ML 2016 Project Abstract
For the Period Ending June 30, 2018

PROJECT TITLE: Biological Consequences of Septic Pollution in Minnesota Lakes
PROJECT MANAGER: Heiko L. Schoenfuss
AFFILIATION: St. Cloud State University
MAILING ADDRESS: 720 Fourth Avenue South, WSB-273
CITY/STATE/ZIP: St. Cloud, MN 56301
PHONE: (320) 308 3130
E-MAIL: hschoenfuss@stcloudstate.edu
WEBSITE: web.stcloudstate.edu/aquatictox
FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2015, Chp. 76, Sec. 2, Subd. 04c

APPROPRIATION AMOUNT: $ 364,000
AMOUNT SPENT: $ 362,650
AMOUNT REMAINING: $ 1,350

<u>**Overall Project Outcome and Results**</u>

All activities proposed for the current study have been completed. The addition of a fifth lake and expansion from 16 to 20 study sites has provided a wealth of chemical and biological data that provide multiple avenues for further analysis and study. Pore-water sampling at all 20 lake sites has been completed and the samples have been analyzed for the presence of Contaminants of Emerging Concern (CECs). In addition, composite surface water samples from all five lakes were collected and analyzed. Composite pore-water samples were also collected and analyzed. Synoptic sampling of septic seepage flow into ground water was completed in the final year of the study.

The chemical analysis of all samples has been completed and included pore-water, surface water, composite samples and laboratory water samples for confirmatory water chemistry. In total, well over 1,000 analyses were conducted to assess the presence and quantify the concentrations of CECs in Minnesota waters. These analyses revealed several key findings. First, CECs are ubiquitous in pore-water samples. Second, concentrations of CECs are higher in sites closer to lakeshore septic systems. Third, in addition to household-source signatures (i.e., CECs most likely used in households and as personal care products), some pore-waters also contain agricultural signatures (i.e., presence of pesticides in pore-water). Fourth, CECs are also ubiquitous in lake surface water -likely as result of incoming ground water flow.

The biological consequences of CEC exposures were evaluated using a combination of field and laboratory assessments. Native sunfish (*Lepomis macrochirus*) were captured near twenty field sites in which pore- and surface water chemistry was assessed for the presence of Contaminants of Emerging Concern (CECs) (Activities 1 and 2). In addition, hatchery-reared sunfish were exposed to mixtures of CECs derived from the pore-water measurements. We also exposed larval and adult fathead minnows (*Pimephales promelas*) in the laboratory to pore water (larvae only) and CEC mixtures. These analyses revealed several key findings. First, male fish taken from septic seepage-influenced lake sites and male fish exposed in the laboratory responded by producing the egg-yolk protein vitellogenin – a well-established biomarker of exposure to estrogenic CECs. Second, larval fathead minnows exposed to either pore water collected from field sites or to a comparable mixture of CECs were less likely to survive than control larvae. Third, higher concentration CEC mixtures, matching those observed in lake porewater produced subtle adverse biological effects. The biological findings identify CECs as a source of concern for the health and sustainability of Minnesota fish populations in lakes impacted by septic seepage.

Project Results Use and Dissemination

One peer-reviewed manuscript has been published, and two additional manuscripts are in preparation. In addition, results of the current study were disseminated widely in a series of presentations at regional and international scientific conferences:

- March 2016 – Society for Environmental Toxicology & Chemistry chapter meeting in Madison, WI
- November 2016 - Society for Environmental Toxicology & Chemistry world congress in Orlando, FL
- February 2017 - Fish & Wildlife Conference in Lincoln, NE
- March 2017 – MN Wastewater Conference, Brooklyn Park, MN
- March 2017 - Society for Environmental Toxicology & Chemistry chapter meeting in Minneapolis, MN
- April 2017 – Thesis defense (Les Warren) at St. Coud State University
- September 2017 – Seminar (Megan Guyader) at St. Cloud State University
- November 2017 - Society for Environmental Toxicology & Chemistry North America meeting in Minneapolis, MN (two presentations)

