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INVESTIGATION OF POTENTIAL BIOMARKERS OF EXPOSURE TO BLEACHED KRAFT MILL EFFLUENT IN NORTH CAROLINA RIVERS

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ABSTRACT

Bleached kraft pulp mills discharge copious amounts of effluent into natural water in the United States. Some components of these effluents are of environmental concern because of their resistance to biodegradation, persistence in the environment and tendencies for bioaccumulation. This study was initiated to investigate aspects of this problem. Its objectives were: (1) to quantitate the relative concentrations of chlorophenolic bile metabolites (CBM's) in fish exposed to bleached kraft pulp mill effluent (BKME) in natural waters and artificial streams, (2) to determine if exposure to BKME results in uroporphyria in feral fish and (3) to investigate correlations between CBM's, levels of hepatic ethoxyresorufin-o-deethylase and porphyrin concentrations. Feral fish were collected at site above and below BKME outfalls at sites on both the Roanoke and Pigeon Rivers in North Carolina. Also, the National Council for Air and Stream Improvement (NCASI) provided fish from their experimental streams in New Bern, NC, which receive controlled amounts of effluent from a mill on-site. Work to determine if uroporphyria could be induced in fish exposed to hexachlorobenzene in the laboratory was also done.

Total concentrations of CBM's as low as 1.8 ng of 2,4,6 trichlorophenol (TClP) equivalents per μl of bile were detected in feral fish from the Roanoke River. On the Pigeon River, a statistically significant correlation between CBM concentration and proximity to an upstream BKME source was observed, as were significant differences in CBM concentrations between samplings in July and November 1992. The detection of individual chlorinated phenolics by use of reference compounds and GC/MS analysis was also possible on samples from the Pigeon River. Samples from the Roanoke River and the NCASI artificial streams exhibited slight, statistically non-significant trends toward increased CBM concentrations with increased exposure to BKME. EROD activities in redbreast sunfish (Lepomis auritus) taken in July from the Pigeon River were not significantly different between sites. EROD activities between sites were significantly different in fish taken in November. A site closer to the BKME outfall than the closest site in the July sampling was sampled in November. EROD activities in white catfish (Ameriurus cattus) and channel catfish (Ictalurus punctatus) taken from sites on the Roanoke River were not significantly different, nor were EROD activities from bluegill (Lepomis macrochirus) and largemouth bass (Micropterus salmoides) from the NCASI artificial streams. No significant correlations among prophyrin concentrations, EROD activities and CBM concentrations within river systems were found, although similar trends between EROD activities and CBM concentrations on the Pigeon River and the NCASI artificial streams were observed.

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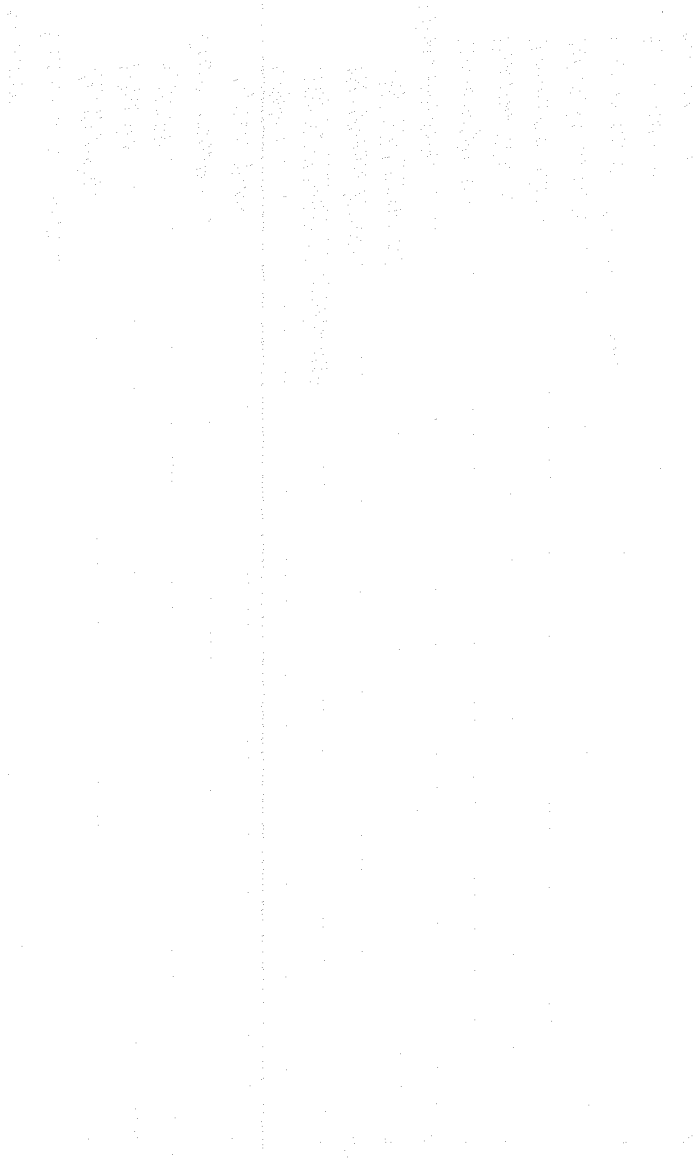

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SUMMARY AND RECOMMENDATIONS

Paper production in the United States results in the discharge of large quantities of bleached kraft pulp mill effluent (BKME) into many surface waters. The effluent is remarkably complex in composition and, while not acutely toxic to most aquatic life, may have sublethal effects on aquatic biota. The compounds in BKME that cause the most environmental concern are chlorinated organics, principally derivatives of phenol and polyaromatic hydrocarbons. BKME has been linked to deleterious changes in blood chemistry, organ size and gross pathology, oxidative stress and other metabolic effects, and reproductive effects and genotoxicity.

The overall objective of the study described here was to establish and quantify specific physiological and biochemical responses in fish to find consistent and robust biomarkers for BKME exposure. The parameters chosen for study were the concentration of chlorinated bile metabolites (CBM's) and uroporphyria, a condition characterized by the accumulation of highly carboxylated porphyrins (HCPs) in the liver. Hepatic ethoxyresorufin-o-deethylase (EROD) activity, which has been previously shown to be a sensitive biomarker for many kinds of chemical pollution, was also measured. The specific objectives of this study were as follows: (1) to determine if feral fish exposed to BKME in North Carolina rivers exhibit uroporphyria; (2) to quantify chlorophenolic bile metabolites in these fish and (3) to assess correlations among hepatic mixed function oxidase activities, hepatic porphyrin concentrations and bile metabolite concentrations in order to assess the effectiveness of these parameters as biomarkers of exposure to BKME.

Two river systems representative of the variety found in North Carolina were chosen. The Pigeon River is a high gradient, fifth order stream in the North Carolina and Tennessee mountains and the Roanoke is a major coastal floodplain river. Different species of fish were sampled from each of these rivers at various times of the year to provide an accurate representation of variability present. The field studies were integrated with laboratory studies and with samples procured from the artificial streams maintained by the National Council for Air and Stream Improvement (NCASI).

Uroporphyria is a condition characterized by the accumulation of highly carboxylated porphyrinogens (HCPs) in the liver that can be induced in mammals and birds by chlorinated organic compounds similar to those found in BKME. The difference in HCP concentrations in animals with and without the condition is considerable, usually 10-fold or more, and the assay for the compounds involved is extremely sensitive, allowing the measurement of femtomoles. Thus it was hoped that the condition would be easily detectable in fish if it was induced by BKME exposure.

Chlorophenolics and other chlorinated organics are lipophilic and show a tendency for bioaccumulation. They have been previously detected in the bile of fish exposed to pure compounds and to BKME. They may also be detected at very low levels, although not as low as the porphyrins. Previous studies have shown CBM levels to be well correlated

with BKME exposure and with distance from the source of exposure. Levels of CBM's have correlated with BKME gradients that extend for distances of over 100 km.

Hepatic EROD activity has repeatedly been shown to be correlated with exposure to any number of toxicants and to be quite sensitive as well. Taken together with prophyria and CBM, it seemed likely that a diagnostic biomarker of BKME exposure could result. As all of these assays are fairly simple and inexpensive to perform, possible extensive use in large scale biomonitoring studies was indicated.

The principal conclusions reached as a result of this work were as follows:

- Uroporphyria is not inducible in channel catfish under conditions that have caused its induction in other animals.
- Uroporphyria is not found in feral fish exposed to BKME.
- Chlorinated bile metabolites were a good indicator of the proximity of feral fish to a BKME outfall on the Pigeon River, but not on the Roanoke.
- Chorinated bile metabolites also indicated that fish in the Roanoke River were exposed to a much lower concentration of chlorophenolics than fish in the Pigeon.
- EROD activity was again shown to be a sensitive indicator of exposure, although a relatively non-specific one.

As a result of this work, we recommend that a suite of biomarkers be used to diagnose BKME exposure. Chlorinated bile metabolites and EROD activity measurements should be included in this suite. While uroporphyria is not a good indicator of exposure in the species used in this study, we recommend that other species of fish be examined for susceptibilty to the condition. If sensitive species are found, uroporphyria could prove a useful biomarker for BKME exposure.

INTRODUCTION

Paper production in the United States results in the discharge of large quantities of bleached kraft pulp mill effluent (BKME) into many surface waters. The effluent is remarkably complex in composition and, while not acutely toxic to most aquatic life, may have sublethal effects on aquatic biota. The composition of BKME varies considerably among mills and there are also large daily variations in effluents from a given mill depending on the stage of the process and the type of wood being processed. The overall objective of the study described here was to establish and quantify specific physiological and biochemical responses in fish to find a consistent and robust biomarker for BKME exposure. The parameters chosen for study were the concentration of chlorinated bile metabolites (CBMs) and uroporphyria, a condition characterized by the accumulation of highly carboxylated porphyrins (HCPs) in the liver. Both of these should be affected specifically by compounds unique to BKME. Additionally, uroporphyria is a recognized condition in humans, which may enable linkage between ecotoxicological effects and human health. Within the last two decades or so, there have been a plethora of investigations into the effects of BKME on aquatic life. The results of some of these will be discussed here.

BKME and its effects on fish

A bleached kraft mill produces high quality pulp from wood to be used in the manufacture of light colored paper and cardboard products. The kraft process involves cooking chipped wood in the presence of NaOH, formed by mixing soda ash or Na_2CO_3 with lime. The mixture also usually contains 20-30% sulphide. The process extracts from the whole wood usable cellulose, which is then bleached with chlorine, chlorine dioxide or a mixture of the two. Chlorine binds to organic products produced by the hydrolysis of the lignin in the wood and these compounds are discharged in BKME. BKME is a complex mixture of thousands of compounds. It has been shown to contain resin acids, fatty acids and chlorinated derivatives of benzene, phenol, guiacol, catechol, cymene and cynemene, naphthalene, fluorene, phenanthrenes and 2,3,7,8 tetrachlorodibenzodioxin (Hrutfiord and Negri 1992; Koistinen et al. 1994a; Koistinen et al. 1994b; McKague et al. 1990; Rantio 1992; Suntio, et al. 1988). In spite of extensive research conducted on BKME, it is estimated that only 10 - 40% of the lower molecular weight components, those readily taken up by fish, have been identified. (Leach and Thakore 1975).

A variety of effects on fish correlated with BKME exposure have been documented. The most ubiquitous effect seems to be the induction of hepatic ethoxyresorufin-o-deethylase (EROD) activity. It has been shown in laboratory-exposed rainbow trout (Oncorhynchus mykiss) (Martel et al. 1994), caged whitefish Coregonus muksun and C. m. x C. peled) and vedace (Coregonus albula) (Lindstrom-Seppa and Oikari 1989; Lindstrom-Seppa et al. 1989) and feral fish, including white sucker (Catostomus commersoni)

(Munkittrick et al. 1994), longnose sucker (Catostomus catostomus) and mountain whitefish (Prosopium williamsoni) (Kloepper-Sams and Benton 1994), and carp (Cyprinus carpio) (Ahokas et al. 1994), and in fish collected at distances of up 100 km from the source of the effluent (Gagnon et al. 1994b). Induction has been correlated with chronic exposure to BKME, as measured by muscle dioxin content, rather than acute exposure, as measured by the resin acid and chlorinated phenolic content of bile (Kloepper-Sams and Benton 1994), and with absorbable organic halogen (AOX) levels (Ahokas et al. 1994). It was found to be poorly correlated with organic halogens extracted from muscle tissue or sediments (Ahokas et al. 1994). In a laboratory study, replacement of the bleaching agent chlorine by chlorine dioxide or peroxide did not effect the EROD-inducing property of the effluent, but it was found that some non-kraft effluents failed to cause induction. (Martel et al. 1994). In one species, burbot (Lota lota), EROD was found to be uninduced after exposure of fish to a highly treated BKME (Kloepper-Sams and Benton 1994).

Other effects, some more deleterious, have also been documented. Blood chemistry and cellular characteristics in fourhorn sculpin (Myoxocephalus quadricornis) and lake trout (Salmo trutta) appear to be affected by exposure (Bengtsson et al. 1988; Hardig et al. 1988; Oikari et al. 1988). Decreased gonad size and increased liver size have been observed in exposed fish (Larsson et al. 1988, Munkittrick et al. 1994); decreased liver size has also been reported (Adams et al. 1993).

There is evidence that exposure to BKME increases oxidative stress on channel catfish (Ictalurus punctatus) as indicated by increases in catalase, reduced glutathione levels and fatty acid oxidase activity (Mather -Mihaich and Di Giulio 1991), suggesting that BKME may act a peroxisome proliferator. A later study confirmed the decrease in GSH levels in another species, the bullhead (Cottus gobio), and found also that glucose-6-phosphate dehydrogenase and vitamin C levels are also decreased, leading to enhanced risk of oxidative damage (Bucher, et al. 1993).

There are numerous reports of reproductive effects of BKME exposure. Perturbations in levels of circulating sex steroids have been reported. In white sucker, 11-ketotestosterone levels were reduced in males and testosterone levels were increased in females while 17 β-estradiols in females were unaffected (Gagnon et al. 1994a). Another study by the same group found 17 β-estradiol levels in females depressed (Gagnon et al. 1994b). White sucker at Jackfish Bay on Lake Superior showed delayed sexual maturity, reduced gonadal size, diminished secondary sexual characteristics and depression of the levels of circulating steroids (Van der Kraak et al. 1992) Similar results were also obtained in a study which examined 12 different mills in Canada (Munkittrick et al. 1994). Both groups attribute the steroidal depression to decreased capability of gonadal tissue to synthesize the compounds.

Evidence of the genotoxicity of BKME is also extant. BKME has been shown to be mutagenic by the Ames test (Rao et al. 1994) with the majority of the mutagenic activity associated with the polar fraction. Acidic fish extract from BKME exposed fish was also found mutagenic in the Ames test (Blevins 1991). Increased stand breakage in DNA from exposed fish was also found (Adams et al. 1993).

Gross pathology, exemplified by fin erosion in goldfish (Carassius auratus) and perch has been demonstrated (Lindesjoo and Thulin 1994; Sharples et al. 1994). The pathology is identical for both species. Skeletal deformities have been reported to occur in exposed perch (Lindesjoo et al. 1994) and pike (Esox lucius) (Lindesjoo and Thulin 1992). Effects on behavioral patterns in vendace, illustrated by schooling behavior, have also been documented (Myllvirta and Vuorinen 1989). One report attributes the disappearance of entire species, including lake trout and whitefish, to the sublethal toxicity of BKME discharge (Oikari et al. 1988).

However, it is apparently possible to greatly reduce or eliminate all of these effects by proper treatment of effluent. A recent study of a mill using 100% chlorine dioxide bleaching and primary and secondary effluent treatments, at a site with little prior environmental degradation, found no effect on organ sizes, condition indices, blood chemistry, reproductive indices or population parameters in mountain whitefish or longnose sucker (Kloepper-Sams et al. 1994; Swanson et al. 1994).

Of the components of BKME that have been identified, the chlorinated organics are of particular importance because they are produced in large quantities, are lipophilic, persistent and toxic to aquatic organisms. The lower molecular weight chlorinated phenolics (phenols, catechols and guiacols) have been identified as the major toxic constituents of BKME (Leach and Thakore 1975). Another report shows that 85-95% of the chlorinated organics present in BKME are less than 1000 grams/mole in molecular weight (Jokela and Salkinoja-Salonen, 1992). These compounds have also been shown to be highly resistant to biological wastewater treatment (Jones 1991)

Quantitative structure-activity relationships have been obtained for the chlorophenols both in vitro (Saito and Shigeoka 1994) and in vivo (Smith et al. 1994) for fish. These studies show that toxicity increases with increased chlorination, with pentachlorophenol (PCP) being the most toxic, and that the meta isomer of the monochlorinated phenol (MCP) is more toxic than the ortho. Indeed, even the bile of fish exposed to sediment spiked with PCP was found to be 10-fold more toxic to Dapnia magna that that of control fish (Andreasson and Dave, 1994). Smith's group found the major cause of toxicity to be polar narcosis.

