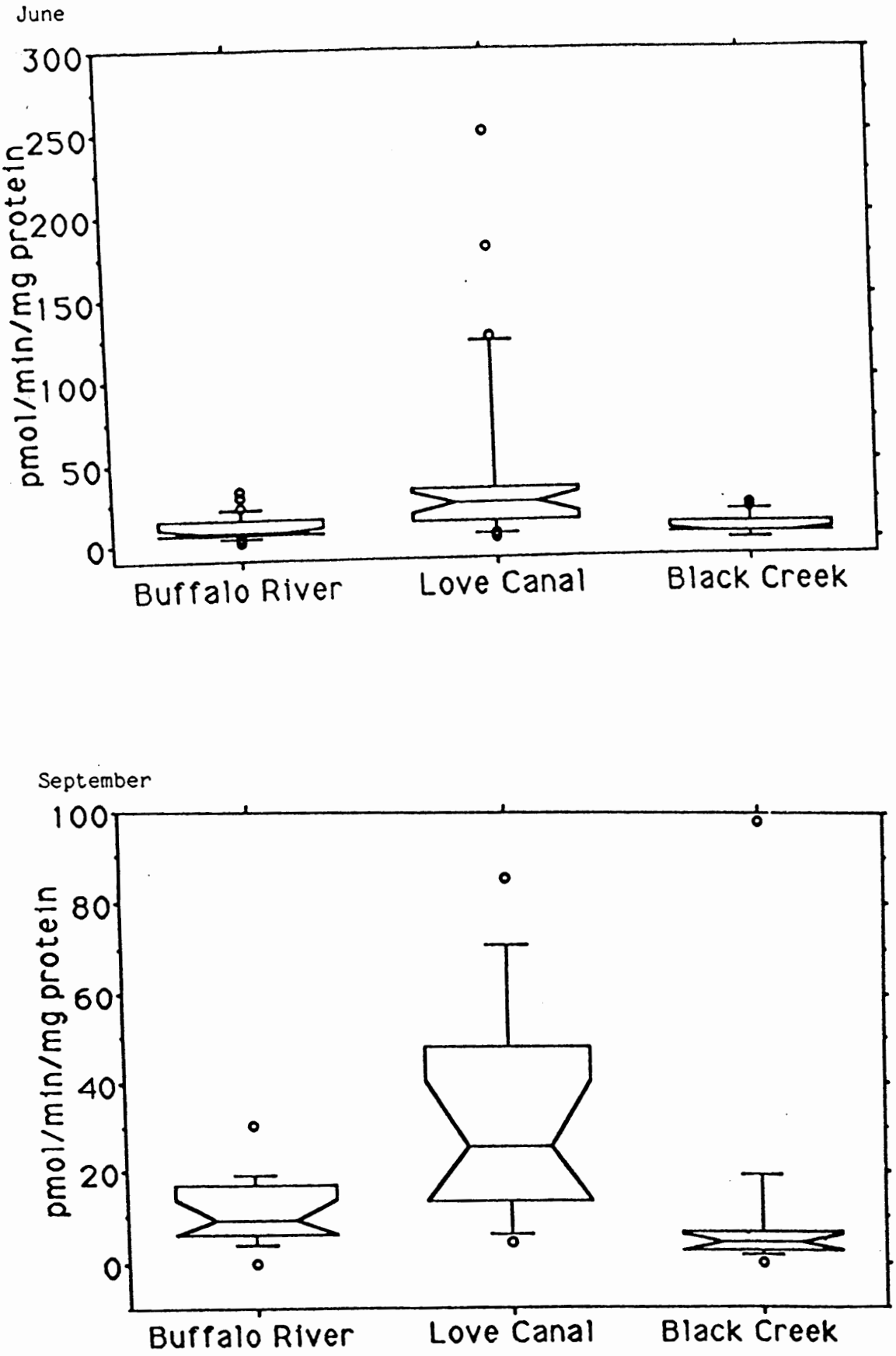

Fig. 16. Hepatic EROD activities by site for June and September samplings

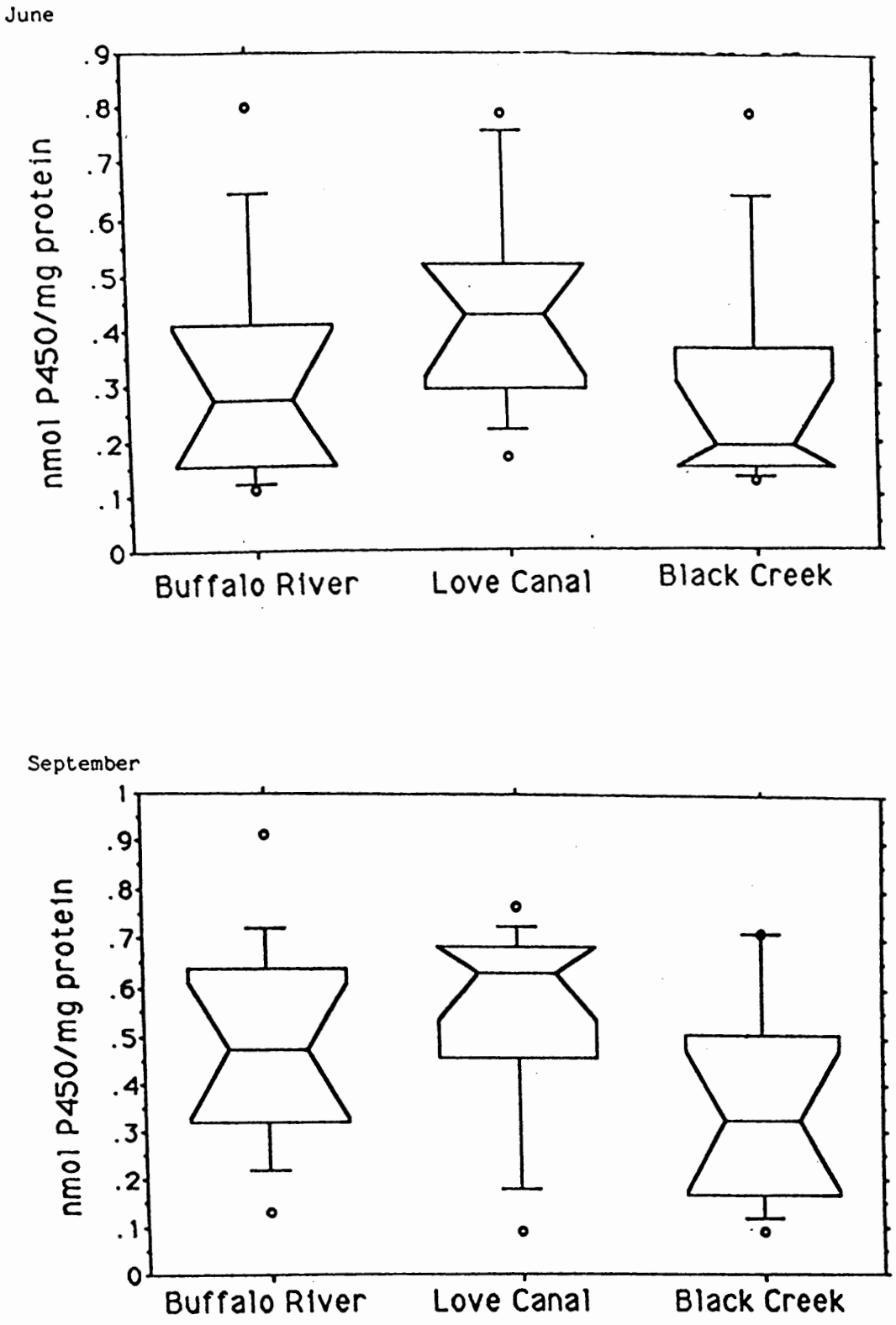

Fig. 17. Hepatic total cytochrome P450 content by site for June and September samplings

Cytochrome P4501A. Median P4501A concentrations, as measured by an ELISA, followed similar trends to those observed for EROD activities and levels of cytochrome P450 measured spectrophotometrically (Fig. 18). Both June and September values in fish from Love Canal (293.6 & 241.9 nmol P450 equivalents/mg protein) were significantly higher than those from fish from the reference site (187.4 & 84.8 nmol P450 equivalents/mg protein). Levels observed in Buffalo River fish in June (253.2 nmol P450 equivalents/mg protein) was also significantly higher than for the reference site, but the September value (114.2 nmol P450 equivalents/mg protein) was not. All values measured in the field-collected fish were lower than those observed in laboratory-induced fish. Median values for fish exposed to the low dose of BNF ranged over the time course from 326 to 580 nmol P450 equivalents/mg protein; values for fish exposed to the high dose ranged from 582 to 646 nmol P450 equivalents per mg protein.

