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Effects of Septic Seepage in Minnesota Lakes on Resident Fish Species

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Effects of Septic Seepage in Minnesota Lakes on Resident Fish Species

by

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Abstract

The potential of On-site Wastewater Treatment Systems (OWTSs) being a non-point source of contaminants into lake systems is a growing concern. Since many lakes are down gradient of OWTSs, the septic seepage easily contacts surrounding groundwater and enters the shallow waters though the hydrological process. It is also in these shallow areas that many native fish species spawn. Five study lakes were established that included two septic-influenced sites and two reference sites each. Water sampling throughout the early spring and summer established the presence and absence of Contaminants of Emerging Concerns (CECs) at each respective site. Adult male sunfish were collected off their spawning beds between May and July to explore the effects of these contaminants on the native fish species. The fish were euthanized and sampled for blood and internal organs. To explore the effects of these contaminants on the larval fathead minnow, a 21-day static renewal exposure was completed using groundwater collected from the same septic influenced and reference sites in each study lake. Following the 21-day exposures, larvae underwent behavioral testing that included the analysis of predator avoidance as well as feeding performance. Two CEC mixtures were also created from the water chemistry results to replicate seepage from different OWTSs. Adult sunfish and fathead minnows were exposed to these mixtures at a range of concentrations for a 21-day period. Larval fathead minnows were also exposed to these mixtures at the same concentrations. Laboratory exposures assessed the same endpoints as the resident male sunfish and larval groundwater exposures to observe if the same pathologies and behaviors would occur. The assessment of biological endpoints in resident sunfish and laboratory exposed sunfish and fathead minnows provides a rich data matrix to test the hypothesis that septic seepage causes adverse health effects in resident fish populations in northern lakes.

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For my parents,

Thank you for the continuous support and the push to always go the extra mile.

For my advisor and mentor Dr. Heiko Schoenfuss,

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CHAPTER 1: LITERATURE REVIEW

With Contaminants of Emerging Concerns (CECs) becoming more widespread in freshwater systems, the concern over their biological impacts is growing in the aquatic toxicology community (Ferrey et al., 2012; Kolpin et al., 2012). Many studies of CECs have focused on the biological effects of CECs in streams and rivers, but little is known about the sources and impacts of these chemicals in lake systems (Baker et al. 2014). **The objective of the current study was to examine the biological effect of one potential source of CECs, On-site Wastewater Treatment Systems (OWTS), on two common fish species, the fathead minnow (*Pimephales promelas)* and the bluegill sunfish (*Lepomis macrochirus),* at two life stages.**

There are many non-point sources that may provide entry of CECs into lakes including agricultural and road run off. In addition, the increased construction of lake shore homes relying on OWTSs may be an important source of CECs to lakes (Baker et al., 2014). The hydrological cycle of groundwater inflow into lakes may interact with CECs when groundwater passes through the drain-fields of OWTSs. CECs are not easily broken down by OWTS and can easily leach into groundwater and into the nearby lakes (Godfrey et al., 2007; Verstraten et al., 2005). OWTS stand out among sources for CECs addition into lakes for multiple reasons: 1) they are commonly used in residences surrounding lakes; 2) they are not designed to remove CECs from household waste water; and 3) previous studies identified distinct chemical signatures that are associated with human household consumption (Baker et al., 2014, Phillips et al., 2015).

The inflow of groundwater frequently occurs in the shallow, near shore areas of the lake (littoral zone) (Dobson, 2005). The littoral zones in lakes provide important spawning and

habitat grounds for many fish species including fathead minnows and bluegill sunfish (Becker, 1983). These species can spawn for several months during the spring and summer (Becker, 1983) and, therefore, may be exposed to CECs during that period. The exposure to CECs can feminize males (Sumpter and Jobling, 1995; Thorpe et al., 2007; Dammann et al., 2011; Elliot et al., 2014), reduce fecundity (Dammann et al., 2011), and diminish larval predator escape response (McGee et al., 2009).

Past studies have examined the presence and biological effects of CECs in surface water (Writer et al., 2010) and groundwater (Baker et al., 2014) of lake systems, but there is still a lack of understanding of the actual source of these CECs into lakes. The current study is focused to detect a range of CECs that include compounds used in household items like detergents, herbicides, insecticides, and pharmaceuticals in groundwater down-gradient of OWTS and connect biological pathologies observed in fish to this source of exposure. By combining CEC characterization with assessments of biological responses in exposed fish, we will improve the interpretive power for identifying sources, exposure scenarios, and fates of CECs.

1.1: Biology of the Fathead Minnow

The fathead minnow, *Pimephales promelas*, is a teleost fish belonging to the family Cyprinidae (Helfman et al., 2009). Body length ranges from 41 to 71 mm (Pflieger, 1975) with males being larger than females (Becker, 1983). Both sexes are deep-bodied, with a single, soft-rayed dorsal fin, slightly forked caudal fin, and a blunt head (Nelson and Paetz, 1992). The dorsal color ranges from a pale brown or yellowish-olive color with the lateral aspects of the body having a silvery tone with a dusky stripe (Pflieger, 1975). A dark spot can also be seen at the base of the caudal fin, but none of the fins have definite markings (Pflieger, 1975). During the breeding season, strong sexual dimorphism is present with the male developing broad, black vertical bands, a thick dorsal pad in front of the dorsal fin, and three rows of tubercles on the snout (Smith, 1979). The fathead minnow differs from other species in the same genus by having a considerably darker color, a pre-dorsal strip, and having a substantially deeper body (Smith, 1979). The species is distributed widely throughout Central North America and has been introduced beyond its native range as a result of its popularity as a baitfish among anglers (Pflieger, 1975). Although it is a very wide-spread species, it has been observed that the fathead minnow is very intolerant of competition and is seldom seen in habitats that support numerous species of fish (Pflieger, 1975). Fathead minnows inhabit a variety of habitats and can be found in small ponds, streams, lakes, and sluggish creeks (Pflieger, 1975; Becker, 1983; Smith, 1979). These habitats include a variety of substrates including sand, silt, rubble, and silt (Pflieger, 1975; Becker, 1983; Smith, 1979). Fathead minnows are omnivorous feeders and can tolerate high temperatures, extreme turbidity, and periods of low oxygen (Pflieger, 1975).

As a fractional spawner, fathead minnows reproduce continuously starting in mid-May through most of the summer months (Smith, 1979, Pflieger, 1975). Spawning begins when temperatures reach approximately 15.6°C (60˚F) and continues into the fall until temperatures drop below that threshold (Duda, 1989; Danylchuk and Tonn, 2001). Although it has a wide range of spawning temperatures, optimal temperatures have been observed to be 15.6˚C to 18.4˚ C (60-65.1˚F) (Becker, 1983). Male fathead minnows exhibit distinct, territorial behavior during the breeding season and the species is often recognized as being one of the most nest defensive and egg attentive species (Smith, 1979). The male fathead minnow will find a small submerged overhanging rock or fallen debris and use its tubercles to clean the overhead surface before attempting to attract females (Becker, 1983). A female can lay upwards of 80 to 370 eggs at a time and will spawn several times (Thomsen and Hasler 1944). The male then guards the eggs aggressively using its tubercles to drive away intruders and the spongy dorsal pad to clean and keep eggs free of sediments (Markus 1934). To minimize the spread of infections, the male will occasionally nibble at the egg mass to remove any fungus-infected eggs to protect the rest of the batch (McMillian 1972).

The eggs will hatch in 4 to 6 days in water temperatures at 25˚C (Hasler et al., 1946). Once hatched, the larvae will remain near the nest until their entire yolk-sac is absorbed and continue to stay in the shallows as growth continues at a rapid rate (Becker, 1983). The larvae are an average size of 4.75 mm at hatching (Markus, 1934). After the first 20 days, larvae are an average of 20 mm in length and by 60 days are an average of 29 mm in length (Becker, 1983). By the end of the first summer, larvae from the first hatching of the summer can be fully mature and sexually active if environmental conditions are permissive (Becker, 1983).

Because of its wide geographical range, rapid sexual development, ecological relevance, and sensitivity to environmental pollutants fathead minnow is a popular model for toxicity testing (Denny 1987; Geiger et al., 1988; Jensen et al., 2001). Effects of CECs have been widely studied on the fathead minnow and endpoints include histopathology of the liver and gonadal tissues (Elliot et al., 2014; Barber et al., 2011, Writer et al., 2010), survival (Ankley et al., 2001; Bistodeau et al., 2006), and the predator escape response in larval fathead minnows (McGee et al, 2009; Rearick et al., 2014). For this purpose, cultured populations of fathead minnow are reared in laboratory cultures (Becker, 1983).

1.2: Biology of the Bluegill Sunfish

The bluegill sunfish, *Lepomis macrochirus*, is a teleost fish belonging to the family Centrarchidae (Helfman et. al, 2009). Sunfish may grow to lengths in excess of 95 mm and a body weight of 340 grams (Pflieger, 1975). Bluegill sunfish have a compressed body spotting many different color variations (Smith, 1979). A common variation of body color consists of a dark olive green to brown on its sides with 5 to 9 vertical bars and sometimes can have purple to blue reflections (Becker, 1983). The belly and throat can include a white variation, while the breast can range in different intensities of a bright yellow to reddish orange (Pflieger, 1975). A black blotch will also be present in the posterior rays of the soft dorsal fin (Smith, 1979). Parental males (males who build nests and defend eggs and larvae) are often lighter in color with a prominent, bright yellow and orange breast (Gross and Charnov, 1980).

Due to the wide-spread practice of stocking, bluegill sunfish are now reproducing in most rivers and lakes in Midwest and Central North America (Becker, 1983). Bluegill sunfish have

one of the highest abundances among lake fish species (Poff and Threinen 1963) with habitats consisting of mostly clear, well vegetated waters (Becker, 1983). Bluegill sunfish can also be found many other aquatic habitats including swamps, streams, and ponds (Becker, 1983, Smith, 1979).

The optimal spawning temperature for bluegill sunfish ranges from 19 to 26˚C (67 to 80˚ F). Spawning will begin in mid-May and last throughout the summer months ending generally in late August (Becker, 1983). If water temperatures stay above 20˚C for prolonged periods of time, the spawning season may extend further into the fall (Becker, 1983). Similar to the fathead minnow, male Bluegill sunfish are responsible for nest building and parental care of the eggs and larvae (Gill, 1906; Gross and Charnov, 1980). When waters begin to reach optimal temperature, male bluegill sunfish will leave the deeper wintering waters and enter the shallow littoral zone to select a sand or gravel bar to build its nest (Becker, 1983). The preferred spawning water depths have been observed to be in waters about 0.3 to 0.6 meters in depth (Becker, 1983; Pflieger, 1975; Carlander, 1977). The substrate type, substrate firmness, vegetation density, and dissolved oxygen content have been reported to be the important factors for bluegill sunfish nesting colonies (Gosch et al., 2006). Bluegill sunfish are observed to desire a hard-bottomed gravel substrate with a low density of vegetation and dissolved oxygen levels being moderate at 2.7 mg/l. Areas with the lowest (1 mg/l) and highest (6 mg/l) levels of dissolved oxygen were not found to contain bluegill nests (Gosch et al., 2006). Bluegill sunfish will spawn in colonies and have bene observed to build 40 to 50 nests in a 1250m^2 section of the littoral zone (Harlan and Speaker 1956). Once the male finds its spawning site, it will excavate a round depression in the

sediment ranging from 5 to 15 cm in depth and, on average, about twice its body length in diameter (Becker, 1983; Pflieger, 1975).

Once the nest is built, the male will defend it vigorously from other male sunfish, and even females throughout spawning, embryonic development, and larvae growth (Becker, 1983; Pfieger, 1975). Net egg productivity per nest has been estimated to average of 4,800 eggs (Churchhill, 1976). The eggs are small, demersal, and very adhesive (Becker, 1983). The male keeps the eggs aerated and clean of debris with gentle fanning motions of the pectoral and pelvic fins (Becker, 1983). It has been reported that more than one female may spawn in one nest or one female may spawn in multiple nests (Pflieger, 1975). Small non-nesting males may also intrude the area and release sperm before being chased off by the defending males (Gross and McMillian, 1989). These sneaker males may become darker in color and mimic female behavior to gain access to nesting areas (Miller, 1963).

1.3: Potential Sources of Contaminates in Lakes

During the spawning season, male fish who defend their nests and provide parental care are present in these littoral areas for weeks or months (Bartlett et al., 2010) and may be exposed to CECs along with the eggs they are guarding. During these summer months while spawning is occurring, many homes around the lake are more frequently visited and lawn maintenance is increased providing greater input of CECs into OWTS and through runoff. CECs may remain in surface waters for days, weeks, or even months depending on the time it takes for certain CECs to break down (Barber et. al, 2011). This increases the possibility of longer exposure to CECs

then most other systems of water like rivers that have a much higher water turnover rate then lakes.

Studies on the effects of CECs on lake systems are becoming more relevant, especially in light of an experiment in Canada resulted in the collapse of an entire fish population after exposing a lake with a synthetic estrogen over three consecutive summers (Kidd et al. 2007; Palace et al. 2006). Previous studies have investigated CECs in lakes using lake-wide sampling methods that incorporates surface water grab samples the lake shoreline and lake, but the impacts of near-shore hydrological processes may not be fully captured by these sampling techniques. CECs are not easily broken down by OWTS and can easily leach into the groundwater system that inflows into the nearby lakes (Fig. 1; Godfrey et al., 2007; Verstraten et al., 2005).

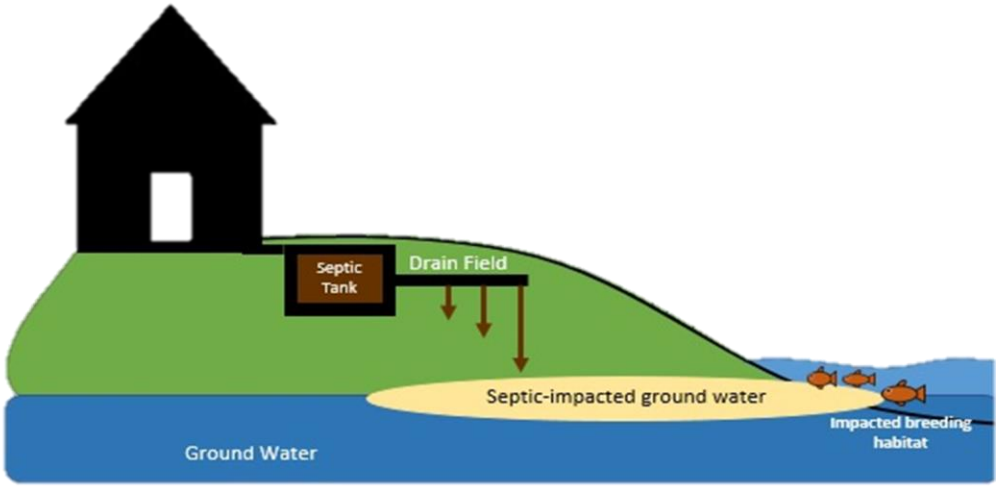


Figure 1.1. Diagram of the process contaminates may leach into the groundwater from septic systems and inflow into nearby lakes.

CEC exposures are classified in two categories: 1) acute toxic exposures often produce a lethal effect in a short period of time (killing or severely damaging the organism); 2) chronic toxic exposure effects organisms over a long period of time and generally at lower concentrations then acute exposures (Dodson, 2005), that produces effects that are sub-lethal including behavioral changes, histopathological changes in the liver and gonads and changes in the appearance of the secondary sexual characteristics (Dammann et al., 2011; Elliot et al., 2014).

Subedi et al. (2015) found in both lake and septic water samples that all ten of their targeted PPCPs were found (included two antibiotics, two antimicrobials, an antihypertensive, an antiseizure, an analgesic, a plasticizer, an ultraviolet filter, and a stimulant). Along with the ten PPCPs found, Subedi et. al (2015) also found eleven PFASs and eighteen PCBs. PFASs are found in multiple products including non-stick cookware, cosmetics, and textiles (Giesy and Kannan, 2002). PCBs are found in plasticizers, adhesives, pesticides and inks (IL Dept. of Health, 2009). OWTSs are a source for these contaminates and further studies are needed to research the biological effects in freshwater systems and work to eliminate these contaminates from the environment (Subedi et al., 2005; Baker et al. 2014).