The genotoxicity of these compounds is debatable. One group found neither 2-chlorophenol, 4-chlorophenol nor the oxidation products arising from enzymatic treatment to be mutagenic in the Ames test (Massey et al. 1994). Another group found that 4-chlorophenol was mutagenic in the Ames test at 100 ppm (Ono et al. 1992). This group also found PCP to be mutagenic via the Ames test in the presence of S9, and mutagenic in both the absence and presence of S9 in an assay using the induction of *umu*DC genes fused to the *lacZ* gene in Salmonella thyphimurium, indicating the potential for bioactivation of this compound. PCP was also shown potentially genotoxic by causing the induction of prophage lambda in the presence of S9 (De Marini et al 1990). This group also found no evidence of the mutagenicty of the MCPs. PCP has also been found to be potentially immunosupressive in fish, having been shown to inhibit phagocytosis and

superoxide production in two different phagocytic cell populations derived from killifish (Fundulus heteroclitus) (Roszell and Anderson 1994).

BKME and CBM's

The lipophilicity of the chlorophenolics leads to a tendency for bioaccumulation (Wesen et al. 1991), so detection of their presence in fish can provide a useful biomarker of exposure to BKME. The reported detection limit for these compounds in bile is 20 ng/ g bile fluid, corresponding to a concentration in the water of 400 pg/ l (Soderstrom and Wachtmeister 1991). Laboratory studies have shown an increase of conjugated chlorophenolics in fish bile with increasing concentrations of BKME from 0.6% to 2% (Oikari and Nittyla 1984). In that study, conjugated resin acids and chlorophenolics comprised 93 - 99.9% of the total conjugates in bile. Similar results were obtained in an analysis of water contamination which compared bile samples taken from fish in the laboratory, kept in cages in the field, and from feral fish. Levels of bile conjugates were well correlated with BKME concentrations in the laboratory studies and with distance from the effluent source in field-exposed fish (Oikari and Holmbom 1986). In a more recent study, gradients of 3,4,5 trichloroguiacol and tetrachlorocatechol were detected up to 18 km from the effluent source and varied in a linear fashion with distance from the source. (Soderstrom et al. 1994). Another group detected chlorophenolic and resin acid compounds in the bile of mountain whitefish and longnose sucker when they could not be detected in muscle tissue and demonstrated a spatial gradient of these compounds which stretched 230 km downstream from the effluent source. They also detected these compounds after acute exposure to BKME in an 8 day depuration study in the laboratory. (Owens et al 1994).

Porphyria

Porphyrias are conditions characterized by the accumulation of porphyrins in the liver. Uroporphyria, a condition distinguished by the accumulation of highly carboxylated porphyrins (HCPs) in the liver, appears to be induced by exposure to some polyhalogenated aromatic hydrocarbons (Kennedy and James 1993) which can occur in BKME. The condition has been extensively investigated in mammals and birds, although only two studies have been published to date concerning fish. Its potential efficacy as a biomarker of BKME exposure is due to the fact that it occurs as a specific response to chlorinated organic compounds in BKME and that when it occurs there is usually a 10-fold or greater increase in hepatic HCPs, allowing easy detection. The assay as performed on avian liver tissue requires only small amounts of liver, making it useful for studies on small animals with limited quantities of liver tissue. HCPs can persist in the liver for months or years and are not sensitive to easy degradation due to conditions which can arise during field collection. The assay is also very sensitive, with a detection limit approaching 0.05 pmol porphyrin/g liver tissue.

Uroporphyria develops from a perturbation of the heme biosynthetic pathway; in particular, from a dysfunction in the enzyme uroporphyrinogen decarboxylase (UROD). Mammilian studies indicate that the mixed function oxidase (MFO) system and its regulation are involved in uroporphyria and that the presence of iron is also important.

While investigated in great detail, the exact mechanism of the development of uroporphyria remains unresolved. Various theories are discussed below.

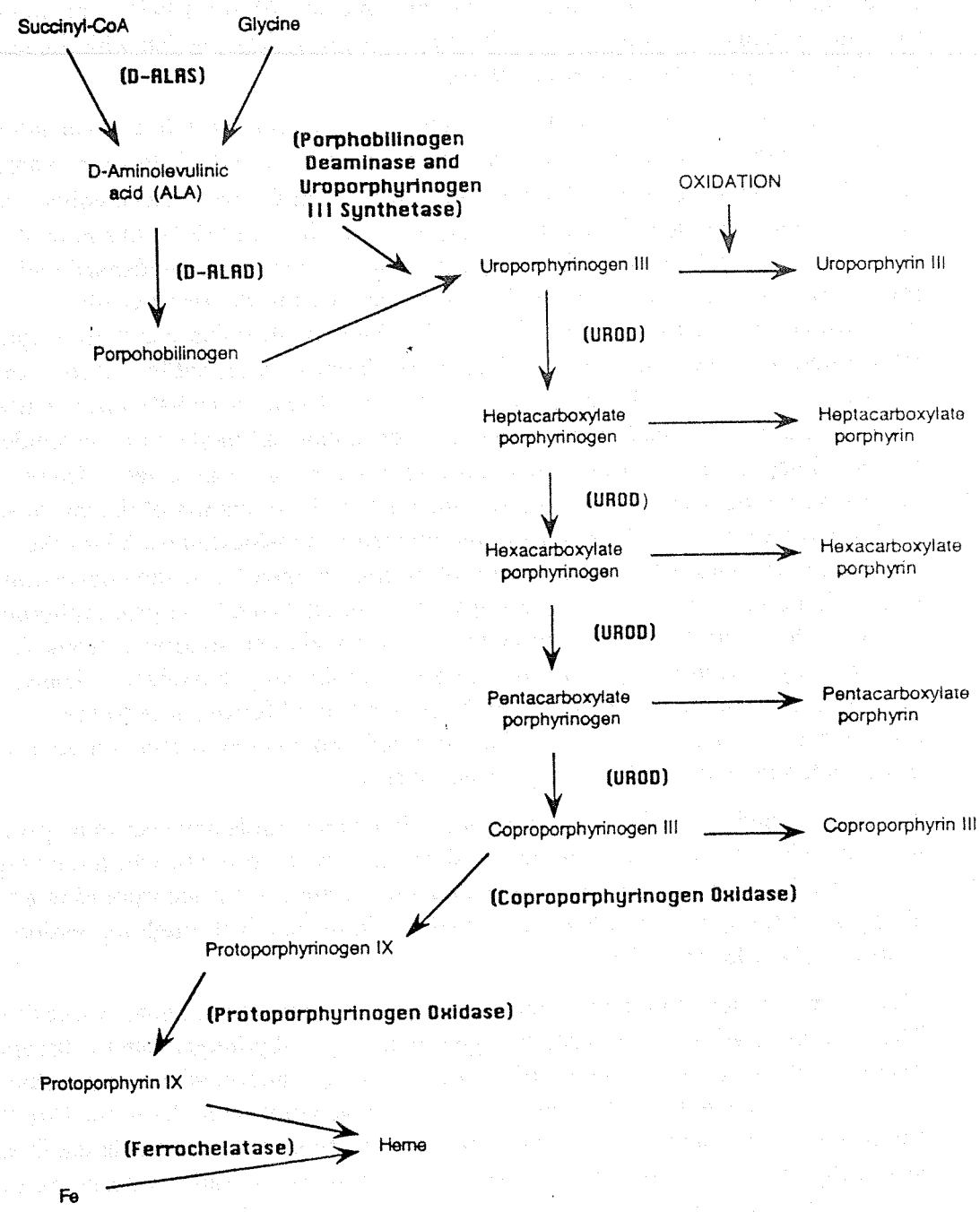
Heme synthesis. Heme is a ubiquitous prosthetic group that is found in many proteins such as cytochromes, oxidases, hemoglobin, peroxidases and catalase. It is integral to cellular functioning and a blockage in its synthesis could have extensive organismal effects. The primary sites of heme biosynthesis in mammals are erythroid cells and in hepatic tissue, but it is believed that all cells have this capacity (Marks 1985). The kidney appears to be the primary site of erythropoesis and heme biosynthesis in fish with the liver the next most active organ. (Addison et al. 1990).

Figure 1 shows a schematic of the pathway. In the mitochondrion, glycine and succinyl-CoA are condensed by δ-aminolevulinic acid synthetase (ALA-S) to form δ-aminolevulinic acid (ALA). This step is rate-limiting in mammals, but the next step involving δ-aminolevulinic acid dehydratase (ALA-D) is rate-limiting in fish (Addison et al. 1990). After ALA is translocated to the cytoplasm, two molecules are condensed with ALA-D to form porphobilogen (PBG), which is in turn converted to the tetrapyrrole uroporphyrinogen III by sequential catalysis with PBG deaminase and uroporphyrinogen III synthetase. Next, four sequential decarboxylations, catalyzed by UROD, form coproporphyrinogen III for uroporphyrinogen III. The intermediate formed after each decarboxylation is named for the number of remaining carboxyl on the molecule — they are called hepta-, hexa-, penta-, and coproporphyrinogen, respectively. These decarboxylations occur at a rapid rate which deters the oxidation of the intermediates. (Elder and Urquhart 1987) Coproporphyrinogen is translocated back into the mitochondrion where it is changed to protoporphyrinogen IX by the conversion of two of its propionic acid groups to vinyl groups, which is catalyzed by coproporphyrinogen-oxidase. Protoporphyrin IX is formed by the removal of six hydrogen atoms from protoporphyrinogen IX by catalysis with protoporphyrinogen oxidase. Finally, heme is formed through the ferrochelatase-catalyzed addition of ferrous iron to the protoporphyrin. Heme can then be incorporated into a hemoprotein, stored in free pools or degraded through the heme oxygenase system.

The identity and intracellular location of the free heme pools have yet to be precisely defined. Theoretically, they can be visualized as a small pool into which the newly synthesized heme is fed and out of which heme is removed for incorporation into proteins. This pool is thought to regulate heme synthesis in mammals through repression of ALA-S synthesis (De Matteis 1988)

Many different xenobiotic compounds are known to disrupt the heme synthesis pathway. The decarboxylation of uroporphyrinogen to coproporphyrinogen can be disrupted in a manner that causes the oxidation of the porphyrinogen intermediates to porphyrins, which are extremely stable in the liver and increase in concentration in the urine. (Smith and De Matteis 1990; Kennedy et al. 1986). This accumulation of porphyrins in the liver is known as porphyria. The pattern of porphyrin accumulation or the ratio of porphyrins in different carboxylation stages present can serve as a fingerprint for the presence of particular compound affecting the system. Polyhalogenated aromatics such as hexachlorobenzene (HCB) and 2,3,7,8-tetrachlorodibenzodioxin (TCDD) are known to cause an

Figure 1 - The heme biosynthetic pathway



accumulation of the HCPs uro- and heptacarboxyporphyrin. This accumulation is called uroporphyria (Kennedy et al. 1986; Marks 1985).

Uroporphyria. Hepatic uroporphyria is a condition caused by an interruption in heme synthesis and characterized by the accumulation of HCPs in the liver. Chlorinated hydrocarbons have been known to cause this condition in mammals since the 1950's when hexachlorobenzene-contaminated grain was mistakenly consumed by humans (Peters 1976). Hundreds of people developed porphyria, and some continued to show symptoms 25 years after the incident (Peters et al. 1982). Research has shown that uroporphyria may be induced in mammals (De Matteis 1988: Marks 1988: Cantoni et al. 1981; Cantoni et al. 1987), in birds (Kennedy and Fox 1990), and in various cell cultures (Lambrecht et al. 1988b; Kawanishi et al. 1978) by some polyhalogenated aromatic compounds. Chlorinated hydrocarbons such as TCDD, HCB pentachlorophenol (PCP) and some tetra- and hexachlorobiphenyls have been used to induce uroporphyria in research animals (Goldstein et al. 1977; Krishnan et al. 1991; Lambrecht et al. 1988a; Van Ommen et al. 1989). There have been a few studies published concerning uroporphyria in fish. The accumulation of HCPs and reduction of in vitro UROD activity in feral fish was found in pike (Esox lucius) from the Rhine River in Germany, a river highly contaminated with a variety of toxicants (Koss et al. 1986). These fish had higher levels of halogenated organics in their tissue than fish from a reference site, as well as higher lead and mercury levels. It was unclear whether the observed uroporphyria was induced directly by the organohalogens or whether the metals had a synergistic effect. Such synergism has been observed in rats (Koss et al. 1986). Another group found increased levels of HCPs and significant differences in the ratios of HCP to protoporphyrin and coproporphyrin to protoporphyrin in BKME exposed whitefish (Coregonus clupeaformis), which they attribute to the inhibiton of porphyrinogen decarboxylase (Xu et al. 1994). Other unpublished results indicate that exposure of carp and trout to TCDD failed to induce uroporphyria. These exposures were of limited duration and it was the opinion of the researcher that additional studies might produce different results. (Sean Kennedy, Environment Canada, personal communication, 1993).

Other researchers have shown that co-planar compounds, or compounds with relatively flexible conformations that have a high probability of co-planarity are active inducers of uroporphyria, while similar, non-planar chlorinated hydrocarbons are not. (De Matteis et al. 1989; Kawanishi et al 1978; Sassa et al. 1986). Specifically, para and meta substituted isomers were found to be more potent inducers — for example, 3,4,3',4'-tetra- and 3,4,5,3',4',5'-hexachlorobiphenyl isomers are very active inducers of uroporphyria, while 2,3,2',3'-tetra- and 2,6,2',6' tetrachlorobiphenyl are weak inducers. The compound 2,5,2',5'-tetrachlorobiphenyl is inactive in inducing the condition. The most active commercial PCB investigated was Kanechlor-400. (Kawanishi et al., 1978). Other compounds found to induce uroporphyria in chick embryo hepatocytes were parathion, carbamazepine and nifedipine. (Lambrecht et al. 1988b) The mechanism by which these compounds affect heme biosynthesis is unknown. An examination of the factors which appear to be important follows.

UROD. A decrease in UROD activity enhances the accumulation of uroporphyrin in the liver (Visser et al. 1989). The concentration of immuno-reactive enzyme does not change in porphyric liver, demonstrating the inhibition rather than the repression of UROD (Elder and Sheppard 1982). Other work determined that [^{14}C]HCB-radioactivity did not bind covalently to UROD, indicating that a form of the uroporphyrogenic compound does not bind to the UROD protein (Smith et al. 1986). There is considerable debate over whether UROD inhibiton occurs before (Smith et al. 1981), or after (Visser et al. 1989) the accumulation of HCPs. The mechanism of the inhibition is currently unknown and under investigation and the various theories pertaining to this subject are discussed in a later section of this report. One group has even demonstrated the development of uroporphyria without any inhibition of UROD (Lambrecht et al. 1988b). Hence, the importance of UROD inhibition in the development of uroporphyria has not been unequivocally ascertained, even though the enzyme catalyzes the step in which heme synthesis is blocked.

The mixed-function oxidase (MFO) system. Most of the compounds that induce uroporphyria also induce the MFO system, which is responsible for the metabolism and excretion of xenobiotic and endogenous non-polar compounds. The enzymes that constitute the MFO system, the cytochrome P450 isozymes, NADPH-cytochrome P450 reductase, cytochrome b_5 and NADPH-cytochrome b_5 reductase, occur primarily in the membrane of the smooth endoplasmic reticulum and act to create a redox cycle which causes the oxidation of non-polar xenobiotics by the cleaving of diatomic oxygen with the concomitant production of water. (Stegeman and Kloepper-Sams 1987). The oxidized products may be excreted directly or further metabolized by phase II pathways. Some of these biotransformations may create compounds much more toxic than the parent compounds.

