

**Tumor Prevalence and Biomarkers of Exposure and Response in
Brown Bullheads (*Ameiurus nebulosus*) from the
Tidal Potomac River Watershed**

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ABSTRACT

Four groups of thirty brown bullheads (*Ameiurus nebulosus*) were collected from the tidal Potomac River watershed to survey tumor prevalence in relation to contaminant exposure. Fish were obtained from the Quantico embayment, near a Superfund site that released polychlorinated biphenyls (PCBs) and DDT compounds; Neabsco Creek, a tributary with petroleum inputs from upstream areas and marinas; and the Anacostia River (both in spring and fall), where sediment is contaminated with polynuclear aromatic hydrocarbons (PAHs), PCBs, and organochlorine pesticides. Fish were also collected from the Tuckahoe River, on the Eastern Shore of Maryland, as a reference. Fish were necropsied and examined grossly and histopathologically for skin and liver neoplasms. Cytochrome P450 activity, bile PAH metabolites, and muscle organochlorine pesticide/PCB concentrations were determined in randomly selected individuals. There were significant differences among sites in liver tumor prevalence: Anacostia (spring)-50%, Anacostia (fall)-60%, Neabsco-17%, Quantico-7%, Tuckahoe-10%. Skin tumor prevalences were also significantly different: Anacostia (spring)-37%, Anacostia (fall)-10%, Neabsco-3%, Quantico-3%, Tuckahoe-0%. Tumor prevalences in Anacostia fish were comparable to those at contaminated sites in the Great Lakes. PAH concentrations were higher in Anacostia sediments than at the other sites and there were significantly higher concentrations of PAH metabolites in bile of the Anacostia fish. At present, there are insufficient data, however, to establish a cause-effect linkage with a particular class of contaminants. Tumor surveys in selected species are recommended for monitoring the status and remediation of Regions of Concern and other areas in the Chesapeake Bay watershed.

INTRODUCTION

The prevalence of tumors in wild fish has been used as an indicator of environmental quality in saltwater (Malins *et al.* 1987; Vogelbein *et al.* 1990; Myers *et al.* 1994) and freshwater (Baumann *et al.* 1987, 1991, Smith *et al.* 1994) ecosystems. The strongest evidence for chemical etiology exists for polynuclear aromatic hydrocarbons (PAHs) in sediments, which have been implicated in the development of liver carcinogenesis (e.g., Baumann *et al.* 1991; Malins *et al.* 1987; Vogelbein *et al.* 1990). A cause and effect relationship between PAHs and liver tumors or preneoplastic lesions in fish has been established by experimental studies (Schiewe *et al.* 1991; Metcalfe *et al.* 1988). Further evidence linking PAHs in sediments with liver tumors was developed by Baumann and Harshbarger (1998) from surveys conducted in the 1980s and 1990s with bottom-feeding brown bullheads (*Ameiurus nebulosus*) in the Black River, Ohio. They observed that liver tumor prevalence increased and decreased according to changes in sediment PAH concentrations. During the 1980s, tumor prevalence decreased after closure of a coking plant and a dramatic decline in sediment PAH concentrations. In 1992 and 1993, there was an increase in tumor prevalence in age-3 fish exposed to highly contaminated sediments resuspended from remedial dredging that occurred in 1990. In 1994, age-3 fish were free of tumors, and Baumann and Harshbarger (1998) theorized that this was the first year class that would not have been present during the dredging.

The relationships between skin and oral (orocutaneous) tumors and environmental contaminants in bullheads are less conclusive. Grizzle *et al.* (1984) observed an increased prevalence of papillomas in black bullheads (*Ameiurus melas*) exposed to chlorinated wastewater effluent. Black *et al.* (1985) induced papillomas in brown bullheads by repeatedly painting the skin with sediment extracts containing high PAH concentrations. Black and Baumann (1991) summarized several Laurentian Great Lakes surveys with brown bullheads in which higher oral and cutaneous tumor prevalence occurred in PAH-contaminated tributaries relative to uncontaminated creeks or lakes. Poulet *et al.* (1993) concluded that there was no evidence for viral etiology because they were unable to transmit orocutaneous tumors to healthy brown bullheads. Poulet *et al.* (1994), however, noted the occurrence of orocutaneous tumors in 94 brown bullheads collected from 17 locations (both contaminated and uncontaminated) in New York State and stated that the distribution of lesions did not suggest a strict correlation with chemical carcinogens.

Environmental managers in the Great Lakes have used the presence of tumors (especially liver tumors) as a criterion for identifying and prioritizing contaminated areas or Areas of Concern. They consider prevalence greater than 2% to be $\frac{1}{2}$ elevated (Hartig *et al.* 1990). Baumann *et al.* (1996) summarized liver and skin tumor data from the Great Lakes and reported that liver tumor prevalence exceeding 9% and skin tumor prevalence exceeding 20% were nearly always observed in chemically contaminated areas.

Here we report a survey of tumor prevalence in brown bullheads from three locations in the tidal Potomac River watershed, with varying degrees and types of contaminant inputs. A reference site, in an agricultural and forested watershed on Maryland's Eastern Shore, was also

sampled. The objectives were to compare the prevalence of skin and liver tumors among fish from these areas and examine the possible association between tumor prevalence and indicators of contaminant exposure and response.

MATERIALS AND METHODS

Site Selection and Sampling

Groups of 30 brown bullheads (≥ 260 mm total length) were collected in 1996 from the following locations in the Potomac River watershed: Anacostia River, Neabsco Creek, and the Quantico embayment (Figure 1). The Anacostia River (Washington, DC) is one of three Regions of Concern identified by the U.S. Environmental Protection Agency's Chesapeake Bay Program (U.S. EPA 1997). Sediments are contaminated with PAHs, polychlorinated biphenyls, chlordane, and heavy metals, and there is a health advisory restricting fish consumption (ICPRB 1996). A preliminary survey of 20 brown bullheads (> 280 mm total length) from the lower portion of the river, revealed that 15 fish had hepatocellular carcinomas (May and Harshbarger 1992). Neabsco Creek, a Potomac River tributary 35 km down river from Washington, DC, borders the Featherstone National Wildlife Refuge. There are moderately high levels of PAHs in sediments in some areas of Neabsco Creek, most likely due to inputs from marinas in the tidal area and small spills in the non-tidal portion. Pinkney *et al.* (1995a) reported a 10% prevalence of hepatocellular carcinomas and a 33% prevalence of squamous carcinomas in brown bullheads from this site. The Quantico embayment is a crescent-shaped, 78-hectare area of the Potomac River, 56 km down river from Washington, DC. It borders a U.S. EPA Superfund hazardous waste site, the Old Landfill at the Marine Corps Combat Development Command, which released PCBs and DDT compounds that accumulated in embayment sediments (Pinkney *et al.* 1995b). An additional group of 30 fish was collected from the Tuckahoe River (Figure 1), which drains a forested and agricultural watershed on Maryland's Eastern Shore of the Chesapeake Bay and served as a reference area for the survey.

