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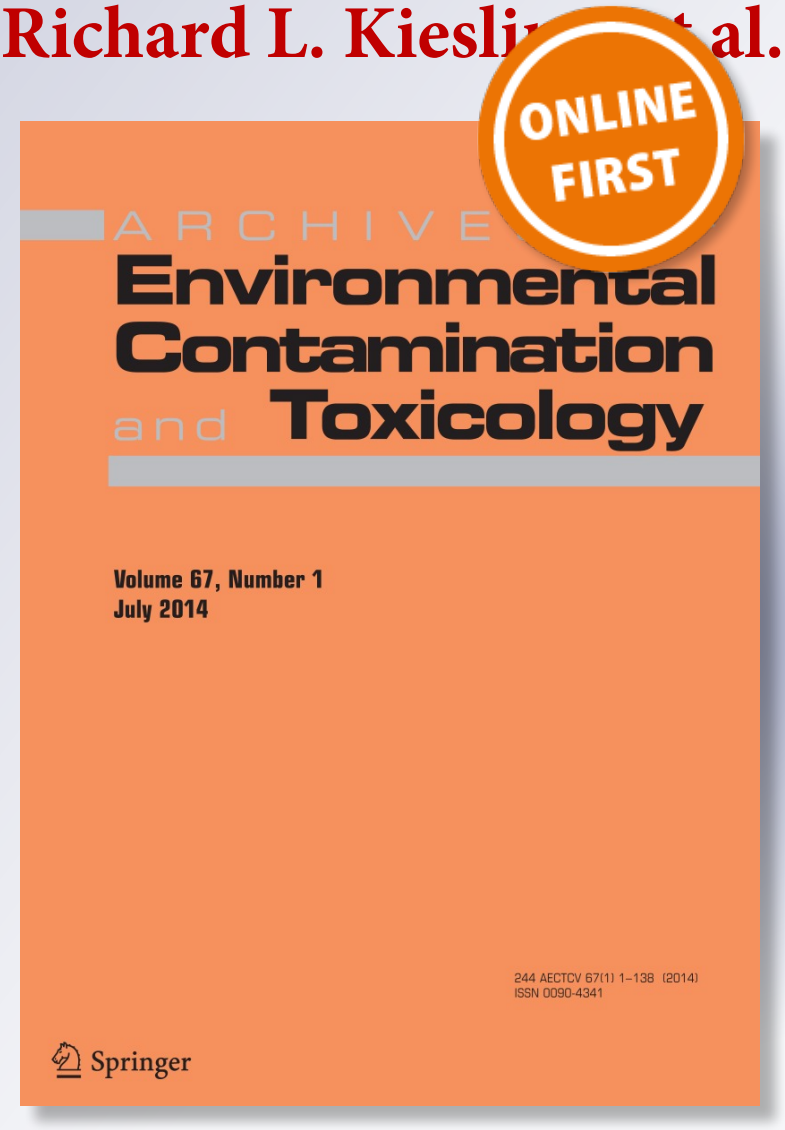

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Identifying Non-point Sources of Endocrine Active Compounds and Their Biological Impacts in Freshwater Lakes

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Abstract Contaminants of emerging concern, particularly endocrine active compounds (EACs), have been identified as a threat to aquatic wildlife. However, little is known about the impact of EACs on lakes through groundwater from onsite wastewater treatment systems (OWTS). This study aims to identify specific contributions of OWTS to Sullivan Lake, Minnesota, USA. Lake hydrology, water chemistry, caged bluegill sunfish (*Lepomis macrochirus*), and larval fathead minnow (*Pimephales promelas*) exposures were used to assess whether EACs entered the lake through OWTS inflow and the resultant biological impact on fish. Study areas included two OWTS-influenced near-shore sites with native bluegill spawning habitats and two in-lake control sites without nearby EAC sources. Caged bluegill sunfish were analyzed for plasma vitellogenin concentrations, organosomatic indices, and histological pathologies. Surface and porewater was collected from each site and analyzed for EACs. Porewater was also collected for laboratory exposure of larval fathead minnow, before analysis of predator escape performance and gene expression profiles. Chemical analysis showed EACs present at low concentrations at each study site, whereas discrete variations were reported between sites and between summer and fall samplings. Body condition index and liver vacuolization of sunfish were found to differ among study sites as did gene expression in exposed larval fathead minnows. Interestingly, biological exposure data and water chemistry did not match. Therefore, although results highlight the potential impacts of seepage from OWTS, further investigation of mixture effects and life history factor as well as chemical fate is warranted.

The widespread occurrence of endocrine active compounds (EACs) in freshwater systems throughout the United States is a growing concern in aquatic toxicology (Ferrey et al. 2012; Kolpin et al. 2002). Research has, however, largely

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focused on point sources of EAC pollution in rivers and streams (Barber et al. 2000, 2007; Jobling et al. 1998; Jobling and Sumpter 1993; Lee et al. 2010; Lozano et al. 2012). The importance of investigating effects of EACs in lake communities was recently highlighted when an experiment in Canada resulted in the collapse of fish populations after dosing of the lake with the synthetic estrogen 17α-ethinylestradiol (Kidd et al. 2007; Palace et al. 2006). The reliance of many lakeshore homes on onsite wastewater-treatment systems (OWTS) and the long residence times of lakes raises the specter that EACs may affect the viability of lake fish communities.

To assess EAC presence and potential effects on the freshwater systems in Minnesota, Writer et al. (2010) performed a survey of 11 trophically diverse lakes across the state. The study documented widespread occurrence of trace amounts of EACs in lakes across trophic classes, and fish populations were found to exhibit biological responses (vitellogenin production in male fish and histopathological changes to reproductive organs) consistent with estrogenic exposure (Hemmer et al. 2002). However, the investigators also noted much heterogeneity of chemical occurrence and biological responses among lakes. These results suggest that further investigation and characterization of non-point sources of EACs and transport mechanisms is warranted.

Lawn or road runoff, agricultural runoff, recreational activities, and OWTS are all potential sources of EAC contamination to lakes. Previous investigations of EACs in lentic systems have frequently used a lake-wide integrated sampling approach, which may not adequately capture the possible impacts that near-shore hydrological processes may have on lake water concentrations and composition of EACs (Palace et al. 2006; Writer et al. 2010). Littoral (near-shore) zones are, however, of particular biological value within lentic environments because they provide spawning habitats, forage, and cover for various life stages of many fish species.

Assessing non-point sources of pollution requires detailed geographic analysis of landscape in the watershed surrounding and supplying the lake. Variable land use, water-quality characteristics, hydrologic flux, vegetation, sediment, and resident aquatic species within lakes have the potential to influence points of entry (and exit) for EACs. Hydrological residence times influence contaminant concentrations: Longer residence times in the lake or in the groundwater system may allow greater abiotic and biotic contaminant degradation, but they also may result in the accumulation of EACs that are recalcitrant to degradation. Exchange between lake water and groundwater also can influence contaminant concentrations. For example, areas with greater residential density and greater numbers of OWTS (or other sources of contamination) may generate groundwater plumes that discharge to adjacent lakes (Gilliom and Patmont 1982; McCobb et al. 2003; Robertson et al. 2005; Standley et al. 2008; Stanford and Weinberg 2010; Swartz et al. 2006) potentially contributing EACs in the littoral zone through groundwater discharge. Because many fish species preferentially spawn and forage in the littoral zone, the potential for exposure to site-specific EAC contamination throughout the spawning period exists. Consequently, variations in geography, geology, hydrology, and land use may create varying exposure scenarios for fish populations located within the same lake.

