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A reconnaissance of the intersex condition in Micropterus dolomieu of the Upper Ohio River Basin as an indicator of anthropogenic endocrine disruptors

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A reconnaissance of the intersex condition in *Micropterus dolomieu* of the Upper Ohio River Basin as an indicator of anthropogenic endocrine disruptors

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Abstract

The intersex condition has been used as a marker of endocrine disruption in West Virginia. Fifty-two male smallmouth bass from 6 basins on the upper Ohio River were examined for the presence and severity of intersex. Vitellogenin (VTG) RNA levels were quantified as marker of estrogenic contaminants. A significant increase in intersex ($p=0.02$), ranging from 83.3% to 100%, corresponds with higher population densities and agricultural land ($p=0.003$). At reference sites, smallmouth bass had intersex frequencies of 16.7% to 33.3%. Vitellogenin results were significantly lower ($p< 0.001$) at impacted sites, consistent with previously reported results in female fish. VTG levels in males were not significantly different. Increased intersex occurrence in males and lowered VTG expression in females have shown that agricultural chemicals and common residential wastewater constituents are likely targets for exploring the relationship between individual EDCs and intersex. There may be a natural background level of the intersex condition.

Summary

Since the publication of the first nationwide reconnaissance of emerging contaminants in 2002 by the USGS, the apparent ubiquity of endocrine disrupting contaminants (EDCs) in the surface waters of the US has spurred research into the various endpoints that can be used to quantify their effects. The list of known EDCs is long, with various sources and intended uses, not to mention the many chemicals in our surface waters whose endocrine disrupting potential is unknown. Analysis of concentrations of EDCs in aquatic systems, the effect of these mixtures on responsive cell types, and controlled exposure studies in vertebrates are being conducted, but replicating complex environmental conditions and mixtures presents myriad difficulties when attempting to transfer this knowledge to real-world situations. However there are several clear markers of feminization, a specific type of endocrine disruption, which account for the net effect of all EDCs acting in concert in aquatic organisms.

The intersex condition, or ovotestis, was observed for the first time locally in smallmouth bass (*Micropterus dolomieu*) of the Potomac River basin in 2003, and has been used as an indicator of endocrine disruption in the surface waters of West Virginia and beyond. Several mechanisms have been shown to result in ovotestis in controlled experiments including direct interaction with the estrogen receptor, and interference in hormone biosynthesis, metabolism, bioavailability and transport. The observable endpoint is evident at the histological level as immature oocytes in the gonads of male fish. However, little is known about the prevalence of this condition and as a result, inferring its most significant causes has been a slow process. An intersex survey, of a broad geographic scale, is useful to show where higher incidences of intersex correspond to specific environmental variables.

In this study, 52 male smallmouth bass from 6 basins on the upper Ohio River were examined for the presence and severity of intersex including the Kanawha (including upper Elk, upper Gauley, and upper Greenbrier) and Muskingum (including Tuscarawas, Walhonding, and main stem) watersheds. West Virginia sites (Kanawha watershed) are intended reference sites with relatively unimpacted water quality while Ohio sites (Muskingum watershed) represent surface waters moderately impacted by agricultural land uses and higher population densities. Vitellogenin (VTG) RNA levels were also quantified in collected fish as a second proven marker of aquatic environments with estrogenic activity. Research has shown this egg yolk precursor protein, normally expressed in female fish, is often elevated in male fish exposed to estrogenic contaminants, and lowered in female fish in the same environments. As hypothesized, a significant increase in intersex occurrence ($p=0.02$), ranging from 83.3% to 100%, was found to correspond with markers of anthropogenic stressors including higher population densities and significantly increased ($p=0.003$) agricultural land at impacted sites. However, it was also discovered that even at reference sites, smallmouth bass were found to have intersex at frequencies of 16.7% to 33.3%. Vitellogenin results were only consistent with expectations based on previously reported results in female fish. That is, VTG levels were significantly lower at impacted sites in female fish ($p< 0.001$). VTG levels in males were not significantly different.

While the predicted increase of intersex occurrence in males and lowered VTG expression in females at impacted sites supports the use of ovotestis as an indicator of anthropogenic EDCs, explaining other results might require further research. With a minimum intersex occurrence of 16.7% at reference sites, investigation of additional sites, perhaps in roadless wilderness areas, could show whether we should ever expect a non-zero natural baseline

occurrence of ovotestis. The existence of reference sites with zero intersex would then require an explanation of causes of endocrine disruption at our reference sites. If current terrestrial sources of EDCs appear insignificant, as at these reference sites, other routes of their introduction such as atmospheric deposition or highly-persistent historical sources may need to be investigated. Concurrent time-integrated water sampling should also be conducted at future fish collection sites for chemical and/or bioassay analysis so that significant correlations between estrogenic activity, specific EDCs, intersex, and VTG can be shown when they exist. The results of this study have shown that agricultural chemicals and common residential wastewater constituents are likely targets for exploring the cause/effect relationship between individual EDCs and intersex, but that there may still exist a natural background level of the intersex condition.

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Introduction and Literature Review

What are EDCs and how do they work?

Endocrine disrupting compounds (EDCs) comprise a broad spectrum of chemicals that interfere with an organism's endocrine system or its chemical communication network responsible for regulating nearly all of the body's physiological processes. EDCs can be organized by their intended use, such as disinfectants, plasticizers, and pharmaceuticals or by chemical structure, including PAHs (polyaromatic hydrocarbons), PCBs (polychlorinated biphenyls), phenols, and phthalates[1, 2]. A representative set of suspected EDCs is presented in the appendix[3, 4].

Hormones, the chemical messengers of the endocrine system, are often structurally mimicked by EDCs and thus can modulate biochemical signaling pathways, even in the absence of the endogenous hormone. In other cases, the EDC works to disrupt normal hormone-receptor binding by antagonistically blocking access to a particular receptor, preventing the natural hormone from performing its intended function[5]. Two of the better-understood hormones in the human endocrine system are estrogen and androgen. The commonly female-associated sex steroid estrogen and the male-associated sex steroid androgen are responsible for sexual differentiation and control essential functions of the reproductive system in individuals of *both* sexes. Of these, estrogen has received more attention in environmental EDC studies. For example, in the estrogen pathway EDCs are known to affect hormone synthesis, intermediate transport mechanisms, metabolism and destruction[5].

Routes of exposure

While the release of most EDCs into surface waters is currently unregulated, untracked, and sometimes unintentional, they may enter the environment *via* point or non-point sources. Point sources such as effluent from industrial discharges may be monitored depending on the type of permit, though accidental spills and other point sources may go completely untracked. Non-point sources too, including atmospheric deposition and runoff from impervious surfaces and land applications of waste products, are largely unmonitored. When they do exist, guidelines for responsible emissions of EDCs are determined on an individual basis, using what is known about the additive effects of single chemicals on humans and wildlife, and not taking into account synergistic or antagonistic effects where, for example, two or more EDCs have been shown to have effects different in magnitude than the simple addition of their individual effects alone [6]. Furthermore, because EDCs are so varied in chemical nature and intended use, multiple routes of their introduction into surface waters must be considered before solutions can be explored. For example, in this study, where we seek to find if endocrine disruption is occurring where there are no known point or non-point sources directly introducing EDCs into the streams, perhaps another route, such as atmospheric deposition, plays an important role. Because some EDCs are combustion byproducts, this is not an unreasonable proposition.

History of EDC studies in aquatic systems

The history of known endocrine disruptors in the environment began in 1976 with dichloro-diphenyl-trichloroethane (DDT) [7] Introduced in 1947, DDT was later banned in the United States in the 1970s. It has been shown to have endocrine disrupting properties through both estrogen receptor(ER)-dependent and ER-independent mechanisms in humans [8, 9], as well as to have feminizing effects in fish[10] and other vertebrates[11,12] up to and including permanent sex reversal from male to fertile female[13]. Since DDT and the modern industrial chemical revolution, myriad pollutants have been introduced into our waterways, air, and other environmental media. While initial regulations were aimed at reducing the amount of contaminants with obvious and acute toxic effects, we have recently seen more obscure chemically-induced effects reported in the news. There is a recent example of this locally in the upper Ohio River Basin (ORB) with legal battles and medical research on the carcinogenic properties of the chemical C8, or the ammonium salt of PFOA (perfluorooctanoic acid), which is used in the manufacture of Teflon and has been shown to have leaked into the water supplies of several communities [14]. Endocrine disruptors are similar in that their effects will likely take years to manifest themselves in humans, and, with all the confounding factors, may not be easily traced back to point or non-point sources. Still, they are already one of the next big issues in water quality news[15, 16].

Geographically, endocrine disruptors have been studied all over the globe, though more intensively in North America, Europe, and Australia, leaving conditions and levels in the developing world largely unknown [4].

The most comprehensive survey of EDCs in the United States was done as part of a larger study on an extended selection of organic wastewater contaminants (OWCs), conducted by the US Geological Survey (USGS) in 1999-2000 [3]. Closer to the ORB, there have been relatively few large river studies focusing on the Mississippi River Basin.

Research carried out by various state, federal, and academic research groups in the upper ORB, and in West Virginia specifically, is easier to fully describe. . The most extensive research was conducted by fish pathologist Dr. Vicki Blazer's fish health laboratory at the USGS

Leetown Science Center. Dr. Blazer has found numerous occurrences of the intersex condition throughout the Potomac watershed in West Virginia, Maryland, and Virginia[17], [18]. In 2003 and 2004, when their group began seeing oocytes in many of the male gonads, the intersex issue was brought to the public's attention and has caused much concern in West Virginia, and perhaps even more in downstream communities in Maryland, Virginia, and Washington DC[15,16,19]. Since that first discovery, collaborations have been formed with government and academic researchers across the state. The USGS Charleston, WV office has conducted water chemistry sampling of potential EDCs using semipermeable membrane device and polar organic compound integrative sampler types of passive samplers[20]. The West Virginia Department of Environmental Protection (WVDEP) has provided funding and support for fish collections. The US Army Corps of Engineers (USACE), Huntington District has also secured funding for this thesis project in particular, as well as participating in fish collection. Vitellogenin analyses have been conducted by the US Fish and Wildlife Service (USFWS) [21] (see below for more on vitellogenin).

Endpoints for detection and quantification

The effects of EDCs have been studied in a wide variety of animals other than fish including invertebrates (mussels, snails), reptiles, amphibians, birds and mammals[18].

Histology

The characteristic endpoint of endocrine disruption in this study is the intersex condition, also known as ovotestis. It is a condition where female reproductive cells, namely oocytes, are found within the male gonad. In species such as *Micropterus dolomieu* and *Ambloplites rupestris*, individuals are normally dioecious, or gonochoristic, and ovotestis is thought not to occur in the absence of anthropogenic stimuli. Therefore, the presence of oocytes in the testis is considered a response to estrogenic contaminants which interfere with normal sexual development and differentiation. Many questions remain, including: Is it possible that there *is* a low natural level of the intersex condition in these fish as shown in other species [22]? If so, the increased frequency of this condition is likely an indication of endocrine disruption, but further surveys are required to establish baseline expected levels of intersex occurrence in pristine waters. Also, which species are most likely to develop intersex given comparable concentrations of environmental EDCs? Studies examining multiple species from the same location do not show an even distribution of intersex, indeed some species show no signs of the condition[23].

<u>Biochemical assays</u>

Other endpoints of endocrine disruption being used to assess these contaminants are biochemical markers such as vitellogenin, the sex steroids estradiol and testosterone[24] , detection of bioaccumulated EDCs *via* antibody and enzyme techniques[25,26, 27], and even cortisol as a measure of immunosuppression *via* endocrine disruption. Vitellogenin (VTG) is an egg yolk protein precursor which is normally synthesized in the liver of female fish after estrogen stimulation [28]. It is used as a quantifiable endpoint for endocrine disruption by estrogen-mimics. RNA extracted from the fishes' livers is reverse-transcribed to DNA, and then with a primer specific to smallmouth bass vitellogenin, the amount of RNA for this protein can be quantified.

Analytical chemistry

More directly, chemical detection of EDCs in bulk or temporally integrated water samples is also possible *via* biochemical and analytical chemistry techniques[29]. Myriad EDCs have been studied in relation to endocrine disruption effects (intersex and feminization), including instances where these physiological effects have been intentionally induced in lab and field exposure studies[30,31]. Less focus was given to this approach in the current project because, with so little information about effective concentrations for impacts in fish, which EDCs exactly are responsible for the impacts from river to river, the effect of complex mixtures, and what exactly those effects are on each species, finding individual contaminants in the waters surveyed provides little help in predicting the prevalence of the intersex condition.

Examining human impact more broadly

Because larger scale surveys for EDCs have shown them to be surprisingly ubiquitous [3], it seems very likely that endocrine disruption in aquatic wildlife is not the result of isolated spills or point sources of contaminants alone. More likely, the contaminants themselves are also widespread in occurrence and use. For this reason we look for broader relationships here between different types of human influence and endocrine disruption in fish. Potential contributors of endocrine disrupting contaminants (EDCs) to the watershed were targeted using a spatial analysis of land cover types and known point sources. Suspected land-use types contributing EDCs include urbanized and agricultural areas because previous studies biased toward sampling downstream of these influences have shown high prevalence of organic wastewater contaminants [3]. More specifically, wastewater treatment plants (WWTPs) have been suspected as a major source within urbanized areas and shown to be such [32-33].

I hypothesize that increased population density and land development at impacted sites (referred to as the Ohio site group) will result in higher incidences of intersex, increased vitellogenin levels in males, and decreased vitellogenin levels in females when compared to reference sites located in West Virginia.