Environment and Natural Resources Trust Fund (ENRTF) M.L. 2015 Work Plan

Date of Report: July 31, 2018

Date of Next Status Update Report: July 31, 2018

Date of Work Plan Approval: June 11, 2015

Project Completion Date: June 30, 2018

Does this submission include an amendment request? __No__

PROJECT TITLE: Biological Consequences of Septic Pollution in Minnesota Lakes

Project Manager: Heiko L. Schoenfuss

Organization: St. Cloud State University

Mailing Address: 720 Fourth Avenue South, WSB-273

City/State/Zip Code: St. Cloud, MN 56301

Telephone Number: (320) 308 3130

Email Address: hschoenfuss@stcloudstate.edu

Web Address: web.stcloudstate.edu/aquatictox

Location: Statewide

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$364,000**
	Amount Spent:	**$362,650**
	Balance:	**$1,350**

Legal Citation: M.L. 2015, Chp. 76, Sec. 2, Subd. 04c

Appropriation Language:
$364,000 the first year is from the trust fund to the Board of Trustees of the Minnesota State Colleges and Universities System for Saint Cloud State University to assess the presence and possible sources of contaminants of emerging concern in Minnesota lakes in order to determine their effects on fish health, understand the potential contribution from septic systems, and inform options for remediation and prevention to protect Minnesota lakes from these contaminants in the future. This appropriation is available until June 30, 2018, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Biological Consequences of Septic Pollution in Minnesota Lakes

II. PROJECT STATEMENT:
Contaminants of Emerging Concerns (CECs), which include hormones, pharmaceuticals and compounds found in personal care products, have the potential to harm lake fish populations. We will link the established presence of CECs in Minnesota Lakes to observed biological effects in lake fish and provide the foundation needed for subsequent remediation efforts. The ENRTF has been instrumental in establishing that CECs are present and cause biological effects in Minnesota rivers and lakes. Riverine research has progressed to identifying sources and assessing the feasibility of remediation. However, for Minnesota lakes, only the presence has been established: the actual **sources of CECs** to the lakes have yet to be conclusively elucidated. **Understanding the sources of CECs is a crucial and necessary step to remedying the problem of CECs in Minnesota lakes.** Lake environments respond to pollution very differently than rivers as the sources of pollution are usually more diffuse (non-point sources) and as the long residence time of lake water (months to years) prevents the dilution of incoming pollution often observed in riverine environment. As a consequence, biological effects already observed in riverine fish exposed to CECs are likely more pronounced in lake fish and are more difficult to assess. In many lakes, obvious point sources of pollution are usually lacking.

Among potential CEC sources to Minnesota Lakes, onsite septic systems stand out for three reasons: they are commonly used around Minnesota lakes; they are not designed to remove CECs from household waste water; and CEC composition and concentrations measured in Minnesota Lakes in previous studies had distinct human "signatures" with many compounds present that are usually only associated with human household consumption (for example mood altering drugs, fragrances). The goals of this project are, therefore, to (i) validate the presence and biological effects of CECs in representative lake-types in Minnesota; (ii) identify a broad suite of approximately 50 CECs and their currently unknown metabolites that are contributing to the observed biological effects, and (iii) and conduct detailed analysis of potential sources with particular focus on onsite septic systems. The outcome of the proposed study is the identification of specific sources of CECs to Minnesota lakes and the documentation of the hydrologic pathways that result in discharge to lakes. As part of this approach, we will examine traditional and advanced (aerated) onsite septic system to assess their potential to treat CECs more effectively than is the case with traditional septic systems. This knowledge will aid natural resource managers in water conservation districts, watershed associations, and county zoning offices in identifying sources of CECs in their aquatic resources and will provide the information needed for water treatment specialists to assess potential remediation and/or preventative actions.