NADPH-cytochrome P450 reductase. This activity was measured only in fish collected in June. No significant differences between any of the sites were observed. Values were, Buffalo River, 29.65, Love Canal, 30.8 and reference site, 30.9 nmol cytochrome c reduced/min/mg protein. This activity was not measured in the laboratory study.

Hepatic xenobiotic-DNA adducts. Median, maximum and minimum concentrations of hepatic xenobiotic-DNA adducts, as measured by ^{32}P postlabeling, are shown in Table 9. Levels were significantly higher at Buffalo River, followed by Love Canal and the reference site, at both sampling periods. Differences between Love Canal and the reference site were not significant at either sampling time. Differences between the polluted sites and the reference site were more pronounced in September than in June. Values for the June sampling were Buffalo River , 94, Love Canal 61 and reference, 20 nmol adducts/mol nucleotides. Values for September were Buffalo River, 64, Love Canal, 11.5 and reference, 4.5 nmol adducts/ mol nucleotides.

8-OHdG concentrations. No significant differences in 8-OHdG concentrations in hepatic DNA from fish caught at any of the sites, at either sampling time, were observed. However, the rank order of the median values from the sites is the same at both sampling times and higher in samples from all sites in September than in June. Median values were, Love Canal, 5.7, Buffalo River, 5.6 and reference, 3.8, in June and Love Canal, 10.6, Buffalo River, 8.2 and reference, 7.2 in September. Values represent a ratio between 8OHdG and 2'dG concentrations and thus are dimensionless.