Methods

Study site description

Sites were coded using an adaptation of the WV DEP's "AN code" which identifies each site uniquely and describes where its water flows. For example, OKNG-79-(2.5) stands for "Ohio – Kanawha - New - Greenbrier – 79 – (2.5 miles) because the site was 2.5 miles from the mouth of the West Fork (of the Greenbrier), where it forms the main stem of the Greenbrier and then passes 78 other tributaries on the way to the mouth. The Greenbrier then flows into the New River, which flows into the Kanawha River, which finally flows to the Ohio. Census and land cover data aided in site selection to ensure a clear difference in population densities and degree of land development between the impacted site group and reference sites.

Two large watersheds within the Ohio River Basin were studied. The further upstream of the two watersheds sampled, the Muskingum River watershed contains three sites intended to represent agriculturally and residentially impacted waters including the Walhonding River at Warsaw, OH [OMW-(11.3)] and the Tuscarawas River at New Philadelphia [OMT-(57.9)] and at Dover, OH [OMT-(54.5)]. The Muskingum is the largest watershed in the state of Ohio, draining approximately 8,038 square miles. The Tuscarawas and Walhonding rivers are the two primary tributaries of the Muskingum, with drainages of 2,590 and 2,252 square miles, respectively. Muskingum sites comprise more agricultural land uses and moderate-density residential areas to contrast with less agriculture and low population density in the West Virginia sites (Figure 1).

The other tributary of the Ohio sampled was the Kanawha River watershed, the largest basin in West Virginia with a drainage area of approximately 12, 236 square miles. Intended to represent reference conditions, Kanawha basin sites were selected higher in the watershed and perceived to contain little to no human impacts. They include: the upper Gauley River (above Lake Summersville) at Cowen, WV [OKG-(86.9)]; the Back Fork of the Elk River near its mouth at Webster Springs, WV [OKE-111-(0.5)]; and the West Fork of the Greenbrier River at Durbin, WV [OKNG-79-(2.5)]. The Durbin site on the West Fork of the Greenbrier was sampled as a combination of two sites, separated by less than a mile. The West Fork of the Greenbrier River was predicted to have the best water quality and lowest human influence because of its location in the relatively undisturbed Monongahela National Forest. Indeed, the West Fork is a known trout stream carrying brook trout according to WV DEP records.

We also examined more traditional water quality indicators of human influence available for West Virginia sites such as a biological index based on benthic invertebrate diversity and abundance (WVSCI = West Virginia Stream Condition Index), and fecal coliform counts. These data come from the WV Department of Environmental Protection's Watershed Assessment Program database (WAPbase) and are a result of various sampling programs all over the state dating back over 10 years.

The WVSCI data used include all data points available for each sampled river within 10 miles of the fish sampling location. WVSCI scores associated with the Back Fork Elk River, Gauley River, and West Fork Greenbrier sites, are plotted in Figure 2. As might be expected, all WVSCI scores for these reference sites were indicative of biologically unimpaired water (> 60.6).

In the case of the fecal coliform data, because all WV sites were intended to be reference sites, low (<400 cfu/100 mL) fecal counts were verified. Data points where fecal counts may have been artificially elevated due to higher turbidity caused by runoff events were removed.

Both total suspended solids (levels > 10 mg/L) and proximate precipitation notes (significant rainfall within the previous 24 hours) were considered inappropriate conditions for representative fecal coliform sampling and grounds for removal of that data point. Average fecal coliform counts associated with each site are plotted against intersex occurrence in Figure 3. Again, counts are low for all reference sites, well below the limit for water contact recreation, which is 400 colony forming units (cfu) per 100 mL in more than 10% of samples collected.

Fish collection and necropsy

In late summer and early fall 2005, *Micropterus dolomieu* were collected at Ohio and West Virginia sites, respectively. Stream reaches of up to 1,000 meters were sampled, targeting best fish habitat, until anywhere from 9 to the preferred goal of 20 total fish were obtained. The season was chosen based on previous intersex sampling in WV.

Muskingum and Kanawha watershed sites differed significantly in that all Muskingum sites were non-wadeable streams; whereas Kanawha sites were all wadeable. Therefore, the collection method at Ohio sites was electrofishing from a boat platform custom-built and maintained by Steve Foster of the US Army Corps of Engineers (US ACE). The sampling approach was typical of daytime, large river sampling techniques. At the wadeable WV sites, backpack units were Smith-Root (Vancouver, WA) model 12-B POW Electrofishers or similar battery powered models. Sites were electrofished until enough fish were collected or until it was determined that the desired number could not be collected in a reasonable amount of time. Such a determination was made based on a) time already spent and density of *M. dolomieu* encountered thus far, b) suitability of habitat encountered, and c) suitability of habitat remaining in sample reach. Only fish larger than 6 inches were included. Necropsies were conducted in accordance with USGS Biomonitoring of Environmental Status and Trends (BEST) procedures. Fish were anesthetized and sacrificed in tricaine methane sulfonate (MS-222). Fish length, total mass, and organ mass were recorded in addition to notation of signs of a healthy fish (e.g. red gills, unfrayed fins) as well as comments on any signs of poor health such as external or internal parasites and lesions. Male gonads and livers were dissected, excised, and preserved. Bass testis samples were preserved in a buffered formalin solution containing zinc called Z-Fix™ (Anatech Ltd., Michigan) to preserve morphology, and stored until they could be further processed at the fish laboratory at the Leetown Science Center. Liver samples were stored in a preservative eliminating RNase activity so that vitellogenin RNA could be later analyzed by the US FWS lab.

Histological analysis

Histological analyses were conducted by Dr. V. Blazer at the Leetown Science Center. Briefly, tissues were dehydrated, paraffin-embedded, slide-mounted, hematoxylin and eosin (H&E)-stained, and sliced into 6 μm sections. For each fish, the presence or absence of intersex in the tissue, as well as the degree of intersex, was scored using a method adapted from that developed by Bateman et al.[34] for use in European Flounder (*Platichthys flesus*) called the ovotestis severity index (OSI). Examination of five cross sections, approximately equally spaced along the length of the testis, has been shown to accurately diagnose the presence or absence of intersex [17].

The OSI allows for more detailed severity information beyond the simple presence or absence of intersex. Briefly, gonadal tissue sections are viewed at 100× magnification. Within a single section, the field of view is panned until the most extreme evidence of intersex is located. The five-level scoring was based solely on distribution of oocytes within the testis in the sections observed. The scoring consists of the following ratings: 0 indicates the complete absence of oocytes, a score of 1 indicates the presence of a single oocyte, a score of 2 indicates the presence of 2 or more oocytes which are not associated with one another, a score of 3 indicates the presence of more than 1 but less than 5 oocytes which are grouped together, and a score of 4 indicates the presence of more than 5 oocytes grouped together. The score for each of the five sections was used to calculate an average OSI score for each fish.

Vitellogenin

Vitellogenin analyses were conducted by the US Fish and Wildlife Service laboratory. RNA was purified from liver samples, reverse-transcribed to DNA, and used in quantitative polymerase chain reactions (qPCR) to quantify relative concentrations of vitellogenin RNA in each liver. The protocol used was DyNAmo SybrGreen Protocol (BMERB032 Rev 4) with the following parameters: R^2 values > 0.990 for vitellogenin, > 0.987 for 18S; C(T) for positive control = 12.0; Pos. ctrl Vg/18S =1.09 .

GIS

Non-point source information comes from land use/land cover data from the USGS Land Cover Institute (LCI) derived from 1992 LandSat data. Land use/land cover types provide an indication of potential non-point sources and include: developed land (broken down into low and high intensity residential, and commercial/industrial/transportation), agricultural land (broken down into hay/ pasture & grass, row crops, and probable row crops), undisturbed or forested land (consisting of conifer forest, mixed forest, deciduous forest, woody wetlands, and emergent wetlands), and mine lands (including "barren, quarry" and "barren, transitional"). Population density was estimated using census population density data for each county (as a whole) making up the majority of each site's watershed in OH and WV (i.e. counties with very small portions inside the watershed were often disregarded). In addition to non-point sources, known point-source contributors of EDCs include the waste water treatment plants mentioned above, permitted industrial discharges, and combined sewage outfalls. Information on point sources was obtained from the WV Department of Environmental Protection's permitting databases. Because these point sources are commonly associated with more highly developed and densely populated areas, the analysis was simplified to compare land use types in the watershed. However, for the WV reference sites, all permitted discharges were included on a map of the entire watershed to demonstrate the lack of major point-source stressors on these sites. These maps are shown in Figures 4-6, with an example of the information typically included with the permit in Figure 7.

To focus the analysis on regions expected to affect the samples, a simplified approach was used to approximate a "region of influence." Collection site coordinates were used to generate a zone with a five mile radius (centered on the collection site), or hypothetical immediate "sphere" of influence for the purpose of this model. This enabled calculation of land cover percentages. We also attempted a more precise representation of the region of influence by delineating watersheds from the sample collection coordinates *up*stream. This model is more realistic in one sense because it represents all the land which collects the water that will eventually flow through the collection site. Conversely, by extending the region of influence, we are considering locations that are farther from the collection site. The impact of a location's land cover should probably be expected to decrease with increasing distance from the sample site, but the amount of this decrease is complicated to predict. This is a result of several factors including increased dilution of pollutants as the river's size increases, decreased concentrations of pollutants due to their degradation, and partitioning of pollutants out of the water column and into sediments where they're less bio-available. Finally, the underlying data for delineating watersheds was not available for OH sites. As a result, the watershed-delineated region of influence model was only used as supplementary information to the five mile zone of influence model, and only for WV sites.

All geographical analyses, including watershed delineations were conducted in ESRI's (Environmental Systems Research Institute, Inc.) ArcMap 9 software from the ArcGIS suite. In addition to standard functions from this application, tools from the Watershed Characterization and Modeling System (WCMS) developed by the Natural Resource Analysis Center (NRAC) at West Virginia University with support from the WV Department of Environmental Protection (WVDEP) were used for applications specific to watershed monitoring in West Virginia. The region of influence data layer was then used as a mask for the various pollutant and land cover data layers to generate a smaller subset of data which contained information on each of the land cover types for each region of influence. Percentages of each land cover type were then calculated from the total number of pixels of land cover use divided by the total number of pixels within the zone. With the knowledge of potential land cover influences around each collection site, the degree of each land cover type was plotted against the degree of intersex found in that population to examine the relationships.

Results

Fish collection

A total of 52 male fish were sampled from the sites in 2005. Mean lengths were 28.2 cm for intersex fish and 26.8 cm for normal fish at impacted sites, and 26.1 cm for intersex fish and 23.8 cm for normal fish at reference sites. The apparent greater mean length of intersex fish at both site groups is not statistically significant. No attempt was made to rate the significance of these differences between site types since impacted sites were all larger, non-wadeable rivers while reference sites were smaller, wadeable streams and habitat size could have an obvious effect on size regardless of intersex prevalence. Sex, length, and weight data are listed for all fish collected in Table 1, with site location information in Table 2.

Histology and Vitellogenin Data

Most fish samples were diagnosed with ovotestis. Occurrence of intersex males at impacted OH sites was greater ($\bar{x}$ = 88.2 %) than at WV reference sites ($\bar{x}$ = 31.4 %) (Figure 8). This is a significant increase ($z = 1.96$, $p = 0.02$) at impacted sites when compared with reference sites as demonstrated by a non-parametric Mann-Whitney U Test using NCSS (v. 2001, Kaysville, Utah, USA). The numbers of fish found having any degree of the intersex condition are also listed in Table 1. Mean OSI scores were 1.0 for impacted sites and 0.1 for reference sites, again significantly higher ($z = 5.08$, $p < 0.001$) at impacted sites than reference sites, using the Mann-Whitney U test. Mean OSI scores are also detailed in Table 1. Totals by site are listed in Table 3. These OSI data are plotted in Figure 9. There was at least one fish with the intersex condition found at every site sampled.

A one-tailed Mann-Whitney U test of the vitellogenin assay data showed significantly higher ($z = -4.32$, $p < 0.001$) VTG levels in females at reference sites than at impacted sites as expected (Figure10), but no significant difference (NSD) between average VTG expression in males at impacted sites when compared to reference sites ($z = -4.76$, $p = 0.99$, Figure 11).

GIS

Agricultural land cover appears to be the strongest predictor of intersex occurrence under our model. Figure 12 shows the linear regression results. The regression equation is: [Intersex = 0.2027 + 1.7577 × (% agricultural land)], with $r^2 = 0.913$ and a t test for the significance of the slope of the line being significantly different from zero yielding: $t = 6.4778$, $p = 0.0029$.

Contrary to our expectations, developed land, hypothesized as most likely to be strongly contributing to the level of anthropogenic endocrine disruptors, is not a significant predictor of intersex occurrence. Figure 13 shows the regression analysis of intersex occurrence versus the percentage of developed land cover (as represented by categories 21, 22, and 23, or "low intensity residential," "high intensity residential," and "commercial/industrial/transportation" respectively). The regression equation is: [intersex occurrence= 0.4126 + 3.275 × (% developed land)] and the r^2 value is 0.53. A t test comparison with a zero slope yields: $t = 2.1238$, $p = 0.1009$, highlighting the lack of a significant relationship between developed land cover and intersex in our study.

Figure 14 shows percentage undisturbed land cover as represented by naturally vegetated forested land (deciduous, coniferous, and mixed) plotted against intersex occurrence. Applying the same analysis gives the regression: [intersex occurrence = 1.3201 -1.0013 × (% naturally vegetated land)] with $r^2 = 0.4$. The t-test comparison of this slope with zero gives $t = -2.463$, p =

0.036, indicating that naturally vegetated land is a fair predictor of intersex (inverse relationship), but still not very strong with a low r^2 value. The variety of land cover types and percentages as calculated using the five mile radius sphere of influence model are tabulated in Table 4.