The Anacostia site was sampled twice -- in April (ANA-S) and October (ANA-F) to provide information on the variability in response across seasons. Fish were collected by electrofishing (Anacostia) or otter trawling (Neabsco (NEAB), Quantico (QUAN) and Tuckahoe (TUCK). A Hydrolab Surveyor 2 water quality meter (Hydrolab Corporation, Austin, TX) was used to record temperature, dissolved oxygen, pH, and conductivity at each location (Table 1).

Laboratory Procedures

Fish were maintained overnight in coolers with aerated collection-site water. The next day, they were measured for total length, weighed to the nearest gram, and necropsied. Condition factor, $K = (\text{wt (g)} \times 10^5) / \text{length (mm)}^3$, was calculated. Gross lesions were noted and the liver was excised and weighed so that the hepatosomatic index ($\text{HSI} = \text{liver wt/body wt}$) could be determined. Livers (except a 0.5 gram portion used for biochemistry) and skin areas with

visible lesions were preserved in 10% buffered formalin and transported to George Washington University Medical Center for histopathological examination and tumor diagnosis. All materials from the study were maintained and the case reports were entered into the Registry of Tumors in Lower Animals. The following organs -- head and caudal kidney, spleen, brain, muscle, eye, stomach, skin, and gill-- were preserved in zinc formol alcohol, prepared and examined histopathologically at the Maryland Department of Natural Resources laboratory. The pectoral spines were removed, sectioned, and aged (Devries and Frie 1996).

The tissues were processed according to standard histological procedures, sectioned at 6 μm , and stained with hematoxylin and eosin (Luna 1968). At least two sets of slides were prepared from each tissue block for histopathological examination.

Biochemical and chemical analyses were done on randomly-selected fish from each group. A 0.5 g section of the liver was flash frozen in liquid nitrogen and stored at -80 $^{\circ}\text{C}$ for analysis of cytochrome P450 enzyme activity (CYP1A) with the ethoxyresorufin-*O*-deethylase (EROD) assay (Melancon 1996). Induction of these enzymes in fish livers is used as an indicator of exposure to PAHs and chlorinated hydrocarbons (Schrank *et al.* 1997). Bile was collected in disposable syringes, placed in cryotubes in liquid nitrogen, and stored at -80 $^{\circ}\text{C}$ for analysis of PAH metabolites. Because PAHs are rapidly metabolized and eliminated by fish, measurement of PAH metabolites in bile has been used as an indicator of recent (within several days) exposure to PAHs (Collier and Varanasi 1991). Fish were filleted and muscle tissue samples were placed in chemically cleaned glass jars and stored at -20 $^{\circ}\text{C}$ for analysis of organochlorine pesticides and PCBs.

Sediment Sampling

Three sediment samples were collected (July and August 1997) from each location using a stainless steel petite ponar grab. Samples were collected along a transect through the fish sampling area, with one sample near each shore and one in the channel area of the river. For the Quantico embayment, the samples were collected along the length of the trawling transect. Each sample consisted of a single grab. The top 2-4 cm was removed with a stainless steel spoon, homogenized in a stainless steel bowl, and placed in chemically cleaned glass jars. Samples for organic analysis were stored at -20 $^{\circ}\text{C}$ and samples for total organic carbon and grain size were stored at 0-4 $^{\circ}\text{C}$ prior to analysis.

Chemical Analysis

Sediment PAH and bile PAH-metabolite analyses were performed at the Geochemical and Environmental Research Group Laboratory at Texas A & M University (GERG; College Station, TX). Sediments were analyzed for PAH compounds by capillary gas chromatography/mass spectrometry in the selected ion mode (Wade *et al.* 1988). Metabolites of benzo(a)pyrene, naphthalene, and phenanthrene were analyzed by high performance liquid chromatography, with a fluorescence detector, according to the method of Krahn *et al.* (1986). These data were reported as the amount of B(a)P, naphthalene, and phenanthrene equivalents in

ppm wet weight. Sediments were analyzed for grain size and total organic carbon by GERG methods 006 and 009. Organochlorine pesticide and PCB analyses of sediments and tissues were performed at the Patuxent Analytical Control Facility (Laurel, MD) according to the methods of Cromartie *et al.* (1975).

Biochemical Analysis

EROD activity was determined according to Melancon (1996), using an amount of microsomes equivalent to 2.6 mg of liver, 0.125 mM NADPH, and 1.25 μ M substrate at 37°C. Assays run on different days were normalized using mallard duck reference microsomes. Protein was determined by the Lowry *et al.* (1951) method at 50% reduced volume and EROD activity was reported on a picomole per minute per mg microsomal protein basis.

Data Analysis

Histological data were summarized as the prevalence of the various types of lesions among fish from each of the locations. It cannot be inferred that exposure occurs at the location of capture unless it is known that brown bullheads tend to remain in specific locations. In tributaries of the Laurentian Great Lakes, brown bullheads tend to remain in specific creeks with minimal crossover (P. Baumann, U.S. Geological Survey, personal communication). A tagging study conducted in the Presque Isle Bay area of Lake Erie, which is approximately 1500 hectares, indicated that nearly all tagged brown bullheads were recaptured within the Bay (Obert 1997). There are few published data, however, on the movements of brown bullheads in river systems and none for the Chesapeake watershed.