This study aimed to better understand the contribution of OWTS as a non-point source of EACs to lakes and to compare their contributions to effects on fish. Investigations included sampling of surface and porewater in an effort to compare concentrations of EACs in areas where groundwater flow may be transporting EACs to the sediment–water interface of the littoral zone with areas where no such flow was measured. Combining EAC characterization with assessments of biological responses of exposed fish, ranging from genetic to behavioral scales, will provide interpretive power for identifying sources, exposure scenarios, and fates of EACs while accounting for intralake variability. Based on accounts of critical windows of exposure to EACs during periods of development (Ankley and Johnson 2004) and the occurrence of spawning behavior in littoral zones (Cooke and Philipp 2009), we hypothesized that areas that have OWTS-affected groundwater discharge to the lake would have a greater occurrence of EACs and thus offer a greater potential risk for fish exposure.

Material and Methods

Lake Hydrogeology and Land-Use Patterns

Geographic, geological, and chemical data for Sullivan Lake (Fig. 1 were obtained as part of a larger statewide study (Writer et al. 2010). In June and October 2010, additional field studies were performed to evaluate variable non-point source influences on Sullivan Lake. Topographic and land-use maps of the area surrounding Sullivan Lake were used to determine land-use and the location of OWTS. In June 2010, zones of potential upwelling groundwater to the lake were identified through the assessment of temperature variations between surface water and groundwater (residual temperature), pH, and nitrate concentrations (Supplemental Table S1). Beginning at the northern end of the eastern shoreline (Fig. 1) and moving south, surface and porewater temperatures were measured using a thermal probe that was inserted approximately 10 cm into the soft sediment and recorded approximately every 7.6 m. In instances where temperature

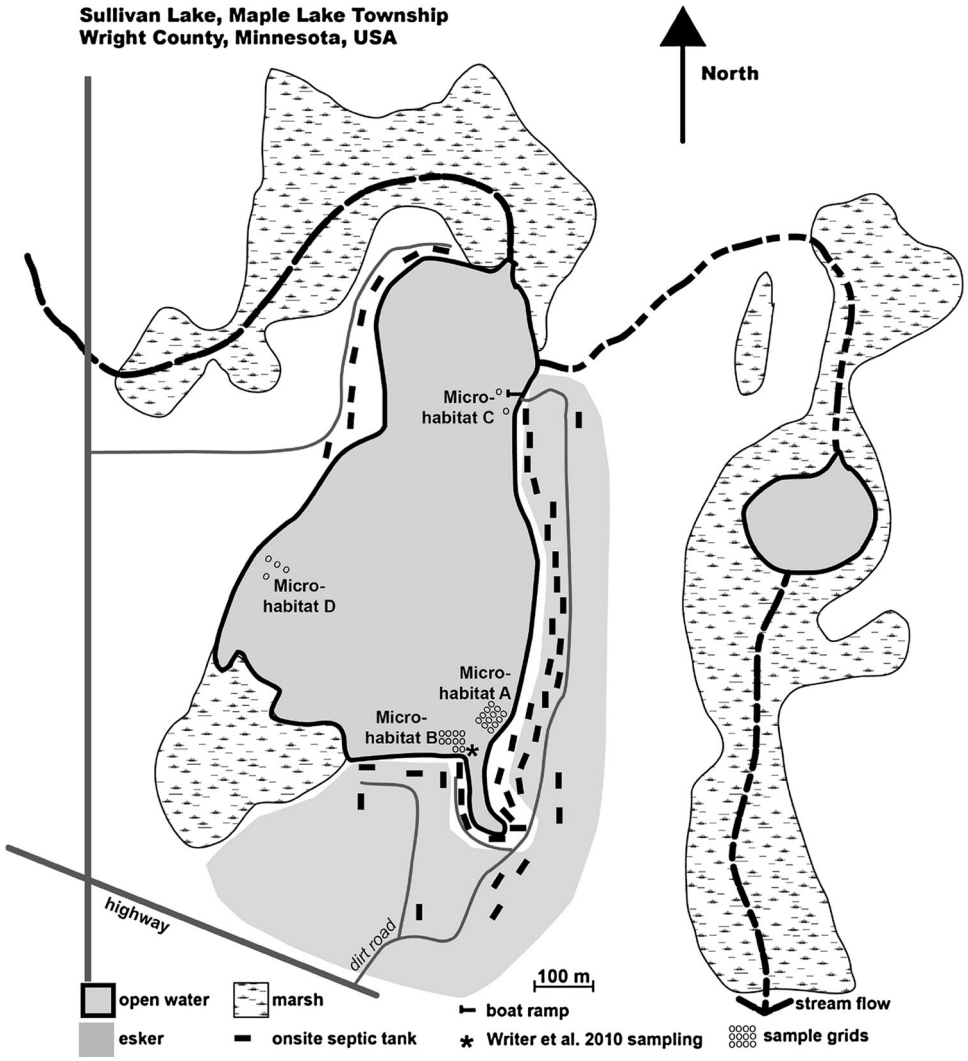

Fig. 1 Outline of Sullivan Lake with inflow from the northwest and outflow through a smaller pond in the east. Streams are indicated by *dashed lines*; *gray lines* are roads within the watershed of Sullivan Lake. Microhabitat sampling occurred in the vicinity of the labeled sites

differences were >2 °C (in most instances when temperature differences were observed they were unequivocal), measurements were taken every 1.5–3 m. Global positioning system coordinates, lake bed composition, and detailed descriptions of the area where measurements occurred also were recorded. Surface water temperature and lake-sediment temperatures at the interface were used to delineate groundwater and surface water interactions, a method that has been successfully used previously (Jones 2006). Reaches with the warmest sediment temperatures indicated locations where lake water was flowing to groundwater, and reaches where sediment temperatures were intermediate suggested a mix of flow directions (Supplemental Table S2). However, intermediate temperatures could also be indicative of OWTS discharge into groundwater. Based on surrounding land use and potential sources of contamination, four microhabitats (sites A through D) were identified for detailed study (Fig. 1).

Study sites A and B are likely to be influenced by OWTS discharges. At these locations, surface water chemistry, porewater chemistry, and subsurface hydraulic head were measured along three transects parallel to the shoreline (Fig. 1). Samples were collected at 2-m intervals (1, 3, and 5 m) perpendicular to the shoreline along transects, and transects were located at distances of 1, 5, 10, and 15 m parallel to the shoreline. Study site C is a control site because no OWTS are within the study area, although potential impacts from road runoff and recreational activities associated with a public boat ramp exist. Study site D is also a control site; it is located on a reach of the shoreline with no obvious impacts from surrounding land use. At sites C and D, dense emergent vegetation limited sampling (Fig. 1). At site C, two locations were sampled near the boat ramp: one near-shore site and one site located 15 m from the shoreline. At site D, two parallel transects were established, and measurements were made at four locations

along each transect. Both sites C and D had a thick layer of organic-rich sediments in the littoral zones resulting in poor hydraulic conductivity and decreased potential for exchange between the lake and groundwater.

Hydraulic head gradient measurements and porewater samples were obtained using a potentiomanometer (Rosenberry and LaBaugh 2008) and a retractable well screen inserted to a depth of 30 cm below the water–sediment interface. A peristaltic pump was used to draw water from the surrounding sediments at a rate <10 mL/min to minimize hydrodynamic disturbance and avoid excessive degassing or inadvertent contributions of lake water to the sample. A manometer was used to measure hydraulic-head difference between the piezometer screen and the lake surface after collection of 60 mL of porewater. Sediments at site C were too fine for pulling water through the well screen and precluded measurements of hydraulic gradients. Groundwater flux also was determined at site A using seepage meters (Rosenberry and LaBaugh 2008).