Discussion

Histology

This study has shown that both the presence of intersex and the OSI scores increase significantly at impacted sites with higher percentages of agricultural development within a five mile radius, and with higher population densities in the counties within the watershed. While high population densities are not often associated with agricultural development, this is the case with impacted sites in this study when compared with the reference sites. Also, recall that the comparison of agricultural development between site groups relies on a very focused window of only 5 miles in radius from each sample site, while the population density comparison evaluates all major counties within the watershed (excluding only counties whose majority is outside the watershed). Other studies have verified these links between intersex and residential, industrial, and agricultural wastewater effluents, and specific EDCs in lab exposures. However, the link between intersex and EDCs may not be as simple as measurable exposure concentrations, but likely also includes chronological components. Not only must we consider duration of exposure, but also the life stage during which an organism was exposed. Finally, it is important to understand which aspects of fish health, other than ovotestis, are being affected by these EDCs.

<u>EDCs and intersex</u>

In the UK, the relationship between WWTP effluents and gonadal intersex in wild fish has been demonstrated[22]. In Japan, intersex could be stimulated in mature medaka (*Oryzias latipes*) when treated with estradiol[35]. Several other studies have demonstrated intersex induction with both natural and synthetic hormones, as well as other estrogenic contaminants[36, 37, 38, 39].

A similar fish collection and histological analysis for intersex, conducted in 2004, surveyed rock bass (*Ambloplites rupestris*) instead of smallmouth bass. The main river sampled was the Kanawha River of West Virginia, a large river which is mainly impacted by mining and rural wastewater effluents in its upper reaches, heavily industrial and urbanized in the vicinity of the city of Charleston, and moderately agricultural in the broad valley it forms near its mouth. Results show that the highest incidences of intersex (present in the majority of fish sampled) were found on the upper Kanawha River, with occurrences around 50% in the industrialized reaches and even lower in the agricultural reaches[40]. This might suggest that rural wastewater discharges could be greater contributors to intersex than industrial discharges or agricultural runoff. It is also possible that dilution plays the dominant role, as the size of the river also of course increases going downstream. However, because a different species was used, a rigorous comparison with these sites was not attempted. As described below, there is evidence that different species respond to various degrees to EDCs, and some not at all when using intersex as the endpoint of detection.

A survey of white perch in lakes affected by "domestic and industrial" effluents showed that several other co-sampled species were completely unaffected when using ovotestis as an endpoint[23]. Great care must be taken in comparing intersex in fish of one species to intersex in another species. In addition to showing intersex prevalence in perch as high as 83% in impacted sites, this study was also able to locate populations at a hatchery and reference site with 0% prevalence. In contrast, the current study found no fish populations with 0% intersex. Again however, these are two different studies with different species.

Locally, a USGS EDC survey of the South Branch Potomac, Cacapon, and Williams Rivers of West Virginia revealed detectable levels of antibiotics and their degradation products[20],

but only in direct grab samples from WWTP discharges, not in any of the surface water passive sampling devices or blood plasma. This result helps emphasize the need for alternate and/or additional approaches to discrete or integrated chemical sampling because, even with EDC sources already identified, sediment partitioning, degradation, and dilution may render certain species undetectable in surface water samples. It is for this reason the GIS analysis discussed below was used. Smallmouth bass from several of these sites were also surveyed for, and found to exhibit the intersex condition[17]. Other EDCs were detected in passive samplers and smallmouth bass blood plasma samples, including diethyl-hexyl phthalate and the flame retardants BDE 47 and BDE 99. The South Branch and Cacapon Rivers represent moderately impacted watersheds with poultry farming, residential and commercial development, but not the intensive row crops and high population densities present in the current study's impacted sites. The USGS study reveals 3 categories of EDCs: the ubiquitous diethyl-hexyl phthalate, found in plasma samples from all sites; the absolutely indicative flame retardants, found in plasma samples at impacted sites only; and less indicative EDCs, detected more often, and in higher concentrations at impacted sites, but occasionally also found at the reference site.
Another surface water chemistry study in the South Branch Potomac deemed the antimicrobial Triclosan as an EDC of concern because of its detection frequency at sites with high prevalence of intersex[41].

Chronologies of exposure and detection

Studies in watersheds affected by wastewater discharges have shown elevated levels of EDCs. One study using gonadosomatic index as an endpoint of endocrine disruption demonstrated some species were only susceptible in their larval stage, not as juveniles[42].

Another study also suggests that, when compared with fish with no history of exposure, fish that have grown and developed in contact with EDCs are at higher risk of developing intersex characteristics later in life either because of their long term exposure, or because of exposure during a critical developmental stage[43].

It has been suggested that, like the spermatocytes normally present in the male gonad, the oocytes found in intersex male smallmouth may be shed during spawning; fish sampled during spawning months (spring) were found to have 25 – 40 % higher incidence of intersex than fish sampled post-spawning (summer) [18]. [17]. This would create an obvious disconnect when comparing the presence/absence of oocytes found in fish sampled post-spawning with fish sampled pre-spawning, or even from any two fish sampled at temporally disparate intervals. Therefore, questions remain concerning the validity of comparing results as detailed as intersex severity as assessed *via* the OSI and exactly how this severity reflects on both the concentrations of endocrine disruptors in the fishes' environment and the chronological regime of their exposure. Studies are needed, and are currently underway, addressing the effects of known exposure concentrations, frequencies, and durations to fish in various stages of development in controlled environments[44,45]. In the mean time, most analyses here will treat intersex as an "all-or-nothing" condition for simplicity.

EDCs, intersex, and fish health

A study examining sperm quality in smallmouth bass of the South Branch Potomac found a significant correlation between decreased sperm motility and viability and intersex[46]. Similar linkages were shown between intersex and sperm quality [47], and intersex and fertility[48] in the UK.

Vitellogenin expression

In trying to compare vitellogenin expression with the intersex condition as two biomarkers for endocrine disruption, it is apparent that these two endpoints are not directly correlated in our case (Figure 11). Whether this is a result of each marker indicating different types of endocrine disruptors at different sample sites, or simply that one (or both) markers are inadequate as comparable bioindicators due to the complexity of the physiologies behind each is unknown. Increases in VTG expression in males have been induced *via* exposure to estrogen and estrogenic agricultural runoff samples[42]. It was also hypothesized, based on evidence in the literature[49], that impacted sites with higher levels of vitellogenin in males should have corresponding lower-than-average levels of vitellogenin in females, i.e.: the opposite direct effect on vitellogenin levels as is seen in males, but a similar end result of reduction in health and reproductive capacity. However, laboratory exposures have also shown that VTG expression in response to EDCs can be highly variable[50]. In fact, a more recent study found that VTG increases in females, just as in males, albeit by a different hormonal mechanism[51]. Unexpectedly, it is also clear that vitellogenin expression in males was not significantly different at our reference sites when compared to impacted sites. While vitellogenin levels have been shown to be elevated in estrogenic EDC-affected fish, it is not clear what this tells us beyond indicating the presence of estrogenic EDCs. In one species studied, elevated VTG levels did not correlate with reduced reproductive success[52]. Therefore, even with elevated VTG levels, the smallmouth bass in the present study might have normal reproductive function.

A US study in cunner (*Tautogolabrus adspersus*) found that, while increased VTG expression in male fish may be an indicator of EDC exposure, it does not necessarily correlate with a decrease in male reproductive health as measured by sperm motility and fertilization[52]. However, the same study suggests that increased VTG induction in males may be indicative of reduced reproductive health in female fish in the same population.

GIS

Micropterus dolomieu inhabits complex, heterogeneous, lotic environments with both point and non-point sources of pollution that can be highly temporally and spatially variable. Although a comprehensive water quality assessment was unavailable, I analyzed the water quality environment at the watershed scale using a top-down method. This method allowed me to account for fish migration, varying flow regimes, sediment-water partitioning, and land use types by characterizing the whole stream reach, and general contamination by focusing on potential sources.

<u>Agricultural land cover</u>

The five mile radius circular sphere of influence used in the land cover analysis showed the strongest expected correlation between agricultural land cover and intersex occurrence (Figure 12). The watershed-delineated region of influence model, run solely on WV (reference) sites (Figures 15-18), was useful only in confirming the suitability of reference sites as undisturbed. Forested land cover was over 80% for all sites and agricultural, developed, and barren land were all less than 1.5% of each watershed. Without more variability in land cover types between sites, any attempt at correlating these with intersex occurrence would not be scientifically prudent.

Undisturbed land cover

The inverse correlation between undisturbed or naturally vegetated (forested) land cover and intersex occurrence is shown in Figure 14. As expected, decreasing the amount of forested land around a sample site corresponds with an increase in intersex occurrence, though this is not as strong a predictor as agricultural land cover. Forested land provides filtration and biodegradation capacity of permeable soil layers, creating a buffer zone between a contaminant source and where it enters the surface waters.

Developed land cover

Contrary to the hypothesis, developed land cover was not a significant predictor of intersex occurrence. One weakness of this indicator within our research model is that, unlike agricultural and undisturbed land cover types, which comprise up to 40% or more of the region of influence, the developed land cover indicator, even at its maximum, comprises less than 20% of the region of influence. Without sites where this factor could be allowed to exert more influence (e.g. denser urban regions), perhaps it shouldn't be surprising that it is a poor predictor of intersex occurrence. In addition, the land cover data that is the basis for this modeling is derived from 1992 LandSat data, making it quite possible that the degree of development has changed in the 17 years since then.

Population density

Population density in the counties that make up each site's watershed was plotted along with intersex occurrence (Figure 19). The linear regression produces an r^2 value of 0.695, indicating that population density can be a fair predictor of intersex occurrence, but not as strong as agricultural land uses. This is intended to be a further justification of the designation of reference sites and impacted sites as having low, and high human influence, respectively. However, additional sites are required with single impacts only (e.g. high population density with no agriculture or industrial development, or high agricultural development with low population density and no industry) in order to determine which of these factors is dominant in driving intersex occurrence, or if stressors are synergistic in their effects.

Conclusions and future work

This project has demonstrated the prevalence of the intersex condition at all sites sampled in 2005 and the correlations with increased agricultural development and population density and decreased forested land. Future studies should include thorough chemical sampling of the surface water environment and fish tissue to help identify potential causal EDCs, especially those that recur from site to site. I would also include bioassays to capture any unexpected or untargeted EDCs with estrogenic activity. Site selection should include more remote locations in roadless areas with no history of human development and outside of any significant plumes that would allow for atmospheric deposition. In addition to including stronger reference sites, I would seek access to any museum samples from historical collections that would allow us to look further back in time at the intersex condition in relevant fish species.

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Literature Cited

1. Crews, D., Willingham, E. & Skipper, J. Endocrine disruptors: present issues, future directions. *The quarterly review of biology* **75**, 243-60(2000).
2. Kümmerer, K. *Pharmaceuticals in the environment : sources, fate, effects, and risks*. (Springer: Berlin; New York, 2004).
3. Kolpin, D.W. et al. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. *Environmental science & technology* **36**, 1202-1211(2002).
4. Damstra, T. et al. *Global assessment of the state-of-the-science of endocrine disruptors : an assessment prepared by an expert group on behalf of the World Health Organization, the International Labour Organisation, and the United Nations Environment Programme*. (World Health Organization: Geneva, 2002).
5. Mueller, S.O. Xenoestrogens: mechanisms of action and detection methods. *Analytical and bioanalytical chemistry* **378**, 582-587(2004).
6. Mu, X. & LeBlanc, G.A. Synergistic Interaction of Endocrine-Disrupting Chemicals: Model Development Using an Ecdysone Receptor Antagonist and a Hormone Synthesis Inhibitor. *Environmental toxicology and chemistry* **23**, 1085-1091(2004).
7. Kupfer, D. & Bulger, W. Studies on the mechanism of estrogenic actions of o,p'DDT: interactions with the estrogen receptor. *Pesticide biochemistry and physiology* **6**, 561-570(1976).
8. Jaga, K. What are the implications of the interaction between DDT and estrogen in the body? *Medical hypotheses* **54**, 18-25(2000).
9. Frigo DE et al. DDT and its metabolites alter gene expression in human uterine cell lines through estrogen receptor-independent mechanisms. *Environmental health perspectives* **110**, 1239-45(2002).
10. Tyler, C.R., Jobling, S. & Sumpter, J.P. Endocrine Disruption in Wildlife: A Critical Review of the Evidence. *Critical reviews in toxicology* **28**, 319 - 361(1998).
11. Guillette, L.J. et al. Alligators and Endocrine Disrupting Contaminants: A Current Perspective. *American zoologist* **40**, 438-452(2000).
12. Milnes MR et al. Altered neonatal development and endocrine function in Alligator mississippiensis associated with a contaminated environment. *Biology of reproduction* **73**, 1004-10(2005).
13. Edmunds JS, McCarthy RA & Ramsdell JS Permanent and functional male-to-female sex reversal in d-rR strain medaka (Oryzias latipes) following egg microinjection of o,p'-DDT. *Environmental health perspectives* **108**, 219-24(2000).
14. Ward, K. C8 Study backs up lawsuit, judge told. *Charleston Gazette-Mail* (2008).
15. Montgomery, R. Does girly bass = girly men? *Bassmaster magazine.* 14(2005).
16. Sex-changing chemicals found in Potomac River. *Reuters (Washington)* (2007).
17. Blazer, V. et al. Intersex (Testicular Oocytes) in Smallmouth Bass from the Potomac River and Selected Nearby Drainages. *Journal of aquatic animal health* **19**, 242-253(2007).
18. Myers, M. & U.S. Geological Survey, Department of the Interior *Oversight Hearing on "Ova-Pollution in the Potomac: Egg-Bearing Male Bass and Implications for Human and Ecological Health"*. (House Committee on Government Reform, 2006).

