U.S. Fish & Wildlife Service

Assessment of Endocrine Disruption in Smallmouth Bass and Largemouth Bass in the Potomac River Watershed

Final Report

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Assessment of Endocrine Disruption in Smallmouth Bass (*Micropterus dolomieu*) and Largemouth Bass (*Micropterus salmoides*) in the Potomac River Watershed

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EXECUTIVE SUMMARY

The Potomac River is the second largest tributary to the Chesapeake Bay. It is an important spawning and nursery ground for both migratory and resident fish species including American eel (*Anguilla rostrata*), striped bass (*Morone saxatilis*), white perch (*Morone americana*), shad and herring (*Alosa sp.*), smallmouth bass (*Micropterus dolomieu*), largemouth bass (*M. salmoides*), sunfish (*Lepomis sp.*), and carp (*Cyprinus carpio*). The upper portions of the Potomac watershed in West Virginia and parts of Virginia are dominated by rural communities and animal agricultural facilities. Closer to the Chesapeake Bay, the Potomac watershed becomes more urbanized with agricultural inputs being replaced more frequently with municipal wastewater treatment plant discharges. According to the Maryland Department of the Environment, there are 747 surface water discharge permits to Maryland waters of the Potomac watershed, 117 of which are municipal wastewater treatment plant discharges.

Since 2003, scientists at the U.S. Geological Survey, National Fish Health Research Laboratory (USGS-NFHRL) in Kearneysville, West Virginia and the West Virginia Department of Natural Resources have been evaluating the reproductive health of smallmouth bass in the upper Potomac River and its tributaries, including the Shenandoah River. They noted the presence of immature female germ cells (oocytes) in the testes of some of the male fish. This condition, a type of intersex, is evidence of a disturbance in the hormonal system of the fish (i.e., endocrine disruption). The USGS studies, in several areas in Virginia and West Virginia, found a wide range in the prevalence of intersex in male smallmouth bass (14% to 100%).

The U.S. Fish and Wildlife Service's mission is to conserve, protect, and enhance fish and wildlife resources and their habitats for the continuing benefit of the American people. The USGS studies in the Shenandoah River, South Branch of the Potomac River, and other tributaries within the Potomac Basin identified a potential threat to these resources and led to several questions:

- Is there a relationship between these reproductive abnormalities and proximity to wastewater treatment plant discharges?

- How widespread is this problem within the Potomac watershed?

- What chemicals can be detected at fish sampling locations; which ones are considered to be endocrine disruptors; and how do the concentrations relate to land use?

To address these questions, a collaborative partnership was formed among scientists from the U.S. Fish and Wildlife Service, Chesapeake Bay Field Office, USGS-NFHRL, USGS Columbia Environmental Research Center, and the Maryland Department of Natural Resources. The team prepared a successful Off-Refuge Proposal that was funded by the USFWS Division of Environmental Quality for 2005-2007 as Project 5F41. The primary objectives were to determine: (1) if bass exposed to endocrine disrupting compounds were exhibiting intersex or other gonadal abnormalities; (2) if male bass exposed to endocrine-disrupting compounds had elevated concentrations of vitellogenin (the protein precursor for egg yolk production); and (3) if wastewater treatment plants were releasing detectable concentrations of endocrine disrupting compounds using passive samplers.

Here we report the results of this multi-year study. The report is presented in three chapters. The first chapter describes the biological findings from the 2005 field collections. The second chapter describes the chemical results from the passive sampler deployments in 2005 and 2006. These two chapters are identical to the published versions, which appear in the May 2009 issue of *Environmental Toxicology and Chemistry*. The third chapter describes the results of a caged *in situ* exposure of hatchery-raised smallmouth bass in selected locations in 2006.

In the fall of 2005, male and female smallmouth bass were collected from two Potomac River tributaries in Maryland, the Monocacy River and Conococheague Creek. For each river, one location was immediately downstream of a wastewater treatment plant discharge and one about 15 kilometers upstream. Largemouth bass were also collected near the discharge of the Blue Plains Wastewater Plant in Washington, DC. For each location about 10 males and 10 females were collected and examined. Blood samples were taken and hormones and vitellogenin were analyzed. Microscope slides were prepared of testes and ovaries for histopathology.

In the fall of 2005 and in the spring of 2006, passive water sampling devices (the semi-permeable membrane device (SPMD) and polar organic chemical integrative sampler (POCIS), were placed at these sites for one to two months. These devices accumulate different types of organic contaminants, including both legacy and emerging compounds, allowing scientists to identify contaminants at very low concentrations.

A high prevalence of intersex (82% to 100%) was identified in male smallmouth bass at all sites. Intersex (23%) was identified in male largemouth bass collected at the site near Blue Plains. Baseline prevalence for smallmouth is uncertain but may be in the range of 14% to 22%; baseline for largemouth may be closer to 0%. Vitellogenin, normally absent from males, was found in the plasma of 33% to 90% of the male smallmouth and 85% of the male largemouth bass. In Conococheague Creek, there was more than a tenfold decrease in the concentration of vitellogenin in the females collected downstream vs. those collected upstream.

Analysis of the passive samplers resulted in the detection of 84 out of 138 targeted chemicals. The agricultural pesticides, atrazine and metolachlor, had the greatest seasonal changes in water concentrations with a 3.1 to 91-fold increase in the spring than in the previous fall. Coinciding with the elevated concentrations of atrazine in the spring were increasing concentrations of the atrazine degradation products, desethylatrazine and desisopropylatrazine, in the fall following spring and summer application of the parent compound. Other targeted chemicals (organochlorine pesticides, polycyclic aromatic hydrocarbons, and organic wastewater chemicals) did not indicate seasonal changes in occurrence or concentration; however, the overall concentrations and number of chemicals present were greater at the sites downstream of wastewater treatment plant discharges. Several fragrances and flame retardants were identified in these downstream sites which are characteristic of wastewater effluent and human activities. The bioluminescent yeast estrogen screen (BLYES) *in vitro* assay of the POCIS extracts indicated that there were chemicals capable of producing an estrogenic response at all sampling sites.

No single chemical or sources that may be causing the intersex and vitellogenin induction were identified. Multiple chemical stressors that are not solely associated with agriculture or

wastewater treatment plant effluent may be responsible for the observed reproductive impairment.

In spring 2006, hatchery-raised smallmouth bass were deployed in cages in Conococheague Creek (both fish sampling sites) and the Monocacy (downstream only). In addition, fish were also caged at a reference pond near the NFHRL in Kearneysville, WV. As a control, a subset of 12 males and 14 females from the hatchery-raised bass were processed for blood plasma vitellogenin analysis and histopathology prior to exposure. The objective of this study was to attempt to induce endocrine disrupting effects in hatchery-raised fish in the same locations where wild-caught smallmouth bass exhibited evidence of endocrine disruption. After 50 days of *in situ* exposure, blood was sampled for plasma vitellogenin and histopathology performed on approximately 10 males and 10 females from each location. Passive chemical samplers, SPMDs and POCIS were placed next to the cages during exposure. The passive sampler analysis demonstrated that the fish in the cages were being exposed to endocrine disrupting compounds in detectable concentrations. However, 83% of the male smallmouth bass had testicular oocytes prior to exposure, making it impossible to determine the effects of the *in situ* exposure.

The caged fish study revealed a high prevalence of intersex in the hatchery-raised fish before they were deployed in the rivers. This result, which will preclude a journal publication of the study, nevertheless warrants further examination on a broader scale. It is important to document the prevalence of intersex and other reproductive impairment in hatchery-reared smallmouth and largemouth bass since these fish are released into the environment on a regular basis. A key research question is whether smallmouth and largemouth bass populations are adversely affected by the presence of testicular oocytes in sexually mature males.

To gain a greater understanding of the reproductive health of bass in the northeast United States, USWFS Region 5 Contaminants Biologists and USGS-NFHRL are sampling fish from rivers near National Wildlife Refuges (from Virginia north to Maine). This On-Refuge Investigation (Project 5N44) was initiated in 2008 and will be completed in 2011. One of the sample locations will be centered around the Potomac River National Wildlife Refuge Complex in the tidal

Potomac. The study is using a similar upstream/downstream design in relationship to potential sources of endocrine-disrupting contaminants.

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CHAPTER I: Reproductive Health of Bass in the Potomac, USA Drainage: Part 1. Exploring the Effects of Proximity to Wastewater Treatment Plant Discharge

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ABSTRACT

Intersex, specifically testicular oocytes, has been observed in male smallmouth bass (SMB) and other centrarchids in the South Branch of the Potomac River and forks of the Shenandoah River during the past five years. This condition is often associated with exposure to estrogenic endocrine disrupting chemicals in some fish species, but such chemicals and their sources have yet to be identified in the Potomac. In an attempt to better understand the plausible causes of this condition, we investigated the reproductive health of bass sampled up- and downstream of wastewater treatment plant (WWTP) effluent point sources on the Potomac River located in Maryland, USA. Smallmouth bass were sampled from the Conococheague Creek and the Monocacy River, and largemouth bass (LMB) were collected near the Blue Plains WWTP on the mainstem of the Potomac River. Chemical analyses of compounds captured in passive sampler devices at these locations were also conducted. A high prevalence of intersex (82 to 100%) was identified in male SMB at all sites regardless of collection area. Intersex (23%) was identified in male LMB collected at the Blue Plains site. When up- and downstream fish were compared, significant differences were only noted in fish from the Conococheague. Differences included condition factor, gonadosomatic index, plasma vitellogenin concentration and estrogen: testosterone ratios. In general, chemicals associated with wastewater effluent, stormwater runoff and agriculture were more prevalent at the downstream sampling sites. An exception was atrazine (and associated metabolites) which was present at greater concentrations at the upstream sites. While it appears that proximity to effluent from WWTPs may influence the reproductive health of bass in the Potomac watershed, inputs from other sources likely contribute to the widespread, high incidence of testicular oocytes.

INTRODUCTION

During 2003, a high prevalence (33 to 80%) of intersex, specifically testicular oocytes (TOs), was identified in male smallmouth bass (SMB, *Micropterus dolomieu*) collected from numerous sites within the South Branch of the Potomac River, West Virginia, USA. Subsequent surveys of SMB in a regionally distinct section of the Potomac River drainage (the Shenandoah River, Virginia) revealed an even higher prevalence of TOs (80 to 100%) than that found in the South Branch [1]. While the observation of TOs in gonochoristic fishes is not an unprecedented finding in wild fishes, it is uncommon and frequently used as a biomarker of exposure to estrogenic endocrine-disrupting chemicals (EDCs) [2-4]. Thus, the apparent widespread prevalence of this condition in the Potomac River drainage suggests the presence of biologically relevant point and non-point sources of these chemical compounds. Interestingly, given that other centrarchids such as largemouth bass (LMB, *M. salmoides*) at these sites have a lower prevalence of TOs than SMB (unpublished data from our lab), SMB may be particularly sensitive to exposure to putative EDCs. However, the cause(s) for the condition and presence of other adverse responses is currently unknown.

The Potomac River basin is located in four U.S. mid-Atlantic states (Maryland, Pennsylvania, Virginia, West Virginia) and the District of Columbia, and is the second largest tributary of the Chesapeake Bay. The basin encompasses over 23,600 km^2 and receives effluent from multiple industrial sources and urban combined sewer overflows as well as non-point sources [5]. Wastewater treatment plants (WWTPs) are included as contributors of point source effluent into this system. While not previously examined in the Potomac River basin, WWTPs are recognized as a general source of EDCs. Examples include both natural and synthetic substances such as polychlorinated biphenyls (PCBs), phthalates, pesticides, heavy metals, alkylphenols, polycyclic aromatic hydrocarbons (PAHs), 17β-estradiol, 17α-ethinylestradiol and bisphenol A [6-8]. Treatment of wastewater prior to discharge differs between WWTPs and not all common strategies effectively remove EDCs. Consequently, different WWTPs vary in their ability to remove particular classes of EDCs [9, 10].

Wastewater treatment plants are only one potential source of chemicals that may adversely affect general and reproductive health of fishes. Run-off from agricultural land has been shown to contain hormones and pharmaceutical chemicals [11, 12]. Additionally, pesticides and herbicides applied on agricultural, public and residential lands are transported to aquatic ecosystems during rain events [13, 14]. Leachates from landfills [15], PCBs [16, 17], PAHs [18, 19] and other compounds from industrial effluents, atmospheric deposition, spills and stormwater runoff are also known to contribute endocrine-modulating chemicals to the aquatic environment.

While intersex may be used as biomarker of reproductive health, other bioindicators of reproductive health in fish populations are frequently evaluated as well. Examples of these include morphological indicators such as changes in gonadosomatic index (GSI) and secondary sex characteristics [20, 21]. Histologic observations within the gonads such as atresia, foci of pigmented cells, and gamete stage are also useful [22]. Lastly, physiological measures of plasma vitellogenin (Vtg) [23] and sex steroid hormone concentrations are common end-points of endocrine disruption [24, 25].

Here we present the results of a study designed to investigate the reproductive health of bass inhabiting waters near three WWTPs. The primary goals of this research were to further evaluate the extent of reproductive abnormalities in bass within the Potomac drainage and investigate potential causes for the high prevalence of TOs and other adverse effects using a suite of biological endpoints. A companion study [26] quantified the presence of polar and non-polar water-borne chemical contaminants and their *in vitro* estrogenicity to define the differences in chemical fingerprints between the sample locations.

Methods and Materials

Sampling Sites

A list of the 66 major WWTPs, defined as those with capacity >0.5 million gallon per day (mgd; 1 mgd = 3.78 million liters per day) in the Maryland and D.C. portions of the Potomac watershed was generated and reviewed. Site selection was governed by the principle criteria that bass were present, could be collected without adversely impacting the local populations, and that collection

could occur immediately downstream of a WWTP and at least 15 km upstream. Sites along Conococheague Creek and the Monocacy River were selected as they best conformed to these criteria. Water temperatures ranged from 21.1 to 23.1^{o}C at the four tributary sites and was 25.8^{o}C at the lower mainstem site. All water quality parameters (Table 1) were similar among sites and within acceptable ranges for bass growth, survival and health [27].

Conococheague Creek (Figure 1A) originates north of Chambersburg, PA, flows through Fairview, MD (site of a U.S.Geological Survey gauging station, 01614500) and into the Potomac River at Williamsport in Washington County, MD. Land-use within the 911 km^2 watershed is agricultural (61%), forested (34%) and urban (5%). The estimated 7Q-10 (minimum base flow for 7 days over a 10 year period) for Conococheague Creek, using data from the nearest USGS gauging station, is 40.1 mgd [28]. The discharge flow of the WWTP (Figure 1B), that utilizes a modified Ludzack-Ettinger treatment process is 4.1 mgd or approximately 10% of the minimum base flow at the downstream site. Agricultural run-off is the primary anthropogenic input at the upstream site. A privately-owned structure, Kemps Mill Dam, is located immediately upstream of the WWTP outfall. This structure is considered to be a major impediment to upstream fish migration.

The Monocacy River (Figure 1A) is approximately 93 km in length and forms near the Maryland and Pennsylvania border. From there it flows south between Frederick and Carroll counties, through the City of Frederick and into the Potomac River. Approximately 60% of the Monocacy watershed is agricultural, 33% forested and 7% urban. Two WWTPs are suspected to influence the selected downstream Monocacy site (Figure 1B). The larger is the Frederick WWTP plant that utilizes the anaerobic-anoxic-aerobic treatment process and has an average daily flow capacity of 8.0 mgd. The Fort Detrick WWTP has a discharge of 1.0 mgd [29]. Fish collections for this site occurred from the outflow of the Frederick City WWTP to approximately 1.5 km downstream. Although a 7Q-10 is not available for the Monocacy River at this site, a USGS gauging station (01643000) is located just upstream [30]. A conservative estimated contribution of 1.4 % volume of stream flow from these two WWTPs was determined by dividing their combined maximum discharge rates by the annual flow rate for 2005. Average annual flow was calculated using historical flow rates measured at this gauging station. In drought years, such as

2002, it is likely that the contribution to stream flow by these WWTPs may approximate 4% volume. The upper Monocacy River site is primarily influenced by agriculture similar to the upper Conococheague Creek site.

A site at the Blue Plains WWTP, Washington, DC (Figure 1A), was also selected for sampling because it is the largest plant in the Potomac River watershed and the world's largest advanced (tertiary) WWTP. It serves the District of Columbia, Montgomery and Prince Georges counties in Maryland and Fairfax and Loudon counties in Virginia and has a design capacity to treat 370 mgd [31]. The percent effluent during baseflow conditions could not be estimated as the area is in the tidal region of the Potomac River and no USGS stream gauges were located nearby.

Fish Collections

Bass were collected by boat or barge electrofishing between September 6 and September 14, 2005. Fish were collected at this time as it marks the seasonal onset of reproductive recrudescence, collections would have less impact on the population than during the spring when nest-building and spawning occurs, and the data could be compared to other field studies that included assessment of reproductive health of bass and were conducted in the fall [32-36]. Thirty mature bass (greater than 200 mm total length) were collected at each site in an attempt to obtain 10 males and 10 females. Smallmouth bass were collected at the sites on Conococheague Creek and the Monocacy River. Because no SMB were caught on the mainstem site of the Potomac near the Blue Plains plant, LMB were collected at this site (Figure 1). Largemouth bass were only captured downstream of the Blue Plains site. Fish collected at the downstream WWTPs and Blue Plains were collected within 1 km of the discharge. Upstream and downstream sites on the same river were separated by at least 15 km.

Fish were euthanized with a lethal dose of tricaine methane sulfonate (Finquel™, Argent, Redmond, WA), weighed on a portable scale to the nearest 0.1 gram, total length measured to the nearest mm. Gonads and livers were weighed on an analytical balance with a calibrated tolerance of 0.01 g. Blood was drawn from the caudal vein with a heparinized syringe, transferred to vacutainers containing 62U sodium heparin (Fisher Scientific, Pittsburgh, PA), and stored on wet ice. Blood was centrifuged for 10 min at 1000 X g and 4°C to hasten plasma separation within 3

hours of collection. Plasma was removed, aliquoted into cryovials and stored at -80^{o}C until assayed for vitellogenin and reproductive hormones.

A necropsy-based assessment similar to that described by Schmitt et al. [37] accompanied the sample collection. Grossly visible lesions and abnormalities were recorded. Gonads were removed and weighed for gonadosomatic index (GSI) determination as follows:

[Gonad weight/ (body weight – gonad weight)] X 100

Gonads were fixed in Z-fix™ (Anatech LTD, Battle Creek, MI) for histological evaluation. Otoliths were removed from the SMB and used for age determination [38].

Histopathological and Biochemical Procedures

Fixed gonads were dehydrated in alcohol, infiltrated with paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) [39]. Gonad sections were examined microscopically, staged and any abnormalities as described in Blazer [22] ranked from 0-4 (absent to severe). Individual oocyte were staged as: stage 1 - immature (nucleolar); stage 2 - early vitellogenic (corticol alveolar); stage 3 - mid-vitellogenic (yolk droplet); stage 4 - mature (yolk hydrates) and stage 5 - postovulatory follicles. The stage for an ovary was based on the most prevalent oocyte stage present. At least 5 sections along the ovary were examined. The percent of atretic eggs was determined by counting 200 oocytes and calculating the percent of degenerating or necrotic oocytes. At least 5 pieces along the length of the testes were sectioned and testicular oocyte prevalence and severity scored as described by Blazer et al. [1]. Male gonad stage was scored as: stage 1 - predominantly spermatogonia or spermatocytes; stage 2 - approximately equal portions of spermatocytes, spermatids and spermatozoa; stage 3 - primarily spermatozoa; stage 4 - post-spawn.

Plasma Vtg concentrations were measured using a direct enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody 3G2 and were carried out at the University of Florida, Center for Human and Environmental Toxicology as described by Denslow et al. [40]. Concentrations of the unknowns were determined from the standard curves and using the Softmax Pro TM

Program (Molecular Devices, Sunnyvale, CA). The limit of detection was 0.001 mg/ml. Inter and intra-assay variability are <10%.