In this study, seven CECs were assessed in the laboratory studies. These CECs were chosen as they had the highest detection rate and highest concentrations in groundwater samples collected. These CECs included N,N-Diethyl-meta-toluamide (DEET), 2,4-Dichlorophenoxyacetic Acid (2,4-D), Tris(2-chloroethyl) phosphate (TCPP), Bisphenol-A (BPA), 4-Octylphenol, Oxybenzone, and Benzophenone. DEET is a common ingredient found in

insect repellents and has been found to reduce erythroblasts in red blood of the common carp (Slaninova et al., 2014). Along with the reduction in erythroblasts, it has also been found to reduce the number of triacylglycerides (Slaninova et al., 2014), which is one of the most important energy-storing lipids and provides a major energy sources to fish (Haluzova et al., 2011). 2,4-D is a commonly used pesticide for many homeowners (Seiler, 1978). To observe if pesticides similar to 2,4-D causes oxidative stress in fish, Oruc and Uner (2003) exposed two species of fish and monitored multiple enzymes related to oxidation in the gills, kidneys, and brain. The authors found that superoxide dismutase was upregulated in the gills after exposure (Oruc and Uner, 2003). Superoxide dismutase is one of the most responsive indicators when observing oxidative stress due to contaminant exposure (Palace, 1996). BPA is used in plastics and has been found to be an estrogenic mimic and common endocrine disruptor. Vajda et al. (2008) observed reduced sperm abundance, induction of vitellogenin, and intersex in males downstream of wastewater treatment outflow that included high estrogenic compounds including BPA. Oxybenzone and Benzophenone are both used commonly in cosmetic products and sunscreens. These compounds have also been found to be estrogenic and induce vitellogenin in males and reduce fecundity of females (Coronado et al., 2015).

1.4: Endocrine Disruption in Fishes

Interfering with reproduction and development, CECs can affect both wildlife and humans (Colborn et al., 1993). Estrogens, including their mimics, are potent CECs that interfere directly with the hypothalamic-pituitary-gonadal axis (Sumpter and Jobling, 1995, Panter et al., 1998). Although silent, male hepatocytes (the main parenchymal tissue of the liver) contain the

genes necessary for synthesis of the egg-yolk precursor protein vitellogenin (Sumpter and Jobling, 1995). Vitellogenesis is the process in which the liver produces vitellogenin (a process induced by estrogen) that is then up taken by growing oocytes and later processed into yolk proteins that are used for growth (Reading and Sullivan 2011; Sumpter and Jobling, 1995). Estrogens in the environment have the ability to bind and activate the estrogen receptors since the estrogen receptors in both mammals and fish are very similar (Sumpter and Jobling, 1995). The concentrations of vitellogenin circulating in blood plasma can be used to assess estrogen exposure in male fish (Sumpter and Jobling, 1995; Panter et al., 1998). Since males lack a normal repository for vitellogenin and its intended target is the ovary in females, scientists have suggested that it becomes concentrated in the kidney and liver, whereby organ failure can occur (Thorpe et al., 2007). Dammann et al. (2011) observed that as concentrations of estrogens increased so did vitellogenin concentrations in exposed male *Fathead minnow*. Other CECs such as the herbicide Diazion can reduce vitellogenin concentrations in female bluegill sunfish resulting in reduced fecundity (Maxwell and Dutta, 2005) and also reduced fertility in both sexes (Dutta and Meijer, 2003). By controlling gonadotropin secretion, vitellogenesis, and the synthesis of eggshell proteins, estrogens are most likely to affect reproduction in females (Sumpter and Jobling, 1995). Females exposed to estrogens may delay spawning (Elliot et al., 2014) have a lower initial rate of egg production, or cease egg production at high estrogen concentrations (Dammann et al., 2011). Dammann et al. (2011) observed that egg production decreased in *Fathead minnow* at concentrations as low as 25 or 50ng/L of estrone.

Microscopic analysis of tissues from estrogen-exposed male fish suggests reduced masculinity (percentage of mature spermatozoa) in the testis (Vajda et al., 2011). This process

can also reveal hepatocyte hypertrophy due to vitellogeninic activity (Wester et al., 2003; Wolf et al., 2005). Kidd et al. (2007) observed a population collapse and concurrent elevations in plasma vitellogenin concentrations following exposure to ethynylestradiol (a synthetic estrogen) in a lake-wide study. Ethynylestradiol is approximately six times as potent as estrone (Schultz et al., 2013). Writer et al. (2010) observed in 90% of lakes studied that endocrine disruption occurred in both caged *Fathead minnow* and collected resident fish.

Morphological and behavioral changes have also been observed in association with CEC exposure (Dammann et al., 2011; Rearick et al., 2014). Estrone has been shown to reduce the escape response of exposed fathead minnow involved in predator avoidance (McGee et al., 2009). Exposure can play a vital role in the anatomical development of fish if exposed at a juvenile age and may result in slower growth, greater susceptibility to predation and being reproductively outcompeted (Elliot et al., 2014).

Reduction in the prominence of male secondary sex characteristics has been observed in association with estrogen receptor agonists in male fathead minnow (Rearick et al., 2014; Dammann et al., 2011; Elliot et al., 2014). Elliot et al. (2014) observed that smaller dorsal pads and lighter banding occurred after males were exposed to estrogens, but also that 80% of the males did not have visible tubercles.

1.5: Conclusions

With CEC concentrations on the rise in freshwater systems, it is more important than ever to look at the potential sources into lakes. Lakes provide valuable habitat for resident fish populations, but also provide the resources necessary for these populations to sustain themselves.

CECs with signatures common to household use are being seen in lakes and OWTSs provide a potential source of contamination through drain fields. Without knowing the sources of these CECs, it is difficult to reduce the biological impact on lake systems.

CHAPTER 2: IDENTIFYING ON-SITE WASTEWATER TREATMENT SYSTEMS AS A NON-POINT SOURCE AND THEIR BIOLOGICAL EFFECTS ON RESIDENT FISH

2.1: Introduction

Contaminants of Emerging Concerns (CECs) are widespread in freshwater systems throughout the United States (Ferrey et al., 2012; Kolpin et al., 2012). CECs have been extensively studied in rivers and streams downstream of wastewater treatment facilities, but little is known about their sources, pathways and impacts in lake systems (Baker et al. 2014). There are many non-point sources of CECs to lakes including agricultural and roadway run off, but the increased construction of lake shore homes relying on On-Site Wastewater Treatment System (OWTS) or "Septic Systems" could be an important contributor of CECs to these lakes (Baker et al., 2014). These systems are being used in majority of residences since urban treatment facilities are not accessible and approximately 500,000 of them are considered outdated under current laws (MNPCA, 2008). OWTSs are not designed to remove CECs and previous studies have connected household chemistry signatures with groundwater contamination from nearshore sites in lakes (Writer et al., 2010; Baker et al., 2014; Phillips et al., 2015).

Writer et al. (2010) studied eleven lakes that varied in in trophic conditions and surrounding land uses and CEC presence in all of them. This suggested that CEC sources may originate from multiple sources but identifying these sources was beyond the scope of the study. Groundwater flow may interact with CECs while passing through the drain fields of OWTSs before moving down gradient into the littoral zone of the lake. CECs are not easily broken down by OWTSs and can easily leach into the groundwater system that inflow into the nearby lakes (Godfrey et al., 2007; Verstraten et al., 2005). A recent study in New York found increased

contaminants down gradient of drain fields in shoreline wells and concluded that groundwater is directly impacted by septic systems (Phillips et al., 2015).

The increasing occurrence of CECs in lake systems is an important issue as the littoral zones of lakes provide important spawning and habitat grounds for many fish species including the fathead minnow (*Pimephales Promelas)* and bluegill sunfish (*Lepomis macrochirus).* These species can spawn for months at a time (Becker, 1983) and may be exposed to CECs during that period. The male bluegill sunfish builds his nest in the early spring and will protect it as long as water and temperature conditions are optimal for reproduction (Becker, 1983). Eggs deposited in nest sites are the directly impacted as the inflowing groundwater potentially contaminated with CECs passes through sediment where these nests are located. After hatching, larvae stay in the littoral zones for several months until large enough to inhibit deeper waters. This may lead to further exposure for several additional months.

To highlight the importance of CEC exposure into lakes, a study in Canada exposed an entire lake to 17α-eithinylestradiol for a multiple year period, which lead to the collapse of the entire fish population (Kidd et al., 2007). Multiple other studies have found that the exposure to CECs can lead to a growing list of biological pathologies in fish. CEC exposure in male fish has been documented to induce vitellogenin production, an indicator of CEC exposure and feminization (Sumpter and Jobling, 1995, Thorpe et al., 2007, Shappell et al., 2010). Males exposed to CECs have also experience a reduction in mature spermatozoa (Vajda et al., 2011) and a reduction in the expression of secondary sexual characteristics (Elliot et al., 2014).

It is not only the adult stage of the lifecycle that can affect these fish species. Several authors have reported that the CEC exposure during the larval and juvenile developmental stages may affect molecular and behavioral change that can include reduction in growth, changes in sexual differentiation (Panter et al. 2002; van Aerle et al. 2002), and a reduction in the predator escape response (McGee et al. 2009).

The current study tests the following two hypotheses: 1) resident male sunfish collected from spawning beds in septic-influenced littoral areas will exhibit biological alterations consistent with CEC exposure and 2) larvae exposed to groundwater collected from these septic-influenced sites will see a reduction in survival, growth, and predator escape responses when compared to fish exposed to reference site groundwater.

2.2: Methods and Materials

2.2.1: Lake and Site Selection. Lakes surveyed were selected from the Minnesota Pollution Control Agency's National Lake Assessment Project (NLAP) and a list of previously studied lakes. Before being surveyed, lakes in the size of 100-1000 acres were chosen as these are lakes that are not too over populated with homes (~50-75% of lake shore) and provided public boat access points. The lakes were then surveyed for sunfish spawning in areas of the lake that included possible septic-influence (homes and OWTS within sight) and reference areas (buffer areas of lake that included shorelines without homes). Once sunfish spawning was observed, a ground water sample was taken nearby using a piezometer and peristaltic pump to measure the temperature, dissolved oxygen, and conductivity. Groundwater generally has a lower temperature, lower dissolved oxygen, and high conductivity when compared to the surface lake water during the spring and summer months. From the summer 2015 surveys, Sullivan Lake (Wright County, MN), Pearl Lake (Stearns County, MN), Cedar Lake (Wright County, MN), Lake Mary (Wright County, MN) and Lake Franklin (Otter Tail County, MN) were selected as study lakes (Figure 2.1).

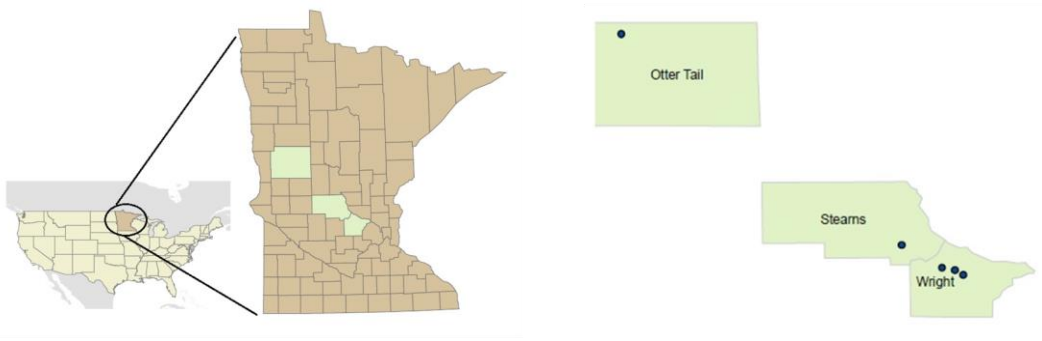


Figure 2.1. Study location within central Minnesota. Study lakes were located within three Minnesotan counties (Otter Tail, Stearns, and Wright Counties).

Figure 2 2. Aerial imagery of study lakes in central Minnesota. (a) Sullivan Lake located in Wright County, MN. (b) Pearl Lake located in Stearns County, MN. (c) Cedar Lake located in Wright County, MN. (d) Lake Mary located in Wright County, MN. (e) Lake Franklin located in Otter Tail County, MN.

2.2.2: Water Sampling. At each site, ground water samples were collected during the summers of 2015 and 2016. To collect groundwater samples, a piezometer was driven through the sediment until the groundwater aquifer was reached (~0.5-1 meters in depth). Once the piezometer was placed, it was connected to a peristaltic pump (Geotech Environmental Supply, Denver, CO) and groundwater was pumped at a slow rate as to not exceed the rate of groundwater replenishment. A multi-parameter water chemistry sonde (YSI Instruments, Ohio) was used to record temperature, conductivity, and dissolved oxygen. At each site, four liters were collected in baked 1L amber glass bottles and immediately placed on ice. Water samples were analyzed at the Higgins Lab (Colorado School of Mines, Golden, Colorado) for an array of 56 CECs including compounds found in cleaning products, steroid hormones, pharmaceuticals, herbicides, and insecticides. Upon arrival in Colorado, water samples were immediately solid phase extracted to purify samples and concentrate samples 50-fold for better instrument signals. Concentrated samples were analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS) run with both positive and negative electrospray ionization (ESI) methods.

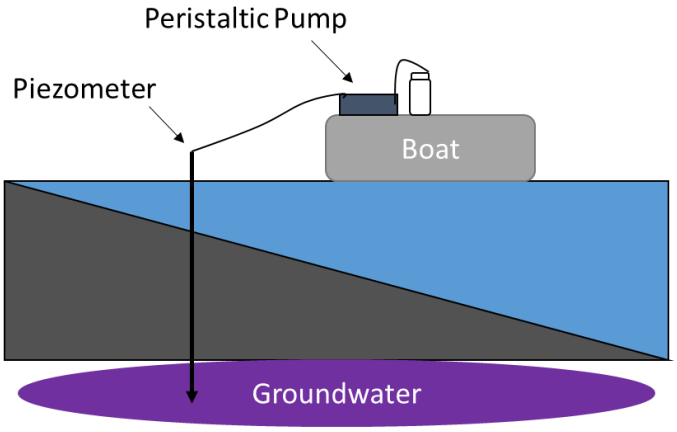


Figure 2.3. Water sampling methods of groundwater at each lake site. A piezometer was pushed though the sediment until the aquafer was detected. The piezometer was then attached to a peristaltic pump to draw groundwater to the surface and into sample bottles.

2.2.3: Resident Fish Collection and Assessment. In the summers of 2015 and 2016, sexually mature male sunfish were collected from their nest sites from each of the study sites. The genus *Lepomis* was chosen as they are common in Minnesota lakes and previous studies have assessed this genus species (Baker et al. 2014; Writer et al. 2010). Fish were collected from all lakes except Pearl Lake as the early ice-off in the spring of 2016 disrupted spawning activity. Males were taken directly off spawning beds by rod and reel (permitted by Minnesota Department of Natural Resources) and immediately euthanized using a buffered MS-222 solution approved by the St. Cloud State University IACUC Committee. A whole blood sample was taken by using a 22-gauge needle to draw blood from the caudal vein on the boat and placed immediately on ice for transfer to the laboratory. In the laboratory, whole blood was centrifuge at 4° Celsius at 8000 × *g* for 12-minutes. Plasma was then pipetted off and placed into a separate vial and stored at -80 Celsius for later analysis of vitellogenin concentrations. Fish carcasses were also placed on ice to be later dissected at the St. Cloud State Aquatic Toxicology Laboratory, St. Cloud, MN for liver and gonadal tissues.