Lake depth and water temperature were measured at the time of water sample collection. A thermistor installed at the end of a rigid probe was used to measure water temperature just above the water–sediment interface and 30 cm below the water–sediment interface (T30 cm). In situ water-quality measurements (specific conductance, dissolved oxygen, and pH) were taken along a single five-point transect starting at the shoreline (Supplemental Table S3) in the lake water column at each site with a field probe according to standard procedures [United States Geological Survey (USGS) 2008].

Water Sampling and Chemical Analysis

Surface-water samples were collected at each of the four study sites in the littoral zone of Sullivan Lake in the July and October 2010. A boat was used to collect grab water samples 10–15 m offshore using a stainless steel bucket. Water samples were split into separate containers and preserved according to guidelines established by the USGS (2008). Water samples were collected in cleaned and burned amber glass bottles for organic analysis. Samples for carboxylic acid compound analysis were preserved with 1 % (v/v) formalin. Water samples were analyzed at the USGS laboratory in Boulder, Colorado. Analyses included total and dissolved organic carbon, acidic organic compounds, neutral organic compounds, steroids and steroidal hormone compounds, and pesticides (Barber et al. 2012).

Porewater samples collected from each site in July 2010 were analyzed for linear alkylbenzene sulfonate (LAS) and alkylphenolethoxylate (AP) compounds using enzyme-linked immunosorbent assay (ELISA) according to manufacturer protocols (http://www.abraxiskits.com). Additional porewater samples were collected for larval fathead minnow exposure studies using a drive point piezometer. Within each study site, the piezometer's stainless steel tip was driven approximately 15 cm into the sediment, and porewater was drawn from the lake bed and collected in 1-L Nalgene containers disinfected with 95 % ethanol and frozen for later use.

Bluegill Sunfish Field Exposure and Assessment

Bluegill sunfish (*Lepomis macrochirus*) were chosen because they are an integral component of many lake ecosystems and because previous studies have described effects of EAC exposure in this species (Writer et al. 2010; Schultz et al. 2013). Biological end points used to assess fish exposure to EACs include determination of plasma vitellogenin concentrations (an egg yolk precursor protein), morphometric indices, and histopathology. Bluegill sunfish exposures were performed at each of the four study sites. At each site, 40 bluegill sunfish (8–12 cm total length; 10,000 Lakes Aquaculture, Osakis, MN, USA) were deployed in a 1.2 × 0.6-m enclosure made of 1.3-cm plastic mesh for 21 days in September 2010. The fish were fed a supplement of frozen brine shrimp on day 7 of exposure. The fish were collected on day 21, placed into aerated coolers, and transported to the St. Cloud State University Aquatic Toxicology Laboratory for biological assessment (<3 h transport). Caging of fish was permitted by the Minnesota Department of Natural Resources and approved by the St. Cloud State University Institutional Animal Care and Use Committee (IACUC).

Fish were killed in 0.2 g/L buffered MS 222, weighed, and measured for total body length (BL) to calculate the body condition factor (weight/(total length)^3 × 100,000) (Fulton 1904). Livers and gonads were then removed and weighed to calculate the gonadosomatic index (mass testes/mass fish × 100) and the hepatosomatic index (mass liver/mass fish × 100) (Allen et al. 2009). Samples of livers and gonads were placed in histological cassettes and stored in 10 % buffered formalin until histological processing.

Blood samples were drawn by way of capillary tube from the caudal vein and centrifuged (5,500×g for 5 min) for plasma separation. Plasma samples were stored at −80 °C until vitellogenin analysis. Measurement of vitellogenin concentrations followed ELISA protocols by Schultz et al. (2013) using purified sunfish vitellogenin and sunfish-validated vitellogenin antibody. All plasma samples were analyzed at three dilutions and referenced against a multi-point standard curve (acceptable standard curve r^2 >0.95).

For the histological analysis, tissue samples were dehydrated and embedded in paraffin using a Jung TP1050 automated tissue processor (Leica, Wetzlar, Germany). Tissues were sectioned at a thickness of 5 µm on a Reichert–Jung 2030 microtome. At least six sections (separated

by 10 μm) were mounted on two microscope slides and stained in a Leica Autostainer XL using standard haematoxylin and eosin techniques (Gabe 1976). Testes and ovarian maturity were each assessed on a five-point scale (US Environmental Protection Agency 2008). All gonads were also assessed for histopathologies, such as fibrosis, presence of proteinaceous fluid, testicular feminization (loss of testicular structure and/or presence of oogonia), ovarian masculinization, and occurrence of intersex (presence of testicular oocytes). Occurrence and abundance of liver (hepatocyte) vacuolization was assessed on a five-point scale (Wolf and Wolfe 2005). All biological samples were coded to ensure "blind" analysis of plasma and histological data. A second observer independently verified histological samples, and all incidences of testicular pathologies were photographed for future reference and analysis.

Larval Fathead Minnow Exposure and Analysis

Posthatch (<24 h old) fathead minnow larvae were obtained from a laboratory rearing facility (USEPA, Cincinnati, OH, USA). Larvae were exposed to porewater collected from the four Sullivan Lake microhabitats. Before the daily 50 % static renewal of exposure waters, the needed volume of water was thawed and warmed to room temperature and filtered through glass fiber filter (Reeve Angel; Whatman, Clifton, NJ, USA) to remove organic particulates. Treatment groups consisted of 25 larvae exposed for 21 days in 1-L Pyrex glass beakers (5 larvae/beaker, 5 replicates). Larvae were treated with porewater collected from study sites A, B, and C and surface water from site D. All larvae were maintained at a constant temperature of 23 ± 0.8 °C and constant photoperiod (16:8 h [light to dark]). Larvae were fed twice daily with 1 mL of hatched brine shrimp (Brine Shrimp Direct, Ogden, UT, USA). Larval maintenance throughout the experiment followed established culturing guidelines (Denny 1987) and was approved by the St. Cloud State University IACUC.

We assessed predator escape performance of exposed larval fathead minnows at 21 days after hatch (McGee et al. 2009). The predator escape behavior is a reflexive response to predators observed across teleost lineages (Eaton et al. 2001). Because predator escape performance is inherently linked to survival, quantification of such behaviors provides important insights to EAC exposure effects at the population level (McGee et al. 2009). The assay quantifies multiple performance variables, including reaction time (latency period), velocity during the first 40 ms after the initiation of a maneuver (BL/ms), and total escape response [BL/(latency in ms + 40 ms)] (McGee et al. 2009) as an overall assessment of the larva's ability to effectively detect and escape a threatening stimulus. For the performance evaluation, one larva at a time is placed into a filming arena (5 cm-diameter Petri dish) and startled by the trigger-activated vibration originating from below the center of the arena. A high-speed (1,000 frames/s) digital video camera mounted above the filming arena is activated to capture the activity of the fish after the vibrational stimulus. Predator escape performance videos were analyzed as standardized by Blob et al. (2007) except that velocity was adjusted to BLs per millisecond to exclude size differences among individual fish as a confounding variable. Larvae were killed after performance analysis, placed in RNAlater (Ambion, Austin, TX, USA), and frozen for subsequent RNA isolation.

Analysis of Gene Transcription

EACs have been documented to impact expression of genes involved in hypothalamic–pituitary–gonadal axis functioning, which regulates many aspects of behavior and reproduction in teleosts (Ankley et al. 2009; Villeneuve et al. 2006, 2007). Changes in gene transcription patterns after exposure to either single EACs or their mixtures provide molecular evidence for exposures and can aid with identification of mode of action of the chemicals responsible for these changes (Garcia-Reyero et al. 2011). Evaluation of changes in gene transcription was performed on larvae exposed to porewater or surface water from Sullivan Lake. Real-time polymerase chain reaction (RT-PCR) analysis was used to examine effects on the transcription of genes that play a role in estrogen signaling (estrogen receptor α [ER]), that are indicators of chemical-induced oxidative stress (glutathione-S-transferase [GST] and glutathione reductase [GSR]), that are involved in the regulation of steroidogenesis (steroidogenic acute regulatory protein [StAR]), or that are indicative of exposure to metals (metallothionein-binding protein [MT2]).