Plasma hormone concentrations, 17β-estradiol (E2) and testosterone (T) were measured using radioimmunoassay according to Sower and Schreck [41]. Briefly, plasma samples were extracted twice in a ten-fold excess of diethyl ether, blown to dryness and solubilized in 200 µl of room temperature Dulbecco's phosphate buffered saline (Sigm-Aldrich, St. Louis, MO) containing 0.1% knox gelatin. One hundred microliters of anti-estradiol antiserum (anti-17β-estradiol Ab - #244 anti-estradiol-6-BSA) purchased from the lab of Gordon Niswender, Colorado State University, Fort Collins, CO, and diluted 1:65000 in PG buffer or anti-testosterone antiserum (R156/7, purchased from Coralie Munroe, University of California, Davis, CA) and diluted 1:30000 in PG buffer was added to each sample tube, vortexed and incubated at room temperature for 30 min. The same volume of PG buffer was added to tubes designated to determine non-specific background and total counts per minute (CPM). Following incubation, 100 µl of tritiated 17β-estradiol or testosterone (5000 CPM in PG buffer) was added to all tubes, vortexed and incubated at room temperature for 60 or 30 min, respectively. Samples were immediately cooled in an ice bath for 30 min and 500 µl of ice-cold charcoal-dextran solution (0.63% alkaline charcoal and 0.4% dextran in PG buffer) was added. Samples were vortexed, incubated on ice for 15 min and centrifuged at 2200 X G for 20 min at 4^{o}C. The supernatant was then decanted into scintillation vials containing 4 ml of OptiPhase HiSafe 2 scintillation fluid (Perkin Elmer, Waltham, MA) and mixed by inversion. Sample CPM were measured using a Tri Carb Liquid Scintillation Counter (Perkin Elmer) and mean sample CPM was determined over an eight min integration time. All samples were run in duplicate and plasma steroid values were interpolated from a standard curve using curve fitting algorithms in Prism for Windows 4.03 (GraphPad Software, San Diego, CA). Standard curves consisted of 9 dilutions of 17β-estradiol (Cat # E0950-000; Steraloids Inc., Newport, RI) or testosterone (Cat # A6950-000; Steraloids Inc.). Sample values were rejected and re-evaluated if the coefficient of variation between duplicate tubes exceeded 10%.

Data Analysis

Prior to comparative statistical analyses all data were tested for normality using the Shapiro-Wilks W test and homogeneity of variance with Levene's test. Given that all data did not conform to the assumptions of parametric statistics, the conservative, non-parametric Kruskal-Wallis test was selected. Data from the Blue Plains site were not included in statistical analyses because LMB rather than SMB were collected. Differences in the prevalence of male bass with intersex and fish with measurable vitellogenin were analyzed using Fisher's Exact Test. All statistical analyses were performed using SyStat 11 for Windows (SyStat Software Inc., San Jose, CA). Differences were considered significant when $P \leq 0.05$.

RESULTS

Morphometric and Fish Health Indicators

At both Conococheague sites, an equal number of male and female SMB were collected. Only seven males were obtained at the Monocacy lower site despite sampling 30 fish. At the upstream site one male fish was misidentified as a female in the field and hence unequal numbers were sampled. At the mainstem site only seven LMB females were collected (Tables 2 and 3).

Female SMB collected at the upstream Conococheague site were larger ($P = 0.008$), but not statistically older than those collected downstream. Female bass collected at the upstream and downstream sites on the Monocacy were similar in size, age and condition factor (Table 2). The LMB collected at the Blue Plains (mainstem of the Potomac River) site were larger than the SMB collected from the Conococheague or Monocacy Rivers; unfortunately they were not aged.

Male SMB collected upstream on the Conococheague were heavier, with a significantly higher median condition factor ($P = 0.009$) at approximately the same age as those collected downstream. On the Monocacy, the males downstream were similar in size (length and weight), had a similar condition factor, but were significantly older than those collected at the upstream site (Table 3).

Reproductive Indicators

All fish collected were sexually mature. The median GSI of female SMB collected at the upstream Conococheague site was approximately double and significantly higher ($P = 0.005$) than that of females from the downstream site (Table 4). Vitellogenin ranged from not detected to 3.378 mg/ml upstream and not detected to 0.802 mg/ml downstream and was detected in 8 of 10 (80%) females at each site. The median concentration of Vtg from downstream females was significantly lower ($P = 0.003$) than that of fish collected upstream (Table 4). This coincided with 90% of the female gonads collected at the upstream site being at stage 2, while only 60% of the downstream gonads were at stage 2 (Figure 2A). The remaining 10% and 40% ovaries respectively, contained oocytes at stage 1 (Figure 2B). Atretic follicles (Figure 2C) were present in females from both collection sites. The average percent atretic follicles per ovary was 8.6% (sd = 4.9) and 4.2% (sd = 4.1) for females collected at the up- and downstream sites, respectively.

On the Monocacy, the median GSI of the upstream and downstream female SMB was similar. Only 56% of the female SMB collected upstream and 77% collected downstream had measurable vitellogenin, and there was no significant difference in concentrations between sites. However, those collected downstream had only 32% (21% if using the mean of only those with measurable levels) the amount of those collected upstream. Ovaries with stage 2 oocytes were observed in 77% and 85% of the females collected at the upstream and downstream sites, respectively. The average percent atretic follicles per ovary was 3.8% (sd = 4.2) and 6.0 (sd = 5.4) for females collected at the up- and downstream sites, respectively.

At the mainstem site the mean GSI of the female LMB was the lowest observed (0.43) and while 100% had measurable vitellogenin the mean concentration was lower than that measured in SMB at the two upstream sites (Table 4). All female gonads collected at this site were stage 1.

Male SMB collected at the upstream site on the Conococheague had approximately triple the GSI as those collected downstream. Sixty percent of the upstream male bass had measurable vitellogenin (ranging from not detected (ND) to 0.337 mg/ml) while 90% of the male downstream SMB had circulating vitellogenin (ranging from ND to 0.306). All testes (100%)

were at stage 2 at the upstream site, while only 60% male gonads were at this stage downstream. The remaining 40% were stage 1. There was no significant difference in the mean concentration of plasma vitellogenin, however the mean concentration in the upstream bass was more than double that of those collected downstream (Table 5). There no statistical difference in the number of males with detectable vitellogenin (P=0.303). A high percentage of SMB with testicular oocytes (Figure 2D, Table 2) was observed at both upstream and downstream sites on the Conococheague (100% and 90% respectively).

On the Monocacy, the median GSI between males up- and down stream were similar (Table 5). Male gonads from the upper Monocacy were predominantly stage 1 (55%), with the remaining 45% at stage 2. While testes from the lower site were mostly stage 2 (75%) with 25% at stage 1. Vitellogenin was observed in 45% of male SMB collected upstream (ranged from ND to 0.081 mg/ml) and 33% of those collected downstream (ranged from ND to 0.278) and 80% and 100%, respectively, had testicular oocytes. Eighty-five percent of the LMB collected at the Blue Plains site had measurable vitellogenin and the mean concentration was the highest measured, while in only 23% was the presence of testicular oocytes noted (Table 5).

Median plasma estradiol concentrations of the SMB were significantly lower in the male fish at both sites when compared to the females. On the Conococheague the downstream males had approximately double the median plasma estradiol of those upstream, while on the Monocacy upstream and downstream levels were similar. Females on the Conococheague were similar upstream and downstream, while on the Monocacy bass from the downstream site had higher concentrations compared to the upstream females. Median male and female estradiol concentrations were similar in the LMB from Blue Plains. (Figure 3).

Median plasma testosterone concentrations of LMB were similar between males and females. On the Conococheague, the upstream males had a lower mean concentration than those downstream, as did the females. On the Monocacy, the median concentrations of the upstream and downstream males were similar. The upstream females had a lower concentration than the downstream females (Figure 4). These differences were not statistically different, however.

The median E/T ratio of the female SMB were all similar and approximately 1, however the median E/T ratio of the female LMB was lower at 0.73. Male SMB on both rivers had a higher median E/T ratios downstream than upstream. The SMB males at the downstream sites had a similar E/T ratio to that of the male LMB (Table 5).

DISCUSSION

Abnormal reproductive physiology resulting from unavoidable exposure to wastewater effluent has been documented in wild fish populations for over a decade [2, 6, 42-44]. Although WWTP effluent contains a milieu of biologically active substances, those with estrogenic potential have garnered most attention from the scientific community [45, 46]. Numerous plasma-associated physiological measures (i.e. vitellogenin and steroid hormones) have been utilized as indicators of exposure to estrogenic chemicals; however, the presence of intersex (principally testicular oocytes in males) continues to rival these measures as it appears to be a less transient indicator of estrogenic exposure. Experimental evidence purports that intersex may be induced early in development, during critical stages of sexual differentiation in gonochoristic fishes. Certainly there are exceptions. That same research demonstrates that intersex is not induced by similar estrogenic stimuli at later life-stages in some species [47]. Interestingly, exposure to estrogenic stimuli later in life may exacerbate the severity of TO in instances when primary exposure occurred at the critical stages of sexual development [43]. In the present study, estrogenic endocrine disruption, evidenced by the presence of TO, was observed at all study sites. The incidence of testicular oocytes in male SMB collected from Conococheague Creek and the Monocacy River exceeded 80% which is higher than that generally observed in SMB from the South Branch of the Potomac, but similar to that observed in SMB from the Shenandoah River [1]. While it is unknown when intersex is induced in male smallmouth bass from this watershed, but it is possible that it occurs during critical developmental stages. Male LMB collected at the Blue Plains site had a much lower prevalence of TO than SMB at the other samples sites. Considering that the Blue Plains site generally had higher levels of most contaminants (Table 6), this may indicate differences in sensitivity to estrogenic chemicals in these closely related species or differences in chemical concentrations in preferential spawning habitat for the two species. Unfortunately a “baseline” occurrence of TO has not been determined for either large-

or smallmouth bass. Currently, the best data for SMB is from "out of basin" sites with both low human population and low to moderate agricultural land-use, at which 14-22% of male SMB were observed to have TO [1]. From 1995-2004 the U.S. Geological Survey conducted fish health assessments, including reproductive endpoints, at large river sites in the Mississippi [32], Rio Grand [33], Columbia [34], Colorado [35] and Savannah, Pee Dee, Mobile, Apalachicola-Chattahoochee-Flint [36] river basins. In these studies LMB and common carp (*Cyprinus carpio*) were the target species. At a total of 55 sites, LMB males were collected and at 32 of these sites (58%) no TO were reported. Hence, suggesting the baseline prevalence for LMB may be zero. It is interesting that smallmouth bass appear to have a fairly high baseline prevalence of this condition. Unfortunately, there is relatively little information regarding sexual development and sex determination in this species. For this reason at the present time it is not possible rule out factors other than or in addition to contaminant exposure as contributing factors to this condition.

The induction of Vtg in male fish is perhaps the most frequently applied biomarker of estrogenic exposure, particularly near WWTP discharges [48-51]. Hepatic synthesis of this glycolipoprotein is estrogen dependent. It is produced in females and males alike given the appropriate stimulus; however, under normal physiological conditions males do not produce Vtg. To this effect, Vtg has no known biological function in males, although antimicrobial actions have been suggested [52]. Unlike induction of TO, the induction of Vtg in male fish is rapid and the protein is detectable in the plasma within days post-exposure. Consequently, the presence of plasma Vtg is more indicative of recent and/or continued exposure to estrogenic compounds than TO. In the present study, plasma Vtg in male SMB ranged from undetectable to 0.337 mg/ml. When considering males with measurable Vtg, mean concentrations of this protein were highest at the upstream Conococheague and downstream Monocacy sites. These concentrations were considerably lower than those measured in male LMB at the Blue Plains site (Table 5). The significance of elevated concentrations of Vtg in these species is unknown, but its presence serves as a useful indication that estrogenic chemicals are present at biologically relevant concentrations. Recently it has been shown that adverse effects are observed in male fathead minnows with Vtg concentrations above 0.5 mg/mL, but not below this concentration [53]. Only five male LMB from our study had concentrations exceeding this threshold, and four (80%) of these had levels exceeding 1mg/ml. Most striking at this site was the observation that males had

comparable concentrations of Vtg to females. The detection of measurable Vtg in male SMB and LMB indicates that in addition to early life exposure to estrogens (suggested by TO) these fish are also likely are exposed to estrogenic chemicals as adults. The extent and frequency of this exposure is still unknown. Additionally, the kinetics of Vtg clearance in bass is unknown. This is of particular importance as male fish do not appear to have specific mechanisms to excrete Vtg [54]. Consequently, Vtg remains detectable in the plasma of male fish for weeks to months post exposure [53, 54]. Thus, in the current study, the presence of Vtg may be the result of exposure weeks to months prior to our sampling. The observed variability of plasma Vtg in male fish includes the possibility of recent immigration of fish from less impacted up-river areas or simply differences in the concentration of chemicals within the river based on flow dynamics. Unfortunately, published home range studies of SMB in the Potomac drainage are not available. However, home ranges of less than 200 m have been reported in rivers systems in Missouri [55]. Other studies have indicated that some populations/individuals are relatively sedentary while others are migratory and this may be affected by season [56]. While our upstream and downstream sites were separated by a much greater distance than the reported home range for some SMB populations, without specific regional data regarding bass movement we can not rule out the possibility of some migration between sites. This is less likely on the Conococheague due the dam immediately upstream of the WWTP outfall.

Species of the genus *Micropterus*, like most gonochoristic fishes, experience an annual reproductive cycle in which recrudescence initiates during the fall and spawning occurs in the spring. Specific dates of these events are largely governed by water temperature and photoperiod. Research conducted on Florida largemouth bass (*M. salmoides floridans*) maintained in tanks and ponds in Texas has established the baseline reproductive cycle for this (sub)species. Recrudescence is evident in October through December [57] and peak GSI occurs between late February and late April (immediate pre-spawn to spawn). The GSI declines rapidly post-spawn in May, and continues to decrease until reaching a minimum in September. A similar cycle has been documented for hatchery pond-raised LMB in Florida [58]. In both studies, the GSI was approximately 0.8 – 1.0 and 0.1 - 0.2 in females and males, respectively during September (water temperature ≈ 15^{o}C). In the current study the mean GSI was 0.4 for female and 0.06 for male LMB. This is considerably lower than the September values reported above.

However, this difference is likely the result of the warmer water temperature at the Blue Plains site. The onset of recrudescence at this site likely occurs later in the year when water temperature decreases. This would explain the low GSI and sex steroid values which were likely at base-line values during sampling. If this is the case, however, the presence of Vtg in both male and female LMB is likely the result of exposures to estrogens in the water rather than endogenous stimuli. Biological evidence of estrogenic substances in the water at this site is confirmed by results of the bioluminescence yeast estrogen screen results reported in Alvarez et al. [26]. Additionally, concentrations of plasma Vtg in male LMB are normally below 0.05 mg/ml at any given time during the year [58]. Of particular relevance to the current work, the presence of TO was not noted in either of the above studies.

Few studies have documented the normal changes in GSI, vitellogenin or reproductive hormones throughout the reproductive cycle of SMB. The GSI of naturalized lake SMB in Japan is lowest in August and begins to rise during September (water temperature approximately 20^{o}C) to 2.0 in females and 0.8 in males [59]. In the current study, fish collected from the downstream Conococheague had significantly lower GSI site when compared to the upstream Conococheague for both females and males (p=0.005 and p<0.001, respectively). These sites experience the same photoperiod and water temperatures are similar (Table 1). The GSI values at this site were also lower than those measured in fish from the Monocacy sites particularly in the case of males (Table 5). Since water temperature was similar among all sites, chemical exposure from site specific inputs is a likely explanation for the delayed onset of spermatogenic recrudescence. The GSI of females collected downstream of the WWTP on the Conococheague was significantly lower than that of females collected upstream is consistent with the lower plasma vitellogenin concentrations (Table 4). Again, this observation further corroborates differences in the timing of recrudescence at the lower Conococheague site compared to that upstream. Since vitellogenin production is related to fecundity and egg quality in individual female fish [60, 61], the lower concentrations in female bass collected downstream could adversely affect egg quality and subsequent survival of fry. However, since the bass were collected in the fall, early or at the on-set of recrudescence, further work is required to confidently document reproductive impairment and population-level effects.

Previous research has demonstrated that roach (*Rutilus rutilus*) exposed to sewage treatment plant effluent had an increased incidence of atresia, decreased estradiol in females, but increased estradiol and testosterone in males [49]. In the current study differences in steroid hormones were subtle, and significant differences were only observed in males from the Conococheague. Males downstream had significantly higher (p=0.004) plasma estradiol concentrations than those at the upstream site on the Conococheague. While concentrations of reproductive hormones are useful indicators of disruption of the reproductive cycle [24], these concentrations are often confounded by natural fluctuations and environmental factors such as water temperature influenced by rain and run-off events [25]. Concentrations of testosterone and estradiol in male SMB have previously been reported at 1 ng/ml or less at this time of year in Japan. In female these concentrations are higher, but below 2 ng/ml [59]. These are considerably higher than those measured at any site during the current study indicating that SMB in the Potomac watershed initiate recrudescence later in the year. This is likely due to geographical differences. To best interpret the significance of different hormone concentrations in these fish, additional multi-year, multi-season data are required to define the normal regional reproductive cycle of SMB.

While no individual chemicals were identified as the singular cause of testicular oocytes and vitellogenin induction in male bass, a number of EDCs were measured all sites (Table 6). Passive samplers, both polar organic chemical integrative samplers (POCIS) and semipermeable membrane devices (SPMD), were deployed at the sites for approximately one month during the sampling period. More detailed information regarding the chemical data is available in Alvarez et al. [26]. Chemical profiles differed between the sample sites and may be partially explained by the type of treatment used at specific plants. This may have also contributed to biological differences observed between bass collected at the upstream and downstream sites in the Conococheague, but not the Monocacy. For instance, three wastewater-associated chemicals commonly used in fragrances (celestolide, tonalide and galaxolide) were identified at the downstream site on the Conococheague. Only one of these three chemicals (tonalide) was found at the downstream site on the Monocacy. None were found at either upstream site. Similarly, tri (2-chloroethyl) phosphate, found in plasticizers and flame retardants, was not found at either upstream sites but was measured at both downstream sites although at a lower concentration on the Monocacy (Table 6). In a study of three WWTPs, Thomas and Foster [62] found the majority

of acidic pharmaceuticals, caffeine and the antimicrobial triclosan were removed during secondary treatment. The difference in the secondary treatment processes (modified Ludzack-Ettinger; MLE versus anaerobic-anoxic-aerobic; A_2O) at the two WWTPs in this study may explain some of the observed differences. The Conococheague plant uses a the MLE process which utilizes activated sludge consisting of anoxic and oxic phases for biological nutrient reduction, while the Frederick WWTP recently upgraded to the A_2O activated sludge process that includes an anaerobic phase as well as the anoxic and oxic cycles [63]. Additional pharmaceuticals may be removed during this phase. In addition, factors such as overall removal efficiencies, operational practices, the nature and concentration of the wastewater influent and the percent of river base flow contributed by effluent. Estimated average annual contribution of WWTPs to flow in the Conococheague, and Monocacy was approximately 1.1 and 1.4 %. While these are annual averages and times exist when WWTP contribution is higher, in general the percent contribution is fairly low compared to other parts of the country and world where intersex has been identified. The fact that female sex steroids were at or below the MDL (see Alvarez et al. [26], and WWTP contribution to river flow is low, suggests that other or additional factors are in part responsible for the disrupted reproductive parameters.

Hexachlorobenzene (HCB), a chemical shown to alter sex hormone concentrations in crucian carp (*Carassius auratus gibelio*) [64], was present in the Conococheague, but not the Monocacy. This chemical was historically used as a pesticide to protect the seeds of onions and sorghum, wheat, and other grains against fungus through the early 1960's. Currently, there are no commercial uses of HCB in the United States, but it is formed as a byproduct during the manufacture of other solvents and some pesticides [65]. While the presence of HCB is a clear difference between the two rivers, its presence does not explain biological differences in SMB at the up- and downstream sites in the Conococheague. In general concentrations of chemical compounds were found at higher or similar concentrations at downstream sites compared to those upstream. The most notable exception was atrazine and its metabolites which were generally higher at upstream sites where agricultural input was greatest. Atrazine is a chlorotriazine herbicide used to control annual grasses and broadleaf weeds prominently applied where corn is grown. It is associated with intersex and reproductive anomalies in anurans [66]. The exposure of adult fishes to atrazine resulted in suppressive effects on plasma androgens,

induction of estrogen (dose and time-related) in goldfish [13] but no strong estrogenic effects or overt reproductive toxicity in fathead minnows [67]. To our knowledge exposure studies during critical periods of sexual differentiation have not been conducted and it is unknown if atrazine could induce intersex in SMB. However, the widespread distribution of this herbicide within the Chesapeake watershed [68], particularly the concentrations measured during spring at the current sites [26] may explain the high prevalence of intersex in the Potomac watershed and should be studied in more depth.

