St. Cloud State University Project Final Report

June 2011

Assessing the Contribution of Microhabitat Differences on the Biological Effects of Endocrine Active Compounds in Bluegill Sunfish in Sullivan Lake, MN

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Synopsis

Our 2008-09 MN Statewide Lake study revealed the ubiquitous presence of endocrine active compounds (EACs) in many Minnesota lakes. The presence of EACs in many lakes was matched by the occurrence of biological responses in resident and caged fish that was consistent with exposure to EACs. However, little correlation was observed between EAC concentrations and observed biological effects. Furthermore, the occurrence of biological effects exhibited substantial variability within sampled lakes and between fish species sampled within a lake. These findings suggest two potential knowledge gaps in our understanding of EACs and their effects in lake environments. First, the sources of EACs and their entrance points into lakes need to be better defined than was possible in our previous statewide lake study. Second, fish habitats within the littoral zone of lakes where greatest biological production occurs, need to be matched with detailed, site-specific exposure patterns.

In the current study, we addressed these knowledge gaps by conducting a detailed analysis of the occurrence and biological effects of potential sources of EACs in one lake that was part of the previous study. Specifically, we used lakeshore owner surveys and geological data to identify three types of land use characteristics that were hypothesized to have the potential to introduce EACs into the lake: (i) on site septic systems (2 study sites); (ii) agriculture; and (iii) public access sites. Furthermore, we used biological characteristics of the limnic environments to identify sites in which native fishes are likely to forage and reproduce, enhancing their chances to encounter EACs

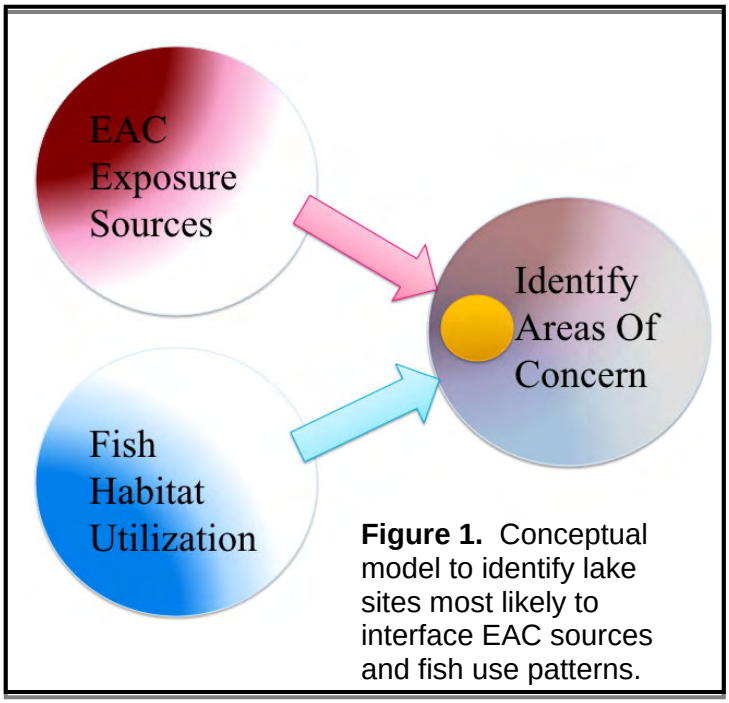


Figure 1. Conceptual model to identify lake sites most likely to interface EAC sources and fish use patterns.

(Figure 1).

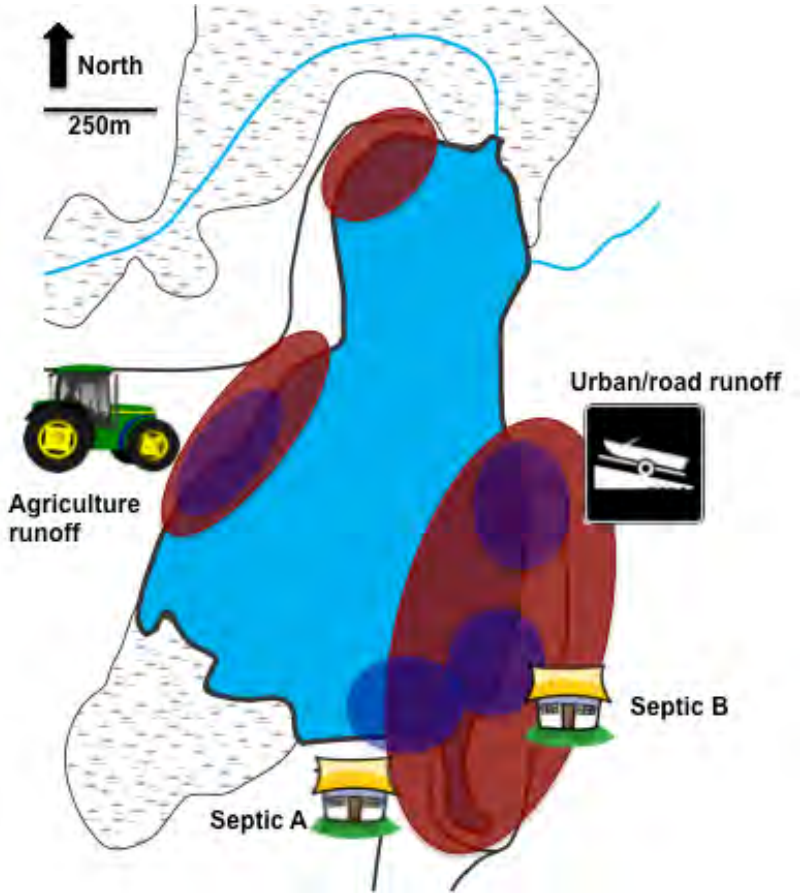


Figure 2. Sullivan Lake, Wright County, MN. Blue shapes indicate areas identified as likely being entry points for EACs using lakeshore owner survey data and geological and hydrological methods; brown ellipsoids indicate areas of high sunfish density to spawning or foraging grounds. Four study sites (septic 1, septic 2, public access; and agricultural sites are indicated as well.

