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Reproductive consequences of exposure to sediment extracts from the South Branch of the Potomac River on Japanese medaka (*Oryzias latipes*)

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**Reproductive Consequences of Exposure to Sediment Extracts
from the South Branch of the Potomac River
on Japanese Medaka (*Oryzias latipes*)**

Seth R. Davis

Thesis submitted to the
Davis College of Agriculture, Forestry and Consumer Sciences
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in partial fulfillment of the requirements
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Master of Science
in
Wildlife and Fisheries Resources

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Abstract

Reproductive Consequences of Exposure to Sediment Extracts from the South Branch of the Potomac River on Japanese Medaka (*Oryzias latipes*)

Seth R. Davis

An intersex condition, defined as the presence of oocytes in the testes of male gonochoristic fish, has been observed in smallmouth bass in the South Branch of the Potomac River, West Virginia, which indicates exposure to exogenous estrogens. Endocrine disrupting chemicals (EDC's) are generally hydrophobic and would tend to be found within the sediment of aquatic environments. Few studies have attempted to show the effects of exposure to EDC's on fish using sediment chemical extracts. We have developed a mass sediment extraction technique using 2 solvents (hexane, ethyl acetate:acetone 50:50) to determine the effects of extracted chemicals from three sites (Springfield, Petersburg, and Franklin, WV) on reproductive performance and physiological parameters of adult mating pairs of Japanese medaka (*Oryzias latipes*) for 14 days. Sediment aliquots (200g dry weight) were mixed with solvent 3 times, sonicated, and filtered. Pairs were subjected to extracts at the ratio of 10g of extracted sediment in 1L of water. Hatching success significantly decreased due to exposure to Franklin extract (both solvents) and Petersburg extract (ethyl acetate:acetone). Springfield exposed females had a significantly increased hepatosomatic index (HSI) and showed a trend towards increased vitellogenin (Vtg) production. Results indicate that non-polar and moderately polar compounds may be disrupting development of fertilized eggs and this is most likely due to mixture effects because results were similar with both extracts. Smallmouth bass in the South Branch are likely experiencing chronic exposure or periodic exposure at critical life periods, which may explain why estrogenic effects were not observed in a 14-d exposure. We suggest this method be used in combination with *in vitro* assays and analytical chemistry in order to investigate mixtures within sediment.

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Foreword

This thesis has been formatted for submission to Chemosphere according to the journals preparation guidelines. Chapter 2 and 3 will be combined into one paper for publication.

Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Forward.....	iv
Table of Contents.....	v
List of Tables and Figures.....	vii
Chapter 1: Literature Review.....	1
Literature Cited.....	13
Chapter 2: Hexane extract exposure	
Abstract.....	20
Introduction.....	20
Methods.....	23
Results.....	28
Discussion.....	30
Literature Cited.....	34
Figures.....	37
Tables.....	39
Chapter 3: Ethyl acetate:acetone exposure.	
Abstract.....	45
Introduction.....	46
Methods.....	48
Results.....	48

Discussion.....	51
Literature Cited.....	55
Figures.....	58
Tables.....	61
Conclusion.....	67

List of Figures and Tables

Chapter 2: Hexane extract exposure

Figures

Figure 1. South Branch of the Potomac River, WV, and the 3 sites where sediment was collected in Spring 200637

Figure 2. The percent of fertilized eggs that hatched of medaka exposed to hexane sediment extracts. Franklin exposed pairs spawned eggs with significantly lower hatchability compared to controls (KW test, $\chi^2=10.3925$, $p=0.0155$)38

Tables

Table 1. Percent water of sediment collected in Spring 2006 and the wet weight of aliquots used to achieve the equivalent of 200g dry weight for extraction39

Table 2. Body condition measures, fecundity, egg diameters, and percent of spawned eggs fertilized of medaka pairs exposed to hexane sediment extracts.....40

Table 3. Hepatosomatic index (HSI) and gonadosomatic index (GSI) of male and female medaka exposed to hexane sediment extracts.....41

Table 4. Mean percent of oocytes in each stage of development42

Table 5. Mean number of macrophage aggregates and bile ducts of medaka exposed to sediment extracts.....43

Table 6. Hepatic vitellogenin concentration (ng Vtg/mg protein) of male and female medaka exposed to hexane extracts44

Chapter 3: Ethyl acetate:acetone extract exposure

Figures

Figure 1. South Branch of the Potomac River, WV, and the 3 sites where sediment was collected in Spring 200658

Figure 2. Hepatosomatic index (HSI, liver weight(g)/body weight(g) \pm SE) of male (shaded bars) and female (clear bars) medaka exposed to ethyl acetate:acetone (50:50) sediment extracts. Franklin medaka had significantly greater HSI's than freshwater controls, but not different than solvent controls, and Springfield exposed medaka had significantly greater HSI's than both controls (KW test, $\chi^2=10.5085$, $p=0.0147$).....59

Figure 3. The percent of fertilized eggs that hatched of medaka exposed to ethyl acetate:acetone (50:50) sediment extracts. Franklin and Petersburg exposed pairs spawned eggs with significantly lower hatchability compared to controls (KW test, $\chi^2=13.4153$, $p=0.0038$).....60

Tables

Table 1. Percent water of sediment collected in Spring 2006 and the wet weight of aliquots used to achieve the equivalent of 200g dry weight for extraction61

Table 2. Body condition measures, fecundity, egg diameters, and percent of spawned eggs fertilized of medaka pairs exposed to hexane sediment extracts.....62

Table 3. Hepatosomatic index (HSI) and gonadosomatic index (GSI) of male and female medaka exposed to hexane sediment extracts..... 63

Table 4. Mean percent of oocytes in each stage of development. 64

Table 5. Mean number of macrophage aggregates and bile ducts of medaka exposed to sediment extracts.....65

Table 6. Hepatic vitellogenin concentration (ng Vtg/mg protein) of male and female medaka exposed to ethyl acetate:acetone extracts.....66

Chapter 1: Literature Review

Recently much effort in the scientific community has been directed toward identifying water systems that contain a newly recognized group of environmental contaminants called endocrine disruptors (Jobling and Tyler 2003). Studies are being performed to identify and understand the action of these endocrine disrupting chemicals (EDC's) on individual organisms in an attempt to determine potential population affects in preparation for future management. The list of EDC's is ever-expanding, and the occurrence of abnormalities in fish and wildlife populations are being identified at a significant rate. The chemicals are now a global issue. Significant levels of organochlorines which may act as endocrine disruptors have been found in polar bears in Svalbard, Canada (Lie et al. 2005).

The list of EDC's is numerous and includes natural and synthetic hormones as well as organochlorines, polycyclic aromatic hydrocarbons, dioxins, byproducts of plastic manufacturing, heavy metals, etc. (Arcand-Hoy et al. 1998). These classes of chemicals may be significantly different structurally from the hormones that they inhibit or mimic (Arcand-Hoy et al. 1998), which makes predicting which ones will cause endocrine disruption difficult without controlled experiments. In addition, single compounds can exert an array of affects. In studies with Atlantic salmon (*Salmo salar*), 4-nonylphenol (NP) was shown to act as an estrogen mimic, a steroid metabolism disruptor, and by modulating estrogen receptor levels (Goksoyr 2006). Coupled with the fact that wild animals are exposed to a mixture of chemicals which may have profound affects at extremely low concentrations, their detection requires somewhat intensive

investigation, and their potential sources in our water systems are hard to determine (Jobling et al. 1998).

A critical fact regarding endocrine disruption is that signs of exposure to EDC's have been identified globally in wild fish populations. Here in the U.S. white perch (*Moronea americana*) in Lake Ontario (Kavanagh et al. 2004), common carp (*Cyprinus carpio*) sampled near St. Paul, Minnesota (Folmar et al. 1996), and smallmouth bass (*Micropterus dolomieu*) located in the South Branch of the Potomac river and the Shenandoah river (Blazer et al., 2008) have shown definite signs of endocrine disruption. Also, endocrine disruption is believed to be the cause of fish abnormalities in waters of Sweden (Larsson et al. 1999), the Elbe River, Germany (Hecher et al. 2002), South Africa (Barnhoon et al. 2004), France (Minier et al. 2000), the United Kingdom (Jobling et al. 2002a), and Tokyo Bay, Japan (Hashimoto et al. 2000). These studies indicate that the effects of endocrine disruption are wide reaching, and the actions of EDC's are affecting a variety of species.

Endocrine disrupting chemicals are synthetic or naturally occurring chemicals that modify or disrupt normal hormonal functions. They can alter the quantities of circulating hormones within organisms, change hormone messages and alter cell activity, amplify the effects hormones have on receptor cells, inhibit the ability of normal hormones to bind to receptor sites, or mimic normal hormones and cause unpredictable cell activity (Arcand-Hoy et al. 1998). Because receptors for hormones are found in multiple tissues and organs within organisms, and because chemicals may act directly through receptors, or via indirect mechanisms, the potential effects of EDC's on individuals are virtually limitless. Larsson et al. (1999) reported that environmental estrogens can disrupt normal

physiological functions at various sites, including mammary glands, bone tissue, cardiovascular tissue, and gonadal tissue. This illustrates the difficulty in determining EDC's direct and indirect effects on organisms.

Induction of vitellogenin in male and juvenile fishes has become a biomarker for exposure to estrogenic EDC's (Sumpter and Jobling 1995). Vitellogenin is an egg yolk protein precursor that is expected to be only expressed in females. Estrogen receptors are found in the liver and once activated lead to the transcription of vitellogenin, which travels through the blood to the ovaries where it is sequestered. Vitellogenin was recently suggested to be physiologically involved in defense reactions in the rosy barb (*Puntius conchonius*) as it displayed antibacterial activity and was induced in male fish challenged with *E. coli* (Shie et al. 2006). Despite this, induction of vitellogenin is regarded as an estrogen receptor-mediated pathway, which is dependent on an estrogenic ligand (Fujiwara et al. 2005). Laboratory studies have shown that a number of EDC's induce vitellogenin in male fish including estrone (E1) (Routledge et al. 1998), estradiol (E2) (Folmar et al. 2000, Gimeno et al. 1998, Routledge et al. 1998, Brion et al. 2004), ethynylestradiol (EE2) (Hill and Janz 2003, Folmar et al. 2000, Nash et al. 2004, Jobling et al. 1996), NP (Hill and Janz 2003, Harries et al. 2000), octylphenol (OP) (Gronen et al. 1999), and diethylstilbestrol (DES) (Folmar et al. 2000).

Field studies have reported that vitellogenin induction occurs in a variety of species. Male white perch in the lower Great Lakes region showed significant levels of plasma vitellogenin according to Kavanagh et al. (2004). The list of fish species found in field studies with significantly increased vitellogenin production when exposed to EDC's includes common carp in a Minnesota metropolitan area (Folmar et al. 1996), rainbow

trout (*Oncorhynchus mykiss*) in Sweden (Larson et al. 1999), wild bream (*Abramis brama* L.) in Germany (Hecker et al. 2002), and flounder (*Pleuronectes yokohamae*) in Tokyo Bay, Japan (Hashimoto et al. 2000). One consideration that cannot be ignored in long-term or field studies is that the induction of vitellogenin has been shown to diminish over time. Nash et al. (2004) found that zebrafish (*Danio rerio*) showed no vitellogenin induction after life-long exposure to <5ng/L EE2, indicating the fish had acclimated. Male Japanese medaka (*Oryzias latipes*) also showed a disappearance of vitellogenin production following OP exposure (Gronen et al. 1999). These studies indicate that the induction of vitellogenin can be useful in determining if short-term exposure to EDC's has occurred.