Although natural and synthetic estrogens are most commonly associated with the induction of testicular oocytes [2, 3, 4, 47] and vitellogenin in male fishes [23, 40, 61], no steroid hormones were detected in the passive extracts in this study. However, it is important to note that the detection limits for 17β-estradiol and 17α-ethynylestradiol were 2.5 ng/L [26]. Concentrations as low as 1 ng/L 17α-ethynylestradiol have been shown to induce intersex [69]. A dose-dependent increase in vitellogenin was induced in juvenile zebrafish (*Danio rerio*) exposed to 2 ng/L 17α-ethynylestradiol and above [70] and levels as low as 0.1 ng/L induce vitellogenin synthesis in immature rainbow trout [42]. Thus the fact that sex steroids were not identified does not imply their absence at biologically relevant concentrations. In fact, estrogenic activity was identified in POCIS samples from all sites using a yeast reporter assay [26]. In short, these findings emphasize the importance of examining both biological and chemical endpoints in field studies, and consideration of the potential biological effects that chronic or intermittent exposure to complex chemical mixtures may influence in aquatic organisms.

Based on the physiological and morphological measures, potential adverse effects of WWTP effluent were observed, particularly downstream of the Conococheague WWTP. These included decreased GSI, decreased circulating vitellogenin in female bass, and altered reproductive hormone concentrations. Vitellogenin in male bass and a high prevalence of TO were noted at both upstream and downstream sites indicating that other sources of endocrine-modulating chemicals such as agricultural, suburban and urban runoff may also influence these populations. In conclusion, while it appears that effluent from some WWTPs may impact reproductive health of fishes in the Potomac watershed, inputs from other sources likely contribute to the widespread, high prevalence of testicular oocytes.

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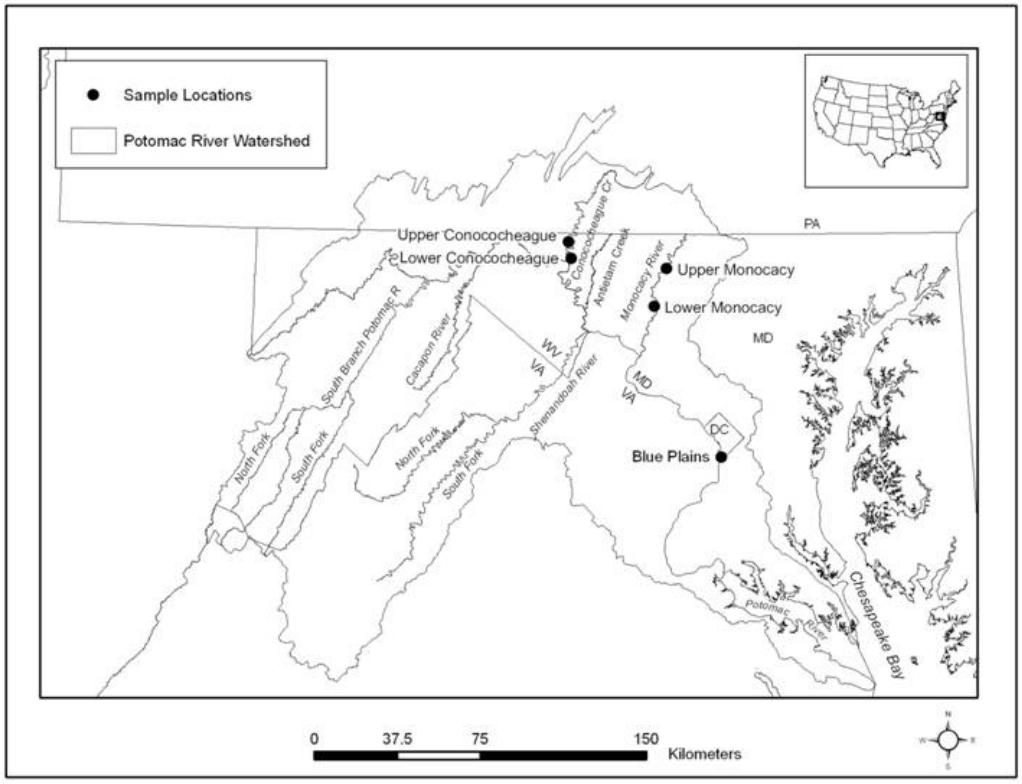
Figure I-1. A. Collection site locations for smallmouth and largemouth bass within the Potomac drainage. B. Sample locations in special reference to wastewater treatment plants.



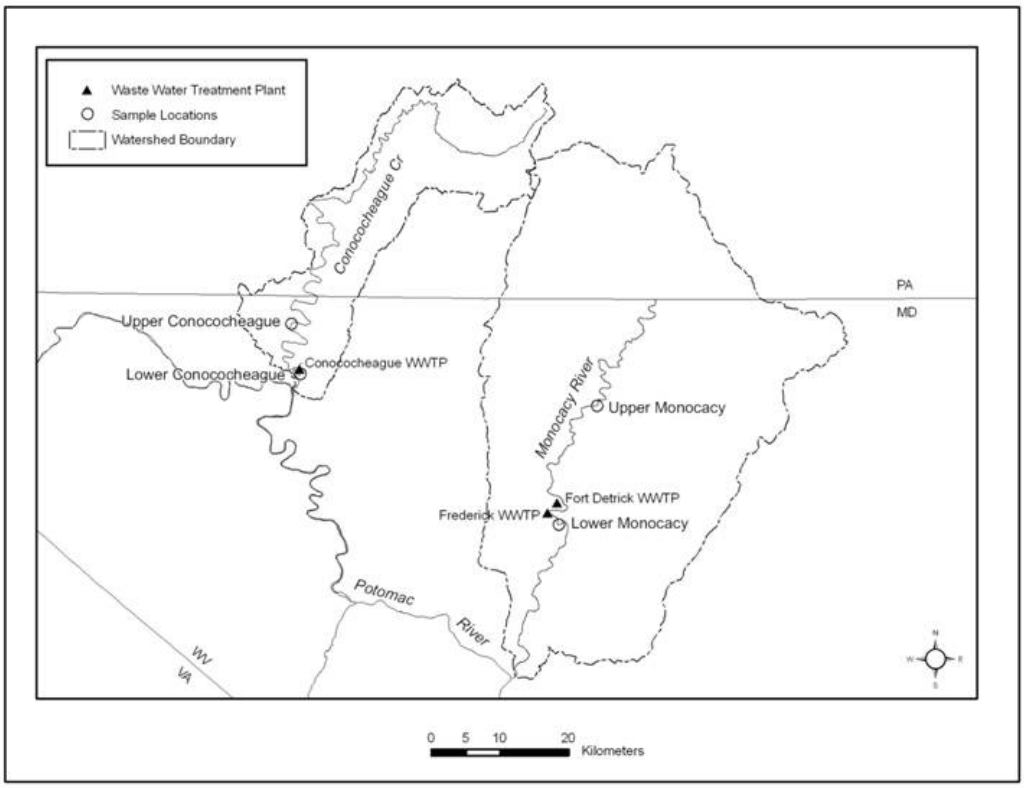


Figure I-2. Microscopic observations in bass gonads. A. Stage 1 ovary with oocytes that have only progressed to the perinucleolar stage (arrows). Scale bar = 200 µm. B. Stage 2 ovary containing oocytes that have progressed to the cortical alveolar stage (a). Scale bar = 200 µm. C. Atretic oocytes (a) within a stage 2 ovary. Scale bar = 200 µm. D. Oocytes (arrows) within the testis of a bass. Scale bar = 100 µm. H &E stain.

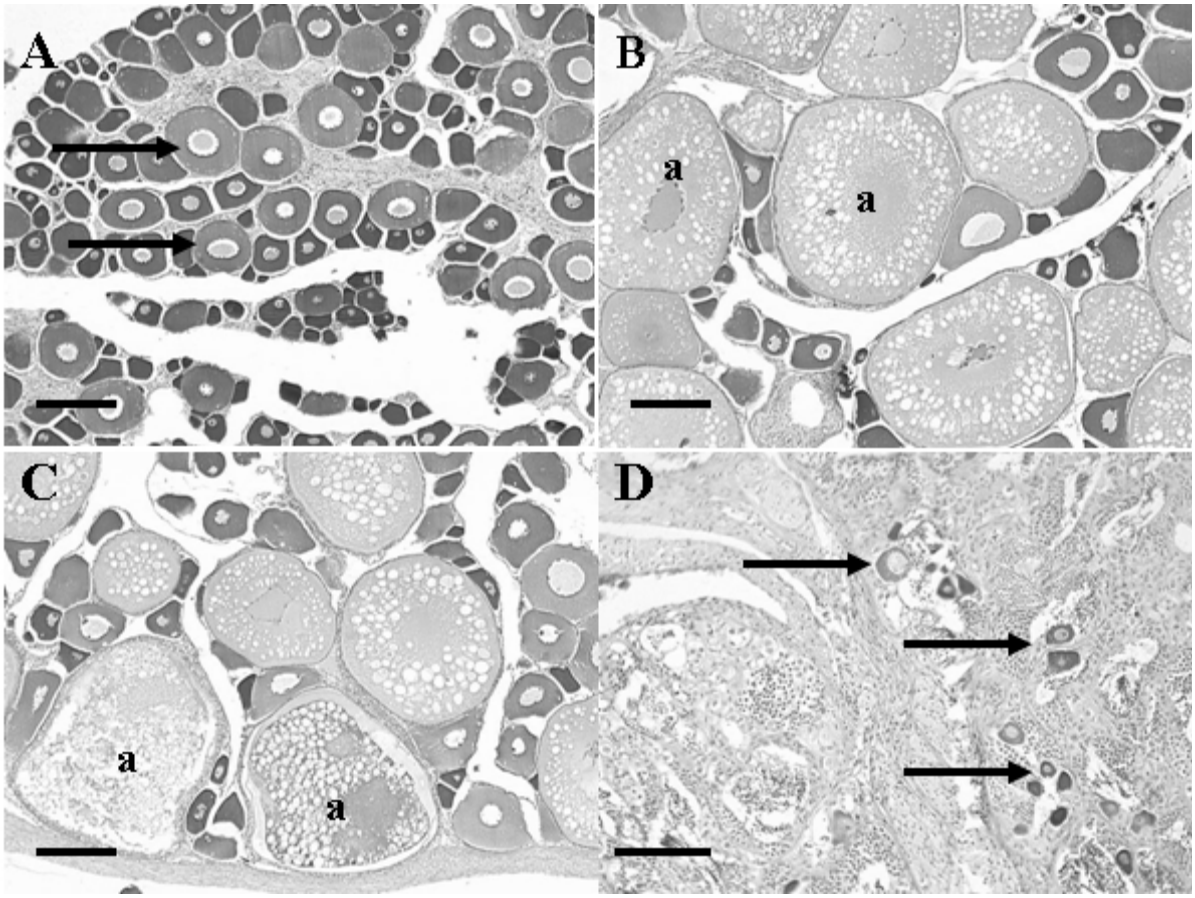


Figure I-3. Plasma 17β-estradiol concentrations (pg/ml) of smallmouth and largemouth bass. Upstream/downstream pairs marked with an asterisk are significantly different. Dots represent outliers and the whiskers mark 5th and 95th percentiles.

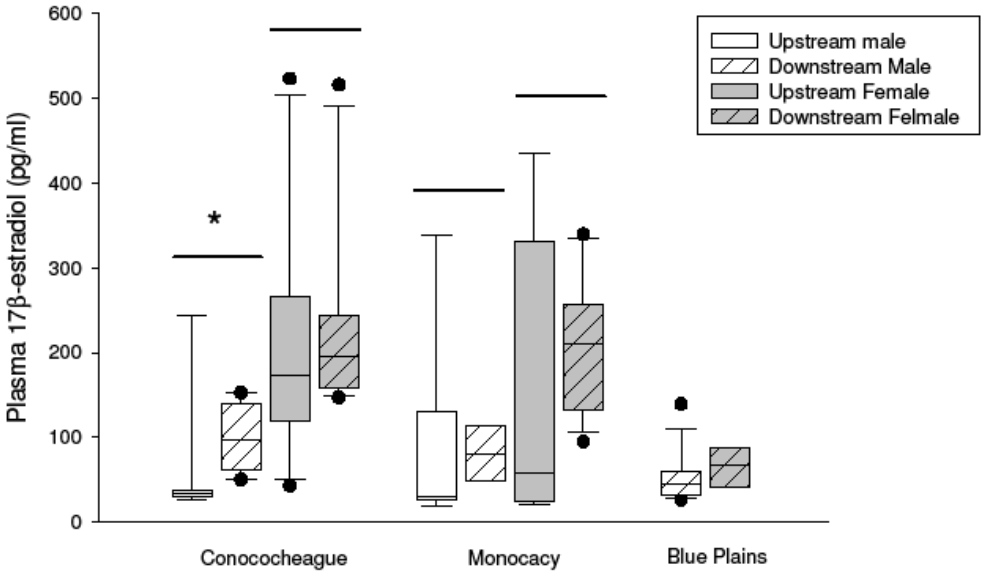


Figure I-4. Plasma testosterone concentrations (pg/ml) in smallmouth and largemouth bass captured in the vicinity of wastewater treatment facilities. Dots represent outliers and the whiskers mark 5th and 95th percentiles.

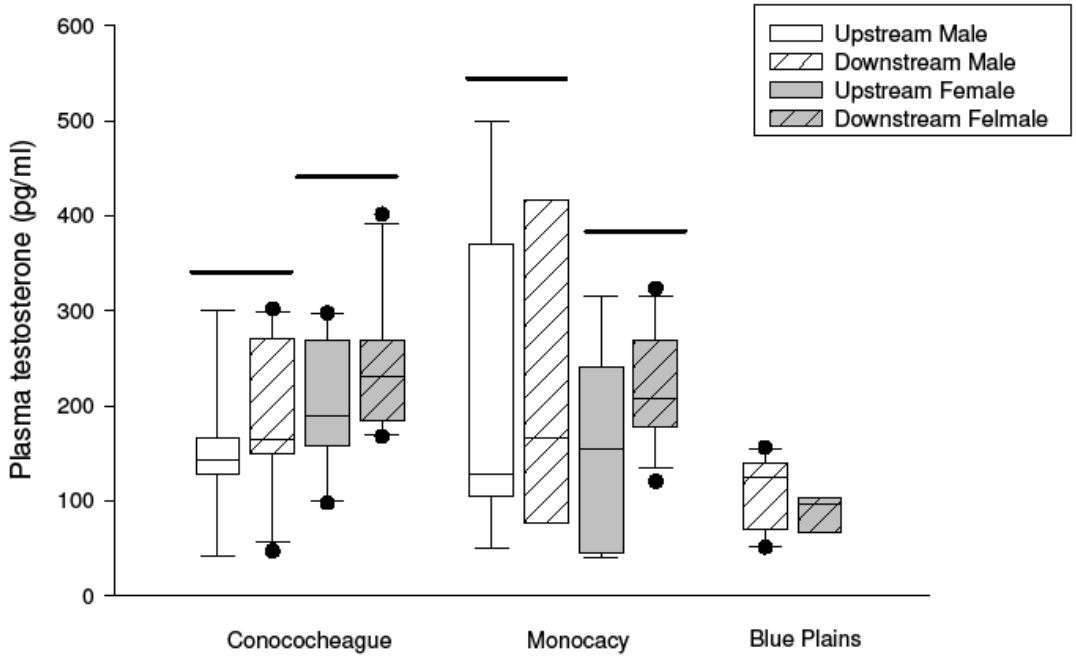


Table I-1. Water quality parameters at collection sites within the Potomac drainage measured at the time of fish collections.

Site	Water Quality Parameters				
	Temperature ^{0}C	Conductivity mS/cm	Dissolved Oxygen mg/L	% Dissolved Oxygen Saturation	pH
Conococheague Upstream	21.1	0.511	7.1	77.4	6.5
Conococheague Downstream	21.7	0.441	9.0	101.4	7.3
Monocacy Upstream	23.1	0.365	10.9	124.2	7.0
Monocacy Downstream	21.8	0.487	10.5	117.2	7.0
Blue Plains	25.8	0.615	6.8	100.0	6.8

Table I-2. Morphometric results for female bass from collection sites within the Potomac drainage.

Site	n	Length (mm)	Weight (gm)	Age	Condition Factor
Conococheague Upstream	10	321 ± 17.3[A]	484.3 ± 87.0[A]	3.6 ± 0.5[A]	1.35 ± 0.03[A]
Conococheague Downstream	10	261 ± 8.8[B]	233.2 ± 27.0[B]	3.1 ± 0.2[A]	1.27 ± 0.03[A]
Monocacy Upstream	9	271 ± 18.8[a]	284.2 ± 61.8[a]	2.6 ± 0.4[a]	1.26 ± 0.03[a]
Monocacy Downstream	13	269 ± 14.2[a]	280.0 ± 51.0[a]	2.7 ± 0.4[a]	1.27 ± 0.04[a]
Blue Plains	7	370 ± 21.1	831.4 ± 180.5	ND[1]	1.48 ± 0.01

[a]Data are presented as means ± S.E. and values followed by different letters indicate the upstream and downstream sites are significantly different (p <0.05) by the Kruskal-Wallis test.
[1]No data available.

Table I-3. Morphometric results of male bass from collection sites within the Potomac drainage.

Site	n	Length (cm)	Weight (gm)	Age	Condition Factor
Conococheague Upstream	10	292 ± 12.7[A]	342.7 ± 40.2[A]	2.9 ± 0.3[A]	1.33 ± 0.03[A]
Conococheague Downstream	10	255 ± 10.4[A]	207.7 ± 24.1[B]	2.6 ± 0.4[A]	1.21 ± 0.03[B]
Monocacy Upstream	11	245 ± 11.3[a]	200.1 ± 32.0[a]	1.7 ± 0.2[a]	1.27 ± 0.02[a]
Monocacy Downstream	7	264 ± 10.6[a]	247.2 ± 28.2[a]	2.6 ± 0.4[b]	1.22 ± 0.03[a]
Blue Plains	13	353 ± 10.1	743.4 ± 87.3	ND[1]	1.61 ± 0.05

[a]Data are presented as means ± S.E. and values followed by different letters indicate the upstream and downstream sites are significantly different (p <0.05) by the Kruskal-Wallis test.

[1]No data available.

Table I-4. Gonadosomatic indices (GSI), plasma vitellogenin and E/T (estrogen/testosterone) ratio of female bass from collection sites within the Potomac drainage.

Site	GSI	% with Vitellogenin	Plasma Vitellogenin mg/ml*	E/T ratio
Conococheague Upstream	1.26 ± 0.35^{A}	$80\%^{A}$	1.247 ± 0.485^{A}	0.98 ± 0.14^{A}
Conococheague Downstream	0.63 ± 0.06^{B}	$80\%^{A}$	0.119 ± 0.105^{B}	0.91 ± 0.05^{A}
Monocacy Upstream	0.94 ± 0.11^{a}	$45\%^{a}$	1.885 ± 1.014^{a}	0.89 ± 0.17^{a}
Monocacy Downstream	0.82 ± 0.09^{a}	$77\%^{a}$	0.395 ± 0.118^{a}	0.95 ± 0.07^{a}
Blue Plains	0.43 ± 0.05	100%	0.465 ± 0.251	0.76 ± 0.09

[a]Data are presented as means ± S.E. and values followed by different letters indicate the upstream and downstream sites are significantly different ($p < 0.05$) by the Kruskal-Wallis or Fisher's Exact test.

*Mean only of those fish with a measurable amount of vitellogenin.

Table I-5. Gonadosomatic indices (GSI), testicular oocytes (TO), plasma vitellogenin and estrogen/testosterone (E/T) ratio of male bass from collection sites within the Potomac drainage.

Site	GSI	Prevalence TO	TO Severity Index	% with Vitellogenin	Plasma Vitellogenin mg/ml*	E/T ratio
Conococheague Upstream	0.39 ± 0.06^{A}	$100\%^{A}$	2.1 ± 0.3^{A}	$60\%^{A}$	0.117 ± 0.051^{A}	0.37 ± 0.10^{A}
Conococheague Downstream	0.13 ± 0.06^{B}	$90\%^{A}$	1.8 ± 0.4^{A}	$90\%^{A}$	0.050 ± 0.036^{B}	0.58 ± 0.08^{B}
Monocacy Upstream	0.30 ± 0.04^{a}	$82\%^{a}$	1.2 ± 0.3^{a}	$45\%^{a}$	0.059 ± 0.010^{a}	0.46 ± 0.13^{a}
Monocacy Downstream	0.30 ± 0.15^{a}	$100\%^{a}$	1.9 ± 0.3^{a}	$33\%^{a}$	0.143 ± 0.085^{a}	0.51 ± 0.15^{a}
Blue Plains	0.06 ± 0.003	23%	0.2 ± 0.2	85%	0.577 ± 0.165	0.51 ± 0.07^{a}

[a]Data are presented as means ± S.E. and values followed by different letters indicate the upstream and downstream sites are significantly different ($p < 0.05$) by the Kruskal-Wallis or Fisher's Exact test.