19. Okie, S. Assessing hormone-mimicking chemicals: some scientists are alarmed about pesticides, contaminants and natural plant estrogens. *The Washington Post* Z.08(1999).
20. Chambers, D. & Leiker, T. *A Reconnaissance for Emerging Contaminants in the South Branch Potomac River, Cacapon River, and Williams River Basins, West Virginia, April-October 2004*. (USGS : Reston, VA , 2006).at <http://pubs.usgs.gov/of/2006/1393>
21. McGee, B. et al. *Evaluation of Endocrine Disrupting Effects in Potomac River Fish*. (National Fish and Wildlife Foundation: Washington, D.C., 2003).
22. Jobling, S. et al. Widespread Sexual Disruption in Wild Fish. *Environmental science & technology*. **32**, 2498(1998).
23. Kavanagh, R.J. et al. Endocrine Disruption and Altered Gonadal Development in White Perch (Morone americana) from the Lower Great Lakes Region. *Environmental Health Perspectives* **112**, 898-902(2004).
24. Scippo , M. et al. Recombinant human estrogen, androgen and progesterone receptors for detection of potential endocrine disruptors. *Analytical and bioanalytical chemistry* **378**, 664-9(2004).
25. Scrimshaw, M.D. & Lester, J.N. In-vitro assays for determination of oestrogenic activity. *Analytical and bioanalytical chemistry* **378**, 576-581(2004).
26. Estevez-Alberola, M. & Marco, M. Immunochemical determination of xenobiotics with endocrine disrupting effects. *Analytical and bioanalytical chemistry* **378**, 563-575(2004).
27. Rodriguez-Mozaz, S. et al. Biosensors for environmental monitoring of endocrine disruptors: a review article. *Analytical and bioanalytical chemistry* **378**, 588-598(2004).
28. Tyler, C.R. et al. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology*. **166**, 418(1996).
29. Heisterkamp I, Gandrass J & Ruck W Bioassay-directed chemical analysis utilizing LC-MS: a tool for identifying estrogenic compounds in water samples? *Analytical and bioanalytical chemistry* **378**, 709-15(2004).
30. Rodgers-Gray TP et al. Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development. *Environmental science & technology* **35**, 462-70(2001).
31. Jobling, S. et al. Inhibition of Testicular Growth in Rainbow Trout (Oncorhynchus mykiss) Exposed to Estrogenic Alkylphenolic Chemicals. *Environmental toxicology and chemistry* **15**, 194-202(1996).
32. Heberer, T., Reddersen, K. & Mechlinski, A. From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. *Water science and technology* **46**, 81-88(2002).
33. Rutishauser, B.V. et al. Comparative Analysis of Estrogenic Activity in Sewage Treatment Plant Effluents Involving Three In Vitro Assays and Chemical Analysis of Steroids. *Environmental toxicology and chemistry* **23**, 857-864(2004).
34. Bateman, K.S., Stentiford, G.D. & Feist, S.W. A Ranking System for the Evaluation of Intersex Condition in European Flounder (Platichthys flesus). *Environmental toxicology and chemistry* **23**, 2831-2836(2004).
35. Kang IJ et al. Effect of 17beta-estradiol on the reproduction of Japanese medaka (Oryzias latipes). *Chemosphere* **47**, 71-80(2002).

36. Koger CS, Teh SJ & Hinton DE Determining the sensitive developmental stages of intersex induction in medaka (Oryzias latipes) exposed to 17 beta-estradiol or testosterone. *Marine environmental research* **50**, (2000).
37. Metcalfe, C.D. et al. Estrogenic Potency of Chemicals Detected in Sewage Treatment Plant Effluents as Determined by In Vivo Assays with Japanese Medaka (Oryzias latipes). *Environmental toxicology and chemistry /* **20**, 297(2001).
38. Gray, M.A. & Metcalfe, C.D. Induction of Testis-Ova in Japanese Medaka (Oryzias latipes) Exposed to p-Nonylphenol. *Environmental toxicology and chemistry* **16**, 1082-1086(1997).
39. Gronen S et al. Serum vitellogenin levels and reproductive impairment of male Japanese Medaka (Oryzias latipes) exposed to 4-tert-octylphenol. *Environmental health perspectives* **107**, 385-90(1999).
40. Foster, S. *Monitoring for the presence of intersex anomalies in the Muskingum and Kanawha River drainage basins.* (US ACE - Water Resources Engineering Branch - Water Quality Team: Huntington District, US Army Corps of Engineers, 2005).
41. Wells, M.J. *An assessment of the occurrence of chemicals causing endocrine disruption in fish in the South Branch of the Potomac River*. (Center for the Management, Utilization and Protection of Water Resources, and the Dept. of Chemistry; Tennessee Technological University: Cookeville, Tennessee, 2005).
42. Yonkos, L.T. Poultry litter-induced endocrine disruption: laboratory and field investigations. (2005).
43. Liney KE et al. Assessing the sensitivity of different life stages for sexual disruption in roach (Rutilus rutilus) exposed to effluents from wastewater treatment works. *Environmental health perspectives* **113**, 1299-307(2005).
44. McArdle, M.E., McElroy, A.E. & Elskus, A.A. Enzymatic and Estrogenic Responses in Fish Exposed to Organic Pollutants in the New York-New Jersey (USA) Harbor Complex. *Environmental toxicology and chemistry* **23**, 953-959(2004).
45. Liney KE et al. Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environmental health perspectives* **114**, 81-9(2006).
46. Henderson, H. The effects of contaminants on sperm quality and intersex condition of smallmouth bass (Micropterus dolomieu) in the Potomac River. (2007).
47. Jobling S et al. Altered sexual maturation and gamete production in wild roach (Rutilus rutilus) living in rivers that receive treated sewage effluents. *Biology of reproduction* **66**, 272-81(2002).
48. Jobling, S. et al. Wild Intersex Roach (Rutilus rutilus) Have Reduced Fertility. *Biology of reproduction* **67**, 515-524(2002).
49. Klaper R et al. Gene expression changes related to endocrine function and decline in reproduction in fathead minnow (Pimephales promelas) after dietary methylmercury exposure. *Environmental health perspectives* **114**, 1337-43(2006).
50. Ackermann, G.E. et al. Effects of long-term nonylphenol exposure on gonadal development and biomarkers of estrogenicity in juvenile rainbow trout (Oncorhynchus mykiss). *Aquatic toxicology - Amsterdam* **60**, 203 -221(2002).
51. Scott AP et al. Relationship between sex steroid and vitellogenin concentrations in flounder (Platichthys flesus) sampled from an estuary contaminated with estrogenic endocrine-disrupting compounds. *Environmental health perspectives* **114**, 27-31(2006).

52. Mills, L.J. et al. Relationship between Reproductive Success and Male Plasma Vitellogenin Concentrations in Cunner, Tautogolabrus adspersus. *Environmental health perspectives* **111**, 93-99(2003).

Tables

Table 1 – Individual fish sample information.

site	fish ID	species	sex	length (cm)	weight (g)	Intersex (males only)	avgerage OSI score
OMT-(57.9)	1	SMB	M	29.8	388.0	Y	1.2
	2	SMB	M	28.1	254.0	N	0
	3	SMB	F	23.6	168.0		
	4	SMB	M	34.2	520.0	Y	1
	5	SMB	F	22.0	140.0		
	6	SMB	M	29.7	360.0	Y	0.4
	7	SMB	M	27.3	280.0	Y	0.6
	8	SMB	M	28.6	273.0	Y	0.4
	9	SMB	F	33.7	498.0		
OMT-(54.5)	1	SMB	M	27.2	281.0	Y	0.6
	2	SMB	M	29.2	248.0	Y	1.4
	3	SMB	F	24.4	204.0		
	4	SMB	F	22.6	142.0		
	5	SMB	F	31.0	377.0		
	6	SMB	M	23.2	161.0	Y	1.6
	7	SMB	F	23.7	171.0		
	8	SMB	F	28.2	344.0		
	9	SMB	M	25.0	197.0	Y	0.8
OMT-(15.5)	(none)						
OMW-(11.3)	1	SMB	F	37.6	698.0		
	2	SMB	M	27.4	286.0	Y	1.4
	3	SMB	F	37.8	706.0		
	4	SMB	M	26.1	232.0	Y	0.8
	5	SMB	M	26.2	249.0	Y	1.8
	6	SMB	F	21.0	117.0		
	7	SMB	M	39.1	887.0	Y	1.2
	8	SMB	M	25.6	226.0	Y	2.2
	9	SMB	F	25.5	213.0		
	10	SMB	F	29.1	323.0		
	11	SMB	F	33.1	539.0		
	12	SMB	F	39.2	829.0		
	13	SMB	F	30.0	350.0		
	14	SMB	F	30.6	378.0		
	15	SMB	F	24.5	188.0		
	16	SMB	F	28.5	318.0		
	17	SMB	M	24.0	203.0	Y	1.6

site	fish ID	species	sex	length (cm)	weight (g)	Intersex (males only)	avgerage OSI score
	18	SMB	M	25.5	250.0	N	0
	19	SMB	F	18.8	92.0		
OMW-(11.3)	20	SMB	F	31.0	425.0		
OM-(23.8)	(none)						
OKNG-79-(2.5)	1	SMB	F	33.4	446.0		
	2	SMB	F	30.5	339.0		
	3	SMB	F	19.8	95.0		
	4	SMB	F	26.5	206.0		
	5	SMB	F	27.6	261.0		
	6	SMB	M	21.6	115.0	Y	0.4
	7	SMB	M	23.0	158.0	N	0
	8	SMB	F	24.5	172.0		
	9	SMB	F	20.1	93.0		
	10	SMB	F	26.0	236.0		
	11	SMB	M	19.2	85.0	Y	0.4
	12	SMB	F	20.6	109.0		
	13	SMB	M	18.5	78.0	N	0
	14	SMB	M	19.3	93.0	N	0
	15	SMB	M	17.3	67.0	N	0
	16	SMB	M	18.9	84.0	Y	0.2
	17	SMB	M	30.4	353.0	N	0
	18	SMB	M	26.0	194.0	Y	0.2
	19	SMB	F	25.3	187.0		
	20	SMB	F	24.4	170.0		
	21	SMB	M	22.5	122.0	N	0
	22	SMB	M	25.0	171.0	N	0
	23	SMB	M	32.0	382.0	Y	0.6
	24	SMB	M	28.4	260.0	N	0
	25	SMB	F	23.6	156.0		
	26	SMB	M	29.5	387.0	N	0
	27	SMB	M	22.0	135.0	N	0
	28	SMB	M	20.5	108.0	Y	0.2
	29	SMB	M	21.2	105.0	N	0
	30	SMB	M	20.5	96.0	N	0
OKE-111-(0.5)	1	SMB	F	28.0	271.0		
	2	SMB	M	37.0	574.0	Y	0.4
	3	SMB	M	20.0	97.0	N	0
	4	SMB	F	29.1	278.0		
	5	SMB	M	28.5	283.0	N	0

site	fish ID	species	sex	length (cm)	weight (g)	Intersex (males only)	avgerage OSI score
	6	SMB	M	27.0	251.0	N	0
	7	SMB	M	29.7	344.0	Y	0.2
	8	SMB	F	27.1	231.0		
	9	SMB	M	30.4	368.0	N	0
	10	SMB	F	30.0	384.0		
	11	SMB	M	30.1	332.0	N	0
	12	SMB	M	28.9	302.0	Y	0.4
	13	SMB	F	29.0	306.0		
	14	SMB	M	26.6	229.0	Y	0.2
	15	SMB	F	24.0	153.0		
	16	SMB	F	27.4	258.0		
	17	SMB	F	29.9	320.0		
	18	SMB	F	24.2	177.0		
	19	SMB	M	21.0	109.0	N	0
	20	SMB	M	19.7	95.0	N	0
OKG-(86.9)	1	SMB	F	28.0	288.0		
	2	SMB	F	24.1	179.0		
	3	SMB	M	26.5	209.0	Y	0.4
	4	SMB	F	26.3	214.0		
	5	SMB	M	24.2	190.0	N	0
	6	SMB	F	23.6	157.0		
	7	SMB	F	22.5	131.0		
	8	SMB	M	21.0	107.0	N	0
	9	SMB	F	21.0	104.0		
	10	SMB	M	24.1	167.0	N	0
	11	SMB	M	21.0	115.0	N	0
	12	SMB	F	20.5	105.0		
	13	SMB	F	24.0	160.0		
	14	SMB	M	26.1	201.0	N	0
	15	SMB	F	20.3	107.0		
	16	SMB	F	18.2	78.0		
	17	SMB	F	19.2	74.0		

Table 2 – Collection site location information.

