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Assessment Of largemouth Bass (Micropterus Salmoides) Condition Using Liver Histopathology, Health Parameters and Water Quality Of The Central Chattahoochee River Watershed

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ASSESSMENT OF LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) CONDITION USING LIVER HISTOPATHOLOGY, HEALTH PARAMETERS, AND WATER QUALITY OF THE CENTRAL CHATTAHOOCHEE RIVER WATERSHED

by

Kari Carmack Goodwin

This thesis was prepared under the direction of the candidate's thesis advisor, Professor Elizabeth Klar, Department of Biology, and has been approved by the members of her advisory committee. It was submitted to the faculty of the College of Letters and Sciences and was accepted in partial fulfillment of the requirements for the degree Master of Science in Natural Science

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Columbus State University
December 2022

Assessment Of largemouth Bass (Micropterus Salmoides) Condition Using Liver Histopathology, Health Parameters and Water Quality of The Central Chattahoochee River Watershed

A THESIS

SUBMITTED TO THE

COLLEGE OF LETTERS AND SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE IN NATURAL SCIENCE

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ABSTRACT

The health of Largemouth bass from west-central Georgia was assessed using liver histopathology and the degree of tissue change index (DTC), hepatosomatic indices (HSI), relative weight, condition factors, and intersex. These health parameters as well as water chemistry data were compared across four bodies of water, three within the Chattahoochee watershed, and one pseudo-control located in the Flint watershed. Fish from Lake Oliver, Lake Harding, and West Point Lake had severely impacted liver health compared to Warm Springs National Fish Hatchery, with DTC rankings over 100 for the three lakes within the Chattahoochee watershed, demonstrating severely damaged livers. There was a significant difference between the hepatosomatic index and condition factor for fish when grouped into either high (>100) DTC or low (< 58) DTC categories, but not for relative weight. DTC scores between males and females in the study showed no significant difference. Males who had intersex had significantly higher DTC averages. Water chemistry analysis found 12 compounds that are known endocrine disruptors that can cause liver damage, including several types of phthalates in the Chattahoochee Watershed.

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To all the friends I have made here at CSU, I will cherish the time we spent climbing, camping, writing, and researching. You guys made the journey a time in my life I'll not soon forget.

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ACF	Apalachicola, Chattahoochee, Flint River Basin
LMB	Largemouth bass
IBI	Index of Biotic Integrity
H&E	Hematoxylin and Eosin stain
HSI	Hepatosomatic Index
EPA	Environmental Protection Agency (federal level)
NCBP	National Containment Biomonitoring Program
FDA	Food and Drug Administration
EPD	Environmental Protection Division (state level)
EDC	Endocrine Disrupting Compounds
CAFO	Concentrated Animal Feeding Operations
GIS	Geographic Information Systems
DTC	Degree of Tissue Change
RWt	Relative Weight Index
C	Condition Factor
GPS	Global Positioning System
HUC	Hydrologic Unit Code
ppb	Parts per billion

Introduction

Chattahoochee River: A Brief History

The headwaters of the Chattahoochee River begin in the Blue Ridge Mountains and flows 430 miles southwest to ride the state border between Georgia and Alabama until reaching Florida, where it transitions into the Apalachicola River, emptying into the Gulf of Mexico (Couch et al., 1996). The piedmont of north Georgia is separated from the coastal plain by a fall line that runs through Columbus, Georgia (Wikipedia, 2022). The Kolomoki mounds national monument provides evidence of indigenous peoples inhabiting the lands surrounding the Chattahoochee River possibly as early as 1000 B.C (Pluckhahn, 2020). To survive early European occupation in what is now the southeastern United States, after disease and forced relocations killed many among the numerous indigenous tribes that lived along the river, they coalesced language and culture into what eventually became collectively referred to as the Creek Tribe. It was the Creek Indians that gave the Chattahoochee River her name; it was recorded in 1799 by Benjamin Hawkins that *chat-to* meant "stone," and *ho-che* meant "marked" or "flowered" (Willoughby, 2020).

By the 1800s, the Creeks had ceded the land to colonizers who were running shipping routes along the river to transport cotton. Ships could run from the Gulf of Mexico to Columbus, Georgia uninterrupted, but the natural falls made navigating north of the fall line difficult. As such, the city of Columbus became an industrial power in the south known for textile mills and gristmills (Willoughby, 2020) and in 1828 we see the first dam of the Chattahoochee River built to power the mills just north of Columbus by Seaborn Jones (southres.com, 2022; Wikipedia, 2022). Due to the fall line, the natural 124-foot drop in elevation on the river created a flow gradient that invited the development of dams for hydroelectric power production.

As of 2015, there were 16 dams along the Chattahoochee River (Google Maps, 2022), but some of the dams no longer being used have been removed (Chattahoochee.org, 2022). As of now, 13 dams remain on the Chattahoochee (Leitman et al., 1991) and five water reservoirs are maintained by the Army Corps of Engineers: Buford Dam (Lake Lanier), West Point Dam, Walter F. George Lock and Dam, George W. Andrews Lock and Dam, and Jim Woodruff Lock and Dam (Lake Seminole) (Georgia River Network, 2022). Smaller water bodies such as Lake Harding, Lake Oliver, and Lake Eufaula are also located on the river. The Chattahoochee drains an area of 8,770 square miles and is now heavily impacted by urban development, recreation, agricultural practices, and hydroelectric practices (Couch et al., 1996; Wikipedia, 2022).

Ecology and the Watershed

The Chattahoochee River is a part of a large drainage basin in the southeastern United States, which also includes the Apalachicola and Flint Rivers (collectively known as the ACF basin) and drains lands from Georgia, Alabama, and Florida (Figure 1). Despite being heavily impacted by anthropomorphic activity, the basin has been able to maintain a rich degree of species diversity and helps maintain biological productivity in Apalachicola Bay Florida (Couch et al., 1996). Georgia ranks third in the United States for freshwater fish diversity (Dahlberg and Scott, 1971), with an approximated 265 native species of fish, 240 bird species, and 16 species of freshwater turtles (Means, 1977). The forested riparian zones along the river house many species, 52 mammalian species including rabbits, whitetail deer, 21 species of salamanders, 26 species of frogs and toads, and many snakes (Means, 1977; National Park Service, 2021). It is home to five endemic fish species that are found nowhere else in the world: Cherokee darter (*Etheostoma* *scotti*), Etowah darter (*Etheostoma etowahae*), Ocmulgee shiner (*Cyprinella callisema*), Altamaha shiner (*Cyprinella xaenura*), and the Chattahoochee sculpin *(Cottus chattahoochae*) (Georgia DNR, 2022; Means, 1977).

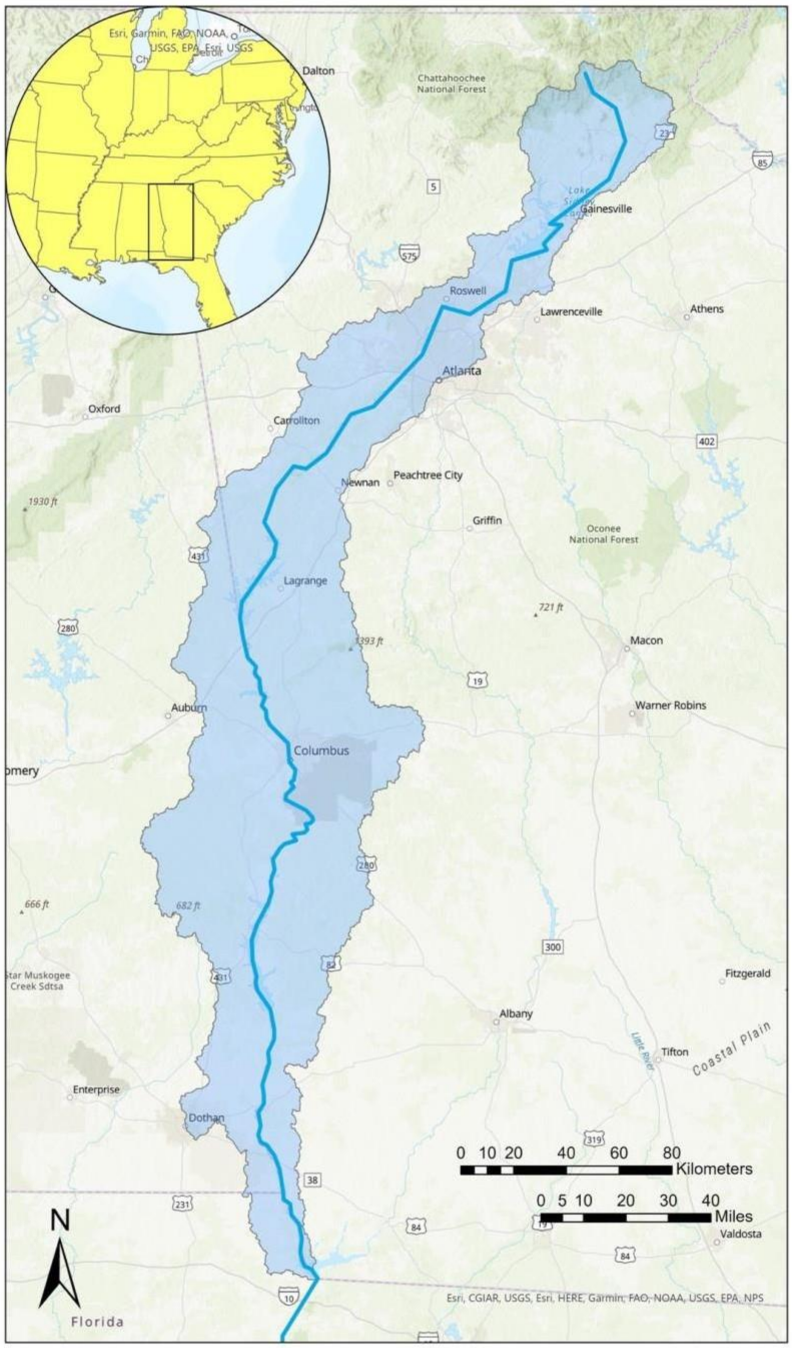

Figure 1. Map of the Chattahoochee River watershed. The Chattahoochee River flows south from the headwaters in the Piedmont, between the states of Georgia and Alabama, south to Florida where it becomes known as the Apalachicola River.

Of those fish native to Georgia, 20 haven't yet been described by ichthyologists, 10 are federally protected under the U.S. Endangered Species Act, 58 are state-listed, (Georgia Department of Natural Resources, 2022) and roughly one-third of all freshwater fish in Georgia are threatened (Straight et al., 2021). There are a total of 76 federally listed species in Georgia, and 145 in Alabama, but some of the most notable endangered species endemic to the Chattahoochee are the shoal bass, alligator snapping turtle, and gulf-coast sturgeon, and bald eagle (U.S. Fish and Wildlife, 2022).

The southeastern U.S. is rich in invertebrate diversity and has more freshwater mussel species than anywhere else in the world (Burch, 1973). The Chattahoochee River is home to several federally threatened and endangered freshwater mussel species including the oval pigtoe and purple bankclimber (Means, 1977). There are 30 species of crayfish that are found in the ACF basin, and six of those are endemic to the Chattahoochee River basin (Hobbs, 1942; 1981). The Jim Woodruff Dam and the hydroelectric dams on the Chattahoochee have severed access to native anadromous fish, such as the gulf sturgeon and striped bass, to spawning grounds in the freshwater of the Apalachicola and Chattahoochee Rivers, from their saltwater estuaries and marsh habitats in the Gulf of Mexico (Couch et al., 1996). This has in turn affected native mussel populations by limiting the ranges of host fish required by their parasitic larvae (Couch et al., 1996). A 1993 survey by the U.S. Fish and Wildlife Service discovered nearly all freshwater river mussel species to be gone from the mainstem of the Chattahoochee River (Williams and Brim-Box, 1993).

Habitat destruction due to dams, channel modifications, and impacted water quality have drastically reduced species diversity in the southeastern United States (Lydeard and Mayden, 1995). Dam operations impact the river hydrology and can create water level fluctuations of 4 ft or more (Couch et al., 1996), which can impact wildlife feeding, breeding, and nesting behaviors. Even though the Chattahoochee River is free flowing, it is heavily regulated and impacted by this series of dams and hydroelectric power structures. Tributaries flowing into the mainstream are freer flowing than the river itself. (Couch et al., 1996). Measurements taken from the USGS monitoring station in Atlanta say that the mean discharge for that location is 2070 cubic feet per second over the last 66 years of historical data (USGS 2022). These measurements vary based on where the monitoring is taking place and can fluctuate dramatically, especially during water surges from dam activities. Georgia's biggest threats to fish health and biodiversity are land use practices, urbanization, agriculture, chemical pollutants, climate change, hydroelectric practices, and invasive species. (Couch et al., 1996; Georgia DNR, 2022).

Human Influence

In addition to the diverse wildlife supported, the river supplies drinking water to millions of Georgia, Alabama, and Florida residents and is the most heavily used water resource in Georgia and the ACF basin (Gregory and Frick, 2000; Couch et al., 1996). The Chattahoochee flows through the densely populated Atlanta metro area which is ranked as one of the 60 most populated urban areas in the world (Wikipedia, 2006) and is relied upon for primary water sources as well as a means for the disposal of treated municipal sewage waste effluent, as are many major rivers (Seabrook, 1997). Urbanization is a two-edged sword, dealing fatal blows to ecosystems and habitats through development, and then polluting those remaining aquatic ecosystems with urban effluent (Coles et al., 2012, Walsh et al., 2005, Walsh et al., 2007).

Pollutants can come from many sources, including agricultural, industrial, and domestic effluents, and can contain a wide range of chemicals, both organic and inorganic such as pesticides, fertilizers, solvents, oils, and heavy metals (Pandey et al., 2003). The ecosystems of urban rivers and streams are hit the hardest by human activity and water contaminations which are known to cause many health problems for fish, including disease and structural tissue damage and alterations (Chang et al., 1998; Paul & Meyer, 2001). Second only to habitat destruction, chemical and organic pollutants are the biggest threat to aquatic ecosystem health and biodiversity and are a great area of concern for human health and water quality monitoring (Allan and Flecker, 1993).

In most water quality violations, it is urban run-off and non-point source pollution that is to blame, but sewage overflows, municipal discharges, and failure to correctly treat and discharge effluents from industrial facilities are also heavily responsible (Couch et al., 1996). In 1996, Couch reported that a staggering two-thirds of the 938 flowing miles of the ACF in Georgia did not meet or only partially met water quality standard requirements for designated-use criteria. In this report, it was noted that of those that did not meet standards, 72% of violations were due to urban runoff and non-point source pollution (Couch et al., 1996). For decades there have been fish-consumption warnings against eating the fish from parts of the Chattahoochee River due to contaminations of chlordane, mercury, and poly-chlorinated biphenyl (PCBs) in edible tissues (Georgia DNR, 1992; NPS.gov, 2021). Largemouth bass from the Chattahoochee River have been shown to contain mercury and should not be consumed more than once per week according to national health advisories (NPS.gov, 2021).

Study Species: Largemouth Bass *(Micropterus salmoides)*

While Largemouth bass *(Micropterus salmoides)* is considered an invasive species worldwide that negatively impacts community structure and reservoir health internationally (Han et al., 2016), it is an endemic species of the Chattahoochee River (Fuller et al., 1999) and is one of the southeast's most popular recreational fish, fueling an angling fishing industry worth billions of dollars (Wikipedia, 2022). Due to its popularity as a sporting fish, Largemouth bass (LMB) have been introduced worldwide as a recreational species and are having detrimental impacts on native fish populations worldwide (Scott and Crossman, 1973; Welcomme, 1988). Due to its morphology and swimming abilities, adult predation is rare, and its preference for warm freshwater environments and aggressive, strategic feeding makes it particularly dangerous to tropical and subtropical aquatic natives (Fuller et al., 1999), as such it is listed in the Global Invasive Species Database (ISSG, 2009).