* Mean only of those fish with a measurable amount of vitellogenin.

Table I-6. Selected concentrations of chemicals that measured above the method quantitation limit (MQL) in passive sampler extracts (more detail available in Alvarez et al. this issue).

Chemical Estimated pg/L	Conococheague Upstream	Conococheague Downstream	Monocacy Upstream	Monocacy Downstream	Potomac Blue Plains
Total PCBs	66	215	<MQL	410	2,550
Total PAHs	4,136	4,114	3,921	16,790	21,825
Total DDTs[1]	94	144	<MQL	655	355
Hexachlorobenzene	83	58	<MQL	<MQL	59
Pentachloranisole	56	225	110	190	310
Lindane	<MQL	620	<MQL	550	<MQL
Total Benzenehexachloride[2]	89	404	<MQL	93	218
Chlorpyrifos	<MQL	115	<MQL	48	475
Total Chlordane[3]	43	136	35	95	620
Dieldrin	175	295	104	195	550
Heptachlor epoxide	<MQL	165	<MQL	150	405
Endosulfan + endosulfan II	630	1,470	830	1,330	6,050
Metolachlor	730	1,115	12,000	10,750	1,850
Atrazine[4]	90,000	46,850	88,000	44,000	30,900
Wastewater-related chemicals Presented as ng/Polar Organic Chemical Integrative Sampler					
Celestolide	<MDL[a]	130	<MDL	<MDL	130
Tonalide	<MDL	120	<MDL	<MDL	515
Galaxolide	<MDL	335	<MDL	210	955
Prometon	100	115	100	<MDL	145
Tri(2-chloroethyl) phosphate	<MQL	165	<MQL	100	360

[a]MDL is the method detection limit.
[1]Sum of *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDD, *o,p'*-DDT, *p,p'*-DDD, *p,p'*-DDT concentrations.
[2]Sum of α-benzenehexachloride, β-benzenehexachloride, δ-benzenehexachloride.
[3]Sum of oxychlordane, *trans*-chlordane, *cis*-chlordane.
[4]Sum of desisoprpylatrazine, desethylatrazine, atrazine

CHAPTER II: Reproductive Health of Bass in the Potomac, USA Drainage: Part 2. Seasonal Occurrence of Persistent and Emergent Organic Contaminants

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ABSTRACT

The seasonal occurrence of organic contaminants, many of which are potential endocrine disruptors, entering the Potomac River watershed was investigated using a two-pronged approach during the fall of 2005 and spring of 2006. The following work describes the measurement of select chemical contaminants at sites potentially impacted by agricultural runoff and wastewater effluent. Passive sampling devices, the semipermeable membrane device (SPMD) and polar organic chemical integrative sampler (POCIS), were deployed in tandem at sites above and below wastewater treatment plant discharges within the watershed. Analysis of the samplers resulted in the detection of 84 out of 138 targeted chemicals. The agricultural pesticides, atrazine and metolachlor, had the greatest seasonal changes in water concentrations with a 3.1 to 91-fold increase in the spring than in the previous fall. Coinciding with the elevated concentrations of atrazine in the spring were increasing concentrations of the atrazine degradation products, desethylatrazine and desisopropylatrazine, in the fall following spring and summer application of the parent compound. Other targeted chemicals (organochlorine pesticides, polycyclic aromatic hydrocarbons, and organic wastewater chemicals) did not indicate seasonal changes in occurrence or concentration; however, the overall concentrations and number of chemicals present were greater at the sites downstream of wastewater treatment plant discharges. Several fragrances and flame retardants were identified in these downstream sites which are characteristic of wastewater effluent and human activities. The bioluminescent yeast estrogen screen (BLYES) *in vitro* assay of the POCIS extracts indicated that there were chemicals capable of producing an estrogenic response at all sampling sites.

Keywords: Potomac River, SPMD, POCIS, emerging contaminants, wastewater

INTRODUCTION

The Potomac River watershed is an important spawning and nursery ground for both migratory and resident fish species. Recent studies on fish health in the Potomac watershed have found sites with alarming numbers of the fish exhibiting external lesions and incidences of intersex, specifically testicular oocytes, in male smallmouth bass (*Micropterus dolomieu*) from areas receiving surface runoff and direct inputs from agricultural, industrial, and other human activities [1-2].

Throughout the Potomac River watershed multiple point and non-point sources exist consisting of largely rural communities and agriculture in the upper regions and industry and municipal wastewater treatment plant (WWTP) discharges in the lower regions [3]. According to the Maryland Department of the Environment, of the 747 permitted surface water discharges within the Maryland portion of the Potomac River watershed, 117 are WWTPs. WWTPs are widely recognized as a source of endocrine-disrupting compounds (EDCs) which cover a wide range of chemical classes including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, phthalates, alkylphenol surfactants, heavy metals, and natural and synthetic hormones [4-6].

Common practices of taking a discrete, "grab" sample of 1-2 L of water for chemical analysis are often insufficient at providing information on the trace, but potentially toxicologically significant, concentrations of anthropogenic organic contaminants. Passive samplers extract contaminants from volumes of water, often tens to hundreds of liters over a typical 30 day deployment, much greater than possible with discrete samples allowing for chemical concentrations in the part-per-trillion to part-per-quintillion (ng L^{-1} to fg L^{-1}) range to be detected. Discrete water samples only represent conditions present at the instant of sampling and as such can miss episodic events (i.e., spills, surface runoff, and meteorological events). Repetitive sampling schemes which would be necessary to detect episodic changes in chemical concentrations can be logistically challenging and expensive, particularly in remote locations or

areas which experience frequent hydrological changes. Passive samplers provide data as a time-weighted average concentration over the deployment period (weeks to months) which are a fundamental part of the ecological risk assessment processes for chemical stressors.

Two of the most widely used and studied passive samplers are the semipermeable membrane device (SPMD) and the polar organic chemical integrative sampler (POCIS). SPMDs consist of a nonporous layflat polyethylene membrane tube containing a neutral lipid (triolein). They are designed to mimic key aspects of the bioconcentration process, which results in elevated contaminant concentrations after exposure to trace hydrophobic organic contaminants in aquatic environments. Sampling of compounds with moderate to high octanol-water partition coefficients (K_{ow}s >3) is integrative (i.e., extracted residues are constantly accumulated without significant losses back into the environment) and analyte concentrations are reported as time weighted average values [7]. The POCIS is designed to mimic an organism's exposure to hydrophilic organic contaminants with low to moderate K_{ow}s (<3). The POCIS consists of a solid phase sorbent or mixture of sorbents contained between two sheets of a microporous polyethersulfone membrane. Sampling of compounds by the POCIS is integrative and analyte concentrations are reported as time weighted average values [8-9]. By using SPMDs and POCIS in concert, it is possible to monitor for large numbers of organic contaminants possessing a wide range of chemical and physical properties.

The versatility of passive sampling devices allows for not only chemical analyses to be performed, but contaminants they sample also can be coupled with *in vitro* reporter system assays. By doing so, the net biological effect of the complex mixtures captured by these devices can be quantified relative to a target standard [10-11]. In the instance of chemicals that may affect reproduction, a handful of assays have been developed that report the binding of chemicals to sex hormone receptors. Many of these assay platforms involve the utilization of estrogen sensitive mammalian cell lines that have been genetically modified to produce specific enzymes (which can then be quantified) following exposure to estrogen [12-15]. Although these assay platforms are sensitive, mammalian cells tend to be affected by the inherent toxicity of many chemicals and can be cumbersome to perform. Recently a bioluminescent yeast estrogen screen

(BLYES) has been developed that is sensitive (≈ 4 x 10^{-11} M) and less susceptible to toxic chemicals than mammalian cell reporter systems [16].

Here we complement the passive sampler technology with a series of chemical analyses and an estrogen reporter assay to assess the contaminant profiles of water receiving input from different land-use practices within the Potomac River watershed. The combination of the chemical analyses and *in vitro* assays along with physical observations on fish health and biological reproductive endpoints [2] will be used to help bridge the gap in understanding the potential causes of intersex and instances of endocrine disruption.

MATERIALS AND METHODS

Sampling Sites

The mainstem of the Potomac River and two of its tributaries, Conococheague Creek and the Monocacy River, which can receive a significant portion of their flow from the effluent of WWTPs, were selected based on their proximity to WWTP discharges and availability of largemouth and smallmouth bass for collection for biological measurements (Figure 1) [2]. In the fall of 2005, passive samplers were placed for 31 days during the months of September and October at two sites on the Monocacy River, two in the Conococheague Creek, and one site in the Potomac River at the Blue Plains WWTP outfall in Washington, DC. The Monocacy River and Conococheague Creek each had sites upstream (UP) and downstream (DS) of known WWTP discharges. In the spring of 2006, a second set of passive samplers were deployed for 49 days during April and May in the Monocacy River (DS), and in the Conococheague Creek (UP and DS). A reference site at the USGS National Fish Health Research Laboratory (NFHRL), Kearneysville, West Virginia, was added to the spring sampling, replacing the Blue Plains site. The UP sites were located at least 15 km upstream of the nearest major WWTP input designated as having a discharge of greater than 1 million gallons per day (mgd, or 3.8 million liters per day, mLd), however, a small WWTP was known to discharge 0.003 mgd (0.011 mLd) approximately 3 km upstream of the UP Conococheague Creek site. The DS sites were located immediately downstream of the WWTP discharges.

Conococheague Creek (Figure 1) originates in Pennsylvania and flows south into the Potomac River at Williamsport, MD. Land use of the 911 km^2 watershed is largely agricultural (61%) and forested (34%) with minor urban influence (5%). Effluent from the Conococheague WWTP comprised of 3.2% and 1.6% of the estimated mean flow at the DS site during the fall and spring sampling periods (USGS stream flow-gage 1614500). The Monocacy River (Figure 1), with a drainage area of 1,927 km^2, forms near the Maryland and Pennsylvania border and flows south through the City of Frederick, Maryland and into the Potomac River. Land use of the Monocacy watershed is similar to the Conococheague with 60% agricultural, 33% forested and 7% urban. Two WWTPs are suspected of influencing the DS Monocacy site with an estimated 3.7% and 2.3% of the mean flow during the fall and spring sampling periods (USGS stream flow-gage 1643000). In Washington, DC, the Blue Plains WWTP is the largest plant in the Potomac River watershed using a combination of nitrification/denitrification, filtration, chlorination/dechlorination, and post aeration. It serves the District of Columbia, Montgomery and Prince Georges counties in Maryland and Fairfax and Loudon counties in Virginia and has an average output of 370 mgd (1400 mLd) of treated wastewater. The percent effluent during baseflow conditions could not be estimated as the area is in the tidal region of the Potomac River and no USGS stream gauges were located nearby. The NFHRL reference site is a research pond with no WWTP input. This pond receives surface water from other research ponds at the facility and may be susceptible to chemical input from surface runoff and transport from nearby farms.

Passive sampler construction

SPMDs and POCIS were fabricated according to established procedures [7-9]. For each site, one deployment canister containing eight POCIS and one deployment canister with four SPMDs were prepared. This provided sufficient samplers to allow for replicate analyses at each site. Field blanks for both sampler types at each site were also prepared.

The POCIS used in this study contained the triphasic admixture of (80:20 W:W) Isolute ENV+ and S-X3 dispersed Ambersorb 1500 enclosed between two polyethersulfone membranes. Each POCIS unit had an effective sampling surface area of 41 cm^2 and a membrane surface area to sorbent mass ratio of $\approx$ 180 cm^2/g conforming to the specification of a standard POCIS [8].

The SPMDs used in this project consisted of 97 cm long (86 cm between the lipid-containment seals) by 2.5 cm wide layflat low-density polyethylene tubing containing 1.0 mL of purified triolein [17]. The membrane surface area to total SPMD volume ratio of SPMDs used in this study was ≈ 86 cm^2/mL, and triolein represented approximately 20% of the mass of the SPMDs conforming to a "standard SPMD" as defined by Huckins et al. [8]. Two of the four SPMDs deployed and one of the two field blank SPMDs at each site were fortified with 1 µg of each of the five perdeuterated polycyclic aromatic hydrocarbons (PAHs) selected as performance reference compounds (PRCs - acenaphthylene-d_{10}, acenaphthene-d_{10}, fluorene-d_{10}, phenanthrene-d_{10} and pyrene-d_{10}). PRCs are analytically non-interfering organic compounds with moderate to high fugacity from SPMDs that are added to the lipid prior to membrane enclosure and field deployment [8]. By comparing the rate of PRC loss during field exposures to that of laboratory studies, adjustments to the sampling rates of targeted chemicals can be made to more accurately reflect the site specific sampling rates. The amount of loss will be dependant on environmental factors such as exposure time, facial flow/velocity at the sampler's surface, temperature, and biofouling. Due to the strong sorptive properties of the adsorbents used in the POCIS, initial attempts to incorporate PRCs into the POCIS to date have failed [9].

Sample processing and chemical analysis

Each SPMD and POCIS was extracted individually prior to designating extracts for specific processing and analysis procedures. A list of the targeted chemicals is presented in Table 1. Neat chemical standards and custom chemical mixtures were obtained from Accustandard (New Haven, CT, USA), ChemService (West Chester, PA, USA), Sigma (ST. Louis, MO, USA), and LGC Promochem (Middlesex, UK). All solvents were Optima grade from Fisher Scientific (Pittsburgh, PA, USA). SPMDs were processed and analyzed for PAHs, organochlorine (OC) pesticides, and total polychlorinated biphenyls (PCBs). Select organic wastewater chemicals (OWCs), agricultural pesticides, and hormones were measured in the POCIS. The extracts from a single deployed POCIS and blank from each site were screened for the potential estrogenic activity of sequestered chemicals using the BLYES assay.

SPMDs

The procedures used for preparing SPMD samples for analysis were similar to published approaches [18-19]. Briefly, the target analytes were recovered by dialysis with hexane and then the dialysates were fractionated by size exclusion chromatography (SEC) prior to class-specific cleanup and analysis.

Polycyclic aromatic hydrocarbons (PAHs). Samples designated for the analysis of PRCs and PAHs were processed using a tri-adsorbent column consisting of phosphoric acid silica gel, potassium hydroxide impregnated silica gel, and silica gel [19]. Analysis of selected PAHs and PRCs was performed using an Agilent 6890 gas chromatograph (GC, Agilent Technologies, Inc., Wilmington, DE) coupled to a 5973N mass selective detector (MSD, Agilent Technologies, Inc., Palo Alto, CA) with a HP-5MS (30 m x 0.25 mm i.d. x 0.25 μm film thickness) capillary column (Agilent Technologies, Inc., Wilmington, DE) as reported by Alvarez et al. [18]. Quantitation was achieved using a seven point calibration curve ranging from 10 to 4000 ng mL^{-1} with 2-methylnaphthalene-d_{10} and benzo[*e*]pyrene-d_{12} as internal standards.

Organochlorine pesticides and polychlorinated biphenyls (PCBs). The OC/PCB SPMD samples were further enriched using a Florisil column followed by fractionation on silica gel [19]. The first silica gel fraction (SG1) contained > 95 % of the total PCBs, hexachlorobenzene (HCB), heptachlor, mirex and 40 to 80 % of the *p,p*'-DDE when present in extracts. The second fraction (SG2) contained the remaining 28 target OC pesticides and ≤ 5 % of the total PCBs (largely, mono- and dichlorobiphenyl congeners). Analysis of the SPMD samples for PCBs and OCs were conducted using a Hewlett Packard Model 5890 series-II GC equipped with an electron capture detector (ECD, Hewlett Packard, Inc., Palo Alto, CA) and a DB-35MS (30 m x 0.25 mm i.d. x 0.25 μm film thickness) capillary column (J&W Scientific, Folsom, CA) [18]. Quantitation of OCs and PCBs were accomplished using a six-point internal standard calibration curve with PCB congeners I-30 and I-207 as internal standards. The concentrations of the OC standards ranged from 1.0 to 80 ng mL^{-1}. The PCB calibration standards were composed of a 1:1:1:1 mixture of Aroclors 1242, 1248, 1254, and 1260 covering the range of 200 to 4000 ng mL^{-1}.

POCIS

The procedures used for preparing the POCIS samples for analysis in this study are similar to published approaches [8-9, 18]. Chemicals of interest were recovered from the POCIS sorbent using 50 mL of 1:1:8 (V:V:V) methanol:toluene:dichloromethane followed by 20 mL of ethyl acetate. The extracts were reduced in volume by rotary evaporation, filtered, and composited into 2-POCIS equivalent samples thereby increasing the amount of chemical present in each sample to aid in detection. It is often desirable to combine POCIS extracts as sampling rates are often low due to their small surface area.

Organic wastewater chemicals (OWCs). Analysis of the waste indicator chemicals was performed on raw POCIS extracts due to the difficulty in adequately "cleaning-up" a sample while maintaining the integrity of such a diverse set of chemicals. Analyses were performed on the GC-MSD system previously described using a temperature program of injection at 40 °C, held for 3 min, then ramped at 9 °C/min to 320 °C and held at 320 °C for 3 minutes. Identification of the targeted chemicals was performed using positive ion electron impact ionization full-scan MS. Quantitation was performed by comparison of unique ions for each chemical to a four-point calibration curve from 100 to 5000 ng mL^{-1} with *p*-terphenyl-d_{14} as the internal standard.

Agricultural pesticides. Details for the processing and analysis of POCIS for agricultural pesticides have been previously reported [18]. Briefly, the extracts were fractionated using SEC, followed by sample cleanup and enrichment by Florisil adsorption chromatography. Analysis was performed using the GC-MSD system previously described [18]. A six-point calibration curve ranging from 10 to 2000 ng mL^{-1} with *p*-terphenyl-d_{14} as the internal standard was used for quantification.

Hormones. Processing methods for selected hormones from POCIS have been reported [20]. Briefly, the extracts were fractionated by SEC with the collect window initiated at 5% of the time between the apexes of the chromatographic reference peaks DEHP and biphenyl [19]. The post-SEC samples were enriched and fractionated by adsorption chromatography using potassium hydroxide impregnated silica gel. Half of each extract was taken to near dryness

under high purity N_2, redissolved in 0.5 mL 1:1 (V:V) water:acetonitrile and analyzed by HPLC. These underivatized extracts were analyzed with a Hewlett Packard 1090 Series II Liquid Chromatograph with a diode array detector (Hewlett Packard, Palo Alto, CA) and a Supelco (Supelco, Bellefonte, PA) Discovery® C_8 analytical column (150 x 4.6 mm, 5 µm d_p). The remaining extract halves were derivatized for GC-MSD analysis. Quantitation of the HPLC analyses was performed using external calibration of an eight-point calibration curve ranging from 10 to 500 ng of each hormone injected on-column. A separate raw extract (no processing) from each site was also derivatized and analyzed by GC-MSD to rule out any unexpected procedural recovery problems.

Derivatization of extracts and calibration standards for GC-MSD analysis was initiated by the addition of 2% methoxyamine-HCL in pyridine followed by heating at 70ºC for 2 hours. Then, a mixture of bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) and triethylamine was added to the samples with an additional 18 hours on the heating block at 70ºC. The derivatized samples were then solvent exchanged into hexane then run through mini silica gel (300 mg) columns to remove color and any precipitate. The derivatized hormones were recovered from the silica gel with hexane prior to analysis. Analysis of the derivatized extracts was performed using the GC-MSD system previously described with the temperature program of injection at 90 °C, ramped at 25 °C/min to 200 °C, then 4 °C/min ramp to 255 °C, ramped at 10 °C/min to 310 °C and held at 310 °C for 3 minutes. A five-point calibration curve ranging from 50 to 5000 ng mL^{-1} with *p*-terphenyl-d_{14} as the internal standard was derivatized concurrently with the field samples and blanks.

In vitro bioluminescent yeast estrogen screen (BLYES)

The BLYES was employed to estimate estrogenic potential of compounds accumulated by the POCIS during the duration of the deployment. Strain BLYES was kindly supplied by the Sayler Laboratory, University of Tennessee. The assay was performed in accordance to the published methods of Sanseverino et al. [16] with slight modifications. In short, strain BLYES was grown in YMM (leu^-, ura^-) at 30°C and 150 rpm shaking to an approximate optical density at 600 nm (OD_{600}) of 1.0. One hundred microliters was transferred to each well of a black 96-well Costar

microtitre plate preloaded with 100 µL of POCIS sample diluted 10% in YMM (leu^-, ura^-). All samples were assayed in triplicate per plate and each plate contained a series of 17β-estradiol (E2) standards ranging from $8.2x10^{-14}$ to 8.0×10^{-7} M. Samples were assayed on four separate occasions to assess repeatability. Stock E2 and POCIS samples were solublized in methanol. Control wells contained YMM (leu^-, ura^-) and the appropriate concentration of methanol to assess baseline bioluminescence (BL) of strain BLYES. Plates were incubated at 30ºC in a humidified chamber at 100 rpm on an orbital shaker for 3 hours and then loaded into SPECTRAFluor Plus plate reader (Tecan) for kinetic BL measurements. The measurements of the test plates were taken every 30 min for 6 hours and induced BL was determined using an integration time of 2 seconds per well and a gain value of 150. Estrogenicity was measured as the fold induction of bioluminescence relative to the 17β-estradiol control. Relative estrogenicity was also determined for each site by subtracting the measured relative light units of deployed POCIS values from the corresponding site specific POCIS control. All relative light units data was assigned a relative estrogenicity via interpolation from the standard curve using a 4-parameter logistic equation using Prism 4 for Windows (GraphPad Software).