Physical abnormalities to gonads represent another group of effects on fish that occur as a result of exposure to estrogenic compounds. In male zebrafish, 50 ng/L EE2 significantly decreased gonadal growth (Nash et al. 2004). Van den Belt et al. (2002) also showed that EE2 had negative physical effects on zebrafish characterized by a significant decrease in gonado-somatic index (GSI), and an absence of mature vitellogenic oocytes within the ovaries, which are generally a major fraction of ovarian volume. Testicular fibrosis was observed in sheepshead minnows exposed to 2 ng/L EE2 (Zillioux et al. 2001), Japanese medaka exposed for 3 weeks to 463 ng/L E2 (Kang et al. 2002), and Japanese medaka exposed to OP (Gray et al. 1999). In addition, Flammarion et al. (2000) showed that just 2 injections of 2mg/Kg E2 over a 14-day period resulted in degenerative and necrotizing sperm cells in the chub (*Leuciscus cephalus*). Atrophied gonads have been reported in mosquitofish (*Gambusia affinis*) exposed from 3 days-post-hatch (DPH) to 78 dph in 0.5-50 µg/L 4-NP (Dréze et al. 2000).

Several more examples of physical abnormalities have been shown to result from exposure to estrogens. Nephrotoxicity was observed in zebrafish as a result of exposure to 10 ng/L EE2 (Weber et al. 2003). Also fathead minnows exposed from their embryo stage to 56-days in EE2 had anal protrusions and distended abdomens in 40-50% of fish exposed to 16 ng/L EE2 and all fish exposed to 64 ng/L EE2 (Länge et al. 2001). Dréze et al. (2000) described an array of liver abnormalities seen in the mosquitofish including lipid content depletion, perivascular fibrosis, and hepatocytes that were fibroblast-like and contained nuclei at various stages of pycnosis. Lastly, Nash et al. (2004) reported that zebrafish did not exhibit acute toxicity to 50 ng/L EE2 demonstrated spinal deformities.

Of particular interest regarding physical abnormalities associated with exposure to estrogenic chemicals is the incidence of intersex. Intersex is characterized by the presence of ovarian tissue within testes of male fish. Although hemaphroditism and sex reversal are common in some families of fish, a majority of fish are gonochorists and distinctive males and females exist and are stable (Nolan et al. 2001). Cases of intersex have been identified in various species around the world including smallmouth bass in the Shenandoah River, VA, and South Branch of the Potomac River, WV (Blazer et al., 2008) and in the Snake River and Columbia River (Hinck et al. 2006), largemouth bass in the Rio Grande Basin (Schmitt et al. 2005), sharptooth catfish (*Clarias gariepinus*) in South Africa (Barnhoon et al. 2004), white perch in Lake Ontario, USA (Kavanagh et al. 2004), roach (*Rutilus rutilus*) in French rivers (Minier et al. 2000), roach in U.K. rivers (Jobling et al. 2002a), and the flounder in Japan (Hashimoto et al. 2000).

Many laboratory studies have shown that Japanese medaka develop an intersex condition when exposed to high enough concentrations of estrogenic chemicals. Metcalfe et al. (2000) saw intersex in medaka from treatments of o,p'-DDT at concentrations of 5, 10, and 50 µg/L from 1-100 dph. Gronen et al. (1999) observed intersex in medaka with a 21-day exposure of 74 and 230 ng/L OP. Additionally, Kang et al. (2002) reported intersex medaka in every treatment group in their investigation of E2, including 5 out of 8 males in the lowest treatment (29.3 ng/L). Gray et al. (1999) illustrated that a critical period exists during a medaka's early life stages when it is most sensitive to exogenous estrogens by showing that a 100 µg/L OP exposure starting at 3 DPH resulted in a greater incidence of intersex than beginning the exposure at 1,5,7,21, or 35 [dph](#). Another species that has shown intersex in laboratory experiments are zebrafish, exposed to 30 and 100 µg/L NP by Hill and Janz (2003), and in a life-long exposure to 5 ng/L EE2 by Nash et al. (2004).

Due to the feminizing effect of estrogenic chemicals, skewed sex ratios have been observed in many laboratory studies. Piferrer and Donaldson (1992) found that Chinook salmon fry (*Oncorhynchus tshawytscha*) developed into all females when immersed in 400 µg/L E2 for eight hours. Similar results were found for salmon in a 2-hour immersion of 400 µg/L EE2 (Piferrer and Donaldson 1992). Lee et al. (2003) reported all female Korean rockfish (*Sebastes schlegeli*) when fry were fed either E2 or 2,4-dinitrophenol at 5, 50, or 100 µg/g food for 29 days. Additionally hybrid sturgeon (*Huso huso* female X *Acipenser ruthenus* male) were all female after being fed a 1 µg/g E2 diet from 3-18 months (Omoto et al. 2002). Lastly Länge et al. (2001) reported fathead minnows skewed towards females in >1 ng/L EE2. With evidence of intersex in wild

fish, it is possible that feminized populations of wild fish exist, and that there may be potential population declines if the problem is severe enough to alter normal reproduction.

Few studies have demonstrated that endocrine disruption, specifically due to exposure to environmental estrogens, have been negative influences on the sustainability of wild fish populations. An understanding of reproductive effects is of greatest importance (Brion et al. 2004). Laboratory studies have indicated that environmental estrogens alter normal reproduction in many ways, and may result in populations that are impacted in the wild. Hill and Janz (2003) showed that zebrafish adults exposed to 10 ng/L EE2 and 100 µg/L NP had significant decreases in percent viable eggs produced. The same investigators reported that resulting eggs hatched at a significantly lower rate and swim-up success decreased as a result of the exposure (Hill and Janz 2003). In another study, zebrafish subjected to a life-long exposure to 5 ng/L EE2 showed complete reproductive failure (Nash et al. 2004). A study with medaka revealed that males exposed to OP for 21-days and then matched with healthy females showed significantly decreased reproductive success illustrated by decreased numbers of eggs, percent of eggs fertilized, and survival of the embryos (Gronen et al. 1999). Medaka were also reported to show decreases in courtship intensity which resulted in reductions in fertilization rates when exposed to OP from early development (Gray et al. 1999b). The above studies indicate that wild populations may be experiencing reproductive failure in some fashion. Jobling et al. (2002b) reported a significant negative correlation between fertilization success and degree of feminization of the intersex gonads in wild roach.

Considering all of the effects of EDC's, it should be noted that exposure to estrogenic chemicals sometimes leads to mortality in fish. Herman and Kincaid (1988) observed 50% mortality in rainbow trout orally administered E2 in food at a dose of 30 mg E2/Kg food over a 76-day experiment. Also in the presence of E2, male fathead minnows exogenously exposed to the hormone showed concentration-dependent mortality (Kramer et al. 1998). At somewhat high concentrations of 400-3,200 ng/L, EE2 caused a significant decrease in survival in subadult sheepshead minnows (*Cyprinodon variegates*) (Zillioux et al. 2001). Mortality in laboratory settings due to exposure to EDC's is often a result of relatively high concentrations of chemicals compared to those generally observed in actual field sampling. It is conceivable that due to the process of bioaccumulation, and because of the physical abnormalities believed to result from exposure to EDC's, mortality in wild fish is certainly a possible consequence of exposure to EDC's.

Few studies have attempted to perform laboratory exposures with environmental samples or *in situ*. Harries et al. (1997) and Pawlowski et al. (2003) caged male rainbow trout at various distances downstream of sewage treatment works (STW) effluent and used vitellogenin induction as a biomarker for estrogen exposure. Similarly Nichols et al. (1999) caged fathead minnows in the effluents of waste water treatment plants in central Michigan to determine the effects on the fish's reproductive physiology. Xie et al. (2004) combined 3 liters of wastewater with 6 liters of tap water to expose rainbow trout and evaluate for estrogenicity. To our knowledge a large scale sediment study to demonstrate the cumulative affects of chemicals within the benthos has not been attempted.

Many studies use *in vitro* assays to quantify estrogenicity of chemicals, mixtures of compounds, tissue samples, and water or sediment samples. These include competitive binding with E2 (Shelby et al. 1996), the yeast estrogen assay (YES) (Huggett et al. 2003), and several others which utilize cells transfected with human or mouse estrogen receptors, such as human embryonal kidney (HEK 293) cells (Schreurs et al. 2002) and MCF-7 cells (Schlumpf et al. 2004). *In vitro* assays allow for a measure of the total potency of complex mixtures (Snyder et al. 2001) and provide information on mechanisms of action (Shelby et al. 1996). Whole-animal studies are hard to use to determine mechanisms due to organism's complex regulatory processes and the possibility of a chemical or mixture having multiple hormonal activities (Sohoni and Sumpter 1998). The drawbacks of *in vitro* assays are that they don't mimic whole animal metabolism and distribution (Shelby et al. 1996) and chemicals may act independently of the receptor and indirectly result in estrogenicity (Beresford et al. 2000).

In vivo and *in situ* exposure of fish to chemicals represents the alternative to using *in vitro* estrogenicity screenings. The chemicals are either injected, given orally in food, or administered via the water through exogenous exposure, and often include measuring vitellogenin or luciferase activity in sacrificed fish as measurements of estrogenic activity. Studies have often shown *in vivo* assays to be more sensitive than *in vitro* assays. Routledge et al. (1998) found that OP was 10 times more estrogenic *in vivo* when measuring plasma vitellogenin in exposed rainbow trout and roach, than using the YES assay. They attributed this to the bioaccumulation occurring over the course of the 21-day exposure. Samples from 4 municipal wastewater facilities in New York and 1 from Texas showed 10-fold greater estrogenicity *in vivo*, when plasma vitellogenin was

measured after a 7-day medaka exposure, compared to *in vitro* YES assays (Huggett et al. 2003). These investigators pointed out that non-estrogen receptor ligands were probably eliciting effects via indirect mechanisms, and the animal cells most likely absorbed the chemicals more efficiently than the yeast cells (Huggett et al. 2003). Pawlowski et al. (2003) caged rainbow trout at 3 sites downstream of municipal treatment plants in Germany and compared vitellogenin induction data to three *in vitro* assays. This group reported vitellogenin induction at 2 of the 3 sites, although estrogenic activity was observed at all sites using the *in vitro* assays, a finding likely explained by the presence of anti-estrogenic compounds that masked the estrogenic activity *in vivo*. Care should be taken when interpreting estrogenicity data *in vivo* and *in vitro*. Also *in vivo* assays take longer, but often represent a more inclusive look at how fish will respond to chemicals in their natural environment.