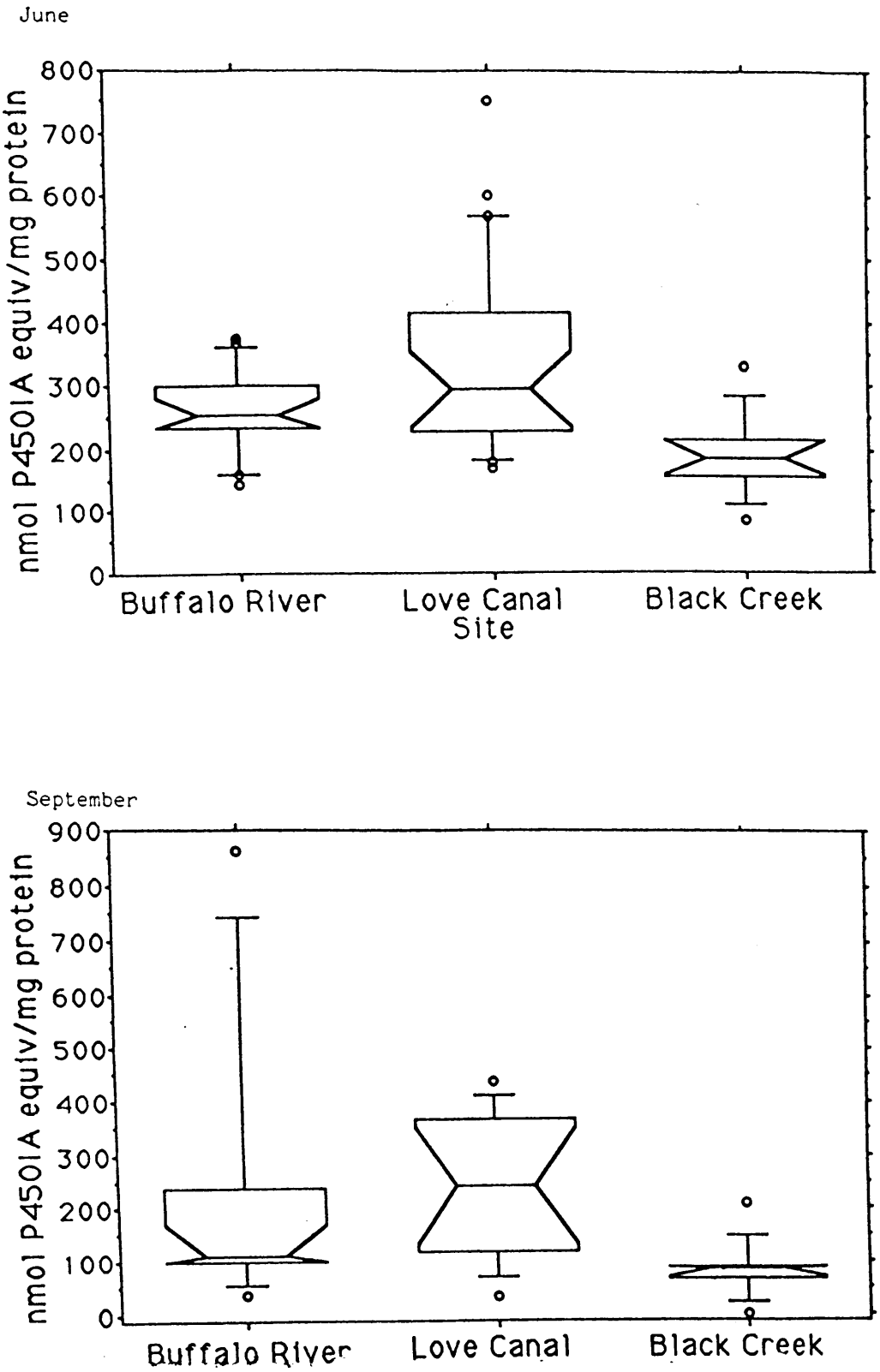

Fig. 18 Hepatic total cytochrome P4501A quantitation by site for June and September samplings