Statistical analyses were performed with SyStat 11 at $\alpha = 0.05$. One-way analysis of variances (ANOVAs) examined differences in BL between sites and rivers. The Tukey-Kramer *post hoc* test was executed if the general ANOVA model was significant.

Quality Control

Method limits of detection (MDL) and of quantification (MQL) were estimated from the average signal-to-noise ratio of the response of targeted chemicals from the instrumental analysis of the laboratory and field matrix blanks (SPMD or POCIS). A detailed discussion of the types of blanks used has been reported elsewhere [7, 9, 18] MDLs were determined as the mean plus three standard deviations of the response of a coincident peak present in the blanks [21]. The MQLs were determined as the mean plus 10 standard deviations of the target chemicals [21]. In cases where no coincident peak was present, the MQL was set at the low-level calibration standard and the MDL was estimated to be 20% of the MQL. This process of determining MDL/MQL values from the blanks accounts for any bias due to the sampler's materials, handling, shipping, storage, and processing.

Throughout the passive sampler processing and procedural steps, matrix spikes and instrumental verification checks were employed to monitor for potential problems. Radiolabeled surrogates of model compounds were added to select quality control samples and immediately measured using a liquid scintillation counter (Beckman Coulter, model LS6500, Fullerton, CA, USA) at specific steps in the processing scheme to rapidly determine processing recoveries and to identify potential problems. Select SPMDs from each study period were fortified with ^{14}C phenanthrene (a common PAH) with recoveries of 91% and 89% for fall and spring, respectively. Select POCIS were spiked with ^{3}H ethynylestradiol (a widely used synthetic hormone) in both fall and spring with recoveries of 94% and 84%. In spring, a POCIS was spiked with ^{14}C diazinon (a common organophosphate insecticide) resulting in a recovery of 66%. Recovery of chemicals throughout the SEC system, monitored using ^{14}C phenanthrene, averaged 97% with 3.7% relative standard deviation (n=4).

Matrix (i.e., fabrication and field) blanks for the passive samplers were processed and analyzed concurrently with the field deployed samplers. Overall, the blanks did not indicate there were any problems of sample contamination due to the materials and/or processing and handling of the samplers in the laboratory or field. The fall SPMDs did show a slightly elevated background of OC pesticides during the GC-ECD analysis which contributed to somewhat higher MDLs and MQLs for that sample set. The interfering peaks were determined not to be the chemicals of interest but rather co-eluting materials originating from the polyethylene membrane of the SPMDs as these peaks were present at a similar intensity and retention time in SPMD matrix blanks run concurrently.

For reporting purposes, the MDLs and MQLs for each sample set were calculated as the approximate ambient water concentrations based on the average PRC data across the sites for each sampling period. When sampling rate information was not available, the MDLs and MQLs were expressed as the mass of chemical sequestered by a single sampler (i.e., ng/POCIS or ng/SPMD).

Estimation of Ambient Water Concentrations

Using models previously developed [7-9], PRC loss data, chemical sampling rates (when available), and amounts of chemicals sampled, the average water concentrations of selected chemicals can be estimated. Uptake of chemicals into passive samplers generally follows linear, curvilinear and equilibrium phases of sampling. Integrative (or linear) sampling is the predominant phase for compounds with log K_{ow} values ≥ 5.0 and exposure periods of up to one month in SPMDs and for most of the chemicals tested in the POCIS. During the linear uptake phase the ambient chemical concentration (C_w) is determined by

$$C_w = N/R_s t \quad (1)$$

where N is the amount of the chemical accumulated by the sampler (typically ng), R_s is the sampling rate (L/d), and t is the exposure time (d). Previous data indicates that many chemicals of interest sampled by the POCIS remain in the linear phase of sampling for at least 56 d [8-9], therefore, the use of a linear uptake model (eq. 1) for the calculation of ambient water concentrations was justified.

For SPMDs, regression models have been created which estimate a chemical's site specific R_s and its C_w based on the log K_{ow} of the chemical, the PRC's release rate constant (k_e) and SPMD-water partition coefficient (K_{sw}) [7]. A PRC's k_e is determined from the amount of PRC initially added to the SPMD (N_o) and the amount remaining (N) as shown in equation 2. The log K_{sw} is determined from a regression model of the PRC's log K_{ow} as shown in equation 3 where a_0 is the intercept determined to be -2.61 for PCBs, PAHs, nonpolar pesticides and -3.20 for polar pesticides. The $R_{s\text{-}PRC}$ can then be calculated as shown in equation 4 where V_s is the volume of the SPMD.

$$k_e = - [\ln(N/N_o)]/t \quad (2)$$

$$\log K_{sw} = a_0 + 2.321 \log K_{ow} - 0.1618 (\log K_{ow})^2 \quad (3)$$

$$R_{s\text{-}PRC} = V_s K_{sw} k_e \quad (4)$$

The extrapolation of C_w from measured values of N requires knowledge of a chemical's site-specific sampling rate (R_{si}) which is determined from a third-order polynomial (eq. 5) where $\alpha_{(i/PRC)}$ is the compound-specific effect on the sampling rate and the relationship between the R_{s-PRC} and R_{si} (eq. 6).

$$\log \alpha_{(i/PRC)} = 0.0130 \log K_{ow}^3 - 0.3173 \log K_{ow}^2 + 2.244 \log K_{ow} \quad (5)$$

$$R_{si} = R_{s\text{-}PRC}(\alpha_i / \alpha_{PRC}) \quad (6)$$

The C_w of a chemical in the water can then be calculated by

$$C_w = N/(V_s K_{sw}[1-\exp(-R_s t/V_s K_{sw})]) \quad (7)$$

RESULTS

Chemical Analyses

In this work, 138 individual chemicals (not including the ~120 individual PCB congeners used to estimate total PCBs) were selected as representative anthropogenic organic chemicals which may be present from agricultural, industrial, and municipal inputs (Table 1). Analysis of the passive samplers resulted in the detection of 84 of these targeted chemicals. Chemicals which were detected in a passive sampler from at least one site are shown as the mean of replicate samples in Tables 2-5. In cases where the value of one replicate was <MDL, the value of the other replicate was given representing the maximum observed value. In general, the replication was quite good with an average relative percent difference of 17% (n=458). Based on the availability of chemical sampling rates and the PRC data, water concentrations were estimated from the chemical residues sampled by the SPMDs and POCIS [7-9]. If the sampling rate for a chemical was unknown, the result was given as mass of chemical per sampler to be used for comparing the relative loading between sites.

The number and relative water concentrations of the OC pesticides were similar between the fall and spring samplings (Table 2). Pentachloroanisole, a degradation product of pentachlorophenol, chlorpyrifos, *cis/trans*-chlordane, dieldrin, and endrin were commonly measured across the sampling sites and study periods. Endosulfan and its degradation product, endosulfan-II, were present at the greatest concentrations of up to 5 ng L^{-1} at the Blue Plains site. As expected, the highest concentrations for most of the targeted chemicals were found at the Blue Plains site which is heavily influenced by urbanization. Up to 80% of the targeted PAHs, including the priority pollutant PAHs, were identified in SPMDs from the fall and spring samplings (Table 3). In the fall, the DS Monocacy River and Blue Plains sites were the most heavily contaminated with PAHs which concentrations up to 4.7 ng L^{-1} (phenanthrene). The DS Monocacy River site continued to be the most contaminated with PAHs in the spring with fluoranthene having the maximum concentration of 5.4 ng L^{-1}.

A screen for chemicals potentially originating from wastewater inputs identified several OWCs, such as fragrances, plasticizers, and flame retardants (Table 4). The Blue Plains site had the greatest number of detections and the highest concentrations of OWCs from the fall sampling. Surprisingly, the UP Conococheague Creek samples also had detectable levels of fragrances and flame retardants indicating a potential wastewater input. Atrazine, also identified at all sites in the agricultural pesticides screen, was confirmed by the OWC screen. In the spring sampling, the DS Monocacy River site had the greatest number of OWCs which was consistent to the chemical data from OC pesticide and PAH analyses.

Several chemicals associated with agricultural practices were found during both the fall and spring samplings (Table 5). Atrazine, metolachlor, desisopropylatrazine and desethylatrazine (DIA and DEA, both atrazine metabolites) were the most commonly identified. In the fall, atrazine concentrations ranged from 23 ng L^{-1} (DS Monocacy River) to 110 ng L^{-1} (DS Conococheague Creek). DEA concentrations in the fall peaked at 59 ng L^{-1} in the UP Conococheague Creek site. In the spring, atrazine concentrations were greatest with a maximum concentration of 2100 ng L^{-1} at the DS Monocacy River site.

Initial analyses of the hormones in the POCIS extracts using HPLC were inconclusive, therefore a portion of the extracts were reanalyzed by GC-MS after derivatization to gain sensitivity and selectivity. No hormones were identified using either method. Since it was suspected that natural and/or synthetic hormones may have been present at the sites, a raw extract from a separate POCIS from each site was derivatized and analyzed by GC-MS. As with the previous analyses, none of the targeted hormones were identified above the estimated MQL of 2.5 ng L^{-1}. Concentrations of E2 in the fall DS Conococheague Creek, spring UP Conococheague Creek, and E2 and 17α-ethynylestradiol in the spring DS Monocacy River POCIS were at the MDL.

In vitro bioluminescent yeast estrogen screen (BLYES)

Analysis of POCIS extracts with strain BLYES indicated that all sites surveyed contained chemicals with measurable estrogenicity (Figure 2). Extracts collected during the fall sampling (corrected to their respective field blank) induced 2.50-6.22 fold more BL than estrogen-free growth medium alone. Statistically significant differences were observed between the study sites (One-way ANOVA, $f = 55.99$, $p < 0.001$). Sampling sites upstream and downstream of targeted WWTPs within the same river did not statistically differ (Figure 2a). Induction at the Blue Plains sampling site was nearly twice the amount observed at the other sites in the fall (Figure 2a). In the spring, induction was lowest at the NFHRL reference site while induction was greatest in the UP Conococheague and DS Monocacy (Figure 2b). Extracts from all sites during both sample years induced statistically elevated BL relative to responses to the estrogen-free controls ($p<0.001$). Estimated estrogenicity relative to E2 for all sites was in the nanomolar range. Estrogenic activity was detected in the field blanks as BL was induced 1.1 - 3.2 fold higher than estrogen-free controls during the fall season and 1.0 – 2.9 fold during the spring. In all cases induction by extracts from deployed POCIS devices were statistically greater than their corresponding field blanks.

DISCUSSION

Evaluation of chemical occurrence and relative concentrations were used to determine seasonal patterns, degradation of chemicals, and differences between sampling sites in common waterways (UP versus DS sites). Comparison of the data from the fall and spring samplings

revealed no substantial differences between the occurrence or concentrations of OC pesticides, PAHs, or other OWCs. The BLYES indicated that the only significant difference in the total estrogenicity of sampled chemicals between the fall and spring samplings was at the DS Monocacy River site (two sample t-test, P=0.001). Kolpin et al [22] reported decreasing concentrations of OWCs as stream flow increased largely due to dilution, however, this effect was not observed in this study as the ratio of WWTP effluent to mean stream flow was largely unchanged between sampling periods.

The greatest changes in concentration between the sampling periods were for the agricultural pesticides, atrazine and metolachlor. For both chemicals, the concentrations were 3.1 to 91-fold greater in the spring sampling which was expected due to increased pesticide application corresponding to spring crop planting in the largely agricultural reaches of the watershed. Considering that the mean stream flow only increased 2-fold between the fall and spring (flow was measured at the DS sites only), any variation in the POCIS R_s was considered to be negligible. The estimated water concentrations were similar to those reported by Alvarez et al. [20] from a sampling on the nearby North Fork of the Shenandoah River in northern Virginia during the spring and early summer of 2007.

Corresponding to the differences in atrazine concentrations are the changes in the occurrence of two of atrazine's main degradation products, DEA and DIA. At the three sites with both fall and spring samplings (UP Conococheague Creek, DS Conococheague Creek and DS Monocacy River), DIA concentrations were below the MQL in the spring but present at quantifiable levels in the fall. DEA was present at quantifiable concentrations at all three sites in both the spring and fall with a 3-fold increase in concentration in the fall UP Conococheague Creek sample. Greater concentrations of DIA and DEA in the fall can be attributed to degradation of the parent compound (atrazine) following spring and summer application.

A relative measure of residence time and mode of transport of agricultural chemicals in the system was determined using the deethylatrazine-to-atrazine ratio (DAR). The DAR is calculated by dividing the concentration of DEA by that of atrazine [23-24]. A DAR value

greater of 1.0 indicates primarily groundwater transport to the river where atrazine is converted to DEA via metabolic activity of soil bacteria and fungi [23]. DAR values less than 1.0 are an indicator of point-source contamination as transport to the river is mainly through surface runoff. Calculation of DAR ratios for the study sites shows that only UP Conococheague Creek (1.4) during fall had a value indicative of a non-point source contamination. A substantial decrease in the DAR was observed at all sites between the fall and spring sampling (UP Conococheague Creek 1.4 to 0.05; DS Conococheague Creek 0.2 to 0.05; and DS Monocacy River 0.4 to 0.01) which clearly shows the fresh application of atrazine and subsequent runoff during the spring planting season (Table 5). The NFHRL reference pond had a DAR of 0.63 which was likely due to overspraying and surface runoff from adjacent farms.

Generally, concentrations and numbers of chemicals detected were greater in water collected from sites downstream of WWTP discharges. In particular, OWCs had the greatest occurrence and concentrations in the DS sites influenced by WWTP discharges. Similarly, the DS Monocacy River site had much greater PAH concentrations than the corresponding UP site indicating that the WWTPs may have been a major source of PAHs in the Monocacy River. In contrast to these findings, the levels of PAHs and OWCs were relatively constant between the UP and DS Conococheague Creek sites. At both the Monocacy River and Conococheague Creek no substantial differences were found for the agricultural pesticides between the UP and DS sites. The BLYES assay also showed elevated estrogenicity in samples from the UP Conococheague Creek site suggesting the presence of a WWTP or other waste discharge. A combination of a WWTP approximately 3 to 5 km upstream of the UP site and leachate from septic tanks in this largely rural region of the watershed may have contributed to the elevated concentrations. A previous study showed that water concentrations of many OWCs remain largely unchanged over distances of 3 km [25].

The BLYES assay indicated that there were chemical(s) present at each site which were capable of promoting an estrogenic effect at a level statistically greater than the background response observed in the blanks. It is not clearly understood which chemicals associated with the sampler matrix or sample processing may have been responsible for the observed response in the field

blanks, however, it has previously been reported that the estrogenic response is likely due to impurities in the POCIS membrane [18]. Chemical analysis of select natural and synthetic steroidal hormones found levels to be at or less than the MDL. However, due to the strong responses observed in the BLYES, it is likely that one or more estrogens or estrogen mimicking chemicals contributed to the response. A definitive identification of the estrogen mimics would involve a combination of analytical chemistry methods and *in vivo* or *in vitro* estrogenic assays in a manner similar to toxicity identification and evaluation (TIE) tests. Such methods were beyond the scope of this study.

Iwanowicz et al. [2] found that intersex had occurred in 82-100% of the male smallmouth bass collected at both the UP and DS sites during the fall sampling. This suggests that multiple chemical stressors may be responsible for reproductive impairment in fishes which are not solely associated with agriculture or WWTP effluent. Little is known about the long-term chronic effects due to exposure to trace concentrations of OWCs [26]. Atrazine is a likely suspect due to its widespread use in the region and elevated concentrations at the study sites, however, direct effects on the reproductive health of various fish species have not been found [27-29]. Although a direct link between intersex and organic contaminants has not been identified, this work provides important information on the types and relative concentrations of chemicals which were present in areas where intersex in fish occurs.

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Figure II-1. Map of Potomac River watershed indicating the 2005/2006 sampling locations. NFHRL – National Fish Health Research Laboratory, Kearneysville, West Virginia (reference site).

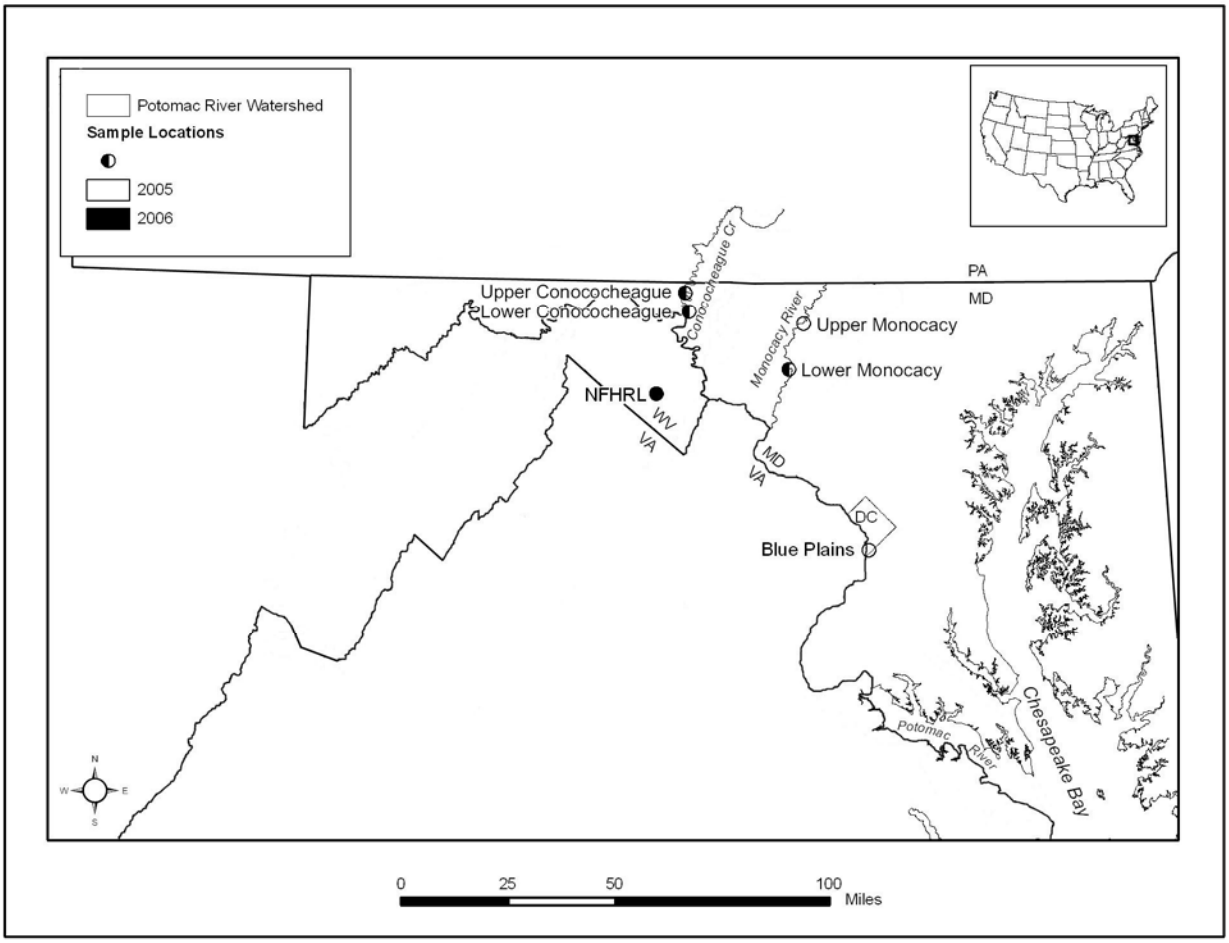

Figure II-2. Response of strain BLYES to POCIS extracts. POCIS extracts from 2005 (a) and 2006 (b) diluted to a working concentration of 10% in growth medium were incubated with 1 OD600 of strain BLYES at 30C for 6 hours. Induction of bioluminescence here is depicted as the difference of field deployed POCIS to the site specific field blanks. Induction is relative to the 17β-estradiol control (E2). Data were compared via one-way ANOVA (Tukey-Kramer *post hoc* test). Sites denoted with different letters are statistically different while those with the same letters are not ($P < 0.05$).