Once four study sites had been identified (Figure 2), we deployed a multitude of techniques to test the hypothesis that ***Biological Responses in Fish are Correlated with Microhabitat Exposure to Endocrine Active Compounds (EACs) Within a Lake.*** Specifically, we complete the following objectives:

1. We assessed short- and long-term EAC exposure effects on caged adult sunfish in confined habitats in the littoral zone of a lake. (two caged sunfish fish exposure experiments)
2. To differentiate physiological effects in adult native sunfish based on microhabitat differences in sources and concentrations of EACs. (wild fish collection)
3. To characterize the exposures of fish during embryonic and larval development to EACs within nest sites in different microhabitats as defined by EAC sources.
4. To measure the consequences of embryonic and larval exposures of fish in microhabitats to EACs of various sources.

Methodology & Results

Identification of Microhabitats through Lakeshore Owner Surveys, Geology & Hydrology.

Geographic, geological, and chemical data from Sullivan Lake were obtained from the previous study conducted by Writer et al. (Anthropogenic tracers, endocrine disrupting chemicals, and endocrine disruption in Minnesota lakes. *Science of the Total Environment* 409: 100-111). Topographic maps of Sullivan Lake were analyzed by hydrologist Don Rosenburry to identify land use characteristics of the lake's interactions with its surrounding watershed. Identification of land formations has the potential to predict areas in which groundwater discharge is likely to occur. In June 2010, thermal probes were used to measure ground water flow along the littoral zone of the lake in parallel with measuring pH and nitrate concentrations to determine the physio-chemical characteristics of pore and surface water (Table 1). Water temperature and pore water temperature were measured and recorded approximately every 25 feet along the eastern shoreline of Sullivan Lake (shoreline with highest density of single family dwellings and location of public access to the lake), in instances where temperature differences were more severe, measurements were taken every 5-10 feet. GPS coordinates and lakebed composition, as well as detailed descriptions of the area where measurements occurred, were also recorded. Temperature data was then used to determine residual temperature differences, indicating ground water flow into the lake, out of the lake, or a mixture of both.

Table 1. Field data collected to identify microhabitats of interest/ groundwater inflow; data collected during June 1st, 2010 survey. Numbers in red suggest increased septic inflow. NR-not recorded

Field station	*Waypoint*	*Matrix*	*Lakebed*	*pH (units) **	*SC (umhos/cm)*	*T (deg C)*	*$T_{residual}$*	*NO3*
Surface water and pore water physio-chemical characteristics of the Eastern Shoreline of Sullivan Lake (from southwest [top] to northeast [bottom])	Southwest of Septic Site A	Pore	Silt/Clay	NR	1395	18.6-19.1	3.4 to 3.9	0.2
		Lake	Silt/Clay	7.16	416	22.5		0.2
		Pore	Silt/Clay	NR	548	16.0 to 16.9	5.6 to 6.5	0.2
		Lake	Silt/Clay	7.89	379	22.5		0.2
	Septic Site A	Pore	Sandy	NR	590	16.6	7.1	NR
		Lake	Sandy	7.87	403	23.7		NR
		Pore	Sandy	NR	610	21.4	2.3	0.1
		Lake	Sandy	8.19	402	23.7		NR
		Pore	Sandy	NR	754	NR	NR	ND
		Lake	Sandy	7.85	424	23.7		NR
		Pore	Sandy	NR	721	NR	NR	ND
		Pore	Sandy	NR	871	NR	NR	ND
		Pore	Sandy	NR	916	NR	NR	ND
		Lake	Sandy	7.75	400	24.39		0.2
		Pore	Sandy	NR	1229	NR	NR	ND
	Septic Site B	Pore	Sandy	NR	876	NR	NR	0.3
		Pore	Sandy	NR	692	NR	NR	0.3
		Pore	Sandy	NR	678	NR	NR	0.3
		Pore	Sandy	NR	2053	NR	NR	0.2
		NR	Silt/Clay	NR	NR	NR	NR	NR
	Between Septic Site B and	Pore	Silt/Clay	NR	1868	NR	NR	4.6
		Pore	Silt/Clay	NR	1454	NR	NR	ND

	Urban/Runoff							
	Urban/Runoff	Lake	Silt/Clay	8.4	389	24.3		NR

Using this data, we identified sites in which ground water inflow was indicated (Table 2). Piezometers and septic seepage meters were used to further confirm ground water flow patterns. A 40m x 20m perimeter was established at septic sites A and B and were considered septic-inflow microhabitats; all further water sampling, fish sampling, and fish caging took place within these plots. Using geographic data and observations, we also identified areas of the shoreline where land use patterns indicated possible urban/road-runoff and agricultural run-off contamination.

Table 2. Groundwater and surface water temperature differences along the eastern shoreline of Sullivan Lake (residential shoreline); data collected during June 1, 2010 reconnaissance survey. "Down" indicates lake water outflow into the sediment; "up" indicates inflow of groundwater into the lake through the sediment; "mixed" indicates flow in both directions based on diurnal patterns.

	T_{lake}	T_{pore}	$T_{residual}$	*Interpretation*
Surface water and pore water temperature characteristics of the Eastern Shoreline of Sullivan Lake (from southwest [top] to northeast [bottom])	23.2	19.2	4	Mixed/down
	23.2	20	3.2	Mixed/down
	23.2	19.4	3.8	Mixed/down
	23.2	17.1	6.1	Mixed/up
	23.2	17.1	6.1	Mixed/up
	23.2	17.1	6.1	Mixed/up
	23.2	16.8	6.4	Mixed/up
	23.2	18.3	4.9	Mixed/down
	23.2	17.3	5.9	Mixed/down
	23.2	17.6	5.6	Mixed/down
	23.2	16.8	6.4	Mixed/up
	23.2	16.5	6.7	Mixed/up
	23.2	16.3	6.9	Mixed/up
	23.2	14.4	8.8	Mixed/up
	23.2	14.1	9.1	Mixed/up
	23.2	15.7	7.5	Mixed/up
	23.2	17.4	5.8	Mixed/down
	23.2	20.8	2.4	Mixed/down
	23.2	21.7	1.5	Down
	23.2	21.6	1.6	Down
	23.2	21.3	1.9	Down
	23.2	23.2	0	Down
	23.2	21.6	1.6	Down
	23.2	21.7	1.5	Down
	23.2	20.4	2.8	Mixed/down
	23.2	21.3	1.9	Down
	23.2	21.9	1.3	Down
	23.2	20.3	2.9	Mixed/down
	23.2	19.5	3.7	Mixed/down
	23.2	19.7	3.5	Mixed/down
	23.2	19.6	3.6	Mixed/down
	23.2	19.6	3.6	Mixed/down
	23.2	20.8	2.4	Mixed/down
	23.2	21.4	1.8	Down