Frozen larvae were transferred into 1 mL of TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) for homogenization with tools treated with RNAseZap (Ambion, Austin, TX, USA) between each sample. RNA was isolated from 10 larvae/treatment group (chosen at random) following protocols established by Life Technologies (Carlsbad, CA, USA) using an Ambion RNA RiboPure Kit (Life Technologies). Isolated RNA was suspended in 100–200 μl of elution buffer. RNA quality and quantity was assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA samples were stored at −80 °C until gene expression analysis by way of RT-PCR. Isolated RNA with 260/280 absorbance ratio >1.8 was diluted to 10 ng/mL and analyzed using a one-step SYBR Green RT-PCR Reagents Kit following manufacturer's

protocols (Life Technologies). Quantification of ER and Star mRNA was performed using existing primers (Garcia-Reyero et al. 2009; Villeneuve et al. 2007). Quantification of oxidative stress response genes (GST, GSR) and metal exposure indicator gene (MT2) was also performed using published primers (Beggel et al. 2011; Jovanović et al. 2011). All samples were analyzed in duplicate. A melting curve analysis for each reaction determined product specificity. Fold-changes in mRNA abundance for genes of interest were calculated using the comparative C_t and $\Delta\Delta C_t$ methods, and 16S ribosomal protein L8 (RPL8) was used as a reference gene (Livak and Schmittgen 2001). One-sample Student *t* test was used to evaluate whether fold-changes in gene expression of treated versus control fish were statistically significant (Prism 4.01 statistical package; GraphPad Software Inc, La Jolla, CA, USA).

Statistical Analysis

Assumptions of normality were tested using Kolmogorov–Smirnov test for all data before any additional analysis (IBM SPSS Statistics 19, Armonk, NY, USA). When parametric standards were not met, data were either transformed (Ln) to normalize, or Kruskal–Wallis (non-parametric) tests were used to assess analysis of variance (ANOVA) about population means. Dunn's or Tukey's post hoc tests (dependent on appropriateness) followed Kruskal–Wallis analyses. Gene expression data were analyzed for relative fold induction of the expression of genes under estrogenic control compared with the control gene (RPL8) to determine upregulation or downregulation of genes. Mean fold induction relative to the control mean $\Delta\Delta C_t$ value was calculated for each gene and used for statistical analysis. Using one-sample Student *t* test, all fold inductions were analyzed against a test value of 1, which would indicate no induction of gene expression; $p < 0.05$ was considered significant for all statistical analyses.

Results

Land Use and Hydrogeology

Sullivan Lake covers approximately 300,000 m^2 and has a maximum depth of 18 m (http://www.dnr.state.mn.us, accessed June 2011). The bottom substrate varies from sand to organic material, clay, and silt throughout the lake. Resident fish species include northern pike (*Esox lucius*), largemouth bass (*Micropterus salmoides*), and bluegill sunfish (*L. macrochirus*). Predominant land use in the surrounding watershed is 49 % cropland, 11 % deciduous forest, 8 % residential use, and 7 % wetland (Writer et al. 2010). Homes line the eastern and western shorelines and rely on OWTS. The lake is used for recreational activities such as boating and fishing and is accessible by way of a public boat ramp near study site C (Fig. 1).

The landscape surrounding Sullivan Lake is characterized by Pleistocene glacial deposits and may have been part of a larger lake that during glacial times was dammed up behind the 15 m-high esker-like ridge along the eastern shoreline (Don Rosenberry, personal communication; Fig. 1). The presence of marsh adjacent to the north, northwest, and southwest shorelines indicates that hydraulically driven groundwater discharge likely is shifted to the margins between uplands and wetlands rather than occurring at the boundary between wetlands and the open-water portion of the lake. The predominant geologic feature, from the perspective of groundwater discharge into the lake, is the esker-like ridge along the eastern and southeastern shoreline. Higher groundwater head beneath the ridge creates a hydraulic gradient that increases the potential for groundwater discharge along the eastern shoreline, which coincides with the areas of maximum residential density and creates the greatest potential for OWTS discharge into the lake. Storm water and agricultural runoff along the western shoreline is potentially buffered by the wetlands, which may attenuate chemicals before they enter the lake.

Microhabitat Water-Column Chemistry

The aminocarboxylic acid metal-complexing compounds, ethylenediamine-tetraacetic acid (EDTA) and nitrilotriacetic acid (NTA), and nonionic surfactant degradation products, 4-nonylphenolmonoethoxycarboxylic acid and 4-nonylphenol-diethoxycarboxylic acid (NPEC), were detected in the lake water column samples at all study sites in July, whereas only EDTA was detected at all study sites in October (Table 1). Several neutral organic compounds were also detected at all study sites in July and October samplings, including the antioxidants 2,6-di-*tert*-butyl-1,4-benzoquinone and 2,6-di-*tert*-butyl-4-methylphenol. The insecticide *N*,*N*-diethyl-*meta*-toluamide (DEET) was detected at all study sites in July and at sites A, B, and D in October. Other compounds detected at sites A and B included fragrances acetylhexamethyltetrahydronaphthalene (AHTN) and hexahydrohexamethylcyclo-pentabenzopyran (HHCB), the plasticizer bisphenol A, (BPA) caffeine, the natural product and disinfectant 4-methylphenol, and nonionic surfactant degradates (4-nonylphenol, 4-nonylphenolmonoethoxylate [NPEO], and 4-*tert*-octylphenol). Steroid and steroidal hormone analyses of July and October samples detected cholesterol and coprostanol at all sites, and 4-androstene-3,17-dione and estrone were detected in July only. Pesticide analyses of July samples detected 2,4-dichlorophenoxyacetic acid, acetochlor ethane sulfonic acid (ESA), acetochlor

Table 1 Lake water chemistry and calculated EEQs

Compounds	Units	July 2010				October 2010			
		A	B	C	D	A	B	C	D
Acidic organic compounds									
Ethylenediaminetetraacetic acid	ng/L	200	200	200	600	300	200	200	300
Nitrilotriacetic acid	ng/L	100	100	100	100	100	<dl	<dl	<dl
4-Nonylphenolmonoethoxycarboxylic acid[a]	ng/L	500	400	450	400	<dl	<dl	<dl	<dl
4-Nonylphenoldiethoxycarboxylic acid[a]	ng/L	300	<dl	<dl	<dl	150	<dl	<dl	<dl
Neutral organic compounds									
Acetylhexamethyltetrahydro-naphthalene	ng/L	<dl	<dl	<dl	<dl	15	15	<dl	<dl
BPA[a]	ng/L	25	30	<dl	<dl	10	18	<dl	<dl
Caffeine	ng/L	15	<dl	<dl	<dl	35	25	<dl	<dl
2,6-Di-*tert*-butyl-1,4-benzoquinone	ng/L	30	35	35	30	75	50	40	20
2,6-Di-*tert*-butyl-4-methylphenol	ng/L	30	15	35	15	10	5	10	20
N,*N*-Diethyl-*meta*-toluamide	ng/L	145	130	75	60	85	80	<dl	20
Hexahydrohexamethylcyclo-pentabenzopyran	ng/L	<dl	<dl	<dl	<dl	15	10	<dl	<dl
4-Methylphenol	ng/L	25	30	35	30	<dl	15	<dl	<dl
4-Nonylphenol[a]	ng/L	90	50	<dl	<dl	55	<dl	<dl	<dl
4-Nonylphenolmonoethoxylate[a]	ng/L	185	85	<dl	<dl	95	<dl	<dl	<dl
4-*tert*-Octylphenol[a]	ng/L	30	15	15	<dl	75	40	<dl	<dl
Steroids and steroidal hormones									
4-Androstene-3,17-dione	ng/L	<dl	0.7	0.5	1.3	<dl	<dl	<dl	<dl
Cholesterol	ng/L	5700	6000	20500	23000	3300	3400	2800	3200
Coprostanol	ng/L	47	34	31	585	42	17	17	30
17α-Estradiol[a]	ng/L	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
17β-Estradiol[a]	ng/L	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
Estrone[a]	ng/L	0.8	0.5	1	0.9	<dl	<dl	<dl	0.6
17α-Ethinylestradiol[a]	ng/L	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
Progesterone	ng/L	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
Testosterone	ng/L	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl
Mean EEQ	ng/L	0.15	0.11	0.21	0.18	0.01	0.003	n/a	0.12
Maximum EEQ	ng/L	1.07	0.74	0.85	0.72	0.53	0.18	n/a	0.48
Pesticides									
2,4-Dichlorophenoxyacetic acid	ng/L	67	68	79	79	Not measured			
Acetochlor ESA	ng/L	82	87	108	95				
Acetochlor OXA	ng/L	45	54	52	52				
Alachlor ESA	ng/L	ND	51	19	139				
Metolachlor ESA	ng/L	17	19	20	18				
Hydroxyatrazine	ng/L	139	148	149	140				