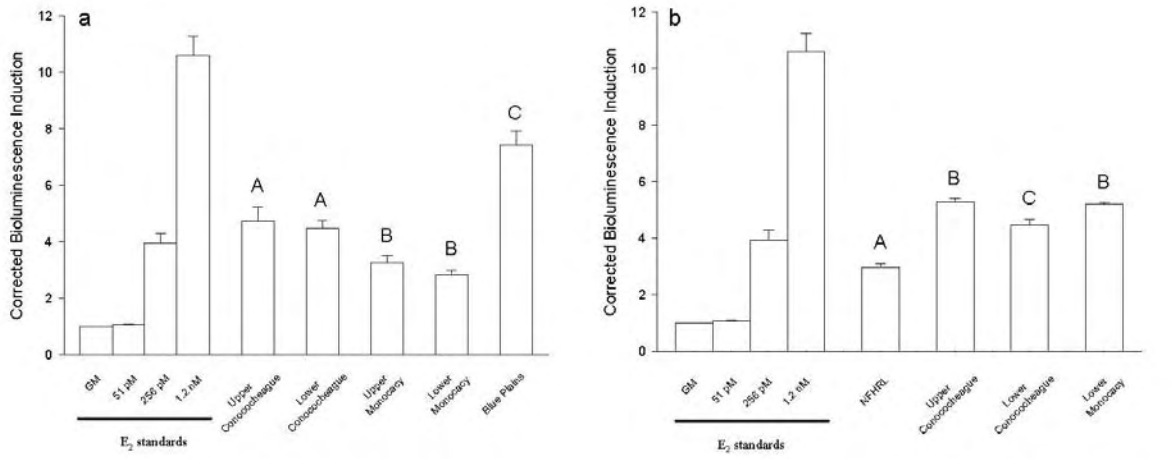

Table II-1. Selected chemicals targeted for analysis in passive samplers deployed in the Potomac River watershed during the Fall 2005 and Spring 2006 samplings.

Organochlorine Pesticides and PCBs[a]	**Polycyclic Aromatic Hydrocarbons (PAHs)[a]**	**Organic Wastewater Chemicals[b]**	**Agricultural Pesticides[b]**
α-Benzenehexachloride	Acenaphthene	1,4-Dichlorobenzene	Acetochlor
β-Benzenehexachloride	Acenaphthylene	4-*n*-Octylphenol	Alachlor
δ-Benzenehexachloride	Anthracene	4-*tert*-Octylphenol	Ametryn
cis-Chlordane	Benz[*a*]anthracene	Acetophenone	Atraton
trans-Chlordane	Benzo[*a*]pyrene	Anthraquinone	Atrazine
Chlorpyrifos	Benzo[*b*]fluoranthene	Atrazine	Chlorpyrifos
Dacthal	Benzo[*g,h,i*]perylene	Benzophenone	Dacthal
Diazinon	Benzo[*k*]fluoranthene	Bromacil	Desethylatrazine
Dieldrin	Chrysene	Bromoform	Desisopropylatrazine
o,p'-DDE	Dibenz[*a,h*]anthracene	Caffeine	Diazinon
p,p'-DDE	Fluoranthene	Camphor	EPTC
o,p'-DDD	Fluorene	Carbaryl	Fipronil
p,p'-DDD	Indeno[1,2,3-*c,d*]pyrene	Carbazole	Fonofos
o,p'-DDT	Naphthalene	Celestolide (ADBI)	Malathion
p,p'-DDT	Phenanthrene	Chlorpyrifos	Methyl Parathion
Endrin	Pyrene	Cholesterol	Metolachlor
Endosulfan	1,2-dimethylnaphthalene	Cotinine	Metribuzin
Endosulfan-II	1-ethylnaphthalene	Diazinon	Pendimethalin
Endosulfan Sulfate	1-methylfluorene	Dichlorvos	Prometon
Heptachlor	1-methylnaphthalene	Diethyl phthalate	Prometryn
Heptachlor Epoxide	2,3,5-trimethylnaphthalene	Diethylhexylphthalate	Propazine
Hexachlorobenzene	2-methylfluoranthene	D-Limonene	Simazine
Lindane	2-methylnaphthalene	Ethyl citrate	Simetryn
p,p'-Methoxychlor	2-methylphenanthrene	Galaxolide (HHCB)	Terbuthylazine
Mirex	3,6-dimethylphenanthrene	Indole	Terbutryn
cis-Nonachlor	4-methylbiphenyl	Isophorone	Trifluralin
trans-Nonachlor	9-methylanthracene	Isopropylbenzene (cumene)	
Oxychlordane	Benzo[*b*]naphtho[2,1-*d*]thiophene	Isoquinoline	
Pentachloroanisole	Benzo[*b*]thiophene	Menthol	
cis-Permethrin	Benzo[*e*]pyrene	Metalaxyl	
trans-Permethrin	Biphenyl	Methyl salicylate	**Hormones[b]**
Trifluralin	Dibenzothiophene	*N,N*-diethyltoluamide (DEET)	
	Perylene	*para*-Cresol	17β-Estradiol
Total PCBs		Phantolide (AHMI)	17α-Ethynylestradiol
		Phenol	Estriol
		Prometon	Estrone
		Tetrachloroethylene	
		Tonalide (AHTN)	
		Traseolide (ATII)	
		Tri(2-chloroethyl) phosphate	
		Tri(butoxyethyl) phosphate	
		Tri(dichloroisopropyl) phosphate	
		Tributyl phosphate	
		Triphenyl phosphate	

[a] Chemicals under this category were analyzed for in SPMD extracts.
[b] Chemicals under this category were analyzed for in POCIS extracts.

Table II-2. Estimated water concentrations of detected[a] organochlorine pesticides in SPMDs from the 2005/2006 sampling periods in the Potomac River watershed. Reported values are the mean of replicate samples.

Site identification[b]	UP C Creek		DS C Creek		UP Mon River		DS Mon River		Blue Plains		NFHRL	
Sampling year	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}
α-Benzenehexachloride	<130[c]	<210	*250*[d]	<210	*220*	-----[e]	*180*	<210	*230*	-----	-----	<210
β-Benzenehexachloride	<4.6	<140	<4.6	<140	<4.6	-----	*5.5*	*170*	*9.3*	-----	-----	<140
δ-Benzenehexachloride	**89**[f]	<2.5	**94**	**29**	<49	-----	**93**	**29**	**220**	-----	-----	<2.5
cis-Chlordane	**21**	**24**	**72**	**52**	**17**	-----	**38**	**35**	**330**	-----	-----	*7.1*
trans-Chlordane	**22**	**20**	**64**	**67**	**18**	-----	**46**	**30**	**240**	-----	-----	*10*
Chlorpyrifos	<11	**120**	**120**	**180**	*19*	-----	**48**	**160**	**480**	-----	-----	**280**
Dacthal	<9.5	<150	**21**	<150	*15*	-----	*16*	<150	<9.5	-----	-----	<150
Dieldrin	**180**	**130**	**300**	**200**	**100**	-----	**200**	**150**	**550**	-----	-----	**19**
o,p'-DDE	<12	**13**	<12	**8.9**	<12	-----	<12	**11**	*15*	-----	-----	*4.7*
p,p'-DDE	**78**	**80**	**83**	**88**	*44*	-----	**70**	**57**	**87**	-----	-----	*34*
o,p'-DDD	**29**	*37*	**30**	*46*	<8.8	-----	**110**	*40*	**61**	-----	-----	<19
p,p'-DDD	*22*	**22**	*41*	*36*	<18	-----	*33*	*26*	*47*	-----	-----	**9.1**
o,p'-DDT	*41*	<8.4	**62**	*15*	<38	-----	**480**	**98**	**180**	-----	-----	<8.4
p,p'-DDT	<74	*110*	*170*	*110*	<74	-----	*100*	<90	*160*	-----	-----	<90
Endrin	*48*	**54**	**81**	88	**55**	-----	**70**	**59**	*51*	-----	-----	**21**
Endosulfan	*85*	*270*	*74*	**550**	*80*	-----	*96*	*300*	**1100**	-----	-----	*420*
Endosulfan-II	**550**	<900	**1400**	**2900**	**830**	-----	**1200**	*1000*	**5000**	-----	-----	<900
Heptachlor	<0.8	<1.9	<0.8	**54**	<0.8	-----	<0.8	**6.9**	**25**	-----	-----	<1.9
Heptachlor Epoxide	*69*	*44*	**170**	**64**	*68*	-----	**150**	*37*	**410**	-----	-----	*35*
Hexachlorobenzene	**83**	**38**	*54*	**41**	<22	-----	<22	*18*	*55*	-----	-----	<14
Lindane	*440*	<540	**620**	<540	*460*	-----	**550**	<540	*470*	-----	-----	<540
p,p'-Methoxychlor	<88	<20	*94*	*21*	<88	-----	*97*	*28*	**140**	-----	-----	<20
Mirex	**26**	**5.3**	*6*	<0.8	**19**	-----	<1.3	*3.8*	<1.3	-----	-----	<0.8
cis-Nonachlor	*7.1*	<10	**9.8**	*14*	*6.8*	-----	**11**	<10	**35**	-----	-----	<10
trans-Nonachlor	**35**	*47*	**52**	*58*	<25	-----	**49**	*45*	**110**	-----	-----	<37
Oxychlordane	<2.2	*1.6*	**7.2**	**9.4**	*3*	-----	**7.4**	**3**	**60**	-----	-----	*1.4*
Pentachloroanisole	**56**	<120	**230**	<120	**110**	-----	**190**	<120	**310**	-----	-----	<120
cis-Permethrin	<240	*8.5*	<240	<7.0	<240	-----	*270*	<7.0	<240	-----	-----	<7.0
Trifluralin	*120*	**3.4**	**180**	<0.6	<110	-----	**200**	<0.6	**230**	-----	-----	<0.6
Total PCBs[g]	<210	**3900**	*220*	**580**	<210	-----	**410**	**790**	**2600**	-----	-----	<210

[a] Only compounds detected in at least one sample are listed. A full list of compounds analyzed for is given in Table 1.
[b] UP C Creek – upstream Conococheague Creek; DS C Creek – downstream Conococheague Creek; UP Mon River – upstream Monocacy River; DS Mon River – downstream Monocacy River; Blue Plains – Potomac River at Blue Plains WWTP, Washington, DC; NFHRL – National Fish Health Research Laboratory, Kearneysville, West Virginia.
[c] Less than (<) values are below the MDL.
[d] Italic values are estimates greater than the method detection limit (MDL) but less than the method quantitation limit (MQL) and shown for informational purposes only.
[e] Site was not sampled during this study year.
[f] Bold values are reportable values greater than the MQL.
[g] Total PCBs determined from a 1:1:1:1 (by weight) mixture of Aroclors 1242, 1248, 1254, and 1260.

Table II-3. Estimated water concentrations of detected[a] polycyclic aromatic hydrocarbons (PAHs) in SPMDs from the 2005/2006 sampling periods in the Potomac River watershed. Reported values are the mean of replicate samples.

Site identification[b]	UP C Creek		DS C Creek		UP Mon River		DS Mon River		Blue Plains		NFHRL	
Sampling year	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}	2005 pg L^{-1}	2006 pg L^{-1}
Acenaphthene	**210**[c]	**370**	**170**	**340**	**220**	-----[d]	**360**	**480**	**410**	-----	-----	**320**
Anthracene	*52*[e]	**130**	*60*	**99**	*55*	-----	**250**	**230**	**160**	-----	-----	*40*
Benz[*a*]anthracene	**37**	<1.8[f]	**39**	**140**	**23**	-----	**140**	**230**	**370**	-----	-----	<1.8
Benzo[*a*]pyrene	*25*	*29*	*16*	*24*	<9.5	-----	*29*	**130**	**78**	-----	-----	<6.0
Benzo[*b*]fluoranthene	**74**	<5.2	**77**	<5.2	**30**	-----	**210**	<5.2	**260**	-----	-----	<5.2
Benzo[*g,h,i*]perylene	*49*	<7.9	*52*	<7.9	*13*	-----	**65**	**92**	**130**	-----	-----	<7.9
Benzo[*k*]fluoranthene	**54**	**120**	**53**	**96**	**23**	-----	**130**	**750**	**130**	-----	-----	<5.7
Chrysene	**230**	**240**	**230**	**160**	**130**	-----	**880**	**1700**	**1200**	-----	-----	*13*
Dibenz[*a,h*]anthracene	<10	<6.4	<10	<6.4	<10	-----	<10	<6.4	*13*	-----	-----	<6.4
Fluoranthene	**950**	**890**	**730**	**810**	**980**	-----	**4400**	**5400**	**4000**	-----	-----	**100**
Fluorene	**200**	**160**	**170**	**130**	**190**	-----	**420**	**300**	**570**	-----	-----	**101**
Indeno[1,2,3-*c,d*]pyrene	*41*	*34*	*35*	*21*	<12	-----	*37*	**76**	*40*	-----	-----	<7.2
Naphthalene	*730*	<140	*910*	<140	*760*	-----	*760*	<140	**1200**	-----	-----	<140
Phenanthrene	**1200**	**1200**	**950**	**980**	**1400**	-----	**4700**	**3300**	**2400**	-----	-----	**510**
Pyrene	**620**	**500**	**770**	**2800**	**540**	-----	**2600**	**3500**	**4000**	-----	-----	<21
1,2-dimethylnaphthalene	*46*	<18	*60*	<18	*40*	-----	*78*	*61*	**120**	-----	-----	<18
1-ethylnaphthalene	<17	<15	*38*	<15	*19*	-----	*59*	<15	**85**	-----	-----	<15
1-methylfluorene	**150**	**51**	**300**	<6.9	*96*	-----	**390**	**230**	**1000**	-----	-----	<6.9
1-methylnaphthalene	**2500**	*300*	**260**	*260*	*210*	-----	**300**	*190*	**540**	-----	-----	<180
2,3,5-trimethylnaphthalene	**87**	<7.4	**100**	<7.4	*42*	-----	**220**	<7.4	**410**	-----	-----	<7.4
2-methylfluoranthene	**37**	**34**	**40**	**36**	**25**	-----	**110**	**220**	**220**	-----	-----	<5.4
2-methylnaphthalene	*240*	<270	*310*	<270	<230	-----	*330*	<270	*530*	-----	-----	<270
2-methylphenanthrene	**120**	**180**	**120**	**160**	**150**	-----	**580**	**660**	**560**	-----	-----	<7.4
3,6-dimethylphenanthrene	*34*	<5.4	**42**	<5.4	**40**	-----	**160**	<5.4	**420**	-----	-----	<5.4
4-methylbiphenyl	<130	<9.2	<130	**600**	<130	-----	<130	**260**	<130	-----	-----	**360**
9-methylanthracene	<8.6	<6.1	<8.6	<6.1	<8.6	-----	<8.6	*29*	<8.6	-----	-----	<6.1
Benzo[*b*]naphtho[2,1-*d*]thiophene	**21**	*25*	**20**	**31**	**15**	-----	**140**	**290**	**180**	-----	-----	<5.6
Benzo[*e*]pyrene	**95**	**86**	**100**	**74**	**32**	-----	**170**	**390**	**330**	-----	-----	<6.1
Biphenyl	*60*	<42	*98*	<42	*75*	-----	*83*	<42	*180*	-----	-----	<42
Dibenzothiophene	*68*	**75**	*56*	*57*	*71*	-----	**220**	**210**	**220**	-----	-----	<15
Perylene	**64**	**55**	**97**	**46**	**61**	-----	**56**	**45**	**240**	-----	-----	<5.5

Only compounds detected in at least one sample are listed. A full list of compounds analyzed for is given in Table 1.

[b] UP C Creek – upstream Conococheague Creek; DS C Creek – downstream Conococheague Creek; UP Mon River – upstream Monocacy River; DS Mon River – downstream Monocacy River; Blue Plains – Potomac River at Blue Plains WWTP, Washington, DC; NFHRL – National Fish Health Research Laboratory, Kearneysville, West Virginia.

[c] Bold values are reportable values greater than the MQL.

[d] Site was not sampled during this study year.

[e] Italic values are estimates greater than the method detection limit (MDL) but less than the method quantitation limit (MQL) and shown for informational purposes only.

[f] Less than (<) values are below the method detection limit (MDL).

Table II-4. Amounts of waste indicator chemicals detected[a] in POCIS from the 2005/2006 sampling periods in the Potomac River watershed. Reported values are the mean of replicate samples.

Site identification[b]	UP C Creek		DS C Creek		UP Mon River		DS Mon River		Blue Plains		NFHRL	
Sampling year	2005 ng/POCIS	2006 ng/POCIS	2005 ng/POCIS	2006 ng/POCIS	2005 ng/POCIS	2006 ng/POCIS	2005 ng/POCIS	2006 ng/POCIS	2005 ng/POCIS	2006 ng/POCIS	2005 ng/POCIS	2006 ng/POCIS
Atrazine	**350**[c]	**4450**	**400**	**5100**	**690**	-----[d]	**170**	**25000**	**400**	-----	-----	**1400**
Benzophenone	*30*[e]	*30*	*30*	<20[f]	<20	-----	*30*	*45*	*40*	-----	-----	<20
Carbazole	<20	<20	<20	<20	<20	-----	<20	**200**	**200**	-----	-----	<20
Celestolide (ADBI)	<20	<20	**130**	<20	<20	-----	<20	**130**	**130**	-----	-----	<20
Diethylhexylphthalate	*320*	*360*	*300*	**610**	**400**	-----	<280	*340*	**3500**	-----	-----	**570**
Ethyl citrate	**100**	**110**	**250**	**130**	<20	-----	**120**	**330**	**330**	-----	-----	**100**
Galaxolide (HHCB)	<20	<20	**340**	**30**	<20	-----	**210**	**1900**	**960**	-----	-----	<20
Metalaxyl	*40*	<20	<20	<20	*40*	-----	<20	<20	<20	-----	-----	<20
N,*N*-diethyltoluamide (DEET)	*50*	*55*	*55*	*65*	*50*	-----	*50*	**120**	**110**	-----	-----	*40*
Phantolide (AHMI)	<20	*70*	*70*	*70*	<20	-----	<20	*80*	*80*	-----	-----	<20
Prometon	*95*	***95***	**120**	**110**	**100**	-----	<20	**120**	**150**	-----	-----	<20
Tonalide (AHTN)	<20	<20	**110**	<20	<20	-----	*30*	**230**	**520**	-----	-----	<20
Traseolide (ATII)	<20	<20	<20	<20	<20	-----	<20	**150**	<20	-----	-----	<20
Tri(2-chloroethyl) phosphate	*75*	*60*	**170**	*80*	*85*	-----	*95*	**160**	**360**	-----	-----	*60*
Tri(dichloroisopropyl) phosphate	<20	**250**	**300**	**260**	**260**	-----	**280**	**500**	**500**	-----	-----	**220**
Tributyl phosphate	<20	<20	**210**	**200**	<20	-----	**200**	**220**	**290**	-----	-----	<20
Triphenyl phosphate	*60*	<52	<52	<52	<52	-----	*60*	*70*	*70*	-----	-----	<52

[a] Only compounds detected in at least one sample are listed. A full list of compounds analyzed for is given in Table 1.
[b] UP C Creek – upstream Conococheague Creek; DS C Creek – downstream Conococheague Creek; UP Mon River – upstream Monocacy River; DS Mon River – downstream Monocacy River; Blue Plains – Potomac River at Blue Plains WWTP, Washington, DC; NFHRL – National Fish Health Research Laboratory, Kearneysville, West Virginia.
[c] Bold values are reportable values greater than the MQL.
[d] Site was not sampled during this study year.
[e] Italic values are estimates greater than the method detection limit (MDL) but less than the method quantitation limit (MQL) and shown for informational purposes only.
[f] Less than (<) values are below the MDL.

Table II-5. Estimated water concentration of detected[a] agricultural pesticides in POCIS from the 2005/2006 sampling periods in the Potomac River watershed. Reported values are the mean of replicate samples.