The Largemouth bass is in the black bass (*Centrarchidae*) family and is a piscivorous, freshwater gamefish native to the eastern U.S. and extends north into southern Quebec and as far south as northern Mexico (Wikipedia, 2022).). It is one of several black bass species that are indigenous to the southeastern U.S. and readily found in their native habitat of the Chattahoochee River. Although LMB are more tolerant of turbidity than other black bass species (Etnier and Starnes, 1993), they prefer warm, calm, clear water in lakes, ponds, swamps, rivers, and creeks with plenty of underwater vegetation and overgrown banks that both serve as shelter and provide a landscape for predation and are predatory feeders that survive primarily on fish, crayfish, and frogs, and can be cannibalistic (Froese and Pauly, 2019, Fuller et al., 1999). LMB reach sexual maturity around one year old (Davis and James, 1997) and their spawning season typically occurs in the spring when water temperatures are between 55 -65 degrees Fahrenheit, but it has been found that they can also tolerate ice cover in their native range for up to six months, demonstrating that there is no cold inhibition to breeding if warmer waters are available during spawning season (CABI, 2022). The largest and most robust LMB specimens can be found in their native range of the southern United States where the spawning season stretches from March until June (Whitcomb, 2016).

Due to its worldwide dispersion, LMB is uniquely qualified to serve in the capacity of a bioindicator species and can tell us many things about the biological integrity of streams and rivers (Karr, 1981; Hocutt, 1981). The flexibility of the LMB regarding range tolerances and localized habitat preferences and its undiscriminating feeding behaviors have deemed this species an “intermediate” to “tolerant” species in terms of the Index of Biotic Integrity (IBI) (Hocutt, 1981; Karr, 1981; Halliwell et al., 1999; Pirhalla, 2004, Grabarkiewicz and Davis, 2008). Established by Dr. James Karr in 1981, this index has been widely used to evaluate surface water integrity since the 1980s and is adaptable to many regions and habitats (Simon and Emery, 1995).

An indicator organism has characteristics that allow us to discover more about the presence or absence of environmental conditions that are difficult to measure in other ways or with other taxa (Landres et al., 1988) and ecological health is determined by the actualization of an environment's natural biological potential, stable biotic conditions, and a capacity for self-repair when disturbances occur, all without the necessity of management efforts (Grabarkiewicz and Davis, 2008; Karr 1986). In aquatic environments that are heavily impacted, where inhabitants are under chronic exposure to pollutants and toxic chemicals, using bioindicators can answer many health questions, especially when internal filtering organs and morphological alterations are examined (Poleksic and Tutundzic, 1994).

Liver Pathology

While gills, kidneys, and livers are all particular interest when studying the toxic effects of water contamination and environmental pollutants in fish (Poleksic and Tutundzic, 1994;

Fernandes and Mazon, 2003; Thophon *et al.*, 2003), our organ of interest is the liver. The liver is known to be a homogeneous organ regarding its tissue, so issues occurring in one part are very likely to also be occurring in subsequent parts. Livers are filtering organs and since liver disease is the leading cause of morbidity in humans worldwide (Lozano et al., 2010), this organ is well studied and methods for diagnosing liver issues are of particular importance in the field of pathology. These pathological tools can be adapted to fish when examining their livers because there are very few morphological differences between human and fish livers (Phillips, 2014; Wilkins, 2013).

The role of the liver in biofiltration and detoxification makes it particularly sensitive to water contamination (Rodrigues & Fanta, 1998; Van der Oost *et al.*, 2003). Since it is the most affected organ by chemical pollutants (Camargo and Martinez, 2007), it is an efficient indicator organ to study. If the liver tissue is being affected, the kidney and gills are also suffering from similar stress (Camargo and Martinez, 2007), and the organism is suffering from widespread deleterious effects on epithelial tissues through chronic contamination, interrupting important functions like detoxification, metabolism, immunity, digestion, vitamin storage, hormone regulation, reproductive potential, fat storage, and glucose homeostasis (Kalra et al., 2022). The liver is also sensitive to gonadal hormones like estrogen, which has been connected to altered gene expressions and liver carcinogenesis (Baldissera et al., 2016).

Damage to liver tissues disrupts the normal function of this important filtrating organ and can cause cascading effects of harm to the organism. The metabolism of estrogens occurs in the liver and when there is damage present, these hormones do not get properly metabolized or inactivated, which results in an increased number of estrogens circulating and accumulating in the bloodstream (Camargo and Martinez, 2007). All blood leaving the stomach and intestines passes through the liver for filtering before being transported by the hepatic veins to the heart. When there is significant liver damage many cascading issues start to appear. When cellular transport of iron to the blood can't occur after chronic cytoplasmic degeneration and cellular rupture is present over long periods, it can lead to a buildup of iron in the liver, causing anemia (Currie et. al., 2022).

Hepatic fibrosis is a condition that begins as a healing response mechanism to inflammation or injury, but under chronic conditions, leads to an unnatural buildup of extracellular matrix and fibrous scars in the liver (Bataller & Brenner, 2005; Roehlen et al., 2020). This causes a collapse of normal hepatic structure and a loss of hepatocytes which can lead to cirrhosis, or the end-stage liver disease where normal function and detoxification is drastically impaired by excess scar tissue (Aydin & Akcali, 2018; Nishikawa, 2015). Hepatic damage is reversible in animal and clinical models, unless there is already cirrhosis present (Troeger et al., 2012), and many of these issues have been shown to be heavily impacted by diet (Wu et al., 2022). Evidence of this degradation can be seen through an examination of the tissues using histological imaging (Poleksic and Tutundzic, 1994), and as such, histology can be a very useful tool in environmental monitoring by the examination of vital organs which are chronically exposed to potentially polluted waterways (Gernhofer et al., 2001).

Liver Histology

The use of histopathological analysis to observe tissue alterations found in the liver is a less complicated means of health assessment than trying to identify functional impairment for the organism (Fanta et al., 2003) while also providing insight into the health of aquatic environments (Hinton and Lauren, 1990). In both laboratory and field studies, the health of fish who have experienced persistent pollutant exposure has been determined using histological evaluation

(Hinton et al., 1992; Schwaiger et al., 1997; Teh et al., 1997; Wester and Canton, 1991;). The need for more efficient, accurate, and diverse stains has driven the development of staining techniques that can highlight specific cellular structures with a degree of efficiency and accuracy that makes histology an indispensable scientific and diagnostic tool (Alturkistani et al., 2015, Black, 2012).

Histological staining includes five basic steps: fixation, processing, embedding, sectioning, and staining (Titford, 2009), but the particulars can vary based on the type of tissue being examined. For our purposes, these general steps sufficiently explain the process. Fixation retains the chemical composition of the tissues and prevents degradation by hardening the tissues, leaving them preserved, yet solid enough to cut thin cross sections, and processing involves removing all the water from the tissue with ethanol (Titford, 2009; Shostak, 2013). Then, to enhance and preserve the morphology of the cells, tissues are embedded into paraffin wax and hardened into wax blocks (Musumeci, 2014) which can be easily cut with a microtome. Sectioning is the term used for this process of cutting the tissues into very thin “ribbon-like” cross sections with the microtome and mounting them on slides before staining, which is the process of infiltrating the tissues with chosen dyes to illuminated desired parts of the cell structure (Musumeci, 2014).

Histological Stains

Histological staining is used to demonstrate the contrast between different components of the tissue with monochromatic and metachromatic dyes, depending on chemical affinity, density, and permeability (Victor, 2014). Different visual patterns are associated with different stains, and target tissue constituents can be identified by carefully choosing which stain is used in the analysis (Gupta, 2009). If one stain doesn’t show all the necessary structures, it is common to use multiple different staining techniques to confirm or support a diagnosis (Alturkistani et al., 2016; Krishna, 2013; Musumeci, 2014). Stains used in this study can be seen in Figure 2.

Hematoxylin and Eosin (H&E), seen in Figure 2A, is the most widely used stain in histopathology (Bancroft and Layton, 2013) because most processes taking place can be studied and illuminated using this one resourceful stain (Titford and Bowman, 2012). Hematoxylin is a deep purple / blue color and stains nucleic acids whereas eosin is bright pink and stains proteins. In normal tissues, hematoxylin stains the cell nuclei a purplish color, eosin stains the cytoplasm and extracellular features pink, and other structures will stain in a combination of these two hues that allow for the identification of structures by a practiced pathologist (Fischer et al., 2008).

Iron stain (Figure 2B) uses a Prussian blue reaction to stain iron cells. Iron can be stored in hepatocytes in either soluble form (ferritin) or insoluble form (hemosiderin). The iron stain colors the soluble ferritin a faint blush in the cytoplasm and the non-soluble hemosiderin as coarse blue granules. In the presence of hepatocellular damage, the mix of soluble and insoluble iron forms takes on certain detectable patterns in hepatocytes and Kupffer cells (Batts, 2007; Krishna, 2013).

Trichrome stains (Figure 2C) were created from a need by pathologists to have multicolored, differentiated tissue samples (Shostak, 2013) and as such, it has the effect of staining collagen fibers blue against a red background of hepatocytes to demonstrate the presence and thickness of collagen fibers (Mitchell, 2018). This stain helps delineate patterns of collagen fibers to highlight structures that are either a part of a healthily functioning liver or damaged because of chronic liver disease (Krishna, 2013; Lefkowitch, 2006; Thompson, 2011).

Reticulin stain (Figure 2D) is another useful stain for analyzing a type 3 collagen fiber that utilizes silver to saturate reticulin fibers that appear black or gray against a pinkish background of liver tissue. (Krishna, 2013). Reticulin fibers form the extracellular matrix of the cell, so studying this hepatic architecture can help assess the health of the cellular framework (Xerri et al., 1989) as well as the expression in regenerative and neoplastic conditions (Hartleb et al., 2011). A collapse of reticulin fibers can form a scaffold that helps to hold diseased liver tissue together, so seeing these structures can indicate the degree of damage or the stage of repair related to necrosis (Wen et al., 2016).

The Periodic Acid Schiff (Figure 2E) stain identifies glycogen by staining it a bright magenta color. Evaluation of glycogen storage in hepatocytes can be an indication of healthy or damaged liver tissue, for instance, excessive glycogen storage in hepatocytes can be an indication of chronic liver damage (Cho et al., 2015; Hui et al., 2017). The use of several different staining techniques can be very useful in determining hepatic structure and function and choosing which stains would be most effective for the desired outcome of the analysis.

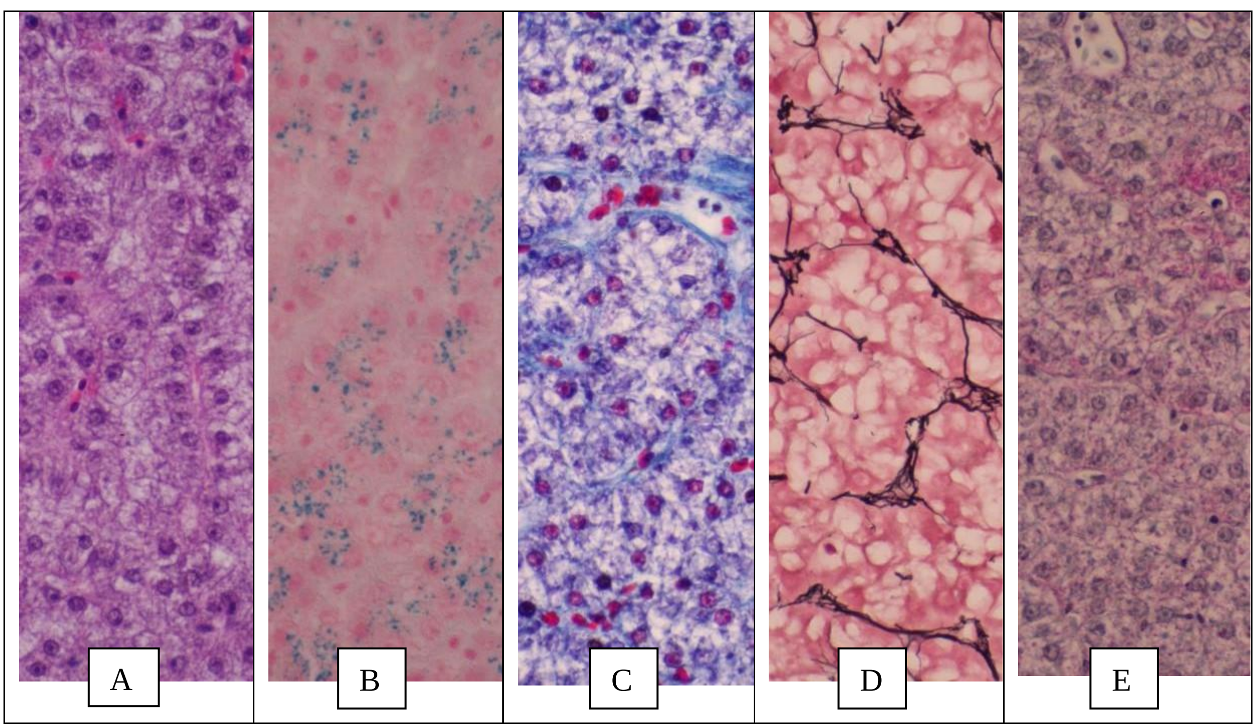

Figure 2. Histological stains used on the fish livers. These include A. Hematoxylin and Eosin; B. Iron Stain; C. Trichrome Stain; D. Reticulin Stain; E. Periodic Acid Schiff Stain.

Liver Degree of Tissue Change

The foundation for this work was pioneered by Polesksic and Tutundzic. They were the first scientists to publish work which quantified damage to the liver using histopathology and fused that data to make conclusions about the health of the aquatic ecosystems. This study applied a rating system to fish organs that had been examined histopathologically and divided the fish into three categories; first stage issues included changes that while present under current environmental conditions, would be fully recoverable and full structure and function could recover if removed from the environmental triggers (Poleksic and Tutundzic, 1994). These changes occur mostly on the cellular level. If the environment remains unchanged, however, the issues will probably worsen in severity and advance to stage two. Stage two changes were more severe and likely to have effects on healthy tissue function and appear mostly as nuclear changes. In environments with persistent pollutants, these changes would most likely not recover and the issues would advance to stage three. If water quality could be fully mitigated, lesions could be repairable if they are not already too advanced. Stage three tissue damage is non-recoverable, even with an improvement in habitat and water quality, and in the absence of toxic exposures. Stage three changes will eventually result in the cessation of vital tissue functions. (Camargo and Martinez, 2007; Poleksic and Tutundzic, 1994).

Other Health Parameters Under Consideration:

Hepatosomatic Index (HSI)

Using general health parameters is very important in trying to establish the health of a specimen since they show the organism's environmental interactions and the ability to get the necessary resources from their environment. Health parameters can also indicate the physiological state of the organism based on developmental markers, fat storage, or general well-being (Nikolsky, 1963). Because the liver is a metabolic organ, the HSI is a useful bioindicator of potentially perilous conditions that arise because of environmental stressors on the organism (Pait and Nelson, 2003). Liver damage often leads to an increase in the weight of the liver, so a high HSI indicates an enlarged liver, which is often associated with liver damage in the form of excess iron storage, fat storage, and an increase in nucleic activity due to an increased demand for genomic transcription that precedes tissue repair (Arukwe and Goksoyr, 2003; Nikolsky 1963). It has been found that endocrine disruptors may affect males and females differently, leading to more adverse effects in the livers and higher HSI in males of the same species than females due to endogenous estrogens present in female tissues (Orlando et al., 1999).