The MFO system is induced by a variety of compounds (Payne et al. 1987). In mammals, with which most research concerning the MFO system has been done, there are four distinct patterns of induction. Induction of the type caused by 3-methylcholanthrene (3-MC) is the primary mechanism that functions in fish (Stegeman and Kloepper-Sams 1987). Other compounds causing this type of induction are polycyclic aromatic hydrocarbons (PAHs) PCBs and polychlorinated dioxins and furans. Compounds which induce a given isozyme may not necessarily be good substrates for that isozyme — TCDD has one of the highest affinities known for the Ah receptor (Smith et al. 1981) and is one of the most potent inducers of the MFO system, but is a poor substrate for the cytochrome P450 induced (Lambrecht et al. 1988a).

Cytochrome P450 induction usually accompanies uroporphyria (Cantoni et al. 1987; Lambrecht et al. 1988a; Smith et al 1990) and has been shown to enhance uroporphyria in liver cell cultures (Debets et al. 1981) Studies with mice show that strains that were Ah responsive developed uroporphyria earlier and more severely than other strains non-responsive. Treatment with MC caused increased uroporphyrin accumulation in rats treated with ALA and TCDD (Lambrecht et al. 1988a; Smith et al. 1981). Piperonyl butoxide, a P450 inhibitor, curtails the production of HCPs in HCB-treated chick embryo hepatocytes (Sinclair et al. 1981).

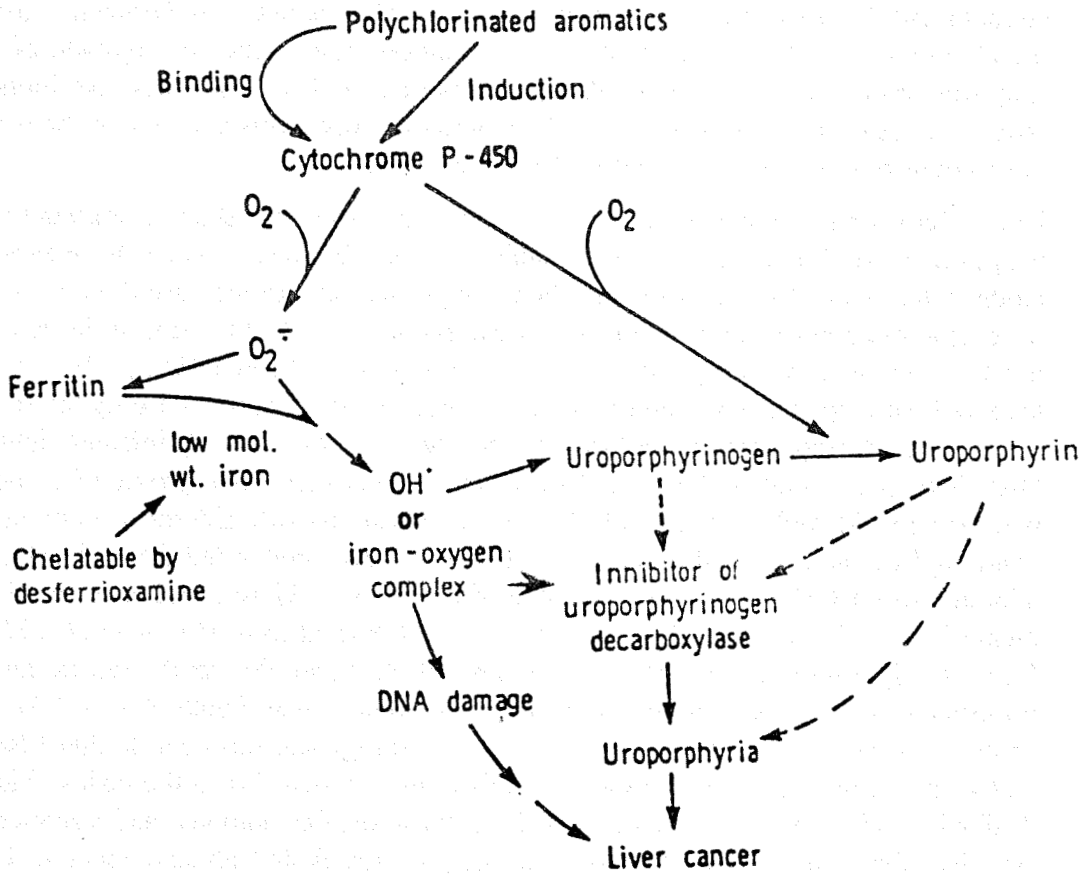
While P450 induction appears to be crucial in the initiation of uroporphyria, highly uroporphyrogenic compounds appear to be better inducers of the MFO system than substrates for it. TCDD is a more potent initiator of uroporphyria than tetrachlorobiphenyl (TCB), but less than 1% of TCDD and 29% of TCB were converted to water soluble metabolites in 19 hours by chick embryo hepatocytes (Lambrecht et al. 1988a), indicating that the uroporphyrinogenicity of these compounds is not related to their rates of metabolism by the MFO system. The same group found that EROD activity rose before uroporphyrin accumulation was seen, then fell as HCP content increased. They suggested that this might be due to limited heme availability which caused a concomitant decrease in the P450 protein moiety. Conversely, Van Ommen's group found cytochrome P450 based biotransformation essential to the development of uroporphyria in rats treated with HCB (Van Ommen et al. 1989). They also found a strong correlation over time between the amount of porphyrins excreted in the urine and the amount of oxidative metabolites of HCB excreted.

Iron. Many studies indicate that the presence of iron enhances the development of uroporphyria and that the condition is inhibited in its absence. Iron has been shown to inhibit UROD and ALA-D, to induce heme oxygenase and to increase the rate of uroporphyrinogen oxidation to uroporphyrin. (Bonovsky 1991). Uroporphyria has been produced in mice by dosing with iron and ALA (Deam and Elder 1991). This study involved Ah-responsive and non-responsive mice, demonstrating that only the responsive mice acquired uroporphyria. In other studies, ferrous iron alone significantly inhibited UROD in vitro (Smith and Francis 1983) and mice became uroporphyric when dosed only with iron (Smith and De Matteis 1990). HCB-dosed rats with siderosis, a condition resulting from an excess of iron, displayed significantly greater and faster developing inhibiton of UROD activity than non-siderotic rats. The siderotic animals also showed higher levels of ALA-S and P450 as a result of HCB treatment. (Louw et al. 1977). Interestingly, iron reduced P450 induction by HCB. In another study, female rats were observed to be more susceptible to uroporphyria than males (Smith et al. 1990). It was shown that the females had greater hepatic iron storage and turnover, higher EROD levels and greater amounts of glutathione peroxidase and vitamin E than the males. Finally, desferrioxamine, an iron chelator caused a delay in the development and a reduction of the severity of uroporphyria in HCB-treated rats (Wainstock de Calmanovici et al. 1986). Theories advanced to explain this phenomenon, as well as other hypotheses concerning the mechanism for the development of uroporphyria are presented below.

Mechanism. The mechanism by which chlorinated hydrocarbons induce uroporphyria is poorly understood. Some hypotheses are shown diagramatically in Figure 2.

Some researchers theorize that the halogenated compound induces P450, then binds to the isozyme to produce the oxidation of uroporphyrinogen to uroporphyrin. (Lambrecht et al. 1988a)

Figure 2 - Possible mechanisms of uroporphyria



A second theory proposes that after the induction of P450, the organochlorine interacts with the isozyme to produce an unidentified oxidizing compound which causes the oxidation of the uroporphyrinogen. (Lambrecht et al. 1988b). The initial P450 induction could also be due to other inducers such as 3-MC or β-napthoflavone (BNF) in mammals (De Matteis et al 1988). Iron may catalyze the P450 mediated oxidation of uroporphyrinogen to a site-specific inhibitor of UROD (Deam and Elder 1991).

Others propose that the induced P450 produces an activated oxygen species that reacts with heme or a precursor which then bind irreversibly to UROD (Urquhart et al. 1988). These researchers prose two roles for iron: 1) ALA-S may be directly induced by iron or may be potentiated by the porphyrogenic compounds or 2) iron may be involved in the production of the UROD inhibitor either as a catalyst or as a component of the compound. The active site of UROD is sensitive to oxidative inactivation and a planar molecule could bind to it and catalyze the production of hydroxyl radicals which would inhibit the enzyme (Elder et al. 1986; Urquhart et al. 1988).

The Pigeon, Roanoke and Neuse River Systems

The Pigeon River. A map of the Pigeon River system is shown in Figure 3. The Pigeon River is a high gradient, fifth order stream which originates in the Shining Rock Wilderness and borders the Great Smoky Mountains National Park on its west bank. It flows west from North Carolina into Tennessee and is dammed for hydroelectric production at Walter's Lake, North Carolina. Two relatively pristine rivers, the Little Pigeon and the Little River, which resemble the Pigeon in location of headwaters, size, flow, bottom substrate and gradient (Adams, 1993) were chosen as reference sites. The Champion mill at Canton, North Carolina is located approximately 30 miles from the headwaters of the Pigeon River and approximately 20 miles from the Tennessee border. The mill is a large bleached kraft operation and had a recent average daily production of 875 tons of bleached softwood pulp and 565 tons of bleached hardwood pulp (Lockwood-Post's Directory 1988). During the time of sampling for this study, the wood types used at the mill were 45-50% hardwood and 50-55% softwood. Bleaching was accomplished with 40% chlorine dioxide (Derrick Brown, Champion International, personal communication). Effluent from the mill undergoes primary activated sludge treatment before discharge into the Pigeon River (Lockwood-Post's Directory 1988). The maximum wastewater design flow for this mill is equal to the 7Q10 low stream flows, or 7-day average minimum flows which recur every 10 years, on average (USGS 1993). Therefore, during seasonal low flow conditions, the bulk of the river flow is effluent.

The Roanoke River System. A map of the Roanoke River system is shown in Figure 4. The Roanoke is a major coastal floodplain river with its source in the mountains of Virginia. It discharges into the western end of the Ablemarle Sound in North Carolina. Its length is 660 km. and it provides 50% of the freshwater input into Ablemarle Sound. The riparian lands of the lower portion of the river are cypress swamp which drain directly

Figure 3 - The Pigeon River system

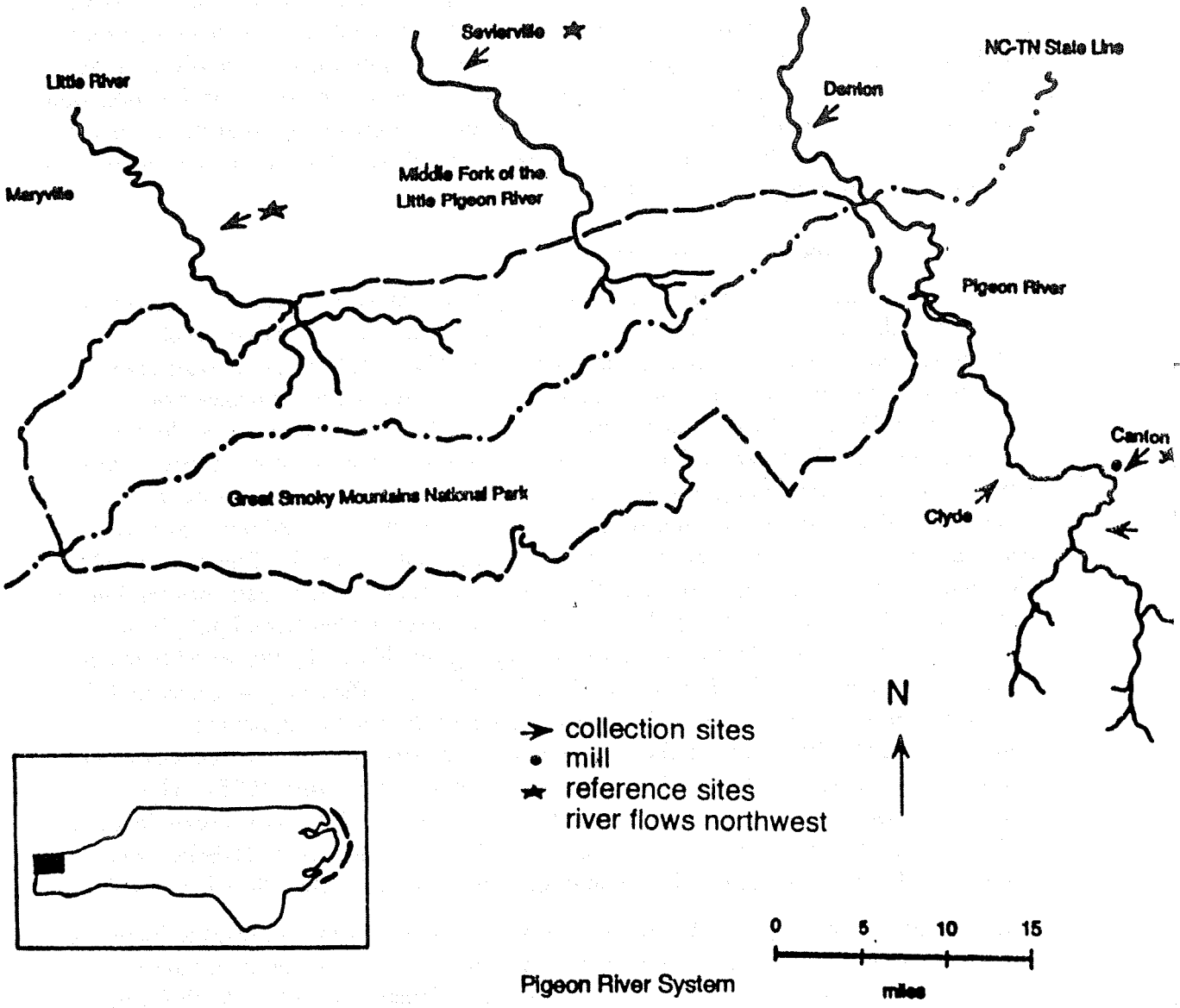
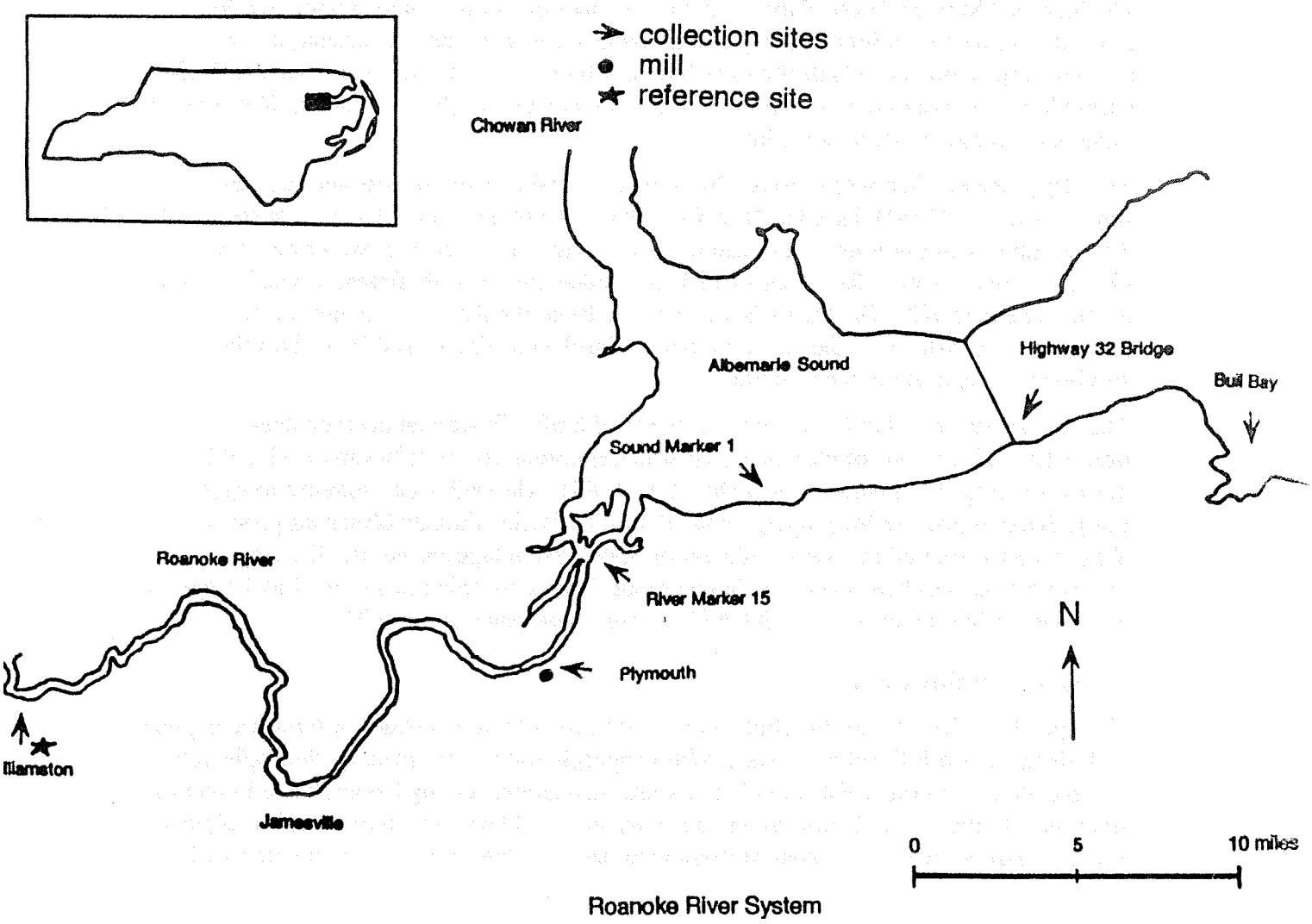


Figure 4 - The Roanoke River system



into the river, lending a brown stain from vegetative tannins and humic acids. River flow is regulated by six upstream reservoirs, the last of which is Roanoke Rapids Lake at river km 220. This hydroelectric facility exerts direct control over the instream flow of the lower Roanoke, controlling approximately 87% of the flow at the mouth of the river (Giese et al. 1987; Rulifson 1990).