Sediment contaminant data were summarized in terms of total PCBs, total DDT (DDT and metabolites) compounds, total chlordane (sum of cis- and trans-nonachlor, alpha- and gamma-chlordane, and oxychlordane), and total PAHs. Concentrations of the eight PAHs identified as carcinogens or suspected carcinogens by Menzie *et al.* (1992) -- benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, and indeno(1,2,3-c,d)pyrene -- were summed as total carcinogenic PAHs.

Concentrations of total PCBs, total DDT compounds, and total chlordane in muscle tissues, bile PAH-metabolites, and differences in EROD activities among the groups of fish were compared using one way analysis of variance (ANOVA) and Tukey's multiple comparison test. If non-parametric statistics were required, we used the Kruskal-Wallis (K-W) test followed by Dunn's method (Sokal and Rohlf 1981; Jandel Corp. 1995). The prevalence of tumors among the five collections of fish was compared statistically using an extension of Fisher's Exact test (Stokes *et al.* 1995). Because the risk of liver neoplasms is known to increase significantly in older fish (reviewed by Moore and Myers 1994) and in females relative to males (Baumann *et al.* 1990), both the whole collections and subsets of same-aged and same-sexed fish were compared.

Correlation analysis (Sokal and Rohlf 1981) was used to determine if there were associations

between EROD activity or HSI and concentrations of PAH-metabolites in bile or organochlorine contaminants in muscle tissue. Logistic regression (Stokes *et al.* 1995) was used to evaluate the relationship between chemical, biological, and biochemical variables and the prevalences of liver and skin tumors. The biological predictor variables included sex, age, length, weight, HSI, and condition factor. The chemical predictor variables were total PCBs, DDT, and chlordane in muscle tissues. The biochemical predictor variables were P450 activity, and benzo(a)pyrene, naphthalene, phenanthrene, and total PAHs in bile. Each predictor variable was examined separately and models were considered statistically significant if the p-value for the Wald statistic was less than 0.05. For all significant regressions, odds ratios were reported, with values greater than one indicating an increased risk for the response variable for an incremental increase in the predictor variable (e.g., per year of age). Stepwise logistic regression was considered but was not feasible because of intercorrelations between predictor variables and limited sample size (because not all fish were analyzed for all of the predictor variables).

RESULTS

Fish Pathology

Gross examination revealed the presence of numerous lesions in the mouth area in some of the fish, particularly those from the spring Anacostia (ANA-S) group. Lesions were generally fleshy-pink colored, oval or round, and solid in texture (Figure 2). After histopathological examination of the skin lesions, four ANA-S fish (13%) were diagnosed with invasive (cancerous) skin tumors, characterized as squamous carcinomas (Table 2; Figure 3) and seven ANA-S fish were diagnosed with non-invasive epidermal papillomas (see Appendix A for a glossary of pathological terminology). Three fall Anacostia (ANA-F) fish (10%), one Neabsco (NEAB) fish (3%), and one Quantico (QUAN) fish (3%) also had epidermal papillomas. There were statistically significant differences ($p=0.001$, based on an extension of Fisher's Exact Test), in the prevalence of skin tumors (of either diagnosis) with the highest rates in the ANA-S and ANA-F fish (37% and 10%, respectively) compared with the other collections (0-3%, Table 2, Figure 4a). Spreadsheets with chemical, biological, and pathological data are provided in Appendix B.

Gross liver lesions, characterized as whitish and pale colored areas up to 0.3 cm in diameter, were observed in several of the Anacostia fish. After histopathological examination of the livers of all sampled fish, neoplastic lesions of various types were diagnosed (Table 2). Prevalence of hepatocellular carcinoma (Figure 5) was significantly different among the collections, with the highest rates in the Anacostia fish (27% -ANA-S, 30%-ANA-F) compared to the other collections (0-7%, $p=0.001$, Table 2, Figure 4b). Prevalence of invasive tumors of bile duct cell origin (cholangiocarcinoma; Figure 6) was more evenly distributed ($p=0.12$) between Anacostia fish (17%-ANA-S, 13%-ANA-F), NEAB fish (10%), and the other two collections (0-3%, Table 2). Other fish were diagnosed with non-invasive tumors (hepatocellular adenomas and cholangiomas) and preneoplastic lesions (foci of hepatocellular

alteration). There were statistically significant differences in the prevalence of liver tumors of any type with the highest rate in the Anacostia fish (50%-ANA-S, 60%-ANA-F) compared to the other collections (7-17%, $p=0.001$, Table 2, Figure 4).

In general, there were significant ($p<0.05$; Table 2, Figure 4) differences in lesion prevalence among the groups of fish, with maximum skin and liver lesion prevalence occurring in the two collections from the Anacostia and lesser prevalence in collections from the other three locations. The rankings in lesions and the statistically significant differences were maintained when the analysis was restricted to either same-aged or same-sexed fish (Table 2).

Other Lesions

Parasitic infestations of the liver were frequently observed and were diagnosed as either biliary myxidosis or helminthiasis. The prevalence of liver parasitism and lesions in the brain eye, gill, head and caudal kidney, heart, and spleen are summarized in Table 3. The brain lesions consisted of areas of demyelination and necrosis. The eye lesions were characterized as herniation or trematode infestation of the lens. The gill lesions were predominantly proliferative branchiitis, and atrophy, hypertrophy, and telangiectasis of the lamellae. Kidney lesions were characterized as interstitial necrosis and the spleen lesions were characterized as ellipsoid hypertrophy. Heart lesions consisted of a focal aneurism, an embolism, and mesotheliosis.

Biological Parameters

There were small but statistically significant differences in the length, weight, and condition factors of the fish from the five collections. The median length of the TUCK fish (266 mm) was significantly less than that of the other four collections (272-278 mm; K-W test, $p<0.001$; Table 4). The Tuckahoe fish also weighed less and were younger than the other collections. There were statistically significant differences in the condition factor (K), with the highest mean K in the QUAN fish (1.51) and the lowest in the TUCK fish (1.22; Table 4). The slightly smaller fish from the Tuckahoe were younger (ages 3 and 4) than the Anacostia (ages 3 through 7) and Neabsco and Quantico fish (ages 3 through 6). Sex ratios were also variable, with mostly females collected from the fall in the Anacostia, whereas mostly males or nearly equal numbers were determined for the other collections.