Relative Weight

Relative weight is an index used to determine the condition of a fish and how it compares to others of the same size for the same species and was first developed as a measure of fish health (Wright, 2000). It is calculated by dividing the weight of the fish by the expected weight for the same species at the same length, grown under ideal conditions and with ample food. This index can be helpful in the fisheries industry to determine if fish are not growing sufficiently or losing weight (Blackwell et al., 2000). Lack of high-quality food, poor water conditions, water temperatures that are too hot or too cold, crowding and resource competition, or disease can contribute to stress that inhibits ideal growth (Murphy et al., 1991). While growth may be difficult to measure, the relative weight of the fish is easy to measure and indicates if the fish are thriving or living under stressful conditions (Le Cren, 1951; Nikolsky, 1963). A relative weight below 0.8 is very thin, whereas 0.9 is considered average for well-balanced ponds, and greater than 1.0 is typically associated with excess fat storage (ACES, 2018).

Condition Factor

Condition factor (K) is a bioindicator tool that expresses the ratio between fish body weight and length that is widely used to ascertain the survival, reproductive success, maturity, and general health of fish (Blackwell et al., 2000, Le Cren, 1951). As such, it can often be a good indicator of water quality and aid in understanding the complex interplay between biotic and abiotic factors in an ecosystem and the overall health of fish populations inhabiting specific habitats (Tsoumani et al., 2006). For example, in areas with high productivity due to high nutrient availability, such as downstream from sewage overflows, elevated condition factors are often detected (Prado et al., 2011; Weber et al., 2013).

One problem with relying heavily on the condition factor is that it assumes isometric growth for fish populations, which is not always the case, but it can still be a useful parameter for examining fish health between similar populations (Bolger & Connolly, 1989). Another difficulty is that the condition factor is heavily affected by the reproductive stage of the fish and their reproductive organs. When comparing condition factor values, it is important to sample the individuals or populations at the same time of the year so that they are experiencing the same pressures on food resources and reproductive cycles (Baxter & Barnham, 1998). Condition factors can range from 1.60 (excellent condition, trophy fish); 1.40 (a healthy, well-proportioned fish), 1.20 (a decent size fish, acceptable to most anglers), 1.00 (a long, thin fish, poor condition), 0.80 (extremely poor fish, very thin) (Baxter & Barnham, 1998).

Males vs Females

It can be beneficial to compare male and female health parameters to see if one sex is more harmed by environmental conditions than the other, thereby impacting reproductive success, resource acquirement, and survivability. It is known that chemical pollutants impact the liver health of males more than females for instance, due to the higher energy expenditures and metabolic rate of male fish in natural environments (Madenjian, 2011). Therefore, the bioaccumulation rate of organic chemicals can be distinct between males and females (Johnston et al., 2022).

A study that compared the polychlorinated biphenyl (PCB) and mercury levels of eight different fish species found that for every species, the PCB levels were higher in males. Mercury levels were nearly equal between the sexes, although it is thought that this is due to a naturally occurring phenomenon whereby endogenous androgens such as testosterone and 11-ketotestosterone, enhance mercury elimination in male fish. (Madenjian et al., 2016). While males have higher energy expenditures due to faster and more frequent swimming and therefore eat more and ingest more mercury, they are also capable of ridding it from their systems more rapidly than their female counterparts. These elimination patterns between the sexes may also apply to higher vertebrates including humans. (Madenjian et al., 2016). Whole fish body mass is also correlated to a higher level of bioaccumulation for males than females, meaning that larger males take up more of the same chemical than a female of the same body mass (Johnston et al., 2002).

Intersex

Teleost fish have flexible and adaptive reproductive strategies and exhibit a wide range of sexual plasticity, ranging from determinate and unchanging to hermaphroditic (Bahamonde et al., 2013). Largemouth bass are gonochoristic, meaning that they develop either as males or females and do not change their sex throughout their lifetime (Iwanowicz et al., 2016), unlike hermaphroditic fish species that can produce both male and female gametes at asynchronous points in their natural and typical life cycle (Delvin and Nagahama, 2002). Intersex refers to the divergent condition that occurs when both male and female reproductive stages are present in the same gonad at the same time and has typically only been documented in males (Bahamonde et al., 2013; Blazer, 2002; Iwanowicz et al., 2016; Nolan et al., 2001; Tyler and Jobling, 2008).

Intersex is observable through histological examination of male gonads, when oocytes, or the precursor to reproductive egg cells, are detected in the testes. (Blazer, 2002). It can also be detected in males by the presence of oocytes in the testes or feminization of the gonadal ducts, with the severity of feminization depending on the intensity of exposure to aquatic contaminants (Nolan et al., 2001). Intersex is the result of estrogenic compounds, endocrine disruptors, and estrogen mimickers in the environment. (Blazer, 2002; Iwanowicz et al., 2016; Metcalfe et al., 2010) and has been documented in ecologically vulnerable taxa such as fish, aquatic invertebrates, reptiles, and amphibians (Hecker et al., 2006). There is still some uncertainty in this field as to the rate of occurrence of intersex in natural fish populations, and whether it could be an underlying molecular mechanism of sex determination (Bahamonde at al., 2013).

Largemouth bass demonstrate a certain sensitivity to aquatic contamination through the development of intersex conditions when their carp counterparts in the same aquatic habitat do not (Hinck et al., 2009). Southeastern fish species that appear more sensitive to changes in these sexual morphologies include largemouth bass (*M. salmoides*), smallmouth bass (*M. dolomieu*), and channel catfish *(Ictalurus punctatus*; Hinck et al., 2009). Gizzard shad *(Dorosoma cepedianum*), brown bullhead (*Ictalurus ameiurus*), pumpkinseed (*Lepomis gibbosus*), bluegill (*Lepomis macrochirus*), bullhead (*Ameiurus nebulosus*), white bass (*Morone chrysops*), striped bass (*M. saxatilis*), flathead catfish (*Pylodictus olivaris*), northern pike (*Esox lucius*), burbot (*Lota lota*), brown trout *(Salmo trutta*), largescale sucker (*Catostomus macrocheilus*), longnose sucker *(C. catostomus*), and white sucker (*C. commersoni*) demonstrated no intersex when counterparts in the same habitat did, indicating that they may be less sensitive to aquatic pollutants that act on gonadal alterations (Baldigo et al., 2006; Hinck et al., 2009; Kavanagh et al., 2004). Common carp (*Cyprinus carpio*) and smallmouth bass (*M. dolomieu*) have less consistent results from intersex reports, with some studies demonstrating intersex and other studies showing resilience (Hinck et al., 2008; 2009; Kavanagh et al., 2004; Schmitt et al., 2005; Viganò et al., 2006). While results from intersex reports indicate that some species are more resilient than others to aquatic pollutants that cause changes in gonadal morphologies (Bahamonde et al., 2013), studies have consistently demonstrated the sensitivity of largemouth bass to these disruptive chemicals in the environment, with evidence of intersex being the highest in the southeastern United States (Hinck et al., 2009; Iwanowicz et al., 2016).

Water Quality and Assessment

Chemical Pollution

Chemical pollution has been acknowledged as one of the planetary thresholds that could impact the sustainability of life on earth (Steffen et al., 2015); however, establishing where these thresholds lie is difficult to quantify, especially when considering the introduction of novel entities and challenges to studying the impact of pollution on living organisms (Carvalho et al., 2014; Wang et al., 2020). While the exact number of man-made chemicals currently in use worldwide is unknown, according to recent chemical inventories it is approximately 350,000 according to the collaborative efforts of several major countries (Wang et al., 2020). The U.S. Environmental Protection Agency (EPA) defines a contaminant as any physical, chemical, biological, or radiological substance in water or matter, with no specificity regarding whether that contaminant is natural or manmade (EPA, 2022). While there are challenges in determining exact amounts, widespread use, and difficulty in eliminating chemicals once utilized make it very likely that there are a great many chemical pollutants circulating in the environment (EPA, 2022). Another challenge is that chemicals present in low quantities that may not cause significant harm singularly could combine with other compounds to create mixtures that are more toxic and many of these interactions are not well investigated. (Carvalho et al., 2014; WHO.int, 2022).

Discovering which chemicals are present in the environment that pose notable risks and which organisms are the most vulnerable to their toxic exposure are two very important questions that have been at the forefront of this field for decades. In 1967, the National Contaminant Biomonitoring Program (NCBP) was founded by the U.S. Fish and Wildlife Service to document trends in environmental contaminant concentrations that threaten fish and wildlife, but it, unfortunately, ended after two decades (Schmitt et al., 1999). In 1987, the Food and Drug Administration (FDA) started a pesticide monitoring program that tests for over 800 chemicals in our food, which is an ongoing program that monitors contaminants and nutrients in the average U.S. diet (U.S. FDA, 2022), and the EPA monitors drinking water contaminants in a national monitoring program as well (U.S. EPA, 2022).

There are numerous ways chemical pollution can end up contaminating watersheds. Industrial discharge can contribute a significant number of pollutants that legally fall within discharge limits set by state and federal EPD and EPA standards, but still pose risks to aquatic ecosystems (Gillion et al., 2006). Nonpoint source pollution from urban run-off, suburban landscapes, and airborne particulates can contribute a higher chemical load into the environment than point-source loads and are directly correlated to urbanization (Gillion et al., 2006; Rose, 2007; Stamer et al., 1979). Non-point source pollution seeps into the groundwater in urban areas and diffuses throughout urban and suburban areas, carrying contaminants with it. Pinpointing the exact source of these contaminants can be more difficult to determine than storm event contamination like roads and storm drains because of the diffusive, non-point source seepage in urban areas (Rose, 2007).

Chemicals found in a variety of cleaning products, surfactants, lubricants, defoaming agents, dyeing assistants, pesticides, fertilizers, and emulsifiers can enter water systems and degrade into base chemicals, all of which can remain in bodies of water for indeterminate periods (Gilliom et al., 2006; Bennie, 1999). As soil is depleted of nutrients through years of continual agricultural use, fertilizer is needed to maintain the growth of crops. For example, from 1950-1951 in India alone, the agricultural use of over 69 thousand tons of fertilizers was required to keep up with increased food demands from growing populations (Parikh & Sadekarpawar, 2013; Patel & Singh, 2010). In a separate report released by the Food and Agriculture Organization of the United Nations, it is estimated that the total nutrient demand from fertilizers is growing at a rate of 1.9% each year, and based on numbers from 2015 and 2016, the estimated use of fertilizers worldwide in 2020 would be 201.66 million tons (2017).

Endocrine Disruptors

The endocrine system regulates the hormonal balance within the body. This messenger system is constantly signaling between organs in the body, using hormones as its means of communication. Chemicals in the environment can mimic hormones, causing disruptions to normal endocrine function. The liver plays a huge role in this because one of its most important roles in the body is the regulation of hormones.

While effects of chemical pollution cause broad harm to the ecosystems, physiological impacts on living organisms are equally as harmful and expansive. Anthropogenic contaminants in water can impose risks to reproductive health by acting as endocrine disrupting compounds

(EDC). Chemical pollutants in aquatic environments are cytotoxic and act as endocrine disruptors that can alter gonadal development and function (Kavanagh, et al., 2004). Steroid hormones, while essential in the natural reproductive cycles of all vertebrates, controlling essential processes such as sexual maturity, reproduction, stress responses, osmoregulation, and physiological processes, are quickly becoming one of the most widespread aquatic contaminants (Ojoghoro, 2021; Weber et al., 2015).

Sources of polluting contamination in water are diverse, originating from byproducts formed during water disinfection processes, released from industry, livestock activity, or therapeutic drugs released into sewage systems (Gonsioroski et al., 2020, Figure 3). Synthetic and natural hormones from humans, the pharmaceutical industry, and agriculture have been identified and quantified in recent studies of aquatic environments, showing their presence in these habitats is widespread (Boxall et al., 2012; Ojoghoro, 2021; WHO.int, 2022).

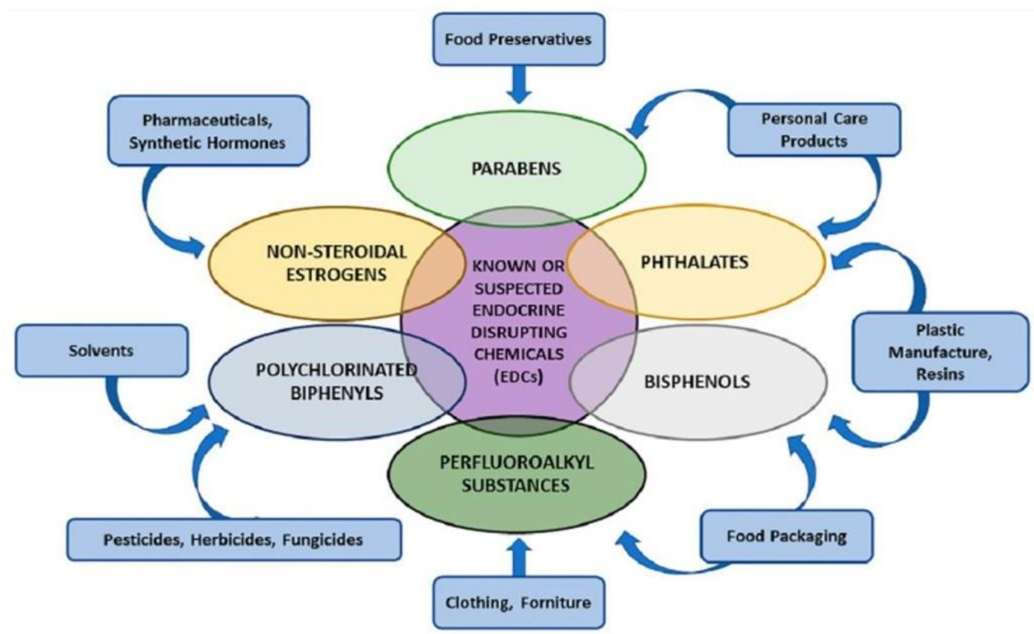

Figure 3. Contaminant diagram showing where EDCs originate. Taken from open-source internet images.

The most notable compounds in this study relate to pesticides, fertilizers, plasticizers, and hormones, which are all known for their endocrine-disrupting capabilities (Kavanagh et al., 2004). A study done by the U.S. Geological Survey in 2008 found 85 man-made chemicals in the tap water from nine different states, including pharmaceuticals like medication, antibiotics, anticonvulsants, mood stabilizers, synthetic hormones, and oral contraceptives (Fisher et al., 2016; USGS, 2022). There is evidence that hormones are not effectively removed during wastewater treatment and as a result, significant amounts of these hormones are present in drinking water (Silva et al., 2012). Estrogens and androgens are potent endocrine disruptors, perturbing normal hormone system functioning when individuals are chronically exposed to these compounds from external sources (NIEHS.NIH.gov, 2022). Concern over the potential side effects of potential exposure to these compounds is what prompted the EPA to begin its national program to monitor the most common hormones found in drinking water.

The Safe Drinking Water Act, passed in 1996, requires the EPA to update its list of no more than 30 unregulated contaminants that are of highest concern in public water systems every five years (U.S. EPA, 2022). Currently, there are seven volatile organic compounds and seven hormones that are routinely monitored by the EPA as a part of their Unregulated Contaminant Monitoring Program (U.S. EPA, 2022).

The worldwide human population releases approximately 30,000kg of natural steroidal estrogens and 700kg of synthetic estrogens from birth control annually, and the discharge of estrogen from livestock is more than twice this much, nearly 83,000kg/yr. for just the United States and European Union combined (Adeel et al., 2017). These estrogens from concentrated animal feeding operations (CAFOs) often find their way into aquatic environments (Shrestha et al., 2012). There are three different types of estrogens produced naturally in the female body: 17

β-estradiol, estrone, and estriol. 17 β-estradiol (E2) is synthesized naturally in the ovaries of premenopausal females. Estrone (E1) is a metabolite of E2 and is a third less estrogenically potent than its parent compound (DrugBank, 2022; Ezhilarasan, 2020; NIH, 2012).