During dissection, fish weighed (grams) and lengths along with liver and gonad weight were recorded (grams). From these values, body condition factor (weight/(total length)^3 x 100,000) (Fulton 1904), hepatosomatic index (liver weight/ mass fish x 100) (Allen et al., 2009), and gonadal somatic index (gonad weight/ mass fish x 100) (Allen et al., 2009) were calculated.

Vitellogenin concentration values were determined through an enzyme linked immunosorbent assay (ELISA) using purified sunfish vitellogenin and sunfish validated vitellogenin antibodies. Protocol followed parameters as used in Schultz et al. 2013. All samples

were analyzed at three dilutions (1:50, 1:250, and 1:1,000). An eight-point standard curve was used to reference absorbance readings of samples.

2.2.4: Larval Fathead Minnow Exposures and Assessment.

Exposure Organisms. Larval fathead minnows (<24 hours) were obtained from Environmental Consulting and Testing, Inc. (Superior, WI, USA). Exposure temperature (23 ± 2° Celsius) and photoperiod (16:8hrs light:dark) were held constant throughout the exposures. Larvae were fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct, Ogden, OT). All fish maintenance was performed in accordance with St. Cloud State University's Institutional Animal Care and Use Committee (IACUC) policies.

Experimental Design. Water for the exposures was collected concurrently and using the sample method described above for collecting water chemistry samples. Samples for fish exposures were stored frozen at the Aquatic Toxicology Laboratory, St. Cloud State University until fish exposure experiments could commence. Exposure water was thawed and brought up to room temperature before each daily exchange. Before exposure water was used for exchanges, it was filtered through a paper-filter to remove solid particulate. Static renewal of 50% exposure water was performed daily. Treatments included 20 study sites (10 septic, 10 reference), a blank well water control, and a positive control consisting of 625 ng/L estrone. Each treatment consisted of 10 glass jars containing 20 larvae exposed for 21 days.

On day 21, survival, growth, the predator escape response (McGee et al. 2009), and feeding efficiency were assessed. The predator escape response is a natural, reflexive response performed by an individual when in presence of a predator (Easton et al. 2001). By introducing the test subject to a vibration stimulus, the response is triggered and can then be recorded by

high-speed (1000 frames/sec) camera for analysis. The assay quantifies the reaction time (latency to the response) (msec), velocity of movement for 40 ms after the initial movement (BL/ms), and the total escape response [BL/(latency + 40 ms)] as an overall assessment of the response (McGee et al. 2009). During the assay, a larvae is placed into a 5 centimeter petri dish over a trigger-operated vibrational pad under the high speed camera. Once the stimulus is triggered, the camera is synced to capture the response. Videos were analyzed using ImageJ (https://imagej.nih.gov/ij/). Seven data points were recorded during analysis. These included the time the stimulus was initiated (light indicator), two points to measure one millimeter from the grid below the larvae, first larval head movement, the tip of the nose of the larvae, tip of the tail of the larvae, tip of the nose 20/ms after first movement, and tip of nose 40/ms after first movement. From these points, the final endpoints could be calculated. After the assay, larvae were euthanized using a 0.1% MS-222 solution (St. Cloud State University IACUC approved protocol).

To assess the feeding efficiency of larvae, two larvae were placed into a feeding arena containing 10 mL aerated well-water 18-24 hours before the assay. This was to ensure larvae were withdrawn from food and. Two larvae were placed into each arena to ensure a competitive environment. At the beginning of the assay, 25-35 newly hatched brine shrimp were counted on a microscope slide. After counting, brine shrimp were washed into the feeding arena using well-water and larvae were allowed to feed for 1 minute before being euthanized in 0.1% MS-222 solution (St. Cloud State University IACUC approved protocol). Remaining brine shrimp were counted under a dissection microscope.

2.3: Results

2.3.1: Lake and Site Selection. Five lakes were chosen as study lakes: Sullivan Lake, Pearl Lake, Cedar Lake, Lake Mary, and Lake Franklin. For each lake, two putative septic-influenced and two putative reference sites were chosen. Putative septic and reference site determination was based on groun water temperature, conductivity and visual indicators (presence of spawning beds, presence/absence of nearshore OWTS). Table 2.1 lists the groundwater characterization data for each site on each lake. In total, 20 sites were chosen on the five study lakes.

Table 2.1. Results of groundwater and surface water characterization during lake surveys. The four possible study sites follow each selected lake. At each site, groundwater was collected using a piezometer and peristaltic pump. Temperature, Conductivity, and Dissolved Oxygen were recorded to characterize groundwater inflow.

Lake	Site ID	Projected Site Type	Type	Temp [C]	Conductivity [SC]	Dissolved Oxygen (%)	Dissolved Oxygen (mg/L)
Sullivan Lake	*Site A*	*Septic*	*Pore*	14.4	822	28.6	2.94
			Lake	15.63	423	112.8	11.2
	Site B	*Septic*	*Pore*	13.99	1644	41.5	4.25
			Lake	14.43	430	109.2	11.14
	Site C	*Reference*	*Pore*	12.53	700	31	3.29
			Lake	15.79	422	114.1	11.31
	Site D	*Reference*	*Pore*	11.87	1208	25.2	2.72
			Lake	15.45	421	121.7	12.18
Pearl Lake	*Site A*	*Septic*	*Pore*	14.05	474	29.8	3.06
			Lake	14.29	443	146	15.48
	Site B	*Septic*	*Pore*	12.58	464	29.9	3.11
			Lake	13.11	418	151.9	15.92
	Site C	*Reference*	*Pore*	9.91	721	825.5	8.99
			Lake	13.75	467	131.3	13.59
	Site D	*Reference*	*Pore*	11.38	693	56.4	5.97
			Lake	14.34	456	109.3	11.12
Cedar Lake	*Site A*	*Reference*	*Pore*	13.55	602	43	4.39
			Lake	14.79	413	143.2	14.43
	Site B	*Septic*	*Pore*	14.3	1008	38.8	3.75
			Lake	16.78	419	132.5	12.84
	Site C	*Reference*	*Pore*	12.46	1239	51.6	5.42
			Lake	15.18	418	110.7	11.02
	Site D	*Septic*	*Pore*	15.38	1226	42.2	4.11
			Lake	16.83	418	130.7	12.61
Lake Mary	*Site A*	Septic	*Pore*	21.3	--	11.6	1.04
			Lake	21.6	--	97.2	8.46
	Site B	Septic	*Pore*	20	--	11.3	1.03
			Lake	20.1	--	94.2	8.6
	Site C	Reference	*Pore*	18.4	--	11	1.03
			Lake	18.9	--	97.3	9.39
	Site D	Reference	*Pore*	20.7	--	12.7	1.13
			Lake	22.7	--	99.3	8.64
Lake Franklin	*Site A*	Septic	*Pore*	17.22	1929	42.3	4.05
			Lake	19.48	840	61.7	95.1
	Site B	Septic	*Pore*	17.34	2628	26.9	2.55
			Lake	19.9	839	101.5	9.26
	Site C	Reference	*Pore*	14.43	1990	27.6	2.8
			Lake	22.57	939	157.8	13.7
	Site D	Reference	*Pore*	14.34	2037	21.3	2.19
			Lake	19.69	789	126.6	11.62

2.3.2: Water Chemistry. Groundwater chemistry showed a total increase in CECs (ng/L) observed at septic sites then reference sites for each lake (Figure 2.1). Although greater total CEC concentrations were observed at the septic-influenced sites, many of the lakes still observed the same number of detections at each of the site types. When reviewing the water chemistry for the septic- influenced sites, the top seven detected compounds included N,N-Diethyl-meta-toluamide (DEET), 2,4-Dichlorophenoxyacetic Acid (2,4-D), Tris(2-chloroethyl) phosphate (TCPP), Bisphenol-A (BPA), 4-Octylphenol, Oxybenzone, and Benzophenone.

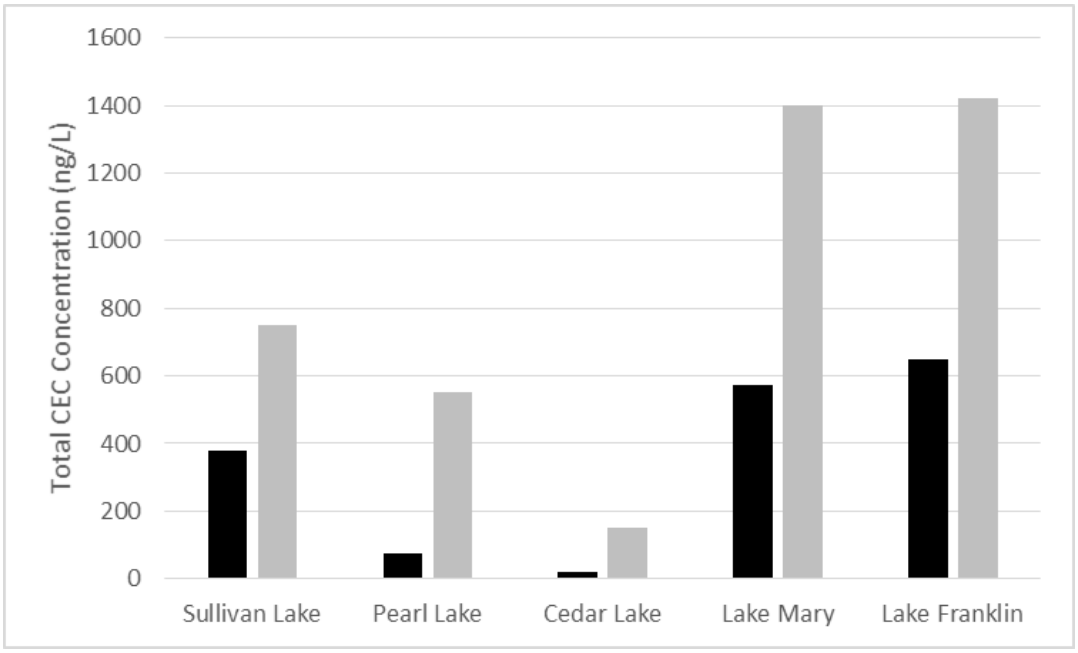


Figure 2.4. Total Nano-gram per liter (ng/L) concentrations of CECs at each lake by reference (black) or septic-influenced (gray) sites. Septic-influenced sites show a greater ng/L concentrations then the respective reference sites at each lake.

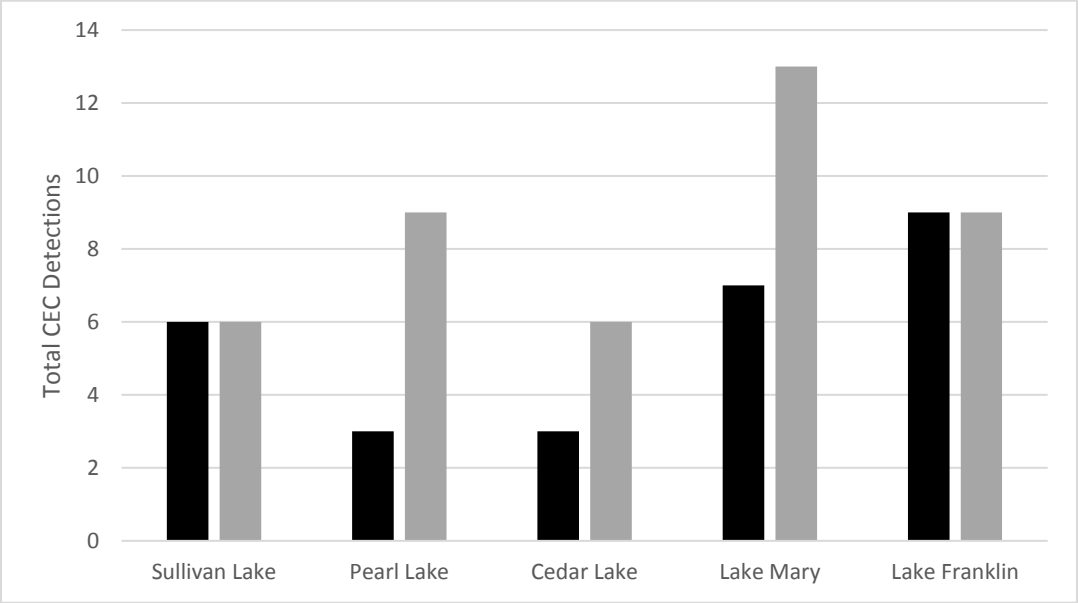


Figure 2.5. Total number of CEC detections at each lake by reference (black) or septic-influenced (gray) sites.

2.3.3: Resident Fish Collection and Assessment. During the summers of 2015 and 2016, a total of 286 resident male sunfish were collected off of their nesting grounds from the five study lakes. A total of 186 fish were collected from septic-influenced sites and 84 from reference sites. On a lake basis, 128 were collected from Sullivan Lake, 81 from Lake Mary, 35 from Cedar Lake, and 26 from Lake Franklin (Table 2.1).

Table 2.2. Total fish collected from reference and septic influenced sites from their respective lakes.

Lake	Total Sunfish Collected from Reference Sites	Total Sunfish Collected from Septic-Influenced Sites
Sullivan Lake	17	110
Pearl Lake	0	0
Cedar Lake	10	24
Lake Mary	43	38
Lake Franklin	5	21
TOTAL	**84**	**184**

When comparing glucose concentrations in resident male sunfish, no significant differences were observed between fish from reference (mean glucose: 50.12 mg/dL) and septic-influenced sites (45.05 mg/dL) (two-tailed t-test, $p>0.05$; Figure 2.2).

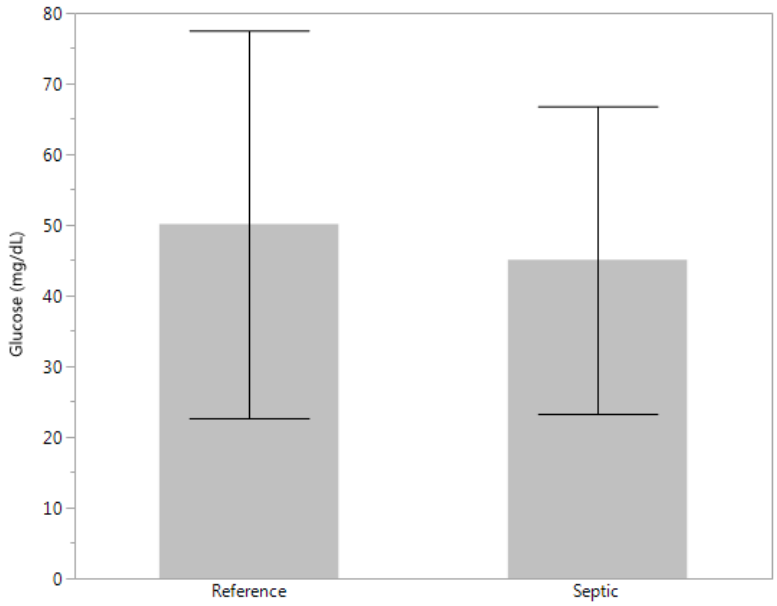


Figure 2.2. Mean glucose concentration (mg/dL) of resident males from study lakes in septic-influenced and reference sites. Bars and error bars represent mean ± standard deviation.

Prior to analysis, vitellogenin concentrations (ug/mL) were log10 transformed to normalize the data. Vitellogenin concentrations of resident fish from septic-influenced sites and reference sites were significantly different from each other (two-tailed t-test, p=0.0108). Higher concentrations of vitellogenin were observed in fish from septic-influenced sites (Mean= 1268.64 ug/mL) when compared to fish from reference sites (Mean =495.02 ug/mL). When analyzing the data by site type (septic-influenced or reference) in each lake, Sullivan Lake and Lake Mary had significantly higher vitellogenin concentrations in fish from septic-influenced sites then reference sites (Tukey's HSD, p=0.02 Sullivan Lake, p=<0.001 Lake Mary; Figure 2.3).