* $T_{residual} = T_{lake} - T_{pore}$; $T_{residual} < 2°C$ indicates probable lake water loss downward to groundwater;

2°C < $T_{residual}$ < ~10°C indicates likely mixture of ground water and surface water $T_{residual}$ > ~ 10°C indicates probable ground water discharge to lake.

Geography of the Lake. Analysis of topographic maps of Sullivan Lake indicated a glacial geology. Wetlands to the north, northwest, and southwest of the lake, suggests less gradient to drive flow and making groundwater discharge occurring along the western shoreline of Sullivan Lake less likely. The esker-like ridge along the eastern shoreline creates potential for groundwater discharge driving septic leachate plumes into the lake if a substantial groundwater mound is present beneath that ridge. This would drive flow of groundwater into the lake along the eastern shoreline, indicating the greatest likelihood of anthropogenic constituents in the sediments. High terrain to the south may result in a similar groundwater flow along that shoreline.

Geographic analysis of land use patterns was obtained from the Minnesota DNR Data Deli (http://deli.dnr.state.mn.us/). Land approximately 0.25 miles west, north, and south of the lake and 0.5 miles east of the lakes was analyzed. Data reported that as of 2009, forests and farmland surrounded 26% of Sullivan Lake, 18% was attributed to residential areas, 17% to wetland/grassland, to other lakes, and 2% to roads. This data was used to predict potential sources of pollution.

Population surveys approved by the St. Cloud State University Institutional Review Board were used to collect further information regarding residential land use. Household participation in the survey amounted to 24 household out of the 60 that were sent surveys, accounting for 64 individuals. Eleven surveys were reported from the south and east shorelines, three surveys were reported from the northwest shoreline, and ten surveys were reported from residents that live on the far side of the lakeshore roads (not directly on the lake). Approximately 27% of residential participants living on the southeast shoreline have on-site septic systems that that were installed more than 15 years ago, 27% were installed between 5 and 15 years ago, 18% were installed within the last 5 years and approximately 27% of respondents were unsure when their septic system was installed. Of the 3 returned surveys from residents of the northwest shoreline, one household reported having an on-site septic system that was installed longer than 15 years ago, while the other two households reported having septic systems between 5 and 15 years old. Of the returned surveys from households living on the opposite side of the lakeshore road, 70% reported having septic systems that were installed longer than 15 years prior, 10% reported having septic systems between 5 and 15 years old, and 20% don't know how old their septic systems are. Surveys also indicated that 58% of all participants currently have household pets.

Objective 1. *We assessed short- and long-term EAC exposure effects on caged adult sunfish in confined habitats in the littoral zone of a lake.*

Objective 2: *To differentiate physiological effects in adult sunfish based on microhabitat differences in sources and concentrations of EACs.*

Following microhabitat assessment and selection, we utilized the chosen microhabitats to carry out ground and surface water analysis, fish exposures, and the collection of resident fish to determine EAC exposure and effects. The caging of bluegill sunfish and collection of resident sunfish was permitted by the Minnesota Department of Natural Resources, and the St. Cloud State University Animal Care and Use Committee (IACUC) approved all procedures. Bluegill sunfish (supplied by 10,000 Lakes Aquaculture, a certified disease free facility) were caged once during the spawning season and once after the spawning season ended. For each caging event, 40 fish were caged at 4 sites, which had been characterized as being influenced by septic, urban, or agricultural run off (Figure 1). The fish were deployed for 21 days, and were fed brine shrimp on day 7 of the exposure

period. Water samples for each site were collected on day 7 for estrogenicity quantification. The fish were collected on day 21, placed into aerated coolers and transported to the St. Cloud State Aquatic Toxicology Laboratory for biological assessment.

Resident sunfish were collected using seining techniques during the spawning season (July 2010). The target collection size was 20 sunfish per microhabitat (resident fish were not collected from the agriculturally influenced site). Fish were sacrificed in the field using MS 222 (Finquel, Argent Chemical Laboratories, Redmond, WA) and stored in containers of 10% formalin until biological analysis and dissection.

Body Condition Indices. The same biological assessment procedures were used for both resident and caged bluegill sunfish. Fish were sacrificed in MS 222, weighed and measured for total body length to calculate the body condition factor (BCF) (weight/ (total length)^3 x 100,000). Livers and gonads were then removed and weighed to calculate the gonadosomatic index (GSI; mass testes/mass fish x 100) and the hepatosomatic index (HIS; mass liver/ mass fish x 100; Allen et al. 1999). Tissue samples were placed in histocassettes and stored in 10% buffered formalin until histological processing and analysis.

Vitellogenin Analysis. Blood samples were drawn via capillary tube from the caudal vein and centrifuged (3500 rpm for 5 min) for plasma separation. Plasma samples were stored at -80° C until analysis. As the egg-yolk protein vitellogenin (VTG) is normally produced below the detection limit in the plasma of male fish, its use as a biomarker of endocrine disruption has become a common procedure in assessing estrogenic exposure in fish species. VTG concentrations were determined using an enzyme linked immunosorbent assay (ELISA). Microtiter plate wells were coated with bluegill sunfish VTG at an approximate concentration of 4 µg/mL in coating buffer (0.5 M carbonate, pH 9.6). One well on each plate was coated with 5% bovine serum albumin in coating buffer for a non-specific binding subtraction. Samples and standards were prepared and mixed with a polyclonal anti-sunfish VTG antibody (provided by Steve Bartell, St. Cloud State University) at a 1:2500 dilution and incubated at 37°C for 2 hours. At the end of the incubation period, plates were washed 3 times in an automated plate washer; incubated samples and standards were added to the wells and incubated for 1 hour at room temperature. Plates were washed 3 times and probed with an anti-rabbit secondary antibody (Sigma Aldrich) at a dilution of 1: 10000 for 1 hour at room temperature. Plates were washed and 3,3', 5,5,'-tetra-methylbenzidine enzyme substrate was added and incubated under tin foil for approximately 20 minutes. Substrate absorbance was read at 620 nm on a Thermo Multiskan EX microplate reader (Vantaa, Finland), and logistic regression of the standards was used to calculate VTG concentrations. All plasma samples were analyzed at 3 dilutions and referenced against a multi-point standard curve (acceptable standard curve r^2>0.95).