The greatest HSI values were calculated for the two collections of Anacostia River fish (Table 4). Significantly greater HSI values were observed in the ANA-S fish (0.025 ± 0.001 ; mean \pm one standard error) compared to all of the other groups of fish, including the ANA-F fish (0.023 ± 0.001 ; ANOVA, $p<0.001$). Both of the groups of Anacostia fish had significantly greater HSI values than the NEAB (0.019 ± 0.005) and QUAN fish (0.019 ± 0.001). HSI values were positively correlated with bile PAH-metabolite concentrations: benzo(a)pyrene ($r=0.61$, $p<0.0001$), naphthalene ($r=0.52$, $p<0.0001$), phenanthrene ($r=0.58$, $p<0.0001$), total PAH ($r=0.56$, $p<0.0001$, all based on 70 observations), and with muscle total chlordane concentrations ($r=0.42$, $p=0.003$, $n=46$).

Fish Tissue and Bile Contaminants

Median concentrations of total PCBs were significantly higher in the ANA-S, ANA-F, and NEAB fillets compared with the TUCK fillets ($p < 0.001$; Table 5). Nearly identical median concentrations (0.30-0.34 ppm) were found in the Anacostia and Neabsco fish, whereas PCBs were detected in only one of the ten Tuckahoe fish. The median Tuckahoe concentration used for statistical purposes was 0.025 ppm, one half of the detection limit. The median concentration of total PCBs was 0.16 ppm in the Quantico fish. Mean total DDT concentrations were also significantly higher in the ANA-F, NEAB, and QUAN fish compared with the TUCK fish ($p < 0.001$). The sum of total PCB, DDT, and chlordane concentrations was termed total organochlorines and compared among the collections of fish. Median concentrations were significantly higher ($p < 0.001$) in all of the Potomac/Anacostia watershed fish (0.28-0.46 ppm) compared to the Tuckahoe fish (0.090 ppm, Table 5).

Concentrations of total PAH metabolites (sum of benzo(a)pyrene, naphthalene, and phenanthrene) in bile were significantly higher in the two Anacostia collections compared with those from the other three sites ($p < 0.001$; Table 5). Median concentrations were 2939 ppm in ANA-S and 391 ppm in ANA-F compared with concentrations of 92-198 ppm in the other collections of fish. In general, ANA-S fish had considerably higher concentrations of the individual PAH metabolites than fish from the other collections.

Cytochrome P450

EROD activity was significantly elevated in the ANA-S fish (median: 79.4 picomoles/minute/mg microsomal protein) compared with the QUAN fish (median: 36.5) and TUCK fish (median: 39.0) fish ($p < 0.001$; Table 5). Although the highest median values were in fish from the Anacostia collections, the two individuals with the highest activities were from Neabsco Creek (405.4 and 174.1 pmoles/min/mg). There were no significant correlations between EROD activity and concentrations of PAH metabolites in bile or organochlorine contaminants in muscle.

Sediment Contaminants

Sediments from the four locations were similar in terms of grain size and total organic carbon content (Table 6). Mean total PAH concentrations, however, were significantly higher in the Anacostia sediments (26.8 ppm dry wt) compared with the three other collection sites (1.8-5.1 ppm; $p < 0.001$). Mean carcinogenic PAH concentrations were also significantly higher in the Anacostia sediments (9.0 ppm) compared to the other sites (0.5-2.2 ppm; $p < 0.001$). The differences in mean total PCB concentrations among the Anacostia (0.89 ppm), Quantico (0.31 ppm), and Neabsco (0.26 ppm) were not large enough to be statistically significant. PCBs were not detected in the Tuckahoe River sediments.

Logistic Regression Analysis

With liver hepatocarcinoma as the response variable, increased risks were associated with the following predictor variables: sex, age, length, K, HSI, bile-benzo(a)pyrene, -naphthalene, and -total PAH (Table 7). Based on the odds ratio, the strongest effects (greatest relative risks) were for sex (females had 4.514 x the odds for males) and age (3.525 x per year). There was an increased risk with increasing concentrations of bile benzo(a)pyrene (1.401 x per 1.0 ppm), naphthalene (1.063 x per 100 ppm), and total PAH (1.048 x per 100 ppm). The logistic regression results for total liver tumors were similar to those for hepatocellular carcinoma. Increased risks for total skin tumors were associated with age (2.457 x per year), HSI (1.231 x per 0.001 units), and bile PAH-metabolites (1.086-1.846 x).

DISCUSSION

Lesion Prevalence in Relation to Contaminant Exposure

The liver tumor prevalences in the Anacostia River bullheads (50 and 60%) warrant concern, in that they greatly exceeded the 9% prevalence that Baumann *et al.* (1996) suggested was indicative of contaminant exposure. Liver tumor prevalence in Neabsco Creek bullheads (17%) was also above the 9% prevalence, whereas the 10% prevalence in Tuckahoe bullheads was essentially equivalent.

Increased odds for liver and skin tumors were associated with increased concentrations of several bile PAH metabolites and were not associated with concentrations of organochlorine pesticides or PCBs in muscle, or EROD activity. Of the PAH metabolites, the highest odds ratios were for benzo(a)pyrene, known to be carcinogenic in mammals (Menzie *et al.* 1992), and reported to be transformed to carcinogenically-active intermediates in brown bullheads (Steward *et al.* 1990). Studies conducted by the National Oceanographic and Atmospheric Administration (NOAA) with marine fish on the U.S. West Coast have also reported positive statistical associations between bile PAH metabolites and neoplastic and preneoplastic liver lesions (Krahn *et al.* 1986; Myers *et al.* 1994). Based on the West Coast NOAA studies, Horness *et al.* (1998) used regression analysis to propose 2.8 ppm total PAHs (confidence limit of 0.011-5.5 ppm) as a threshold sediment concentration above which there was an increased prevalence of hepatic lesions in bottom-dwelling English sole (*Pleuronectes vetulus*). While such an analysis has not been performed for brown bullheads, the average total PAH concentration in the three Anacostia River sediments near the fish collection site was 26.8 ppm, nearly 10 times the proposed threshold.