The Weyerhauser mill in Plymouth NC is a large one with multiple operations, including a bleached kraft mill. Recent average daily production at this mill utilized 450 tons of unbleached softwood, 520 tons of bleached softwood and 435 tons of bleached hardwood. The bleached kraft mill uses chlorine dioxide as the primary bleaching agent, and the effluent is subjected to both primary and secondary treatment before discharge. On average, approximately 1% of the river flow is effluent; this can rise to as much as 10.5% during low flow conditions which occur primarily in autumn (Steve Woock, Weyerhauser Paper Co., personal communication).

The Neuse River. Samples received from the National Council for Air and Stream Improvement (NCASI), based in New Bern, North Carolina, were also used in this study. This group has an outdoor experimental stream setup which receives water diverted directly from the Neuse River, into which controlled amounts of effluent are added from an on-site paper mill. The Neuse River, like the Roanoke River, is a major coastal floodplain river which originates in the North Carolina piedmont and flows into the southwestern end of Pamlico Sound.

The Weyerhauser mill in New Bern is a bleached kraft mill with an average daily production of 725 tons of bleached pulp which is comprised of 75% softwood and 25% hardwood pulp (Lockwood-Post's Directory 1988). The mill uses a modern oxygen delignification process for pulping coupled with a chlorine dioxide bleaching process. Wastewater is treated in a series of aeration stabilization lagoons before discharge, involving 5 days of heavy aeration in one pond, 5 days polishing in a second and 5 days in a settling basin (Dennis Borton, NCASI, personal communication 1993)

Objectives of this study

The specific objectives of this study were as follows: (1) to determine if feral fish exposed to BKME in North Carolina rivers exhibit uroporphyria; (2) to quantify chlorophenolic bile metabolites in these fish and (3) to assess correlations among hepatic mixed function oxidase activities, hepatic porphyrin concentrations and bile metabolite concentrations in order to assess the effectiveness of these parameters as biomarkers of exposure to BKME.

MATERIALS AND METHODS

Laboratory Studies

Fish. Juvenile channel catfish, 6" to 8" long were obtained from either Aquaculture Advisory Service, Garner, NC, or Blue Ridge Fish Hatchery, Kernersville, NC. Fish were kept in 100 liter aquaria under flow through conditions at 28°C and allowed to acclimate to laboratory conditions for three weeks before exposure. Fish were fed Purina Catfish Chow.

Chemicals. All chemicals were of the highest purity available and acquired from Sigma Chemical Co., St. Louis, Mo., except for tetrachlorguiacol and tetrachlorocatechol, which were obtained from Helix Biotechnologies, Inc.

Laboratory exposure of channel catfish to hexachlorobenzene and iron. Two separate experiments to determine if channel catfish acquire uroporphyria under conditions which have induced the syndrome in other animals were done. (Kennedy and Wigfield 1990; Krishnan et al. 1991). The fish were exposed to hexachlorobenzene, a known prophyrogenic compound (Bonkovsky 1991; Krishnan et al.1991) and a constituent of BKME (Bjorseth 1979). The fish were pretreated with iron, a known synergist in the presence of HCB in the induction of uroporphyria (Masini et al. 1988; Smith and Francis 1983).

The dosing and sacrifice regimes for the first experiment are shown in Table 1. Fish pretreated with iron were dosed with 400 mg Fe/kg injected as 4 ml iron-dextran/kg. The low dose of HCB was 500 mg/kg and the high dose was 1 g/kg. HCB was suspended in corn oil at appropriate concentrations so that the fish received 5 ml corn oil/kg.

The dosing and sacrificing regimes for the second experiment are shown in Table 2.

Laboratory exposure of channel catfish to 2,4,6 trichlorophenol. This experiment was done to insure that a chlorophenolic metabolite could be detected in the bile of fish exposed to a chlorophenol in the water. It was decided to use 2,4,6 trichlorophenol for this experiment because: (1) it has been shown to be a dominant compound present in the bile of fish exposed to BKME (Oikari and Holmbom 1986; Soderstrom and Wachtmeister 1991; Roges et al 1988); (2) data concerning its metabolism in fish are available; (3) it is a persistent constituent of biologically treated mill effluent (Leunberger et al.1985); (4) the acetyl derivative has been successfully analyzed in fish bile and displays good chromatography.

The study consisted of 3 treatment groups: (1) a control, which received acetone only, (2) low dose, receiving 15 μg TClP per liter of tank water; (3) high dose, receiving 300 μg TClP per liter of tank water. Exposure was by static renewal where TClP was added to the water in an acetone carrier and renewed every 24 hours for four consecutive days. Sampling was done at 24, 48 and 96 hours, with four fish taken from each treatment group at each time point.

Table 1 - Design for laboratory experiment 1

	Dosing Regimen			Sacrificing Regimen			
Trt/Time	Day 0	Day 3	Day 10	Day 10	Day 25	Day 60	Day 70
none				n = 3	n = 3	n = 3	n = 3
Iron[1] only	iron			n = 1	none	n = 3	none
Iron + corn oil	iron	corn oil	corn oil	n = 2	n = 3	n= 3	n = 1
Iron + HCB (low)[2]	iron	HCB + corn oil	HCB + corn oil	n = 2	n = 2	n = 2	
Iron + HCB (high)[3]	iron	HCB + corn oil	HCB + corn oil	n = 3	n = 1	n = 4	n = 2

[1]Injected IP at 400 mg/kg as 4 ml/kg iron dextran

[2]Injected IP at 500 mg/kg as suspension in 5 ml/kg corn oil

[3]Injected at 1 g/kg as suspension in 5 ml/kg corn oil

Table 2 - Design for laboratory experiment 2

	Dosing Regimen			Sacrificing Regimen			
Trt/Time	Day 0	Day 3	Day 10	Day 0	Day 15	Day 45	Day 100
none				n = 4			
saline[1] + corn oil	saline	corn oil	corn oil		n = 4	n = 4	
saline + HCB[2]	saline	HCB + corn oil	HCB + corn oil		n = 4	n = 6	n = 6
Iron[3] + corn oil	iron	corn oil	corn oil		n = 3	n = 2	
Iron + HCB	iron	HCB + corn oil	HCB + corn oil		n = 4	n = 6	n = 6

[1]Injected IP at 4 ml/kg

[2] Injected at 1 g/kg as suspension in 5 ml/kg corn oil

[3] Injected IP at 400 mg/kg as 4 ml/kg iron dextran

Sacrificing of fish and sample storage. Fish were stunned by a blow to the head and killed by cervical dislocation. Livers were immediately removed and frozen in liquid N_2 where they were stored until analysis. Bile was taken with a disposable syringe and frozen on liquid N_2 and stored until use.

Porphyrin analysis. Total hepatic porphyrins, uroporphyrins and hepta porphyrins were determined according to methods developed by Kennedy and associates. (Kennedy and James 1993; Kennedy et al 1986). Briefly, a 100 mg sample of liver tissue was homogenized with 6 ml of 1:1 (v/v) 1N HCl/ acetonitrile (ACN) and centrifuged at 8124 x g. The supernatant was collected and an additional aliquot of HCl/ACN was added and the pellet was resuspended and centrifuged again. The supernatant from this centrifugation was added to the first, diluted to 50 ml with water, then concentrated on Sep-Pak Plus tC18 cartridges (Waters, Inc., Tauton, Mass.). Porphyrins were eluted with 2.5 ml ACN which was evaporated under N_2. The residue was stored at -70 °C until analysis.

The residue was prepared for HPLC analysis by the addition of 50 µl of concentrated HCl, followed by 10 seconds of vortexing. The HCl solution was allowed to sit for 5 minutes before being vortexed again as before, then diluted with 450 µl of water and sonicated for 5 minutes.

Samples were analyzed on a Perkin-Elmer HPLC using a 3 cm C18 column (Perkin-Elmer) and a mobile phase consisting of 1:1 (v/v)1M ammonium acetate, pH 5.2: methanol at a flow rate of 2.0 ml min. Porphyrins were detected wit a Perkin-Elmer LS50b fluorescence spectrometer equipped with a Hammamatsu 928 photomultiplier tube. Fifty µl of sample were injected using an overfill technique. Two injections were done per sample and the results averaged.

Standards were prepared from a kit obtained from porphyrin products (Logan, Utah). Standards were run at regular intervals during the analysis to check column stability.

Results were obtained for total HCPs (the sum of uro- and heptaporphyrins), total porphyrins (the sum of uro-, hepta and coproporphyrins) and the ratios of HCP:total and HCP:total plus isocoproporphyrin were calculated. Results are expressed as pmol porphyrin/10 mg tissue.

An experiment to determine the efficiency of recovery of porphyrins from liver homogenate was done before any samples were analyzed. A liver homogenate from channel catfish was divided into 12 aliquots. Four of these were not spiked and were analyzed to assess the absolute background levels of porphyrins, then 4 concentrations were added to the other aliquots in duplicate, and the samples analyzed as above.

Samples from the Roanoke River were analyzed by Dr. Sean Kennedy at Environment Canada. This was done near the beginning of this work to determine if methods developed for other species could be successfully used for fish tissues. Results from this analysis are expressed as pmol porphyrin/ g tissue.

EROD analysis. Microsomes were prepared by a modification of the method of Eriksson, et al. (1978). Tissue was homogenized as a 20% (w/v) solution with 0.25M

sucrose/0.05M Tris HCl, pH 7.4 and centrifuged at 10,000 x g for 20 minutes. The supernatant from that spin was centrifuged at 105,000 x g for 1 hour, the pellet resuspended in sucrose-Tris and centrifuged again as above. The second pellet was resuspended in 0.15 M KCL/0.1 M Tris-HCl (pH 7.4 in 20% glycerol and stored at -70°C.

EROD was analyzed according to the method of Burke and Mayer (1974), using a Perkin-Elmer LS50b fluorescence spectrometer. Protein concentrations of the microsomal preparations were determined with the bicinchoninic acid kit from Sigma Chemical Co. (St. Louis, MO).

Bile analysis. Chlorinated bile metabolites (CBMs) were analyzed by a modification of methods of Voss and Oikari and Holmbom (Voss et al. 1981; Oikari and Holmbom 1986). Briefly, a 2-100 µl bile sample was diluted to 1 ml with water and 200 µl of concentrated HCl was added. Samples were hydrolyzed at 70°C for 2.5 hours, then cooled and set to pH 6.9 with 12N NaOH after the addition of 200 µl of 0.5M Na-phosphate buffer, pH 6.9. The samples were then acetylated by the addition of 5µl of acetic anhydride in the presence of 0.1M K_2CO_3. This was followed by sequential extraction with two-1 ml aliquots of hexanes.

CBM's in field samples were analyzed with a Hewlett-Packard gas chromatograph equipped with an electron capture detector and an Alltech Econo-cap column (30 m long, 0.25 mm i.d.) containing an SE-30 stationary phase. 2 µl of hexanes solution was injected in splitless mode and a temperature program from 100°C to 280°C at 4°C/min was run. Standards prepared from spiked bile were run with each set of analytes. Samples from the laboratory exposure contained much higher concentrations of CBMs and were analyzed with the instrument in split mode with a temperature program that varied from 80°C to 280°C at 10°C/min. The retention times of acetylated reference compounds were used to determine the time period over which the acetylated chlorophenolics eluted, so chromatograms were integrated between 7.5 and 25 minutes only. Integration was performed using the EZChrom software package version 4.7 (Scientific Software, San Ramon, CA). Total peak area units were expressed as ng TClP equivalents based on standard curves run with TClP.

Dioxin and furan analysis. Dioxin and furan concentrations in muscle tissue were determined by Weyerhauser Corporation according to Woock. (Woock 1993). Each sample was a composite of 3 to 9 fish of the same species from the same site, and some of the same fish used for the liver and bile analyses in our laboratory were used. The toxic equivalence concentrations so determined were used for correlation analysis in this study.

Field Sites and Sampling

Pigeon River. Three sites were sampled July 7 through July 9, 1992. These sites were: (1) Denton, NC, at approximately 70 km below the effluent outfall; (2) the Little River near Maryville, Tennessee, a reference site; (3) the middle fork of the Little Pigeon River near Sevierville, Tennessee, another reference site. Two additional sites were sampled on 19 November, 1992: (1) Clyde, NC, approximately 8 km downstream from the mill; (2))

Canton, NC, about 2 km upstream from the BKME outfall, another reference site. All of these collection sites are shown on the map in Figure 3.

Thirty redbreast sunfish (Lepomis auritus) were taken at Denton and from the Little River and the middle fork of the Little Pigeon River with a boat mounted electroshock device. Thirteen male redbreast sunfish were caught at Canton and 12 at Clyde. The fish were held in aerated water until sacrifice, which occurred no more than 30 to 45 minutes after capture. Liver samples were shared with a group from Oak Ridge National Laboratory who required a larger portion of liver than we did, so liver samples for this study were not collected from all fish captured. Bile was taken when possible, but there were fish that had none to take.

Roanoke River. Sites sampled on the Roanoke River are shown in Figure 4. Collections were done between September 16 and December 9, 1992, from a total of 7 sites in North Carolina. These were: (1)Williamston, a reference site, 47 km upstream from the BKME outfall; (2) Jamesville, a second reference site 24 km upstream; (3) Plymouth, 3 km downstream; (4) River Marker 15, 8 km downstream; (5) Sound Marker 1, in the Ablemarle Sound, 16 km downstream; (6) the Highway 32 bridge across the Ablemarle Sound 31 km downstream; (7) Bull Bay in the Ablemarle Sound, 40 km downstream.

White catfish (Ameriurus cattus) were caught at all sites, channel catfish at 4 sites and largemouth bass (Micropterus salmoides) at 2 sites. A total of 89 fish were collected using a variety of techniques including gill nets, trot lines, hoop nets, rod and reel and trawling.

Liver and bile were taken from these fish. Due to unforeseen problems during field collections, some of the liver samples thawed for an indeterminate time which could have been as much as 2 days. Since freeze thawing can destroy P450 activity, EROD analyses were not done on these samples. Porphyrin analyses were done since porphyrins are stable compounds unlikely to have been affected by the thaw (Sean Kennedy, Environment Canada, personal communication, 1993).

NCASI artificial streams. Bluegill sunfish (Lepomis macrochirus) and largemouth bass that were exposed to 0%, 4% or 15% effluent for 10 months were collected from the artificial streams by dropping the water level. The fish were kept in aerated water for up to 2 hours before being anesthetized in MS-222 then sacrificed. Liver and bile samples were collected.

Statistical treatment of data. Some of the data from the field experiments were analyzed with Microsoft Excel or SAS, and some were done by hand. Data from the field samples were non-normally distributed, so non-parametric methods were used.

Intersite comparisons of EROD measurements were made using a two-tailed Mann-Whitney U-test for ranked and unpaired comparisons. Results were not considered significant unless the null hypothesis could be rejected for $p < 0.05$. When multiple Mann-Whitney tests were used within a study, a Bonforoni correction factor for the α value was applied in order to reduce the risk of a type I error. CBM results were analyzed with a single factor ANOVA and Scheffe's multiple comparison method was used to identify means that were significantly different, if such were indicated by the ANOVA.

RESULTS

Laboratory Studies

Porphyrins. Table 3 shows the results of the study done to assess the recovery of known amounts of porphyrins from channel catfish liver homogenate. Since uroporphyria in other species is usually characterized by at least a 10-fold increase in all porphyrin levels, these recoveries were deemed adequate to detect the condition in fish.