III. OVERALL PROJECT STATUS UPDATES:

Project Status as of January 1, 2016:
We developed a list of sixteen candidate lakes for this study and visited eight of those lakes to determine their suitability for the proposed study. Franklin Lake (Pelican Rapids, MN); Pearl Lake (Kimball, MN); Cedar Lake (Annandale, MN); and Sullivan Lake (Buffalo, MN) were found to meet most of the study requirements (sunfish habitats present, separation of septic influenced and reference sites; knowledge of ground water flow patterns) and will likely be included in subsequent field seasons of this study. Sunfish nesting habitats, zones of septic influence and reference sites were mapped for Pearl, Cedar and Sullivan Lake. Pore water (ground water sampled in the interstitial layer in the littoral zone of the lake) was sampled at two reference sites and two septic influenced sites in each of the three lakes (a total of 12 sampling events). Pore water samples were subjected to preliminary analytical chemistry. Larval fathead minnows were exposed for 21 days to pore water collected from the twelve field sites as well as blank and positive control water samples. Logistical preparations for the 2016 field season are underway.

Project Status as of July 1, 2016:
With the onset of the fish spawning season in May 2016 we began implementing our experimental study design. We collected pore water samples from four sites in each of five study lakes to gather a total of 10 sites likely to

be influenced by groundwater inflow tainted with septic discharge and ten sites that will serve as reference locations. Pore water samples from all 20 sites have been sent to our collaborators at the Colorado School of Mines for extraction and analysis, which is ongoing. Fish collections at all 20 study sites is ongoing and mostly driven by local lake water temperatures. A second round of pore water collections will focus on collecting samples that integrate over a longer period of time. Laboratory exposure experiments have begun and will continue for the remainder of the year. An experimental design for the integration of water samples across space and time has been developed and will be implemented during the remainder of the summers field season.

Project Status as of January 1, 2017:

Temporally integrated water samples as well as grab samples for the 2016 sampling season were fully processed for the targeted CECs. An additional method for the nontargeted analysis of these samples is being developed and will be implemented in the next reporting period. Spatially integrate water samples were collected from all 20 sites: four sites for each of five lakes. A total of ten composite samples were submitted for analysis of targeted CECs: one reference and one septic-influenced sample from each lake. All larval fathead minnow pore water exposures and preliminary analyses were completed during the current reporting period. In total, larval fish were exposed to pore water from all 20 sites (10 septic-influenced and 10 reference) from the five study lakes. Analysis of of tissues from the wild-caught male sunfish is ongoing. Water chemistry of pore water samples were used to create two separate synthetic mixtures to resemble the chemical signatures of two septic systems. These two mixtures were used to exposure mature adult fathead minnows and sunfish at four concentrations (plus blank and carrier controls) including the measured concentrations of the compounds in pore water along with a 1/10, 10x, and 100x the environmental concentration. Planning for the 2017 sampling season is underway which will focus on a range of on-site septic treatment technologies and an in-depth sampling along the septic flow pathway.

Project Status as of July 1, 2017:

Larval and adult fathead minnow and sunfish exposure experiments using pore water and laboratory mixture solutions have been completed. All data have been analyzed and have been subjected to quality control measures. Statistical analysis is ongoing in an attempt to link water chemistry results (Activities 1 and 2) with observed biological effects (Activity 3). This approach allows for a more nuanced assessment of the impacts of CECs on larval and adult fish. Our laboratory exposure experiments using mixtures of CECs similar in composition and concentrations to those measured in pore water suggest a significant role of several CECs in the development of adverse effects in exposed sunfish and fathead minnows. Additional integration of laboratory fish exposure results and confirmatory water chemistry (Higgins Lab at the Colorado School of Mines) are ongoing. Particularly noteworthy is the apparent linkage of active ingredients in sunscreen and estrogenic effects in exposed sunfish and fathead minnows. We are conducting follow-up experiments this summer and fall to strengthen these connections prior to manuscript submission. A Master's Thesis (Les Warren, Spring 2017, St. Cloud State University) summarizing results to-date has been prepared and defended in May 2017. Results of this effort are currently being composed for publication in a peer-reviewed journal.