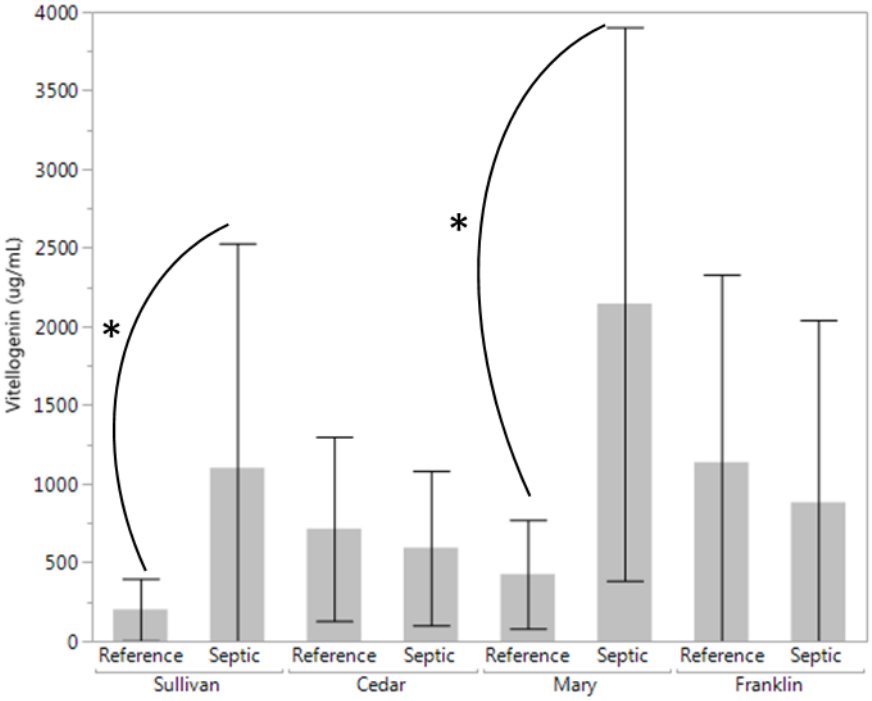


Figure 2.7. Vitellogenin concentration (ug/mL) of resident fish in study lakes by septic-influenced and reference sites. Bars represent mean +/- standard deviation.

Body condition factor of resident males from septic-influenced and reference sites were not significantly different from each other (two-tailed t-test, $p>0.05$) (Figure 2.4). The Hepatic somatic index was found to be significantly different from septic-influenced resident fish (Mean= 0.947) and reference site fish (Mean= 1.05) (two-tailed t-test, $p=0.0104$) (Figure 2.5). Livers from resident males from septic-influenced sites were significantly smaller then of reference site fish. No significant difference was observed when comparing the gonadal somatic index between fish from reference and septic-influenced sites (two-tailed t-test, $p>0.05$; Figure 2.5).

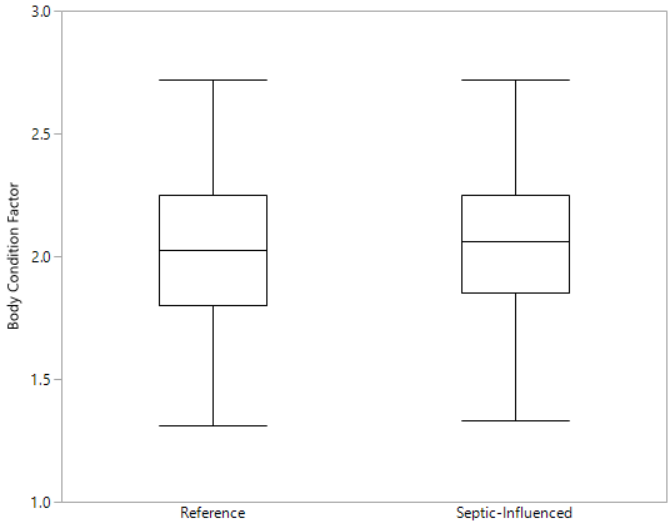
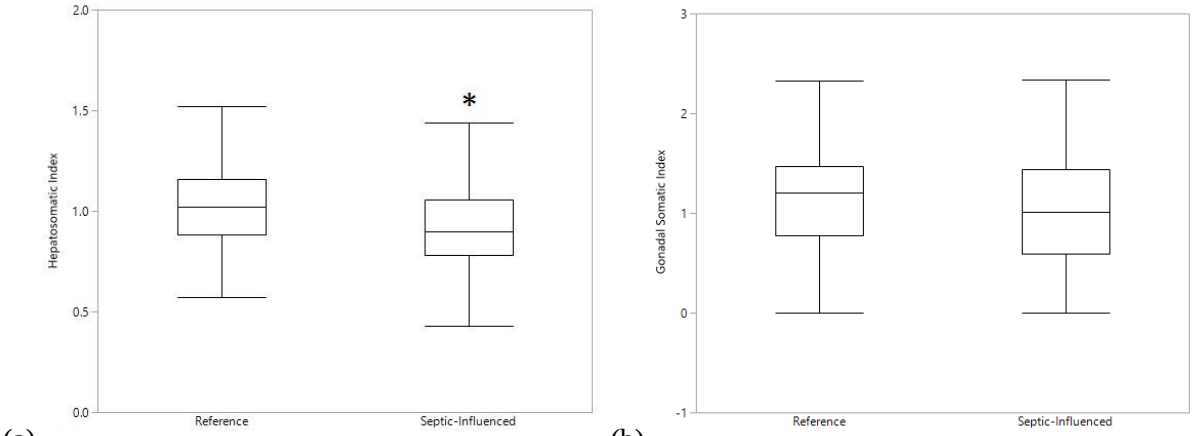


Figure 2.8. Body condition factor of resident male fish collected from septic-influenced sites and reference sites. Body condition factor is calculated as (body weight/total length)^3 x 100,000. Boxes represent mean and range. Whiskers represent 95% confidence intervals.



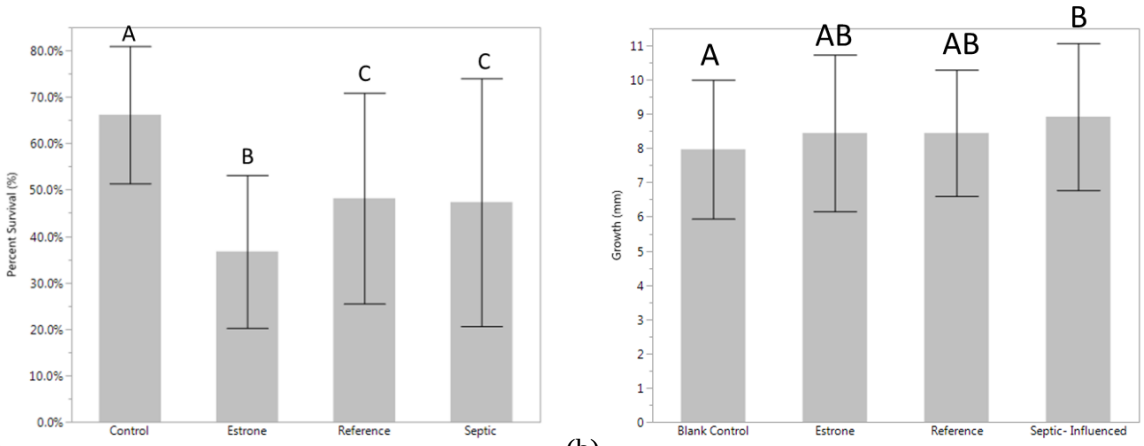
(a) (b)

Figure 2.9. Hepatosomatic Index (a) and Gonadal Somatic Index (b) of resident male fish collected from septic-influenced and reference sites of the study lakes. Hepatosomatic Index calculated as liver weight/body weight *100. Gonadal Somatic Index calculated as gonadal weight/body weight *100. Boxes represent mean and range. Whiskers represent 95% confidence intervals.

Table 2.3. Means, standard error, and sample sizes of endpoints for reference and septic-influenced sites.

Endpoint	**Reference Sites**		**Septic-Influenced Sites**	
	(Mean +/- SE)	**Sample Size (n)**	**(Mean +/- SE)**	**Sample Size (n)**
Glucose (mg/dL)	49.66 +/- 27.41	84	45.17 +/- 21.77	184
Plasma Vitellogenin (ug/mL)	495.02 +/- 512.39		1268.64 +/- 1504.97	
Body Condition Factor	2.03 +/- 0.29		2.07 +/- 0.32	
Hepatosomatic Index	1.05 +/- 0.25		.947 +/- 0.33	
Gonadal Somatic Index	1.12 +/- 0.64		1.04 +/- 0.72	

2.3.4: Larval Fathead Minnow Exposures and Assessment. After completion of the 21-day static renewal exposure to the groundwater samples, a blank well-water control, and positive estrone control, survival and growth were assessed. Survival was calculated as the total number of larvae surviving on day 21 divided by 20 (starting number of larvae; Figure 2.5). A significant decrease in survival was observed in the estrone, reference, and septic-influenced treatments when compared to the blank well-water control (ANOVA with Tukey HSD, $F=14.3773$, $df= 3$, $p= <0.001$). Survival in the septic-influenced and reference treatments were also significantly higher than the estrone positive control (ANOVA with Tukey HSD, $F=14.3773$, $df= 3$, $p= 0.03$). A significant increase in growth was observed in larvae in the septic-influenced treatment when compared to the blank well-water control (ANOVA with Tukey HSD, $F= 6.8285$, $df= 3$, $p = 0.002$; Figure 2.5).



(a) (b)

Figure 2.10. Mean survival (a) and growth (b) of larvae exposed to groundwater collected from the 20 lake sites, blank well-water control, and positive estrone control. (a) Mean survival for each treatment in percent-survived. (b) Mean growth (mm) for each treatment. Bars represent mean survival ± standard error.

When assessing the predator escape response, the reaction time was significantly slower in the positive estrone control then of the blank well-water control (ANOVA with Tukey's HSD, $F=0.0353$, $df= 3$, $p=0.0353$). No significant differences were observed when assessing escape velocity or total escape response between treatments (ANOVA, $p>0.05$).

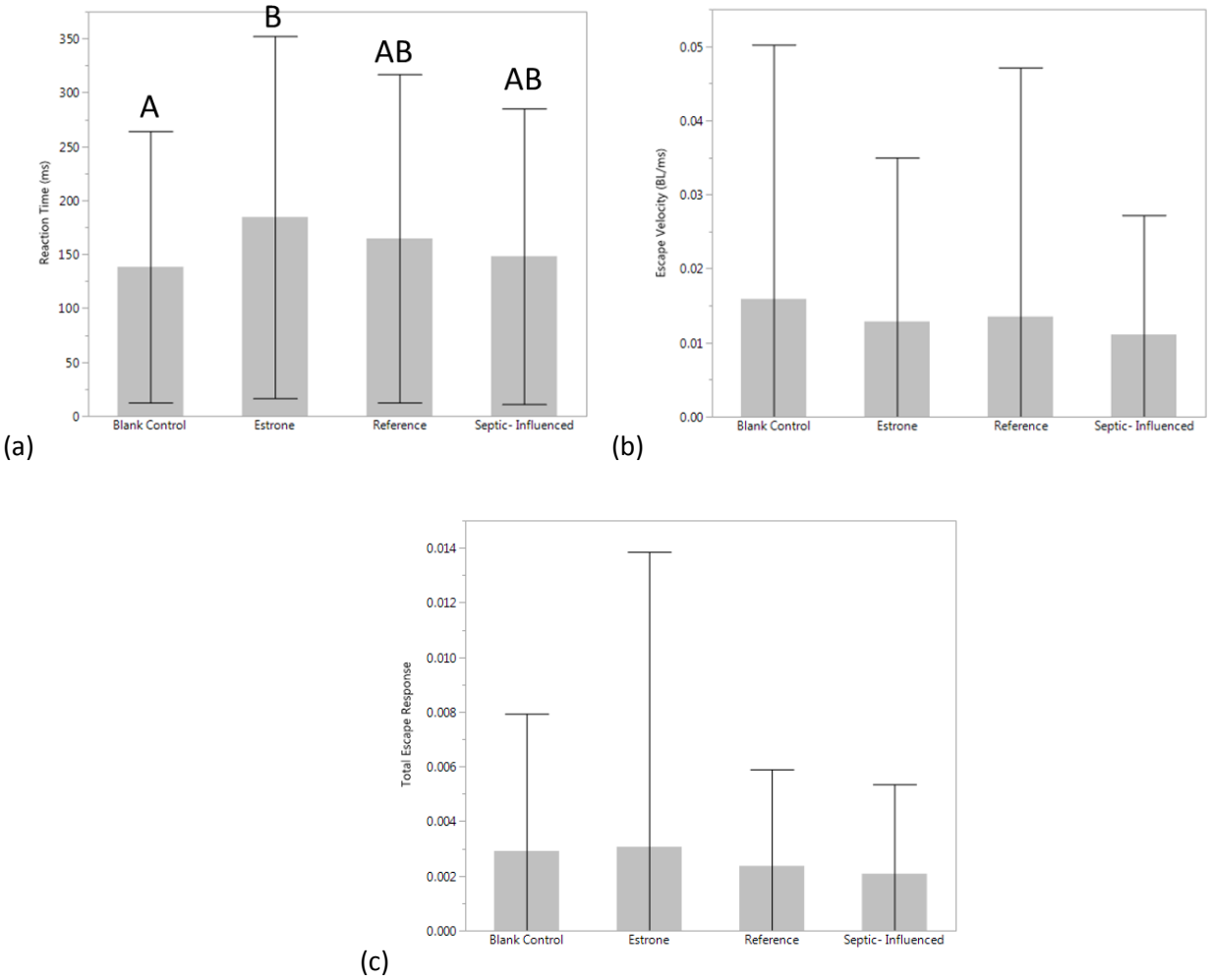


Figure 2.11. Effects of 21-day exposure of larvae to groundwater samples, a blank-well water control, and a positive estrone control on predator escape performance. (a) Mean reaction time to start of stimulus and first movement (ms) (b) Mean escape velocity (BL/ms) in body lengths (BL) for the first 40ms after first movement (c) Total escape response that considers both the latency and escape velocity. Bars represent mean $\pm$ standard error.

A significant decrease in feeding performance was observed in the positive estrone control compared to all other treatments (ANOVA with Tukey HSD, F= 6.2255, df= 3, p= 0.004; Fig 2.12). Positive estrone control larvae consumed 45% of brine shrimp, whereas blank well-water control larvae consumed 59%, septic-influenced larvae 58% percent, and reference larvae 57% of brine shrimp, respectively.

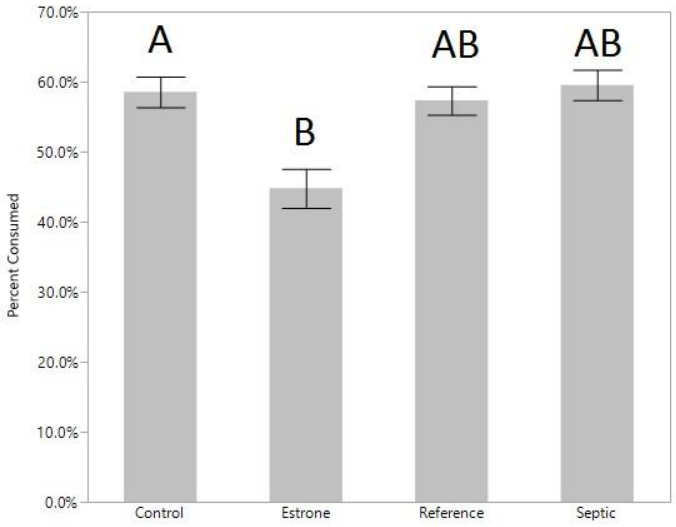


Figure 2.12. Percent of brine shrimp consumed during feeding efficiency assay by treatment. Bars represent mean +/- standard deviation.

Table 2.4. Result summary of 21 day 50% static renewal exposures of larvae to ground water and control treatments.