Waste water treatment works (WWTW's) are considered to be large contributors of estrogenic and androgenic compounds to water systems. Kirk et al. (2002) found effluents from five different WWTW's showed estrogenic activity in the low to medium ng/L range. Desbrow et al. (1998) reported estrogenicity in chemical fractions of domestic wastewater effluents which were found to contain low levels of natural and synthetic steroidal estrogens. Also Murk et al. (2002) found an average reduction in estrogenicity between influent and effluent of 75% for 4 large wastewater treatment plants in the Netherlands, thus estrogenic chemicals are not completely eliminated during treatment, and are being discharged into the aquatic environment. Many studies only use estrogen receptor based *in vitro* assays to detect activity in wastewater effluent, which may underestimate estrogenic activity according to Xie et al. (2004).

Endocrine disrupting chemicals can also enter the aquatic environment due to agricultural practices. Lorenzen et al. (2004) reported chickens sampled throughout Ontario, Canada produced litter with detectable estrogen receptor gene transcription activity. Of greatest concern is the waste runoff that results from these huge operations. Also Paul et al. (2005) showed that exposure of grazing sheep ewes to an experimental sewage sludge treated field following standardized guidelines resulted in a decrease in fetal growth, testicular growth and testicular hormones in male offspring. These results are somewhat controversial and Evans (2006) claims that the inorganic fertilizer applied to the control field contained higher levels of nutrients, explaining the measured differences in body weights of ewes and fetuses. Additionally, the most commonly used herbicide in the U.S., atrazine, has been shown to cause demasculization of the larynx of the African clawed frog (*Xenopus laevis*) and the production of hemaphrodites after larval exposure at environmentally relevant concentrations (Hayes et al. 2002). This is presumably due to a decrease in testosterone, which may be converted to estrogen due to the presence of atrazine.

According to the West Virginia Department of Environmental Protection (WVDEP), fish sampling took place in July 2003 to investigate a high incident of skin lesions, occasional fish kills and a perceived population decrease of smallmouth bass in the South Branch of the Potomac River (SBP). Aided by the U.S. Geological Survey's National Fish Health Laboratory (NFHRL), the WVDEP discovered high occurrences of intersex in male smallmouth bass, including 80% sampled at Petersburg, WV, and 37.5% (including largemouth bass) at Petersburg Gap, WV (V. Blazer, personal communication). The WVDEP reported that there are 73 discharges permitted in the

SBP basin, all of which are potential sources of EDC's. Of these 34 are industrial runoff, 20 for general sewage, 3 for aquaculture facilities, 2 for landfill leachate, and the remaining are individual permits (WVDEP 2006)). Franklin, WV and Petersburg, WV have two of the largest wastewater treatment plants within the basin, and it is believed that up to 60-70% of homes in some areas have "straight-pipe" discharges directly into the river (WVDEP 2006).

Also important is the fact that poultry and cattle farming dominate the basin. Poultry houses and processing plants line the river side in several areas and produce significant amounts of chickens and turkeys annually. In 2002, more than 90 million birds were sold from the eastern panhandle of West Virginia alone (WVDEP 2006). According to Basden et al. (2000) approximately 160,000 tons of poultry litter is produced annually in the state. Most of the waste product is sold to farmers as fertilizers, and ultimately finds its way to our waterways via runoff processes.

Japanese medaka are small rice paddy fish of East Asia that can survive in fresh or saltwater at a wide range of temperatures (~1-36°C). They have been widely used in laboratory settings because they are easily reared, have short life-cycles, and produce viable eggs every day under the correct conditions. This makes the medaka an ideal organism for aquatic toxicology experiments that investigate effects of physiology and reproduction.

We have developed a mass sediment extraction technique using two solvents (hexane, ethyl acetate:acetone (50:50)) in order to expose medaka to environmental samples from the South Branch of the Potomac River. The main objective is to begin to

classify contaminants that may be present using a variety of endpoints including reproductive performance, histological analysis, and vitellogenin induction.

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Chapter 2: Hexane extract exposure

Abstract

Recently intersex has been observed in male smallmouth bass in the South Branch of the Potomac River, West Virginia, which may indicate exposure to exogenous estrogens. Endocrine disrupting chemicals (EDC's) are generally hydrophobic and would tend to be bound to sediment within aquatic environments. Few studies have attempted to show the effects of exposure to EDC's on fish using sediment chemical extracts. We have developed a mass sediment extraction technique to determine the effects of extracted chemicals from three sites (Springfield, Petersburg, and Franklin, WV) on reproductive performance and physiological parameters of adult mating pairs of Japanese medaka (*Oryzias latipes*) for 14 days. Sediments were divided, sonicated with hexane, and filtered three times. After solvent exchange with acetone, pairs were subjected to extracts at the ratio of 10g of extracted sediment in 1L of water. Hatching success significantly decreased due to exposure to Franklin sediment extract, which may indicate the presence of nonpolar contaminants disrupting embryonic development of fertilized eggs. Vtg levels in male and female medaka were not significantly different than controls. Histology showed that tissues and organs were unaffected by exposure. Results indicate that contaminants may be present, but whether these possess endocrine disrupting characteristics requires further investigation.

Introduction

Chemicals that have the potential to alter normal functioning of the endocrine systems of organisms are of global concern (Jobling et al., 1998) and are commonly referred to as endocrine disrupting chemicals (EDC's). Intersex has been observed in

male smallmouth bass (*Micropterus dolomieu*) in the South Branch of the Potomac River, West Virginia (Blazer et al., 2008), which may indicate exposure to exogenous estrogens (Nolan et al., 2001). The list of EDC's includes alkylphenolic compounds, polychlorinated compounds, polybrominated compounds, steroid sex hormones, and phthalates, many of which are of anthropogenic origin (Petrovic et al., 2001). Many of these are hydrophobic compounds and would tend to accumulate in the sediment of aquatic systems.

A variety of endpoints have been used in studies to evaluate the effects of EDC's on fish. Reproductive toxicity has been shown to be a very sensitive outcome to EDC exposure in laboratory studies (Hill and Janz, 2003; Nash et al., 2004; Gronen et al., 1999; Gray et al., 1999), and Jobling et al. (2002) reported a significant negative correlation between fertilization success and degree of feminization of the intersex gonads in wild roach (*Rutilus rutilus*). Physical abnormalities are often reported as consequences of exposure to EDC's and include disrupted oocyte development (Van den Belt et al., 2002), bile duct proliferation (Blom et al., 1998), pigmented macrophage aggregate (MA) accumulation in renal and splenic tissue (Blazer, 1991; Agius and Roberts, 2003; Thilakaratne et al., 2007), and abnormal thyroid follicle histology (Fournie et al., 2005). Additionally, induction of vitellogenin, an egg yolk protein precursor normally only expressed in mature female fish, has become a prominent biomarker to exposure to xenoestrogens (Sumpter and Jobling, 1995) as it has been shown to be an estrogen receptor mediated pathway dependent on an estrogenic ligand (Fujiwara et al., 2005).

Though many studies have shown the effects of single compounds or mixtures on a variety of fish species, environmental exposures, or *in situ* laboratory exposures are rare. Harries et al., (1997) and Pawlowshi et al., (2003) caged male rainbow trout (*Oncorhynchus mykiss*) downstream of sewage treatment works and measured vitellogenin to determine estrogenicity of the effluent. Similarly Nichols et al., (1999) caged fathead minnows (*Pimephales promelas*) in effluent of waste water treatment plants to determine the effects on reproductive physiology. Others have used diluted wastewater (Xie et al., 2004) and solvent extracts (Huggett et al., 2003) to expose fish and test for estrogenicity. To our knowledge a large scale sediment extraction and *in vivo* exposure has not been attempted.

Japanese medaka (*Oryzias latipes*) were used in this study because they are easily reared, become sexually mature in a short period of time (~3-4 months), and produce viable eggs daily under the correct conditions (Yamamoto 1975). Medaka have been used extensively in laboratory studies to demonstrate the effects of EDC's on fish reproduction, physiology, and behavior. For these reasons medaka are ideal organisms for aquatic toxicology studies.

The objective of this study is to determine the effects of hexane sediment extracts from 3 sites on the South Branch of the Potomac (SBP) River on mating pairs of mature medaka exposed for 14 days as an initial attempt at classifying potential contaminants present in the river. Endpoints measured will include reproductive success, histological parameters, and hepatic vitellogenin.

Methods

Extracts

Sediment was collected in May 2006 from three sites (Franklin, Springfield, Petersburg, WV) on the South Branch of the Potomac River (Figure 1) in areas where clay and silt were available. Samples were placed in acetone-rinsed aluminum foil and stored at -20°C for less than 40 days until extraction. To thaw, samples were placed over a mesh screen which allowed water to drain off. Aliquots from each site were thawed, weighed and allowed to dry in order to determine percent water content (Table 1). Wet samples were weighed so that each was the equivalent of 200g dry weight as in Table 1. These aliquots were mixed with 150ml hexane and sonicated for 5 minutes with 3 second pulses and 3 second pauses using a Fisher Scientific SSD Sonic Dismembrator with a Misonix, CL-4 Ultrasonic Converter, capable of 20KHz and equipped with a 1.905cm (¾ inch) flat tip horn. The intensity was sufficient to produce visible mixing. The solvent was poured off through a 0.45µm glass filter with assisted filtration. The sonication-filtration process was repeated two more times with new solvent. The resulting extracts (~4-5L) were combined, reduced in volume to ~10ml under a nitrogen stream, solvent exchanged to acetone, and stored at -20°C.

To determine recovery efficiencies of a common PCB, two 200g sediment aliquots were spiked with decachlorobiphenyl, sonicated, filtered, solvent exchanged to acetone and analyzed according to EPA method 8082 (EPA, 1998) for PCB's on a Varian CP-3800 GC (Ser # 10608, Varian Analytical, Chicago, IL) equipped with a Varian 8410 auto injector with 10 position rack (S/N 20734), dual 1177 split/splitless

injectors and dual Ni⁶³ Electron Capture Detectors (Model number 02-001972-00, S/N 413943 and 413096). These showed recovery efficiencies of 44% and 70% respectively.

Fish

Japanese medaka used in this study were from a breeding stock maintained at West Virginia University for over 3 years. Water quality parameters averaged: pH 6.5, 22°C, 6.1 mg/L dissolved oxygen, 140 mg/L hardness as CaCO₃, and 1.7 g/L salinity. Fish were fed freshly hatched brine shrimp twice daily and commercial flake food (Tetramin©, Blacksburg VA) once daily. Water temperatures were regulated in water baths at 28±1°C, and a 16:8 light:dark photoperiod was maintained. All medaka used in this experiment were sexually mature (at least 4 months of age).

Conditions of Exposure

Fifteen mating pairs of Japanese medaka were exposed to extract for 14-days from either Franklin, Springfield, or Petersburg sediments. Solvent and freshwater controls each contained 15 pairs. Full water changes and extract renewals occurred daily. Pairs were first placed in glass containers in 800ml of Nanopure© reconstituted balanced saline solution (BSS; 86mM NaCl, 2mM KCl, 1mM MgSO₄, 0.24mM NaHCO₃) and allowed to acclimated until egg production was established. Then the pairs were subjected to extracts at a ratio which was equivalent to 10g of dry sediment in 1L water. This concentration was determined to be sub-lethal in 96-hour mortality tests using juvenile (1-4 weeks-post-hatch) medaka and higher concentrations resulted in mortality of juveniles. Acetone amounts added daily were less than 100µl for all treatment groups (>0.0125% solvent) and solvent controls received 100µl acetone daily.