Table 3 - Results of recovery study for porphyrin assay

	% recovered		
Compound/Amt (pmol)	Uroporphyrin	Heptaporphyrin	Coproporphyrin
0.05	51	29	89
0.5	72	55	95
5.0	67	60	72
50.0	71	72	72

Figure 5 shows representative chromatograms of a porphyrin standard and a sample taken from laboratory study number 2. The HPLC conditions chosen were satisfactory for the separation of different isomers of the porphyrins. All of the standard porphyrins were resolved into two peaks, one for each isomer. Three peaks were obtained for coproporphyrin. We did not identify this peak, but it may be a form of coproporphyrin known as isocoproporphyrin (S. Kennedy, Environment Canada, personal communication.).

Some of the results from the laboratory studies are shown in Figures 6 & 7. Uroporphyria was not induced by any of the treatments used in either study. The treatments shown in these Figures are the most extreme – those which are most likely to cause uroporphyria as based on results from other species.

Figure 5 - Representative chromatograms from a porphyrin analysis

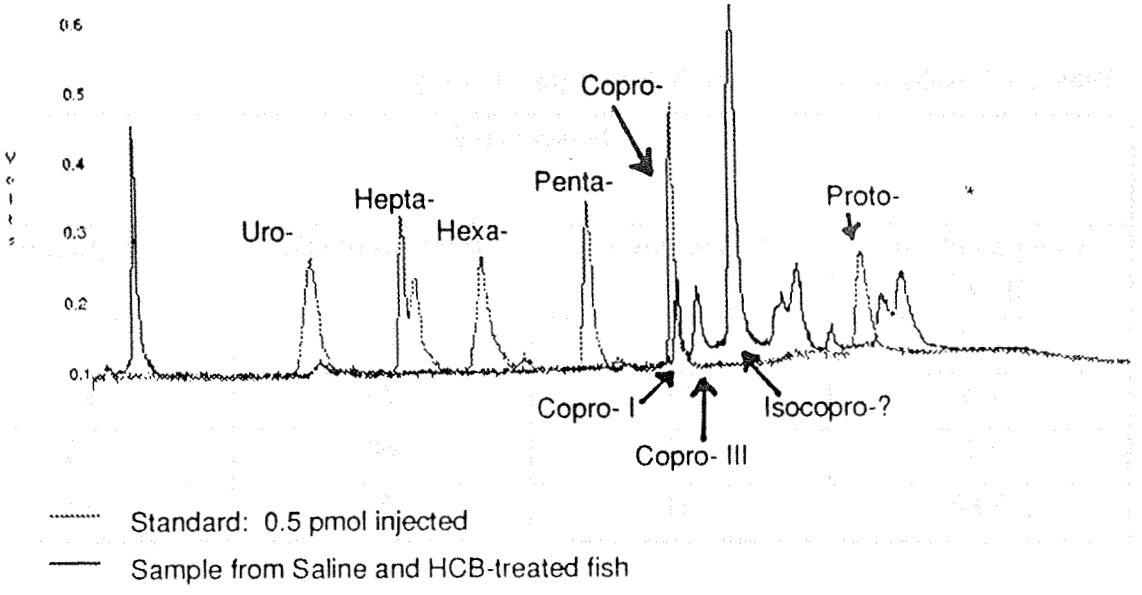


Figure 6 - Hepatic porphyrin levels in channel catfish exposed to hexachlorobenzene and iron (laboratory experiment 1)

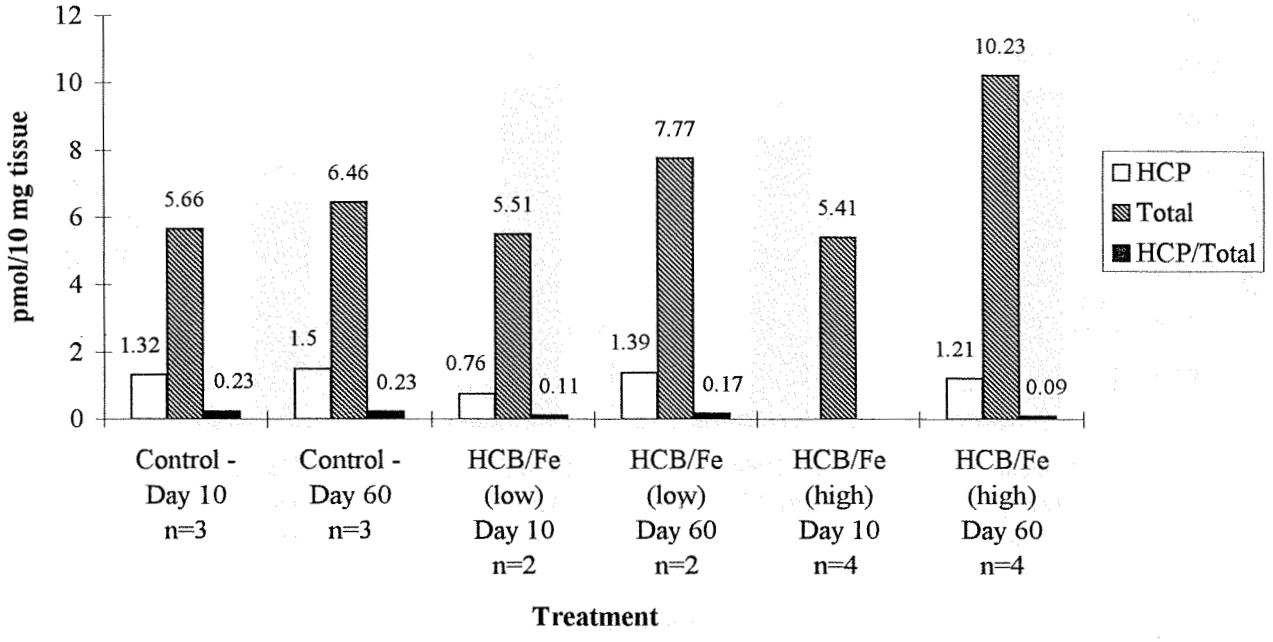
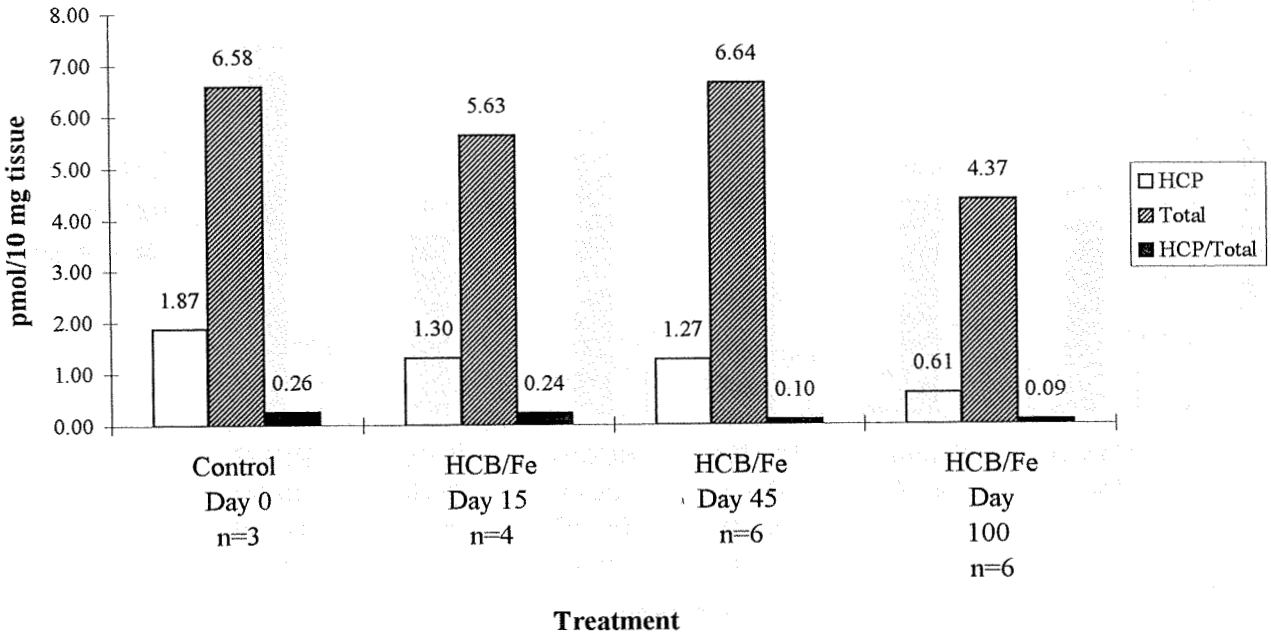


Figure 7 - Hepatic porphyrin levels in channel catfish exposed to hexachlorobenzene and iron (laboratory experiment 2)



EROD. The results of the EROD determinations from laboratory experiments 1 and 2 are shown in Figures 8 &9. They show no induction of EROD by any of the treatments at any time. For reference, in a study done in our laboratory immediately prior to these studies, channel catfish induced using β-napthoflavone show EROD levels above 1000 p mol resorufin /min/mg protein.

CBMs. The results of the bile analysis for channel catfish exposed to TClP in the laboratory are shown in Figure 10 . A representative chromatogram of a field sample is shown in Figure 11. Channel catfish exposed to the high-dose levels of TClP (300 µg/l) had at least ten-fold greater concentrations of TClP in their bile than fish exposed to the lower dose (15 µg/l) at each time point. Fish exposed to the high dose showed a range of 3.07 - 4.99 µg TClP/ µl bile while fish exposed to the lower dose ranged from 275 - 556 ng TClP/ µl bile. In the high dose groups, the average concentration of TClP in the bile increased 62% from 24 to 48 hours and reached a plateau that remained to 96 hours. The results demonstrate that water borne TClP is taken up by the fish and can be detected in the bile, indicating that the method may be useful in the detection of the same compounds in feral fish.

Field Studies

Pigeon River. Porphyrin analysis was done only on samples taken in November. The results are shown in Table 4 and Figure 12. HCP concentrations, total porphyrins and the ratio of HCP/total porphyrin were all not significantly different at the Canton (reference) or Clyde sites, both in North Carolina. The Clyde site was the site sampled closest to the BKME outfall.

Table 4 -Hepatic porphyrin concentrations in redbreast sunfish from the Pigeon River, November, 1992

	porphyrin concentration (pmol/10 mg tissue) median and range		
Site	HCP[1]	Total[2]	HCP/Total
Canton (reference)	0.073	0.612	0.19
n=11	0.01 - 3.82	0.21 - 4.19	0.02-0.91
Clyde	0.019	0.550	0.042
n=12	0.001 - 3.63	0.147 - 8.19	0.001 - 0.443

[1]Highly Carboxylated Porphyrins = uroporphyrin + heptaporphyrin

[2]Total = HCP + coproporphyrin

Figure 8 - Hepatic EROD activities in channel catfish exposed to hexachlorobenzene and iron (laboratory experiment 1)

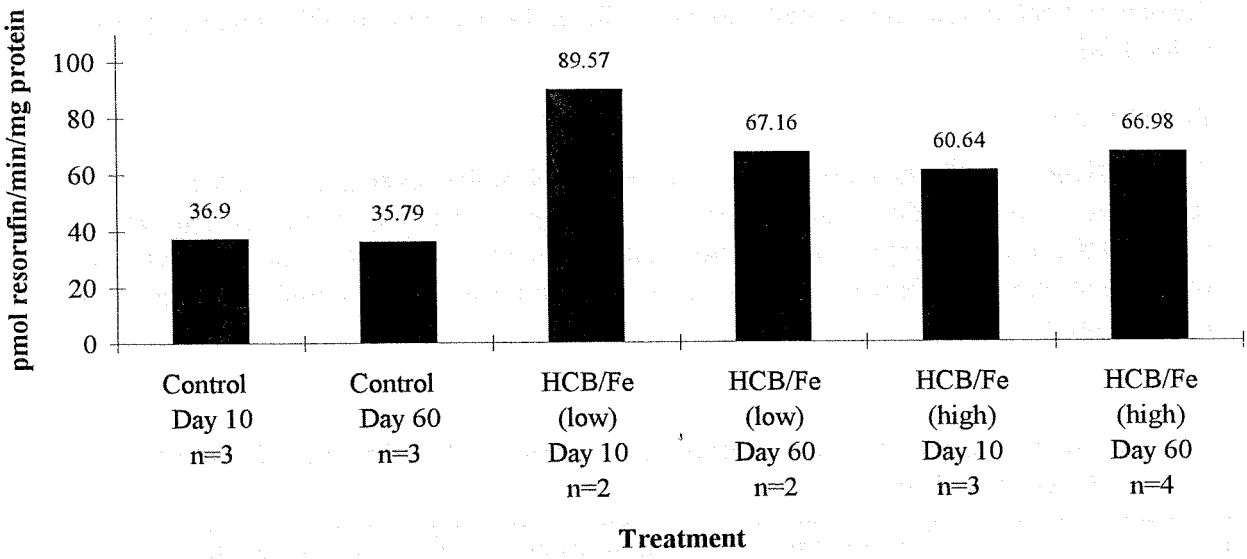


Figure 9 - Hepatic EROD activities in channel catfish exposed to hexachlorobenzene and iron (laboratory experiment 2)

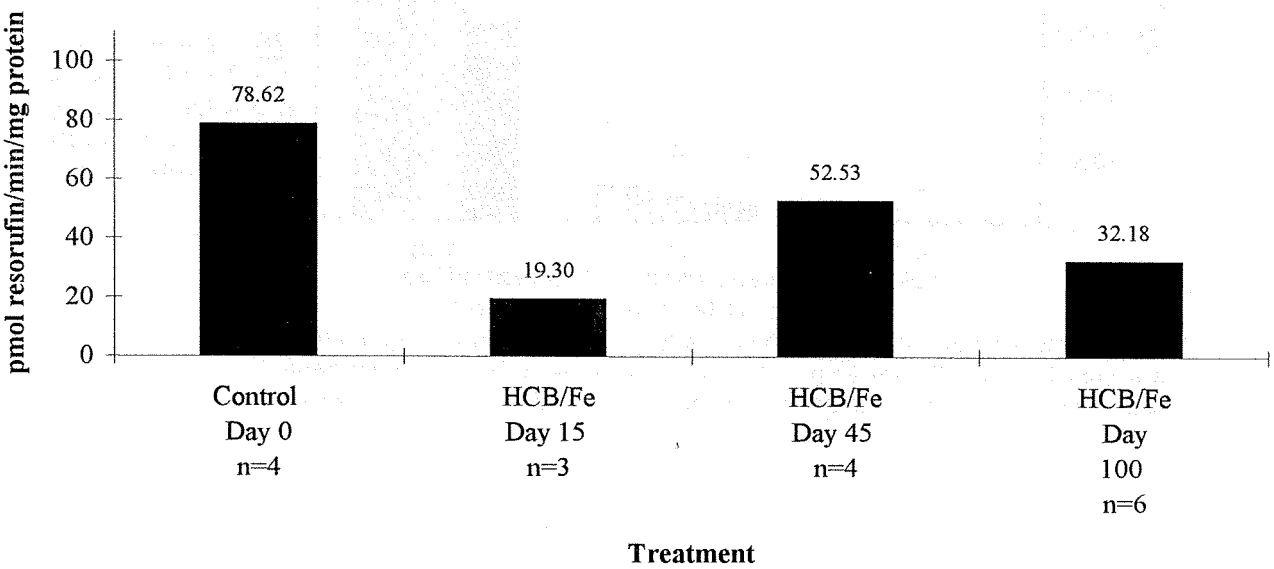
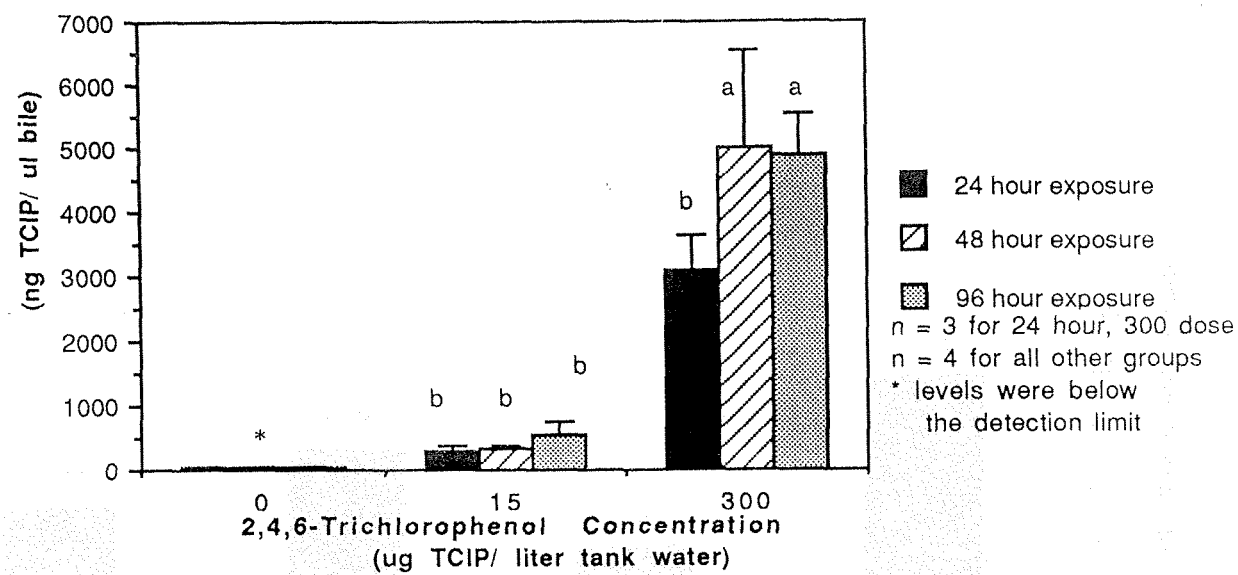


Figure 10 - Hepatic levels of metabolites of 2, 4, 6 trichlorophenol in channel catfish exposed by waterborne route



Sample means not sharing a common letter, a or b, within an exposure group are significantly different at $p<0.05$. Tank water and TCIP concentration were renewed every 24 hours for the four consecutive days of the experiment.