Endpoint	Blank Control (Mean +/- SD)	Estrone (Mean +/- SD)	Reference Sites (Mean +/- SD)	Septic-Influenced Sites (Mean +/- SD)
Survival (%)	66 +/- 14.8	36 +/- 36.7	48 +/- 48.2	47 +/- 47.3
Growth (mm)	7.97 +/- 2.03	8.44 +/- 2.29	8.45 +/- 1.84	8.92 +/- 2.15
Response Time (ms)	138.36 +/- 125.84	184.48 +/- 167.93	165.19 +/- 152.05	148.10 +/- 137.10
Escape Velocity (BL/ms)	0.0159 +/- 0.034	0.0128 +/- 0.022	0.0135 +/- 0.033	0.0111 +/- 0.016
Total Escape Response (BL)	0.0029 +/- 0.005	0.0031 +/- 0.011	0.0024 +/- 0.004	0.0021 +/- 0.003
Sample Size (n)	158	143	261	235

2.4: Discussion

The objectives of this study were to 1) link groundwater interaction with OWTS drain fields before inflowing into lake systems and 2) associate observed biological endpoints of CEC exposure to septic-influenced sites of the study lakes. The first objective utilized groundwater samples from lake sites (septic-influenced and reference areas). For the second objective, resident male sunfish were collected and processed for biological endpoints. In addition, groundwater from each site was collected to expose larval fathead minnows in the laboratory.

When analyzing the groundwater chemistry data, septic-influenced sites contained greater CEC concentrations than reference site for all five study lakes. These results strongly suggest an interaction of OWTS discharge with groundwater before it inflows into nearby lake littoral zones. These results are consistent with the findings of Baker et al. (2014) where groundwater collected from locations in the proximity of OWTS also exhibited higher loads of CECs.

Although our reference sites had the same number of detections as septic-influenced sites, CEC concentrations were much lower at the reference sites. The presence of CECs in reference samples is an indicator that groundwater patterns around these lake systems may be more complex than anticipated, thus influencing all areas of the lake. The top seven compounds detected were commonly used domestic chemicals. These included insect repellent (DEET), herbicide (2,4-D), plasticizer (TCPP and BPA), household cleaners (4-ocytlphenol), sunscreen (oxybenzone), and cosmetics (benzophenone). DEET and BPA were detected in previous studies (Writer et al., 2010; Baker et al., 2014) along with 2,4-D (Baker et al., 2014) and 4-octylphenol (Writer et al., 2010). Using the water chemistry results to provide a final assignment of septic-influeces and reference sites for thee biological analysis, four sites changed classification. These included two reference sites (one on Sullivan Lake and one on Lake Franklin) and two septic-influenced sites (one on Cedar Lake and one on Pearl Lake). Although these four sites changed, it still provided an equal balance of the total number of septic-influenced and reference sites (10 each). The water chemistry shows evidence that complex mixtures are present in lake systems despite the absence of wastewater discharge.

Although not all biological endpoints were significantly different between septic-influenced sites and reference sites, key biological pathologies were still observed. Males from septic-influenced sites had significantly higher vitellogenin concentrations than males from reference sites. This suggests that these males are being exposed to estrogenic compounds (Sumpter and Jobling, 1995). Lakes with highest vitellogenin concentrations included Sullivan Lake and Lake Mary. These two lakes had significantly higher vitellogenin concentrations in males from septic sites than of reference sites. This was not observed in Cedar Lake or Lake

Franklin, but smaller sample sizes were also taken from these lakes compared to Sullivan and Lake Mary. In our water chemistry, three studied estrogenic CECs (Oxybenzone, Benzophenone, and BPA) were observed in the top detections. Oxybenzone and Benzophenone are UV-filters that are commonly used in cosmetics and sunscreens. These compounds induce vitellogenin concentrations in male fish and reduce fecundity of female fish in rainbow trout (Coronado et al., 2015).

Body condition factor and gonadal somatic index did not differ between male fish from septic-influenced sites compared to reference site. As mentioned before, lake environments are at optimal conditions in Minnesota during the early summer as nutrients and reproduction are high. We would expect to find males with high body condition and fully mature gonads of nesting males.

The hepatosomatic index is an indicator of liver health and energy storage. In poor environments, the liver will generally be smaller in size since less energy reserve is present. The male sunfish from the septic-influenced sites on average had smaller livers proportional to body size then of male sunfish collected from reference sites, indicating less suitable environments.

When examining the results of the larval exposures, only a few significant effects were observed. A significant reduction in survival was observed in both the septic-influenced site and reference site exposed larvae when compared to the blank well-water control. CECs were present at all site types and all lake water treatments. This could potentially indicate that the smaller CEC concentrations in the reference site samples are still causing an effect.

The only endpoint in the predator escape response to show significance was reaction time in the estrone positive control. Estrone has been observed to affect reaction time and the predator

escape response at low concentrations when being exposed in the embryonic and larval life stage forms (McGee et al., 2009). Baker et al. (2014) did not find any significant differences in reaction time, escape velocity, or total escape response in larvae exposed to collected groundwater and this could be an indicator that larval fish are not affected by groundwater CEC exposure until a later life stage.

Conclusions. This study investigated the potential for OWTS to interact with inflowing groundwater and act as a non-point source of CECs into lakes. Water chemistry of groundwater collected from the study lakes show an increase in the total concentrations of CECs from septic-influenced sites than reference sites. This is an indicator that OWTS systems do impact groundwater before it enters the surrounding lake. Biological pathologies observed in the resident fish species do indicate a presence of CECs in these lake systems with worsened effects in fish from the septic-influenced sites and reference sites.

CHAPTER 3: LABORATORY EXPOSURES TO CECs COMMON IN SEPTIC SEEPAGE TO BLUEGILL SUNFISH AND FATHEAD MINNOWS

3.1: Introduction

Many studies have confirmed the presence of Contaminants of Emerging Concerns (CECs) in lake systems (Writer et al., 2010; Baker et al., 2014). Although there are many inputs into lake systems including agricultural and roadway runoff, there has still been the potential for septic seepage from On-Site Wastewater Treatment Systems (OWTS) to impact surrounding groundwater before entering the lake system through the lentic substrate (Baker et al., 2014, Phillips et al., 2015). With many homes being built on nearshore slopes around lakes, groundwater may flow past the OWTSs of these homes and into the littoral zone of the lake. Septic systems contain drain fields in which liquid discharge precipitates through before entering the environment. These drain fields are not designed to clean discharge of contaminates like pharmaceuticals, personal care products, or steroids before it enters the environment. This allows the groundwater to then carry the contaminants down gradient into the shallow littoral zone of the lake.

It is in the littoral zone that many native fish species including bluegill sunfish (*Lepomis macrochirus*) and fathead minnows (*Pimephales promelas)* spawn during the spring and summer (Becker, 1983). In both species, the males protect their nests during the entire spawning season that can last for multiple months depending on water temperatures and daylight (Becker, 1983). These shallow waters are also where the eggs are laid directly into the sediment and larvae cling to vegetation and large stones until they are large enough to swim on their own. This provides

the ability for many resident fish to be exposed for their entire lives to CECs entering lakes through groundwater discharge into the lake littoral zone.

Previous studies have observed biological consequences consistent with CEC exposure to caged fathead minnows (Writer et al., 2010) and bluegill sunfish (Baker et al., 2014) in Minnesota lakes. Caged male fathead minnows produced vitellogenin and experienced histopathlogical changes after a 21-day exposure (Writer et al., 2010). Histopathlogical changes included increased liver vacuolization and testicular feminization.

To causally connect biological changes to septic seepage from OWTS, this study's objective was to expose laboratory raised fish to CEC mixtures similar in composition and concentrations to groundwater near septic-influenced sites in Central Minnesota lakes. Water chemistry from groundwater samples taken from septic-influences and reference sites (See Chapter 2) was used to replicate groundwater CEC mixtures. In total, five study lakes were sampled at possible septic-influenced sites that spawning was occurring. Bluegill sunfish and fathead minnows were used to assess the biological effects of these CECs. Sunfish were chosen as they are common to many Minnesotan lakes and spawn in these shallow waters where groundwater is inflowing (Becker, 1983). Fathead minnows were chosen as they are a common model in toxicology testing (Denny, 1987) and many endpoints of endocrine disruption to CECs are recognized in these organisms. Larval and adult life stages of fathead minnows were studied to assess the effect of these CECs across ontogeny.

3.2: Materials and Methods

3.2.1: CEC Mixture. During the summers of 2015 and 2016, groundwater analysis on five central Minnesota lakes confirmed the presence of CECs in groundwater samples from the lentic zones of the lakes. These lakes included Sullivan Lake (Wright County, MN), Pearl Lake (Stearns County, MN), Cedar Lake (Wright County, MN), Lake Mary (Wright County, MN), and Lake Franklin (Otter Tail County, MN). Since the water chemistry contained very complex mixtures of CECs, the top seven detected compounds were targeted and used to produce synthetic mixtures for laboratory exposures. Compounds included: N,N-Diethyl-meta-toluamide (DEET), 2,4-Dichlorophenoxyacetic Acid (2,4-D), Tris(2-chloroethyl) phosphate (TCPP), Bisphenol-A (BPA), 4-Octylphenol, Oxybenzone, and Benzophenone.

Water chemistry data was assessed by site to observe the presence/absence of the respective compound and the concentration detected. To simulate different OWTS systems, two mixtures were created as a presence/absence of 2,4-D was observed in sites. Mix 1 was created to simulate the sites that had a detection of 2,4-D, whereas Mix 2 simulated sites without 2,4-D.

To examine the effects of CECs over a range of concentrations, each mixture was used at four different concentrations. The environmentally highest measurement of each CEC informed the medium treatment for each mixtures. A low treatment consisted of a ten-fold dilution of each CEC, a high treatment was formed by a 10 fold increase over the medium treatment, and finally a super high treatment used a 100 fold increase (10 fold increase of the high concentration).

All CECs were obtained from Sigma Aldrich (St. Louis, MO, USA) and stored per manufacture instruction until mixtures were prepared. Mixtures were prepared at the St. Cloud State University Aquatic Toxicology Laboratory, St. Cloud, MN in 100% ethanol and then sent to the Higgins Lab (Colorado School of Mines, Golden, CO) for analysis and confirmatory water chemistry. All mixtures were stored at 4^{o} C until use.

Table 3.1. Compounds and concentrations of CECs used in each mixture created. Mix #1 includes all seven compounds, whereas Mix #2 excludes 2,4-D since it was not detected at all septic-influenced sites.

	Mix #1 (ng/L)				**Mix #2 (ng/L)**			
	Low	**Medium**	**High**	**Super High**	**Low**	**Medium**	**High**	**Super High**
DEET	40	400	4,000	40,000	8	80	800	8,000
2,4-D	40	400	4,000	40,000	0	0	0	0
TCPP	7.8	78	780	7,800	7.8	78	780	7,800
BPA	3.2	32	320	3,200	3.2	32	320	3,200
4-Octylphenol	1.6	16	160	1,600	1.6	16	160	1,600
Oxybenzone	8	80	800	8,000	40	400	4,000	40,000
Benzophenone	4	40	400	4,000	6.5	65	650	6,500

3.2.2: Adult Sunfish and Fathead Minnow Exposures and Assessment.

Study Organisms. Adult fathead minnows were obtained from Environment Consulting and Testing, Inc. (Superior, WI, USA). These minnows were reproductively mature animals at 6-7 months of age. Mature bluegill sunfish were obtained from 10,000 Lakes Hatchery (Anoka, MN, USA). These sunfish were 12-18cm in length. Fish were fed twice daily with a mixture of frozen brine shrimp (Brine Shrimp Direct, USA) and frozen bloodworms (Brine Shrimp Direct, USA). Exposure conditions were maintained at a constant temperature (23 $\pm$ 2° Celsius) and photoperiod (16:8 light to dark). All organisms were housed at the St. Cloud State Aquatic Toxicology Laboratory, St. Cloud, MN. All fish maintenance was performed in accordance with St. Cloud State University's Institutional Animal Care and Use Committee (IACUC) policies.

Sunfish Exposure Design. Sunfish were exposed using a 21- day flow-through exposure design. Treatments included a low (1/10x), medium (1x; environmental), high (10x), and super high (100x) of each mixture and an ethanol carrier control. Two 114 liter aquaria were used per treatment with 20 fish per aquaria. Each mixture was performed during separate 21-day exposures with ethanol controls.

During exposure, 10L of well water from a dedicated well was spiked with mixture concentrate and pumped into mixing tanks mounted above aquaria at a pump rate of 2.5 mL per minute using a Cole-Palmer Master flex 7523-40 peristaltic pump. Well water was then fed into the mixing tanks at a rate of 200mL per minute to achieve the final concentration for each treatment. Lines from mixing tanks then fed directly into treatment aquaria at a rate of 400mL per minute/aquarium (4.8 exchanges/day).

Fathead Minnow Exposure Design. Fathead minnows were exposure using a 21-day 50% static renewal design. Ten aquaria (7 liter) per treatment were used (10 treatments = 100 aquaria). Exchanges were performed daily to ensure no degradation of compounds and waste would not build up in aquaria. Exchanges were performed by spiking n aliquot of concentrated stock CEC solutions into 40L of well water in a mixture-dedicated container. After mixing, 3.75 liter of solution was then transferred to each aquarium using mixture-dedicated watering cans. Exchanges always started with low treatments and worked up to super high treatments before being rinsed with well water for the following day. Fathead minnows were paired with one male and one female in each aquarium to assess daily fecundity. This exposure experiment was replicated once.

Biological Endpoints. During dissection, fish weight, length, liver and gonad weight were recorded. From these values, body condition factor (weight/(total length)^3 x 100,000) (Fulton 1904), hepatosomatic index (liver weight/ mass fish x 100) (Allen et al., 2009), and gonadal somatic index (gonad weight/ mass fish x 100) (Allen et al., 2009) were calculated. Whole blood was collected from the caudal vasculature and drawn up by a heparinized capillary tube. Glucose concentrations in whole blood was measured using a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT). To separate plasma from whole blood, a centrifuge cooled at 4 degree Celsius was opearted at 8000x*g* for a 12-minute period. Plasma was pipetted off and stored at -80 Celsius for later analysis of vitellogenin concentration. Fathead minnow males were observed for secondary sex characteristics (banding, tubercles, and dorsal pad) and rated on a four-point scale (0-3) with 3 being the most dominant.

Vitellogenin concentrations in sunfish were calculated using an enzyme linked immunosorbent assay (ELISA) using purified sunfish vitellogenin and sunfish validated vitellogenin antibodies. Sunfish protocol followed parameters as used in Schultz et al. 2013. Fathead minnow protocol followed parameters as used in Shapell et al. (2010). All samples were analyzed at three dilutions (1:50, 1:250, and 1: 1,000). An eight-point standard curve was used to reference absorbance readings of samples.

The fecundity of female fathead minnows was assessed by checking egg tiles in aquaria on a daily basis for newly laid eggs. The tank, treatment, and total number of eggs were recorded daily. Eggs were then disposed of and a replacement breeding tile was placed back into the aquarium.

2.2.3: Larval Fathead Minnow Exposures and Assessment.

Exposure Organisms. Larval fathead minnows (<24 hours) were obtained from Environmental Consulting and Testing, Inc. (Superior, WI, USA). Exposure temperature (23 $\pm$ 2° Celsius) and photoperiod (16:8hrs light:dark) were held constant throughout the exposures. Larvae were fed twice daily with newly hatched brine shrimp (Brine Shrimp Direct, Ogden, OT). All fish maintenance was performed in accordance with St. Cloud State University's Institutional Animal Care and Use Committee (IACUC) policies.

Experimental Design. Waters for the larval exposure was collected and stored frozen during the adult sunfish exposures to ensure the same CEC composition in all laboratory exposure experiments. Treatments included both synthetic mixtures at 4 concentrations including of a low (1/10x), medium (1x; environmental), high (10x), and super high (100x) and an ethanol carrier control. Static renewal of 50% exposure water was performed daily. Exposure water was

thawed and brought up to room temperature before each daily exchange. Each treatment consisted of 10 glass jars containing 20 larvae exposed for a 21-day period.