Site identification[b]	UP C Creek		DS C Creek		UP Mon River		DS Mon River		Blue Plains		NFHRL	
Sampling year	2005 ng L^{-1}	2006 ng L^{-1}	2005 ng L^{-1}	2006 ng L^{-1}	2005 ng L^{-1}	2006 ng L^{-1}	2005 ng L^{-1}	2006 ng L^{-1}	2005 ng L^{-1}	2006 ng L^{-1}	2005 ng L^{-1}	2006 ng L^{-1}
Atraton	<0.13[c]	<0.08	<0.13	<0.08	**1.9**[d]	-----[e]	<0.13	<0.08	<0.13	-----	-----	<0.08
Atrazine	**47**	**380**	**110**	**430**	**92**	-----	**23**	**2100**	**54**	-----	-----	**120**
Desethylatrazine	**59**	**18**	**18**	**20**	**52**	-----	**8.3**	**11**	**10**	-----	-----	**66**
Desisopropylatrazine	**18**	*2.8*[f]	**18**	*2.8*	**19**	-----	**18**	*2.8*	**18**	-----	-----	**15**
Metolachlor	**0.73**	**7.5**	**1.1**	**9**	**12**	-----	**11**	**97**	**1.9**	-----	-----	<0.90
Prometon	**1.1**	*1.2*	**3.2**	*1.4*	**2.1**	-----	**1.4**	**1.8**	**6.1**	-----	-----	<0.45
Simazine	**8.1**	**17**	<0.29	**18**	**12**	-----	<0.29	**38**	<0.29	-----	-----	**7.4**
Terbuthylazine	<0.23	<0.72	<0.23	<0.72	<0.23	-----	<0.23	<0.72	**9.1**	-----	-----	<0.72
DAR[g] values	**1.4**	**0.05**	**0.2**	**0.05**	**0.6**	-----	**0.4**	**0.01**	**0.2**	-----	-----	**0.63**

[a] Only compounds detected in at least one sample are listed. A full list of compounds analyzed for is given in Table 1.
[b] UP C Creek – upstream Conococheague Creek; DS C Creek – downstream Conococheague Creek; UP Mon River – upstream Monocacy River; DS Mon River – downstream Monocacy River; Blue Plains – Potomac River at Blue Plains WWTP, Washington, DC; NFHRL – National Fish Health Research Laboratory, Kearneysville, West Virginia.
[c] Less than (<) values are below the MDL.
[d] Bold values are reportable values greater than the method quantitation limit (MQL).
[e] Site was not sampled during this study year.
[f] Italic values are estimates greater than the method detection limit (MDL) but less than the MQL and shown for informational purposes only.
[g] DAR – desethyatrazine to atrazine ratio used as an indicator of pesticide transport. DAR = desethylatrazine (mol/L) / atrazine (mol/L).

CHAPTER III: Reproductive Health of Bass in the Potomac, USA Drainage: *In Situ* Exposures with Hatchery–Raised Smallmouth Bass

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ABSTRACT

In spring 2006, hatchery-raised smallmouth bass were deployed in cages in several Maryland tributaries of the Potomac River. Fish were caged downstream of wastewater treatment plants in Conococheague Creek and Monocacy River. These sites, as well as a site 15 km upstream of the downstream sample on the Conococheague Creek, were chosen because 2005 samples of wild-caught smallmouth bass had evidence of endocrine disruption. In addition, fish were also caged at a reference pond near the U.S. Geological Survey National Fish Health Research Laboratory (NFHRL) in Kearneysville, WV. As a control, a subset of 12 males and 14 females were processed for blood plasma vitellogenin analysis and histopathology prior to exposure. The objective of this study was to attempt to induce endocrine disrupting effects in hatchery-raised fish in the same locations where wild-caught smallmouth bass exhibited evidence of endocrine disruption. After 50 days of *in situ* exposure, blood was sampled for plasma vitellogenin and histopathology performed on approximately 10 males and 10 females from each location. Passive chemical samplers, semi-permeable membrane devices (SPMDs) and polar organic compound integrated samplers (POCIS), were placed next to the cages during exposure. The passive sampler analysis demonstrated that the fish in the cages were being exposed to endocrine disrupting compounds in detectable concentrations. The prevalence of testicular oocytes in males sampled from the cages placed in the three river locations ranged from 56% to 89%; prevalence at NFHRL was 73%. However, 83% of the male smallmouth bass had testicular oocytes prior to exposure, making it impossible to determine effects of the *in situ* exposure. Although this study was inconclusive as to whether short term exposure of hatchery-raised fish can be used to induce the same biological response as the wild-caught fish, it did highlight the fact that hatchery-raised smallmouth bass may have a high prevalence of intersex. Additional investigations are necessary to determine the prevalence of intersex in hatchery-raised bass and whether the presence of intersex affects fish populations.

INTRODUCTION

Since the early 1980s, endocrine disrupting compounds (EDCs) in humans, fish, and wildlife have been recognized as a global environmental concern [1, 2]. Both man-made chemicals and plant and animal hormones have been shown to have endocrine disrupting effects. In aquatic systems, two major sources of endocrine disrupting chemicals are agricultural production, [3, 4] and wastewater treatment plant effluents [5-9]. Kirk *et al.* [10] found that sewage treatment plants with secondary treatment transfer up to 30% of the androgenic and estrogenic compounds into the effluent. In plants that relied solely on primary treatment, up to 93% of the androgenic and estrogenic compounds analyzed in influent were detected in effluent.

Numerous studies have indicated the induction of vitellogenin in male fish either captured or caged near wastewater treatment plants [11-15]. Vitellogenin is produced in the liver and is used by female fish in the formation of egg yolk and is normally not present in the blood of males. Folmar *et al.* [11] reported reduced serum testosterone and vitellogenin induction in male carp (*Cyprinus carpio*) collected near the St. Paul, MN wastewater treatment plant. Similar effects were also observed in male walleye (*Stizostedion vitreum*) from the same location. Although intersex was not observed, gonads showed less advanced stages of spermatogenesis relative to fish from a reference location.

Harries *et al.* [15] placed male trout in cages at varying distances downstream of five wastewater treatment plants in the United Kingdom. After exposure to effluent for three weeks, the authors demonstrated that male fish were synthesizing vitellogenin on a gradient for up to 5 km downstream in direct response to endocrine disrupting chemicals in the wastewater effluent. Generally fish synthesized more vitellogenin the closer they were to the wastewater treatment plant.

In the U.S. Geological Survey's Biomonitoring of Environmental Status and Trends (BEST) Program, fish health surveys have examined carp as the representative bottom-dweller and largemouth bass (*Micropterus salmoides*) as the representative predator [16]. In a pilot study of the Mississippi, Columbia, and Rio Grande Basins, intersex (oocytes in testes) was observed in

bass from 5 of 35 sites, and vitellogenin was present in male bass from at least 9 sites. Smallmouth bass (*M. dolomieu*) was also used as an alternative species in the Mississippi and Columbia basins.

The Potomac River is the second largest tributary to the Chesapeake Bay. It is an important spawning and nursery ground for both migratory and resident fish species including American eel (*Anguilla rostrata*), striped bass (*Morone saxatilis*), white perch (*Morone americana*), shad and herring (*Alosa* sp.), smallmouth bass, largemouth bass, sunfish (*Lepomis* sp.), and carp. The upper portions of the Potomac estuary in West Virginia and parts of Virginia are dominated by rural communities and animal agricultural facilities. Closer to the Chesapeake Bay, the Potomac watershed becomes more urbanized with agricultural discharges replaced more frequently with municipal wastewater treatment plant discharges. According to the Maryland Department of the Environment, there are 747 surface water discharge permits within the Maryland waters of the Potomac River watershed. Of the 747 permitted discharges, 117 are from municipal wastewater treatment plants [17].

In 2005, smallmouth bass were collected at two locations each on the Monocacy River and Conococheague Creek. Largemouth bass were collected near the Blue Plains Wastewater Treatment Plant on the mainstem in Washington D.C. In addition, passive chemical sampling devices, semi-permeable membrane devices (SPMDs) and polar organic compound integrated samplers (POCIS), were placed upstream and downstream of wastewater treatment plants in the Monocacy River and Conococheague Creek, and downstream of the Blue Plains plant. Iwanowicz *et al* [18] (Chapter I of this report) found that intersex in male smallmouth bass ranged from 82% to 100% with a high degree of severity at theses sites and that male largemouth bass exhibited 23% intersex, also with a high severity index. Alvarez *et al* [19] (Chapter II) reported that several classes of suspected or known endocrine disrupting compounds were detected in the water at all sites. The objective of the current study was to determine if evidence of reproductive impairment could be induced in field deployed hatchery-raised smallmouth bass.

Materials and Methods

The Sites

In this study, we selected three of the four smallmouth bass sites that were previously sampled in 2005 and discussed in Chapters 1 and 2 of this report: the Upper Conococheague, the Lower Conococheague, and the Lower Monocacy (Figure III-1). Funding constraints did not allow us to sample all four sites. We chose to sample both the Upper and Lower Conococheague sites because there were statistically significant differences in gonadosomatic index (GSI) in the 2005 wild-caught smallmouth. The Lower Monocacy site was chosen because it is just below a wastewater treatment plant and had the highest prevalence of gonadal intersex in male fish collected in 2005 (100%). In addition, we chose a reference site located on the NFHRL in Kearneysville, WV because it is relatively free from human impact and is in a secure location.

The Fish

Two hundred hatchery-raised smallmouth bass between 13 cm and 20 cm were purchased from the Jones Fish Hatchery in Newtown, Ohio and transported to the caged sites in a 1900 L fiberglass fish hauling tank. The tank was aerated and temperature ranged from 11.8 ^{O}C to 13.7 ^{O}C during transport.

The Exposure

After being allowed to acclimate to ambient temperature for 30-45 minutes, 50 fish were placed into 115 L polyethylene barrels that had been pre-staged at each site. These cages were designed according to McGee *et al* [20]. The barrels had rectangular windows approximately 0.5 m^2 in size to allow maximum water exchange. The windows were covered by a stainless steel plate with 5 cm inch circular holes. The stainless steel plate was covered by a 33 mm^2 polyethelene mesh which allowed water flow without allowing fish to escape (Figure III-2). The barrels were fixed to a support pole such that they could be raised and lowered as necessary. Barrel covers were locked to prevent tampering. Fish were exposed in stream for 50 days, from April 4 until May 25, 2006, which in other studies was a sufficient time to exhibit vitellogenin induction in males and gonadal intersex [14, 21]. SPMDs and POCIS were deployed for the duration of the caged exposure.

Fish were checked and fed twice weekly using wild-caught mummichogs (*Fundulus heteroclitus*) for the duration of the project. The mummichogs were captured in Deep Cove Creek a small tributary to the Chesapeake Bay in Southern Anne Arundel County, Maryland. Water quality parameters (temperature, pH, conductivity and dissolved oxygen), were measured during each site visit using a Quanta Hydrolab meter (Hach Inc., Loveland, TX). On April 4, before site deployment, 50 fish were removed from the transport tank and placed in a water filled cooler. The first 12 males and 14 females were sacrificed and processed as described in Iwanowicz *et al* [18] (Chapter I). Similarly, on May 25, at the end of the 50-day exposure, the first 9-11 male and 8-12 female fish from each site were removed and processed using the same methods. A necropsy-based assessment similar to that described by Schmitt *et al*. [22] was performed. Grossly visible lesions and abnormalities were recorded. Gonads and liver were removed and weighed for GSI and hepatosomatic index (HSI) determination as follows:

$$GSI = [\text{gonad weight(g)}/(\text{body weight(g)} - \text{gonad weight(g)}] \text{ X } 100$$

$$HSI = [\text{liver weight(g)}/(\text{bodyweight(g)} - \text{liver weight(g)}] \text{ X } 100$$

Gonads and liver were fixed in Z-fix™ (Anatech LTD, Battle Creek, MI) for histological evaluation.

Condition Factor (CF) was measured as described in Schmitt *et al*. [22] and is summarized as follows:

$$CF = [100{,}000 \text{ X } (\text{total length(cm)}/\text{weight(g)}^3)]$$

Laboratory Methods

Fixed gonads were dehydrated in alcohol, infiltrated with paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E) [16]. Gonad sections were examined microscopically, staged and any abnormalities as described in Blazer [3] were ranked from 0 to 4 (absent to severe). Individual oocytes were staged as: stage 1 - immature (nucleolar); stage 2 - early vitellogenic (corticol alveolar); stage 3 - mid-vitellogenic (yolk droplet); stage 4 - mature (yolk hydrates) and

stage 5 - postovulatory follicles. The stage for an ovary was based on the most prevalent oocyte stage present. At least five sections along the ovary were examined. The percent of atretic eggs was determined by counting 200 oocytes and calculating the percent of degenerating or necrotic oocytes. At least five pieces along the length of the testes were sectioned and testicular oocyte prevalence and severity scored as described by Blazer *et al.* [3]. Male gonad stage was scored as: stage 1 - predominantly spermatogonia or spermatocytes; stage 2 - approximately equal portions of spermatocytes, spermatids and spermatozoa; stage 3 - primarily spermatozoa; stage 4 - post-spawn.

Plasma vitellogenin concentrations were measured using a direct enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody 3G2 and were carried out at the University of Florida, Center for Human and Environmental Toxicology as described by Denslow *et al.* [13]. Concentrations of the unknowns were determined from the standard curves and using the Softmax Pro TM Program (Molecular Devices, Sunnyvale, CA). The limit of detection was 0.001 mg/mL. Inter and intra-assay variability were <10%.

At the same time the POCIS and SPMD were collected and shipped at 4 ^{O}C to U.S. Geological Survey, Columbia Environmental Research Center, in Columbia MO for analysis using the methods described in Alvarez *et al* [19] (Chapter II).

Data Analysis

Differences among the treatments for gonad developmental stage, (CF), (HSI), (GSI), and intersex severity were assessed using one-way analysis of variance followed by Tukey's multiple comparison test. If necessary, data were log transformed to meet parametric assumptions. If parametric assumptions were not satisfied, we used the nonparametric Kruskal–Wallis test followed by Dunn's method [23]. Differences among the treatments for percent intersex were compared using an extension of Fisher's exact test. For all procedures, a significance level of 0.05 was used.

RESULTS

Passive Samplers

Analysis of the passive sampler extracts indicated that 59 out of 138 chemicals sampled were detected. Table III-1 through III-5 are summaries of the results of the 2006 SPMD and POCIS sampling effort. See Chapter II for a full discussion of the passive sampler results from both 2005 and 2006.

Male Smallmouth Bass

Gonadal stages ranged from 1 to 3 across all groups including the pre-deployed fish. The median stages of the Lower Conococheague, Upper Conococheague and NFHRL groups were significantly more advanced than the pre-deployed fish (Table III-6). The prevalence of intersex in male fish ranged from 56% in the Lower Conococheague to 83% in the pre-deployed smallmouth bass, with no statistically significant differences among the groups. The median severity of intersex in male fish ranged from 0.4 to 1.0 with no statistically significant differences among the groups. Mean CF ranged from 1.04 in the pre-deployed fish, to 1.26 in the Lower Conococheague. The mean CF in the Lower Conococheague was significantly greater than that for the pre-deployed fish. GSI scores for male smallmouth bass ranged from 0.23 to 0.58 with no statistically significant differences among groups. The mean HSI in the pre-study fish was significantly higher (1.88) than that for both the Lower Conococheague (1.22) and the NFHRL (1.00). Vitellogenin was not detected in any male fish.

Female Smallmouth Bass

Gonadal stages ranged from 1 to 2 across all treatments including the pre-deployed fish with no statistically significant differences among groups (Table III-7). Intersex was not observed in female smallmouth bass at any of the sample locations or in the pre-deployed fish. Mean CF ranged from 1.02 in the pre-deployed bass, to 1.14 in the bass caged at the Lower Monocacy site. The means of the Lower Monocacy and the Lower Conococheague groups were significantly greater than the mean of the pre-deployed fish. Mean GSI in the female smallmouth bass ranged from 0.67 in the pre-deployed fish to 1.06 in the Lower Conococheague site. The mean GSI for female smallmouth bass in the Lower Conococheague was significantly greater than that for all

groups except the NFHRL. Median HSI in female fish ranged from 1.11 at the NFHRL control site to 1.44 at the Upper Conococheague site. Although the Kruskal-Wallis test was significant there were no significant differences among the groups based on Dunn's method. Vitellogenin was not detected in any of the female fish sampled in this study.

DISCUSSION

Although the passive sampler results clearly show exposure to known and suspected endocrine disrupting chemicals, this study was compromised because the fish exhibited intersex prior to exposure. Induction of intersex likely occurs during the critical sexual differentiation period early in life [24, 25]. Since pre-deployed male smallmouth bass had a high degree of intersex, it is likely that these fish were exposed to endocrine disrupting compounds at the hatchery.

Neither male nor female fish had detectable vitellogenin concentrations. In this study, smallmouth bass were in cages at the height of the reproductive season in an effort to induce intersex. Since vitellogenin is a precursor to the production of yolk, it usually is found in its highest concentrations just prior to the spawning season and drops off dramatically making it difficult to detect during the spawning season [26]. As a consequence, we expected to measure lower concentrations of vitellogenin than would be seen at other times of the year. However, the lack of vitellogenin may also be attributed to the fact that it is difficult to detect vitellogenin in fish prior to fully mature stage 4 gonad development [27]. In this study, gonad development in females was generally between stage 1 and 2. Males were found to have gonad stages between 1 and 3. Because these fish were young (approximately 1-yr old) and in such early stages of gonad development they were immature and may not yet be capable of producing vitellogenin.

In general, the CF, GSI, and HSI were similar in the pre-deployed fish and NFHRL control in both male and females. In several treatment sites (male bass in Lower Conococheague and Upper Conococheague, and female bass in the Lower Conoococheague and Lower Monocacy) morphometric indicies were greater than in the pre-deployed fish. This is more likely a factor of feeding rather than an effect of endocrine disrupting compounds. Feeder fish were introduced to the cages twice a week. In most cases, feeder fish were found in the cages from the previous

feeding and maintenance event, suggesting an excess of food. The pre-deployed fish were deliberately not fed five days prior to transport in order to minimize waste production during transport.

Although this study was inconclusive as to whether short term exposure of hatchery-raised fish can be used to induce the same biological response as the wild caught fish, it did highlight the fact that hatchery-raised smallmouth bass may have a high prevalence of intersex. To our knowledge, this is the first time that intersex has been identified in hatchery raised fish. Additional investigations are necessary to determine the cause and extent of intersex in hatchery-raised bass and the impact this may have on fish populations in stocked water bodies. In general, more information is needed on possible population impacts of intersex on both hatchery-raised and wild bass.

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Figure III-1 Map of Potomac River watershed indicating the 2006 sampling locations. NFHRL – National Fish Health Research Laboratory, Kearneysville, West Virginia (reference site). 2006 sample.

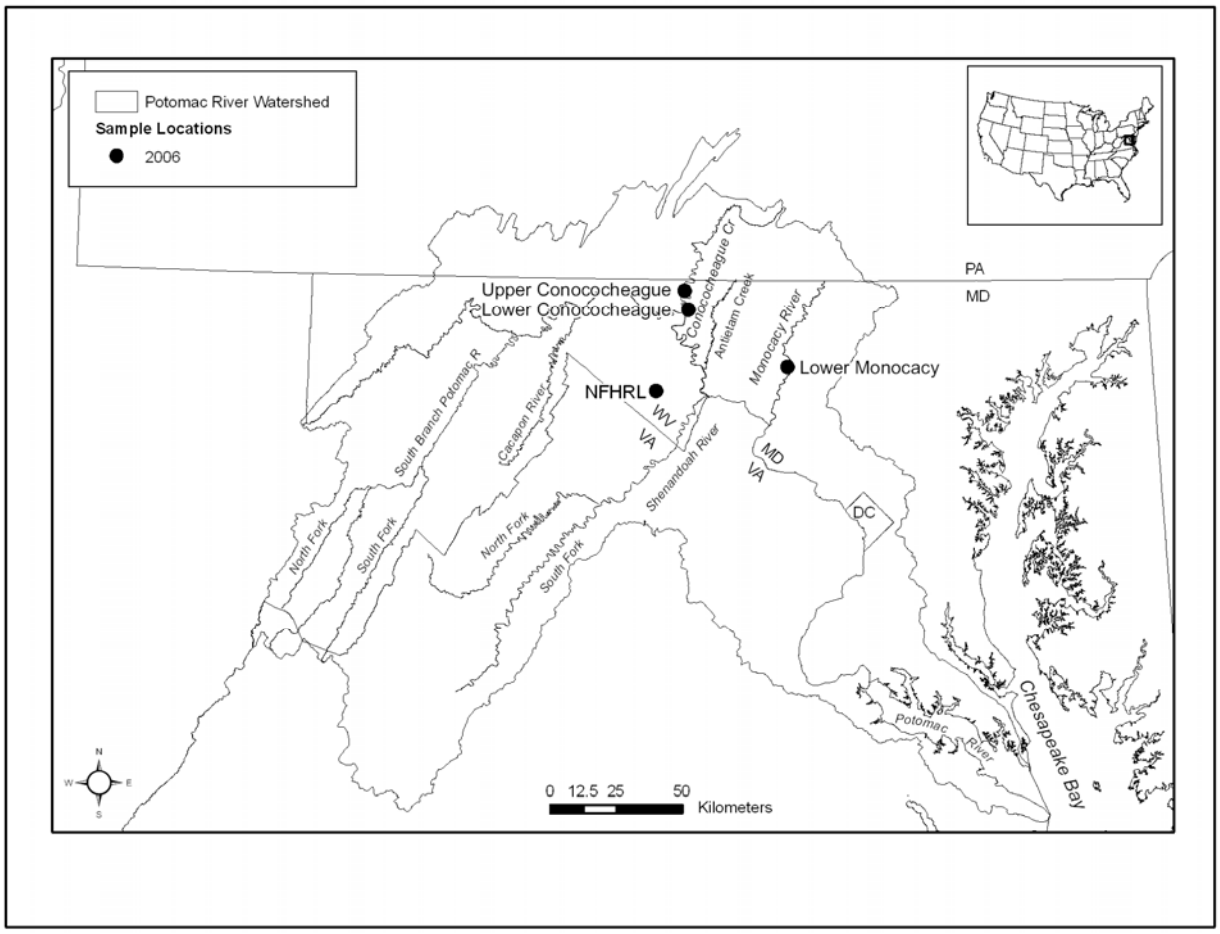

Figure III-2. 115 Liter polyethylene fish cage at the National Fish Health Research Laboratory.