The 50% and 60% liver tumor prevalences observed in the Anacostia River collections were similar to those observed in various studies in the Black River, Ohio, summarized by Baumann and Harshbarger (1998). In this river, tumor prevalence has been linked to sediment PAH contamination from steel operations, including a coke plant. Baumann *et al.* (1996) summarized three collections from the Black River as having total liver neoplasm prevalence

of 60% (1982), 32.5% (1987), and 58% (1992). Concentrations of 11 PAHs in Black River sediment samples were measured over various years and decreased from 1096 ppm in 1980 to 4.27 in 1987. After remedial dredging in 1990 resuspended some of the sediments, concentrations increased to 16.6 ppm in 1992 and declined to 9.8 ppm in 1994 (Baumann and Harshbarger 1998).

PAHs in the Anacostia River are derived from both petroleum discharges and combustion of petroleum products. These chemicals enter the system via point and nonpoint sources, including National Pollution Discharge Elimination System (NPDES) outfalls, hazardous waste sites, combined sewer and storm sewer outfalls, and spills (Wade *et al.* 1994; ICPRB 1996). The mean (\pm SD) concentration of these same 11 PAHs in the three Anacostia River sediment samples collected in the present study was 17.0 ppm. The mean total PAH concentration (sum of 44 analytes including alkylated PAHs) was 26.8 ± 2.9 ppm. Other monitoring studies in the Anacostia River have reported total PAH concentrations in sediments as high as 77.3 ppm (Pinkney *et al.* 1993) and 249 ppm (Hydro-Terra, Inc. 1998). Thus, the present and historical data for the Anacostia River indicate sediment PAH contamination, although there are no reports of concentrations as high as those initially measured in the Black River.

Studies of the possible association between liver neoplasia and PAH contamination in the Chesapeake Bay region have been reported by Vogelbein *et al.* (1990) and Pinkney *et al.* (1995a). Vogelbein *et al.* (1990) reported a 33% prevalence of hepatocellular carcinomas in mummichogs (*Fundulus heteroclitus*) from an area of the Elizabeth River where sediments were contaminated with creosote. Total PAHs in those sediments were considerably higher, 2200 ppm, than those reported in the present study. Pinkney *et al.* (1995a) reported a 10% prevalence of hepatocellular carcinomas in Neabsco Creek where maximum PAH concentrations were 25.5 ppm. This prevalence is similar to that reported in Neabsco Creek in the present study (7% hepatocellular carcinomas, 17% total liver tumors), in which the maximum sediment concentration was 12.7 ppm.

The apparent association between tumor prevalence and PAHs in sediments and PAH-metabolites in bile should not be considered proof of a cause-effect relationship. First, the bile reflects recent exposure (i.e., days) whereas the development of tumors was initiated months to years before collection. Second, fish in the Anacostia and Neabsco are exposed to both PAHs and organochlorines, in addition to other chemicals that were not measured in this study. It is possible that tumors are initiated by PAHs and promoted by exposure to organochlorine pesticides and PCBs (Moore and Myers 1994; Myers *et al.* 1994). It is also possible that movements of the fish may confound the interpretation.

The average Anacostia skin tumor prevalence (23%) slightly exceeded the 20% rate that Baumann *et al.* (1996) suggested as indicative of contaminant exposure. In the two Anacostia collections, liver tumor prevalences (spring-50%, fall - 60%) were similar, whereas skin tumor prevalences were markedly different (spring-37%, fall -10%). There were also similar prevalences of liver tumors in the sampling of Neabsco Creek in 1992 (10%) and 1996 (17%),

whereas the skin tumor prevalences were very different (33% in 1992, 3% in 1996). These findings are consistent with literature that indicates a stronger linkage between liver tumors and chemical contamination than between skin tumors and chemical contamination (e.g., Baumann *et al.* 1996).

Although not observed in the present study, bullheads from the Anacostia River are frequently observed to have clubbed or missing barbels (Jon Siemien, D.C. Department of Health, personal communication). This lesion has been correlated with elevated sediment PAH concentrations (Smith *et al.* 1994). The lesions we observed in organs other than the liver as well as the parasitic lesions in the livers, however, were not related to the tumor prevalence. For example, the prevalence of liver parasitism was equally high (14/30) in ANA-S, ANA-F, and TUCK fish.

Biochemical Indicators of Exposure

According to S. MacDonald (Texas A&M GERG, pers. comm., bile benzo(a)pyrene concentrations above 1 ppm, phenanthrene concentrations above 50 ppm, and naphthalene concentrations above 500 ppm are indicative of highly polluted environments. Benzo(a)pyrene concentrations of ~0.2-1.0 ppm, phenanthrene concentrations of ~10-50 ppm, and naphthalene concentrations of ~300-500 ppm are indicative of moderately polluted environments. Based on the concentrations of PAH-metabolites in bile, the Anacostia bullheads would be in the highly polluted environment classification. These fish had greater recent exposure to PAHs than fish collected at the other sites (Tables 5 and 6) and metabolite concentrations are consistent with those reported from PAH-contaminated sites. For example, the mean concentration of benzo(a)pyrene equivalents in the ANA-S fish (4.1 ppm) was similar to values reported by Arcand-Hoy and Metcalfe (1999) for Black River brown bullheads collected in 1994 (2.8 ppm) and for brown bullheads caged for eight days in the PAH-contaminated Trenton Channel area of the Detroit River (Leadly *et al.* 1999). The benzo(a)pyrene equivalents measured by Leadly *et al.* (1999) in an upstream reference area (0.6 ppm) and in laboratory controls (0.3 ppm) were similar to the mean concentrations in the NEAB and TUCK fish (0.47 and 0.49 ppm, respectively).

Bile PAH metabolite concentrations, however, cannot serve as a simple predictor of neoplasm frequencies for several reasons. First, this biomarker measures response to exposures that occurred over a period of days whereas the critical period for initiating carcinogenesis probably occurred months or years before the fish were collected. Second, there is considerable temporal variability in this response. Leadly *et al.* (1999) reported significant differences in bile PAH concentrations in caged brown bullheads over a course of 16 days.

Levels of EROD activity, indicative of exposure to both organochlorine and PAH contamination, were not consistent with the tumor rankings. For example, although cytochrome P450 activity was significantly elevated in the ANA-S fish relative to QUAN and TUCK fish, the activity in the ANA-F fish was similar to that in all of the other groups of fish (Table 5). Possible explanations for the lack of consistency between the cytochrome P450

activities and liver neoplasm prevalence in the two collections from the Anacostia are: (1) a seasonal effect on enzyme response; (2) differences in the movement patterns (i.e., exposure to contaminants) of the spring and fall collections from the Anacostia; (3) differences between contaminant exposure at the time of tumor induction vs. the exposure that resulted in enzyme response; and (4) evidence that PCBs are stronger inducers of cytochrome P450 than PAHs (Eufemia *et al.* 1997).