Histological Analysis. Tissue samples were removed from 10% buffered formalin, dehydrated, and embedded in paraffin using a Jung TP1050 automated tissue processor (Leica, Wetzlar, Germany). Tissues in paraffin blocks were sectioned at a thickness of 5 µm on a Reichert-Jung 2030 microtome, sections were mounted on slides and stained in a Leica Autostainer XL using standard haematoxylin and eosin techniques (Gabe, 1976). Testicular form and function were assessed on a five-point scale based on microscopic evaluation sperm maturation (spermatagonia, spermatocytes, spermatids, and spermatozoa). Ovarian form and function were assessed on a 5-point scale based on microscopic evaluation of ovarian maturation (oogonia, cortical alveolar oocytes, perinuclear oocytes, and early-late vitellogenic oocytes), similar to US EPA guidelines. All gonads were assessed for histopathologies such as fibrosis, the presence of proteinaceous fluid, testicular feminization (loss of testicular structure and/or presence of oogonia), ovarian masculinization, and the occurrence of intersex (presence of testicular oocytes). The occurrence and abundance of hepatocyte vacuolization was assessed on a five-point scale. All biological samples were coded to ensure "blind" analysis of plasma and histological data. A second laboratory team member

independently verified histological samples and all incidences of testicular pathologies were photographed for future reference and analysis.

Statistical Analysis. Assumptions of normality were tested using with the Kolmogorov-Smirnov test for all data sets prior to any additional analysis (Prism 4.01 statistical package, GraphPad Software Inc, Oxnard, CA). Some data sets that did not meet normality standards were transformed (log) and subsequently tested with an ANOVA. Kruskal-Wallis (nonparametric) tests were used for the majority of data analysis, as data sets did not meet standards of homogeneity. Dunn's post-tests followed Kruskal-Wallis analysis; probability of $p < 0.05$ was set as the level of significance for all comparisons.

Results – Survival. Survival rates of caged sunfish at septic site B were greater than 90% and greater than 35% at the agricultural site in August 2010. Septic site A and the urban/road run-off microhabitat had total mortality of caged sunfish following the 14-day exposure in August 2010 (likely due to high water temperature and low dissolved oxygen content at the caging sites). Sunfish deployment in September 2010 resulted in survival rates greater than 90% for all microhabitats (septic A, septic B, urban/road run-off, agricultural). Wild sunfish were successfully collected from septic site A, septic site B, and the urban/road run-off site (20 fish at each site).

Results – Body Condition Indices. BCF was found to be significantly lower from baseline in fish caged at septic site A, septic site B, and the agricultural site (Figure 3). Fish caged at the public access site were also found to have larger BCFs than those caged at septic site A. When analyzed against wild sunfish, deployed fish caged at septic sites A and B were found to have a significantly lower BCF than sunfish native to those sites. Fish caged at septic site B were found to have significantly lower HSI than those caged at septic site A and baseline fish. No differences were found in HSI between caged and resident fish species at any given site ($p > 0.05$, Kruskal-Wallis, data not shown). Gonadosomatic indices did not reveal any differences between exposure sites or from baseline measurements ($p > 0.05$, Kruskal-Wallis, data not shown). However, analysis of resident sunfish GSI showed significant differences between septic sites 1 and 2 ($p < 0.05$, Kruskal-Wallis).

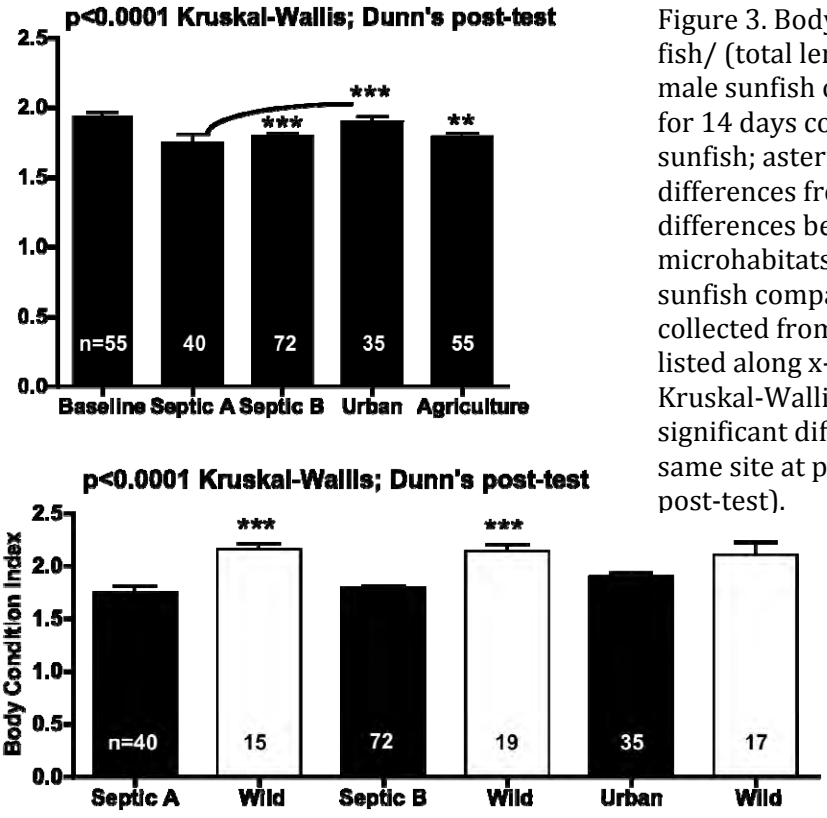


Figure 3. Body Condition Factor: (mass of fish/ (total length)^3) x 100,000. (Top) BCF of male sunfish caged at different microhabitats for 14 days compared to baseline male sunfish; asterix indicates significant differences from baseline fish, arch indicates differences between fish exposed at different microhabitats. (Bottom) BCF of caged male sunfish compared to BCF of wild male sunfish collected from each microhabitat. Sample size listed along x-axis. P-values derived from Kruskal-Wallis analysis; ** indicates significant differences from caged fish at same site at $p<0.01$; *** at $p<0.001$ (Dunn's post-test).