On day 21, survival, growth, the predator escape response (McGee et al. 2009), and feeding efficiency were assessed. The predator escape response is a natural, reflexive response performed by an individual when in presence of a predator (Easton et al. 2001). By introducing the test subject to a vibration stimulus, the response is triggered and can then be recorded by high-speed (1000 frames/sec) camera for analysis. The assay quantifies the reaction time (latency to the response) (msec), velocity of movement for 40 ms after the initial movement (BL/ms), and the total escape response [BL/(latency + 40 ms)] as an overall assessment of the response (McGee et al. 2009). During the assay, a larvae is placed into a 5 centimeter petri dish over a trigger-operated vibrational pad under the high speed camera. Once the stimulus is triggered, the camera is synced to capture the response. Videos were analyzed using ImageJ (https://imagej.nih.gov/ij/). Seven data points were recorded during analysis. These included the time the stimulus was initiated (light indicator), two points to measure one millimeter from the grid below the larvae, first larval head movement, the tip of the nose of the larvae, tip of the tail of the larvae, tip of the nose 20/ms after first movement, and tip of nose 40/ms after first movement. From these points, the final endpoints could be calculated. After the assay, larvae were euthanized using a 0.1% MS-222 solution (St. Cloud State University IACUC approved protocol).

To assess the feeding efficiency of larvae, two larvae were placed into a feeding arena containing 10 mL aerated well-water 18-24 hours before the assay. This was to ensure larvae were withdrawn from food and. Two larvae were placed into each arena to ensure a competitive

environment. At the beginning of the assay, 25-35 newly hatched brine shrimp were counted on a microscope slide. After counting, brine shrimp were washed into the feeding arena using well-water and larvae were allowed to feed for 1 minute before being euthanized in 0.1% MS-222 solution (St. Cloud State University IACUC approved protocol). Remaining brine shrimp were counted under a dissection microscope.

3.3: Results

3.3.1: Adult Sunfish and Fathead Minnow Exposures and Assessment.

Male Sunfish Assessment. No significant differences in body condition factor and hepatosomatic index of the male sunfish were observed between treatments (ANOVA, $p>0.05$; Figure 3.1). Similarly, the gonadal somatic index of male sunfish did not differ between treatments (ANOVA, $F= 2.8846$, $df= 8$, $p=0.0169$; Figure 3.1). The gonadal somatic index for sunfish exposed in Mixture #2 Low, Medium, High, and Super High, Mixture #1 Low, and the Ethanol Control were significantly larger than of Mixture #1 Medium, High, and Super High treatments (Student's t, all $p<0.05$). Glucose readings for the male sunfish were not significantly different from each other (ANOVA, $p>0.05$; Figure 3.1).

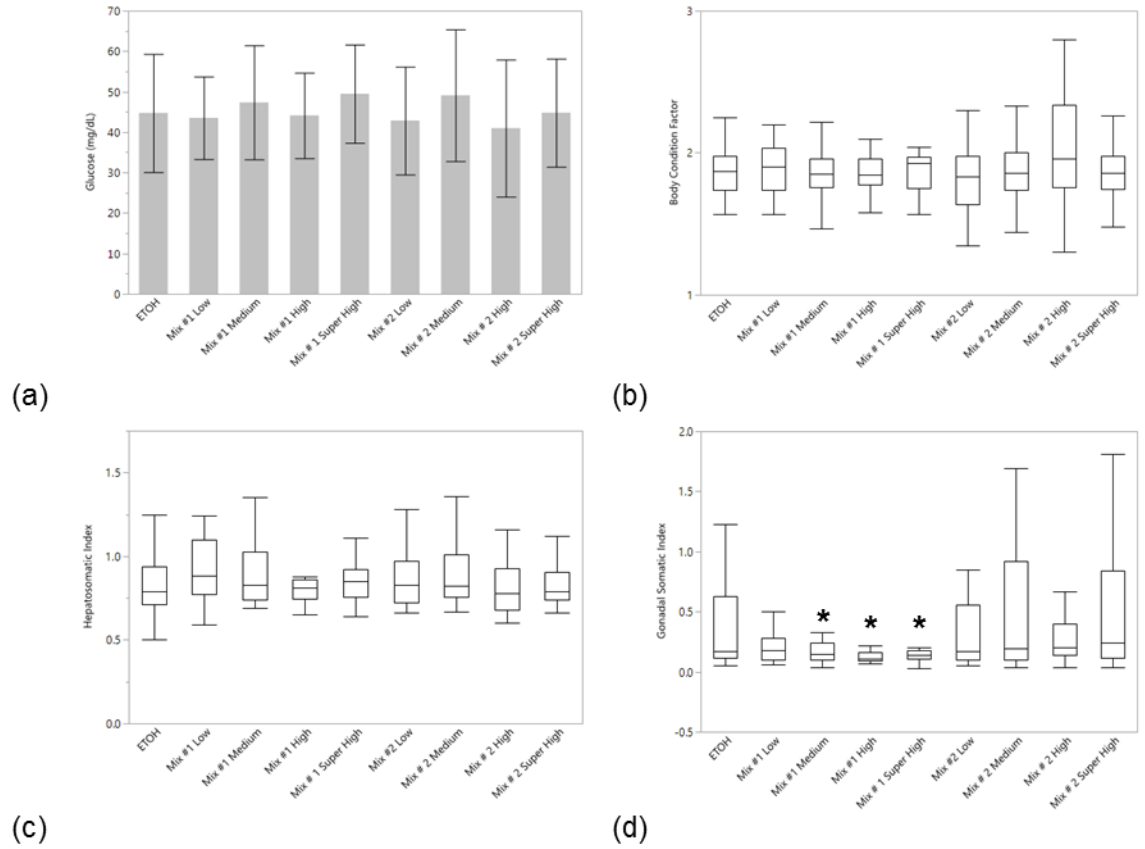


Figure 3.1**.** Male sunfish results for measured glucose and calculated biological indices. (a) Glucose (mg/dL). Bars represent mean glucose +/- standard deviation. (b) Body Condition Factor ((body weight/total length) ^{3} x 100,000). (c) Hepatosomatic Index ((liver weight/body weight) * 100). (d) Gonadal Somatic Index ((gonadal weight/body weight) *100). Boxes represent mean and range of values. Whiskers represent the 95% confidence interval.

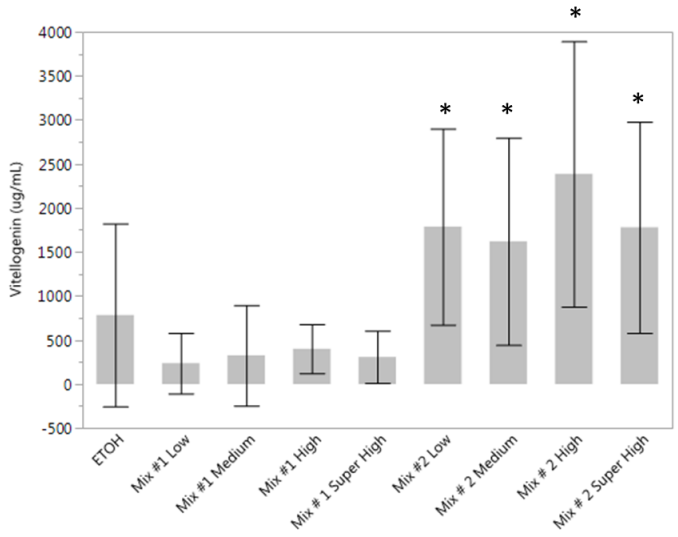


Figure 3.2. Vitellogenin concentration (ug/mL) of male sunfish by treatment. Bar represent mean +/ standard deviation.

A significant difference in plasma vitellogenin concentrations was observed by treatments (ANOVA, $F= 23.4943$, $df= 8$, $p<0.001$) (Figure 3.2). Sunfish exposed in the Mixture #2 Low, Medium, High, and Super High treatments contained higher plasma vitellogenin concentrations than fish in the Mixture #1 Low, Medium, High, and Super High treatments.

Table 3.2. Summary of male sunfish endpoints. Means +/- standard deviation.

Treatment	Glucose (mg/mL) +/- SD	Body Condition Factor +/- SD	Hepatosomatic Index +/- SD	Gonadal Somatic Index +/- SD	Vitellogenin +/- SD
Ethanol Control	44.72 +/- 14.62	1.92 +/- 0.22	0.80 +/- 0.14	0.35 +/- 0.43	784.08 +/- 1039.05
Mix #1 Low	43.55 +/- 10.22	1.90 +/- 0.18	0.92 +/- 0.18	0.29 +/- 0.33	237.62 +/- 344.20
Mix #1 Medium	47.35 +/- 14.11	1.89 +/- 0.21	0.86 +/- 0.27	0.18 +/- 0.11	326.18 +/- 571.75
Mix #1 High	44.11 +/- 10.55	1.85 +/- 0.17	0.82 +/- 0.12	0.12 +/- 0.04	401.05 +/- 277.40
Mix #1 Super High	49.50 +/- 12.14	1.89 +/- 0.22	0.84 +/- 0.18	0.19 +/- 0.28	307.95 +/- 296.92
Mix #2 Low	42.85 +/- 13.34	1.81 +/- 0.23	0.84 +/- 0.22	0.41 +/- 0.53	1788.44 +/- 1113.89
Mix #2 Medium	49.10 +/- 16.30	1.88 +/- 0.20	0.80 +/- 0.30	0.56 +/- 0.62	1621.86 +/- 1177.49
Mix #2 High	41.00 +/- 16.94	2.37 +/- 1.37	0.81 +/- 0.23	0.33 +/- 0.32	2385.64 +/- 1508.34
Mix #2 Super High	44.80 +/- 13.37	1.88 +/- 0.20	0.85 +/- 0.19	0.49 +/- 0.50	1780.35 +/- 1199.54

Female Sunfish Assessment. Body condition factor was significantly different between treatments (ANOVA, $p=0.0268$) (Figure 3.4). The body condition factors of Mixture #1 High and Super High were significantly larger than of Mixture #2 High. No significant difference was observed in hepatosomatic index between treatments of female sunfish (ANOVA, $p>0.05$), but a significant difference was observed in the gonadal somatic index (ANOVA with Tukey HSD, $F= 2.7343$, $df= 8$, $p=0.0069$) (Figure 3.3). The gonadal somatic indexes of Mixture #2 High were significantly larger than for Mixture #1 High females. Similar to the male sunfish, there was no significant difference observed in glucose concentrations in the female sunfish between treatments (ANOVA, $p>0.05$) (Figure 3.3).

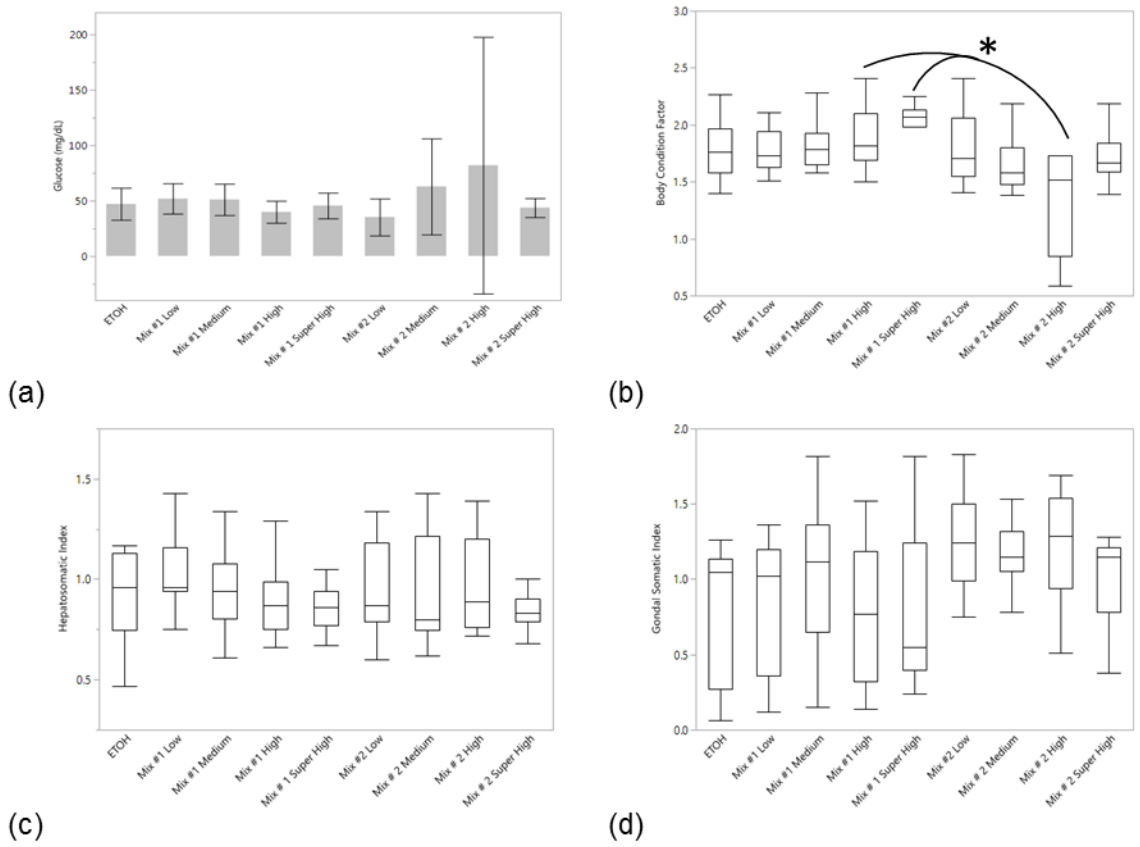


Figure 3.3. Female sunfish results for measured glucose and calculated biological indices. (a) Glucose (mg/dL). Bars represent mean glucose +/- standard deviation. (b) Body Condition Factor ((body weight/total length) ^{3} x 100,000). (c) Hepatosomatic Index ((liver weight/body weight) * 100). (d) Gonadal Somatic Index ((gonadal weight/body weight) *100). Boxes represent mean and range of values. Whiskers represent the 95% confidence interval.

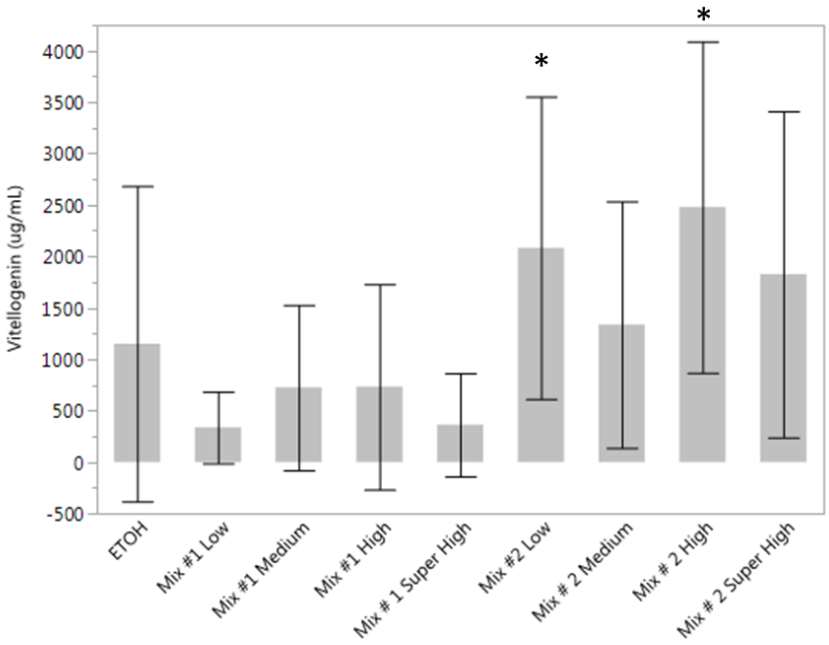


Figure 3.4. Vitellogenin concentration (ug/mL) of female sunfish by treatment. Bars represent mean +/- standard deviation.