Mean EROD activities in the Anacostia and Neabsco collections, ranging from 52.6 to 79.4 pmol/min/mg protein, were similar to those measured by Arcand-Hoy and Metcalfe (1999) in brown bullheads from three contaminated sites in the Great Lakes (42.0-59.5 pmol/min/mg protein). Because the two Great Lakes control site collections, however, had lower mean EROD activities (both 18.9 pmol/min/mg protein) than the Tuckahoe fish (39.0 pmol/min/mg protein), the ratio of activities between contaminated and control sites was greater in the Great Lakes study. At present, there are no available data from other $\frac{1}{2}$ reference sites in the Chesapeake to indicate whether the Tuckahoe fish should be considered to have elevated EROD activity.

Hepatosomatic Index and Condition Factor

The highest HSI rankings occurred in the two Anacostia collections with the highest liver tumor prevalence and the logistic regression analysis identified HSI as a significant predictor variable for both liver and skin tumors. A similar result was reported in a survey of four Great Lakes sites by Baumann *et al.* (1991). The mean HSI value determined for the ANA-S fish (0.025) was similar to the mean HSI (0.026) determined by Baumann *et al.* (1991) for brown bullheads from the Cuyahoga River, where the highest prevalence of liver neoplasms were found. Higher HSI values were reported by Arcand-Hoy and Metcalfe (1999) in collections from two Great Lakes contaminated sites (0.024 and 0.030) compared to a control site (0.020). Fabacher and Baumann (1985) reported HSI values of 0.0466 for male and 0.0570 for female bullheads from the Black River compared with values of 0.017-0.027 with fish from uncontaminated locations. They stated that although there are sex and seasonal effects on liver enlargement, it is still useful as an indicator of contaminant stress.

Condition factors reported in the present study ranged from 1.22 to 1.51, which encompasses the 1.30 value reported by Sinott and Ringler (1987) in a highly productive eutrophic lake. The logistic regression identified K as a significant predictor variable for hepatocellular carcinoma (odds ratio of 0.615 per 0.1 unit increase = 39% decreased odds for 0.1 unit increase) and for being lesion-free (1.318 odds ratio per 0.1 unit increase). This suggests that the more robust fish have less risk of being diagnosed with tumors. The results of the logistic regression, however, are not consistent with the comparisons of mean K for the five collections, in which the highest (Quantico-1.51) and lowest (Tuckahoe-1.22) mean K values were measured in fish from the two sites with the lowest tumor prevalence.

Recommendation

Tumor prevalence surveys are recommended for monitoring the status of areas of the Chesapeake Bay with contaminated sediments, including Regions of Concern. Additional studies should be conducted in remote areas to develop a larger data base on background prevalence and to identify areas needing further investigation. For low salinity areas, the brown bullhead may be an appropriate species; however, further information on its movements is needed to confirm its utility as a site-specific indicator. For higher salinity areas such as the Elizabeth River, the mummichog, (*Fundulus heteroclitus*), a species with a small home range, is recommended (Vogelbein *et al.* 1990). Such surveys should be conducted before and after sediment remediation or source control measures have been implemented. Key indicators of exposure and response are the analysis of PAH metabolites in bile and the determination of DNA-adducts in the liver. Calculation of the hepatosomatic index and observation of barbels are recommended for bullhead surveys.

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Table 1. Brown bullhead collection and water quality data

	Anacostia (4/25/96) (ANA-S)	Anacostia (10/9/96) (ANA-F)	Quantico (6/11/96) (QUAN)	Neabsco (9/25/96) (NEAB)	Tuckagoe (11/13/96) (TUCK)
Location	Near CSX Bridge	Near CSX Bridge	S. of Chopawamsic Island	Upstream of RR Bridge	At river bend
Latitude	38°N52'45"	38°N52'45"	38°N30'24"	38°N36'01"	38°N51'59"
Longitude	76°W58'20"	76°W58'20"	77°W17'59"	77°W15'25"	75°W56'04"
Conduct, (mmhos/cm)	0.304	0.157	0.223	0.295	0.184
Temp. (°C)	18.0	14.0	25.7	18.8	7.8
pH	7.27	7.52	8.72	7.50	7.51
D.O. (ppm)	5.49	8.17	9.89	6.62	8.78
D.O.(%Sat.)	57.8	79.5	121.9	70.9	71.7
Salinity	0.1	0.1	0.1	0.1	0.1
Depth (m)	1.8	3.3	0.5	1.4	2.7

Table 2. Liver and skin tumor prevalence (number affected with percentage in parentheses) for the five collections of brown bullheads

Lesion	ANA-S	ANA-F	NEAB	QUAN	TUCK	Statistics*
Entire Collection (n=30)						
FHA	8 (27%)	6 (20%)	1 (3%)	0 (0%)	1 (3%)	p=0.001
HA	3 (10%)	8 (27%)	1 (3%)	0 (0%)	1 (3%)	p=0.002
HC	8 (27%)	9 (30%)	2 (7%)	0 (0%)	0 (0%)	p=0.001
C	1 (3%)	1 (3%)	0 (0%)	2 (7%)	1 (3%)	p=0.72
CC	5 (17%)	4 (13%)	3 (10%)	0 (0%)	1 (3%)	p=0.12
TLC	12 (40%)	10 (33%)	4 (13%)	0 (0%)	1 (3%)	p=0.001
TLB	15 (50%)	18 (60%)	5 (17%)	2 (7%)	3 (10%)	p=0.001
EP	7 (23%)	3 (10%)	1 (3%)	1 (3%)	0 (0%)	p=0.007
SC	4 (13%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	p=0.002
TS	11 (37%)	3 (10%)	1 (3%)	1 (3%)	0 (0%)	p=0.001
LF	6 (20%)	7 (23%)	24 (80%)	27 (90%)	26 (87%)	p=0.001
Age 4 fish (number affected/total number)						
TLB	1/5 (20%)	4/7 (57%)	2/15 (13%)	0/14 (0%)	1/12 (8%)	p<0.001
LF	1/5 (20%)	2/7 (29%)	12/15 (80%)	13/14 (93%)	11/12 (92%)	p<0.001
Age 4-6 females (number affected/total number)						
TLB	7/8 (88%)	11/16 (69%)	3/8 (38%)	2/8 (25%)	1/7 (14%)	p=0.01
LF	0/8 (0%)	3/16 (19%)	5/8 (62%)	6/8 (75%)	6/7 (86%)	p=0.001

*Extension of Fisher's Exact Test (Stokes *et al.* 1995).