Table III-1: Summary of organochlorine pesticides and total PCBs from the 2006 sampling in the Potomac River watershed

[Up C Creek – Upstream Conococheague Creek, Down C Creek – Downstream Conococheague Creek, Mon River – Monocacy River, NFHRL Ref – National Fish Health Research Laboratory reference site, pg/L – picograms per liter]

	Up C Creek #1	Up C Creek #2	Down C Creek #1	Down C Creek #2	Mon River #1	Mon River #2	NFHRL Ref #1	NFHRL Ref #2
Target Chemicals	pg/L	pg/L	pg/L	pg/L	pg/L	pg/L	pg/L	pg/L
Trifluralin	**3.4**	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
Hexachlorobenzene	**38**	**38**	**40**	**41**	*18*	*17*	<14	<14
Pentachloroanisole	<120	<120	<120	<120	<120	<120	<120	<120
a-Benzenehexachloride	<210	<210	<210	<210	<210	<210	<210	<210
Diazinon	<24000	<24000	<24000	<24000	<24000	<24000	<24000	<24000
Lindane	<540	<540	<540	<540	<540	<540	<540	<540
b-Benzenehexachloride	<140	<140	<140	<140	*170*	*160*	<140	<140
Heptachlor	<1.9	<1.9	**53**	**54**	**7**	**6.8**	<1.9	<1.9
d-Benzenehexachloride	<2.5	<2.5	**29**	**28**	**28**	**29**	<2.5	<2.5
Dacthal	<150	<150	<150	<150	<150	<150	<150	<150
Chlorpyrifos	**120**	**110**	**180**	**170**	**190**	**120**	**290**	**260**
Oxychlordane	*1.6*	<0.5	**8.8**	**9.9**	**4.5**	*1.4*	*2*	*0.8*
Heptachlor Epoxide	*45*	*43*	**62**	**66**	*40*	*34*	*35*	<33
trans -Chlordane	**20**	**20**	**65**	**68**	**33**	**26**	*10*	<7.5
trans -Nonachlor	*48*	*46*	*57*	*59*	*45*	<37	<37	<37
o,p'-DDE	**12**	**13**	**8.5**	**9.3**	**11**	**10**	*5*	*4.4*
cis -Chlordane	**23**	**24**	**50**	**54**	**39**	**31**	*7.1*	<4.7
Endosulfan	<250	*270*	*490*	**610**	*340*	*260*	*420*	<250
p,p'-DDE	**82**	**78**	**87**	**88**	**62**	**52**	*34*	<27
Dieldrin	**120**	**130**	**200**	**200**	**160**	**130**	**22**	*15*
o,p'-DDD	*41*	*32*	*45*	**47**	*46*	*34*	<19	<19
Endrin	**53**	**55**	**87**	**88**	**60**	**58**	**22**	**20**
cis-Nonachlor	<10	<10	*13*	*14*	<10	<10	<10	<10
o,p'-DDT	<8.4	<8.4	*14*	**16**	**110**	**86**	<8.4	<8.4
p,p'-DDD	**21**	**22**	**37**	**35**	**25**	**26**	**9**	**9**
Endosulfan-II	<900	<900	**2700**	**3000**	<900	*1000*	<900	<900
p,p'-DDT	*110*	*100*	*110*	*110*	<90	<90	<90	<90
Endosulfan Sulfate	<670	<670	<670	<670	<670	<670	<670	<670
p,p'-Methoxychlor	<20	<20	<20	*21*	*32*	*24*	<20	<20
Mirex	**5.3**	<0.8	<0.8	<0.8	*3.8*	<0.8	<0.8	<0.8
cis -Permethrin	*10*	*7*	<7.0	<7.0	<7.0	<7.0	<7.0	<7.0
trans -Permethrin	<150	<150	<150	<150	<150	<150	<150	<150
Total PCB	**4300**	**3400**	*590*	*570*	**830**	**750**	<210	<210

< values are below the method detection limit (MDL).

Italic values are estimates greater than the MDL but less than the method quantitation limit (MQL) and shown for informational purposes only.

Bold values are reportable values greater than the MQL.

Table III-2: Summary of polycyclic aromatic hydrocarbons (PAHs) from the 2006 sampling in the Potomac River watershed.

[Up C Creek – Upstream Conococheague Creek, Down C Creek – Downstream Conococheague Creek, Mon River – Monocacy River, NFHRL Ref – National Fish Health Research Laboratory reference site, pg/L – picograms per liter]

	Up C Creek #1	Up C Creek #2	Down C Creek #1	Down C Creek #2	Mon River #1	Mon River #2	NFHRL Ref #1	NFHRL Ref #2
Target Chemicals	pg/L	pg/L	pg/L	pg/L	pg/L	pg/L	pg/L	pg/L
Naphthalene	<140	<140	<140	<140	<140	<140	<140	<140
Acenaphthylene	<28	<28	<28	<28	<28	<28	<28	<28
Acenaphthene	**320**	**420**	**350**	**330**	**530**	**420**	**300**	**340**
Fluorene	**160**	**160**	**120**	**140**	**330**	**260**	**91**	**110**
Phenanthrene	**1200**	**1200**	**960**	**990**	**3500**	**3100**	**520**	**500**
Anthracene	**130**	**130**	**100**	**98**	**270**	**190**	*36*	*43*
Fluoranthene	**900**	**870**	**800**	**810**	**5900**	**4900**	**100**	**99**
Pyrene	**530**	**460**	**460**	**5200**	**3800**	**3100**	<21	<21
Benz[a]anthracene	<1.8	<1.8	**140**	**140**	**240**	**220**	<1.8	<1.8
Chrysene	**240**	**240**	**170**	**150**	**1900**	**1500**	*13*	<7.2
Benzo[b]fluoranthene	<5.2	<5.2	<5.2	<5.2	<5.2	<5.2	<5.2	<5.2
Benzo[k]fluoranthene	**120**	**110**	**100**	**92**	**750**	**740**	<5.7	<5.7
Benzo[a]pyrene	**30**	*27*	*24*	*24*	**130**	**120**	<6.0	<6.0
Indeno[i,2,3-c,d]pyrene	*34*	<7.2	*21*	<7.2	**78**	**74**	<7.2	<7.2
Dibenz[a,h]anthracene	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4	<6.4
Benzo[g,h,i]perylene	<7.9	<7.9	<7.9	<7.9	**98**	**86**	<7.9	<7.9
Benzo[b]thiophene	<530	<530	<530	<530	<530	<530	<530	<530
2-methylnaphthalene	<270	<270	<270	<270	<270	<270	<270	<270
1-methylnaphthalene	*300*	*300*	<180	*260*	*190*	<180	<180	<180
Biphenyl	<42	<42	<42	<42	<42	<42	<42	<42
1-ethylnaphthalene	<15	<15	<15	<15	<15	<15	<15	<15
1,2-dimethylnaphthalene	<18	<18	<18	<18	*61*	<18	<18	<18
4-methylbiphenyl	<9.2	<9.2	**600**	<9.2	**280**	**240**	**320**	**400**
2,3,5-trimethylnaphthalene	<7.4	<7.4	<7.4	<7.4	<7.4	<7.4	<7.4	<7.4
1-methylfluorene	**51**	<6.9	<6.9	<6.9	**250**	**210**	<6.9	<6.9
Dibenzothiophene	**78**	*72*	*53*	*60*	**240**	**180**	<15	<15
2-methylphenanthrene	**180**	**180**	**150**	**170**	**720**	**590**	<7.4	<7.4
9-methylanthracene	<6.1	<6.1	<6.1	<6.1	**30**	*27*	<6.1	<6.1
3,6-dimethylphenanthrene	<5.4	<5.4	<5.4	<5.4	<5.4	<5.4	<5.4	<5.4
2-methylfluoranthene	**36**	**32**	**37**	**34**	**220**	**210**	<5.4	<5.4
d]thiophene	*27*	*23*	**31**	<5.6	**300**	**270**	<5.6	<5.6
Benzo[e]pyrene	**95**	**77**	**73**	**74**	**420**	**350**	<6.1	<6.1
Perylene	**65**	**44**	**44**	**47**	**46**	**43**	<5.5	<5.5

< values are below the method detection limit (MDL).

Italic values are estimates greater than the MDL but less than the method quantitation limit (MQL) and shown for informational purposes only.

Table III-3: Summary of hormones from the 2006 sampling in the Potomac River watershed.

[Up C Creek – Upstream Conococheague Creek, Down C Creek – Downstream Conococheague Creek, Mon River – Monocacy River, NFHRL Ref – National Fish Health Research Laboratory reference site, pg/L – picograms per liter]

	Up C Creek #1	Up C Creek #2	Down C Creek #1	Down C Creek #2	Mon River #1	Mon River #2	NFHRL Ref #1	NFHRL Ref #2
Target Chemicals	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L
17b-Estradiol	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
17a-Ethynylestradiol	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
Estrone	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
Estriol	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5

< values are below the method detection limit (MDL).

Table III-4: Summary of agricultural pesticides from the 2006 sampling in the Potomac River watershed.

[Up C Creek – Upstream Conococheague Creek, Down C Creek – Downstream Conococheague Creek, Mon River – Monocacy River, NFHRL Ref – National Fish Health Research Laboratory reference site, pg/L – picograms per liter]

	Up C Creek #1	Up C Creek #2	Down C Creek #1	Down C Creek #2	Mon River #1	Mon River #2	NFHRL Ref #1	NFHRL Ref #2
Target Chemicals	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L	ng/L
EPTC	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)
Trifluralin	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)	NC (<26 ng/POCIS)
Desisopropylatrazine	*2.8*	*2.8*	*2.8*	*2.8*	*2.8*	*2.8*	**15**	**14**
Desethylatrazine	**20**	**16**	**22**	**18**	**15**	**7.1**	**71**	**60**
Atraton	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
Prometon	*1.4*	*0.9*	<0.45	*1.4*	**1.8**	<0.45	<0.45	<0.45
Simazine	**17**	**17**	**19**	**17**	**54**	**22**	**7.4**	**7.4**
Atrazine	**65**	**54**	**77**	**49**	**240**	**200**	**23**	**23**
Propazine	<8.4	<8.4	<8.4	<8.4	<8.4	<8.4	<8.4	<8.4
Diazinon	<1.9	<1.9	<1.9	<1.9	<1.9	<1.9	<1.9	<1.9
Terbuthylazine	<0.72	<0.72	<0.72	<0.72	<0.72	<0.72	<0.72	<0.72
Fonofos	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)	NC (<20 ng/POCIS)
Acetochlor	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Alachlor	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2
Metribuzin	<0.24	<0.24	<0.24	<0.24	<0.24	<0.24	<0.24	<0.24
Prometryn	<4.2	<4.2	<4.2	<4.2	<4.2	<4.2	<4.2	<4.2
Simetryn	<5.4	<5.4	<5.4	<5.4	<5.4	<5.4	<5.4	<5.4
Ametryn	<7.2	<7.2	<7.2	<7.2	<7.2	<7.2	<7.2	<7.2
Methyl Parathion	<0.33	<0.33	<0.33	<0.33	<0.33	<0.33	<0.33	<0.33
Terbutryn	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4
Malathion	<8.5	<8.5	<8.5	<8.5	<8.5	<8.5	<8.5	<8.5
Metolachlor	**7.9**	**7**	**12**	**6**	**99**	**94**	<0.90	<0.90
Dacthal	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)	NC (<29 ng/POCIS)
Chlorpyrifos	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)	NC (<19 ng/POCIS)
Pendimethalin	<6.9	<6.9	<6.9	<6.9	<6.9	<6.9	<6.9	<6.9
Fipronil	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)	NC (<2 ng/POCIS)

< values are below the method detection limit (MDL).
Italic values are estimates greater than the MDL but less than the method quantitation limit (MQL) and shown for informational purposes only.
Bold values are reportable values greater than the MQL.

Table III-5: Summary of waste water compounds and agricultural pesticides from the 2006 sampling in the Potomac River watershed.

[Up C Creek – Upstream Conococheague Creek, Down C Creek – Downstream Conococheague Creek, Mon River – Monocacy River, NFHRL Ref – National Fish Health Research Laboratory reference site, pg/L – picograms per liter]

Target Chemicals	Up C Creek #1 ng/POCIS	Up C Creek #2 ng/POCIS	Down C Creek #1 ng/POCIS	Down C Creek #2 ng/POCIS	Mon River #1 ng/POCIS	Mon River #2 ng/POCIS	NFHRL Ref #1 ng/POCIS	NFHRL Ref #2 ng/POCIS
Tetrachloroethylene	<20	<20	<20	<20	<20	<20	<20	<20
Bromoform	<20	<20	<20	<20	<20	<20	<20	<20
Isopropylbenzene (cumene)	<20	<20	<20	<20	<20	<20	<20	<20
Phenol	<20	<20	<20	<20	<20	<20	<20	<20
1,4-Dichlorobenzene	<20	<20	<20	<20	<20	<20	<20	<20
d-Limonene	<20	<20	<20	<20	<20	<20	<20	<20
Acetophenone	<20	<20	<20	<20	<20	<20	<20	<20
para-Cresol	<100	<100	<100	<100	<100	<100	<100	<100
Isophorone	<20	<20	<20	<20	<20	<20	<20	<20
Camphor	<20	<20	<20	<20	<20	<20	<20	<20
Menthol	<20	<20	<20	<20	<20	<20	<20	<20
Methyl salicylate	<20	<20	<20	<20	<20	<20	<20	<20
Dichlorvos	<20	<20	<20	<20	<20	<20	<20	<20
Isoquinoline	<20	<20	<20	<20	<20	<20	<20	<20
Indole	<20	<20	<20	<20	<20	<20	<20	<20
N,N-diethyltoluamide (DEET)	*60*	*50*	*70*	*60*	**110**	**130**	*40*	*40*
Diethyl phthalate	<20	<20	<20	<20	<20	<20	<20	<20
4-*tert*-Octylphenol	<20	<20	<20	<20	<20	<20	<20	<20
Benzophenone	*30*	<20	<20	<20	*50*	*40*	<20	<20
Tributyl phosphate	<20	<20	**200**	**200**	**210**	**220**	<20	<20
Ethyl citrate	**110**	**110**	**140**	**120**	**290**	**360**	**100**	<20
Cotinine	<100	<100	<100	<100	<100	<100	<100	<100
Celestolide (ADBI)	<20	<20	<20	<20	**130**	<20	<20	<20
Prometon	**100**	*90*	**120**	**100**	**120**	**120**	<20	<20
Atrazine	**5000**	**3900**	**6100**	**4100**	**24000**	**25000**	**1300**	**1500**
Phantolide (AHMI)	70	<20	*70*	<20	*80*	*80*	<20	<20
4-*n*-Octylphenol	<100	<100	<100	<100	<100	<100	<100	<100
Tri(2-chloroethyl) phosphate	*60*	<20	**100**	*60*	**160**	**200**	*60*	<20
Diazinon	<20	<20	<20	<20	<20	<20	<20	<20
Carbazole	<20	<20	<20	<20	**190**	**210**	<20	<20
Caffeine	<20	<20	<20	<20	<20	<20	<20	<20
Traseolide (ATII)	<20	<20	<20	<20	**150**	**150**	<20	<20
Galaxolide (HHCB)	<20	<20	*30*	<20	**1700**	**2100**	<20	<20
Tonalide (AHTN)	<20	<20	<20	<20	**200**	**250**	<20	<20
Carbaryl	<100	<100	<100	<100	<100	<100	<100	<100
Metalaxyl	<20	<20	<20	<20	<20	<20	<20	<20
Bromacil	<100	<100	<100	<100	<100	<100	<100	<100
Anthraquinone	<20	<20	<20	<20	<20	<20	<20	<20
Chlorpyrifos	<20	<20	<20	<20	<20	<20	<20	<20
Tri(dichloroisopropyl) phosphate	**240**	**250**	**260**	**250**	**470**	**530**	<20	**220**
Tri(butoxyethyl) phosphate	<100	<100	<100	<100	<100	<100	<100	<100
Triphenyl phosphate	<52	<52	<52	<52	*70*	*70*	<52	<52
Diethylhexylphthalate (DEHP)	*390*	*330*	**750**	**470**	*350*	*330*	**680**	**450**
Cholesterol	<100	<100	<100	<100	<100	<100	<100	<100

< values are below the method detection limit (MDL).

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Bold values are reportable values greater than the MQL.

Table III-6: Summary of results for the 2006 caged study: male smallmouth bass[a]

Site	Pre-Study	NFHRL control	Lower Conococheague	Upper Conococheague	Lower Monocacy	Statistic
Sample Size	12	11	9	9	10	
Gonad Stage	1.00B[b,c]	3.00(1.00-3.00)A	3.00(2.00-3.00)A	3.00(2.00-3.00)A	2.00(1.00-2.00)A,B	K-W, p <0.001
HSI	1.88 ± 0.21A	1.00 ± 0.06C	1.22 ± 0.08B,C	1.42 ± 0.06A,B	1.36 ±0.10A,B	ANOVA, p <0.001[d]
CF	1.04 ± 0.01B	1.12 ± 0.04AB	1.26 + 0.05A	1.09+ 0.04B	1.15 ± 0.04AB	ANOVA, p = 0.002
GSI	0.58 ±0.30	0.31 ± 0.05	0.45 ± 0.08	0.37 ±0.08	0.23 ± 0.04	ANOVA, p = 0.15[d]
Prevelance of Intersex	83%	73%	56%	89%	70%	FE, p = 0.50
Severity of Intersex	0.8(0.2-2.6)	1.0(0.0-2.6)	0.9(0.0-2.0)	0.4(0.4-2.4)	0.6(0-1.4)	K-W, p = 0.75

[a] Mean ± Standard Error or median with minimum and maximum in parentheses.

[b] Groups with different letters are significantly different at p= <0.05 Tukey's test (analysis of variance [ANOVA])
Dunn's method (Kruskal-Wallis test [K-W]), or Fisher's Exact test (FE).

[c] All fish in pre-study sample were in stage 1 gonad development.

[d] Data log transformed.

Table III-7: Summary of results for the 2006 caged study: female smallmouth bass[(a)].

Site	Pre-Study	NFHRL control	Lower Conococheague	Upper Conococheague	Lower Monocacy	Statistic
Sample Size	14	8	10	12	10	
Gonad Stage	1.00(1.00-2.00)	1.00(1.00-2.00)	2.00(1.00-2.00)	1.00(1.00-2.00)	1.00(1.00-2.00)	K-W, p =0.078
HSI	1.40(1.18-4.05)	1.11(0.82-1.43)	1.24(0.83-7.60)	1.44(0.89-2.22)	1.12(0.47-1.53)	K-W, p =0.014
CF	1.02 $\pm$ 0.02B[(b)]	1.11 $\pm$ 0.02AB	1.11 $\pm$ 0.03A	1.06 $\pm$ 0.03AB	1.14 $\pm$ 0.02A	ANOVA, p =0.004
GSI	0.67 $\pm$ 0.04B	0.76 $\pm$ 0.06AB	1.06 $\pm$ 0.16A	0.68 $\pm$ 0.07B	0.40 $\pm$ 0.10B	ANOVA, p =<0.001

[(a)] Mean $\pm$ Standard Error or median with minimum and maximum in parentheses.

[(b)] Groups with different letters are significantly different at p= <0.05 Tukey's test (analysis of variance [ANOVA]) or Dunn's method (Kruskal-Wallis test [K-W]).