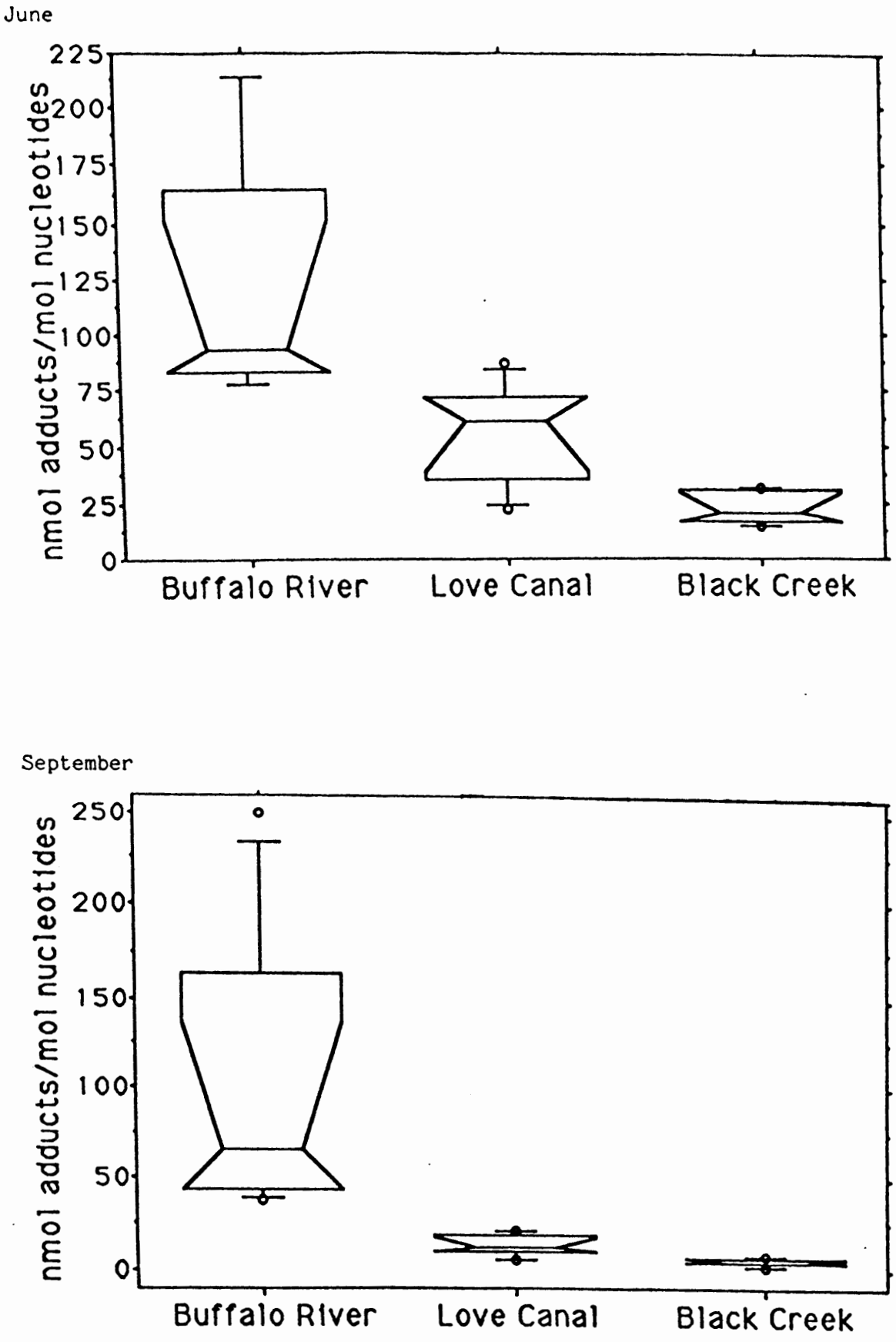

Fig. 19 Hepatic hydrophobic xenobiotic-DNA adducts by site for June and September samplings

Total hepatic glutathione. Median levels of total hepatic glutathione were significantly higher in fish collected in June at Buffalo River (1.57 umol/g tissue) than in those from the reference site (1.14 umol/g tissue). Median values for Love Canal fish were intermediate (1.27 umol/g tissue), though not significantly different from the others. No significant differences were found in fish sampled in September.

Discussion

We initially conceptualized this study with several concrete goals in mind. These were:

(1) An evaluation of several metabolic indices as biomarkers of exposure of catfish to contaminants present in environmental sediments with known contaminant loads.

(2) A comparison of the responses of these indices in feral fish collected from clean and contaminated sites to those in fish exposed to sediments and sediment fractions under controlled, laboratory conditions.

(3) A comparison between two species of catfish, the channel catfish and the brown bullhead, as to their metabolic responses to exposure to the sediments.

The major findings of our study were as follows:

(1) Overall, the most sensitive biomarkers of exposure to the contaminated sediments appeared to be the levels of FACs in the bile of the fish and hepatic EROD activities.

Cytochrome P450 and P4501A levels and levels of hydrophobic xenobiotic-DNA adducts, as detected by ^{32}P-postlabeling (PPL), also showed marked responses in feral fish. Fish from the site with the highest concentrations of PAHs (Buffalo River) showed the highest levels of bile FACs and DNA adducts. Fish from the site dominated by CHs exhibited the highest levels of cytochrome P450, P4501A and had the highest hepatic EROD activities.

(2) There were no essential differences in the responses of channel catfish and brown bullhead to exposure to the sediments under laboratory conditions.

The remainder of this discussion will focus on the results from each of the biochemical assays.

FACs in Bile

Brown bullhead taken from the Buffalo River site had significantly higher levels of FACs in their bile than fish from the other two sites. Elevated levels of phenanthrene-like metabolites were also seen in fish from the Love Canal site sampled in June. These increases were paralleled in fish exposed to sediments in the laboratory, although high levels of FACs in the bile of some control fish makes interpretation of the data equivocal. Similar increases were also noted in channel catfish injected with an extract of the Buffalo River sediment.

Chemical analysis shows that PAH concentrations are two-to twohundredfold higher in sediments from the Buffalo River site than from either of the others. Krahn and co-workers (1986) found no correlation between bile metabolites levels in English sole and sediment aromatic hydrocarbon (AH) concentrations from 11 sites in the Puget Sound. However, Maccubbin's group (1988), working with brown bullhead from the Niagara River region, did find and association between bile FACs and AH concentrations in sediments. This group also found that the mean concentrations of FACs in brown bullhead bile was 10 times higher than that determined by Krahn's group for English sole, despite similar sediment contaminant loads. Our work supports the findings of Maccubbin's group and the hypotheses they promulgated to explain the differences between their results and Krahn's; that brown bullhead might assimilate and/or metabolize AHs more rapidly and completely than English sole do, or that differences in the migratory patterns of the two species may influence both the concentrations of bile FACs and the association between that concentration and sediment AH concentrations. Differences between channel catfish and brown bullhead, as evaluated by the laboratory sediment exposures, are less clear. Channel catfish showed about three-to tenfold more BaP equivalents in their bile than bullhead, but levels in control fish were high indicating that this difference might not be due to exposure to the contaminated sediments. Levels of phenanthrene equivalents were more similar, but again, relatively high levels were seen in control and unexposed fish.