<dl compounds measured lower than the detection limit

[a] These compounds were used to calculate mean EEQ values

oxanilate metabolite (OXA), metolachlor ESA (ethanesulfonate degradate), and hydroxyatrazine in all microhabitats.

Results of chemical analyses were compared with the results from a previous sampling of Sullivan Lake (Writer et al. 2010) to identify variability in EAC concentrations over time as well as among sampling locations. Results indicated that the majority of EAC water-column concentrations and estrogen equivalency quotient (EEQ) reported by Writer et al. (2010) fell within observed ranges of concentrations and EEQ values (Table 1), although concentrations varied among the four study sites.

Microhabitat Water–Sediment Interface

Summer 2010 was dry in the Upper Midwest, and Sullivan Lake received <3 cm of rain in the 2 weeks leading up to

Table 2 Characterization of Sullivan Lake microhabitats and water-quality values for porewater samples collected July 8, 2010

		Microhabitat A				Microhabitat B				Microhabitat C		Microhabitat D	
Location (x,y)		(1,1)	(5,1)	(10,1)	(15,1)	nm	nm	(10,1)	(15,1)	(0,1)	nm	(0,1)	nm
		(1,3)	(5,3)	(10,3)	(15,3)	(1,3)	(5,3)	(10,3)	(15,3)	nm	(15,5)	(0,3)	nm
		(1,5)	(5,5)	(10,5)	(15,5)	(1,5)	(5,5)	(10,5)	(15,5)			(0,5)	(5,5)
Constituent													
Dissolved organic carbon	(mg/L)	3.0	2.6	2.1	2.1	nm	nm	7.6	9.4	11.2	nm	31.8[a]	nm
		5.9	4.0	2.1	3.4	7.8	7.6	8.7	10.4	nm	7.3	12.6[a]	nm
		3.2	7.4	3.6	3.7	10.3	10	11.4	10.1	nm	nm	32.3[a]	5
	Mean	3.6				9.33				9.25		5	
Linear alkyl-benzene sulfonates	(mg/L)	<0.02	0.29	0.04	0.16	nm	nm	<0.02	<0.02	<0.02	nm	0.02	nm
		<0.02	0.92	0.13	0.13	0.02	0.04	<0.02	<0.02	nm	0.03	0.11	nm
		0.38	0.15	0.04	0.1	0.12	0.02	<0.02	0.03	nm	nm	0.02	0.11
	Mean	0.234				0.046				0.03		0.065	
Alkylphenol-ethoxylates	(mg/L)	0.14	<0.02	<0.02	<0.02	nm	nm	<0.02	<0.02	0.11	nm	<0.02	nm
		<0.02	<0.02	<0.02	<0.02	<0.02	0.10	<0.02	0.04	nm	<0.02	<0.02	nm
		<0.02	<0.02	<0.02	<0.02	0.11	<0.02	<0.02	<0.02	nm	nm	<0.02	<0.02
	Mean	0.14				0.083				0.11		<0.02	
Hydraulic head	(mm)	0.0	nm	0.8	0.1	nm	nm	–1	–3	nm	nm	1.2	nm
		15.4	2.1	0.1	0.3	−2	0	–3	1	nm	nm	nm	nm
		3.1	1.4	0.7	2.8	3	–1	–1	0	nm	nm	nm	nm
	Mean	2.68				–0.7				nm		1.2	

X distance parallel to shore in m, *y* distance perpendicular to shore in m, *nm* no measurement

[a] Positive bias due to high inorganic carbon content, not reported

the July sampling and no rain in the 2 weeks before the October sampling. Results from the bed-sediment hydraulic head measurements showed that several values were greater than those for the lake surface (Table 2) indicating a groundwater discharge zone. Hydraulic head was generally less than lake stage on the southern end of the lake (site B) and greater along the eastern shore (site A). Lake water-column temperature, specific conductance, and pH were generally constant with depth (Supplemental Table S3) at site A with the exception of the 15,5 point (Fig. 1). Dissolved oxygen values were either constant within the water column or decreased with depth. Results of site A porewater samples show that dissolved organic carbon ranged from 2.1 to 7.4 mg/L, and LAS ranged from <0.02 to 0.92 mg/L (Table 2). There was one AP detection (0.14 mg/L). Average residual temperature measurements (lake water temperature–porewater temperature) of 6.7 °C indicate that porewater and lake water interactions were generally mixed with some groundwater discharge into the lake (Supplemental Table S2). Based on the surrounding geology and land use, site A was characterized as having possible OWTS influence.

Study site B is located approximately 40 m southwest of site A and also was determined to have potential OWTS influence. The water column temperature, specific conductance, dissolved oxygen, and pH were relatively constant (Supplemental Table S3). Dissolved organic carbon values in the porewater samples from site B ranged from 7.6 to 11.4 mg/L and were greater than those at site A; LAS values ranged from <0.02 to 0.12 mg/L; and there were sporadic detections of AP (Table 2). Residual temperature differences between lake water and porewater were found to range from 2.3 to 7.1 °C, indicating groundwater discharge into the lake (Supplemental Table S1). Bed-sediment hydraulic head values ranged from 3 cm below lake stage to 3 cm above lake stage, indicating mixed flow directions at this location.

Study site C was chosen as an in-lake reference to OWTS sites located in an area prone to storm water and road runoff because the adjacent land slopes steeply toward the boat ramp and adjacent road. Measurements taken within the water column indicated that water temperature and specific conductance were relatively constant (Supplemental Table S3). Dissolved oxygen concentrations decreased with depth,

Table 3 Summary of biomarker analyses for caged bluegill sunfish deployed for 21 days in Sullivan Lake