Estrone can be synthetically or naturally occurring and is very common in pregnant equine urine and menopausal females after ovaries have stopped producing E2 (DrugBank, 2022). Synthetic applications of estrone include treating abnormalities in gonadotropin hormone dysfunction and conditions related to menopause such as vulvar atrophy, as well as osteoporosis from estrogen deficiency (DrugBank, 2022; NIH, 2012). Estrone is classified as an irritant and health hazard due to its acute toxicity (NCBI PubChem, 2022) as well as a class 1A carcinogen by the European Chemicals Agency (ECHA, 2022). It is harmful to aquatic life and has associations with disruptions in fertility and reproductive toxicity as well. (NCBI PubMed, 2022). Because the liver is a hormone-sensitive organ, it is heavily influenced by gonadal hormones like estrogens, which are known to cause injury to the liver and are linked to carcinogenesis of the liver due to genetic alterations and impacts on gene transcription (Baldissera et al.,2016; Chen et al., 2016).

While steroids and estrogenic compounds are of particular importance, there are many other compounds of note that are known to be disruptive to aquatic health and human health. Chlorpyrifos is a widely used, broad-spectrum insecticide that has been unintentionally introduced into terrestrial and aquatic ecosystems whose metabolites induce toxicity through oxidative stress and endocrine disruption that have been linked to memory and IQ deficits in children whose mothers were exposed during pregnancy (Barron & Woodburn, 1995; Nandi et al., 2022; Rauh, 2011). Diethyl phthalate is a commonly used plasticizer that has the highest human exposure of all the phthalates which has been demonstrated to cause low liver weight and cellular damage in laboratory animals (Weaver et al., 2019). Bis (2-Ethylhexyl) phthalate is another common plasticizer used for making materials flexible in household products and has been linked to increased hepatocellular carcinoma, or liver cancer, in animals (NCBI, PubChem, 2022). Bis (2-Ethylhexyl) phthalate was documented by the European Chemicals Agency (ECHA) as a substance of very high concern in 2008 due to the deleterious impacts on human health (ECHA, 2022).

Butyl benzyl phthalate (BBP) is a colorless liquid that is used as a plasticizer for PVC pipe, vinyl flooring, carpet backing, and other plastics (Cameo Chemicals, 2022; Lewis, 2007; National Toxicology Program, 2015). It is known to be very toxic to aquatic organisms, with long-lasting, bio-accumulative effects, a potential threat to fertility and unborn children, and was listed as a substance of very high concern by the ECHA in 2008 (NCBI, PubChem, 2022). In 2012, it was banned by Congress from children's products in amounts greater than 0.1% and causes liver toxicity and central nervous system depression after chronic exposure (NCBI, PubChem, 2022). It is important to note that many of these compounds are derived from cholesterol, which is a lipid, or have lipid characteristics, making them insoluble in water, and therefore more stable, pervasive, and harder to eliminate in aquatic environments.

Pollution in the Chattahoochee Watershed

The Chattahoochee River is the most urbanized and utilized water resource in Georgia and is the most impacted by anthropomorphic activities. In addition to housing the largest and densest human population in the state, it carries the burden of most municipal wastewater discharges in the ACF River basin (Couch et al., 1996; Davis, 1990). In the 1970s the Chattahoochee was classified as grossly polluted with high levels of raw sewage, fecal coliform, and low biotic diversity south of Atlanta. By the 1980s, after significant advancements were made to municipal wastewater treatment facilities and phosphate-detergent chemical bans were made by the state legislature, the quality of wastewater effluent improved significantly (Georgia Water Quality Control Board, 1971).

The Environmental Protection Agency's National Study of Chemical Residues in Fish investigated 60 chemical residues in fish tissues from 388 study sites across the United States, 11 of which were in the ACF River basin. Of those, three sites on the Chattahoochee had fish with contamination levels among the highest in the country, and one of these sites ranked top five in the nation for chemical concentrations of dioxin compounds, pentachloroanisole; 1,2,3 trichlorobenzene; chlordane; chlorpyrifos; and methoxychlor (U.S. Environmental Protection Agency, 1991). In the southeastern U.S., manufacturing and agricultural practices that have historically driven the economy have been a source of organochlorines and mercury in the aquatic habitats of the Chattahoochee (Couch et al., 1996).

Figure 4. Geological regions of Georgia.

To better understand the human impact on the Chattahoochee watershed, it is essential to also consider variations in the underlying geologic features and aquifers that can have a dramatic impact on water quality by means of natural filtration and accessibility to surface level drainage (Cherry, 1961; Couch et al, 1996). The piedmont north of the fall line and coastal plain south of it creates many variations in natural geological filtration along the Chattahoochee River (Figure 4). Stream-water chemistry is largely determined by geochemical processes occurring at the interface between the surface water and groundwater underlying stream beds, as well as between the stream and the land surrounding it along the banks. (Burns et al., 2001; 2003). An example of this is that the contaminant load can be more concentrated and affect the water chemistry of shallow groundwater areas more than others (Burns et al., 2001; 2003).

Septic tank and sewer line leaks are two of the main sources of contamination of shallow groundwater in the Atlanta metropolitan region and the Chattahoochee River basin, leading to higher levels of base flow solute concentrations (Rose, 2007). As of 2001, the sewer system in the city of Atlanta consisted of 15% combined sewage overflow (CSO) systems (Horowitz and Hughes, 2006). The CSOs periodically backup and overflow through manhole covers during heavy rainfall events, discharging large amounts of untreated discharge directly into ground water, streams, and rivers (Horowitz and Hughes, 2006). Several CSO overflows are built into the Chattahoochee Riverwalk at various locations in Columbus as well, introducing contaminants such as heavy metals, pharmaceuticals, hormones, and excess nutrients into the channel directly (Callender and Rice 2000; Neumann et al., 2005; Rose, 2002; 2003). As the populations in urban areas increase, these CSO overflow events become more severe and impactful.

Water Bodies of Concern

West Point Lake is a man-made reservoir located on the border between Georgia and Alabama and was built to help regulate the depth of water in the Chattahoochee River for safe passage of cargo boats traveling upstream from the gulf. Construction began in 1965 and it is approximately 34 miles long and up to 3 miles wide, with a total surface area of 25, 864 acres. West Point Lake, which is buffered by West Point Dam on the southern end, is the northernmost and most rural sample site for this study and has no impoundments separating it from the Chattahoochee River that flows through metro Atlanta (Wikipedia, 2022). It is a popular fishing destination.

Lake Harding is a man-made reservoir located on the Chattahoochee River, built in 1926 for gathering hydroelectric power. It is buffered by Bartlett's Ferry Dam to the south and is located mostly in Harris County, Georgia, while sharing a western border with Alabama. It has a total surface area of 5,850 acres and is 100 feet deep as it approaches the dam to the south (Wikipedia, 2022). Lake Harding is primarily used for recreational activities like boating and fishing and it is not as densely populated as Lake Oliver, even though it is surrounded by some development and housing.

Lake Oliver is a man-made, 2,150-acre reservoir located on the Chattahoochee River and is the smallest, most densely populated of our four study sites. Lake Oliver is located within the city limits of Columbus, Georgia, but the dense population on both the Georgia and Alabama sides of the reservoir means that there are many access points to the lake, which is heavily used for recreation and fishing. It is buffered by two dams, Goat Rock Dam to the north, and Oliver Dam to the south. The fall line that separates the Piedmont of north Georgia and the coastal plain of South Georgia occurs at Oliver Dam, south of Lake Oliver. The river flows through downtown Columbus and continues south until it flows into Lake Eufaula (Wikipedia, 2022).

Warm Springs Hatchery is a part of the Flint River basin and lies in the southern piedmont region of Pine Mountain, Georgia. It has an altitude of 1,200-1,300 feet. The habitat is dominated by Pine Oak Mountain, which is predominately capped with quartzite. It is notable for its natural warm-water springs flowing from the aquifer (Clark and Zisa, 1976). The hatchery is a National Fish Hatchery that pulls water directly from groundwater springs and treats the water with only natural lime salts to adjust for the pH before feeding into tanks and breeding ponds.

Geographic Information System Analysis

Geographic information system (GIS) mapping is a useful tool in determining the percentage of land cover in any chosen area. It is accurate, reliable, and is a resource used worldwide to help determine and understand land use practices. Because the health of aquatic ecosystems is linked to the health of terrestrial ecosystems, the land use practices surrounding a watershed can have a substantial impact. The entire ACF River basin has been subject to varying degrees of forest-cover alteration (Couch et al., 1996). Originally forested by hardwood oak-chestnut-hickory forests in the Piedmont of north Georgia, and longleaf pine forests and wiregrass savannahs of the coastal plain south of the fall line (Wharton, 1978), Georgia's forested ecosystems have been irreparably altered by mining, logging, erosion, and disease (Leigh, 2004). By the 1920s, 87% of Georgia piedmont forest had been cultivated for agriculture (Plummer, 1975) and by 1935, the land was practically infertile and absent of all topsoil which had eroded due to poor land practices in cotton farming (Wharton, 1978).

The secondary forests that cover most of the piedmont of north Georgia today grew from these abandoned agricultural lands, and by the 1970s, 59% of the land cover in the entire ACF River basin was made up of either large pulp wood farms with acres of planted pine or secondary forests that replaced abandoned farmlands (U.S. Geological Survey, 1972-1978). Determining the current state of the land use in the Chattahoochee River watershed will provide one more angle to the river's story, as the health of these aquatic ecosystems is intrinsically tied to the terrestrial ecosystems surrounding them.

Goals and Objectives

The objective of this thesis was to determine and compare the liver health of the Largemouth bass from four aquatic environments: Lake Oliver, Lake Harding, and West Point Lake, and Warm Springs Hatchery. The bodies of Lake Oliver, Lake Harding, and West Point Lake are in the Chattahoochee River watershed, while Warm Springs Hatchery was designated in the Flint River watershed. While this research has been done in other river systems, it has not been done this comprehensively for the Chattahoochee River and is an important preliminary study tracking the health of this key species of the watershed.

Histopathological analysis of the liver of Largemouth bass (*M. salmoides*) was reviewed to calculate an average DTC rating to see how these compare among the sampling locations, and to compare DTC to other health parameters. A preliminary chemical analysis of the water samples from the four aquatic environments was performed using mass spectrometry, looking specifically for organic and hormonal compounds. My thesis aimed to better understand the health of Largemouth bass populations on the middle Chattahoochee River and make inferences about the watershed's condition by using Largemouth bass as a bioindicator species. To address this goal, I asked the following three questions:

1. How does liver health of Largemouth bass, measured by the DTC, compare among four sample sites: Lake Harding, Lake Oliver, West Point Lake, and Warm Springs Hatchery?
2. Does DTC correlate to other Largemouth bass health parameters such as HSI, relative weight, condition factor, gender, or intersex for the four sample populations?
3. Do any of the chemicals detected in the watershed from our analysis have known deleterious effects on liver health?

Methods

Fish sampling and locations

Largemouth bass were previously collected via electrofishing from Lake Oliver (n=69), Lake Harding (n=40), and West Point Lake (n=40) by CSU students and faculty and Georgia and Alabama Department of Natural Resources (DNR). The Largemouth bass from Warm Springs Hatchery (n=25) were received directly from the hatchery. All the Largemouth bass were processed using the appropriate procedures, body weights and lengths measured and liver tissues weighed before being preserved in formalin. Livers were then embedded in paraffin and sectioned at 5 microns for staining and analysis, as per standard histopathological procedures. All collection, handling, and euthanasia procedures followed animal care and use guidelines (American Fisheries Society et al., 2004).

Degree of Tissue Change

Quantifying liver health for research involves applying a rating system to the damages observed for each fish and plugging those values into a formula that ultimately results in a score of damage severity assigned to each fish, also known as the degree of tissue change score or DTC. This model was loosely borrowed and then modified from the medical practice of pathology in diagnosing human liver disease.

Our classifications for scoring have been modified slightly from Camargo and Martinez (2007), which they modified from Poleksic and Tutundzic (1994). A few minor adjustments were made to the scoring system to create a more nuanced system of scoring liver health, such as removing bile stagnation, which is rarely seen, and adding reticulin meshwork collapse to the scoring parameters. Our modified scoring system divides hepatic changes into the three following categories: Stage one issues include nuclear hypertrophy, lateral nuclei, irregularly shaped nuclei, cellular hypertrophy, cytoplasmic vacuolization, cellular atrophy, irregularly shaped cells, and melanomacrophages. Stage two issues include nuclear vacuolization, cytoplasmic degeneration, cellular rupture, nuclear degeneration, or atrophy, and pyknotic nuclei. The third stage includes two indicators of cell death: focal necrosis and reticulin meshwork collapse. DTC values can then be subdivided into five different levels of severity of impact on the functional liver organ of fish: between 0 and 10 indicate normal functioning; between 11 and 20 indicate slight damage; between 21 and 50 indicate moderate changes; values between 50 and 100 indicate severe lesions and values above 100 indicate irreversible damage (Poleksic & Mitrovic-Tutundzic, 1994).

Ten non-adjacent sections from each of the 174 liver specimens were used in this study, which included both males and females. Two of these cross-sections were secured onto a single adhesive slide before staining. Five different stains were used to stain two cross-sections for every fish: hematoxylin and eosin, reticulin, trichrome, periodic acid Schiff, and iron stains. Liver pathology was used to score the resulting tissues and give them a degree of tissue change, or DTC rating, using the formula that was first established by Camargo and Martinez (2007), and was adapted from Poleksic and Tutundzic (1994) as seen in the formula below.

$$DTC = 1\sum_{i=1} a_i + 10\sum_{i=1} b_i + 10^2\sum_{i=1} c_i$$

Equation 1 - Degree of tissue change equation. DTC is calculated by finding the sum of the total number of conditions present from category a (stage 1) and multiplying it by one, adding that to the total number of conditions present from category b (stage 2) multiplied by 10, and adding that to the total number of conditions present from category c (stage 3) multiplied by 100.

DTC Values	Effects (Poleksic and Tutundzic 1994)
0 - 10	Functionally normal tissue
11-20	Slightly to moderately damaged tissue
21 – 58	Moderately to heavily damaged tissue
> 100	Severely damaged tissue

Ten randomly selected, high-powered 40x fields were examined for each cross-section of liver tissue and if the damage was present even once in five out of ten of the high-powered fields, it was marked on the sheet as present and used in the calculations for the DTC. Based on pathological recommendations, if less than five fields out of ten showed an occurrence, the issue was not marked as present or included in the final score. This ensured that the issues that were included in the final score were found consistently in the whole tissue section so that scores were conservative and erred on the side of caution. This method was meant to account for the natural irregularities in tissues that can sometimes lead to misinterpretation of tissue changes and was recommended by the pathologist because it was a clinical model for interpreting tissue changes. The equation for calculating the degree of tissue change can be seen above, where stage one issues were multiplied by 1, stage two issues were multiplied by 10, and stage three issues were multiplied by 100. Scoring sheets were used to calculate the degree of tissue change following histological observations (Figure 5).

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Figure 5 - DTC scoring sheet sample. The hepatic cellular and nuclear changes along the top of the scoring sheet are divided into three categories: Stage I, stage II, and stage III. Scores between 1-10 are classified as healthy liver, 11-21 slightly to moderately impacted liver, 21-58 moderately to severely impacted liver, and above 100-158 are considered severely impacted.

Other Health Parameters Under Consideration

In addition to liver histopathology, the following health parameters of fish health were also considered. The hepatosomatic index (HSI) was calculated by dividing the weight of the liver by the wet weight of the specimen, then multiplied by 100.

$$Hepatosomatic\ Index\ (HSI) = \frac{W_l}{W} \times 100$$

Equation 2 - Hepatosomatic index equation. HSI is found by dividing the weight of the liver by the weight of the specimen, then multiplying by 100.

The relative weight was calculated by dividing the wet weight of a fish by the expected weight for a fish of the same species at the same length growing rapidly and with no restrictions to the food supply, multiplied by 100. A relative weight index score below 80 is considered very thin, 90 is considered average, and greater than 100 is seen as storing more fat than is typical (ACES, 2018). A table with the standard weights of largemouth bass can be found in the supplemental materials.

$$Relative\ Weight\ (RWt) = \frac{W}{W_{standard}}\ x\ 100$$

Equation 3 - Relative weight Index equation. Relative weight index is calculated by taking the weight of the specimen, divided by the weight of a standard fish of the same length from the table in the supplemental materials, then multiplying by 100.