Significant differences between treatments were observed for plasma vitellogenin concentrations in female sunfish (ANOVA with Tukey HSD, F= 27.3686, df= 8, p>0.0001) (Figure 3.4). Females in Mixture #2 Low, Medium, and High Treatments have significantly greater vitellogenin concentrations then of females in Mixture #1 Low, Medium, High and Super High treatments. Mixture #2 Low and High were also significantly higher than of the Ethanol Control.

Table 3.3. Summary of female sunfish endpoints. Means +/- standard deviation.

Treatment	Glucose (mg/mL) +/- SD	Body Condition Factor +/- SD	Hepatosomatic Index +/- SD	Gonadal Somatic Index +/- SD	Vitellogenin +/- SD
Ethanol Control	47.08 +/- 14.52	1.74 +/- 0.34	0.96 +/- 0.33	0.80 +/- 0.45	1151.42 +/- 425.12
Mix #1 Low	51.84 +/- 13.77	1.78 +/- 0.18	1.05 +/- 0.20	0.86 +/- 0.42	336.91 +/- 347.18
Mix #1 Medium	51.05 +/- 14.10	1.82 +/- 0.21	0.95 +/- 0.19	1.01 +/- 0.46	726.51 +/- 803.49
Mix #1 High	39.84 +/- 9.90	1.90 +/- 0.25	0.94 +/- 0.27	0.79 +/- 0.45	732.53 +/- 999.42
Mix #1 Super High	45.53 +/- 11.65	2.00 +/- 0.22	0.89 +/- 0.17	0.79 +/- 0.51	363.87 +/- 502.99
Mix #2 Low	35.14 +/- 16.73	1.79 +/- 0.30	0.92 +/- 0.23	1.27 +/- 0.34	2084.30 +/- 1469.94
Mix #2 Medium	62.45 +/- 43.38	1.66 +/- 0.25	0.96 +/- 0.28	1.17 +/- 0.22	1338.73 +/- 1198.49
Mix #2 High	81.91 +/- 115.9	1.51 +/- 0.24	0.95 +/- 0.25	1.31 +/- 0.53	2480.57 +/- 1610.46
Mix #2 Super High	43.60 +/- 8.57	1.73 +/- 0.22	0.90 +/- 0.24	1.00 +/- 030	1827.01 +/- 1586.15

Male Fathead Minnow Assessment. Body condition factor, hepatic somatic index, and gonadal somatic index of male fathead minnows did not differ between treatments (ANOVA, $p>0.05$) (Figure 3.5). There were also no significant differences for glucose concentrations between treatments of male fathead minnows (ANOVA, $p>0.05$; Figure 3.5). Secondary sexual characteristics did not differ between treatments (Figure 3.6).

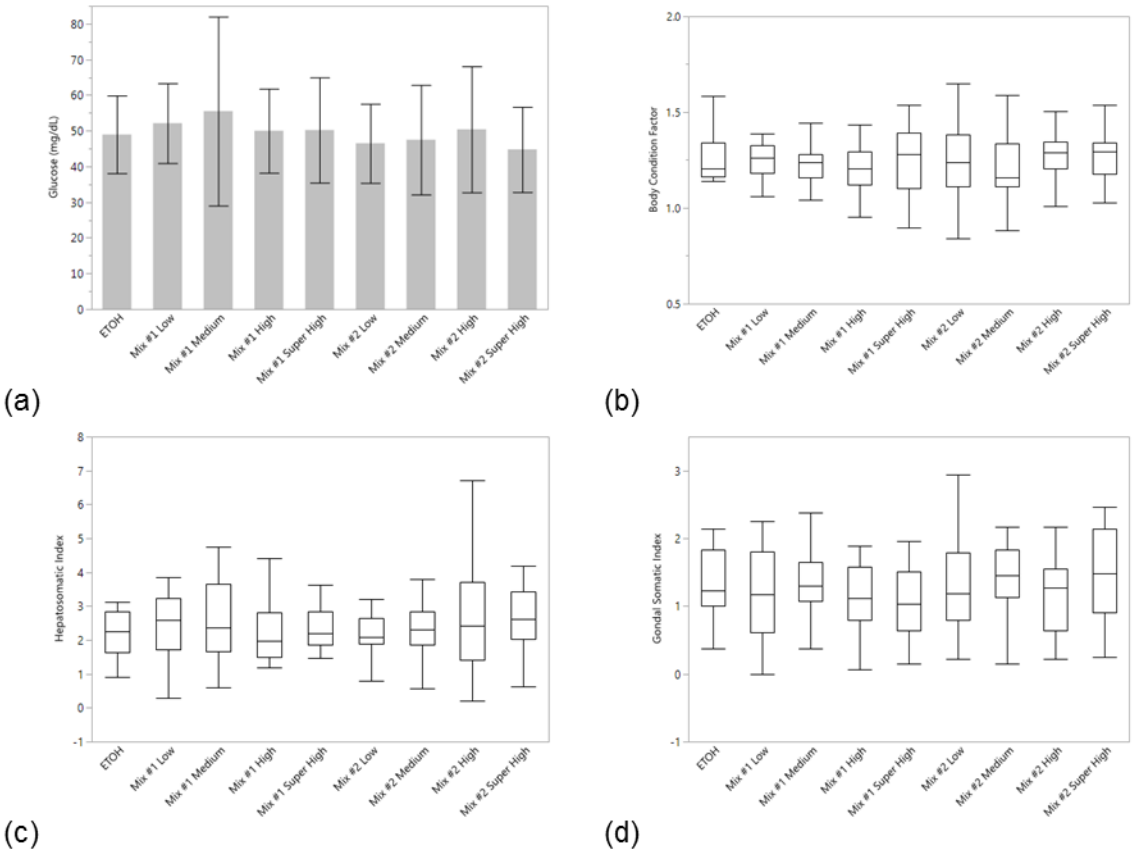


Figure 3.5. Male fathead minnow results for measured glucose and calculated biological indices. (a) Glucose (mg/dL). Bars represent mean glucose +/- standard deviation. (b) Body Condition Factor ((body weight/total length) ^{3} x 100,000). (c) Hepatosomatic Index ((liver weight/body weight) * 100). (d) Gonadal Somatic Index ((gonadal weight/body weight) *100). Boxes represent mean and range of values. Whiskers represent the 95% confidence interval.

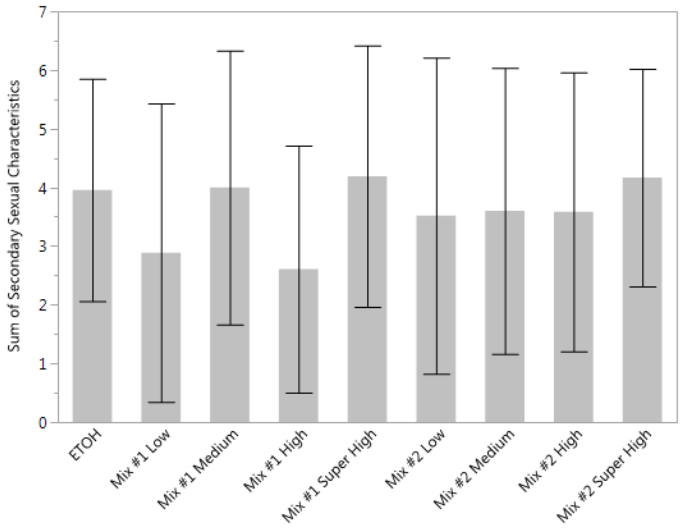


Figure 3.6. Sum of Secondary Sex Characteristics of male fathead minnows by treatment. Bars represent mean of each treatment with +/- standard deviation.

Table 3.4. Summary of male fathead minnow endpoints. Means +/- standard deviation.

Treatment	Glucose (mg/mL) +/- SD	Body Condition Factor +/- SD	Hepatosomatic Index +/- SD	Gonadal Somatic Index +/- SD	Sum of Secondary Sex Characteristics +/- SD
Ethanol Control	48.9 +/- 10.9	1.26 +/- 0.16	2.35 +/- 0.98	1.34 +/- 0.50	3.96 +/- 1.89
Mix #1 Low	52.1 +/- 11.2	1.23 +/- 0.18	2.46 +/- 0.96	1.22 +/- 0.91	2.89 +/- 2.54
Mix #1 Medium	55.5 +/- 26.5	1.22 +/- 0.12	2.49 +/- 1.19	1.36 +/- 0.49	4.00 +/- 2.33
Mix #1 High	50.0 +/- 11.8	1.20 +/- 0.13	2.17 +/- 0.87	1.18 +/- 0.69	2.61 +/- 2.10
Mix #1 Super High	50.2 +/- 14.7	1.30 +/- 0.32	2.35 +/- 0.64	1.07 +/- 0.52	4.19 +/- 2.23
Mix #2 Low	46.5 +/- 11.1	1.24 +/- 0.21	2.15 +/- 0.64	1.25 +/- 0.66	3.52 +/- 2.69
Mix #2 Medium	47.5 +/- 15.4	1.20 +/- 0.17	2.36 +/- 0.72	2.40 +/- 4.21	3.60 +/- 2.44
Mix #2 High	50.4 +/- 17.6	1.28 +/- 0.15	3.69 +/- 5.50	1.16 +/- 0.56	3.58 +/- 2.38
Mix #2 Super High	44.8 +/- 11.9	1.27 +/- 0.13	2.63 +/- 0.92	1.46 +/- 0.65	4.17 +/- 1.86

Female Fathead Minnow Assessment. In female fathead minnows, there were no significant differences in body condition factor, hepatic somatic index, or gonadal somatic index between treatments (ANOVA, all $p>0.005$) (Figure 3.7). There was a significant difference in glucose concentrations of females between treatments (ANOVA with Tukey HSD, $F= 3.6334$, $df= 8$, $p=0.0008$) (Figure 3.7). Mixture #1 Low had significantly higher glucose concentrations then of females in the Mixture #1 Medium and Ethanol Control (Tukey's HSD, $p<0.001$).

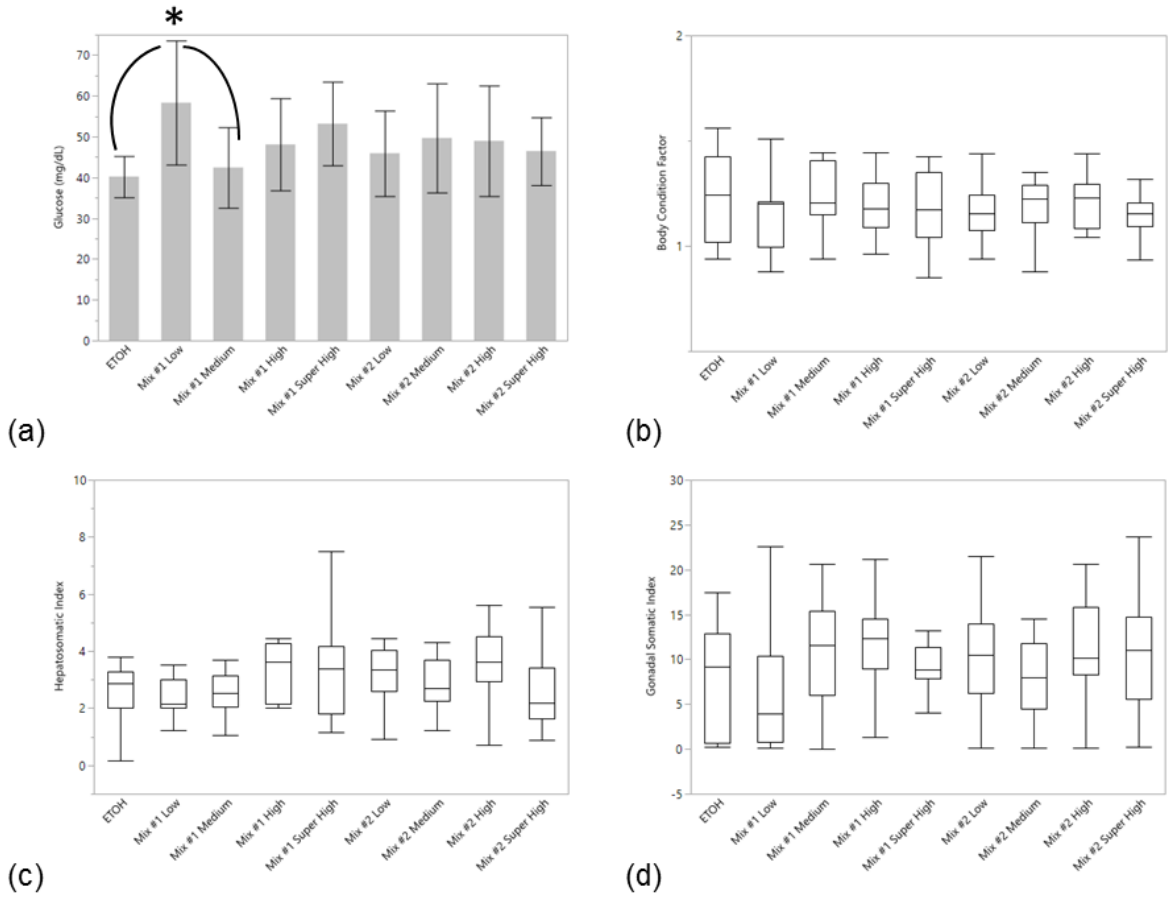


Figure 3.7. Female sunfish results for measured glucose and calculated biological indices. (a) Glucose (mg/dL). Bars represent mean glucose +/- standard deviation. (b) Body Condition Factor ((body weight/total length) ^{3} x 100,000). (c) Hepatosomatic Index ((liver weight/body weight) * 100). (d) Gonadal Somatic Index ((gonadal weight/body weight) *100). Boxes represent mean and range of values. Whiskers represent the 95% confidence interval.

Female fathead minnows were assessed daily for fecundity. The mean cumulative eggs production per day were averaged by the number of females in the treatment (Figure 3.8). Females exposed to Mix #1 High had the highest fecundity whereas in Mixture #2 the Medium concentration produced the most cumulative eggs/day/female.

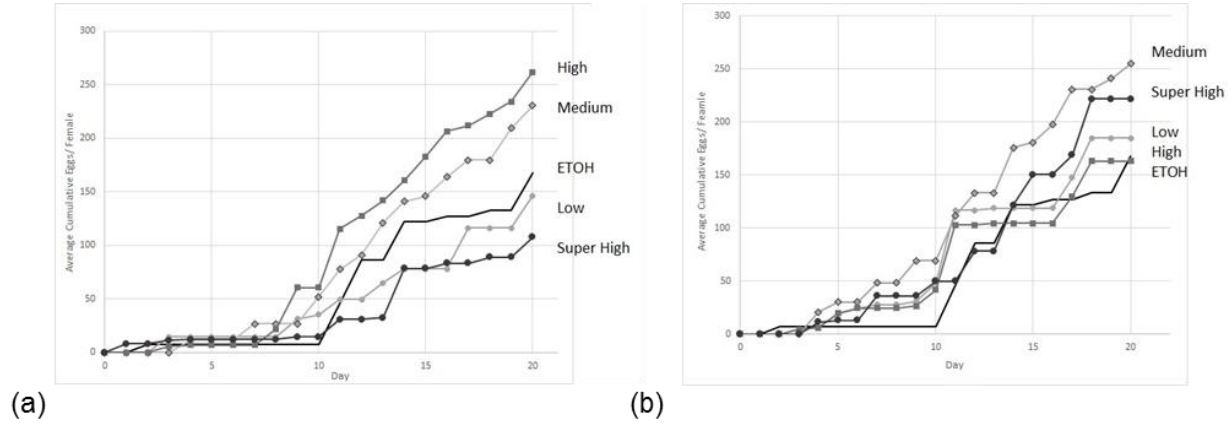
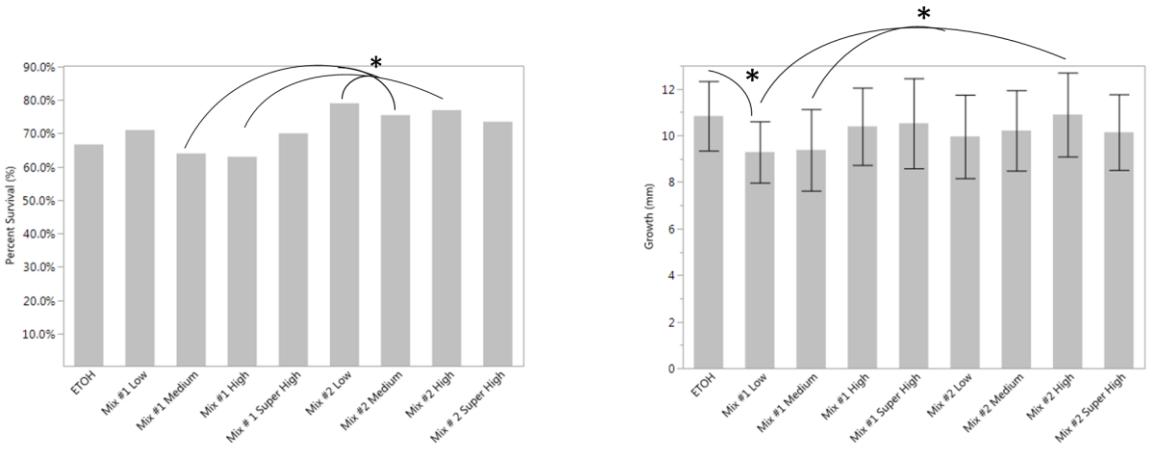


Figure 3.8. Average cumulative eggs per female/day for female fathead minnows. (a) Mix #1 by treatment. (b) Mix #2 by treatment.