MFO Induction Parameters

Values for hepatic EROD activities were consistently higher in feral fish from the two contaminated sites than from the reference site. This activity was six-to eightfold higher in fish from the Love Canal site than in fish from the reference site. Fish injected with an extract of Buffalo River sediment also exhibited marked increases in hepatic EROD activity over uninjected and sham injected controls two days after the injections were given. No increases in this parameter were seen in either channel catfish or brown bullhead

during laboratory exposure to the contaminated sediments, although increases due to the contaminated sediments could have been obscured by high levels in the controls.

Immunoquantitated P4501A and total cytochrome P450 levels increased concomitantly with EROD activities in the feral fish; values were two-to threefold higher in fish from the Love Canal site than from the reference site. Differences between fish collected at the Buffalo River site and the reference site were smaller; cytochromes P450 and P4501A were one-and-a-half-to twofold higher at the contaminated site.

Similar behavior of hepatic EROD activities have been made by other investigators who studied sites with similar contaminant loads (Klotz et al.1983; Elskus and Stegeman 1989a; b; Collier et al. 1992). Our laboratory reported significant increases in hepatic EROD activity in channel catfish exposed to a sediment high in PAHs and PCBs, with peak activities occurring at 7 days exposure and declining thereafter. (Di Giulio et al. in press).

Using both immunological and biochemical methods, we found cytochrome P4501A concentrations and hepatic EROD activities to track each other quite closely in feral fish when data was pooled over sites and sampling periods. This is hardly surprising, as EROD activity has been previously shown to be relatively specific for the P4501A group of isozymes (Stegeman et al. 1992). However, correlations between these parameters in individual fish were weaker. When site and months were pooled, the correlation was fairly strong ($r=0.329$, $p=0.001$ $n=97$). The correlation weakened when sampling times were not pooled (June, $r=0.217$, $p=0.098$, $n=59$: September, $r=0.405$, $p=0.012$, $n=38$). EROD activities showed greater intersite differences than P4501A quantitation, perhaps indicating that EROD is a more sensitive indicator of MFO induction. Other studies have found this not to be the case. Several groups have determined quantitation of P4501A with monoclonal antibodies to be a more sensitive indicator of MFO induction (Stegeman, et al. 1987: Kloepper-Sams and Stegeman 1989). Still other groups have found both methods of similar reliability, whether P4501A is quantitated with mono- or polyclonal antibodies. (Varanasi, et al. 1986a; Goksoyr et al. 1987; Stegeman et al. 1987). Despite the semi-quantitative nature of the polyclonal antibody assay, the P450 isozyme(s) identified in brown bullhead appear to be orthologous with P4501A found in other piscine species. These anomalous results indicate that an a priori determination of whether EROD activity or P4501A quantitation will provide the more sensitve index of MFO induction in a particular study is problematic, and argue that both assays should be used in parallel.

The lack of significant differences in MFO parameters between Buffalo River and the reference site might be due to the higher than expected loads of PAHs and CHs at the reference site. Concentrations of these species ranged as high as 340 ng/g, well above desirable levels for an uncontaminated reference site, and apparently great enough to cause some MFO induction. MFO parameters in bullhead from the reference site were three-to tenfold higher than those in untreated and corn oil-injected fish from the laboratory induction study. We have no satisfactory explanation as yet for the inordinately high levels of EROD activity in unexposed controls during the laboratory sediment exposures., although the bile metabolite data suggests the presence of a contaminant in our flow through system.

Spectral analysis of P450 revealed degradation in some samples from the field study, diagnosed by a shift in the absorbtion maximum of CO-reduced cytochrome P450 from 450 to 420 nm. Forlin and Anderson (1985) noted that P450 degradation occurred over time in rainbow trout (Salmo gairdinari) livers store in liquid nitrogen. Several catalytic activities were also assayed, and acceptable levels of enzyme activities (85% of maximum) were present after 3 days storage. Limitations inherent in the field collection procedure necessitated storage of livers in liquid N_2 for up to 7 days before microsomes were prepared, and these were refrozen before analysis. Also, during the June sampling, fish were preserved alive on ice for up to three hours before sacrifice, again because of inherent difficulties in field collection. This may also explain the observed P450 degradation. Storage studies done in our laboratory, using microsomes prepared from brown bullhead livers, have shown decreases in catalytic activity of up to 30%, which may indicate an underestimate of EROD activities in feral fish. These factors argue for the use of immunological methods of isozyme quantitation, which have been shown to be largely unaffected by decreases in catalytic activity (Goksoyr 1991; Collier et al. 1992).