Condition factor was calculated by dividing the wet weight of the specimen (in grams) by the length cubed.

$$Condition\ Factor\ (c) = \frac{W}{L^3}$$

Equation 4 - Condition factor equation. Condition factor is calculated by dividing the weight of the specimen by the length of that specimen cubed.

The sex of all specimens in our study were determined after preparing the gonads histologically and observing the morphology of the tissue after staining with hematoxylin and eosin (H&E).

The presence or absence of intersex was determined microscopically by Elizabeth Klar (faculty at CSU) by viewing 6 different sections of the gonads from each of the same male fish that were used in the liver analysis. The gonads from the male Largemouth bass were prepared for analysis using the H&E stain according to standard histology procedures. The presence of oocytes, and therefore intersex, was determined by scanning the entire gonadal cross-section and counting the number of oocytes present within each cross-section of tissue.

Water Chemical Analysis

To draw a link between the Largemouth bass liver DTC and the environment, it was important to gather water samples from the study sites to see what chemicals were detected there and see if any of those chemicals have been shown to have health impacts on these key organs. Initial water collections were conducted in September 2021. Samples were processed using mass spectrometry and liquid chromatography at Auburn University hydrology labs. Water samples were collected at Lake Oliver, Lake Harding, and West Point Lake from three approximate locations where some of the fish were collected using GPS coordinates. (Figure 6).

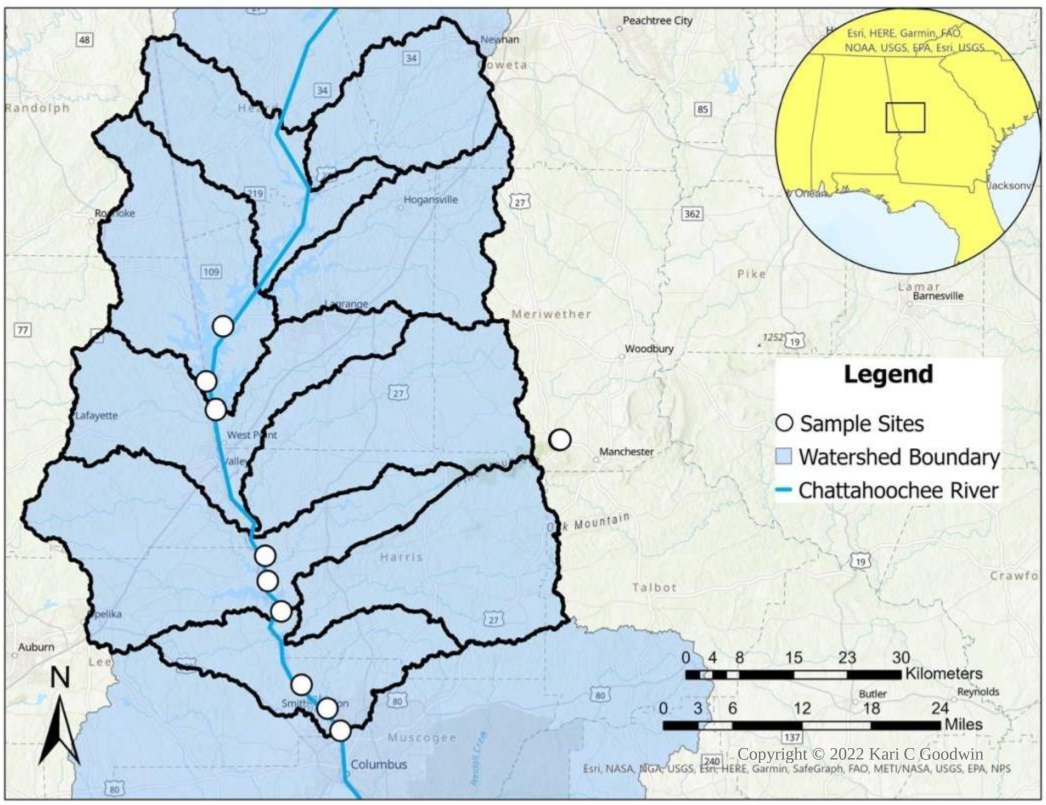

Figure 6. Map of our water sampling locations. The white dots represent individual water collection sites and the black outline shows where each of the sub-watershed boundaries is located. The three southernmost sample sites came from Lake Oliver which had two sub-watersheds flowing into it. The three centrally located collection sites were from Lake Harding, which had three sub-watersheds flowing into it, and the three northernmost sample sites were from West Point Lake, which had four sub-watersheds flowing into it. Warm Springs Hatchery is represented by a white dot, but because it is a part of the Flint River watershed and not the Chattahoochee River watershed, it is not included within the boundaries of our sampling sub-watersheds for GIS analysis.

Samples were collected using a Van Dorn water collection method, following standard operating procedures (U.S. Geological Survey). Depending on the depth at the specific locations, which was determined by the depth finder attached on the boat, samples were taken at surface level, one meter above the bottom depth to avoid gathering excessive sediment, and at a calculated midway point between those two so that water collections from each layer in the water column were included in the sample. Once these samples were all collected, from an upper, middle, and lower water stratum, they were combined into a single bucket and mixed thoroughly before a 500mL sample was collected from the larger composite sample. Distilled water was used to rinse the buckets between water collections at independent locations. Water samples were then stored on ice in light-proof brown glass bottles until further processing.

Warm Springs Hatchery warranted one water sample from the main source spring, one from a subsequent spring holding tank, and a third from water that had been treated by the hatchery before being fed directly into the fishery ponds. Their water treatment involves the addition of lime to adjust for the pH of spring water and then was recombined with natural spring water in a ratio of approximately 50:50 to achieve the desired pH for the fishery habitat. No other chemicals or treatments were added to the water at the hatchery. Water was collected using a dip method from flowing water sources before being saved in brown glass bottles and put on ice until initial filtrations could be performed.

Two sequential filtrations of the samples were performed to remove debris and unwanted solids from the water samples. This was done by using a vacuum filter twice through a Büchner funnel and filter flask, using two pieces of #1 filter paper (110 mm). The debris and solids removed via this method were discarded and the filtered samples were then processed through the steps detailed below. These initial filtration steps were completed by the Columbus State University chemistry department. All samples were treated identically in this and all subsequent procedures, regardless of their source.

To expose and extract as many chemical compounds as possible from the water samples, they were processed according to standard procedures using C-18 filtration columns, using a procedure for quantifying hormones in drinking water using solid-phase extraction (SPE) followed by liquid chromatography-electrospray ionization tandem mass spectrometry. SPE 1000 mg C-18 cartridges were used to concentrate pesticides, herbicides, hormones, and hydrocarbons that were present in the sample, according to methods approved by U.S. EPA guidelines. Our samples were originally 100mL, so all the hormones detected from this procedure represented the quantities present in a 100mL sample. (Thermofisher.com, 2013, Thermofisher.com/chromatography 2016).

The steps used for this process were as follows:

1. In preparing a C-18 cartridge for elution, the cartridge was initially flushed with 10 mL of 100% methanol (MeOH). Once the MeOH exited the column, it was discarded.
2. The column cartridge was then rinsed with 20 mL of distilled water. Once the distilled water exited the column, the water was discarded.
3. Following step 2, the 100mL water sample was gradually added to a C-18 cartridge, over a period of 10 min, until the entire 100mL sample had eluted from the cartridge. A SPE vacuum manifold was used to push the liquids through the C-18 cartridge, and a flow rate of 10-15 mL per minute was maintained throughout, as suggested by standard operating procedures. This discarded water was kept for future use if needed for further testing, but no other steps were taken specifically with the water samples once they were flushed from the column.
4. The C-18 cartridge was then flushed with 5 mL of 100% methanol. This 5 mL sample was placed into 100 mL round bottom flasks and reduced under a slow and steady stream

of nitrogen gas. This step continued until all the methanol evaporated and only solids remained. The remaining solids were then resuspended with 1 mL of 10% methanol. The flask was gently agitated manually until all the solids were resuspended in the methanol, and then pipetted into a small lightproof glass vial and sealed and labeled for analysis at Auburn University Spectrometry Labs.

5. The final step was eluting the cartridge with 5 mL of 100% acetonitrile (ACN). These 5 mL samples were eluted into 100mL round bottom flasks and reduced in the same procedures described above in step 4. This sample was then transferred to a sterile light-proof glass vial and sealed and labeled for further analysis. Each independent water sample from our collection was processed using these methods outlined in steps 1- 4 with a new C18 cartridge.

GIS Land Use

The land-use types included in the GIS analysis were grouped into five categories to simplify the comparison among study sites. Open water stayed as "Open Water"; developed open space, developed low intensity, developed medium intensity, and developed high intensity were all grouped together into "Developed"; barren land remained "Barren Land"; deciduous forest, evergreen forest, mixed forest, shrub, scrub, herbaceous, woody wetlands, and emergent herbaceous were all grouped together into "Undeveloped"; and hay, pasture, and cultivated crops were grouped together into "Agriculture". For this study, only the land use percentages calculated from the five land-use categories mentioned above will be used. The total land use percentages for each study site before recombination can be seen in the supplemental materials.

To reduce the number of sub-watersheds in the analysis, a hydrologic unit code, or HUC10 was used as the base layer in the GIS analysis. HUC10 is a base layer map used in GIS analysis that divides the land features based on water flow in the subwatersheds. A HUC8 for instance would group our watershed into one or two large groups based on flow, and give insufficient detail, whereas a HUC12 would divide the subwatershed into many more units, providing more detail than was needed. For our analysis, a HUC10 was an appropriate fit that allowed us to separate out the subwatersheds and group them according to drainage areas which were the target of this study. This allowed us to clearly define the flow lines of the sub-watersheds feeding directly into each of our study sites, without losing details pertinent to our study. West Point Lake had four HUC10 sub-watersheds, Lake Harding had three, Lake Oliver had two, and Warm Springs Hatchery (Figure 6).

Statistical Analysis

DTC and Other Health Parameters

Statistical tests were run to analyze the DTC of the sample populations. Non-parametric statistics were used to compare DTC values across four study sites, then parametric statistics were used when classifying DTC levels as either Low or High based on their bimodal separations. The DTC scores proved to be a bimodal measure of health, with scores being split between a range from 0-58 possible, then jumping to 100+, due to the logarithmic nature of the equation used to calculate the scores and the total possible parameters within stage one, two, and three. This divided our DTC scores into two groups of fish. Those with a “high” score of over 100, and those with a “low” score of less than 58. Tests were run to compare these metrics to HSI, relative weight, and condition factor for all specimens. For all tests, the significance level was assigned for $p < 0.05$.

The DTC was analyzed for statistical differences between the four study sites using a Kruskal-Wallis non-parametric analysis of variance test, with Dunn’s post hoc test. A Mann-

Whitney test was run to see if the mean DTC was significantly different between males and females, as well as those males who did and didn't have intersex. ANOVA and Tukey's post hoc test was used to compare the mean HSI, relative weights, and condition factors across each of the four study sites. An unpaired t-test was used to see if there was any significant difference of the hepatosomatic index, relative weight, and condition factor between categorical groups of fish with high or low DTC scores. This was done for all study sites combined, as well as each site individually.

A note about the Friedman test, although considered a non-parametric test, was ultimately not used because it only compared the sample means from two populations which was ineffective, for instance when comparing the DTC and HSI, as it was simply comparing the mean DTC to the mean HSI, which was not as good a fit for the data as the Kruskal Wallis test.

To account for discrepancies and try to minimize the possible biases and scoring mistakes in the DTC scores across our sample, we randomly selected 25% of the 174 fish in our sample and did a second DTC scoring of those fish. The two scores were then statistically compared using a t-test and it was found there was no significant difference in the scores ($p=0.178$). This helped to ensure that the DTC scores could be relied upon with confidence and a degree of certainty.

Water Quality Analysis

ANOVA and Tukey's post hoc test was used to compare chemical concentrations in parts per billion (ppb) that were detected using mass spectrometry analysis from our water sample collections across the four study sites.

Results

Degree of Tissue Change

The mean DTC scores were significantly higher between all three lakes compared to Warm Springs Hatchery, $p<0.001$ (Figure 7, Table 1). There were no significant differences in the mean DTC among the lakes. Most of the fish were either in the 21-58 range of moderately to severely impacted liver tissue or the 100-158 range category of severely diseased liver tissue, which had wide variation among the study sites (Figure 8). The bimodal distribution of the DTC score data can be seen in Figure 9, as the scores are split between either low categories below 58, or high categories that fall above 100.

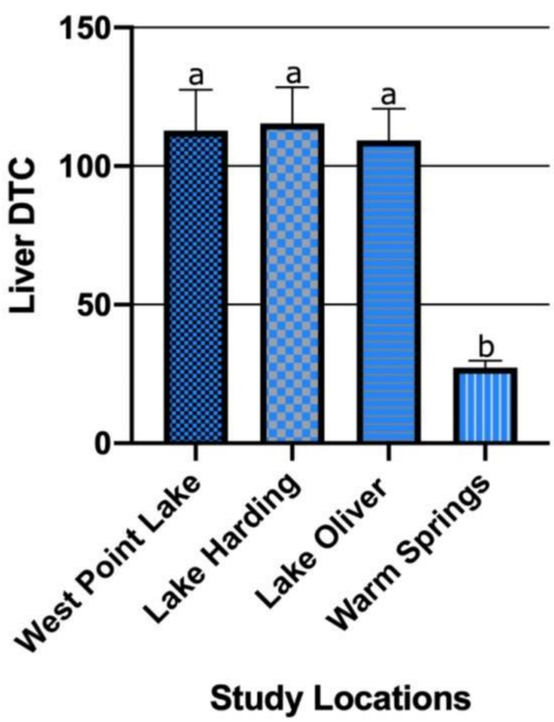

Figure 7. Mean DTC scores at each study location. Significance was detected between all sites and Warm Springs Hatchery p<0.0001 with Tukey's post hoc test.

Table 1. Mean DTC scores and standard deviation values from each study site.

DTC Score	West Point Lake	Lake Harding	Lake Oliver	Warm Springs
Average	112.75	115.475	109.261	27.32

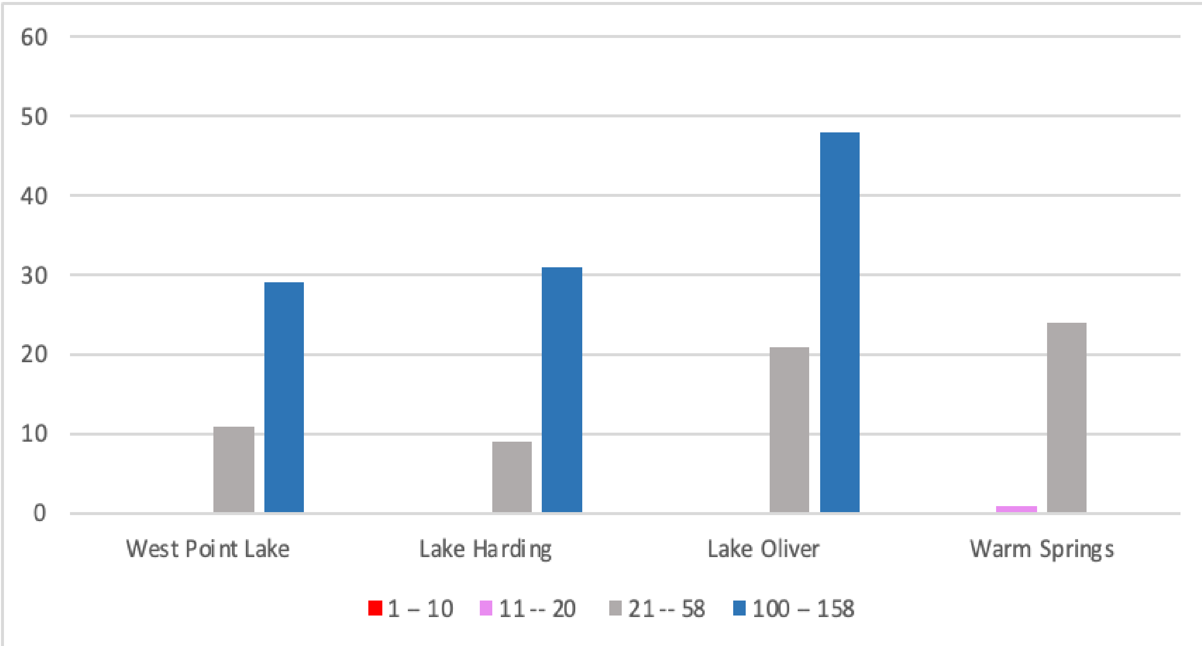

Figure 8. Degree of DTC severity frequency at each study location. This was not a statistical test, but rather an illustration of the liver condition across sites. 1-10 = healthy liver tissue, 11-20 = slightly to moderately damaged liver tissue, 21-58= moderately to severely damaged liver tissue, 100-158= severely diseased liver.