Project Status as of January 1, 2018:

Synoptic sampling (Activity 1) along septic flow paths was conducted in September and October at two locations: a septic flow path and a reference site on the same lake. Samples have been transferred to the Higgins lab (Colorado School of Mines) for further analysis and integration with existing data sets.
All water sample collection has been completed and all environmental water samples have been analyzed using the targeted aqueous chemical analysis (Activity 2). Results of this analysis have recently been accepted for publication ("Trace Organic Contaminant (TOrC) Mixtures in Minnesota Lakes: Effects of On-Site Wastewater Treatment (OWTS) Proximity and Biologic Impact" Science of the Total Environment (STOTEN)). The non-targeted analysis of water samples is using biological results to prioritize features which contribute to the

endocrine activity of environmental TOrC mixtures. All grab, temporally integrated, and spatially integrated porewater samples collected in the Summer of 2016 from Cedar Lake, Sullivan Lake, and Lake Mary were analyzed using high resolution mass spectrometry for this analysis (Activity 2). Additional samples were collected in September of 2017 to address remaining uncertainty regarding the contribution of septic systems to elevated TOrC concentrations near residences. Passive samplers were deployed and gathered 2, 4, and 8 days after initial deployment.

All biological sampling has been completed (Activity 3) and results of fish exposure experiments in the laboratory (Activity 3) are currently being paired with confirmatory water chemistry. Biological results from resident fish collections have been accepted for publication in conjunction with the target aqueous analysis (see above – Activity 2). Results from laboratory exposure experiments are being prepared for journal submission.

Overall Project Outcomes and Results (July 1, 2018):

All activities proposed for the current study have been completed. The addition of a fifth lake and expansion from 16 to 20 study sites has provided a wealth of chemical and biological data that provide multiple avenues for further analysis and study. Pore-water sampling at all 20 lake sites has been completed and the samples have been analyzed for the presence of Contaminants of Emerging Concern (CECs). In addition, composite surface water samples from all five lakes were collected and analyzed. Composite pore-water samples were also collected and analyzed. Synoptic sampling of septic seepage flow into ground water was completed in the final year of the study.

The chemical analysis of all samples has been completed and included pore-water, surface water, composite samples and laboratory water samples for confirmatory water chemistry. In total, well over 1,000 analyses were conducted to assess the presence and quantify the concentrations of CECs in Minnesota waters. These analyses revealed several key findings. First, CECs are ubiquitous in pore-water samples. Second, concentrations of CECs are higher in sites closer to lake-shore septic systems. Third, in addition to household-source signatures (i.e., CECs most likely used in households and as personal care products), some pore-waters also contain agricultural signatures (i.e., presence of pesticides in pore-water). Fourth, CECs are also ubiquitous in lake surface water - likely as result of incoming ground water flow.

The biological consequences of CEC exposures were evaluated using a combination of field and laboratory assessments. Native sunfish (*Lepomis macrochirus*) were captured near twenty field sites in which pore- and surface water chemistry was assessed for the presence of Contaminants of Emerging Concern (CECs) (Activities 1 and 2). In addition, hatchery-reared sunfish were exposed to mixtures of CECs derived from the pore-water measurements. We also exposed larval and adult fathead minnows (*Pimephales promelas*) in the laboratory to pore water (larvae only) and CEC mixtures. These analyses revealed several key findings. First, male fish taken from septic seepage-influenced lake sites and male fish exposed in the laboratory responded by producing the egg-yolk protein vitellogenin – a well-established biomarker of exposure to estrogenic CECs. Second, larval fathead minnows exposed to either pore water collected from field sites or to a comparable mixture of CECs were less likely to survive than control larvae. Third, higher concentration CEC mixtures, matching those observed in lake pore-water produced subtle adverse biological effects. The biological findings identify CECs as a source of concern for the health and sustainability of Minnesota fish populations in lakes impacted by septic seepage.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Integrated Surface and Ground Water Sampling for CECs