Reproductive Trial

Eggs were collected daily 2 hours after the first feeding. Total number of eggs and number fertilized were quantified each day for each pair. Photographs were taken daily of at least 4 eggs from each pair (if available) to calculate average egg diameter. Collected eggs were placed in Yamamoto's hatching solution (Yamamoto, 1975) in 6-well plates and allowed to hatch. Fecundity was defined as the total number of fertilized and unfertilized eggs produced per pair per day. Non-viable eggs and hatched fry were counted and removed for 30 days following collection of eggs, and any developmental abnormalities were noted. Hatchability was defined as the total number of hatched fry divided by the total number of fertilized eggs per pair per day.

Collection of tissues

Following exposure, 10 mating pairs per treatment were sacrificed in tricaine methanesulfonate (MS-222) in order to obtain tissues for analysis. Length and weight of each fish was measured. Livers were removed, weighed, and added to tubes containing 5 μ l phenylmethylsulfonyl fluoride (PMSF, 99% purity, Sigma Aldrich, St. Louis, MO). Gonads were also removed and weighed. Gonadosomatic index (GSI), hepatosomatic index (HSI) and condition factor (CF, a general health index) were calculated using these formulas:

$$\text{GSI} = \text{gonad weight} / \text{body weight}$$

$$\text{HSI} = \text{liver weight} / \text{body weight}$$

$$\text{CF} = \text{body weight} / \text{length}^3 * 10^5$$

Histological examination

The remaining 5 mating pairs per treatment were used for histological examination. Fish were sacrificed in MS-222, fixed in Z-fix ® (zinc-formol solution, Anatech Ltd., Battle Creek, MI), and stored at 4°C. Before sectioning, fish were rinsed with phosphate buffered saline (PBS) and placed in Cal-Ex decalcifier (Fisher Scientific, Fair Lawn, NJ) for 24 hours. Fish were then placed in 30% sucrose in PBS until they absorbed enough of the solution to sink (~24-48 hours). At this time fish were mounted using embedding medium and sagittal sections (10µm in thickness) were cut frozen using a Leica cryostat (Leica Microsystems Inc., Bannockburn, IL). Slides were stored at -20°C until stained with hematoxylin and eosin, washed, dehydrated and coverslipped using standard staining methods. Each was examined with light microscopy. Gonads were viewed to determine the gender of each fish and males were checked for the occurrence of intersex. A random section from each female was used to determine the proportion of oocytes in each stage of development as outlined by Blazer (2002), which includes chromatin nucleolar oocytes, perinuclear oocytes, and 3 stages of vitellogenic oocytes (early, mid, and late). Livers were viewed to count the number of bile ducts in each and to check for bile duct hyperplasia. Three sections were chosen randomly from each fish and all kidney pigmented macrophage aggregates (MA'a) within a 400x field of view were counted. Thyroid tissue was viewed for abnormalities as outlined in Fournie et al. (2005) including follicular cell hyperplasia (simple, nodular, and ectopic), presence of neoplasms (adenomas and carcinomas), altered amounts of follicular colloid, and changes in the thickness of follicular epithelial cells.

Vitellogenin (Vtg)

Frozen liver samples were thawed on ice and homogenized with 10 μ l homogenation buffer (0.1 mol/L Tris base, 1 mmol/L EDTA). Total protein concentration was calculated using a Bio-Rad protein dye reagent and a standard curve of bovine serum albumin (BSA). BSA standards produced a linear range from 0-0.5 mg/ml with an average $R^2=0.99$. A Japanese medaka enzyme-linked immunosorbent assay (ELISA, Biosense Laboratories, Bergen, Norway) for Vtg was used to measure the concentration of Vtg in the samples. Plates were read by a Tecan Genios microplate reader (Phenix Research Products, Candler, NC) at an absorbance of 450nm. Vtg standards produced a linear range between 0-15 ng/ml with an average $R^2=0.91$. Vtg concentration was divided by liver protein concentration to determine ng of Vtg per mg of liver protein. The detection limit was 0.074 ng/mg, and those samples below the detection limit were considered to have a Vtg concentration of zero.

Statistical Analyses

The significance level used was $p<0.05$ for all statistical analysis. The data obtained for the final weights and lengths of fish, average egg diameter, and mean number of eggs spawned per pair were analyzed by ANOVA for significant differences between the controls and the exposed groups. Data that didn't conform to the rules of ANOVA were analyzed using Wilcoxon tests for significant differences between control and exposed groups with post hoc tukey tests. These included CF, HSI, GSI, percent of spawned eggs fertilized, percent of fertilized eggs hatched, Vtg numbers, and histology parameters. F-tests were used to compare the variability of parameters between controls and exposed groups.

Results

Fish

No mortality was observed over the course of the exposure. Fish appeared healthy and showed no obvious signs indicating impaired health. Freshwater and solvent controls were not significantly different in any measured parameter, and were combined for further analysis. Final weights ($F=0.2342$, $p=0.9187$), length ($F=0.1231$, $p=0.9740$), and condition factor (KW test, $\chi^2=5.5803$, $p=0.2328$) were not significantly affected by exposure to hexane extracts (Table 2). HSI of exposed fish were not significantly different from controls for males (KW test, $\chi^2=1.6414$, $p=0.8013$), or females (KW test, $\chi^2=4.4816$, $p=0.3447$)(Table 2). GSI of exposed fish were also not significantly different from controls for males (KW test, $\chi^2=3.8075$, $p=0.4327$) or females (KW test, $\chi^2=2.4778$, $p=0.6486$)(Table2).

Reproductive Trial

The mean number of eggs spawned per pair ranged between 144.9 and 169.5 and no significant difference existed between control and treatment groups ($F=1.3965$, $p=0.2525$) (Table 3). The number spawned each day of the exposure decreased in both control and exposed pairs over the 14-day duration, though there was no statistical difference between controls and treatment pairs in this pattern. The average diameter of eggs (Table 3) was unaffected by exposure ($F=1.1511$, $p=0.3311$) and the occurrence of development abnormalities was minimal for all pairs (<1% of spawned eggs). Eggs from treatment groups and controls were equally likely to be fertilized (KW test, $\chi^2 =7.5710$, $p=0.0558$) (Table 3) though there did exist a strong trend that pairs exposed to Franklin extracts had a lower fertilization rate. In addition, exposure to extract from Franklin

resulted in significantly lower hatchability compared to controls (KW test, $\chi^2=10.3925$, $p=0.0155$)(Figure 2) and the variability in hatchability percentages for Franklin pairs was significantly greater than controls pairs ($F=3.9799$, $p=0.0206$).

Histological Examination

All male testicular tissue appeared normal and no oocytes were discovered within control or treatment male gonads. Ovaries of all female fish contained oocytes in each stage of development, and no significant difference existed between the proportion of each stage of development between control and exposed females ($\chi^2=4.74$, $p=0.97$)(Table 4). The number of bile ducts counted in each liver was not affected by exposure to extracts ($\chi^2=3.4097$, $p=0.3327$) (Table 5). However a Franklin exposed female and a Springfield exposed male had 5 and 4 bile ducts, respectively. The mean number of macrophage aggregates ranged between 85.8 and 188.6 and no significant difference was found between control and exposed groups ($\chi^2=3.6410$, $p=0.3029$) (Table 5). Thyroid follicles of all fish contained normal columnar epithelial cells and no difference in the amount of colloid within follicles could be detected. There was also no noted thyroid follicle hyperplasia (simple, nodular, or ectopic) and there was no adenomas or carcinomas discovered within any fish.

Vitellogenin

A total of 63 liver samples were used in the analysis of Vtg concentration. Ten collected samples could not be used because of the insufficient amount of sample. Seven samples had absorbance values above the standard curve. Four female liver samples analyzed at high dilutions (1:5,000) had absorbance values below the standard curve, but were not considered to have Vtg concentrations of zero. Male liver Vtg levels ranged

from 0-36.95ng/mg, and exposure to extracts had no affect compared to controls (KW test, $\chi^2=4.1951$, $p=0.2412$) (Table 7). This held true when the two male samples that occurred above the standard curve were assigned the highest Vtg concentration measured for males (36.95ng/mg) (KW test, $\chi^2=1.9997$, $p=0.5725$). Female Vtg levels ranged from 1,452-120,014ng/mg, and exposure to Franklin extracts resulted in significantly greater Vtg levels (KW test, $\chi^2=8.1554$, $p=0.0429$) (Table 7), however when the 5 female samples that occurred above the standard curve were assigned Vtg concentrations equal to the highest measured for females (120,014ng/mg) there was no significant difference between Vtg concentration of exposed and control groups (KW test, $\chi^2=5.1361$, $p=0.1621$). Female Vtg levels were two orders of magnitude greater than male Vtg levels for all treatments and controls (Table 7).

Discussion

The presence of male intersex smallmouth bass in the South Branch of the Potomac River indicates that estrogenic EDC's may be present at environmentally relevant concentrations. Many sources of EDC's exist near the South Branch including agriculture, sewage treatment plants, direct residential piping, etc. As an initial attempt at trying to classify the compounds that are eliciting effects in the SBP river, we have extracted sediment from three sites with hexane solvent in order to target nonpolar contaminants to use with a highly sensitive *in vivo* medaka assay to determine consequences of exposure to these extracts. Sediment was used because it acts as a reservoir for the many chemicals of anthropogenic origin that tend to concentrate there. This list includes alkylphenolic compounds, polychlorinated compounds, polybrominated compounds, steroid sex hormones, and phthalates (Petrovic et al., 2001).

Decachlorobiphenyl was used to determine recovery efficiencies because it is one of the many polychlorinated compounds that is very nonpolar. Other compounds that would be extracted using hexane solvent include some PAH's, some polybrominated compounds, and the carbon chain portion of alkylphenolic compounds.

The reproductive trial showed that pairs exposed to extracts from Franklin sediment experienced reproductive impairment as indicated by the significantly lower hatchability and the significantly greater variance in hatchability compared to control pairs. This endpoint with medaka has been shown to be more sensitive to exposure to 17 β -E2, bisphenyl-A, and NP than other *in vitro* studies and comparable to other *in vivo* studies (Shioda and Wakabayashi 2000). Because fertilization rate did not decrease in the Franklin pairs, disruption of embryonic development may have occurred. Two possible exposure routes of the eggs include maternal transfer of lipophilic compounds in the fatty portion of the eggs and direct uptake of compounds due to aqueous exposure after fertilization but prior to egg collection. Ishibashi et al., (2006) reported that 4-NP was maternally transferred in goldfish (*Carassius auratus*). An extensive literature search shows that fish are most sensitive to exposure to EDC's during critical life periods like embryonic development (Gray et al., 1999; Ankley and Johnson, 2004).

The histological examination revealed that extracts failed to induce intersex in male fish. Oocyte development was not altered due to exposure. Other organs, such as liver, kidney, and thyroid gland appeared to be unaffected despite 3 cases of bile duct proliferation. In this study one Franklin exposed female had 5 bile ducts and 2 Springfield exposed fish (1 male, 1 female) had 4 bile ducts. This did not result in a significant difference when mean bile ducts were compared with controls, although no

other fish had more than 3 bile ducts. Sample sizes were low for the histological analysis and proliferation of bile ducts may have been a consequence of exposure. Blom et al., (1998) found that caging juvenile rainbow trout in a PCB-polluted lake caused bile duct proliferation.