Figure 11 - Representative chromatograms of standards and chlorophenolic bile metabolites in a fish from Clyde, NC

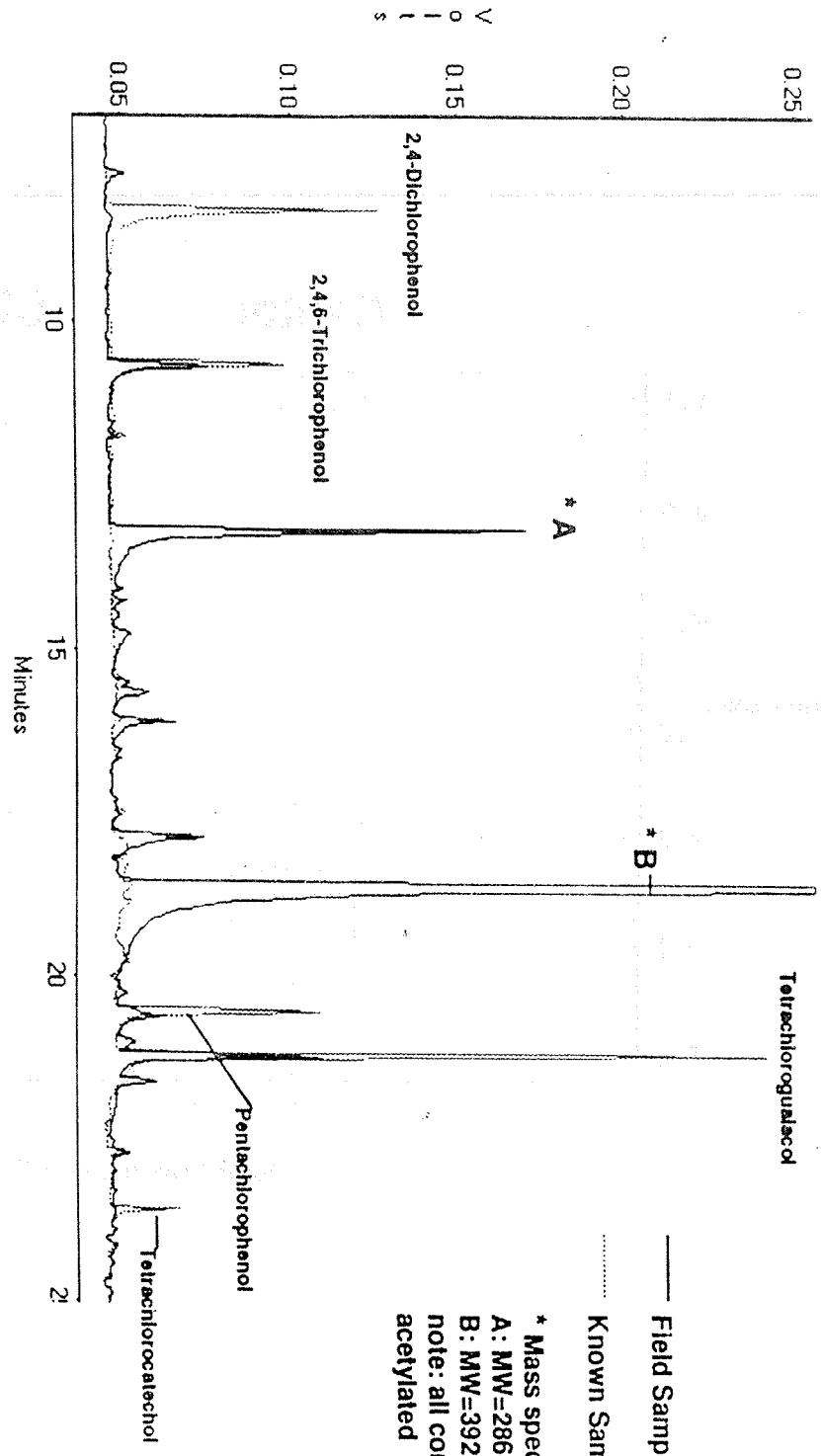
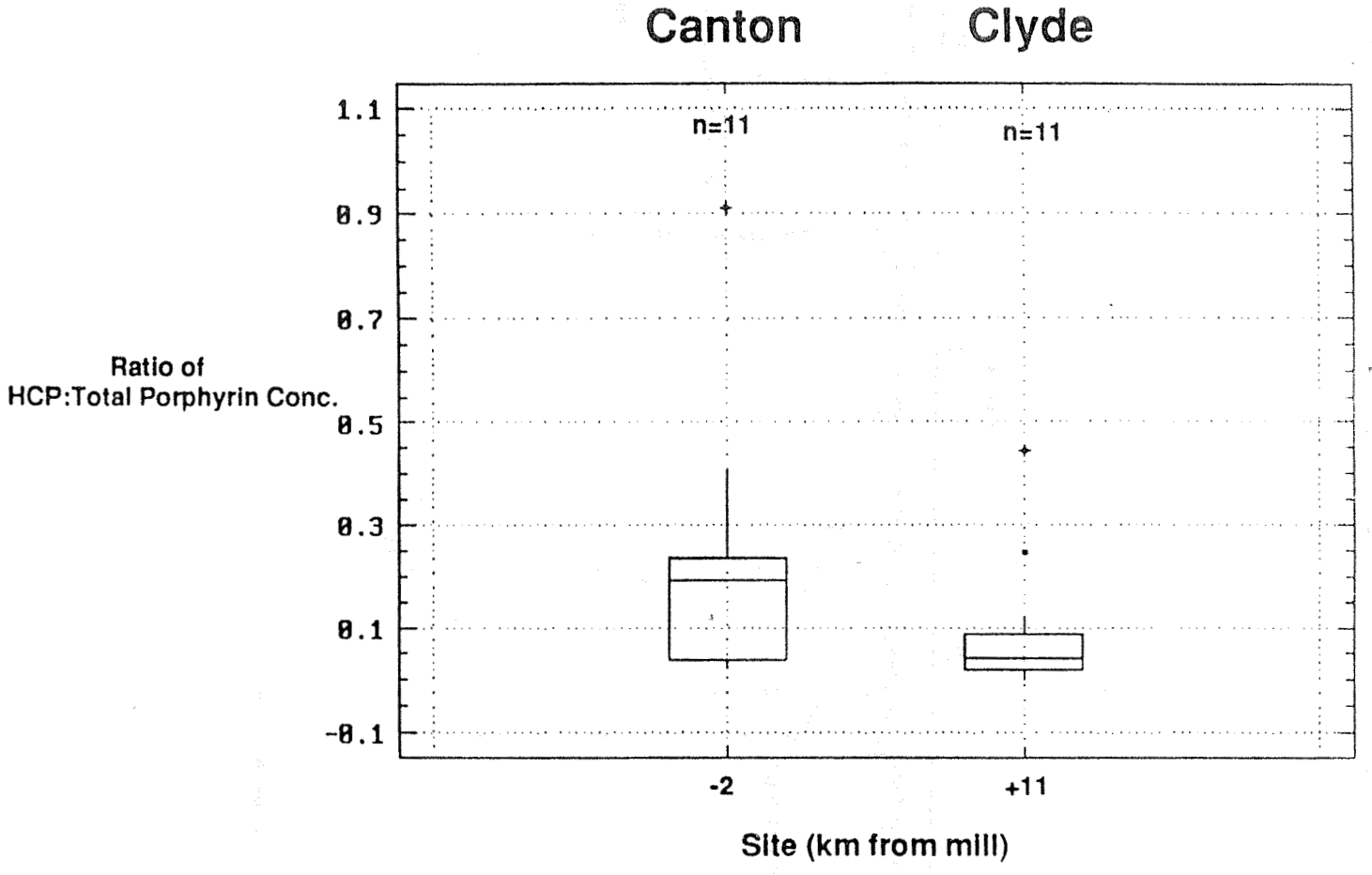


Figure 12 - Hepatic porphyrin levels in redbreast sunfish from the Pigeon River



Results of EROD assays performed on samples taken from the Pigeon River are shown in Table 5.

Table 5 - Hepatic EROD activities in redbreast sunfish from the Pigeon River

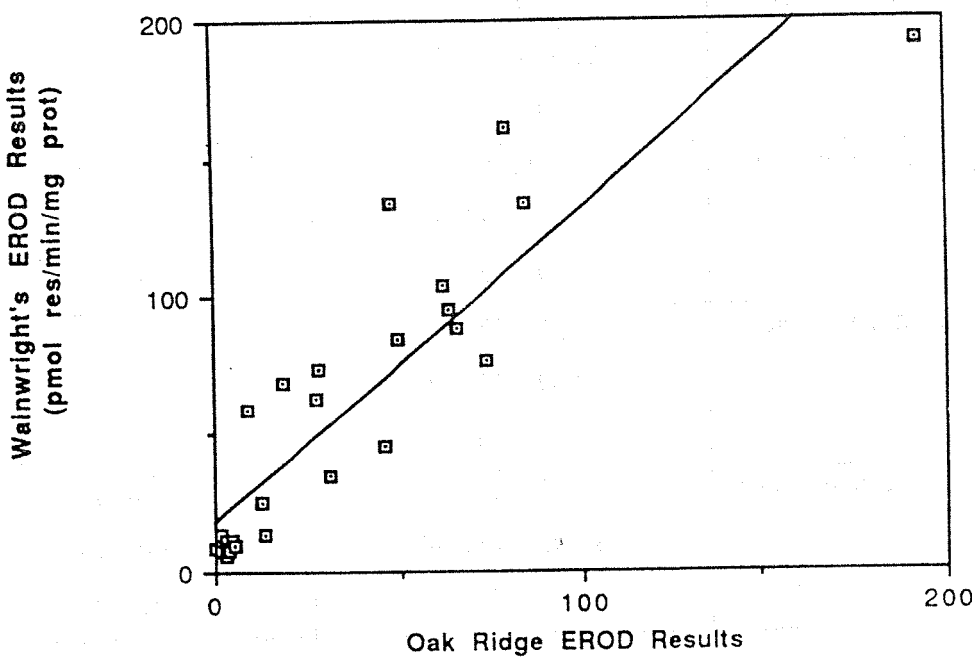
	pmol resorufin/minute/mg protein (median and range)	
Site/Sex	male	female
Canton[1] (reference)	34.33 15.89 - 107.68 n = 11	
Little River[2] (reference)	74.12 25.30 - 104.32 n = 7	
Little Pigeon[2] (reference)	119.19 46.11 - 192.27 n = 2	13.12 n = 1
Clyde[1] (11 km from mill)	81.45 40.63 - 119.76 n = 11	
Denton[2] (70 km from mill)	88 27 35.02 - 161.50 n=7	9.16 5.69 - 13.21 n = 11

[1]November sampling

[2]July sampling

The only significant difference detected between sites was that between Canton, a reference site, and Clyde, the site closest to the mill. Scientists from Oak Ridge National Laboratory (ORNL) performed EROD assays on portions of liver from the same fish used in this study and additionally, from fish taken at the same sites at the same time in July. They were able to detect a significant difference between sites for the July sampling . We performed a correlation analysis of our results with those from ONRL and the results are shown in Figure13. Overall, there was good correlation between the results as determined by the separate groups. EROD activities for males determined in our lab and at ORNL were significantly correlated according to Spearman's rank correlation analysis (r = 0.8088) and Kendall's test (t = .0600). Our results for females from the July sampling are significantly correlated with ORNL's according to Spearman's test (r = 9154) but not

Figure 13 - Correlation of EROD activity measurements done at Duke University and Oak Ridge National Laboratory



according to Kendall's (t = 0.0909). Spearman's test is more appropriate when there is less confidence in the difference between close ranks, a condition which should apply to these data.

CBMs. The samples from the Pigeon River showed a statistically significant increase in total chlorophenolic metabolite concentration with the proximity to the collection site to the BKME outfall. These data are shown in Figure 14. Some compounds present in samples from the Clyde and Walter's Lake, NC, sites and the Denton, TN, site co-eluted with pentachlorophenol, 2,4,6-trichlorophenol and tetrachloroguiacol. Of these, the compound that co-eluted with PCP was present in the greatest concentration at Clyde and Walter's Lake. Another component, which eluted at approximately 18.6 minutes (Figure 11) was the principal compound detected at the Clyde site and was present in all of the downstream samples in detectable quantities. Analysis using GC/MS determined the acetyl derivative of this compound to be monochlorinated with a molecular weight of 392.

EROD/CBM Correlation.There was no significant correlation between EROD activities and CBM concentration for either the July or the November samplings (Figures 15 & 16).

Roanoke River. Porphyrin analysis on these samples was performed by Dr. Sean Kennedy at Environment Canada, and the results are shown in Table 6. No significant difference between sites were found.

Table 6 - Hepatic porphyrin concentrations in channel catfish from the Roanoke River

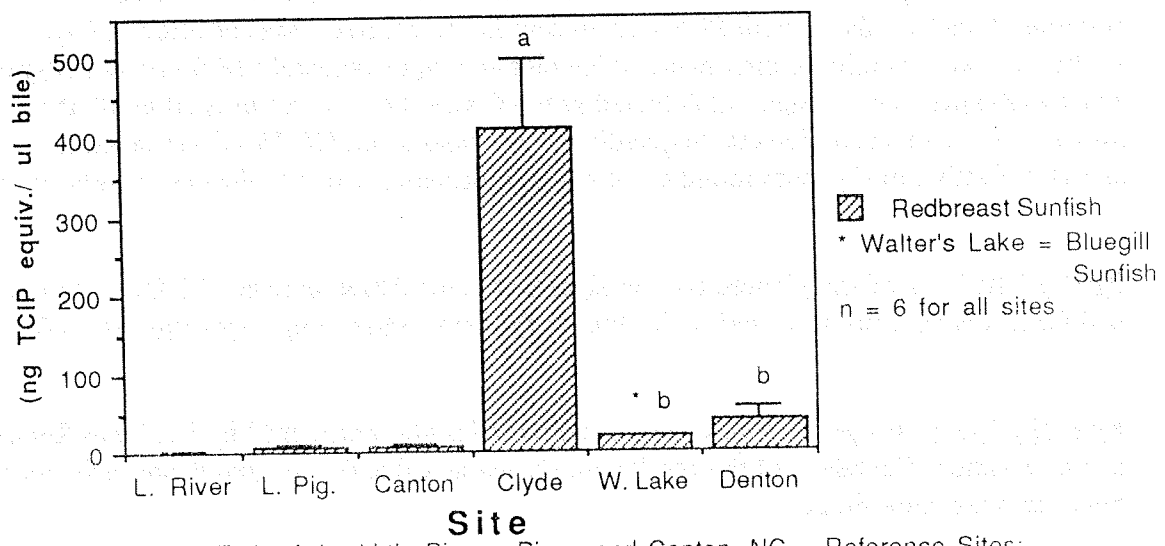
	prophyrin concentration (pmol/g tissue) median and range		
Site	HCP[1]	Total[2]	HCP/Total
Williamston (reference)	6.08 ND[4] - 15.83	88.12 9.78 - 183.52	0.069 ND - 0.5059
Plymouth (2 km from mill)	3.83 ND - 8.29	20.51 87.2 - 111.7	0.278 ND - 0.445

[1]Highly Carboxylated Porphyrins = uroporphyrin + heptaporphyrin

[2]Total = HCP + coproporphyrin

[3]none detected

Figure 14 - Chlorophenolic bile metabolite concentration in redbreast sunfish from the Pigeon River



Little River, Middle Fork of the Little Pigeon River, and Canton, NC = Reference Sites; Clyde, NC = 5 miles, Walter's Lake = 19 miles, and Denton, TN = 29 miles from the BKME Outfall in Canton, NC.