Results – Histological Analysis. Analysis of liver vacuolization indicated differences between exposure sites and baseline ($p < 0.05$, Kruskal-Wallis), however, a post-test did not reveal any significant differences (data not shown). When analyzed against wild sunfish, fish caged at septic site A were found to have a significantly greater amount of liver vacuolization than fish native to the public access site; more importantly, however, are the visible patterns between resident and caged fish from the same sites. Histological analysis of male and female gonads showed a decrease of gonad maturity in fish caged at septic site A as compared to the maturity of baseline fish. Similarly, resident fish from site A also were found to have a significant decrease in maturity. The occurrence of intersex in sunfish was found in a small percentage of baseline and caged sunfish, but not in wild sunfish.

Results – Vitellogenin Analysis. Analysis of VTG production between sunfish caged at each site revealed that those caged at septic site B produced significantly more VTG than sunfish caged the urban/road run-off site (Figure 4).

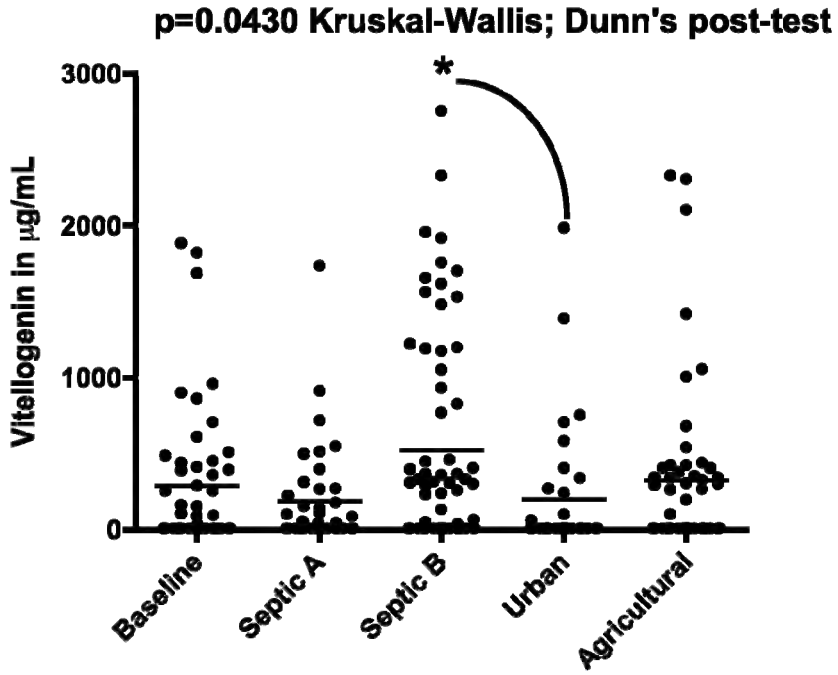


Figure 4. VTG concentrations (µg/mL) of caged male bluegill sunfish. P-values derived from Kruskal-Wallis analysis; * indicates significance at $p < 0.05$ (Dunn's post-test), arch indicates treatments that differed from each other significantly.

Objective 3: *To characterize the exposures of fish during embryonic and larval development to EACs within nest sites in different microhabitats as defined by EAC sources.*

Objective 4. *To measure the consequences of embryonic and larval exposures of fish in microhabitats to EACs of various sources.*

Post-hatch fathead minnow larvae (*Pimephales promelas*) were obtained from the USGS (Cincinnati, OH). Larvae were exposed to pore water collected from Sullivan Lake (duplicate experiments). The

collection of pore water was accomplished by penetrating the lakebed at a depth of 15-20cm with a steel-tipped piezometer. Once securely in the sediment, the external pipe of the piezometer was removed, exposing a nylon mesh filter near the tip of the piezometer. The fine mesh allows pore water to flow into the internal steel pipe while restricting sediment from entering. By connecting a hand pump to the internal pipe above the surface of the water, pore water can be drawn out of the sediment and collected. Pore water could not be collected from the agriculturally influenced site due to water depth; alternatively, surface water was collected following guidelines established by the US Geological Survey. Water samples were collected in 1-L nalgene containers, transported to St. Cloud State University in coolers, and frozen until the exposure was conducted. Prior to larval exposure, water samples were thawed to room temperature and filtered through glass fiber paper (Reeve Angel, Whatman Inc., Clifton, New Jersey) to remove organic particulates. Treatment groups consisted of groups of 25 larvae, exposed for 12 days in conditioned well water in 1-L Pyrex glass beakers. Larvae were treated with either pore water from septic sites A or B, pore water from the urban run-off site, or lake water from the agricultural site; a control group of larvae was exposed to conditioned well water. All water samples were added to the conditioned well water prior to daily water exchange at 50% static renewal and held at a constant temperature of 23°C ± 0.8°C . Exposures were conducted under constant photoperiod (16L: 8D) and larvae were fed twice daily with 1mL of hatched brine shrimp (Brine Shrimp Direct, Ogden, UT).