"AN" code	Impacted or reference	Date sampled	Name	USACE number	Intermediate code	Approx. watershed size (sq mi)	Town
OMT-(54.5)	Impacted	8/23/2005	Tuscarawas	MR2	T2	1800	New Philadelphia, OH
OMT-(57.9)	Impacted	8/23/2005	Tuscarawas	MR3	T1	1400	Dover, OH
OMW-(11.3)	Impacted	8/22/2005	Walhonding	MR1	W	1568	Warsaw, OH
OKE-111-(0.5)	Reference	10/11/2005	Back Fork (Elk River)	WV5	BF	69	Webster Springs, WV
OKNG-79-(2.5)	Reference	10/12/2005	West Fork (Greenbrier River)	WV6	WFG	62	Durbin/Bartow, WV
OKG-(86.9)	Reference	10/11/2005	Top Gauley	WV4	G	72	Cowen, WV
OMT-(15.5)	Impacted	8/22/2005	Tuscarawas	MR7		2400	Orange, OH
OM-(23.8)	Impacted	8/23/2005	Muskingum	MR8		8000	Beverly, OH

Table 3 – Fish sample summary.

site	total fish	species	males	males with intersex	% males with intersex
OMT-(57.9)	9	SMB	6	5	83.3
OMT-(54.5)	9	SMB	4	4	100
OMT-(15.5)	0	SMB	-	-	-
OMW-(11.3)	20	SMB	7	6	85.7
OM-(23.8)	0	SMB	-	-	-
OKNG-79-(2.5)	30	SMB	18	6	33.3
OKE-111-(0.5)	20	SMB	11	4	36.4
OKG-(86.9)	17	SMB	6	1	16.7

* SMB = smallmouth bass

Table 4 – Land cover type percentages as calculated using the 5-mile radius sphere of influence model.

Site	% intersex at each site	21 low intensity residential	22 High Intensity Residential	23 Commercial/ Industrial/ Transportation	total developed (21-23)	11 Open Water	32 Quarries/ Strip Mines/ Gravel Pits	33 Transitional	Total Barren (32-33)	41 Deciduous Forest	42 Evergreen Forest	43 Mixed Forest	Total vegetated, natural forest upland (41-43)	81 Pasture/Hay	82 Row Crops	85 Urban/ Recreational Grasses	Total herbacious planted/ cultivated (81,82,85)	91 Woody Wetlands	92 Emergent Herbaceous Wetlands	Total wetlands (91-92)
OMT-(57.9)	83.33%	10.7%	1.2%	3.5%	**15.3%**	1.9%	1.0%	0.4%	**1.4%**	36.5%	2.9%	2.0%	**41.3%**	26.1%	12.9%	0.0%	**39.0%**	0.1%	0.8%	**1.0%**
OMT-(54.5)	100.00%	10.8%	1.3%	3.4%	**15.4%**	2.1%	0.4%	0.4%	**0.8%**	37.6%	2.8%	2.0%	**42.4%**	26.1%	12.4%	0.0%	**38.5%**	0.1%	0.7%	**0.8%**
OMW-(11.3)	85.71%	0.4%	0.0%	0.1%	**0.5%**	1.1%	0.0%	0.0%	**0.0%**	56.0%	0.7%	0.2%	**56.9%**	22.8%	17.4%	0.0%	**40.2%**	0.5%	0.7%	**1.2%**
OKNG-79-(2.5)	33.33%	0.3%	0.0%	0.1%	**0.3%**	0.5%	0.2%	0.2%	**0.3%**	54.7%	17.8%	19.2%	**91.7%**	4.1%	2.6%	0.0%	**6.7%**	0.4%	0.1%	**0.5%**
OKG-(86.9)	16.67%	0.5%	0.0%	0.1%	**0.6%**	0.7%	0.1%	0.4%	**0.5%**	78.7%	2.0%	11.6%	**92.3%**	4.0%	1.7%	0.0%	**5.8%**	0.1%	0.0%	**0.1%**
OKE-111-(0.5)	36.36%	0.6%	0.0%	0.0%	**0.7%**	0.7%	0.2%	0.2%	**0.3%**	80.4%	1.9%	13.1%	**95.4%**	1.7%	1.1%	0.0%	**2.9%**	0.1%	0.0%	**0.1%**

Figures

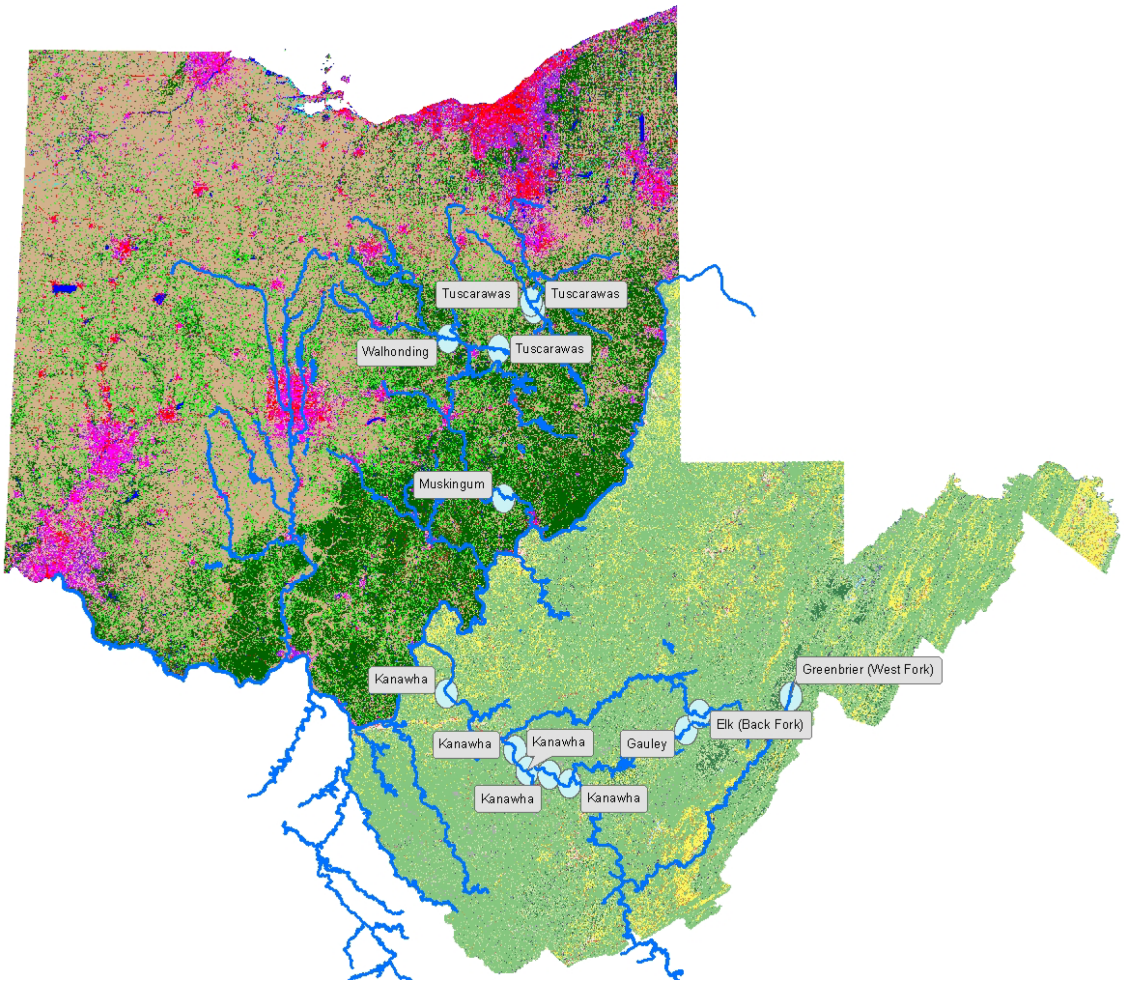

Figure 1 – 2005 fish collection sites in WV and OH plus Kanawha River sites used in S. Foster's 2004 collection of rock bass[1].

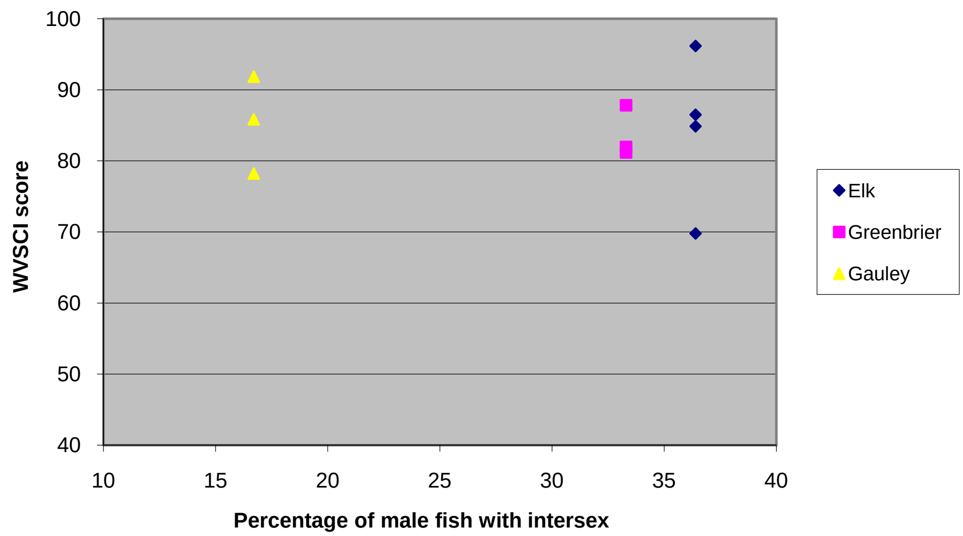

Figure 2 – Occurrence of intersex versus. individual WVSCI scores – In this figure, West Virginia Stream Condition Index (WVSCI) scores from the only 3 sites for which the WVSCI scores are available (reference sites) are plotted on the y-axis against the prevalence of intersex in fish sampled within 10 miles of where the benthic invertebrate sample (from which the WVSCI score is generated) was collected on the x-axis. The high WVSCI scores are indicative of unimpacted water quality and are used as an additional justification for the use of these sites as reference sites.

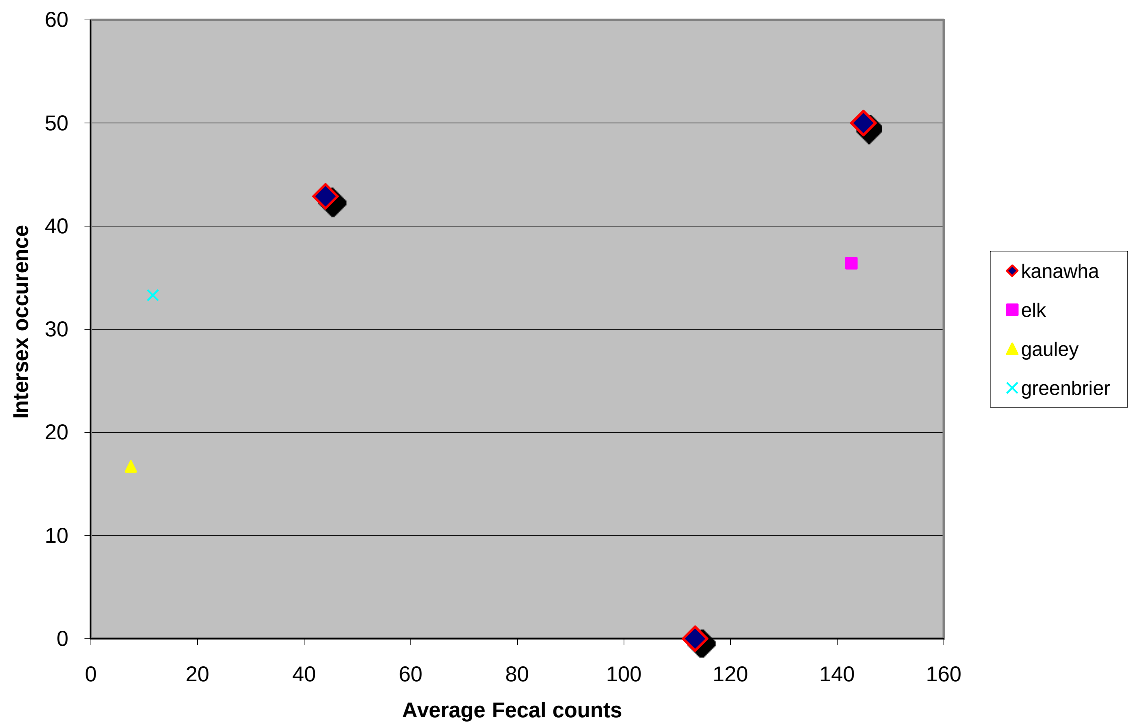

Figure 3 – Average fecal coliform counts versus intersex occurrence – Fecal coliform data (in colony-forming units /100 mL) at the 3 reference sites plus non-reference Kanawha River sites from a previous study are plotted against percentage intersex occurrence. Average fecal counts below the water contact recreation limit of 400 CFU/100 mL were used as an additional justification for the use of these sites as reference sites and a marker of low human sewage impacts.