Differences in NADPH-cytochrome P450 reductase activity were not seen in feral fish. This agrees with previous studies. Goksoyr's group found no induction of this activity in Atlantic cod (Gadus morhua) or rainbow trout despite significant EROD induction with BNF or phenobarbital. Unpublished work done by Carlson in our laboratory also indicated no induction of this activity in bullhead by BNF. However, Winston and co-workers (1988) did find a significant increase in NADPH-cytochrome P450 reductase activity in feral channel catfish from a site polluted with PAHs and PCBs as compared with fish taken from an uncontaminated reference site. Since this enzyme donates electrons to both cytochrome P450 and phase I metabolic intermediates, its activity may not be as precise an indicator of P450 induction as other MFO parameters.

Glutathione Status

In general, hepatic glutathione status was a poor indicator of exposure to contaminated sediments. Channel catfish exposed to sediments showed increased total hepatic glutathione, as did fish collected from the Buffalo River site in June. No differences were seen in laboratory exposed brown bullhead. Some recent studies have shown total hepatic glutathione levels to be elevated by exposure to contaminated sediments (Stein et al. 1992; Di Giulio et al. in press). These response could very well be due to differences in the profiles of contaminants in the sediments. It is also unclear whether these responses indicated increased detoxification via conjugation of metabolites to glutathione, or simply heightened exposure to the xenobiotics.

DNA Single Strand Breaks.

This parameter, as measured by the alkaline unwinding assay, was not determined in feral fish. It was remarkably unresponsive in laboratory exposed fish. Previous results from our laboratory (Di Giulio et al. in press) indicated a lower proportion of double-stranded DNA in fish exposed to contaminated sediment in the laboratory, but we were unable to determine which compound(s) in the sediment was responsible for the DNA damage, and it is entirely possible that such compounds were not present in the contaminated sediments used in this study. Shugart (1988) observed that exposure to BaP at 1 ppm in a flow through system caused a significant reduction of double-stranded DNA in bluegill sunfish livers after 16 days exposure, but the value returned to control levels by day 30. Induction of this type of DNA damage might well require prolonged exposure to relatively specific compounds.

Hepatic Xenobiotic-DNA Adducts

Although a link between sediment contaminant exposure and neoplasia has been reported for several species of fish, the steps in the process of neoplasm development have yet to be elucidated (Meyers et al. 1991). Biochemical indices related to early stages of tumor formation may provide information on the etiology of cancer and further define the association between sediment contaminant exposure and tumor expression (Varanasi et al. 1989b). Of the two biomarkers of genotoxicity examined in the feral fish, the ^{32}P-postlabeling assay appeared to be a good indicator of exposure to sediment-associated genotoxins, while the analysis of 8-OHdG was unresponsive. Further study of the 8-OHdG methodology may be necessary before it is as reliable as the ^{32}P-postlabeling assay.

Elevated concentrations of 8-OHdG in fish hepatic tissue have been reported for acute and chronic exposures to pure compounds, and in fish found in contaminated marine ecosystems (Malins et al. 1990; Malins and Haimnot 1990; 1991; Nishimoto et al. 1991). Those publications report statistically significant increases in hepatic 8-OHdG concentrations, with absolute values ranging from three-to tenfold higher in exposed fish, as compared to controls. Ranking of sites in this study by absolute concentrations of hepatic 8-OHdG was consistent for both sampling times, as follows: Love Canal > Buffalo River > Black Creek (reference site). However, none of the differences between the sites was statistically significant. It is possible that this is due to some species specific variable, such as the rate of 8-OHdG repair, since the studies cited above did not use catfish. Whatever the explanation, the use of 8-OHdG levels as an indicator of DNA damage is of relatively recent origin, and further study is warranted before generalizations as to its utility as a biomarker of toxicant exposure.

DNA adducts as determined by ^{32}P-postlabeling were significantly greater at the Buffalo River site than at the other sites at both sampling times. At present, it is difficult to relate levels of DNA adducts to a single group of environmental contaminants (Varanasi et al. 1989b), although similarities in adduct profiles between feral fish and fish exposed to specific hydrophobic compounds in the laboratory suggest that anthropogenic AHs may be adducted to the DNA of fish from the contaminated sites. (Stein et al. 1989) Xenobiotics most likely to form adducts seem to be PAHs and the highest levels of adducts are found in DNA of fish from sites with heavy PAH loads. (Varanasi, et al. 1987) Comparison of levels of DNA adducts in brown bullhead to sediment concentrations of high molecular weight PAHs (a group of contaminants that contains several known genotoxins) showed a general parallel, implying that the adducts present in brown bullhead are due to PAHs.