Male hepatic Vtg levels were several orders of magnitude lower than female levels within and between all control and exposed groups. The highest male Vtg level and lowest female level were 36.95 and 1,452 ng/mg respectively. Hepatic Vtg concentrations of exposed males were not statistically greater than control males. This is sufficient evidence that induction of Vtg in males was not a consequence of exposure to extracts. The low levels observed in control and exposed males reflect the sensitivity of the ELISA to detect trace amounts of hepatic Vtg as Tatarazako et al., (2004) showed in validation studies of ELISA assays for medaka. Others have shown low levels of Vtg in control males. Goksoyr (2006) reported that Vtg levels in fish plasma can vary from a few ng/ml in unexposed males to >100mg/ml in estrogenized salmonids. The significantly higher hepatic Vtg levels in Franklin exposed females should be interpreted with caution. Three freshwater control females and 2 Petersburg exposed females had absorbance values too high to accurately determine Vtg concentration (Table 4). When these were assigned the highest Vtg concentration measured for females there was no difference between control and exposed groups. Two of the Franklin exposed females had extremely high Vtg levels (66,478 and 120,014 ng/mg), which likely skew the results. For this reason we do not consider the induction of Vtg in Franklin exposed females a significant finding in this experiment.

Van der Ven et al., (2007) used an *in vivo* zebrafish exposure to E2 and tamoxifen to show the effects of an ER agonist and antagonist on several endpoints, and concluded that results of exposures to mixtures should be evaluated carefully due to the complexity of these ligands acting on various ER's in various tissues. With the reproductive toxicity seen as a result of exposure to Franklin extracts, but the lack of Vtg induction and histopathological abnormalities due to exposure, several conclusions are possible. First, nonpolar chemicals, which appear to not be estrogen agonists, may be passed to the eggs via one of the mechanisms outlined above. Secondly, estrogenic chemicals present in the extracts may not elicit a vitellogenic response or induce intersex in males due to the presence of androgenic or antiestrogenic chemicals. Schlenk et al., (2005) found that chemical fractionation of sediments failed to demonstrate relationships between Vtg expression in cultured male California halibut (*Paralichthys californicus*) and 62 analytes including E2, which shows that ER-based assays may underestimate environmental estrogen activity. Lastly the chemicals that have caused the occurrence of intersex in smallmouth bass may not be nonpolar.

Based on these results it would be recommended that further investigation should attempt to target compounds of a more polar nature as they may be impacting smallmouth bass in the SBP River.

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Figures

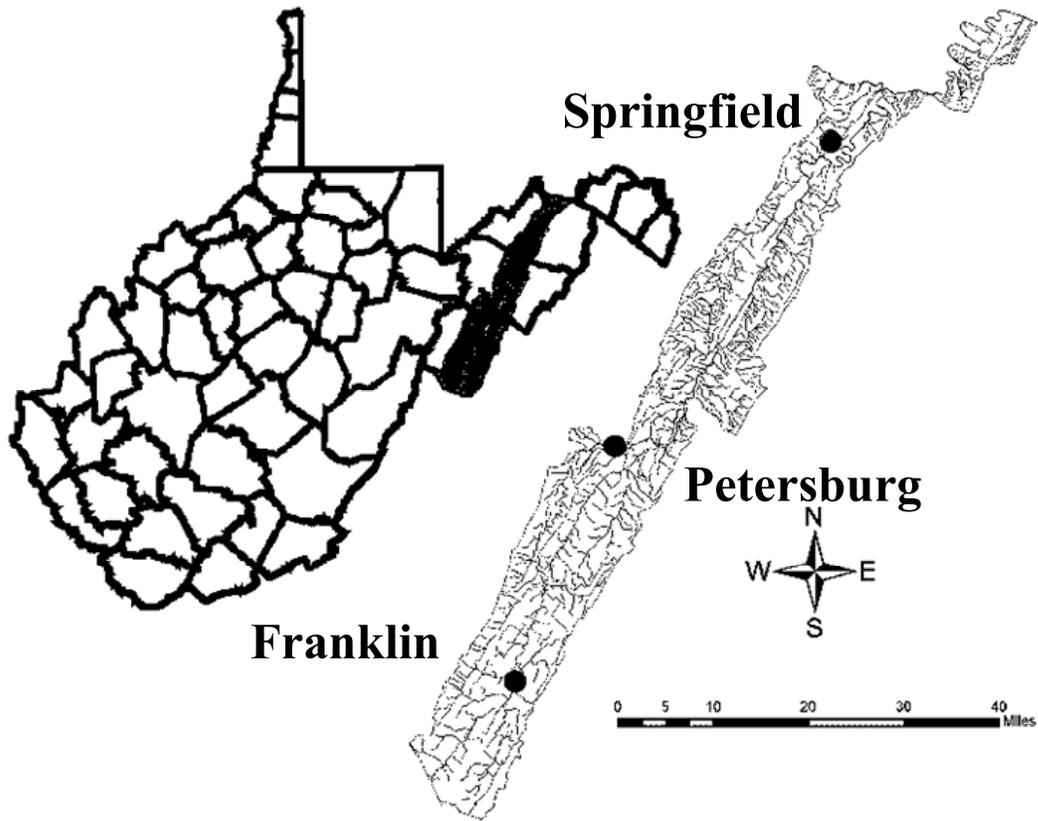


Figure 1. South Branch of the Potomac River, WV, and the 3 sites where sediment was collected in Spring 2006.

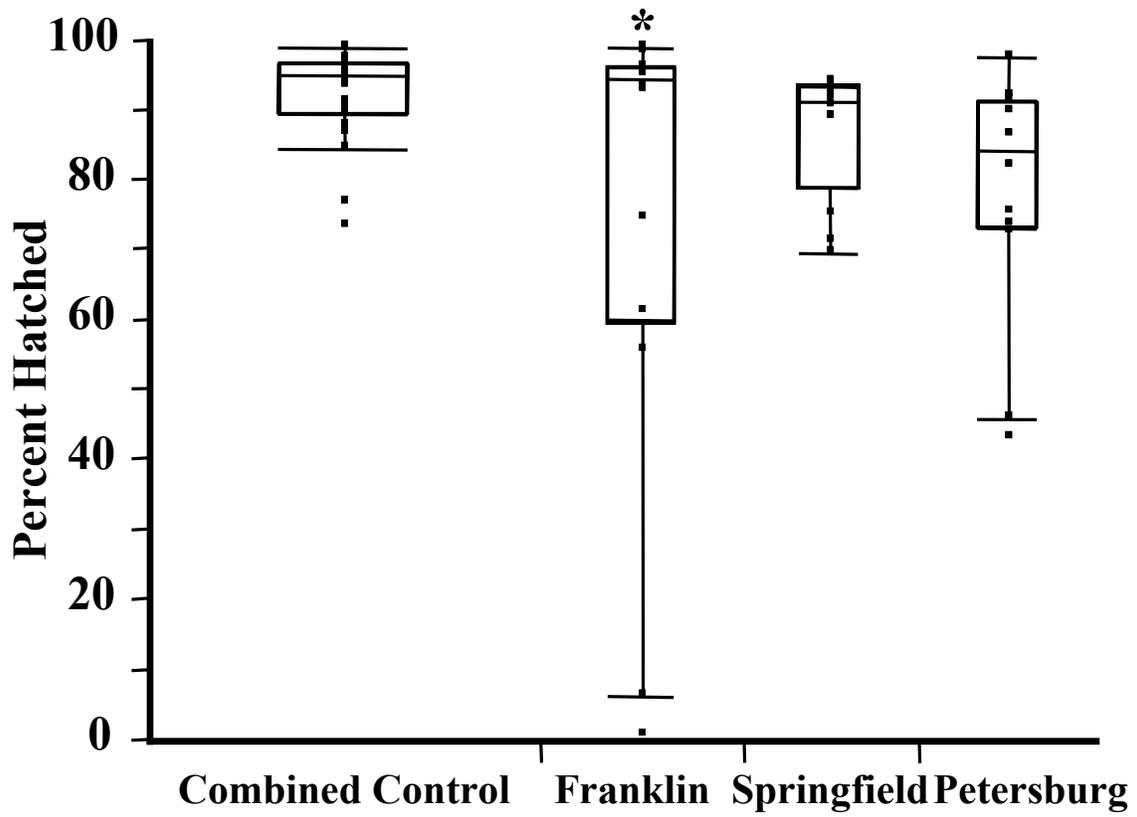


Figure 2. The percent of fertilized eggs that hatched of medaka exposed to hexane sediment extracts. Franklin exposed pairs spawned eggs with significantly lower hatchability compared to controls (KW test, $\chi^2=10.3925$, $p=0.0155$).

Tables

Table 1. Percent water of sediment collected in Spring 2006 and the wet weight of aliquots used to achieve the equivalent of 200g dry weight for extraction

Site	% Water^a	Wet weight of aliquots (g)
Franklin	68.7 ± 0.5	639.0
Springfield	39.8 ± 0.8	332.2
Petersburg	42.0 ± 0.6	344.8

^aData presented as mean ± SE.

Table 2. Body condition measures, fecundity, egg diameters, and percent of spawned eggs fertilized of medaka pairs exposed to hexane sediment extracts^a.

	Weight (g)	Length (mm)	CF (g/mm³ x 10⁵)	Total Eggs/Pair	Avg. egg diameter (mm)	Percent of Eggs Fertilized
Freshwater Control	0.416±0.02	34.7±0.5	0.98±0.01	187.9±12.5	1.260±0.009	97.1±1.1
Solvent Control	0.399±0.01	34.8±0.4	0.95±0.01	172.7±12.4	1.237±0.012	97.4±0.5
Franklin	0.414±0.01	35.1±0.4	0.95±0.01	169.4±9.6	1.234±0.011	93.3±2.6
Springfield	0.413±0.01	34.7±0.4	0.98±0.01	144.9±12.4	1.265±0.014	95.6±0.8
Petersburg	0.412±0.01	34.7±0.3	0.98±0.02	162.1±12.9	1.244±0.011	95.9±1.0

^aAll data presented as mean ± SE.

Table 3. Hepatosomatic index (HSI) and gonadosomatic index (GSI) of male and female medaka exposed to hexane sediment extracts.

	HSI ^a		GSI ^b	
	Male	Female	Male	Female
Freshwater Control	0.033 ± 0.006	0.043±0.003	0.013±0.003	0.075±0.004
Solvent Control	0.022±0.003	0.041±0.003	0.014±0.001	0.070±0.003
Franklin	0.026±0.002	0.035±0.003	0.014±0.001	0.071±0.009
Springfield	0.026±0.002	0.038±0.004	0.011±0.002	0.066±0.007
Petersburg	0.025±0.001	0.043±0.003	0.011±0.001	0.073±0.005

^a HSI = liver weight / body weight; data presented as mean ± SE.

^b GSI = gonad weight / body weight; data presented as mean ± SE.

Table 4. Mean percent of oocytes in each stage of development^a.