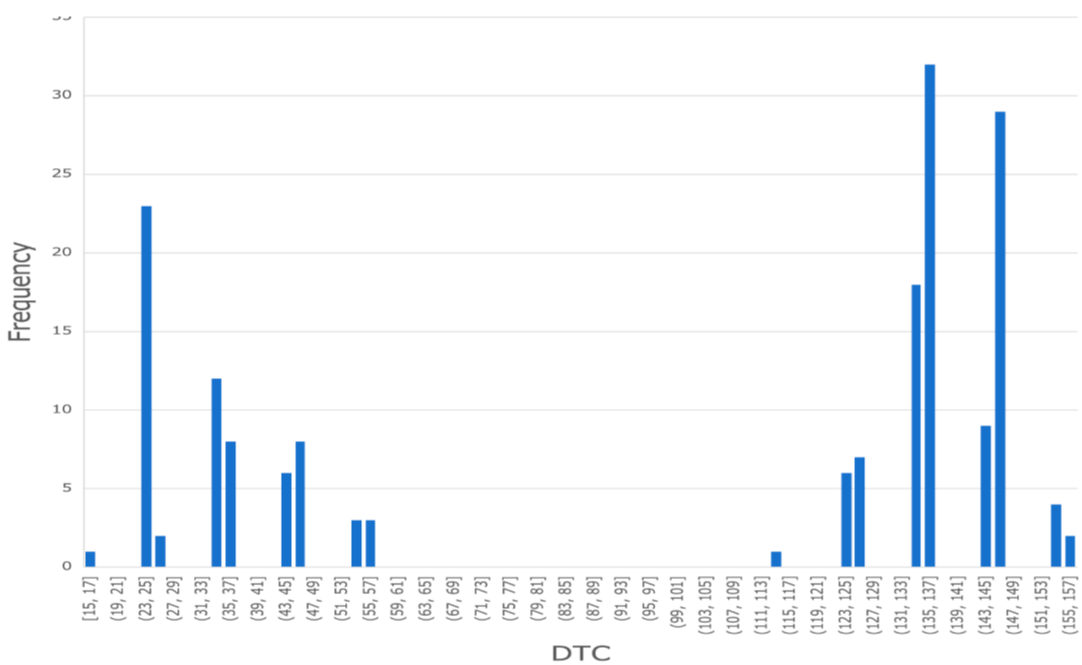

Figure 9. Bimodal frequency of DTC from all fish. This clearly splits our sample population into two distinct categories of low DTC (1-58) and high DTC (100-158).

Degree of Tissue Change and Hepatosomatic Index

Hepatosomatic Index (HSI) Results

The HSI means for our study sites were as follows: West Point Lake = 0.75, Lake Harding = 0.93, Lake Oliver = 0.90, and Warm Springs = 1.27. Among the four locations the HSI was significantly lower in all three lakes versus the hatchery ($p<.0001$). The HSI of Lake Harding was significantly higher than West Point Lake ($p=0.0348$, Figure 10).

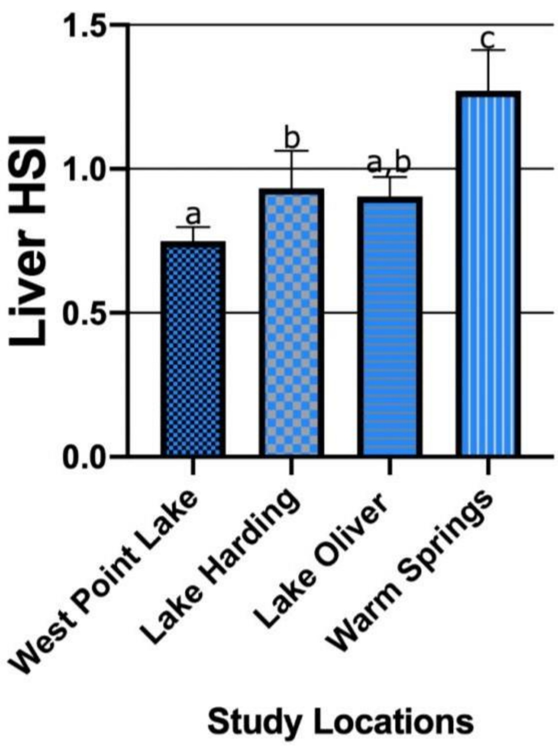

Figure 10. Mean HSI among sampling sites. There was a significant difference between the values of the three lakes and the hatchery (p<0.001), as well as between Lake Harding and West Point Lake, p=0.0348.

Degree of Tissue Change & HSI Results

When subsets of the DTC data, separated into low DTC (n=66) and high DTC (n=108) categories, were used as the grouping variable there was a significant difference between the HSI for fish who had low versus high DTC scores. The mean HSI for the high DTC group (HSI=0.83) and the Low DTC group (HSI=1.10) was significantly different, p<.0001, (Figure 11).

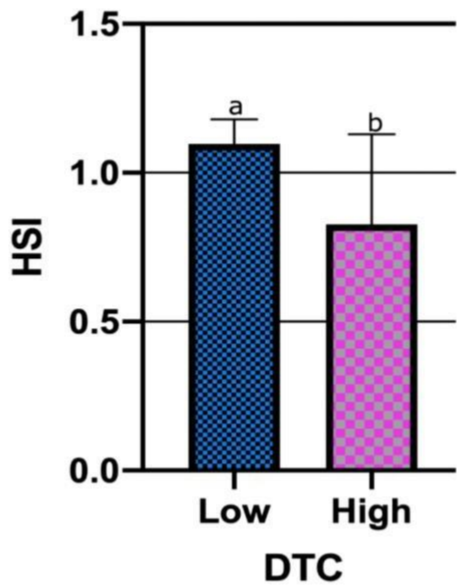

Figure 11. Mean HSI compared between low and high DTC groups for all fish. The mean HSI for High DTC group (>100) was significantly lower than the Low DTC group (<58), p<0.0001.

Degree of Tissue Change and Relative Weight Index

Relative Weight Index Results

The relative weight index means for our study sites were as follows: West Point Lake = 86.45, Lake Harding = 94.01, Lake Oliver = 93.86, and Warm Springs Hatchery = 86.57. For specimens between the four locations, Lake Harding (n=40), Lake Oliver (n=69), West Point Lake (n=40), and Warm Springs Hatchery (n=25), there was no significant difference in the relative weight among the sample populations between study sites (Figure 12).

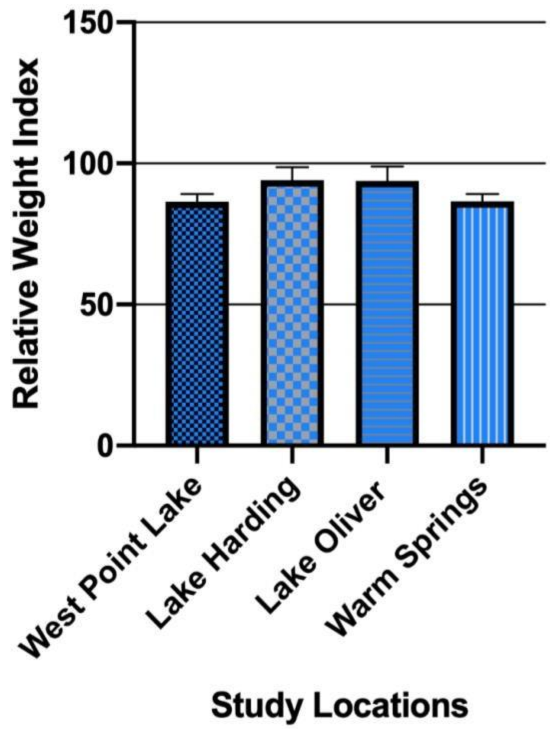

Figure 12. Relative weight index means from each sampling site. Relative weights showed no significant differences among study sites.

Degree of Tissue Change & Relative Weight Results

The mean relative weight for the high DTC group (RWt = 91.80) and the low DTC group (RWt = 90.24) for all specimens showed no significant differences, $p=0.5368$ (Figure 13).

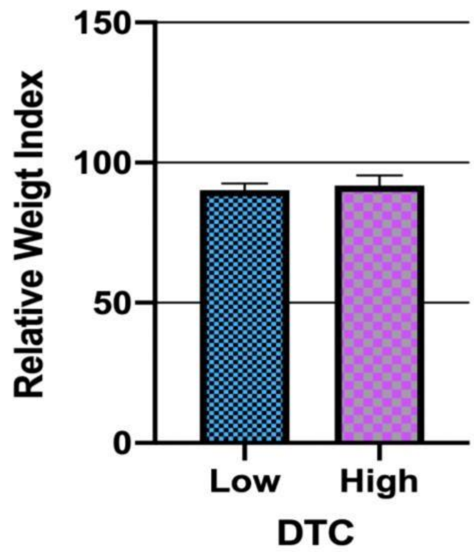

Figure 13. Mean relative weight index between fish separated into low and high DTC groups for all sites combined. There was no significant difference, p = 0.5368.

Degree of Tissue Change and Condition Factor

Condition Factor Results

Mean condition factor for each location was as follows: West Point Lake =1.29, Lake Harding =1.27, Lake Oliver =1.35, and Warm Springs =1.09. The mean condition factors were significantly higher between all three lakes compared to Warm Springs Hatchery. The significant differences between sites were as follows: West Point Lake vs Warm Springs, p=0.0014, Lake Harding vs Warm Springs, p=0.0072, Lake Oliver vs Warm Springs, p<0.0001. (Figure 14).

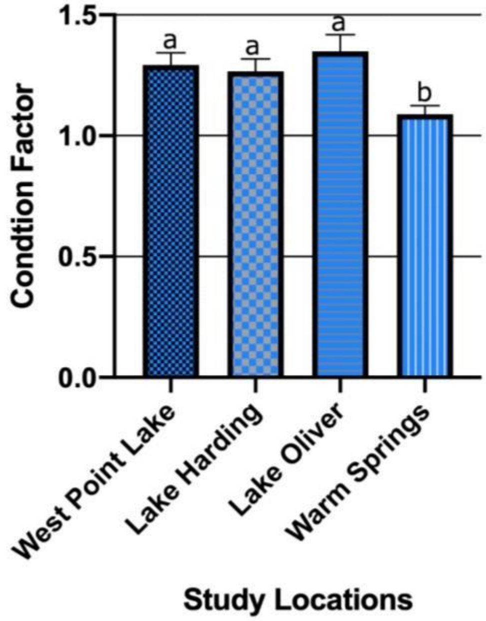

Figure 14. Mean condition factor among all study sites. The condition factor of all three lakes was significantly higher than Warm Springs, p=0.0014, p=0.0072, p<0.0001, respectively.

Degree of Tissue Change & Condition Factor Results

The mean condition factor for the low DTC group was 1.23 and the high DTC group was 1.31. These were significantly different values, with p=0.0362 (Figure 15).

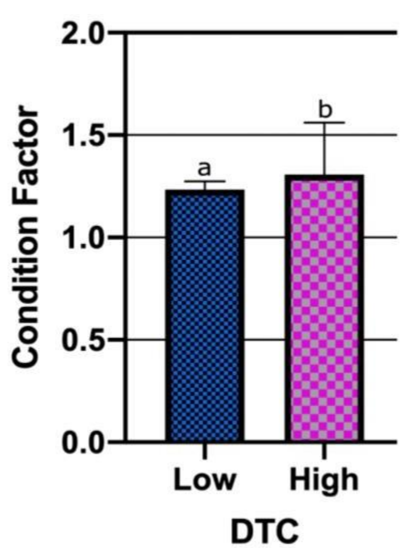

Figure 15. Mean condition factor between all fish separated into low and high DTC groups. Condition factor for the low DTC group was significantly lower than for the high DTC group, p=0.0362.

Males and Females

Males & Females – All Study Sites

There was no significant difference between DTC scores for all males (n=111) and females (n=63) from the study, p=0.0557, (Figure 16).

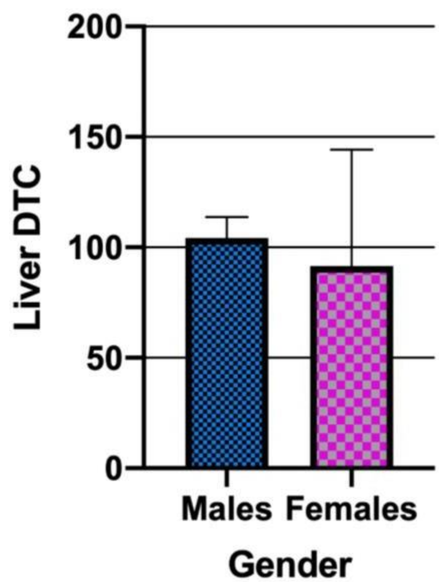

Figure 16. Mean DTC scores between all males and females. There was no significant difference in the DTC between males and females, p=0.0557.

Intersex

Intersex Results

Intersex was examined statistically to see how it related to DTC scores. The results are as follows. These tests looked only at males from the study for all four locations (n=111). Males that did not have intersex, the mean DTC = 85.52, versus those that did have intersex the average DTC = 116.62.DTC scores were significantly higher in males that did have intersex versus those that did not, p=0.0026, (Figure 17).

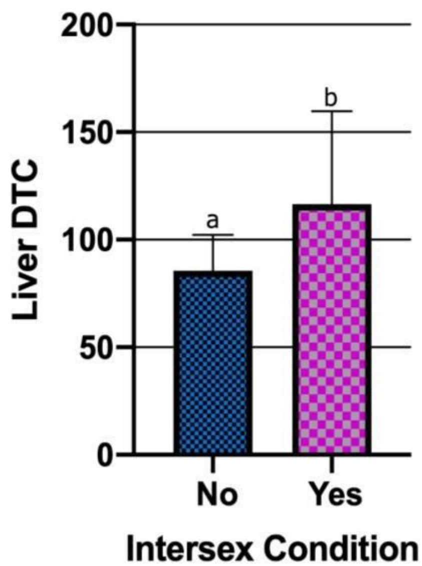

Figure 17. Comparison of the DTC of all male fish with and without intersex. Males with intersex showed a significantly higher DTC, p=0.0026.

Water Chemical Analysis

The mean chemical quantity in parts per billion (ppb) was compared between each of the four study sites. It is important to note that ppb is the equivalent measurement to micrograms per liter, or (µg/L), so these units are used interchangeably. The only mean that showed significant differences was for benzyl butyl phthalate detected at West Point Lake and Warm Springs Hatchery, p=0.036. A complete list of chemicals that we tested for can be seen in Table 2. This does not mean there weren't other chemicals present, but these were the ones that were compared to known EPA standards in our study.