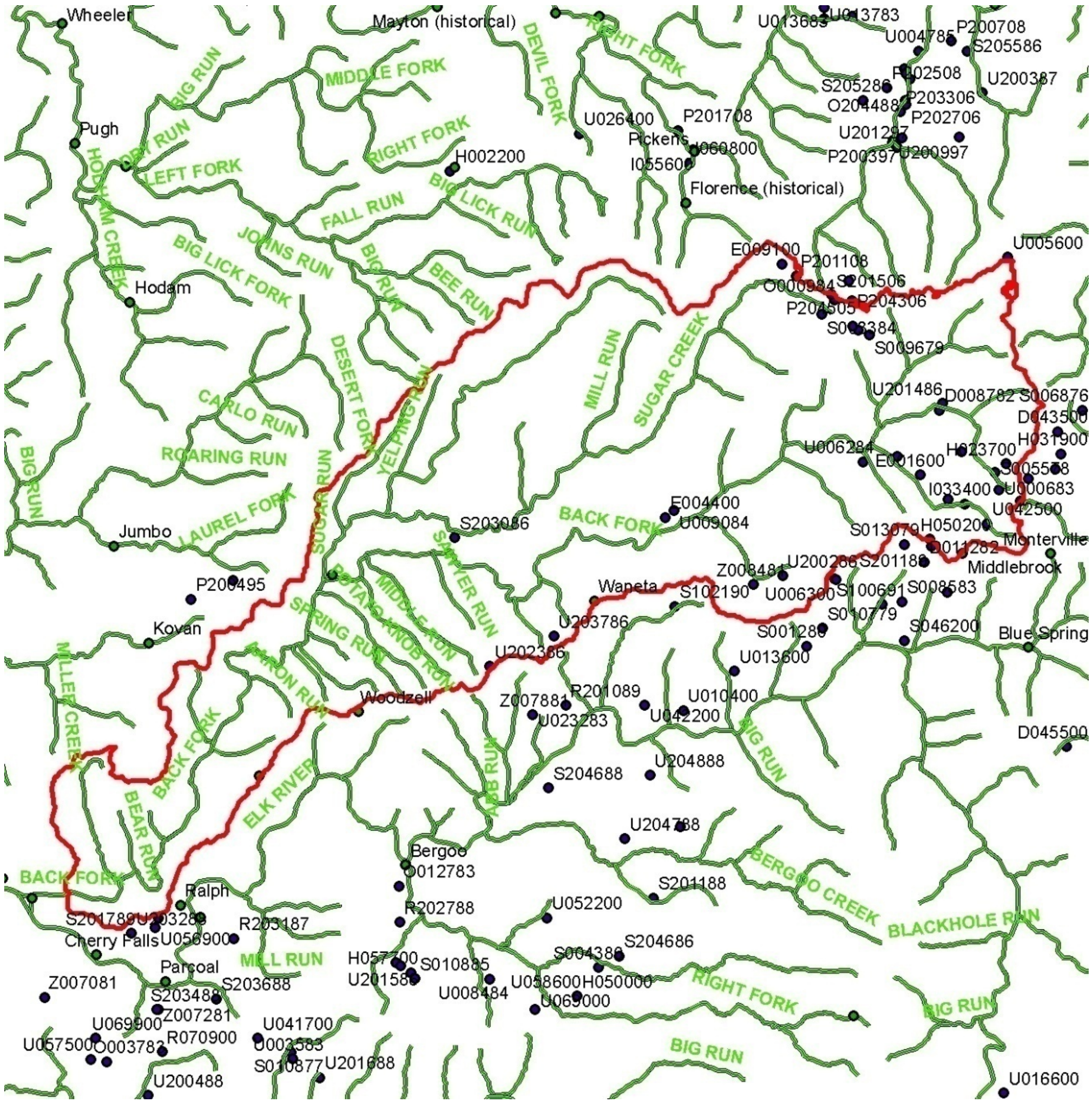

Figure 4 – Point source discharges in Back Fork Elk River site's watershed. The majority of permitted activity was in the headwaters of this site's watershed and is not currently being mined. Individual permit types are listed below the map. This permit record search was used to help justify the choice of these sites as reference sites. Permit types are: S203086, Z008481, S003384, S201506, C000383, S010878, S009679, S021174, S008977, I033400, S013680, S005578, S014576 – coal surface mines; U202386, U203786, E004400, U009084, U200286, E009100, U006284, U203588, E001600, D008782, U201486, E001500, U019583, U042500, U000683 – coal underground mines; P201108, P204505, – resource prospecting; O000984, H039200, O000783, H023700 – other (e.g. haulroads).

Figure 5– Point source discharges in Gauley River site's watershed. The majority of permits listed here are not currently being mined. Individual permit types are listed below the map. This permit record search was used to help justify the choice of these sites as reference sites. Permit types are: S005579, S010877, S200292, S200786, S201086, Z007081 - coal surface mines; U003583, U013883, U041700, U057500, U064800, U069900, U104191, U200488, U201688 - coal underground mines; P201998 - resource prospecting; O003783, O006882, R070900, R072200 - other (e.g. haulroads).

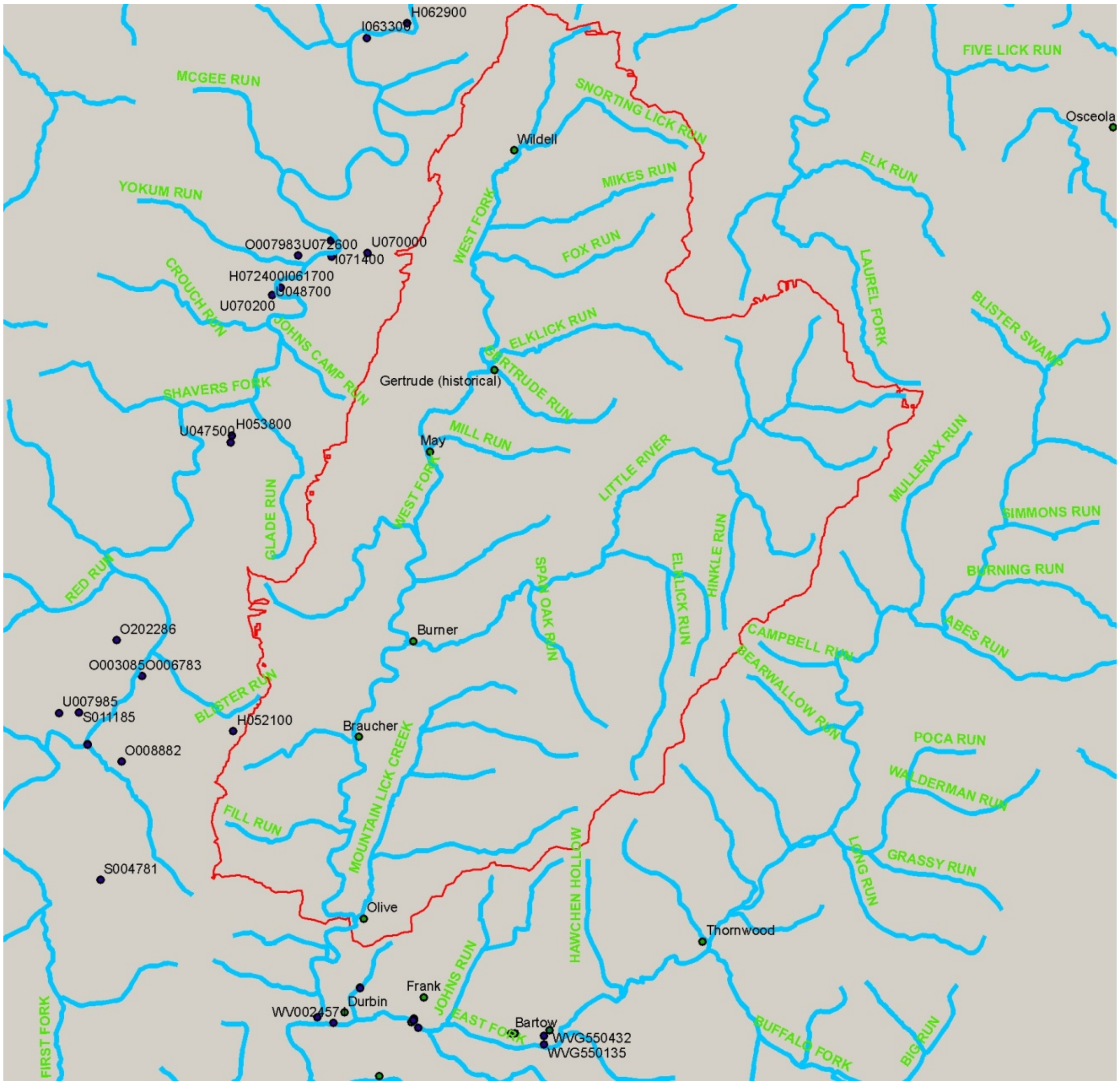

Figure 6 – Point source discharges in West Fork Greenbrier River site's watershed. For this site, there were no permitted discharges at all within the watershed. This permit record search was used to help justify the choice of these sites as reference sites.

FID	9305
Shape	Point
permit_id	S201506
permittee	BOOMERANG COAL INC
facility	Brady Mine
issuedate	5/15/2007
expiredate	5/15/2012
type	Coal Surface Mine
inspstatus	AM-Active, Moving Coal
inspdate	7/16/2008
latitude	38.6208
longitude	-80.1708
acres_new	10
acres_now	10
acres_dist	-1
acres_recl	-1
per_status	New

Figure 7 – Example of information available for each permitted discharge shown in the above maps.

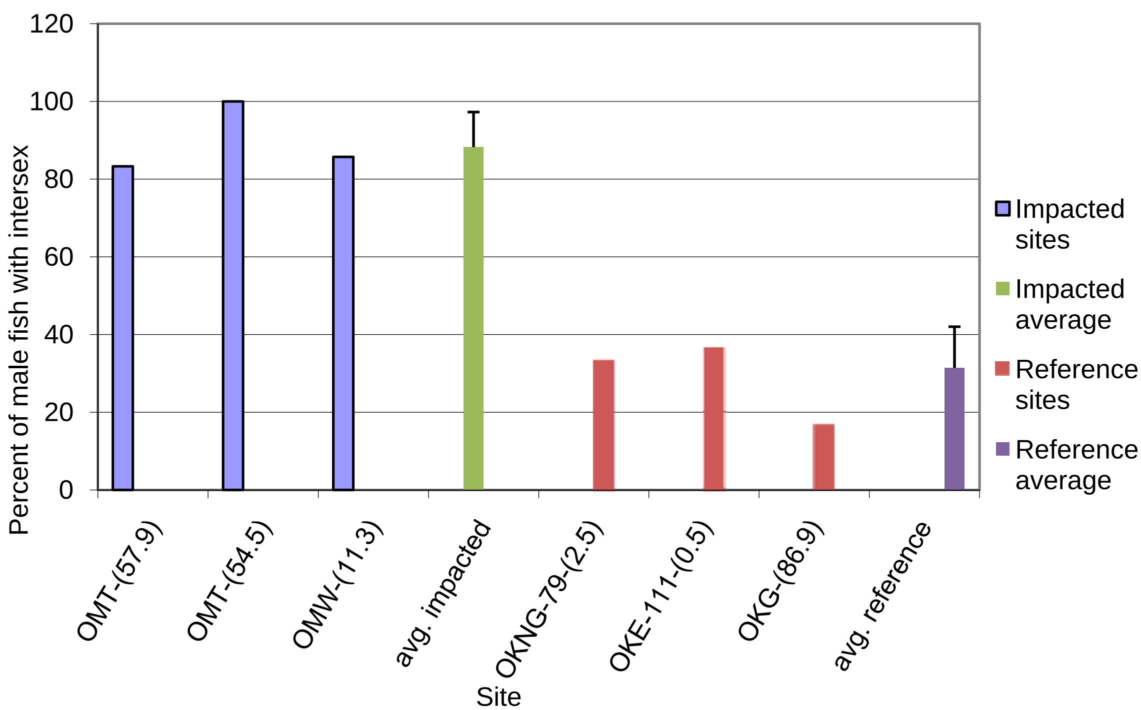

Figure 8 – Average intersex occurrence at each site, grouped by impacted and reference sites. A non-parametric Mann-Whitney U Test ($z = 1.96$, $p = 0.02$) demonstrates a significant increase at impacted sites($\bar{x} = 88.2$ %) when compared with reference sites ($\bar{x} = 31.4$ %).

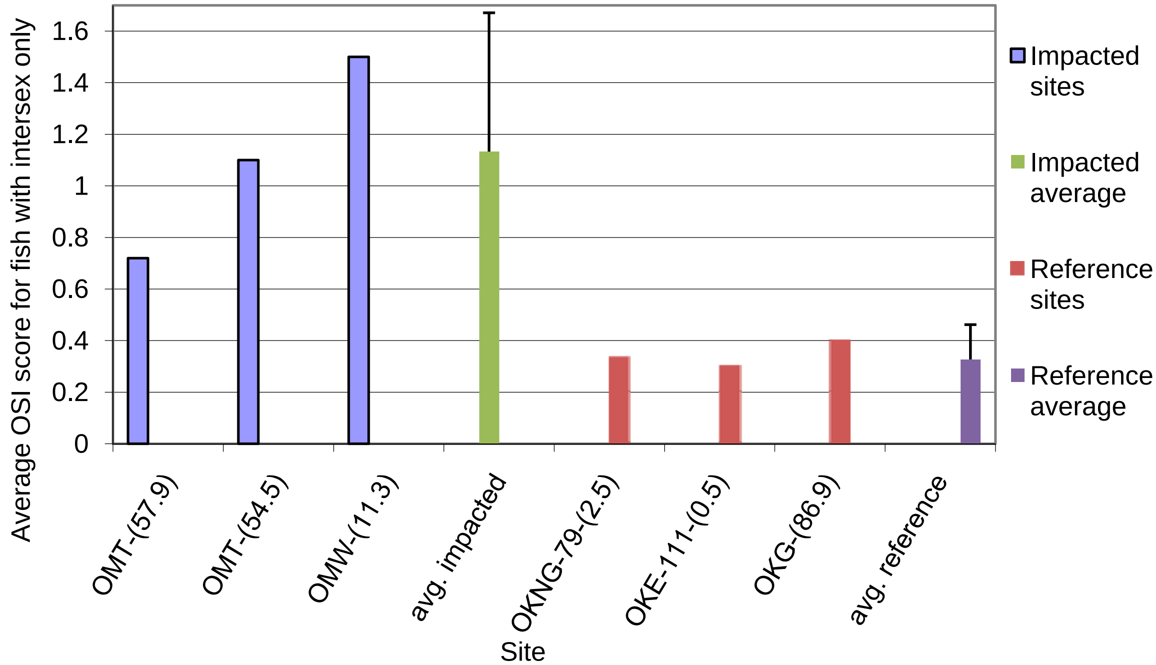

Figure 9 – Average OSI scores for each site, grouped by impacted and reference sites. Mean scores of 1.0 for impacted sites, and 0.1 for reference sites were demonstrated to be significantly different by a Mann-Whitney U test ($z = 5.08$, $p < 0.001$).