Predator Escape Performance. To assess predator escape performance of exposed larval fathead minnows, reflex behaviors to a predatory stimulus were examined. A trigger-activated system was used to measure escape behaviors, as documented by McGee et al. (2009; Aquatic Toxicology). A trigger, wired to a battery-operated chip, causes vibrations to be emitted from the chip resting beneath the filming arena. A light outside of the arena is simultaneously illuminated once the trigger is pushed. Light illumination, in parallel to triggering of the vibratory stimuli, is visible in the field of view of the filming arena to determine time zero of analysis. The filming arena, a plastic Petri dish (5-cm in diameter), sat atop a 1mm grid, above which a high-speed digital video camera was assembled (Redlake MotionScope M1, Tucson, AZ). The morning that exposure ended and filming occurred, larvae were fed 1 hour before escape behavior was analyzed. Each larva was chosen at random, testing treatments in a sequential pattern until all larvae had been filmed. Larva were allowed only one escape behavior recording. Throughout the filming period water temperature in the arena was kept constant at 21°C ± 0.6°C, allowing larvae to acclimate to the arena. Once a larva swam into the center of the arena (marked by a box), the trigger would be pushed to stimulate the vibration and light, consequently stimulating a C-start response. C-start response videos were stored as AVI files, digitized, and analyzed for body length, latency period, escape velocity, and total escape response (following methodology established by McGee et al., 2009, Aquatic Toxicology 91:355-361), except that velocity was adjusted to body lengths per millisecond to exclude size differences between individual fish as a confounding variable. Videos were excluded if the larva did not exhibit a C-start following the stimulus or swam out of the field of view. Larva were sacrificed following performance analysis, set in RNAlater (Ambion, Austin, TX) and frozen for subsequent RNA isolation.

Analysis of Gene Expression. Frozen larvae were transferred into 1mL of TRI reagent for homogenization with tools wipe clean with RNAseZap® (Ambion, Austin, TX) between each sample. RNA was isolated from 10 larvae per treatment group chosen at random, following protocols established by Life Technologies (Carlsbad, CA) using an Ambion RNA RiboPure Kit (Life Technologies, Carlsbad, CA). Isolated RNA was suspended in 100-200µl of elution buffer. RNA quality was assessed with the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA) and the quantity was determined on a NanoDrop spectrophetotometer (NanoDrop Technologies, Wilmington, DE); samples were stored at -80°C until gene expression analysis via real-time polymerase chain reaction (RT-PCR).

Real-time PCR was performed on RNA isolated from larvae exposed to pore (and surface) water from microhabitats within Sullivan Lake. All RNA samples were diluted to 10ng/µL. PCR analysis was used to compare the expression of genes that play a role in normal steroidogenesis in fathead minnows, identified to be potential targets of EACs. The expression of these genes of interest were then compared to the expression of a standard gene which was not exposed to EACs. Expression of the estrogen receptor α gene (ER) and steroidogenic acute regulatory protein (StAR) was compared to the expression of reference gene, rpl8. QPCR reactions were conducted with a one-step procedure using 2.0 µL of diluted RNA combined with 10 µL 2X Power Sybr Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 0.5mM each of either ER, StAR or RPL8 gene-specific primers. All samples were analyzed in duplicate for both genes, and amplified over 40 cycles. A melting curve analysis for each reaction determined probe stringency/specificity. A standard curve was also generated for quantification of transcript in samples using a linear regression formula. Results were analyzed using the Comparative Ct and ΔΔ Ct method.

Statistical analysis. Assumptions of normality were tested using the Kolmogorov-Smirnov test for all data sets prior to any additional analysis (Prism 4.01 statistical package, GraphPad Software Inc, Oxnard, CA). When parametric standards were not met, data was either transformed (log 10) or Kruskal-Wallis (nonparametric) tests were used for data sets. Dunn's post-tests followed Kruskal-Wallis analysis; probability of $p < 0.05$ was set as the level of significance for all comparisons. Gene expression data was analyzed for relative fold induction of the expression of genes under estrogenic control as compared to control genes to determine up- or down-regulation of genes.

Results - Survival Rates and Predator Avoidance Performance. The first exposure resulted in high mortality rates, falling between 100% mortality observed in larvae exposed to pore water from the urban run-off site and 48% mortality observed in control larvae. Analysis of urban run-off pore water indicated high levels of ammonia as the cause of increased mortality; however, the high mortality across treatments remained unexplained.

Survival rates greater than 95% were achieved for all treatments in exposure 2, except for larvae exposed to urban run-off pore water, in which 84% survival was observed. C-start analysis revealed that larvae exposed to pore water from the urban run-off microhabitat had significantly greater body lengths (mm) than larvae exposed to pore water from septic site A and the agricultural run-off site (Figure 5). Analysis of latency period, velocity, and total escape response did not result in any significant differences between treatments (data not shown).

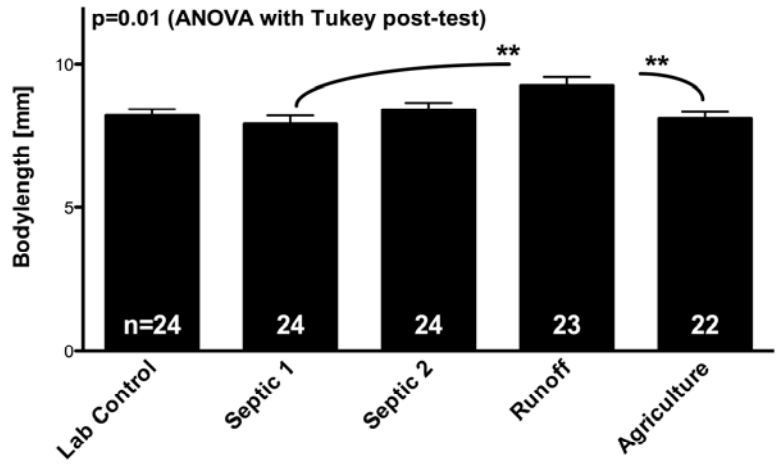


Figure 5. Body lengths (mm) of larval fathead minnows exposed to pore water (or surface water from the agricultural run-off site). P-values derived from an ANOVA; ** indicates a significant

difference from lab control larvae at $p<0.01$ Turkey's post-test, arch indicates significant differences between treatments.