Toxicology biomarker	Microhabitat	Sample Size	Mean ± SD	*p*
Body condition index (measured)	Baseline	25	1.930 ± 0.184	0.001 (ANOVA)
	A	24	1.735 ± 0.133*	
	B	21	1.720 ± 0.158*	
	C	28	1.919 ± 0.218	
	D	20	1.721 ± 0.209*	
HSI (measured)	Baseline	25	1.157 ± 0.354	>0.05 (Kruskal–Wallis)
	A	24	1.052 ± 0.294	
	B	21	0.984 ± 0.284	
	C	28	0.949 ± 0.237	
	D	20	1.167 ± 0.468	
GSI (measured)	Baseline	25	0.149 ± 0.155	>0.05 (Kruskal–Wallis)
	A	24	0.178 ± 0.182	
	B	21	0.137 ± 0.081	
	C	28	0.206 ± 0.182	
	D	20	0.207 ± 0.131	
Vitellogenin (mg/mL) (rank scale)	Baseline	25	620.1 ± 682.9	>0.05 (Kruskal–Wallis)
	A	24	317.1 ± 423.1	
	B	21	376.2 ± 587.1	
	C	28	330.2 ± 533.1	
	D	20	215.8 ± 301.4	
Liver vacuolization (rank scale)	Baseline	25	2.000 ± 1.291	0.026 (Kruskal–Wallis)
	A	23	1.960 ± 1.224	
	B	21	2.380 ± 2.355	
	C	28	1.110 ± 0.685	
	D	20	1.680 ± 1.309	

Statistical tests applied to specific data sets are identified in parentheses below respective values

* Statistical significance as determined by Tukey's post hoc test in conjunction with ANOVA or Dunn's post hoc test in conjunction with Kruskal–Wallis

and near-bottom values were as much as 43 % lower than near-surface values. Porewater dissolved organic carbon values ranged from 7.3 to 11.2 mg/L, whereas LAS (0.03 mg/L) and AP (0.11 mg/L) were detected only in one sample each (Table 2). Depth greater than the length of the thermal probe prevented attaining residual porewater measurements (Supplemental Table S1), and a thick layer of organic matter at the water–sediment interface prevented hydraulic head measurements (Table 2), although the thick layer of organic matter indicates that this site may receive little OWTS influence.

Study site D was chosen as an in-lake reference because the surrounding land use (fallow cropland) did not suggest obvious EAC inputs. Measurements taken within the water column showed that site D had the lowest average temperature, pH, and DO as well as the highest specific conductance (Supplemental Table S3). These conditions indicate that sediment conditions may be anoxic, which could provide suitable habitat for microbial communities that may assist in EAC degradation. Dissolved organic carbon concentrations in the porewater samples had greater values than at the other sites but have the potential for positive bias due to high inorganic carbon levels (Table 2). LAS were detected at concentrations ranging from 0.02 to 0.11 mg/L, and no APs were detected. Measurements to calculate temperature differences between surface water and groundwater were not successful, and a thick layer of organic matter near the water–sediment interface limited hydraulic head measurements (Table 2).

Bluegill Sunfish Exposures

Survival rates of bluegill sunfish exposed in Sullivan Lake were 100 % at site A, 98 % at site B, 93 % at site C, and 100 % at site D. Only reproductively mature male fish (as determined by subsequent histological assessment) were included in the analyses. Body condition index was found to be significantly different between populations ($p < 0.001$, Table 3) with fish exposed at sites A, B, and D having significantly lower values than baseline fish (killed on the day exposure began) and those exposed at site C. The hepatosomatic gonadosomatic indices of the fish were analyzed using Kruskal–Wallis ANOVA and were not significantly different from baseline fish ($p > 0.05$).

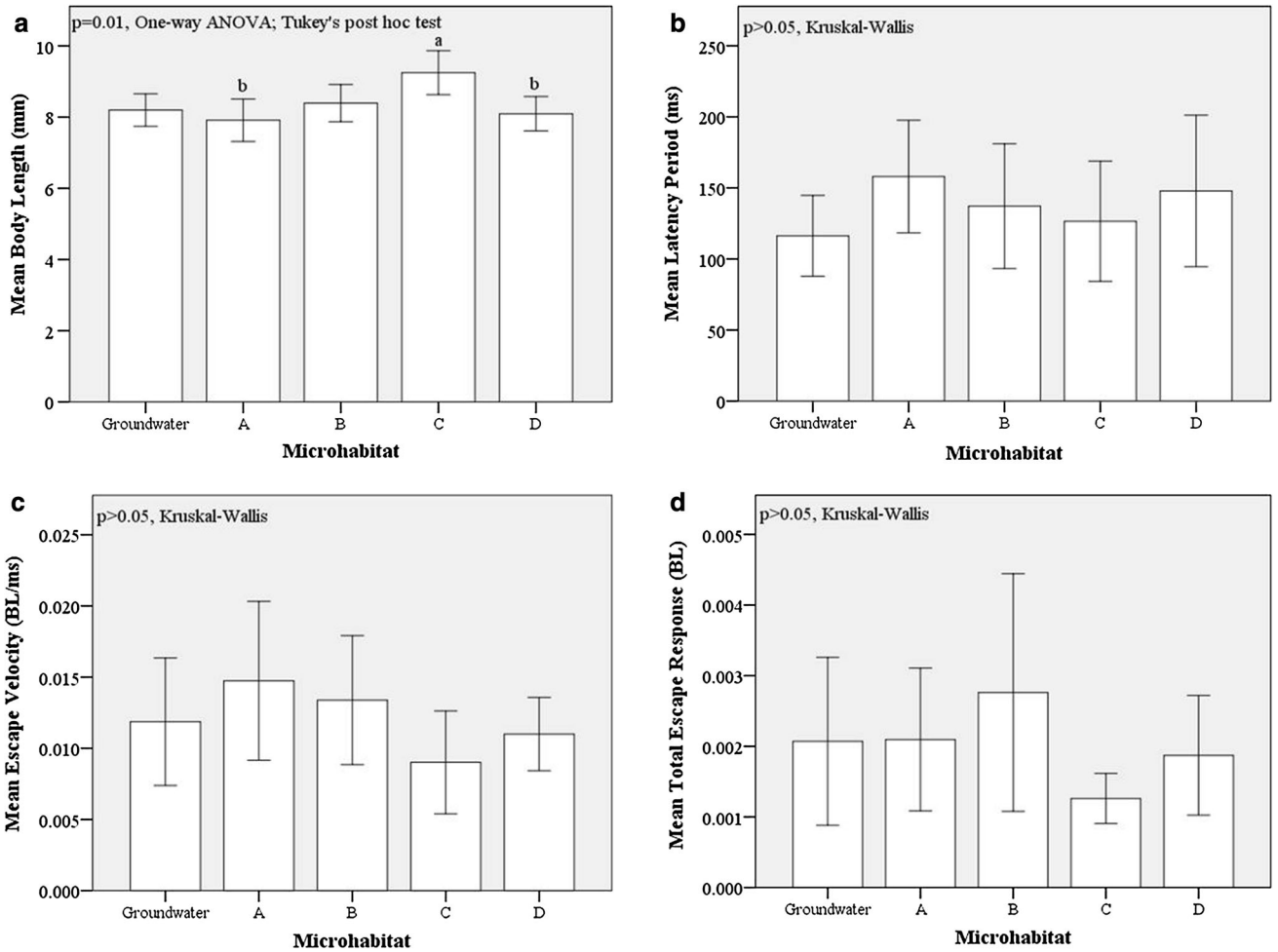

Fig. 2 Effects of 12-day 50 % static renewal exposure of larval (12-day-old) fathead minnows to porewater collected from microhabitats A, B, and C and lake water from microhabitat D, or a condition groundwater control, on predator escape performance (±SE). (*$p < 0.05$). **a** Body length (mm) where *N* values represent sample sizes that are the consistent between graphs **a**–**d**. **b** Latency period (ms) from the time of stimulus to commencement of C-start behavior. **c** Swimming velocity standardized to BL for the 40 ms after commencement of predator escape behavior recorded in BL/ms. **d** Total escape response taking into account latency and escape velocity (BL/ms) from the stimulus to 40 ms after the commencement of C-start behavior

Data from the vitellogenin analysis were log-transformed before analysis and were not significantly different from those of baseline fish ($p > 0.05$, Kruskal–Wallis, Table 3). Results of liver vacuolization showed significant differences ($p = 0.026$, Table 3), however, post hoc analysis of pairwise comparisons didn't reveal any significant differences.