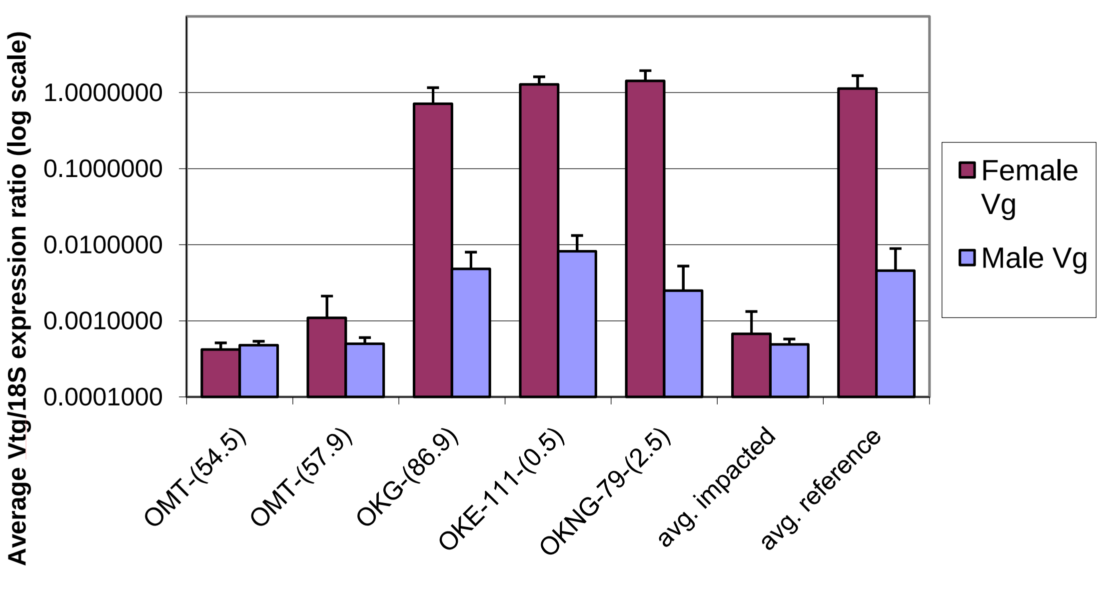

Figure 10 - Average vitellogenin expression at each site with combined averages for impacted and reference site groups. Only the decrease in female VTG at impacted sites was found to be statistically significant by a one-tailed Mann-Whitney U test ($z = -4.32$, $p < 0.001$).

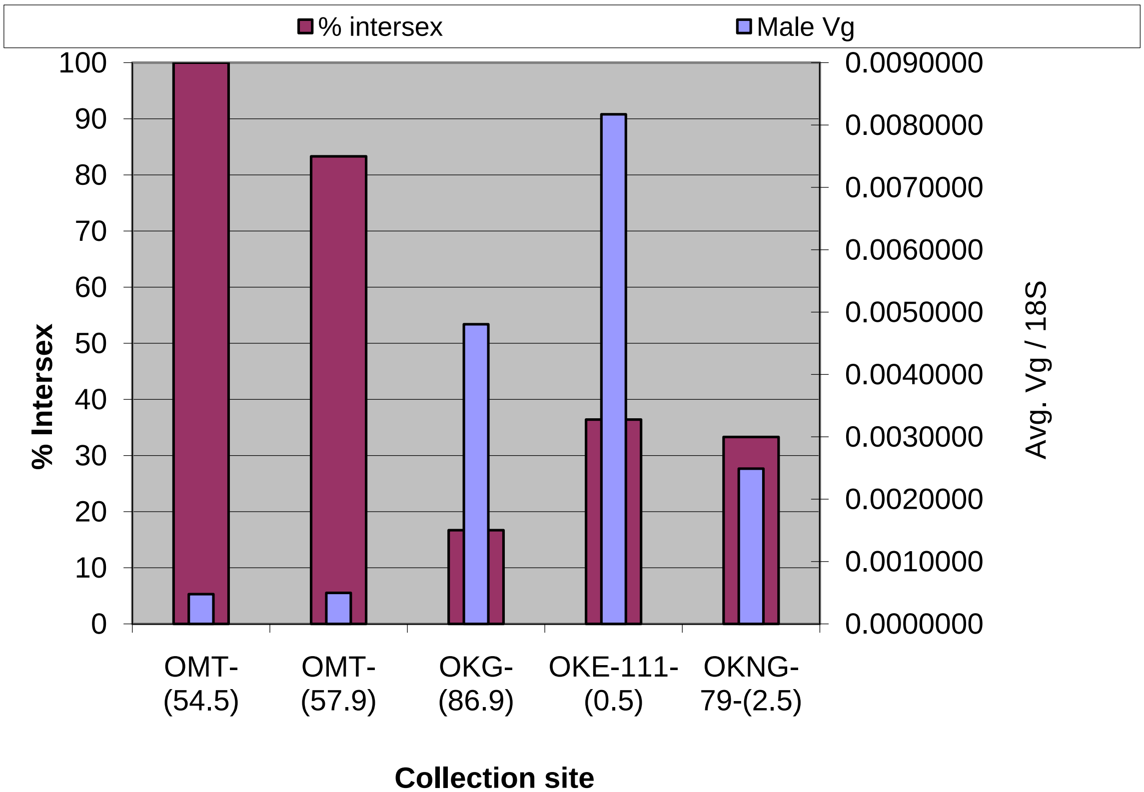

Figure 11 – Intersex occurrence (left axis) and normalized VTG expression in males (right axis) for each site with available VTG data. Though impacted sites (OMT-(54.5) and OMT-(57.9)) appear to have lowered VTG expression, this is not a statistically significant difference.

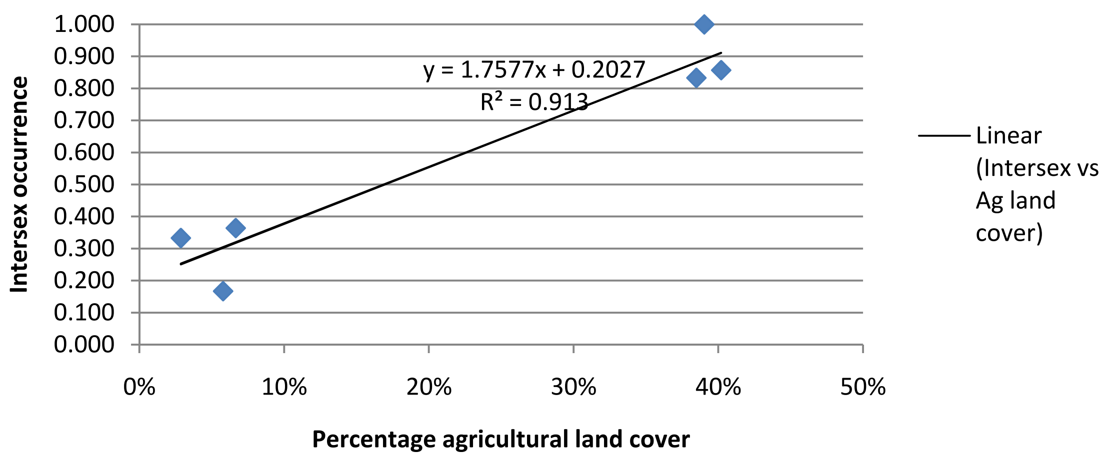

Figure 12 – Intersex occurrence as a function of agricultural land cover (%) with linear regression. These data were generated using the 5-mile radius region of influence model. Agricultural land cover is the strongest predictor of intersex in our model.

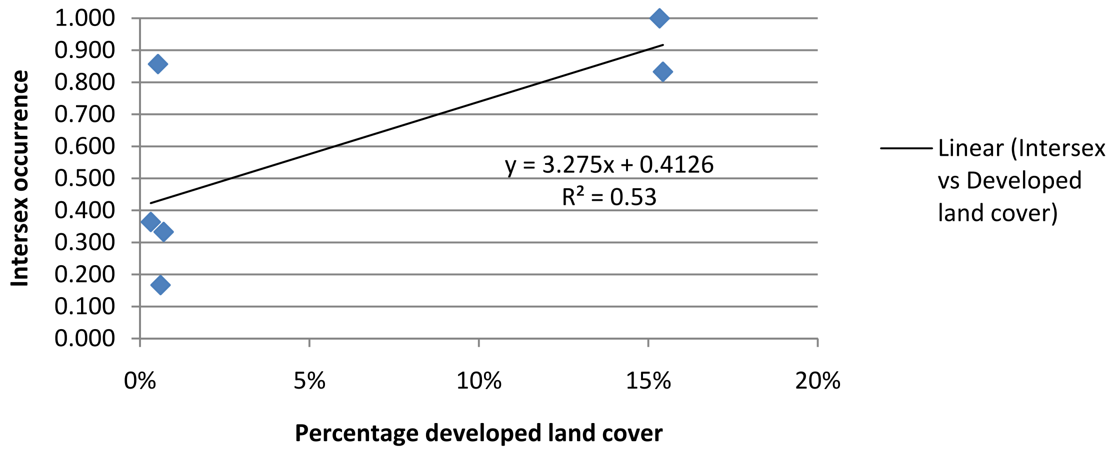

Figure 13 – Intersex occurrence as a function of developed land cover (%) with linear regression. These data were generated using the 5-mile radius region of influence model. Developed land cover was unable to predict intersex occurrence in our model.

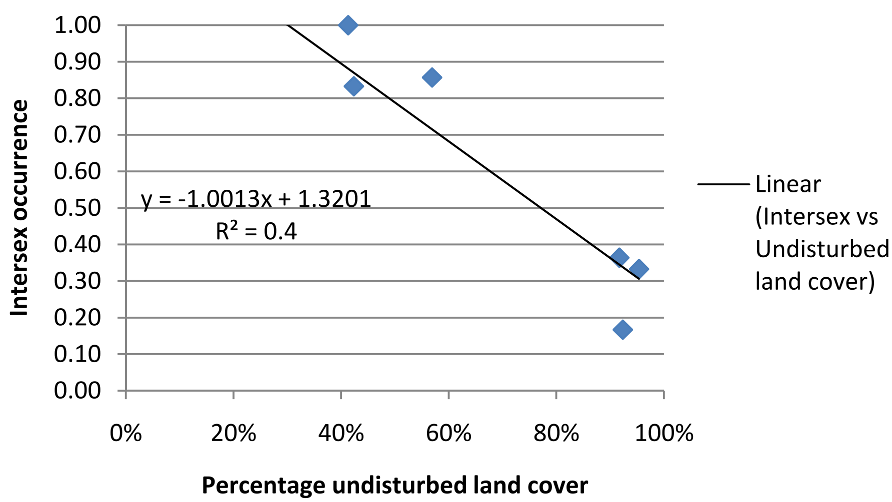

Figure 14 – Intersex occurrence as a function of undisturbed land cover (%) with linear regression. These data were generated using the 5-mile radius region of influence model. While not as strong as agricultural land cover, undisturbed, or naturally-vegetated land cover was found to be a fair predictor of intersex occurrence (inverse relationship) in our model.

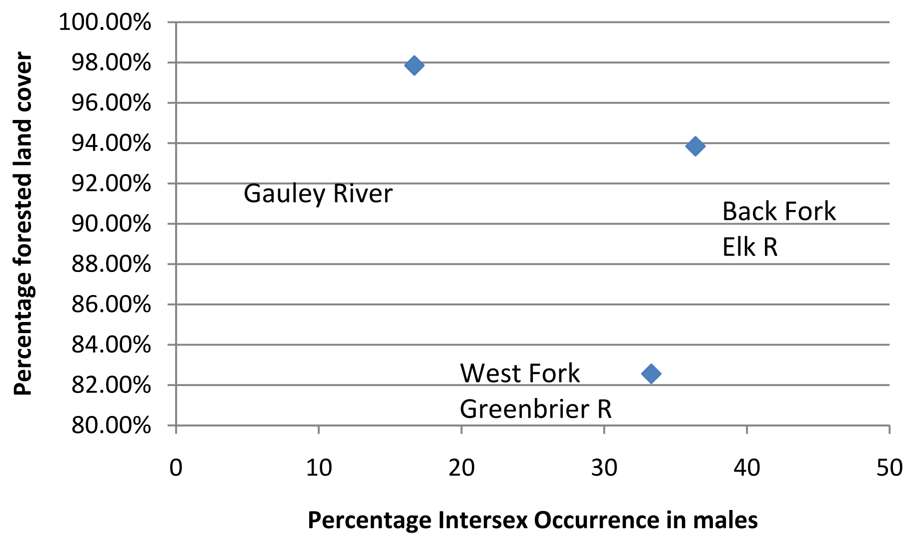

Figure 15 – Intersex occurrence versus forested land cover at WV sites using the watershed delineated region of influence model.