Description: To identify sources of CECs in Minnesota Lakes, both surface and ground water needs to be sampled continuously and across seasons in lakes with varying infrastructure characteristics. The USGS has developed a passive sampling technology that allows for continuous sampling of surface and ground water for a month at a time. This technology will be used to explore the continuous input of CECs through ground water and surface water runoff in four Minnesota lakes (with known concentrations of CECs). Lakes and lake sites will be chosen to encompass different discharge pathways (surface water runoff; sewered vs. onsite septic systems) and septic technologies (traditional systems, advanced aeration). In addition, a preliminary sample of lakes will be assessed in summer 2016 for logistical feasibility of water and fish sampling.

- We will assess four lakes and examine in detail two septic-impacted and two reference sites in each lake (total of 16 sampling sites for the study).
- Passive sampling devices (allowing for the continuous sampling for four weeks at a time) will be installed at two sites in each lake.
- Approximately 128 environmental samples (plus quality assurance/quality control samples) will be screened using inexpensive high-throughput assay technology and augmented with detailed analytical chemistry (Activity 2) when warranted by assay results.
- Due to the potential high temporal variability in water quality of some potential CEC sources (i.e., septic tank leachate), detailed temporal grab samples will also be collected to verify the validity of the passive sampling approach (Activity 2).

These results will inform the biological effects testing (Activity 3).

Summary Budget Information for Activity 1:

ENRTF Budget:	**$ 125,000**
Amount Spent:	**$ 125,000**
Balance:	**$ 0**

Outcome	Completion Date
1. Identify 4 lakes and appropriate study sites and establish sampling devices in lakes	June 30, 2016
2. Sample ground and surface water for eight week periods	December 31, 2016
3. Quantify CEC concentrations in seasonal ground water from 4 lakes using screening methods for model CECs such as estrogens, detergents and pharmaceuticals	June 30, 2017
4. Quantify CEC concentrations in seasonal surface water from 4 lakes using screening methods for model CECs such as estrogens, detergents and pharmaceuticals	December 31, 2017

Activity Status as of January 1, 2016:
Pore water analysis from 12 sites representing two reference and two septic influenced sites in each of three lakes (Pearl, Cedar, Sullivan Lake) indicate the suitability of at least two of the lakes for further analysis (i.e., reference sites are mostly clear of Contaminants of Emerging Concern; septic influenced sites carry higher contaminant loads than reference sites). Results from Cedar Lake will require additional analysis to assess its suitability for this study. Franklin Lake has been examined previously and found to be a suitable study lake. Additional lakes have been examined if substituting Cedar Lake becomes necessary. Logistical preparations for the 2016 field season are underway.

Activity Status as of July 1, 2016
A sampling design to integrate pore water samples across space and time has been developed as the result of a length validation process in which multiple methodologies were explored. Ultimately, the collaborative team settled on compositing samples taking across multiple pore water collection sites in each lake and to supplement this approach with a time integrated sample collected concurrently. This experimental design is currently being implemented across the study sites.

Activity Status as of January 1, 2017
The sampling design to integrate pore water samples across space and time was implemented by synoptic sampling of each lake. Spatial integration was achieved by sample collection at multiple locataions for each septic or reference site in each lake. Samples in each category were combined to produce two compostite samples for each lake. A total of 20 sites were sampled (four for each of five lakes), producing a total of ten composite samples (five in each category of reference or septic-influenced).

Temporal integration was achieve through multiple sub-samples at each sties collected over a number of weeks. A total of 110 samples from two rounds of integrated sampling were submitted to CSM for analysis, and results are summarized below under Activity 2. Current plans for the 2017 ice-free sampling season call for screening of multiple integrated samples from each sites over a number of weeks, based on the results from the 2016 sampling.