Incidence of gonadal pathologies as a percentage of the sample population was calculated. Proteinaceous fluid was found to occur least often in fish from site A (4 %), whereas occurrence in baseline fish and those from sites B, C, and D were similar (frequency of 16, 15, 14, and 10 %, respectively). Incidence of liver fibrosis was more common than any other histopathologies. The greatest occurrence of fibrosis was found in fish exposed at site C with 57 % of the fish affected. At sites B and D, 45 % of exposed fish were affected compared with 44 % of baseline fish and 39 % of fish exposed at site A.

Larval Fathead Minnow Exposures

Larval fathead minnows exposed to porewater from site C had a significantly greater BL than those exposed to porewater from site A and surface water from site D ($p = 0.007$ for A and $p = 0.032$ for D, Tukey's post hoc test) (Fig. 2a). Results from the predator escape assay lacked significant differences among the various study sites and the groundwater control for any variable (Fig. 2b–d).

Gene expression of the ER did not differ among treatment waters and laboratory control (Fig. 3a). However,

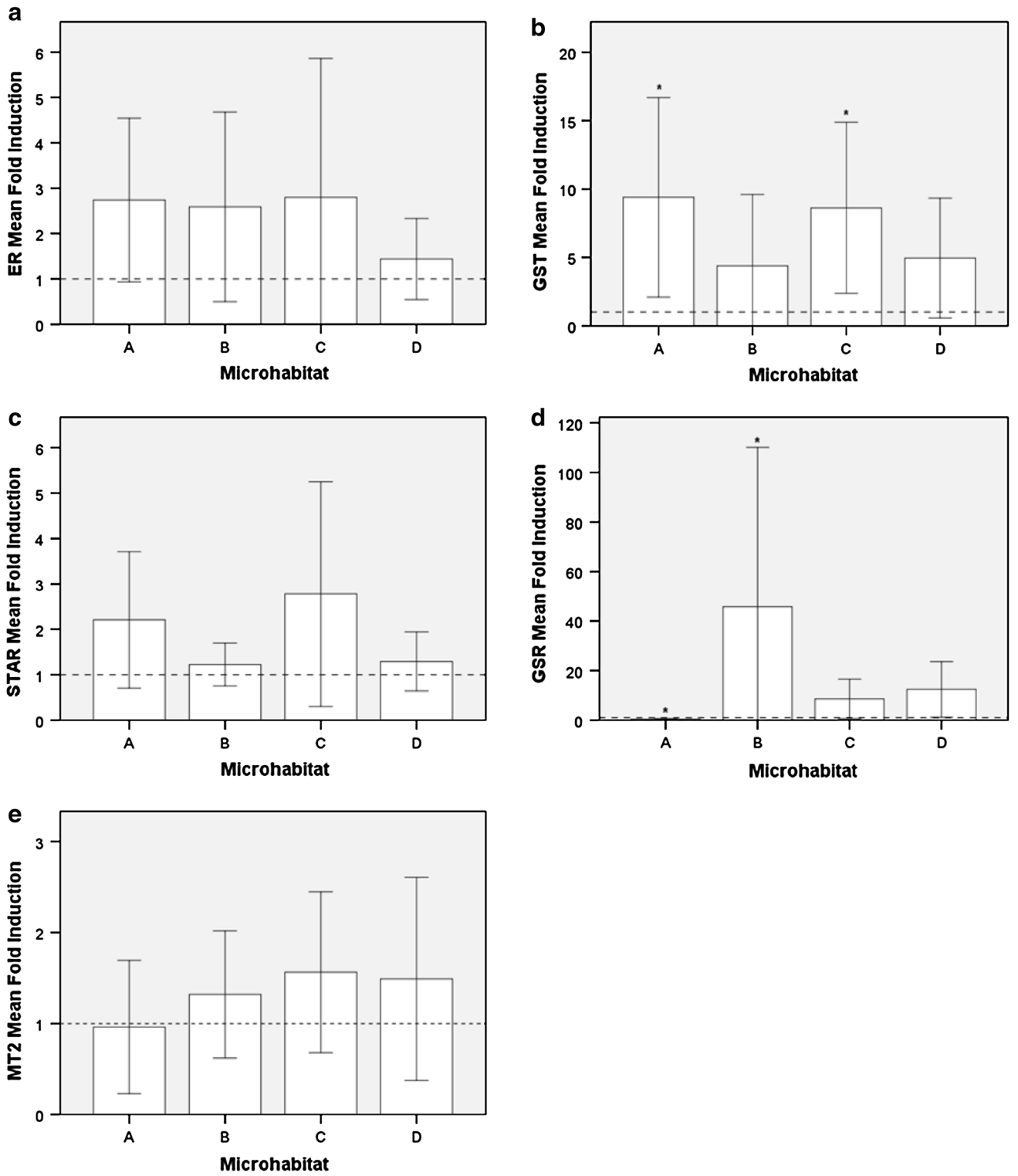

Fig. 3 Results from RT- PCR gene analysis of larval fathead minnows exposed for 12 days to water from the various microhabitats and a conditioned groundwater control. Mean fold induction of $\Delta\Delta C_t$ values of each gene relative to the mean $\Delta\Delta C_t$ value of the groundwater control was analyzed using one-sample Student *t* test ± SE. (*Significantly different from the control mean where $p < 0.05$). *Dashed lines* at mean fold inductions of 1 indicate no differences from the groundwater control mean

GST gene expression was found to be significantly upregulated in fish exposed to porewater from sites A ($p = 0.047$) and C ($p = 0.031$) (Fig. 3b). Fish exposed to waters from sites B and D did not exhibit GST gene expression that was significantly greater than control values. StAR gene mean fold inductions in exposed fish were not significantly different from the control mean (Fig. 3c); however, fish exposed to water from sites A and C also exhibited greater variability with maximum expression of StAR reaching upwards of 3 and 5 times that of control values, respectively. Expression of GSR was significantly upregulated from the control mean in fathead minnow larvae exposed to porewater from site B ($p = 0.004$) and significantly downregulated at site A ($p = 0.005$) (Fig. 3d). Expression of GSR in fish exposed at sites C and D were found to be upregulated as much as 8 times greater than control values despite lacking statistical significance. Analysis of gene MT2 resulted in no significant differences between control and treatment values, although, overall MT2 results exhibited the lowest cumulative expression compared to all other genes (Fig. 3e).

Discussion

Heterogeneity of EAC Occurrence

Lake water chemistry indicated that the occurrence of EACs is not spatially homogeneous throughout Sullivan Lake. Spatial heterogeneity likely is influenced by land-use patterns as has been reported in other recent investigations of freshwater aquatic systems (Veach and Bernot 2011; Writer et al. 2010). The presence of OWTS near study sites A and B represents potential contributing sources of EACs (Conn et al. 2006) to the littoral zone. Hydrologic connection between lake water and OWTS-influenced groundwater can have substantial impacts on measured concentrations of contaminants suggesting that knowledge of flow paths in the hypolentic zone is essential for determining optimal sampling designs in lakes (Conn et al. 2006; Lewandowski et al. 2011). Results from this study confirm that complex mixtures of EACs occur in near-shore lake environments. Multiple compounds were frequently detected in lake water at most of the study sites, including EDTA, NTA, NPEC, 2,6-di-*tert*-butylbenzoquinone, 2,6-di-*tert*-butyl-methylphenol, 4-methylphenol, DEET, 2,4-dichloroacetic acid, acetochlor ESA, acetochlor OXA, alachlor ESA, metolachlor ESA, hydroxyatrazine, androstenedione, cholesterol, coprostanol, and estrone. Contaminants detected in lake water *only* at study sites A and B include the following: BPA, AHTN, HHCB, caffeine, 4-nonylphenol, and NPEO (nonionic surfactant degradates). Lake-water chemistry results indicate complex mixtures at sites with possible OWTS influence, a trend that has been previously reported (Standley et al. 2008). Greater contaminant concentrations and occurrence at residential locations could be attributed to local groundwater flow conditions. In addition, study sites A and B having little vegetation, low organic carbon concentrations in the subsoil, a close proximity to OWTS, and lower rates of biodegradation could contribute to greater EAC contaminant concentrations and occurrence. Sites C and D had, in general, more wetland-like characteristics, which have the potential to filter wastewater by way of removal by vegetative rhizosphere interactions. Differential sediment sorption and microbial degradation potential of contaminants are other possible factors that could influence detection frequency and concentration that remain largely uninvestigated.