ANA -Sreiations: FHA: foci of hepato cellular alteration, HA: hepatocellular adeno ma, HC: hepatocellular carcinoma, C: cholan gioma, CC: cholangiocarcinoma, TLC: total liver or bile invasive tumors, TLB: total liver and/or bile duct invasive or non -invasive tumors, EP: epidermal papilloma, SC: squamous carcinoma, TS: total skin tumors; LF: Lesion free (skin and liver).

Table 3. Non-neoplastic lesions diagnosed in the five collections of brown bullheads

Organ	Diagnosis	ANA-S	ANA-F	NEAB	QUAN	TUCK
Brain	Demyelination, localized	1	1	1	0	0
Brain	Necrosis, localized	0	0	1	4	0
Gill	Branchiitis	1	0	0	14	5
Gill	Lamellar atrophy, hypertrophy, or telangiectasis	5	0	0	2	4
Eye	Lens herniation	0	2	4	0	0
Eye	Lens -- trematode infestation	0	5	6	0	2
Head kidney	Interstitial necrosis	2	0	0	0	0
Caudal kidney	Interstitial necrosis	5	0	0	0	0
Caudal kidney	Nephritis	0	2	3	0	0
Spleen	Ellipsoid hypertrophy	1	0	0	2	0
Heart	Focal aneurism	1	0	0	0	0
Heart	Embolism	0	0	0	1	0
Heart	Mesotheliosis	0	0	0	0	1
Liver	Parasitism (helminthiasis or myxidiosis)	14	14	6	5	14

Table 4. Biological data for the five collections of brown bullheads^{ab}

	ANA-S	ANA-F	NEAB	QUAN	TUCK	Statistics
Length (mm)	272 (260-302) (A,B)	276 (262-301) (A)	278 (261-315) (A)	273 (260-311) (A,B)	266 (261-277) (B)	Kruskal-Wallis (K-W): p<0.001
Weight (g)	269 (198-378) (B)	265 (228-360) (B)	295 (217-399) (A,B)	307 (262-428) (A)	234 (197-270) (C)	K-W: p<0.001
Liver weight (g)	6.78±0.25 (A)	6.20±0.22 (A,B)	5.58±0.21 (B,C)	6.10±0.24 (A,B)	4.87±0.15 (C)	ANOVA: p<0.001
Condition Factor (K)	1.28±0.02 (B,C)	1.28±0.04 (B,C)	1.36±0.02 (B)	1.51±0.02 (A)	1.22±0.02 (C)	ANOVA: p<0.001
Hepato-somatic Index (HSI)	0.025±0.001 (A)	0.023±0.001 (B)	0.019±0.0005 (C)	0.019±0.001 (C)	0.021±0.0005 (B,C)	ANOVA: p<0.001
Sex	17 M, 11 F, 2?	6M, 23F, 1?	13M, 8F; 9 ?	19M; 11 F	14M; 16 F	
Age	5 (4-7) (A) 4+:5;5+:14;6+:4; 7+:2; not aged: 5	5 (3-6) (A) 3+: 2; 4+:7; 5+:12; 6+: 3; not aged: 6	4 (3-6) (A) 4+: 15; 5+: 9; 6+: 3; not aged: 3	4 (3-6) (A) 3+: 1; 4+: 14; 5+: 10; 6+: 1; not aged: 4	3 (3-4) (B) 3+: 14; 4+: 12; not aged: 4	K-W: p<0.001

^a Mean ± one standard error or median with minimum and maximum in parentheses, all based on n=30.

^b Groups with different letters are significantly different at p<0.05 using Tukey's test (ANOVA) or Dunn's method (K-W).

Table 5. Chemical and biochemical analyses^{ab} of brown bullheads (*Ameiurus nebulosus*)

	ANA-S	ANA-F	NEAB	QUAN	TUCK	Statistics ^c
muscle t-PCB	0.33 (ND-0.91) (A)	0.30 (ND-0.61) (A)	0.34 (ND-0.65) (A)	0.16 (ND-0.67) (A,B)	0.025 (ND-0.07) (B)	Kruskal-Wallis (K-W); p=0.002
muscle t-DDT	0.084 ± 0.012 (A,B)	0.088 ± 0.013 (A)	0.10 ± 0.021 (A)	0.10 ± 0.024 (A)	0.042 ± 0.002 (B)	ANOVA ^d ; p=0.01
muscle t-chlordane	0.059 (ND - 0.19) (A)	0.032 (ND - 0.11) (A,B)	0.025 (ND - 0.050) (B)	0.025 (ND - 0.036) (A,B)	0.025 (all ND) (B)	K-W; p<0.001
muscle total OC^e	0.46 (0.080-1.2) (A)	0.44 (0.093-0.81) (A)	0.45 (0.080-0.92) (A)	0.28 (0.086-0.90) (A,B)	0.090 (0.085-0.14) (B)	K-W; p=0.002
bile b(a)p	4.9 ± 0.3 (A)	1.1 ± 0.1 (B)	0.47±0.05 (C)	1.0 ±0.1 (B)	0.49 ± 0.07 (C)	ANOVA ^d ; p<0.001
bile naph.	2200 (1.7-3600) (A)	290 (86-560) (A)	61 (24-210) (B)	140 (59-260) (A,B)	60 (0.3-170) (B)	K-W; p<0.001
bile phen.	863 (480-1164) (A)	100 (34-230) (A,B)	26 (13-80) (C)	57 (27-88) (B,C)	43 (13-160) (B,C)	K-W; p<0.001
bile tPAH	2939 (666-4476) (A)	391 (121-792) (A)	92 (37-291) (B)	198 (86-350) (B)	117 (29-331) (B)	K-W; p<0.001
EROD	79.4 (31.3-139.0) (A)	55.9 (23.8-109.8) (A,B)	52.6 (4.0-405.4) (A,B)	36.5 (10.6-107.5) (B)	39.0 (27.9-59.9) (B)	K-W; p<0.001

^a For calculations, non-detected (ND) values reported as one half of the detection limit (½ d.l.=0.025 ppm for PCBs, 0.005 ppm for DDT and chlordane);

Units: PCBs, DDT, chlordane, bile PAH-metabolites are ppm wet weight of B(a)P, naphthalene, and phenanthrene equivalents; EROD: picomoles per minute per mg protein.