Porewater passive sampling methods were tested with a number of portable DC-powered pumps. None of the pumps tested maintained a constant flow suitable for use with the passive samplers. Equipment tests continue through the current quarter to solve the flow rate issue with the passive samplers.

Activity Status as of July 1, 2017
Successful synoptic sampling along septic flowpaths was twice during the spring at multiple locations along two test sites. Time-integrated sampling at multiple locations along the flow path of two types of systems is planned for September of 2017 or as shallow groundwater levels are conducive to successful sampling.

Activity Status as of January 1, 2018
Synoptic sampling along septic flowpaths was conducted in September and October of 2017 at two locations: a septic flowpath and a reference site on the same lake. Sequential time-integrated samples, collected using passive samplers, were coupled with grab samples from the same locations and submitted for analysis. Samples were collected from shallow groundwater using fixed mini-piezometers at multiple locations along the septic flow path and at one location at the reference sites. Analysis of synoptic and time-ingetrates samples collected at multiple locations along the flow paths is under way under Activity 2.

Final Report Summary:
All sampling has been completed and all samples have been submitted for CEC analysis (Activity 2). Analysis of CEC concentrations have been linked to proximity of septic systems and this analysis has been published (see below).

ACTIVITY 2: Analysis of samples for CECs
Description: Identifying the sources of CECs to Minnesota lakes will require looking for both CECs that are expected to be released to surface waters (i.e., from storm water runoff) and CECs that might leach from onsite septic systems (i.e., pharmaceuticals and personal care products). State of the art liquid chromatography tandem mass spectrometry will be employed for known CEC analysis of selected samples (Activity 1). CECs to be quantified will include approximately 50 known or suspected CECs from all compound classes. These include pharmaceuticals (for example carbamazepine, diazepam, acetaminophen, sulfamethoxazole, etc.), antimicrobial agents (triclosan, triclocarban, etc.), ingredients in personal care products (DEET), detergents (nonylphenol, octylphenol, etc.), flame retardants (TCEP, TCPP) and steroid hormones (estradiol, testosterone, progesterone, etc.). In addition, these samples will be screened for non-target compounds using high throughput LC-time-of-flight MS, which will enable the identification of a broad range of contaminants. This non-target screening is essential, as many known CECs can be biologically or chemically transformed in the environment to often unknown transformation products that retain substantial biological activity, and may confound the biological assays (Activity 3).

Summary Budget Information for Activity 2:

ENRTF Budget:	**$ 142,125**
Amount Spent:	**$ 142,125**
Balance:	**$ 0**

Outcome	Completion Date
1. Develop and validate sampling and extraction protocols	June 30, 2016

2. Accurate quantitation of approximately 50 CECs in passive sampler extracts and grab samples	June 30, 2017
3. Screening of grab samples and extracts for non-target analytes such as metabolites of pharmaceuticals and personal care products	June 30, 2018

Activity Status as of January 1, 2016:
Twelve pore water samples (four sites each in Pearl, Cedar, and Sullivan Lake) have been analyzed to confirm the suitability of the sampling sites for detailed study in 2016. Putative septic influenced sites in Pearl and Sullivan contained higher CEC loads than their respective reference sites. Cedar Lake did not represent an equally clear division between septic influenced and reference sites. Additional analysis is underway to examine the suitability of Cedar Lake for this study. In addition, analysis of these samples allowed for the development of standard sampling, shipping, and extraction protocols.