Xenobiotic-DNA adduct levels showed a similar trend to bile FACs in the feral fish. Previous studies of DNA adduct formation by Dunn and co-workers (1987) revealed DNA adduct levels similar to those we measured. Fish from the Buffalo river site, which was highest in PAH contamination, had both the highest levels of bile FACs and DNA adducts, lending support to the hypothesis that DNA adduct formation is correlated with sediment PAH concentration.

Intersite Comparisons of Biomarkers of Exposure.

Regional differences in prevalent sediment contaminants exist throughout the Niagara River system. Sediments from Buffalo River contain high levels of 2 - 5 ring PAHs related to petroleum industries and combustion, as well as significant levels of heavy metals, probably coming from steel and coke production facilities on the river. (NRTC, 1984) Sediments from the Love Canal - 102nd Street bay site have high concentrations of PCBs, pesticides, dioxins and furans. The Black Creek reference site was selected because we thought it was relatively uncontaminated and had been previously used as a reference site in similar studies, but chemical analysis showed the sediments to have higher PAH concentrations than we found desirable.

Differences in abundance of various sediment contaminants made possible a comparison of the biochemical responses of brown bullhead to various xenobiotics in nature. Metabolic responses in the fish varied between the two contaminated sites, Buffalo River and Love Canal. The observed effects appear to be consistent with the results of the sediment chemical analyses. Chlorinated hydrocarbons such as TCDD and PCBs are potent inducers of P450, and fish from Love Canal, a site with a high load of these compounds, exhibited marked P450 induction. In contrast, The Buffalo River site is mainly contaminated with adduct-forming PAHs; brown bullhead from this site had higher levels of DNA adducts than fish from the other two sites.

Analysis of aromatic metabolites in bile demonstrated that brown bullhead from the Buffalo River site were exposed to much higher concentrations of PAHs than fish from Love Canal. In addition, a strong association between FACs in bile and xenobiotic DNA adducts was evident, while little association between bile FAC concentrations and cytochrome P450 exists. These observations imply that PAHs are primarily responsible for adduct formation in fish from the Buffalo River site (although direct chemical characterization of the adducts would be necessary to confirm this), while other, more potent compounds, are producing the strong cytochrome P450 induction seen in the fish from the Love Canal site.

Laboratory studies comparing metabolic responses due to PAH exposure and CH exposure support the results of this study (Addison et al. 1978; Vodnicnik et al. 1981; Pesonen et al. 1991). In these studies, CHs produced either higher levels or more persistent induction of the cytochrome P450 system than BNF, a model PAH, did. Addison's group (1978) fed brook trout several doses of either Arochlor 1254 or 3-MC and found both compounds to be strong MFO inducers; however, ECOD activity was two-to threefold higher in Arochlor - fed trout.

Both Buffalo River and Love Canal brown bullhead had tumor frequencies which are higher than those observed in bullhead from uncontaminated sites (Black et al. 1980; Hickey et al. 1990). The fact that bulky adduct concentrations at Love Canal were low despite the higher tumor frequencies suggests that some mechanism other than adduct formation may be responsible for the appearance of neoplasia in bullhead from this site. The strong induction of the cytochrome P450 system in brown bullhead from Love Canal shows that these fish are affected by the xenobiotics present at the site, although P450 induction does not always correlate with the presence of neoplasia. The potent P450 inducers present at the Love Canal site are CHs, and these may contribute to tumor formation by a different mechanism than PAHs do. Methods such as 8-OHdG analysis, which detect DNA alterations other than xenobiotic-DNA adducts, may provide some insight into early events in the etiology of neoplasia in fish from this site.

It is difficult to reach definitive conclusions concerning biological responses to contaminants with field samples because of the complex mixtures of contaminants found at polluted sites. The present study applies several commonly used biomarkers as well as some novel approaches to environmental monitoring. These indices showed strong associations between specific contaminants and biochemical effects in exposed organisms, and suggests that the complexity of field studies necessitates the use of several indices of contaminant exposure and effect to differentiate site specific effects. Integrating biochemical and physiological biomarkers with other approaches to contaminant impact assessment, such as sediment chemical analysis, toxicity tests and benthic community analysis, appears to provide a useful and reliable monitoring system for aquatic environments.

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