Figure 15 - Hepatic EROD activities in redbreast sunfish from the Pigeon River

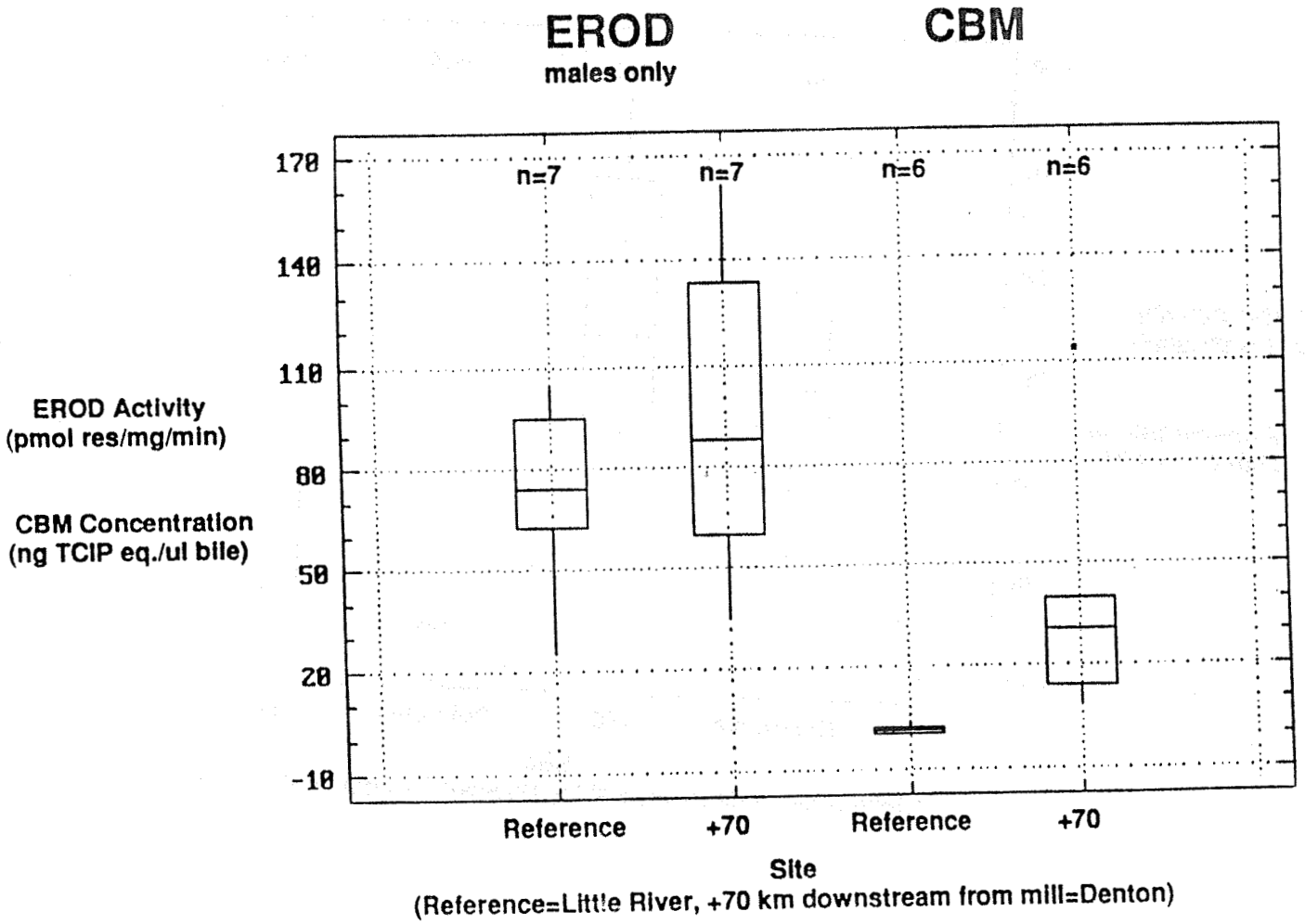
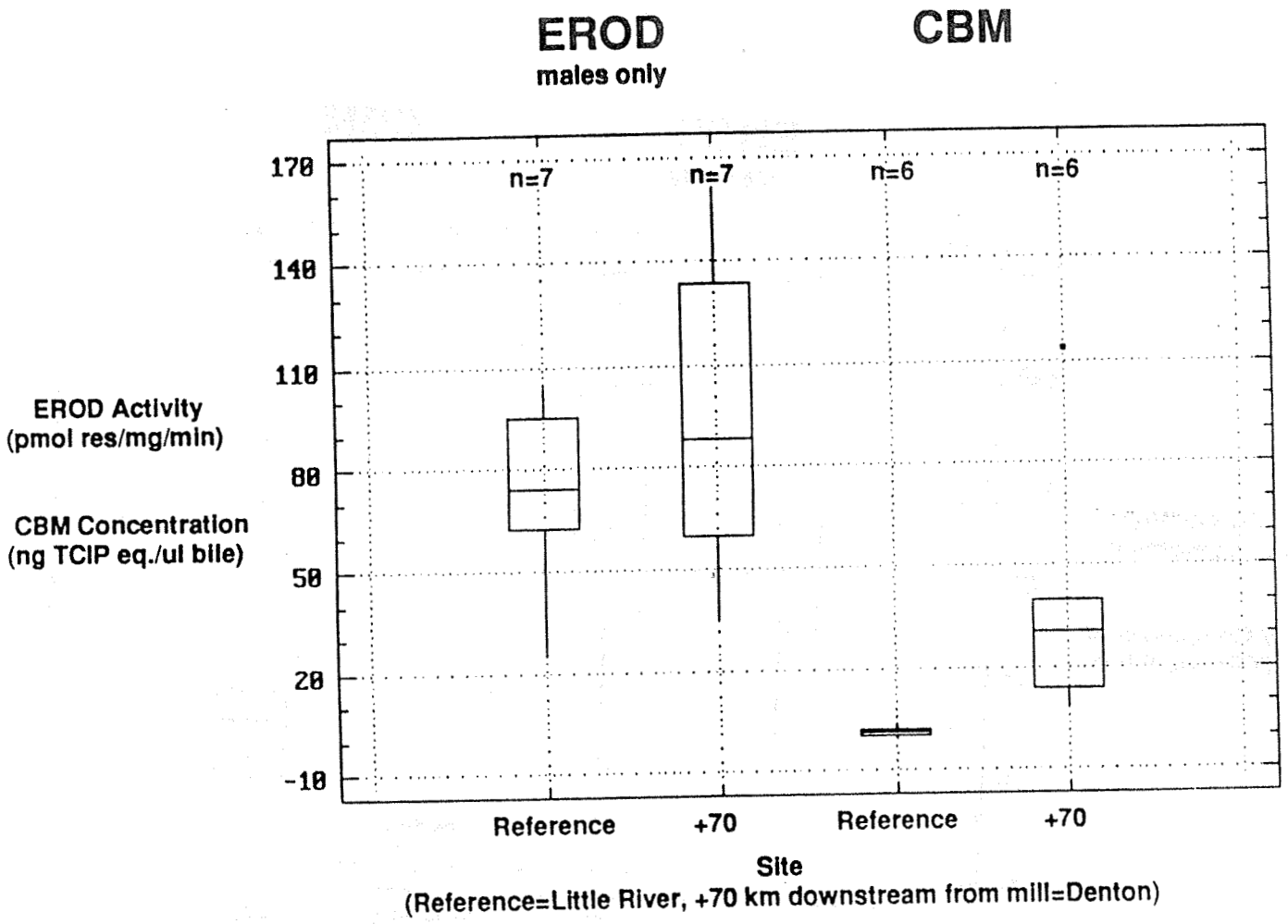


Figure 16 - Correlation between hepatic EROD activities and chlorinated bile metabolite concentrations in redbreast sunfish from the Pigeon River



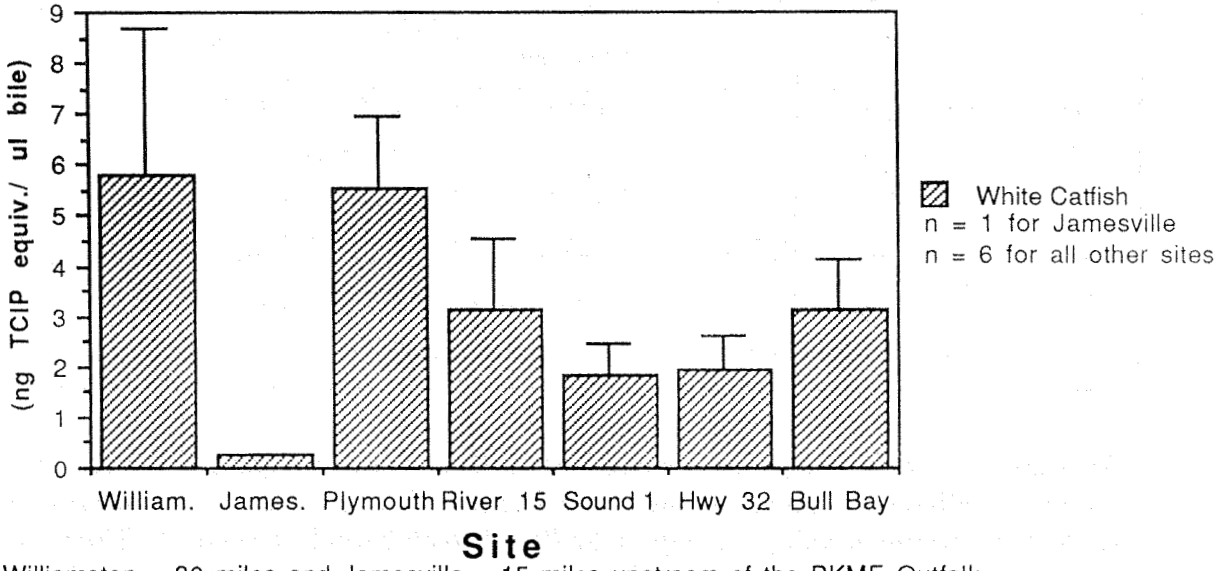
EROD activities determined on fish from the Roanoke River are shown in Table 7. No significant differences among the sites were detected. Comparison between the sexes was restricted by small sample sizes.

Table 7 - Hepatic EROD activities in two species of fish from the Roanoke River

	pmol resorufin/minute/mg protein (median and range)					
Species/Site	**Williamston reference**	**Jamesville reference**	**Plymouth 2 km from mill**	**River Marker 15 5 km**	**Hwy 32 Bridge 19 km**	**Bull Bay 29 km**
Channel catfish	146.30 47.68 - 408.78 n = 7		141.92 n = 1	224.29 178.71 - 372.71 n = 4	187.95 n = 1	
White catfish	150.36 31.45 - 181.48 n = 7	83.91 n = 1	147.47 142-17 - 248.64 n = 6	188.48 120.59 - 233.18 n=7	200.97 115.97 - 290.30 n = 7	54.43 n = 1

Analysis of CBMs in bile from Roanoke River fish are shown in Figure 17. While no significant differences among the sites were determined, a very slight trend towards higher CBM concentrations with proximity to the BKME outfall can be discerned. There was no correlation between CBM concentrations and EROD activities, nor between TEQ concentrations and EROD activities (Figure 19). The TEQs for the two species of catfish exhibited excellent correlation. (Figure 18).

Figure 17 - Chlorophenolic bile metabolite concentrations in white catfish from the Roanoke River



Williamston = 30 miles and Jamesville = 15 miles upstream of the BKME Outfall; Plymouth = 2 miles, River Marker 15 = 4.8 miles, Sound Marker 1 = 10 miles, Highway 32 Bridge = 19 miles, and Bull Bay = 25 miles downstream

Figure 18 - Toxic equivalent concentrations (TEQ) of dioxin in catfish from the Roanoke River

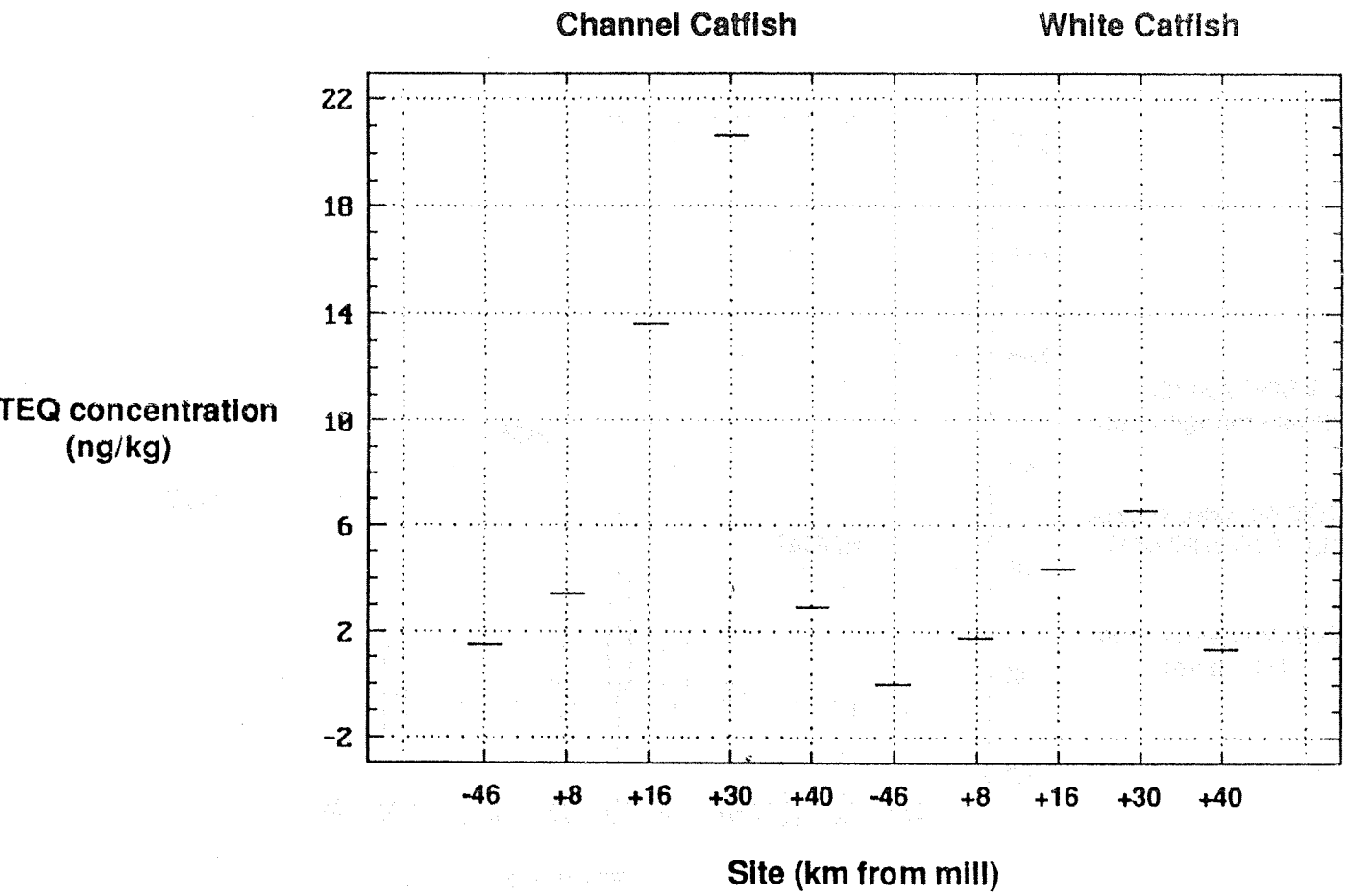
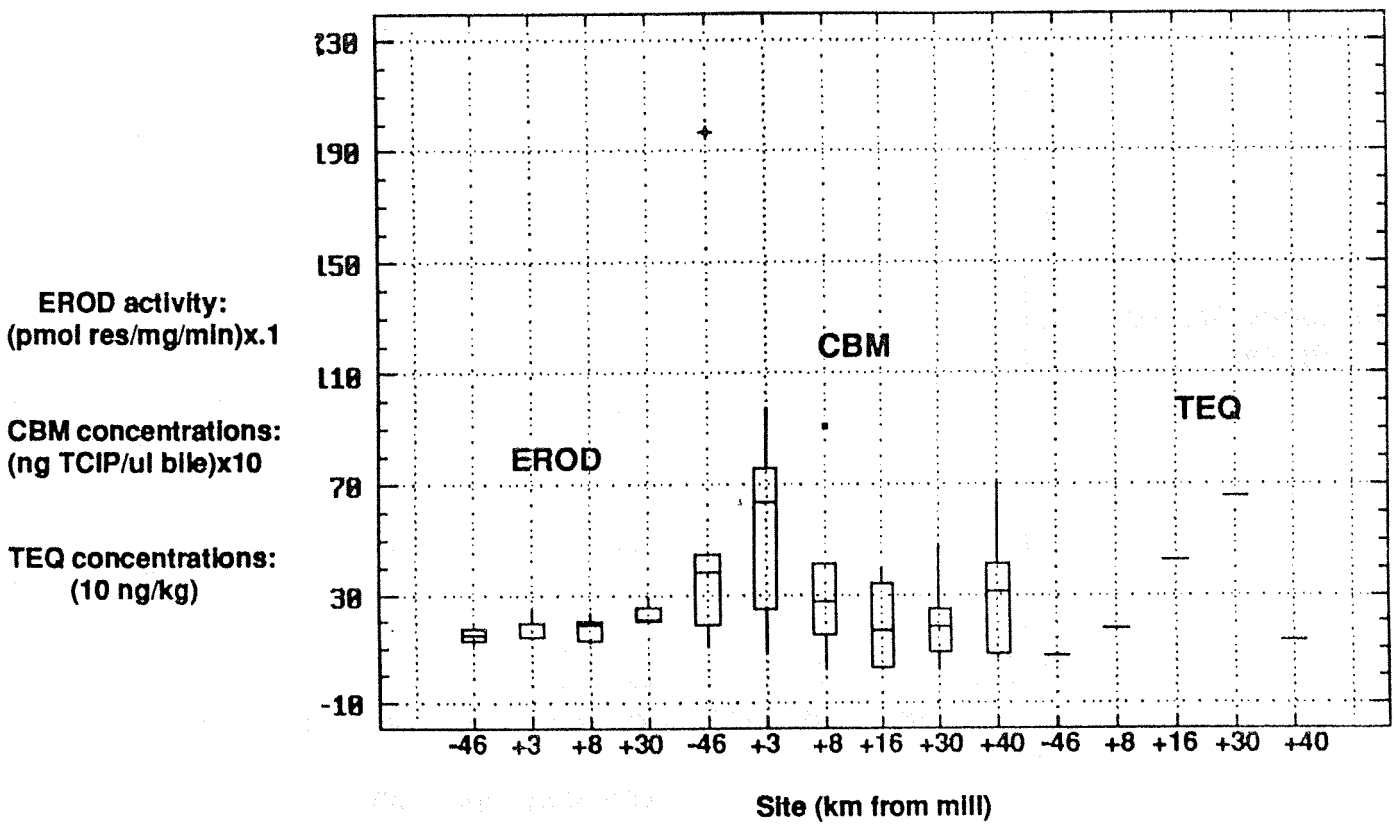