Observed variations of EAC occurrence at study sites between July and October samplings indicates that exposures to fish populations are not uniform over time. NTA and NPEC were found at all sites in July but were only detected at site A in October. Likewise, AHTN and HHCB only were detected at sites A and B in the October sampling. Caffeine was detected only at site A in July, but at sites A and B in October. DEET, 4-nonylphenol, NPEO, and 4-*tert*-octylphenol were detected more frequently in July than in October, possibly related to recreational activities (boating, swimming) being more prevalent in the summer. Androstenedione and estrone were present at more sites in July than October. Cholesterol and coprostanol were detected at all sites during both samplings, but concentrations were greater in the July sampling. These findings suggest that resident sunfish populations may be at greater risk of exposure to EACs during the spawning season, which usually lasts from mid-May to mid-August (Cooke and Philipp 2009). Spawning season also coincides with the greatest site fidelity for male sunfish because these fish practice paternal nest care (Bartlett et al. 2010; Paukert et al. 2004). Temporal variations in EAC occurrence may be attributed to differing sources of contamination during the summer due to increased presence of visitors to the lake, home and lawn maintenance, agricultural practices, or weather patterns as well as natural hormone production of spawning fish populations and changes in microbial degradation. A previous investigation of pharmaceuticals in lakes in Switzerland reported variable concentrations over time (Buser et al. 1998); however, lake characteristics (input sources, sampling locations, hydrologic residence time, trophic status) may vary for individual aquatic ecosystems.

Minimal differences in biological effects were observed among bluegill sunfish deployed at the four study areas. Although our findings of biological responses to EACs are subtle compared with exposure scenarios in other lake environments (Kidd et al. 2007; Palace et al. 2006), it is worth pointing out that the life span of sunfish can exceed a

decade, and our exposures lasted merely 3 weeks. Furthermore, sunfish were only exposed as adults, which does not account for exposure during the vulnerable larval life stage, which occurs by way of direct contact with sediment in the hypolentic zone. Last, our September exposure appeared to have missed the greater EAC concentrations found in July at several microhabitats. Concentrations reported by Kidd et al. (2007), which had led to the collapse of a fish population, were 30–50 times greater than concentrations found in this study, and fish were exposed to a single estrogenic compound rather than a mixture of EACs that could potentially have estrogenic, antiestrogenic, additive, synergistic, or antagonistic effects. Because EACs are potent at low concentrations and have variable effects on fish when present in mixtures, further investigations are necessary to identify how these compounds affect resident fish population under environmental conditions over multiple years and generations.

To better gauge the estrogenicity of the mixtures of various compounds measured in summer 2010, EEQ values were determined using methods described by Vajda et al. (2008). Mean EEQ (see Table 1 for compounds included in calculation values determined from concentrations of steroids and steroidal hormones were found to be highest at study site C and lowest at study site B. Maximum EEQ values were also determined and found to be greatest at study site A and smallest at site D in summer 2010. Mean EEQ values determined for fall 2010 indicated that site D had the greatest and site B the lowest estrogenicity. Similarly, maximum EEQ values were found to be lowest at site B. The highest EEQ values were found at study site A (1.07); however, even this "high" value is low compared with previous reported EEQ values ranging between 4.5 and 38.4 (Legler et al. 2002). Consequently, bluegill sunfish exposed at site A lacked obvious biological responses to estrogenic EACs. Although overall EEQ values were low, and differences in chemistry between the study sites may not be statistically significantly different, concern over observed EACs is still warranted because biological responses in fish species have been detected after exposures to single compounds in quantities of 0.1 ng/L (Purdom et al. 1994). Last, the lake chosen for this study is relatively small, and its hourglass shape promotes water mixing on windy days making any observed differences in microhabitat structure even more noteworthy. Larger, more structured lakes with isolated bays may exhibit even greater heterogeneity in presence and concentrations of EACs in their microhabitats.

At study sites characterized as having OWTS influences (A and B), caged bluegill sunfish showed more adverse responses to exposures than fish caged at other sites. OWTS-influenced sites (A and B) and the fallow agriculture control site (D) were also found to produce sunfish with lower body condition indices than those exposed at site C. Although subtle biological signals of stress within each population may be indicative of acute exposure to environmental contaminants, high variation in biological responses within our experimental populations could be attributed to individual immune responses, additive effects of complex mixtures, and environmental variables such as pH and temperature (Blazer et al. 2010). Although the majority of physical parameters were found to be comparable among study sites, it is possible that subtle differences among study sites affect redox conditions at the water–sediment interface as well as sediment sorption properties, which can influence the bioavailability and degradation rates of EACs.

Larval exposure experiments indicated that porewater quality varied among study sites and may have an impact on surface water quality through hydrologic interactions. Assessments of BL indicate that larval fathead minnows exposed to water collected from sites A, B, and D were smaller than those exposed to water from site C, a finding consistent with results for the exposed bluegill sunfish body condition index. Although gene transcription analyses of ER did not result in significant differences from control values, trends showed induction upward of fourfold at sites A, B, and C. However, GST gene expression increased at all study sites (ranging from 4- to 9-fold) indicating that the larval minnows experienced cellular stress during exposures. Chemicals measured in this study that have been shown to cause oxidative stress in fish species include many of the alkylphenols and pesticides. In addition, GSR gene expressions exhibited 8-, 10-, and 42-fold induction in larval fathead minnows exposed to water from sites C, D, and B, respectively. EACs affecting GSR are characteristically found in detergents and surfactants, which can alter the normal physiology of aquatic organisms and would be more likely to be a product of residential or road runoff. The presence of heavy metals also has been shown to influence the expression of MT2 in zebrafish (*Danio rerio*) (Gonzalez et al. 2005). However, the lack of expression in MT2 gene suggests that heavy metals were not found in sufficiently high concentrations to stimulate expression in larval fathead minnows exposed to water samples from any of the lake microhabitats.

Study Implications

This study investigated the potential of OWTS as a nonpoint source of EACs within lentic systems to gain insight into the spatial distribution of EAC in lakes and key EAC entry points. The lake chosen for this study had been previously identified as being moderately impacted by EAC

presence; therefore, the results obtained in the current study are probably more widely applicable than "worst-case scenario" studies. Water chemistry results suggest that surrounding land use has a measurable impact on the lake habitat heterogeneity. Hydrologic and chemical results suggest that there is potential for groundwater carrying EACs from OWT systems to discharge into lake water. Results from this study suggest that land use can affect fish in associated microhabitats and can help identify non-point source pollutants. Heterogeneous biological responses identified in adult and larval fish between study sites throughout the lake highlight the need to account for life cycle patterns and crucial growth stages in aquatic species when assessing the impact of water quality on biological responses. Further research is warranted to assess biological effects of EAC exposure under variable water-quality conditions in a controlled experiment. Such knowledge would be particularly beneficial to begin deciphering how complex chemical mechanisms influence biological mode-of-action in environmental systems that are in constant flux.

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