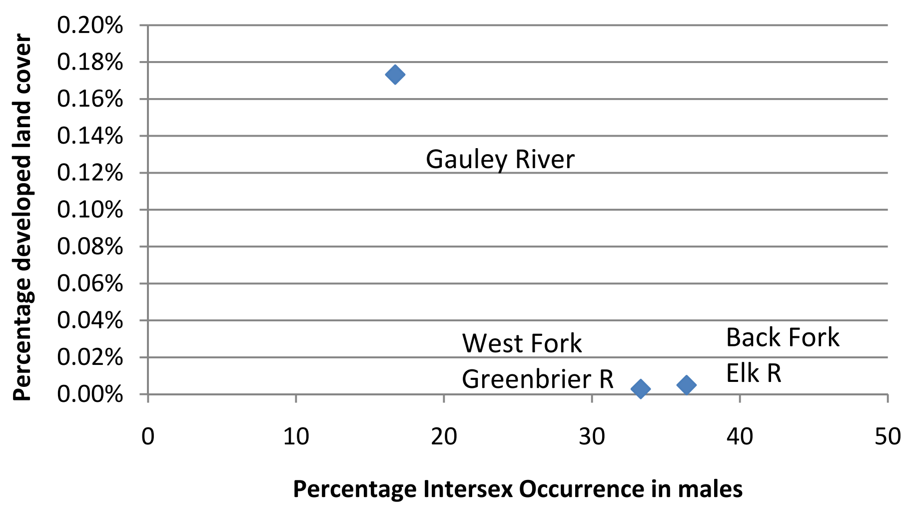

Figure 16 – Intersex occurrence versus developed land cover at WV sites using the watershed delineated region of influence model.

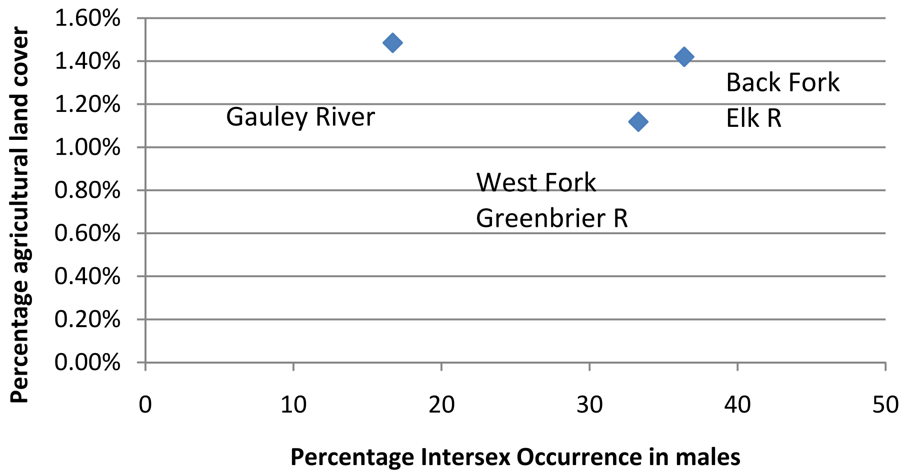

Figure 17 – Intersex occurrence versus agricultural land cover at WV sites using the watershed delineated region of influence model.

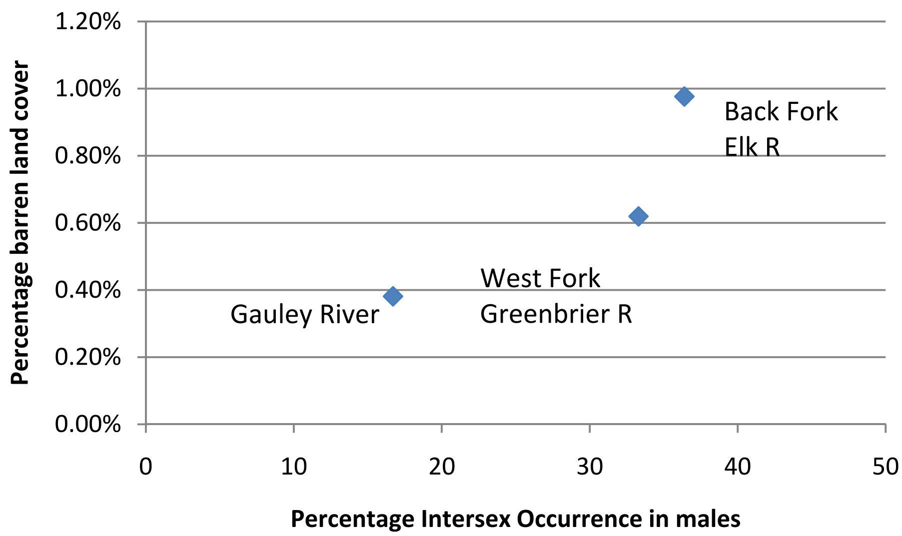

Figure 18 – Intersex occurrence versus barren land cover at WV sites using the watershed delineated region of influence model.

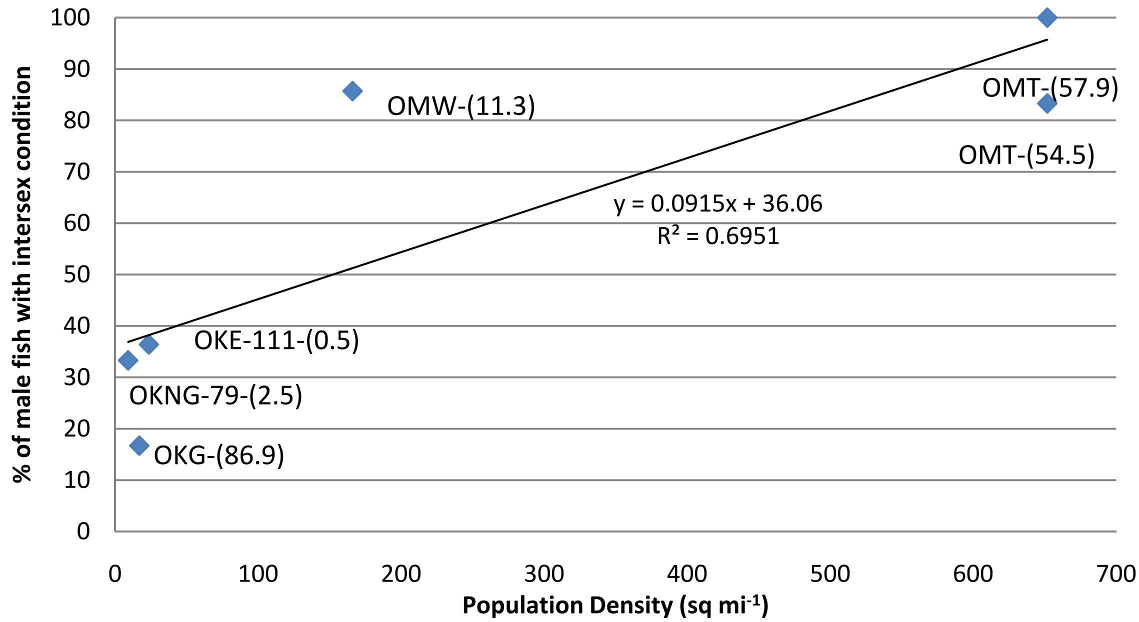

Figure 19 – Population density in watersheds of sample sites. A linear regression line is also plotted and labeled with its equation. Reference sites, clustered in the lower left, are all in sparsely populated watersheds with minimal human impact.

Appendix

Table : List of known and suspected endocrine disrupting compounds.

Category	Examples
…by intended use	
Personal Care Products (PCPs)	-soaps and other disinfectants containing triclosan and other antimicrobials -breakdown products of detergents and other surfactants (nonylphenol, octylphenol)
Pharmaceuticals	-birth control (17β estradiol) -hormone replacement therapy -veterinary hormone supplements -diethylstilbestrol (DES) -cimetidine
Pesticides	2,4,5-T 2,4-D alachlor aldicarb d-trans allethrin amitrole atrazine benomyl beta-HCH carbaryl chlordane chlozolinate -cyhalothrin cis-nonachlor cypermethrin DBCP dichloro-diphenyl-trichloroethane (DDT) DDT metabolites dicofol dieldrin endosulfan esfenvalerate ethylparathion fenvalerate h-epoxide heptachlor iprodione kelthane kepone ketoconazole lindane linurone malathion mancozeb maneb

	methomyl methoxychlor metiram metribuzin mirex nitrofen oxychlordane permethrin procymidone sumithrin synthetic pyrethroids toxaphene trans-nonachlor tributyltin oxide trifluralin vinclozolin zineb ziram
Plasticizers	Phthalates
Natural products	Microcystins – (Peptides produced by cyanobacteria with toxic and potential endocrine-disrupting properties. Microcystins are similar to vasopressin and vasotocin and can therefore react with vasopressin receptors to induce altered expression of sex hormones as well as glucocorticoids.)
…by chemical structure	
Polychlorinated biphenyls (PCBs) – formerly used in electrical equipment, adhesives, and as flame-retardants	3,4,4',5-Tetrachlorobiphenyl
Polybrominated biphenyls (PBBs) – fire retardants	
Phthalates	DEHP BBP DBP DPP DHP DprP DCHP DEP
Phenols	penta- to nonyl- phenols bisphenol A bisphenol F
Styrenes	Styrene dimers and trimers octachlorostyrene

Polyaromatic hydrocarbons (PAHs)	Phenanthrene anthracene
Dioxins (common combustion byproducts)	**2,3,7,8-TCDD – (Tetrachlorodibenzo-p-dioxin)** Carcinogen, toxin, reproductive toxin, suspected endocrine disruptor, most toxic of dioxins – std. for comparison with toxicity of other dioxins **1,2,3,7,8-PeCDD – Pentachlorodibenzodioxin** Found in 2004 study of umbilical cord samples from US babies in 10% of babies at concentration of 3.1pg/g lipid weight in whole blood http://www.ewg.org/reports/bodyburden2/cheminfo.php?chemid=40002 (Env. Working Group website – "BodyBurden – The Pollution in Newborns – a benchmark investigation of industrial chemicals, pollutants, and pesticides in human umbilical cord blood" **1,2,3,4,7,8-HxCDD – Hexachlorodibenzodioxin** **1,2,3,6,7,8-HxCDD – Hexachlorodibenzodioxin** **1,2,3,7,8,9-HxCDD - Hexachlorodibenzodioxin** **1,2,3,4,6,7,8-HpCDD - Heptachlorodibenzodioxin** **OCDD – Octachlorodibenzodioxin**
Furans	**2,3,7,8-TCDF - Tetrachlorodibenzofuran** **1,2,3,7,8-PeCDF - Pentachlorodibenzofuran** **2,3,4,7,8-PeCDF - Pentachlorodibenzofuran** **1,2,3,4,7,8-HxCDF - Hexachlorodibenzofuran** **1,2,3,6,7,8-HxCDF - Hexachlorodibenzofuran** **2,3,4,6,7,8-HxCDF - Hexachlorodibenzofuran** **1,2,3,7,8,9-HxCDF - Hexachlorodibenzofuran** **1,2,3,4,6,7,8-HpCDF - Heptachlorodibenzofuran** **1,2,3,4,7,8,9-HpCDF - Heptachlorodibenzofuran** **OCDF - Octachlorodibenzofuran**
Metals	Bismuth Arsenic Cadmium Lead Mercury

Resume

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Education

2004-2009 Marshall University Huntington, West Virginia, USA

Master of Science Degree in Biological Sciences

- 2 year program – Thesis topic: Environmental Chemistry: Emerging Contaminants in WV Waters

1998 – 2002 Cornell University Ithaca, New York, USA

Bachelor's Degree in Biochemistry

- 4 year degree program in sciences and liberal arts at an ivy league university

Professional experience

2006- present West Virginia Department of Environmental Protection, W.Virginia, USA

Environmental Resource Specialist

- Use of EPA EMAP protocols for the rapid bioassessment of overall stream health. Protocols include sampling of benthic macroinvertebrates, fish, periphyton, water chemistry, and the quality of available habitat. Project leader on the Potomac River fish kill investigations.

2003-2004 Marshall Univ. School of Medicine Huntington, W. Virginia, USA

Research Assistant

- Prepared RNA from tissues via laser capture microdissection as well as various other cellular and molecular biology techniqes in the field of cancer research.

2002 Bechtel Engineering Corporation Atlanta, Georgia, USA

Field Coordinator

- Worked in field office coordinating efforts on complete refurbishment of Cingular Telecom's cellular system.

2000-2002 Cornell University Ithaca, New York, USA

Biophysics Research

- Lipid research in Dr. Gerald Feigenson's physical chemistry lab in the Biophysics Department in the field of thermodynamics using spectroscopy and confocal microscopy techniques.

Posters & Presentations

Barry, C. Biogeophysical Modeling of Water Pollution in West Virginia Waterways Part I: Sources. 8th International Symposium on High Mountain Remote Sensing Cartography. March, 2005. La Paz, Bolivia.

Merritt, D., **Barry, C.,** & Pauley, T. Comparison of age, size and reproductive status of six populations of *Desmognathus quadramaculatus* (Black-bellied salamander) in the southern Appalachians. 66th Annual Meeting of the American Society of Southeastern Biologists. April, 2005. Florence, AL, USA.

Additional Qualifications & Awards

- 2004 - National Aeronautics & Space Administration (NASA) Research Grant recipient
- Nationally registered Emergency Medical Technician (EMT-B)
- SCUBA-certified: Advanced Open Water diving (PADI)

Interests and activities

Nature photography
Backpacking, SCUBA, Caving, Kayaking, Climbing
Traveling (35 countries on 5 continents)
Rugby (9 years experience)