*Table 2. Chemical analysis from water collections. *Benzyl butyl phthalate was only significantly higher at Warm Springs Hatchery compared to West Point Lake, p=0.036. There were no other significant differences in the chemical levels among sites.*

Compounds (µg/L)	West Point Lake	Lake Harding	Lake Oliver	Warm Springs Hatchery
Benzyl butyl phthalate	4.18*	10.28	10.11	12.2*
Dimethylphthalate	19.84	17.31	0.00	32.02
Bis(2-ethylhexyl)adipate	0.45	1.36	6.71	33.17
Testosterone	19.49	13.99	29.69	20.01
Dibutylphthalate	521.05	1218.64	712.14	493.99
Bis(2-ethylhexyl)phthalate	1206.22	33.75	136.59	83.98
Estrone	2416.20	1322.94	4443.96	5062.92
Isophorone	3.48	3.92	5.85	18.49
4-androstene-3,17-dione	0.00	1.90	0.04	8.00
Diethylphthalate	83.15	466.29	202.37	223.12
Equilin	0.00	108.83	0.00	298.21
SSS-tributylphosphorotrithioate(DEF)	0	0	0	0
Phosphamidon	0	0	0	0
Ethion	0	0	0	0
Ethyl_parathion	0	0	0	0
Chlorfenvinphos	0	0	0	0
MethylParathion	0	0	0	0
Phorate	0	0	0	0
Ethoprophos	0	0	0	0
C20H12	0	0	0	0
Profenofos	0	0	0	0
Chlorpyrifos	0	0	0	0
Tetrachlorvinphos	0	0	0	0
Mevinphos2	0	0	0	0
Mevinphos1	0	0	0	0

Phthalates (Figure 18), estrogens (Figure 19), and androgens (Figure 20) were grouped together and displayed on the figures below, even though there was only one significant difference among all chemicals, the illustration helps to understand the relative quantities at each study site. These are not statistical figures.

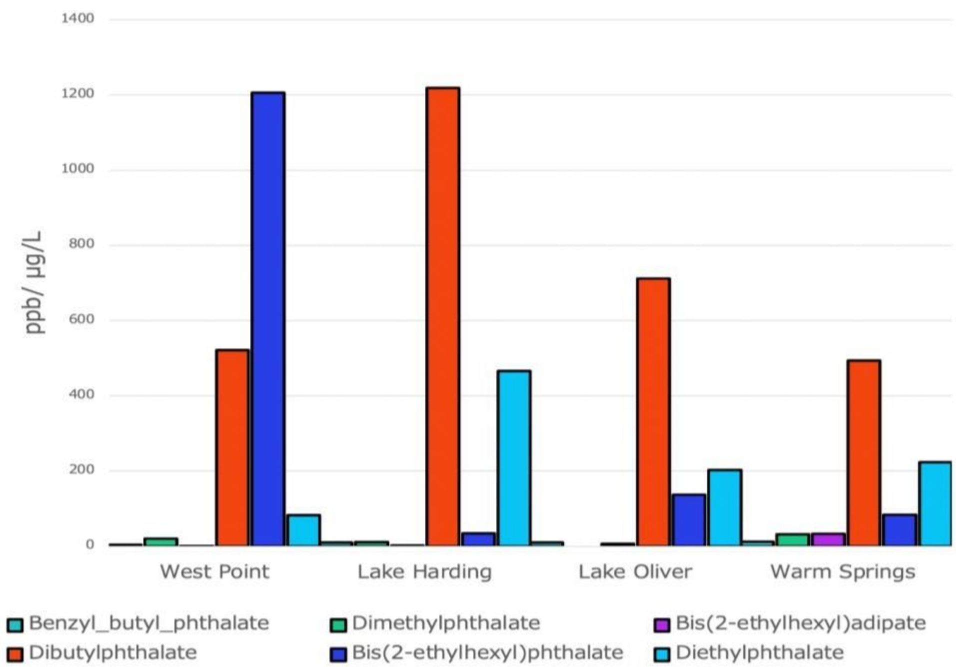

Figure 18. Phthalate levels detected at the study locations.

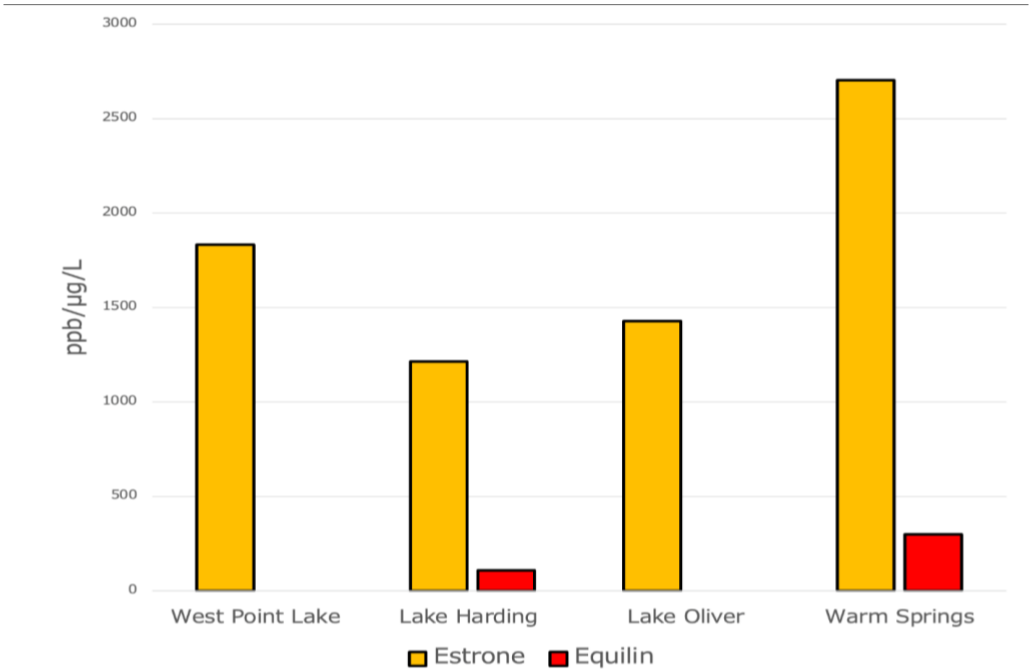

Figure 19. Estrogen levels detected at the study locations.

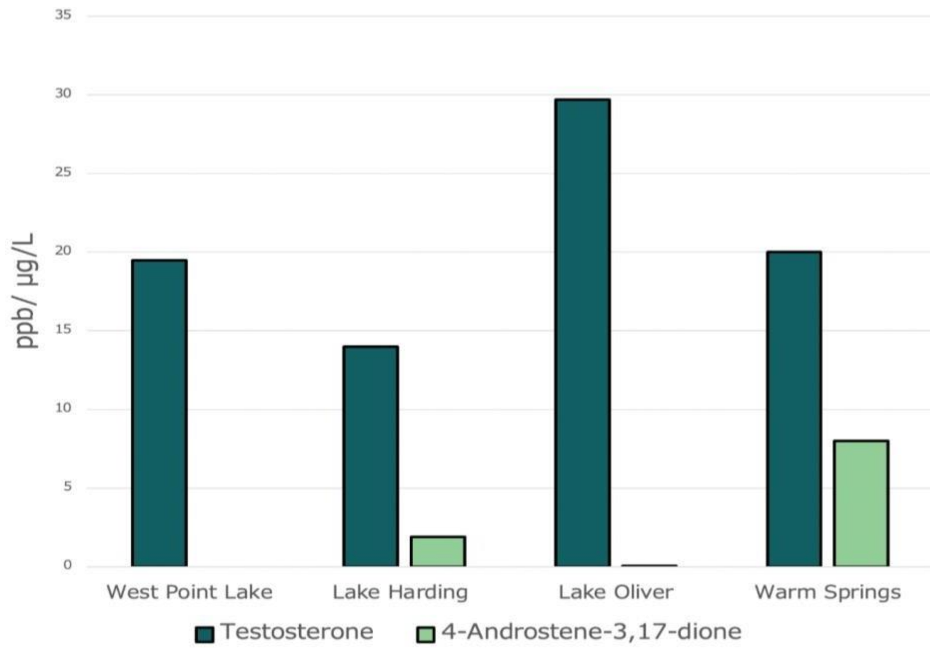

Figure 20. Testosterone levels detected at the study locations.

GIS Land Use Analysis

Statistical tests were not performed for land use results because they were singular values, but the analysis is still important to the study. Figure 21 is a map of our sampling region with a color-coded key for each land use category. The total land use percentages can be seen in Table 3. A table of the raw GIS land use data, prior to condensing the more detailed land use types generated by GIS software down into our five mainland-use categories can be found in Supplemental Materials (Table 4).

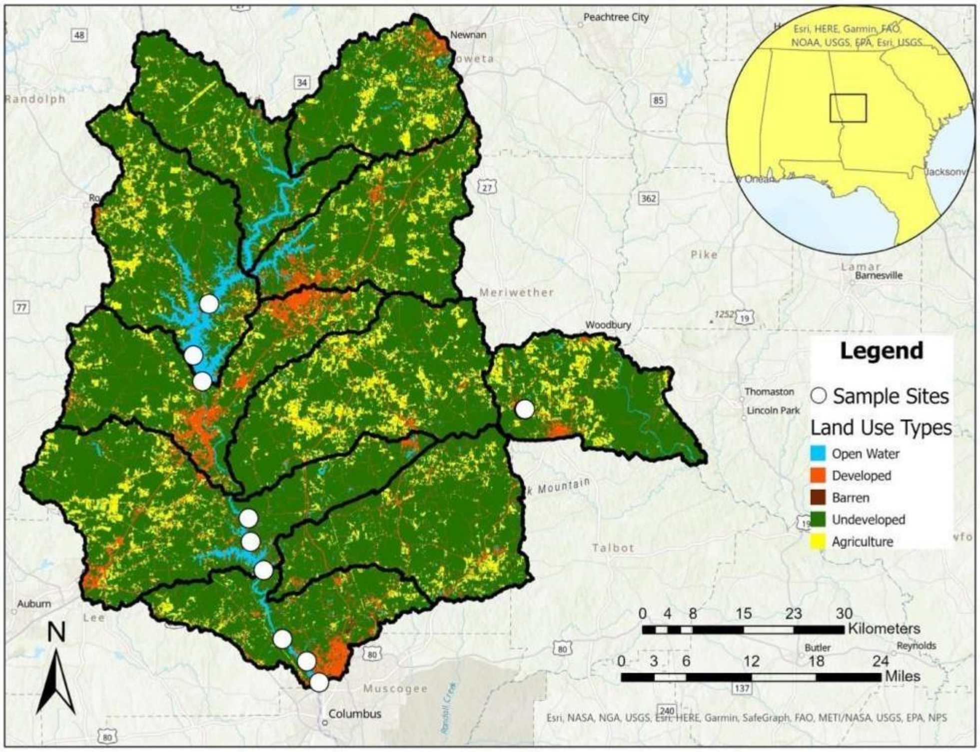

Figure 21 - GIS land use analysis map. The land-use categories used were open water; developed land; barren land; undeveloped land; agriculture.

Table 3 - GIS land use analysis table. The land-use categories used were open water, developed land, undeveloped land, agriculture, and barren land.

Land Use Type	West Point Lake	Lake Harding	Lake Oliver	Warm Springs
Open Water	5.23%	1.84%	2.35%	1.63%
Developed	6.48%	8.83%	9.34%	6.02%
Undeveloped	76.59%	76.32%	79.89%	76.89%
Agriculture	11.45%	12.77%	7.99%	15.38%
Barren Land	0.24%	0.24%	0.43%	0.09%
Total Subwatershed Area	1989.32 sq km	2074.9 sq km	1005.2 sq km	412.01 sq km

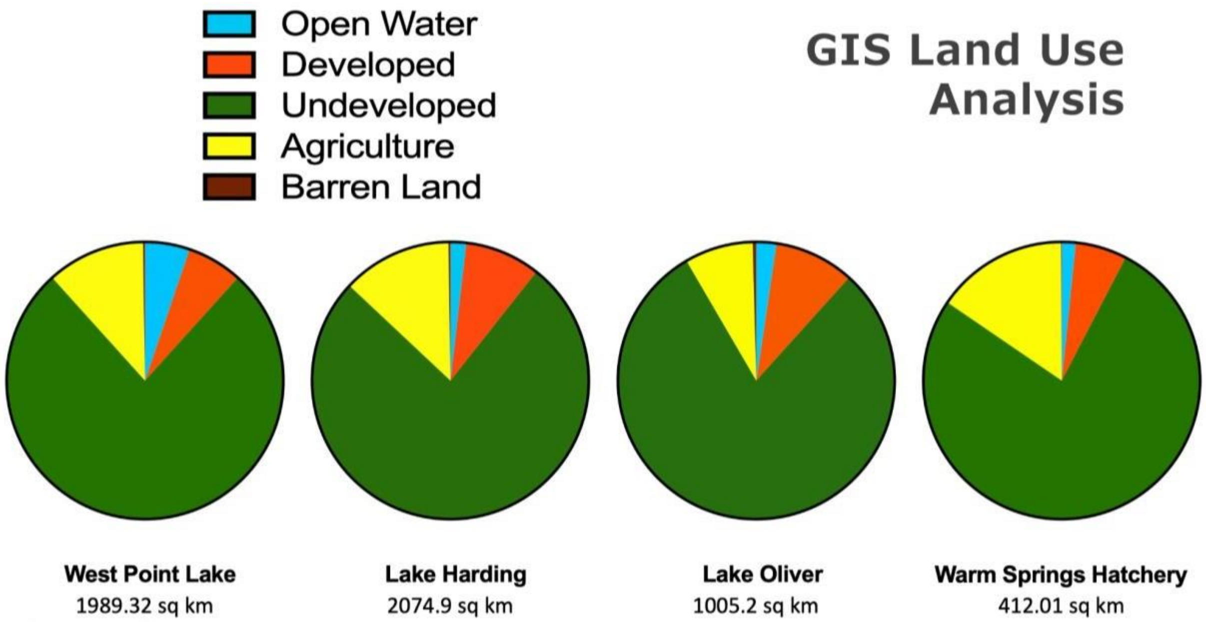

Figure 22 - GIS land use percentages for each study site. Land use was divided into five subcategories: open water, developed, undeveloped, agriculture, and barren land.

Discussion

Degree of Tissue Change

DTC was elevated at all sites located within the Chattahoochee River watershed compared to the Warm Springs Hatchery. DTC is an effective bioindicator of fish health and the overall health of aquatic environments due to its ability to illuminate structural damage to the tissue and therefore functional impairment related to toxic exposure (Camargo & Martinez 2007; Poleksic & Mitrovic 1994). Fibrosis, cellular atrophy, necrosis, reticulin meshwork collapse, and vacuolated nuclei are all examples of tissue abnormalities that are a result of prolonged, chronic exposure to toxins and endocrine disruptors. All these issues and more were detected in our study.

When the mean DTC was compared across study sites, the DTC was statistically higher at the lakes than at Warm Springs Hatchery, indicating that liver health is being affected by different toxic and polluting conditions in the aquatic environments of these fish populations of the lakes. The variation of the DTCs detected between the study sites suggests that this can be a useful bioindicator tool when it comes to assessing the health of aquatic environments, like the study done by Camargo and Martinez, who also found this to be true using liver histopathology to assess fish health after exposure to high levels of contamination in the Cambé Stream (2007). They also discovered that there were few to no changes in the severity of lesions in the liver due simply to seasonal variation, while some changes were observed in glycogen storage and the size of the nucleus (Camargo & Martinez, 2007). This is particularly relevant to our study since fish were collected at different times of the year.

The Chattahoochee River flows through three of our study sites, so it is not surprising that the DTC was consistently high and showed no significant difference among the mean DTC values of the lakes. The dilution factor of West Point Lake could have potentially helped to mitigate the overall effects of the chemical pollution, leading to better liver health, but this is not what was observed, as it was the second highest DTC with a mean value of 112.75. The largest subwatershed area in our study was Lake Harding, which was consistently high in chemical concentrations, despite having the lowest open water land use type among our three lakes. This demonstrates that many of these contaminants are coming from sources other than just the flow of the river, but from seepage from surrounding land through feeder streams and groundwater recharge. Lake Harding had the highest mean DTC of all our study sites, equaling 115.48.