	% Chromatin Nucleolar	% Perinuclear	% Early Vitellogenic	% Mid Vitellogenic	% Late Vitellogenic
Freshwater Control	24.6±2.6	20.3±4.8	15.0±1.6	20.5±4.8	19.6±2.0
Solvent Control	17.5±2.0	27.4±3.4	16.6±2.3	24.6±3.3	13.9±4.4
Franklin	21.3±0.9	27.6±3.1	18.1±2.8	19.2±1.5	13.7±3.0
Springfield	23.8±3.0	29.5±2.3	17.3±3.2	18.0±2.0	11.4±3.4
Petersburg	31.0±6.7	29.8±4.1	15.2±2.0	15.7±4.3	8.3±4.5

^a Data presented as mean ± SE.

Table 5. Mean number of macrophage aggregates (MAs) and bile ducts of medaka exposed to sediment extracts^a.

	MAs	Bile Ducts
Freshwater Control	114.0±11.9	1.4±0.2
Solvent Control	118.6±16.2	1.8±0.3
Franklin	87.6±18.7	2.3±0.6
Springfield	101.2±16.1	2.0±0.5
Petersburg	85.8±16.9	1.3±0.2

^a All data presented as mean ± SE.

Table 6. Hepatic vitellogenin concentration (ng Vtg/mg protein) of male and female medaka exposed to hexane extracts^a.

	Vtg	
	Male	Female
Freshwater Control	7.3±3.9	4840±1575
Solvent Control	2.9±2.9	7294±1419
Franklin	13.4±6.6	32945±16655
Springfield	4.4±2.9	5389±1386
Petersburg	0	6798±1063

^a Data presented as mean ± SE.

Chapter 3: Ethyl acetate:acetone extract exposure

Abstract

An intersex condition, defined as the presence of oocytes in the testes of male gonochoristic fish, has been observed in smallmouth bass in the South Branch of the Potomac River, West Virginia, which indicates exposure to exogenous estrogens. Endocrine disrupting chemicals (EDC's) are generally hydrophobic and would tend to be found within the sediment of aquatic environments. Few studies have attempted to show the effects of exposure to EDC's on fish using sediment chemical extracts. We have developed a mass sediment extraction technique using 2 solvents (hexane, ethyl acetate:acetone 50:50) to determine the effects of extracted chemicals from three sites (Springfield, Petersburg, and Franklin, WV) on reproductive performance and physiological parameters of adult mating pairs of Japanese medaka (*Oryzias latipes*) for 14 days. Sediment aliquots (200g dry weight) were mixed with solvent 3 times, sonicated, and filtered. Pairs were subjected to extracts at the ratio of 10g of extracted sediment in 1L of water. Hatching success significantly decreased due to exposure to Franklin extract (both solvents) and Petersburg extract (ethyl acetate:acetone). Springfield exposed females had a significantly increased hepatosomatic index (HSI) and showed a trend towards increased vitellogenin (Vtg) production. Results indicate that non-polar and moderately polar compounds may be disrupting development of fertilized eggs and this is most likely due to mixture effects because results were similar with both extracts. Smallmouth bass in the South Branch are likely experiencing chronic exposure or periodic exposure at critical life periods, which may explain why estrogenic effects were not observed in a 14-d exposure. We suggest this method be used in combination

with *in vitro* assays and analytical chemistry in order to investigate mixtures within sediment.

Introduction

Chemicals that have the potential to alter normal functioning of the endocrine systems of organisms are of global concern (Jobling et al., 1998) and are commonly referred to as endocrine disrupting chemicals (EDC's). Intersex has been observed in smallmouth bass (*Micropterus dolomieu*) in the South Branch of the Potomac River, West Virginia (V. Blazer, personal communication), which indicates exposure to exogenous estrogens (Nolan et al., 2001). Many of these are hydrophobic compounds and would tend to accumulate in the sediment of aquatic systems.

A variety of endpoints have been used in studies to evaluate the effects of EDC's on fish. Reproductive toxicity has been shown to be a very sensitive outcome to EDC exposure in laboratory studies (Hill and Janz, 2003; Nash et al., 2004; Gronen et al., 1999; Gray et al., 1999), and Jobling et al. (2002) reported a significant negative correlation between fertilization success and degree of feminization of the intersex gonads in wild roach (*Rutilus rutilus*). Physical abnormalities are often reported as consequences of exposure to EDC's and include disrupted oocyte development (Van den Belt et al., 2002), bile duct proliferation (Blom et al., 1998), pigmented macrophage aggregate (MA) accumulation in renal and splenic tissue (Blazer, 1991, Agius and Roberts, 2003, Thilakaratne et al., 2007), and abnormal thyroid follicle histology (Fournie et al., 2005). Additionally, induction of vitellogenin, an egg yolk protein precursor normally only expressed in mature female fish, has become a prominent

biomarker to exposure to xenoestrogens (Sumpter and Jobling, 1995) as its expression has been shown to be estrogen receptor mediated (Fujiwara et al., 2005).

Though many studies have shown the effects of single compounds or mixtures on a variety of fish species, environmental exposures, or *in situ* laboratory exposures are rare. Harries et al., (1997) and Pawlowshi et al., (2003) caged male rainbow trout (*Oncorhynchus mykiss*) downstream of sewage treatment works and measured vitellogenin to determine estrogenicity of the effluent. Similarly Nichols et al., (1999) caged fathead minnows (*Pimephales promelas*) in effluent of waste water treatment plants to determine the effects on reproductive physiology. Others have used diluted wastewater (Xie et al., 2004) and solvent extracts (Huggett et al., 2003) to expose fish and test for estrogenicity. To our knowledge a large scale sediment extraction and *in vivo* exposure has not been attempted.

Japanese medaka (*Oryzias latipes*) were used in this study because they are easily reared, become sexually mature in a short period of time (~3-4 months), and produce viable eggs daily under the correct conditions. Medaka have been used extensively in laboratory studies to demonstrate the effects of EDC's on fish reproduction, physiology, and behavior. For these reasons medaka are ideal organisms for aquatic toxicology studies.

Previously we found that sediment extracted with hexane from Franklin and exposed to mating pairs of medaka resulted in significantly decreased hatchability of fertilized eggs. The objective of this study is to use a more polar solvent (ethyl acetate:acetone 50:50) in order to attempt to further classify the chemicals that may be

causing endocrine disruption in the SBP River. The same endpoints will be measured including reproductive success, histological parameters, and hepatic vitellogenin.

Methods

Sediment was collected in May 2006 from three sites (Franklin, Springfield, Petersburg, WV) on the South Branch of the Potomac River (Figure 1) in areas where clay and silt were available. Extracts were prepared using ethyl acetate:acetone (50:50) solvent according to the methods in Chapter 2. The exposure of mating pairs of Japanese medaka was carried out following methods in Chapter 2 and the same endpoints were used to evaluate the effects of exposure. These included reproductive performance, histological examination of tissues and organs, and hepatic Vtg quantification.

Results

Fish

Three pairs (6 fish) died during the exposure including 2 pairs exposed to Petersburg extract and 1 pair exposed to Franklin extract. The cause of death could not be determined because all 3 pairs were found in the morning at first feeding and significant deterioration had occurred. Freshwater and solvent controls were combined for analysis because significant differences between them were not detected in any of the measured parameters. Final weight (F=0.6619, p=0.6205), length (F=1.0377, p=0.3937), and condition factor (KW test, $\chi^2=0.4122$, p=0.9377) were not significantly affected by exposure to EAA extracts (Table 2). HSI of exposed fish were not significantly different from controls for males (KW test, $\chi^2=0.9146$, p=0.8219) (Table 3), however females exposed to Springfield extract had significantly higher HSI's than controls (KW test, $\chi^2=10.5085$, p=0.0147) (Figure 2). GSI of exposed fish were not significantly different

from controls for males (KW test, $\chi^2=2.4542$, $p=0.4836$) or females (KW test, $\chi^2=7.1377$, $p=0.0676$) (Table 3).

Reproductive Trial

The mean number of eggs spawned per pair ranged between 172 and 209, and no significant difference was detected between control and treatment groups ($F=1.0268$, $p=0.4021$) (Table 2). The number spawned each day of the exposure decreased in both control and exposed groups over the 14-day duration, though there was no statistical difference between control and treatment pairs in this pattern. The average diameter of eggs was unaffected by exposure to extracts ($F=2.1922$, $p=0.0920$) and the occurrence of developmental abnormalities was minimal for all pairs. Hatched fry with crooked spines and partially hatched dead fry were found in each treatment and control group, but composed less than 1% of total eggs in each. Normal looking hatched fry were found dead in control and treatment groups at a frequency less than 2% of total eggs. Franklin and Petersburg had the highest occurrences of opaque, dead eggs (~4 and 6%, respectively), but the rate was not significantly greater than controls (~2.5-3%). Eggs from exposed and control groups were equally likely to be fertilized (KW test, $\chi^2=2.9295$, $p=0.4026$) (Table 2), however exposure to Franklin and Petersburg extracts resulted in significantly lower hatchability compared to controls (KW test, $\chi^2=13.4153$, $p=0.0038$) (Figure 3) and the variability in hatchability percentages for Franklin and Petersburg pairs were significantly greater than control pairs ($F=5.0879$, $p=0.0094$).

Histological Analysis

No oocytes were observed in male testis. Each female had oocytes in every stage of development. The proportion of each oocyte stage was not significantly affected by

exposure to EAA extracts ($\chi^2=0.895$, $p=1.0$) (Table 4). The number of bile ducts was also unaffected by exposure to extracts ($\chi^2=2.4960$, $p=0.4760$) (Table 5). No individual fish had more than 3 bile ducts, and hyperplasia of bile ducts was not a consequence of exposure to extracts. The number of macrophage aggregates in kidneys was unaffected by exposure to extracts ($\chi^2=4.0060$, $p=0.2608$) (Table 5). The amount of colloid in thyroid follicles and the size of epithelial cells were also unaffected, and no thyroid follicle hyperplasia, adenomas, or carcinomas were discovered.

Vitellogenin

Fifty liver samples were used in the analysis. Eight samples had insufficient amounts for the assay. Five female samples had absorbance values that were too high to accurately measure Vtg concentration including 1 freshwater control, 2 solvent controls, 1 exposed to Franklin extracts, and 1 exposed to Petersburg extracts. One sample from a female exposed to Springfield extract had an absorbance value too low to accurately measure Vtg concentration with the much higher dilution factor. The only detectable Vtg in male liver samples was found in 4 solvent control samples (Table 7). Female Vtg levels ranged between 1,099-19,690ng/mg protein, and exposure to extracts had no affect compared to controls (KW test, $\chi^2=4.2823$, $p=0.3691$) (Table 7). When female samples with absorbance values that were too high for quantification were assigned the highest recorded Vtg concentration for female samples (19,690ng/mg), there was still no significant difference between control and treatment groups (KW test, $\chi^2=2.6588$, $p=0.4473$).