Results - Gene Expression Analysis. To assess if larvae exhibited effects of estrogenic exposure, we analyzed rt-PCR expression changes in genes involved in steroidogensis. Following calculation of the relative fold induction of expression of ER, analysis of larvae revealed that those exposed to pore water from septic sites A and B, and the urban run-off site exhibited an increase in expression (equal to or greater than 1.6 relative fold induction) of the ER gene (Figure 6). Interestingly, increased expression of StAR was also observed in larvae exposed to pore water from septic site A and the urban run-off site, but not in those exposed to pore water septic site B (or surface water from the agricultural run-off site).

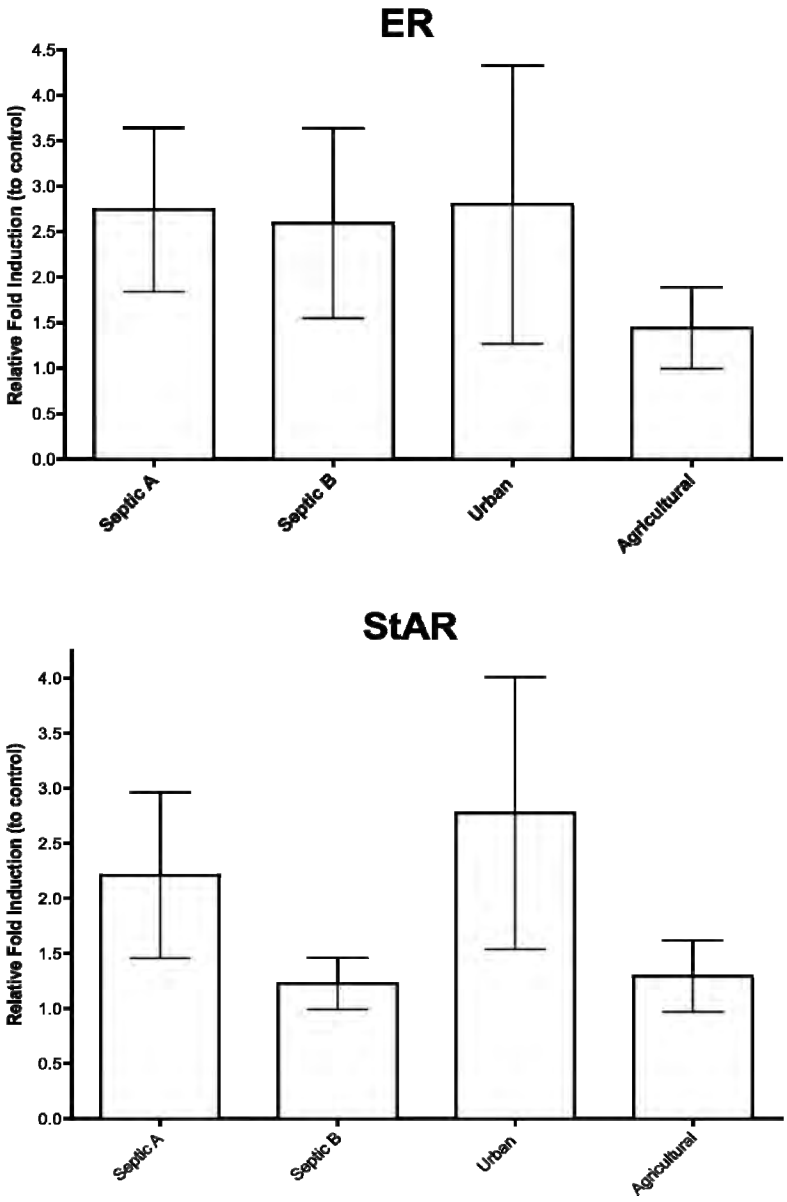


Figure 6. Relative fold induction of gene expression of Estrogen Receptor (ER – top graph) in larval fathead minnows exposed to pore water (surface water from the agricultural run-off site.

(Bottom graph) Relative fold induction of gene expression of StAR in larval fathead minnows exposed to pore water (surface water from the agricultural run-off site.

Gene expression of the target gene is standardized against expression of a "housekeeper" gene that should not be affected by the different treatments applied to the larvae.

Discussion

Limnological data and analysis of Sullivan Lake allowed for the identification of microhabitats that are likely heterogeneous in the occurrence and composition of EACs, due to differential sources of contamination entering this system. These sites were also found to be preferential spawning sites for bluegill sunfish, raising the likelihood of adverse biological effects incurred. Septic Site A and B were identified on the south and southeastern shorelines. Both were found to have higher than average residual temperatures based on lake and pore water comparisons, indicating groundwater inflow. Additional installation of seepage meters and the use of piezometers also indicated likely groundwater inflow at these areas. Sediment in these two microhabitats is a combination of sandy and mucky areas. Large bluegill spawning areas occurred within septic sites A and B.

The urban/road run-off site, identified on the northeastern shoreline, was characterized using geographic data. The major structure surrounding this microhabitat is a boat ramp used by lake residents and the general public. Bluegill nesting was sparse at the site, although resident fish appeared to be significantly less mature than bluegills spawning at septic sites during the spawning season. Sediment at this site was sandy near the shoreline, but sediment particulates became loose (mucky) as depth increased. Much of the shoreline, besides the boat ramp itself, was surround by a thick layer of reeds.

The agricultural runoff microhabitat was identified using geographic data as well, and characterized as having potential influences from agricultural operations based on the land use and drainage patterns for the land surrounding this area of the lake. This site resides on the western shoreline, with no surrounding residential land use. No bluegill nesting was observed within this microhabitat, and mucky sediment conditions make for near impossible spawning conditions. However, mature bluegill sunfish were common in this area and seemed to forage on the abundant zooplankton found among the plant growth.