The overall volume or flow of these bodies of water, the number of non-polluted feeder streams contributing to better water quality, or the amount of pollution being fed into the lake from upriver urban and agricultural areas which filter through the groundwater and eventually feed into the bodies of water, could all be having dramatic impacts on local water quality.

DTC scores above 100 indicate a high degree of hepatic tissue damage, which was the case for West Point Lake, Lake Harding, and Lake Oliver populations. The DTC for each site was significantly different from our semi-control, Warm Springs Hatchery, which was within a healthy range and showed much less cellular damage. This supports our findings of water chemicals detected in the reservoirs of the Chattahoochee River versus the spring-fed hatchery. One possible explanation that could explain the lower DTC score at Warm Springs Hatchery could be fewer contaminants leaching into the streams and groundwater that feed the hatchery in the absence of heavy urban runoff. The underground spring fed water source carries with it less contaminant load and therefore introduces fewer toxins to fish that induce the types of cellular changes that were observed in our lake populations. The number of phthalates were lowest at the hatchery, which I suspect is why the liver health there was better. While they were highest in estrogens and relatively high in androgens, the liver health there was not near as severe as that from the watershed where we saw the highest level of phthalate contamination.

Cellular changes such as cytoplasmic and nuclear degeneration, nuclear vacuolation, and focal necrosis were observed in fish with high DTCs, showing that these livers have been functionally impacted by their environment. Studies done by Chang et al. found similar lesions and hepatic tissue abnormalities in fish exposed to high levels of pesticides from urban runoff on the northeastern US coast (1998). Melanomacrophage aggregates, one of the DTC scoring parameters, were found in many fish also containing parasites in our study and have been shown to be related to severe degenerative and necrotic lesions within hepatic tissues and may be involved in detoxification processes (Haaparanta et al., 1996, Pacheco & Santos, 2002).

Proper liver function is essential to a healthy organism. Poor liver health can lead to low body weight from poorly absorbed nutrients, poor fat digestion, vitamin absorption, and misregulated hormones. Damaged liver tissue cannot properly filter toxins, so fish health will suffer. By the time we see stage 2 or 3 cellular changes in the tissue, there are already functional impairments of the liver, which means that endocrine system regulation would suffer, leading to hormonal imbalances and poor health conditions such as intersex. Direct exposure to endocrine disruptors in the environment not only damage liver tissues and interrupt normal detox and regulatory functions but can act directly within the body to mimic hormones. The functionality of fish livers and human livers is not that different. What is happening to them could be happening to us on a smaller scale. We don't live in this water, but we do drink it, bathe in it, and swim in it.

Degree of Tissue Change & Health Parameters

Fish health was also determined using HSI, relative weight, condition factor, and intersex and results showed that these parameters also revealed information about overall health of LMB. DTC is a good indicator for overall fish health because it has meaningful correlations to other health parameters for Largemouth bass such as HSI, condition factor, and intersex. Our findings show that HSI values were lowest where DTC scores were the highest, indicating liver damage due to low liver weight. Liver damage affects the weight of the liver because cell death, cytoplasmic degeneration, and shrinkage of healthy tissues all contribute to less liver mass. If there is a significant amount of tissue damage and cell death occurring within the liver, this is also one reason there are functional impairments to damaged livers.

Adverse effects on liver weight and accompanying histopathological changes in the liver, were also observed by Parikh and Sadekarpawar (2013). HSI is a relatively simple and cost-effective parameter to test the health of fish, making it a helpful addition to future studies, and has little seasonal variation due to the constant requirements of the liver for detoxification (Rizzo

& Bazzoli, 2020). Liver damage often leads to an increase in the weight of the liver, so a high HSI indicates an enlarged liver, which is often associated with liver damage in the form of excess iron storage, fat storage, and an increase in nucleic activity due to an increased demand for genomic transcription that precedes tissue repair (Arukwe and Goksoyr, 2003; Nikolsky 1963). Our findings however did not support this prior research and it was found that the HSI was the highest in fish with the lowest mean DTC levels, indicating that fish most impacted by liver damage had significantly smaller liver weights. This could be due to loss of healthy liver tissue in those fish, leading to less overall liver mass.

By the time we are seeing stage two or three changes in the tissue, the liver is functionally impaired and can no longer regulate hormones as it should and we see a decrease in fish health, such as the low HSI values, low condition factor, and increased intersex. Intersex may also be occurring from direct exposure to endocrine disruptors and hormones in the water where fish live. Relative weight did not appear to be as useful a metric for analysis in this study, as it was not related to the DTC in any of the statistical tests that were performed.

While our findings did not see meaningful correlations between DTC and relative weight index, we did find significance when looking at condition factor. Factors such as diet, fat storage, the time of year that the fish were collected, spawning season, can all fish weight (Dodds et al., 2013). When comparing condition factor values, it is important to sample the individuals or populations at the same time of the year so that they are experiencing the same pressures on food resources and reproductive cycles (Baxter & Barnham, 1998). Fish from Warm Springs did have the lowest condition factor, while also having the lowest DTC. The mean condition factor for Warm Springs was 1.09, but they were all fed the same controlled diet and were all relatively young at two years old. They could have been leaner due to their developmental stage and diet, leading to a lower condition factor. When statically analyzing these parameters, there were more occasions of significant differences where the condition factor was concerned than relative weight.

Degree of Tissue Change - Males & Females

No significant differences between males and females were observed; therefore, more studies are needed to determine if gender impacts liver health due to differing energy exchanges within habitats and males having higher metabolic rates. The significance value was very close to showing significance, with $p=0.0557$, but due to the unequal number of males and females in our study, having almost twice as many males as females, future studies could try to use an equal number of males and females to get a clearer picture of this relationship.

Degree of Tissue Change – Intersex

Results from these tests showed that for males in our study, DTC scores were significantly higher for those who did have intersex versus those that did not have intersex. This correlation between liver health and intersex suggests that multiple physiological systems are affected by environmental toxicity, and cascading effects could be occurring within the organism, leading to a reduction in the reproductive success of fish populations. Whether the liver damage, with its important role in hormone regulation, could be a causative factor leading to intersex is a question for future studies, but at the very least these findings show that other organs in addition to the liver are being affected from long-term exposure to endocrine disruptors in the aquatic environment and that a correlation exists between the liver health and intersex in the Chattahoochee River watershed.

Water Chemical Analysis

It has been suggested that watersheds have their own unique “urban pesticide signatures”, where even though the same chemicals may be detected in two separate watersheds, the resulting aquatic toxicity could be very different due to different concentrations, geology, rainfall, and flood patterns, etc. (Nowell et al., 2021). This makes it very difficult to predict effects based on chemicals detected in the watershed and makes closer examination of the health of aquatic life important to determine the true impact. This preliminary study focused primarily on notable EDCs which are known to cause cellular disruptions and numerous health issues and, in several cases, exceeded the reporting limits issued by the EPA for hormones. Phthalates are known to damage the liver and kidneys, cause developmental delays and reproductive issues, and are biologically stable and accumulate in the environment. Direct runoff, groundwater seepage, non-point source pollution, and land use all contribute to the chemical load carried in the watershed.

Minimum reporting levels from the EPA for equilin and testosterone were exceeded in our study at all four sites, while estrone was exceeded at Lake Harding and Warm Springs Hatchery, which are both the lowest in open water and the highest in agricultural land use according to GIS land use analysis. Equilin and estrone are both known to be present in high quantities in the urine of livestock and horses (DrugBank, 2022). While the role of estrogens have been shown to play a role in homeostasis of the liver and prevent chronic liver disease, in large quantities, it has been shown to cause liver disease (Ezhilarasan, 2020) so further studies are needed to determine whether these compounds are contributing to liver damage, intersex condition of the fish, or perhaps both due to compounding effects of multiple steroids present in large quantities over long periods of time.

Diethyl phthalate has the highest human exposure of all the phthalates and targets the organ of the liver particularly. A study using laboratory animals that looked at a wide variety of exposure times and intensity found that low liver weight was one of the most quantitative measures of hepatic damage after low dose, long exposure to diethyl phthalate (Weaver et al., 2019). Histopathological changes were also observed under the same conditions, indicating hepatic damage. Dibutyl phthalate, another common plasticizer detected in large quantities in all four locations, has been shown to induce fatty liver disease and abnormal liver function in humans by disrupting signaling pathways in hepatic tissues (Zhang et al., 2021). Benzyl butyl phthalate, detected in all four locations, is known to trigger cellular changes in hepatic tissues leading to cancer and metastasis through disruptions to cellular function (Tsai et al., 2014).

The way these EDCs act individually is not necessarily indicative of how they will work when interacting with other compounds. At the concentrations detected on their own, they may not cause the same measurable effects that they do when combined and over long periods of time (Ojoghoro, 2021). A growing number of biologically active pharmaceuticals are ending up in the environment as well, and we do not know how these will interact with non-target species when they filter into the watershed (Oaks et al., 2004).

Steroids are of particular concern in the aquatic environment, as they have been shown to affect the reproductive organs of fish at even low concentrations in aquatic environments and are therefore of the highest concern (Caldwell et al., 2012; Gunnarsson et al., 2019) Estrone is naturally occurring, but can break down into other forms of estradiol, creating compounding effects that act as endocrine disruptors in fish (Tapper et al., 2020).

Equilin occurs naturally as a horse mare estrogen but is also synthetically made for hormone replacement therapies (NCBI, 2022). Equilin has been shown to have disproportionate effects on the liver compared to human derived estrogens of estradiol and estrone, behaving more similarly to synthetic estrogens. This increased impact on the liver has been shown to affect protein synthesis in the liver (Kuhl, 2005).

GIS Land Use Analysis

Future studies could explore this relationship further, between mean DTC scores of Largemouth bass and land use, but primary analysis can still illuminate differences between each of the watersheds. Because the effects on the liver seem consistent throughout the Chattahoochee River watershed, it would be challenging to pinpoint exactly where some of the worst land use impacts might be coming from based simply on this analysis. The most impacted site of the study was Lake Harding in terms of liver health.

Even though there was not enough data to run statistical analysis on the GIS land use data, GIS analysis showed that Warm Springs Hatchery has the most undeveloped and agricultural land, while having the least open water, developed, and barren land (Table 3). This provides a degree of validation for using it as a semi-control in our study. Not only is it located in the Flint watershed instead of the Chattahoochee, but it is also spring fed and is the least urbanized area of our four study sites.

The increased agricultural land use surrounding the hatchery among our study may help explain why the levels of estrone and equilin detected were so high, especially as evidence suggests that it is common for animal wastes and byproducts to enter surrounding streams (Burkholder et al., 2007). These compounds are found in the urine from livestock and horses, which could be permeating through the rocky substrate of Pine Mountain into the groundwater, finding its way into the stream-fed hatchery. Heavy rainfall causing localized flooding can also lead to dissolved solids from areas immediately adjacent to streams and rivers ending up in aquatic habitats (Cleaves et al., 1970).

Future Considerations

It was outside the scope of this preliminary study to explore the specific impacts of each, and every chemical found in our water analysis, but these initial findings warrant further investigation. The presence of hormones from both natural and synthetic sources in the environment and ending up in drinking water is a large area of concern. More in depth studies are needed to include seasonal changes of chemicals, rainfall effects on concentration of chemicals, and trying to narrow down how they are ending up in the watershed, either through urban runoff, nonpoint source pollution, industrial, municipal, or medical waste. It is also important to determine the impact these chemicals are having on other wildlife in the Chattahoochee watershed as well as potential effects on humans who rely on this for drinking water.

Future studies could also consider if the DTC trend continues southward with the Largemouth bass of Lake Eufaula, which catches all the water flowing from the city of Columbus. The trend seems to indicate that the pollutants having the most severe impacts are being diluted as the Chattahoochee continues to flow south from Atlanta, but we do not know from this study what the potential impacts there are on Largemouth bass populations from Columbus and surrounding areas. While it was outside the scope of this study, it would be very interesting to study the soil quality in these aquatic environments, as sediment holds many of the heavy metals and pollutants that can settle out in slower flowing parts of the river and have been shown to lead to more severe liver alterations on the cellular level (Paris-Palacios et al., 2000). This would be of particular importance to Largemouth bass because populations are relatively stasis in their environments and live primarily in the lower strata of aquatic environments where they would likely be exposed to any such contamination.

To prevent introducing possible bias into the study when scoring fish livers, it would have been useful to have a numbering protocol so that it was a blind scoring system and the study site was not known during scoring. While we did try to minimize the possible variances between different histologists' scoring techniques of the fish by showing no significant differences between a random sample of 25% of all fish in the study, this doesn't really address the issue of possible bias if it was known where the fish were coming from.

Conclusions

Aquatic toxins are having an adverse effect on fish health. Bioindicator species such as the Largemouth bass, as well as bioindicator organs like the liver, give us a glimpse into what is happening on a systemic scale. It is important to take this preliminary study and narrow the scale for future research so these issues can be addressed and mitigated in the Chattahoochee River watershed.

The value of healthy streams and green spaces is both intrinsic and ecological. It is enriching to us all to have healthy natural places to recreate, to farm, to fish, to fuel our own healthy living. The impact of human activities on these spaces seems evermore detrimental and must be curbed to prevent what would be the inevitable cascading effect of environmental degradation. Using indicator species such as the Largemouth bass can serve as a timestamp of environmental conditions as we work as a community of Chattahoochee locals to improve conditions.

Using histopathology on an important organ such as the liver gives us insight into the possible ramifications of these toxins on human health as well, as we also swim, eat, and drink this very same water. Discovering what chemicals are present in our watershed and the impact they have on aquatic ecosystems and potentially human health as well are burdens that must be faced by local, state, and national level organizations supporting water quality and conservation. The widespread use of chemicals and the impacts they have is thought to be one of the newer planetary boundaries (Steffen et al., 2015) within which humanity can only hope to operate safely for some time to come.

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Supplemental Materials

Largemouth Bass	**Column1**
Length (inches)	Standard Weight (lb.)
10	0.5
10.5	0.6
11	0.7
11.5	0.8
12	0.9
12.5	1
13	1.1
13.5	1.3
14	1.5
14.5	1.6
15	1.8
15.5	2

16	2.2
16.5	2.5
17	2.7
17.5	3
18	3.2
18.5	3.5
19	3.9
19.5	4.2
20	4.5
20.5	4.9
21	5.3
21.5	5.7
22	6.2
22.5	6.6
23	7.1
23.5	7.6
24	8.1
24.5	8.7
25	9.3
25.5	9.9

Table 4. GIS Land Use Raw Data

GIS - Land Use Type	West Point Lake	Lake Harding	Lake Oliver	Warm Springs
Open Water	5.2311125	1.835084667	2.347154	1.62546
Developed, Open Space	4.5360195	5.369006	5.7830965	4.164873
Developed, Low Intensity	1.55451925	2.468009667	2.7087235	1.45878
Developed, Med Intensity	0.2621565	0.699940333	0.600547	0.286645
Developed, High Intensity	0.12739725	0.294941667	0.2473	0.110114
Barren Land	0.24131	0.237662333	0.434525	0.086736
Deciduous Forest	27.273239	28.28279667	36.5649375	36.833447
Evergreen Forest	30.3546595	28.31251833	26.253773	23.581358
Mixed Forest	0.67499	0.951763	0.548118	0.77582
Shrub/Scrub	7.508028	8.165051	6.8716015	7.291314
Herbaceous	7.0761465	6.298965667	6.8514405	5.426374
Hay/Pasture	11.436432	12.72936133	7.954386	15.366532
Cultivated Crops	0.01648375	0.041060333	0.0341005	0.009832
Woody Wetlands	3.5777615	4.069665	2.6614645	2.85924
Emergent Herbaceous	0.12933475	0.244173333	0.138831	0.120382
	99.99959	99.99999933	99.9999985	99.996907