Discussion

The occurrence of intersex in smallmouth bass in the South Branch of the Potomac River indicates that estrogenic EDC's may be present at environmentally relevant concentrations. EDC's may arise from a variety of sources and as an initial attempt at classifying the compounds that are eliciting effects in the South Branch of the Potomac River a hexane extraction of sediments from three sites (Franklin, Springfield, and Petersburg) was performed in order to expose medaka to the extracts and determine the *in vivo* effects on several endpoints. Sediment was used because many contaminants tend to accumulate in the substrate (Petrovic et al., 2001). The hexane exposure revealed that a nonpolar contaminant(s) may be present that is interfering with the normal development of fertilized eggs when exposed to Franklin extracts. To further our understanding of what contaminants might be present we have extracted sediment collected on the same day at the same 3 sites with ethyl acetate:acetone (50:50) in order to target chemicals of a more polar (or moderately polar) nature. These include some polychlorinated compounds, some polybrominated compounds, alkylphenolic compounds, pharmaceuticals, personal care products, steroid sex hormones, and phthalates.

The death of 3 pairs of medaka was unexpected due to the fact that a 96-hour mortality test was done with fry (1-4 weeks-post-hatch) to determine a sub-lethal concentration to expose the mating pairs to. It is suspected that the pairs were infected with bacteria that compromised their health and that the extracts alone did not cause the mortality. In all 3 cases both fish died and the fish appeared to have been dead for a considerable time (several hours). Although mortality has been noted as a result of

exposure to EDC's (Herman and Kincaid, 1988, Kramer et al., 1998, Zillioux et al., 2001), the concentrations of E2 and EE2 in these studies were well above typical environmental levels. One possibility is that contaminants in the extracts compromised immune response, making them more susceptible to an infection as a result. Confirming the immune suppression of exposed fish would require further investigation.

Female medaka exposed to Springfield extracts had significantly higher HSI's compared to control females. Ma et al., 2007 found that HSI's were significantly increased in female medaka exposed to 50ng/L EE2 or higher, and suggest the change was due to additional liver burdens, such as vitellogenesis. They also noted that the liver is a detoxification organ and HSI's often increase in response to xenobiotic exposure.

The reproductive trial showed that pairs exposed to Franklin and Petersburg extracts experienced reproductive impairment as indicated by the significantly lower hatching rate of fertilized eggs compared to controls. As discussed in Chapter 2, this endpoint is very sensitive. Fertilization rate was not affected by exposure to extracts, which indicates that disruption of embryonic development may have occurred. Once again two possible explanations for the presence of contaminants in developing eggs might include maternal transfer of lipophilic contaminants to the fatty portion of the eggs or direct uptake of compounds due to aqueous exposure after fertilization but prior to egg collection. The process of embryonic development is mainly a time of rapid cellular division and differentiation of tissues and thus is very sensitive to chemical perturbation. Iwamatsu (2004) has divided the development of medaka embryos into a 39-stage process that takes approximately 9 days to complete for successful hatching to occur.

Early development is a critical life period when fish are most sensitive to exposure to EDC's (Gray et al., 1999).

The only detectable hepatic Vtg in males was found in four solvent control fish. To our knowledge a solvent effect on hepatic Vtg has not been noted before. The levels found in our study (69-77ng/mg) were several orders of magnitude lower than female levels and only slightly higher than baseline levels found in medaka from the hexane exposure (Chapter 2, Table 6). Female hepatic Vtg concentrations were not significantly affected by exposure to EAA extracts. This is sufficient evidence to conclude that Vtg induction in males and altered Vtg production in females was not a consequence of exposure to EAA extracts.

It should be noted that hepatic Vtg levels were higher for Springfield exposed females, although this was not statistically significant. This slight elevation may explain the significant increase in HSI seen in females as a result of exposure to Springfield extracts. The histological analysis didn't reveal any marked proliferation of bile ducts or hyperplasia in Springfield exposed female hepatic tissue, which might have been expected with any increased vitellogenesis.

As with exposure to hexane extracts (Chapter 2), exposure to EAA extracts showed reproductive toxicity, but failed to significantly induce Vtg and histological abnormalities. Once again several conclusions are possible. First, the chemicals disrupting embryonic development, which do not appear to be estrogen receptor agonists, may be passed to the eggs via the mechanisms discussed previously (maternal transport, aqueous exposure). Secondly, estrogenic chemicals in the extracts may not elicit a vitellogenic response or induce intersex in male fish due to the presence of androgenic or

antiestrogenic chemicals. A similar conclusion was reported by Pawlowski et al., (2003) who caged rainbow trout at 3 sites downstream of municipal treatment plants and compared vitellogenin induction with 3 *in vitro* assays. Samples from all of the sites showed significant estrogenicity *in vitro*, but failed to induce Vtg *in vivo* at all sites. This is in agreement with Schlenk et al., (2005) who showed that ER-based assays may underestimate environmental estrogen activity. Lastly, it is possible that the chemicals that have caused intersex in smallmouth bass may not have been present in the sediment collected for this study. All sediment used was gathered on a single day in May 2006. This study represents a “snap-shot” in time and can only attempt to describe the consequences of exposure to the chemicals present at those specific locations at that specific time.

The utility of this method is that it allows for quasi-environmental exposure to actual chemicals from specific sites in the South Branch of the Potomac while eliminating exogenous environmental factors which interfere with most *in situ* exposures. By combining reproductive performance, hepatic Vtg quantification, histological examination, and knowledge of solvent properties this method represents a whole organism response to a specific class (e.g. polar, moderately polar-polar) of suspected contaminants and can help to predict if the combined actions of these contaminants are relevant. Such information would be unobtainable using standard analytical methods that only reveal independent concentrations for individual compounds and no real knowledge of the consequences of the mixture. Also, *in vitro* assays that measure specific receptor-dependent endpoints (yeast estrogen assay (YES)) fall short in their ability to mimic a whole organism response (Shelby et al., 1996). We recommend this assay be used in

conjunction with exposures to sediments extracted with solvents of different properties and at various sites throughout waterways that may contain EDC's.

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Figures

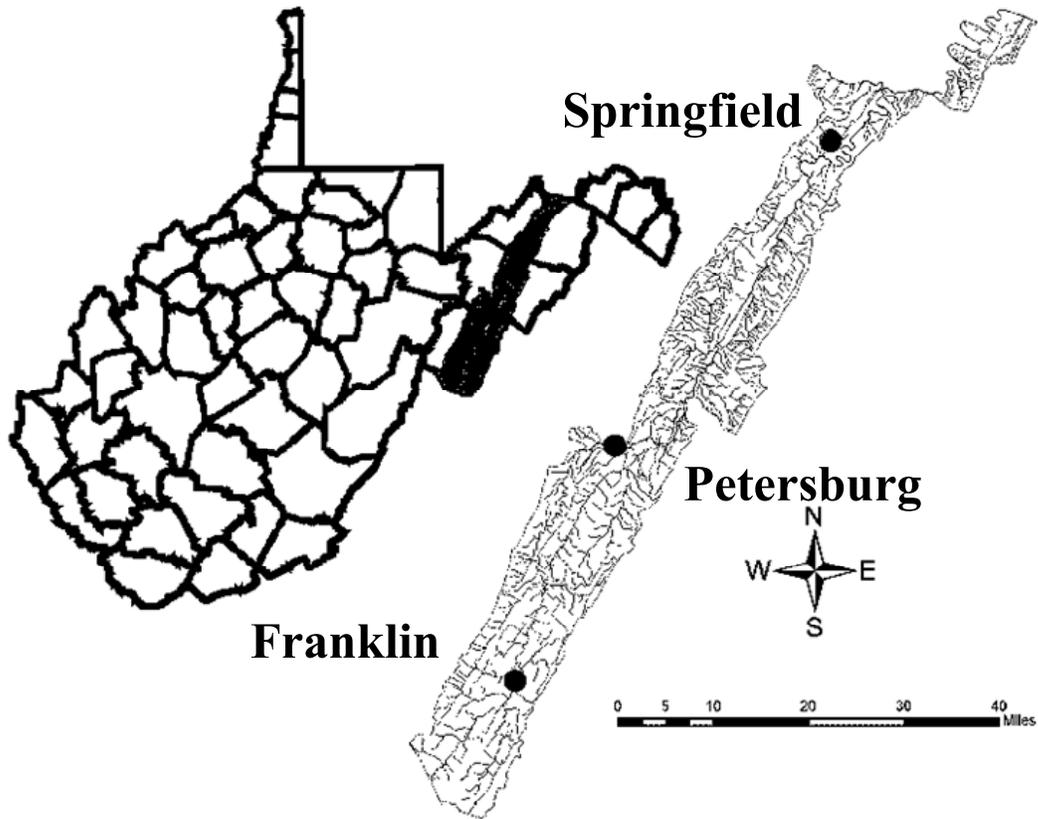


Figure 1. South Branch of the Potomac River, WV, and the 3 sites where sediment was collected in Spring 2006.

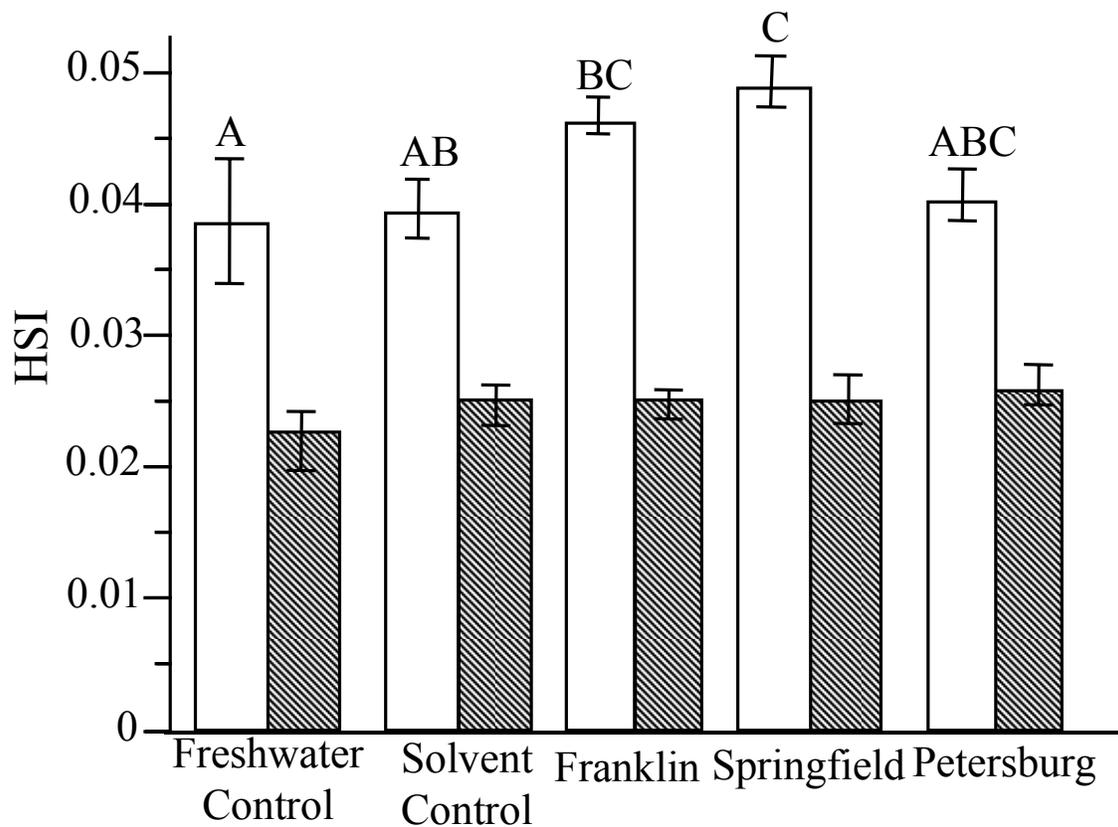


Figure 2. Hepatosomatic index (HSI, liver weight(g)/body weight(g) \pm SE) of male (shaded bars) and female (clear bars) medaka exposed to ethyl acetate:acetone (50:50) sediment extracts. Franklin medaka had significantly greater HSI's than freshwater controls, but not different than solvent controls, and Springfield exposed medaka had significantly greater HSI's than both controls (KW test, $\chi^2=10.5085$, $p=0.0147$).