Differences found between caged bluegill sunfish in the defined microhabitats and between caged and resident sunfish reveals interesting patterns in the observed exposure effects (Table 3). Variation in estrogenic responses in fish between microhabitats was consistent with our predictions that effects of EACs do not elicit homogeneous responses across a lake. At microhabitats predicted to be influenced by septic discharge and agricultural run-off, caged bluegill sunfish were found to have decreased BCFs, while those caged at a site influenced by urban run-off did not. Decreased BCF was the only adverse effect that suggested exposure to EACs within the agricultural run-off microhabitat. Interestingly, no adverse effects were found on sunfish exposed to urban run-off. However, both septic influenced sites showed more evidence of exposure to estrogenic agents. In addition to a decreased BCF, sunfish caged at septic site B had higher levels of VTG, while fish caged at septic site A were found to have an increase in HSI and significantly lower gonad maturity. Variation in biological endpoints between sunfish deployed in the microhabitats suggests that exposure to EACs is not consistent across a lake. A closer look at our results shows that a decrease in BCF occurred alongside other biological effects at both septic sites but not at the agricultural run-off site, suggesting that BCF is a powerful indicator of short-term exposure of fish to aquatic contaminants.

Table 3. Summary of adverse biological effects (indicated by X in the table) observed in caged sunfish; wild-caught sunfish; and larval fish exposed to pore water.

Exposure organism	Biological endpoint	Septic Site A	Septic Site B	Urban runoff	Agricultural runoff
Caged sunfish	Plasma vitellogenin		X		
	Hepatosomatic index	X			
	Body condition factor	X	X		X
	Maturity	X			
Wild sunfish	Gonadosomatic index	X			
	Maturity	X			

Larval fish	**Larval growth**	**X**			**X**
	Expression of ER gene	**X**	**X**	**X**	
	Expression of StAR gene	**X**		**X**	

The analysis of wild fish collected from the microhabitats within Sullivan Lake were indicative of differences in exposure to estrogenic compounds with exposure patterns emerging between resident and caged sunfish. However, differences in biomarker expression also highlight the importance of accounting for exposure history of fish populations when assessing exposure risks. Similar to results from caged sunfish, resident sunfish collected from septic site A were found to have more adverse effects than fish collected from other sites. Maturity and GSI were decreased in resident sunfish collected from septic site A. No adverse effects were observed in resident sunfish exposed to urban run-off, which was also observed in sunfish caged within that microhabitat. Resident sunfish collected from septic site B did not have any significant adverse effects when compared to resident sunfish from other sites. Differences in endpoints between caged and resident fish support our hypothesis that exposure history and resultant length of exposure also plays a role in how susceptible fish are to EACs.

Our results indicate that microhabitats within a lake likely alter EAC exposure scenarios to fish within those environments. Similarities and differences between lake microhabitats should be taken into account when assessing the overall occurrence and risks of EACs. Ignoring limnological differences across a lake or land-use patterns surrounding the lake could result in overlooking the amplified or diminished adverse consequences imposed on lake environments by EACs. Differences in effects between resident and caged fish suggest sampling of wild fish portray the most accurate account of adverse effects of EACs on fish populations. In addition, it appears that assessment effects of EACs based short-term models of exposure of species would result in data that is reduced from actual consequences on fish populations. However, as sampling of resident populations can be more costly, more time consuming, and subject to more experimental errors, patterns between caged and wild fish need to be explored further to develop a better understanding on how short-term exposures of laboratory-reared fish can help predict effects on wild fish populations.

Analysis of predator escape data indicated that, compared to control larvae, larvae did not exhibit effects that are commonly observed following exposure to estrogenic compounds. However, gene alteration in the fish was observed at each site to some degree. Increases in gene expression greater than a 1.6-fold induction relative to the reference gene indicates that larvae exposed to pore water from those sites were experiencing estrogenic-mediated effects. Patterns in the relative fold induction of exposed larvae indicate that those exposed to surface water from the agricultural run-off site exhibited the least amount of gene expression. If water chemistry confirms that estrogenic compounds were found in water samples that larval minnows were exposed to, the increase in expression of ER and StAR would suggest that genetic alterations could occur following a very small exposure period (12 days). If replicates of this data indicate similar effects, it could be an indicator that the proximity of littoral nesting zones of lakes to sources of contamination such as boat ramps and septic inflow areas presents the greatest developmental risks for fish species and populations native to those areas. Complex mixtures of EACs in freshwater systems are likely to induce identical gene alterations across microhabitats, making the comparison of gene induction to water chemistry more valuable. To further ensure validity in the reported data, replications of the experiment would be necessary in light of the failed first exposure in this study.

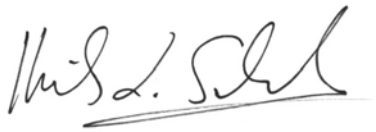
It is reasonable to predict that differences in fish exposure to EACs between microhabitats could elicit developmental effects based on bluegill nesting patterns. Bluegills, which are abundant in Minnesota lakes, are more likely to experience estrogenic effects at the population level, as

spawning and prolonged nesting activities occur in littoral areas where groundwater seepage is most common. More concerning is that theses effects could compromise the ability of embryonic and juvenile bluegill sunfish to survive. Although our research did shed some light on the occurrence and effects of EACs on fish species within a given lake, the results also implicate the need to explore the consequences of exposure to EACs on physiological development to complete a population level effects model.

Conclusions

This study tested the hypothesis that ***Biological Responses in Fish are Correlated with Microhabitat Exposure to Endocrine Active Compounds (EACs) within a lake.*** Our results support the overall hypothesis and provide evidence for localized adverse impacts of EACs on the resident bluegill sunfish population. This study also illustrates the importance of biologically relevant study designs when assessing the sources and effects of EACs in lake environments, which frequently are heterogeneous in both the occurrence of EACs and the use of lake habitat by native fish. Furthermore, this study provides additional evidence that EACs of diverse origins enter lake environments in Minnesota via diverse pathways, including on-site septic systems, public access road runoff and agricultural field runoff. Although none of our biological findings suggests a population level effect of these exposures, the observation of effects across life stages (larvae and adult), across species (sunfish and fathead minnows), and across several orders of organismal functioning (altered gene expressions → altered protein expression → tissue-level alterations in the liver → altered reproductive condition) is reason for concern. If these findings can be replicated in other Minnesota lake systems, as previous studies (Writer et al. 2010) suggest, they would indicate a degradation of lake systems by EACs that would warrant remediation efforts.

Final Report Respectfully Submitted:



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