Table 3.5. Summary of female fathead minnow endpoints. Means +/- standard deviation.

Treatment	Glucose (mg/mL) +/- SD	Body Condition Factor +/- SD	Hepatosomatic Index +/- SD	Gonadal Somatic Index +/- SD
Ethanol Control	40.2 +/- 5.06	1.54 /- 1.36	2.48 +/- 1.08	8.04 +/- 5.85
Mix #1 Low	58.33 +/- 15.22	1.14 +/- 0.18	2.86 +/- 1.97	6.69 +/- 6.70
Mix #1 Medium	42.44 +/- 9.86	1.31 +/- 0.33	3.87 +/- 5.99	10.3 +/- 6.11
Mix #1 High	48.13 +/- 11.28	1.26 +/- 0.37	4.79 +/- 6.22	11.7 +/- 4.66
Mix #1 Super High	53.20 +/- 10.26	1.78 +/- 0.16	3.24 +/- 1.66	9.27 +/- 4.09
Mix #2 Low	45.92 +/- 10.50	1.17 +/- 0.14	3.13 +/- 1.03	10.6 +/- 5.52
Mix #2 Medium	49.67 +/- 13.36	1.20 +/- 0.13	2.85 +/- 0.87	9.25 +/- 7.56
Mix #2 High	49.00 +/- 13.55	1.25 +/- 0.22	3.55 +/- 1.25	11.1 +/- 5.33
Mix #2 Super High	46.44 +/- 8.35	1.18 +/- 0.15	2.68 +/- 1.35	10.9 +/- 6.44

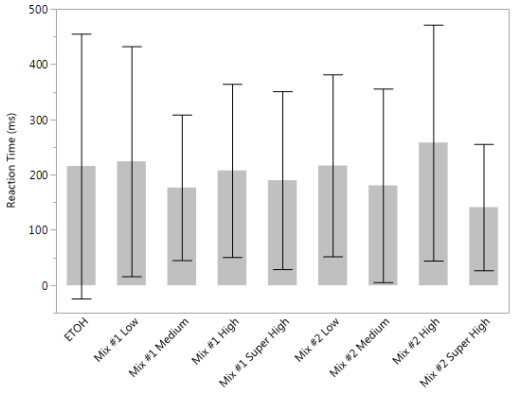
3.3.2: Larval Fathead Minnow Exposures and Assessment. After completion of the 21-day static renewal exposure to the two mixtures and an ethanol control, survival and growth were assessed. A significant decrease in survival was observed in the Mix #1 Medium and High treatments when compared to the Mix #2 Low, Medium, and High treatments. (ANOVA with Tukey HSD, F=2.0639, df= 8, p= 0.0491; Figure 3.9). A significant decrease in growth was observed in the Mix #1 Low and Medium treatments compared to the Mix #2 High (ANOVA with Tukey HSD, F= 3.2673, df= 3, p = 0.0015); Figure 2.5). The ethanol control was also significantly higher than the Mix #1 Low treatment (ANOVA with Tukey HSD, F= 3.2673, df= 3, p = 0.0015).



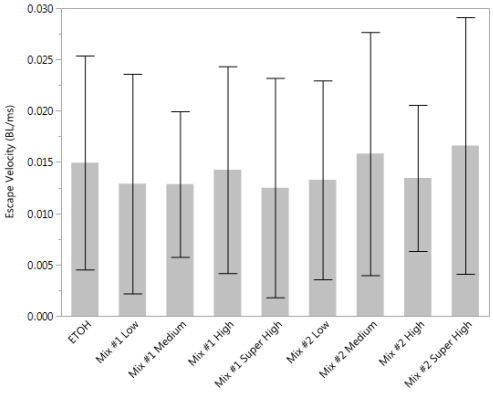
(a) (b)

Figure 3.9. Mean survival and growth of larvae exposed to both mixtures at four concentrations each and an ethanol carrier control. (a) Mean survival for each treatment in percent-survived (number survived/20). (b) Mean growth (mm) for each treatment. Bars represent mean survival $\pm$ standard deviation.

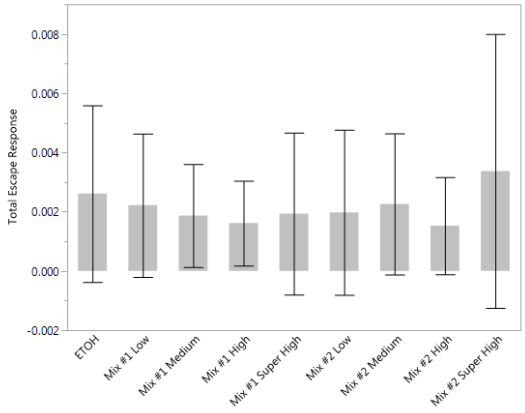
Reaction time, escape velocity, and total escape response did not differ between treatments (ANOVA, $p>0.05$) (Figure 3.10). When comparing the feeding efficiency of exposed larvae, a significant decrease in feeding performance was observed in the Mix #1 High and Super High treatments than of the Mix #2 Medium and High treatments. (ANOVA with Tukey HSD, $F= 4.7389$, $df= 8$, $p= <0.0001$) (Figure 3.11).



(a)



(b)



(c)

Figure 3.10 Effects of 21-day exposure of larvae to both mixtures at four concentrations each and an ethanol carrier control on predator escape performance. (a) Mean reaction time to start of stimulus and first movement (ms) (b) Mean escape velocity (BL/ms) in body lengths (BL) for the first 40ms after first movement (c) Total escape response that considers both the latency and escape velocity. Bars represent mean + standard deviation.

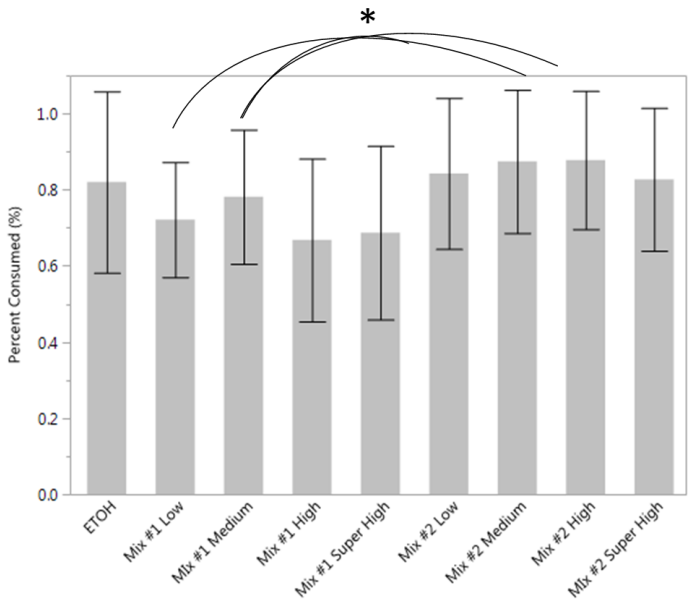


Figure 3.11. Percent of brine shrimp consumed during feeding efficiency assay by treatment. Bars represent mean +/- standard deviation.

Table 3.6. Summary of larval survival, growth, and percent consumed from 21-day laboratory exposures. Mean +/- standard deviation.

Treatment	Percent Survival +/- SD	Growth (mm) +/- SD	Percent Consumed +/- SD
Ethanol Control	66.7 +/- 13.0	10.8 +/- 1.50	82.0 +/- 23.8
Mix #1 Low	71.0 +/- 15.4	9.3 +/- 1.31	72.2 +/- 15.1
Mix #1 Medium	64.0 +/- 9.01	9.4 +/- 1.75	78.2 +/- 17.6
Mix #1 High	63.0 +/- 10.8	10.4 +/- 1.66	66.8 +/- 21.4
Mix #1 Super High	70.0 +/- 10.0	10.5 +/- 1.93	68.7 +/- 22.8
Mix #2 Low	79.0 +/- 8.1	9.9 +/- 1.79	84.2 +/- 19.8
Mix #2 Medium	75.5 +/- 14.9	10.22 +/- 1.72	87.4 +/- 18.8
Mix #2 High	77.0 +/- 14.9	10.9 +/- 1.80	87.8 +/- 18.1
Mix #2 Super High	73.5 +/- 13.5	10.1 +/- 1.63	82.7 +/- 18.8

Table 3.7. Summary of the predator escape endpoints after the 21- day larval exposure. Mean +/- standard deviation.

Treatment	Reaction Time (ms) +/- SD	Escape Velocity (BL/ms) +/- SD	Total Escape Response +/- SD
Ethanol Control	215.65 +/- 239.75	0.015 +/- 0.010	0.0026 +/- 0.0030
Mix #1 Low	224.33 +/- 208.26	0.013 +/- 0.011	0.0022 +/- 0.0024
Mix #1 Medium	176.86 +/- 131.76	0.013 +/- 0.007	0.0019 +/- 0.0017
Mix #1 High	207.62 +/- 156.81	0.014 +/- 0.010	0.0016 +/- 0.0014
Mix #1 Super High	190.09 +/- 161.09	0.013 +/- 0.011	0.0019 +/- 0.0027
Mix #2 Low	216.73 +/- 164.68	0.013 +/- 0.010	0.0020 +/- 0.0028
Mix #2 Medium	180.79 +/- 175.19	0.016 +/- 0.012	0.0023 +/- 0.0004
Mix #2 High	258.00 +/- 213.58	0.013 +/- 0.007	0.0015 +/- 0.0016
Mix #2 Super High	141.13 +/- 114.45	0.017 +/- 0.013	0.0034 +/- 0.0046

3.4: Discussion

The water chemistry results from this study indicate that CECs are present in many if not all groundwater near Central Minnesota lakes. Since factors such as product use, household occupancy, and OWTS condition vary among residences in lakeshore dwellings, we predicted that chemistry would vary from site to site and that multiple mixtures would be needed to assess the biological effects on fish species. Chemistry results indicated that the septic-influenced sites are indeed not homogeneous. Results also suggest that complex mixtures of CEC enter Minnesota lakes through hydrological processes and matched results of Baker et al. (2014).

Minimal differences were observed in bluegill sunfish exposed for 21 days to CEC mixtures derived from the above-mentioned water chemistry. Two significant differences observed in male sunfish among treatments included the gonadal somatic index and plasma vitellogenin concentrations. Males in the Mixture #1 Medium, High, and Super High had significant lower gonadal somatic indexes then of other treatments. Vitellogenin concentrations were significantly higher in all treatments of Mixture #2 compared to all treatments of Mixture #1. Mixture #2 contained higher concentrations of the compound Oxybenzone. Oxybenzone has been show to act as an estrogenic active compound and induce vitellogenin in males of rainbow trout (Coronado et al., 2008).

Female sunfish had a significant reduction in the body condition factor in Mixture #2 High females compared to Mixture #1 High and Super High females. The results of the gonadal somatic index in female sunfish show the Mixture #2 High treatment females had significantly larger gonads in proportion to body weight than of Mixture #1 High females. This is an indicator that these females in the Mixture #2 High are putting more energy into reproduction than of body

condition. Vitellogenin concentrations of Mixture #2 Low, Medium, and High females had significantly higher vitellogenin concentrations then of females in Mixture #1 Low, Medium, High, and Super High. This correlates with the vitellogenin concentrations found in the male sunfish and the higher concentrations of Oxybenzone in Mixture #2.

The effects of CEC exposure are less consistent when assessing the results of the fathead minnow exposures. The only significant difference observed in the adult fathead minnow exposures was an increase in blood glucose concentrations in female fathead minnows from the Mixture #1 Low when compared to Mixture #1 Medium and Ethanol Controls. Mixture #1 Low females also had the lowest fecundity of the Mixture #1 treatments. Higher glucose is an indicator of higher stress, which may suggest why these females produced less eggs.

Survival in larval fish was reduced in all Mixture #1 treatments when compared to all Mixture #2 treatments. Mixture #1 Medium and High treatments had significantly lower survival then of Mixture #2 Low, Medium, and High treatments. When assessing growth, larvae in the Mixture #1 High treatment were significantly larger than larvae in Mixture #2 Low and Medium treatments. This may be due to the lower survival in these treatments and the resultant reduced density in the Mixture #1 Low jars. No significant differences were observed in any endpoints of the Predator Escape Response. This may indicate that exposure to these mixtures may be less influential on the larval stage. However, it is noteworthy that these larvae were not exposed during the embryonic stage. McGee et al. (2009) found that exposure at different life stages (embryonic and larval) had different effects when exposure compound was changed.

In conclusion, biological pathologies observed differed between the two mixtures and the two species. This is a strong indicator that OWTSs and their respective flow paths play an essential role in the effects that one may see in a field study in these lakes. This also indicates that not all species are affected equally and concentrations found in lakes may play an essential role in the pathologies observed for each respective species. Specifically, in our study, Mixture #2 showed worsened effects in the sunfish species with induced vitellogenin levels in both males and females, but also significant reductions in the gonadal somatic index of males and body condition factor of females. In contrast, Mixture #1 showed reduced survival in the larval fathead minnows after the 21-day exposure. Complex mixtures of CECs can act vary differently depending on the life stage, targeted species, and concentrations of CECs in the mixture. Continued research is needed to determine the CECs present in lake systems and their biological effects on the resident fish species.

CHAPTER 4: CONCLUSIONS

When comparing the results from both studies, some similarities, but also may differences were observed. In the resident male sunfish collected from the study lakes, an induction of vitellogenin and the reduction in the hepatosomatic index were observed. Whereas in the laboratory study, the same vitellogenin induction was observed, but no reduction in the hepatosomatic index. But one key indicator of CEC exposure in males, the induction of vitellogenin, was observed in both.

As for larval exposures, a reduction in survival was observed in both the groundwater and laboratory mixture exposures. Lake sites (both septic-influenced and reference sites) had significantly lower survival when compared to the control. But it is also important to remember that CECs were detected at each site, total concentrations were just much lower in reference sites compared to septic-influenced sites. This could indicate that even at lower concentrations, these CECs could be causing on effect. A reduction in survival was also observed in the Mixture #1 Medium and High treatments. No significant differences in the predator escape response was observed in either groundwater or laboratory exposures.

The results of this study indicate that more research is needed on the sources of CECs to lake systems and that the effects on the resident fish living in lakes needs to be further evaluated. Some biological endpoints are shown to be affected by exposure to CECs found in septic seepage, but these lakes are also very complex systems and exposure to these compounds have a lot of variables like nutrition and natural stresses. It is also important to realize that resident fish are possibly exposed for an entire life cycle and our laboratory studies are merely 3 weeks.

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