Figure 19 - Correlations among EROD activities, chlorophenolic bile metabolite concentrations and dioxin TEQs in white catfish from the Roanoke River



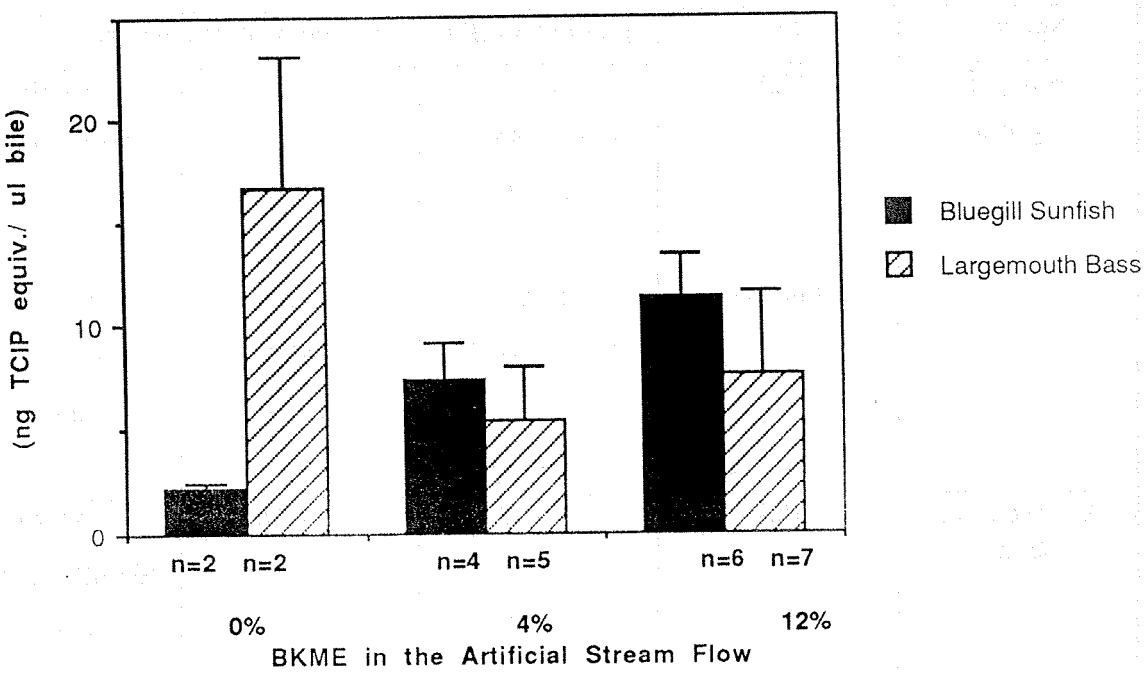
NCASI samples. Porphyrin analyses were not done on these samples. EROD results are shown in Table 8. No significant difference in EROD activity among treatments was found for either largemouth bass or bluegill sunfish.

Table 8 - Hepatic EROD activities in fish from NCASI experimental streams

		% BKME in artificial stream		
		0	4	12
Species	Sex	pmol resorufin/minute/mg protein (median & range)		
Bluegill sunfish	Male	36.4 19.99 - 45.58 n = 4	 n = 0	33.50 20.06 - 45.73 n = 5
	Female	32.57 21.29 - 49.53 n = 3	23.62 0 - 62.25 n = 4	54.14 45.85 - 62.42 n=2
Largemouth Bass	Male	160.88 n = 1	211.26 n = 1	267.94 263.42 - 272.44 n = 2
	Female	123.88 0 - 162.36 n = 5	127.77 102.30 - 152.76 n = 4	119.30 94.12 - 202.21 n = 5

A non-significant trend toward increasing CBMs with increasing effluent concentration was observed for these samples, as shown in Figure 20. The absolute concentrations of CBMs (2 - 16 ng TCIP equivalents/µl bile) was similar to the concentrations observed in the Roanoke River samples. CBM concentrations did not correlate with EROD activities for either species.

Figure 20 - Chlorophenolic bile metabolite concentrations in fish exposed to various concentrations of BKME in artificial streams



DISCUSSION

Uroporphyria

Treatments chosen to induce uroporphyria in channel catfish were expected to have a high probability of success based on results obtained with other species. Laboratory studies with female Wistar rats exposed to 1000 ppm HCB daily in their diet for 43 days resulted in a 4700 fold increase in the levels of hepatic HCPs (Kennedy and Wigfield 1990). Great Lakes herring gull chicks were found to have a 12-fold induction in total porphyrins, and a 38-fold induction in HCPs compared to birds from a reference site.

Feral whitefish exposed to BKME were found to exhibit a 6-fold increase in the ratio of HCPs to protoporphyrin. In comparison with differences of these magnitudes, channel catfish do not acquire uroporphyria as a result of exposure to HCB and/or iron. Given the effectiveness of this treatment in producing the condition in other species, it is unlikely that other chlorinated compounds would produce uroporphyria in channel catfish.

Without knowing the mechanism of uroporphryia induction, it is problematic to speculate as to the reason channel catfish do not behave as other species do. In mammals, ALA-S catalyzes the rate limiting step in heme synthesis, but in rainbow trout, the rate limiting step is the following one, catalyzed by ALA-D (Addison et al. 1990). Some researchers think that an increase in the rate of the reaction catalyzed by ALA-S is important for the development of uroporphyria. Such an increase in an organism where the reaction is not rate-limiting might not cause the imbalance.

It is also interesting that there was a minimal effect by BKME on porphyrin levels in fish from the Pigeon River. The HCP concentration decreased relative to the total concentration of porphyrins, an effect opposite that present in uroporphyria. This might indicate an effect on the reaction catalyzed by coproporphyrin oxidase.

Pigeon River

EROD induction was observed in samples taken from sites downstream from the BKME outfall. As previously mentioned, our results showed significant differences only for the November sampling, but ORNL detected significant differences for both sampling times. The principal reason for the difference in the results obtained by the two groups was probably because ONRL had more samples per site to analyze. We shared liver samples with this group and, in cases where there was only enough liver for one assay, it went to ORNL rather than to us. Many other studies have detected indications in EROD downstream from a BKME source, as noted in the introduction to this report.

We also observed a 10-fold induction in EROD activity in male sunfish as compared with females in the July sampling. This has been previously observed both on the Pigeon River (Adams et al. 1992) and in other BKME receiving waterways. The phenomenon has been seen in white suckers from Lake Superior (Munkittrick, et al. 1992). Others have noted sex differences in EROD induction in rainbow trout that correlate with annual

reproductive cycles (Forlin and Haux, 1990). These differences must be considered when sampling in the field.

Extreme differences between sites were noted for CBMs. A 70-fold increase in the median value of fish from the Clyde site (8 km downstream from the mill) when compared to the Canton reference site was observed, as well as a 30-fold increase in the median value in fish from the Denton site (70 km downstream fro the BKME outfall) when compared to the Little Pigeon River Reference site. These results are in agreement with other studies, as detailed in the introduction to this report, that CBMs are greatly affected by the presence of BKME, even at great distances from the outfall.

It is also interesting that the CBM analysis indicates the Pigeon River was receiving higher concentrations of effluent than the other systems. Flow of BKME in the Pigeon can approach 100% of the river volume during dry conditions. This observation also supports the conclusion that the sunfish species examined here do not acquire uroporphyria as a result of exposure to high concentrations of BKME.

Roanoke River

The results obtained on the Roanoke River are interesting. EROD activities, as well as the concentrations of dioxins and furans in muscle tissue, rose with distance downstream from the mill. Several factors may account for these somewhat anomalous results.

EROD activities in the fish at the reference site may have been induced. There were other undocumented sources of chemical discharge observed near our reference site. Collection was attempted at another reference site, but only one fish was captured. EROD activity in this fish was measured and found to be one-third of the median value from the other reference site.

Different species of fish show different levels of induced EROD activity as a result of BKME exposure. It is possible that the catfish species examined here show a lower level of induction due to BKME than other species and that a difference of that magnitude was undetectable with the sample sizes used in this study.

Although not statistically significant, the trend shows a rise in EROD activity with distance downstream from the mill. There is also a salinity gradient here, increasing with distance from the mill. We are not aware of any studies concerning the effect of salinity on EROD induction, but it is certainly possible that the bioavailabilty of EROD inducing compounds could be affected by salinity.

The results suggest that another source of chlorinated compounds may have been important here. The Roanoke River discharges into the Ablemarle sound adjacent to the mouth of the Chowan River, and the Union Camp bleached kraft mill is on that river. Its presence could influence CBM concentration in fish sampled in the Ablemarle sound, which includes the Sound Marker 1, Highway 32 bridge and Bull Bay sites. The TEQ data indicate that exposure to dioxins and furans was highest at the Highway 32 bridge site, dropping off to either side, possibly indicating another source of pollution at that site.

The CBM concentrations show a trend opposite that of EROD activities and dioxin and furan concentrations again indicating possible multiple sources of contamination. Dioxins

can be potent MFO inducers (Lambrecht et al., 1988a) and the EROD values observed here seem to follow the dioxin gradient rather than the chlorophenolic gradient. The CBM data also indicate that the chlorophenolic concentrations in the Roanoke River are considerably lower than those on the Pigeon, so it makes sense that the EROD activities would follow the trend of the stronger inducer.

NCASI samples

The sampling design for these samples allowed only for reliable statistical analysis of data gathered for female largemouth bass. No significant differences in EROD activities were found among treatments for these fish. Researchers at the Institute for Marine Science also did EROD assays on these same samples and was unable to find differences (Laura Bankey, Institute of Marine Sciences, personal communication, 1993). CBM analysis indicated a slight, non-significant trend of increasing concentration with exposure to an increased percentage of effluent. Absolute levels (2 - 16 ng TClP equiv./ µl bile) were similar to those measured on the Roanoke River; significant differences were not detected in that system either. Significant differences in EROD levels in largemouth bass were detected during a previous sampling from these tanks by NCASI personnel. In the interim, the mill began operation of an oxygen delignification system in place of the chlorine delignification system that had been previously used (Dennis Borton, NCASI, personal communication, 1994). This process reduces the production of chlorinated phenolics (Bonsor, et al. 1988). Also, EROD levels in control fish were higher during the later sampling period, possibly obscuring smaller differences.

Constraints of field studies

Much of the work done here was done in collaboration with others. This allowed a much broader project to be conducted than if we were working on our own. Since we sometimes used tissues from the same fish analyzed by others, it allowed comparison and correlation with the results obtained by other researchers.

However, collaboration also imposed limitations on our work. Since we were guest on the collecting expeditions, we had little control over the design of the field samplings. The experimental designs of the studies conducted on the Roanoke River and at NCASI were not optimized for the goals of this study, resulting in low numbers of samples. Collecting at NCASI and on the Pigeon River necessitated the sharing of liver sample s from small fish. The hosting group received first call on the liver samples, again leaving us with small sample sizes in some cases and also with small amounts (typically less than half a gram) of liver for our assays.

Some fish, particularly on the Roanoke River, were caught using more stressful methods (i.e. hook and line) than we would have desired and some were kept under stressful conditions (unaerated water) before sacrifice. Any assay sensitive to such stresses may show a higher background variance than if less stressful methods were used, allowing small differences to be obscured. The time course of the collections was also less than optimal. Collecting on the Roanoke spanned a time period from mid-September to early December, allowing for the introduction of seasonal variability. The Pigeon River was

sampled in July and November, using separate reference site for the two collections, so data sets could not be analyzed as a group due to seasonal differences.

Conclusions

The principal conclusions reached as a result of this work were as follows:

- Uroporphyria is not inducible in channel catfish under conditions that have caused its induction in other animals.
- Uroporphyria is not found in feral fish exposed to BKME.
- Chlorinated bile metabolites were a good indicator of the proximity of feral fish to a BKME outfall on the Pigeon River, but not on the Roanoke.
- Chorinated bile metabolites also indicated that fish in the Roanoke River were exposed to a much lower concentration of chlorophenolics than fish in the Pigeon.
- EROD activity was again shown to be a sensitive indicator of exposure, although a relatively non-specific one.

Uroporphyria seems, at best, a variable biomarker for BKME exposure. It is not a sensitive indicator of exposure in the species examined in this study, although it has been found in other species. EROD, on the other hand, appears to be a much more sensitive indicator, but perhaps subject to confounding by more extraneous factors as well. CBM concentrations are very sensitive to BKME concentrations, both in this study and in others. However, it is not clear that the assay always measures biologically active compounds in the system, as evidenced by the results for white catfish on the Roanoke River. While EROD activities and TEQs for dioxins and furans increased with distance from the mill for 31 km downstream, CBMs did not. In conclusion, it seems that the complexity of the interactions between effluent, river and fish are best characterized by a suite of indices (Adams et al., 1992) which more fully illuminate various interactions. This suite should be chosen with some foreknowledge of principal sources of chemical pollution present.

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