^b Mean ± one standard error or median with range in parentheses; muscle data, n =10 per collection; bile data, n =14-15 per collection; EROD, n=18-20 for all except QUAN (n=29)

^c Groups with different letters are significantly different at p<0.05 using Tukey's test (ANOVA) or Dunn's method (K-W).

^d log-transformed data.

^eSum of total PCBs, total DDT, total chlordane.

Table 6. Sediment chemistry data (ppm dry wt) based on three samples per location, mean \pm one standard error or median with range in parentheses^a

Chemical	Anacostia	Neabsco	Quantico	Tuckahoe	Statistics
Total PAHs	26.8 \pm 1.6 (A)	4.8 \pm 4.0 (B)	5.1 \pm 2.7 (B)	1.8 \pm 1.3 (B)	ANOVA, p<0.001
Carc. PAHs^b	9.0 \pm 0.2 (A)	1.4 \pm 1.3 (B)	1.2 \pm 0.6 (B)	0.5 \pm 0.4 (B)	ANOVA, p<0.001
Total PCBs	0.89 \pm 0.25 (A)	0.26 \pm 0.10 (A)	0.31 \pm 0.13 (A)	ND ^c (B)	ANOVA, p=0.02
Total DDT	0.18 \pm 0.02	0.10 \pm 0.02	0.32 \pm 0.15	ND ^c	ANOVA ^d (p=0.08)
Total chlordane	0.16 (0.13-0.18)	ND ^c	ND ^c	ND ^c	Kruskal-Wallis, p=0.10
Fraction fines^e	0.56 \pm 0.09	0.62 \pm 0.22	0.71 \pm 0.16	0.61 \pm 0.22	
% total org. C	4.5 \pm 0.6	2.9 \pm 0.9	3.9 \pm 1.2	3.8 \pm 1.6	

^a Groups with different capital letters are significantly different, based on Tukey's test (p<0.05).

^b Total of benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, and indeno(1,2,3-c,d)pyrene.

^c Not detected; for calculations, values reported as one half of the detection limit ($\frac{1}{2}$ DL=0.043-0.118 ppm for PCBs, 0.052-0.142 for DDT, 0.052-0.119 for chlordane).

^dLog₁₀ transformed data.

^efraction fines: fraction silt + fraction clay.

Table 7. Results of logistic regressions showing only significant ($p < 0.05$) associations

Response variable	Predictor variable	Frequency of response	p value^a	Odds ratio^b
Hepatocellular carcinoma (HC)	Sex	19/138	0.0001	4.514
	Age	16/128	0.0004	3.525
	Length	19/150	0.029	1.527
	K	19/150	0.018	0.615
	HSI	19/150	0.0001	1.315
	Bile b(a)p	11/74	0.030	1.401
	Bile naph	11/74	0.048	1.063
	Bile tPAH	11/74	0.044	1.048
Total liver tumors (TLB)	Sex	42/138	0.001	3.654
	Age	38/128	0.0002	2.652
	HSI	43/150	0.0001	1.334
	Bile b(a)p	17/74	0.034	1.349
	Bile phenan.	17/74	0.032	1.184
Total skin tumors (TS)	Age	13/128	0.009	2.457
	HSI	16/150	0.001	1.231
	Bile b(a)p	10/74	0.0005	1.846
	Bile naph.	10/74	0.003	1.102
	Bile phen.	10/74	0.0003	1.445
	Bile tPAH	10/74	0.001	1.086

^a Based on Wald statistic.

^b Scaling of odds ratios: sex (female:male); age (per year); length (per 10 mm); K (per 0.10 units); HSI (per 0.001 units); bile benzo(a)pyrene (b(a)p; per 1.0 ppm); bile naph, phenan., tPAH (per 100 ppm).

APPENDIX A

Glossary of Pathological Terminology

Glossary of Pathological Terminology

SKIN

Epidermal Papilloma (EP): The normal linear stratified squamous skin or lip epidermis is thickened due to an increase in cell number, resulting in a buckling pattern of intertwining epidermal pegs which interdigitate with fibrovascular stromal papillae. The basement membrane separating the basal layer of the pegs from the stroma is intact.

Squamous Carcinoma (SC): Consists of an epidermal papilloma that has undergone squamous metaplasia, often characterized by the presence of squamous pearls, and which has or appears about to breach the basement membrane and invade the adjacent connective tissue.

B ILIARY

Cholangioma (C): A cluster or small mass of well-differentiated bile ducts without increased periductular fibrosis and with a banal appearance.

Cholangiocarcinoma (CC): A mass of poorly-formed bile ducts with significant increase in periductular fibrosis and an aggressive appearance with may include interdigitating with the normal liver. CCs are sometimes centrally necrotic.

HEPATIC

Focus of Hepatocellular Alteration (FHA) (pre or incipient neoplasms): a small, <1.0 mm chromophilic focus without cytologic or pattern atypia that blends into the cords of the normal liver. Believed to be in the neoplasm sequence but at a stage where they may still be reversible. Special stains would show reduced iron and glycogen.

Hepatocellular Adenoma (HA): A chromophilic lesion usually <1.5 mm with subtle cytologic and/or pattern atypia.. Has a banal appearance.

Hepatocellular Carcinoma (HC): A lesion usually >1.5 mm with frank cytologic and pattern atypia. Appears to be replacing adjacent liver tissue.

OTHER TERMS

Squamous: Scaly or platelike.

Metaplasia: Change in the type of cells in a tissue to a form which is not normal for that tissue.

APPENDIX B

Spreadsheet with Biological and Chemical Data

FIGURES