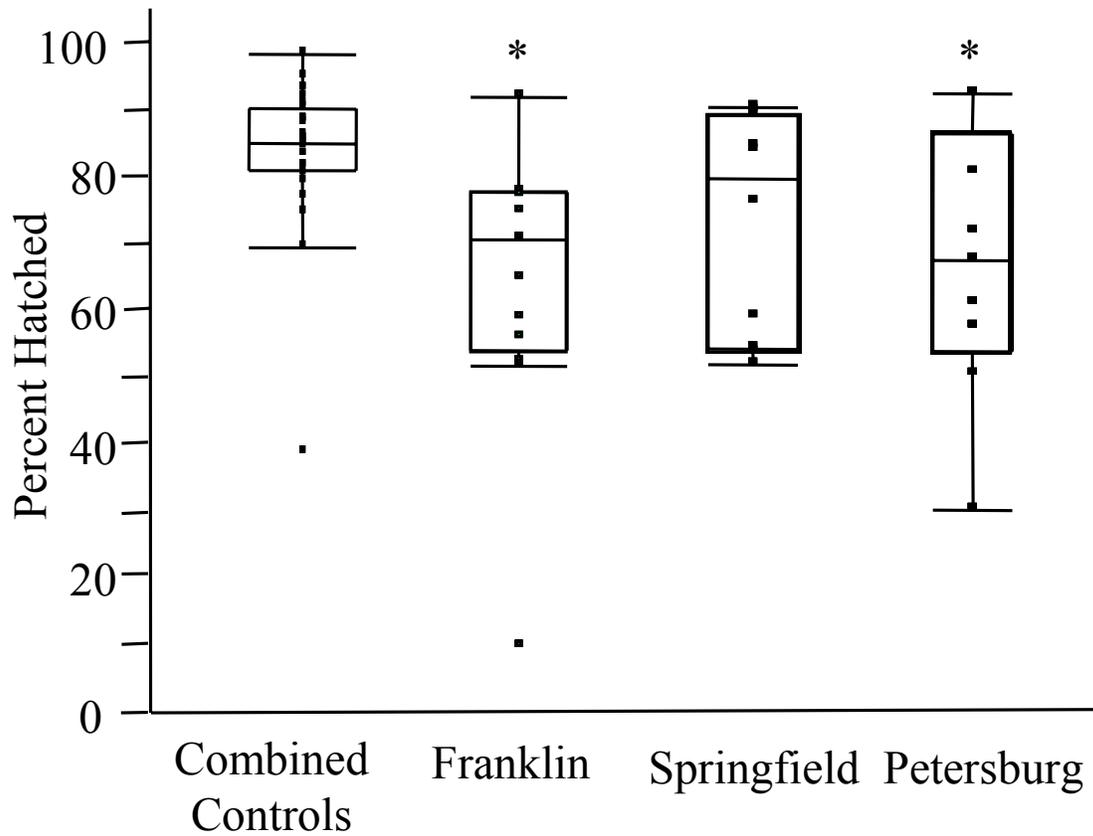


Figure 3. The percent of fertilized eggs that hatched of medaka exposed to ethyl acetate:acetone (50:50) sediment extracts. Franklin and Petersburg exposed pairs spawned eggs with significantly lower hatchability compared to controls (KW test, $\chi^2=13.4153$, $p=0.0038$).

Tables

Table 1. Percent water of sediment collected in Spring 2006 and the wet weight of aliquots used to achieve the equivalent of 200g dry weight for extraction.

Site	% Water^a	Wet weight of aliquots (g)
Franklin	68.7 ± 0.5	639.0
Springfield	39.8 ± 0.8	332.2
Petersburg	42.0 ± 0.6	344.8

^a Data presented as mean ± SE

Table 2. Body condition measures, fecundity, egg diameters, and percent of spawned eggs fertilized of medaka pairs exposed to ethyl acetate:acetone (50:50) sediment extracts^a.

	Weight (g)	Length (mm)	CF (g/mm³ x 10⁵)	Total Eggs/Pair	Avg. egg diameter (mm)	Percent of Eggs Fertilized
Freshwater Control	0.443±0.02	36.0±0.5	0.96±0.02	209.0±8.3	1.237±0.012	96.4±1.1
Solvent Control	0.456±0.02	36.1±0.6	0.95±0.02	198.6±11.7	1.259±0.009	92.9±0.9
Franklin	0.408±0.02	34.9±0.5	0.96±0.01	181.0±18.6	1.268±0.010	90.1±2.3
Springfield	0.435±0.04	35.0±0.9	0.96±0.02	181.3±15.2	1.251±0.012	94.2±1.7
Petersburg	0.446±0.02	36.0±0.5	0.94±0.01	172.3±16.8	1.229±0.010	90.1±3.3

^a Data presented as mean ± SE.

Table 3. Hepatosomatic index (HSI) and gonadosomatic index (GSI) of male and female medaka exposed to ethyl acetate:acetone (50:50) sediment extracts.

	HSI ^a		GSI ^b	
	Male	Female	Male	Female
Freshwater Control	0.022±0.002	0.038±0.005	0.008±0.001	0.065±0.004
Solvent Control	0.025±0.001	0.039±0.002	0.012±0.001	0.072±0.006
Franklin	0.025±0.001	0.046±0.001	0.011±0.001	0.075±0.007
Springfield	0.025±0.002	0.048±0.002 ^c	0.011±0.001	0.086±0.010
Petersburg	0.025±0.002	0.040±0.002	0.009±0.001	0.094±0.013

^a HSI = liver weight / body weight; data presented as mean ± SE.

^b GSI = gonad weight / body weight; data presented as mean ± SE.

^c Significantly higher HSI compared to controls (KW test, $\chi^2=10.5085$, $p=0.0147$)

Table 4. Mean percent of oocytes in each stage of development^a.

	% Chromatin Nucleolar	% Perinuclear	% Early Vitellogenic	% Mid Vitellogenic	% Late Vitellogenic
Freshwater Control	20.5±1.0	23.4±3.2	22.8±2.8	22.3±6.4	11.0±1.1
Solvent Control	24.1±1.1	30.9±5.3	18.6±3.0	15.3±3.5	11.1±1.8
Franklin	22.9±1.7	24.9±5.7	19.2±3.0	19.3±1.7	13.7±3.3
Springfield	20.2±4.1	30.0±3.3	21.3±3.3	18.2±4.1	10.3±1.5
Petersburg	21.3±3.1	24.7±2.5	20.6±1.9	22.1±2.4	11.3±1.0

^a Data presented as mean ± SE.

Table 5. Mean number of macrophage aggregates and bile ducts of medaka exposed to sediment extracts^a.

	MA's	Bile Ducts
Freshwater Control	109.9±12.8	1.9±0.1
Solvent Control	123.6±14.0	2.1±0.3
Franklin	157.6±19.1	1.8±0.2
Springfield	120.7±17.7	2.0±0.3
Petersburg	154.2±21.3	2.3±0.2

^a All data presented as mean ± SE.

Table 6. Hepatic vitellogenin concentration (ng Vtg/mg protein) of male and female medaka exposed to ethyl acetate:acetone extracts^a

	Vtg	
	Male	Female
Freshwater Control	0	6766±1250
Solvent Control	32.1±12.7	6176±1338
Franklin	0	5390±1088
Springfield	0	9155±2907
Petersburg	0	3701±1629

^a Data presented as mean ± SE.

Conclusion

Investigating endocrine disruption in fish has traditionally involved single chemical toxicity testing. Understanding mixture toxicology is of utmost importance because organisms in the environment are rarely exposed to single compounds. By combining *in vivo* responses in fish with mechanistic *in vitro* assays and analytical methods, it should become increasingly easier to understand the properties of mixtures and parse out those compounds or combinations of compounds that are of greatest concern to organisms, including humans. Investigating sediment mixtures is an obvious starting point as many of the chemicals of concern tend to accumulate there. Our method of using different solvents to fractionate chemicals with sediment is useful in that it is a top-down approach. Analytical methods may yield exact concentrations of compounds within the sediment but lend little insight into the activity that a mixture might possess. Using different solvents to prepare extracts for exposure to live organisms represents a fractionation technique that may determine which portions (ie: polar v. nonpolar) elicit responses in a whole organism.

The major drawback of this method is that it assumes that chemicals of different polarities are not interacting in such a way that would influence the toxicity of the whole mixture and is a potential branch for future research. Another limitation is that large amounts of sediment (~2.5kg/sight/solvent dry weight) had to be extracted in a single event. This meant careful planning and handling to ensure that each extract was prepared similarly and within a short amount of time to avoid differences in degradation rates. I would recommend that similar large scale sediment extractions be accomplished with the aid of several people working with more than one sonic dismembrator. Also the presence

of water within the sediment was an issue of concern. Initial attempts to dry the sediment with sodium sulfate were not carried out due to the large amount of sediment and the logistics of grinding with kilograms of sodium sulfate.

The results of this investigation reveal that compounds are present that may interfere with development in medaka embryos. Aside from that endpoint the 14-day exposure did not elicit significant effects. The lack of estrogenic activity may point to a variety of explanations (see discussion in Chapter 3 for review). I believe the 14-day exposure of adult medaka may have been too short in duration, and fish in the natural environment experience potentially chronic exposure to a variety of compounds at critical time periods. Future studies may attempt longer exposures, or expose fish at various life stages to be more representative of environmental conditions. Also retaining resulting fry to determine F1 generation effects on sex ratios, maturity, survival, and successful production of an F2 generation would be useful. Although it would be logistically impossible to maintain all resulting F1 fry (I counted nearly 20,000 for this experiment), a subset of each treatment could be kept and used.

The management implications from this study are vague. The results indicate that contaminants are likely present but whether they are active as endocrine disruptors is unclear, and whether compounds that cause intersex in smallmouth bass were present cannot be determined. Future studies should attempt to understand whether the occurrence of intersex in smallmouth bass is an estrogen-mediated pathway or via some other mechanism (ie: influencing enzyme activity). Also the natural occurrence and consequences of intersex on populations should be investigated. Further, the efforts to identify compounds of concern within the SBP River (and any other aquatic environment

where signs of endocrine disruption are prevalent) should consist of collaborations between many investigators looking at a variety of endpoints. Various analytical and *in vitro* studies have been accomplished, or are underway, in the SBP but drawing conclusions with a synthesis of this information from the various studies is difficult because initial planning did not allow for collection of samples at the same places and times. It is suggested that all investigators involved take part in sampling together so that collaboration is made simple and any results from study-to-study don't have the confounding limitations of temporal or location differences. This to me seems to be a common theme in scientific research where there is a lack of communication between investigators.