Activity Status as of July 1, 2016
Preliminary porewater samples from Sullivan Lake, Cedar Lake, and Pearl Lake were submitted to the Colorado School of Mines (CSM) in 2015. The samples were used to develop two analytical methods on a Sciex 3200 QTRAP liquid chromatograph tandem-mass spectrometer (LCMS/MS) for the targeted analysis of 57 organic wastewater contaminants (OWCs). The methods encompass two ionization modes, both positive and negative, and include two transitions for each analyte. Calibration limits for most of the compounds range from 50 ng/L to 20,000 ng/L on column. A solid phase extraction (SPE) method was also prepared to concentrate the anticipated 1 L field samples by 50-fold. Therefore, the LC-MS/MS lower calibration limits of most analytes in the environmental samples range from 1 ng/L to 400 ng/L. Matrix spike and surrogate recovery analyses were performed on a composite of the preliminary samples. With the exception of acesulfame and testosterone propionate, the SPE method spike recoveries ranged from 69% to 181%, while the surrogate recoveries ranged from 11% to 170%. Analytes included in the positive ionization method demonstrated more favorable matrix spike recovery and surrogate recovery results. The artificial sweetener acesulfame was the only analyte for which exceptionally poor surrogate recovery was routinely observed, likely due to its polar nature (log K_{ow} = 1.33 [Anal. Bioanal. Chem. 403 (2012) 2503-2518.]). This property of acesulfame hinders it's retention onto the Oasis hydrophilic-lipophilic balance (HLB) cartridges used in the SPE method. For this reason, acesulfame was omitted from analysis of the SPE extracts; instead, it will be analyzed by direct aqueous injection. Testosterone propionate had a spike/recovery of 347%. The analyte does not have a directly analogous surrogate in this method; rather, it is quantified using testosterone-d2 which only had a surrogate recovery of 28%. An isotope of testosterone propionate would likely have a higher surrogate recovery and, thus, a more accurate quantitation for the analyte. We plan to keep testosterone propionate in the analysis of the SPE extracts; however, we will closely monitor the matrix spike recoveries in the field samples and adjust reported concentrations accordingly. A suspect screening of unknowns was also performed on samples prepared by both direct aqueous injection and SPE via a preliminary method that concentrated by a factor of 5. The transformation products atrazine-hydroxy and atrazine-desethyl were the only conclusive library matches identified using this approach. We concluded that only concentrated samples should be used for future non-target and suspect analyses. More method development on the Sciex TripleTOF 5600 quadrupole time of flight (QTOF) system is necessary before implementing it for field sample analysis.

A total of 296 aqueous samples from the five lakes selected for the study were submitted to CSM as of June 2016. Targeted analysis of the 56 OWCs was performed on the 1L samples after they were concentrated using the developed SPE method. Supporting inorganic analyses was also executed on separate (but concurrently collected) 50 mL samples, with particular interest in chloride, bromide, boron, and nitrogen species. Analysis of the artificial sweetener acesulfame will be accomplished using samples from a third set of concurrently collected samples. These data, along with basic water parameters taken in the field, will be reviewed soon. The integrated samples from each lake are still in progress. We anticipate 125 additional samples from two rounds of integrated sampling to be submitted to CSM. Analysis techniques will remain the same for these samples. Non-target method development and analysis of unknowns will not occur until all samples have been collected and extracted.

Activity Status as of January 1, 2017

A total of 110 temporally integrated samples were received by CSM and processed using the SPE and targeted LC-MS/MS methods described previously. Targeted analyte data for both temporally integrated and grab samples was processed using AB Sciex's Multiquant software using rigorous quality assurance and quality control (QA/QC) procedures. Common detections in samples included: Benzophenone, DEET, TCPP, Oxybenzone, 2,4-D, Bisphenol A, and 4-tert-octylphenol (Figure 1). Several samples are currently being reanalyzed for 2,4-D, Benzophenone, Caffeine, and DEET at concentrations higher than the calibration limit, 400 ng/L. According to the current data set, "septic" designated sites had higher total OWC concentrations in each lake. However, detection frequency of targeted OWCs varied between each lake with Lake Mary having the most "hits" and Cedar Lake having the least. Further statistical analysis will be conducted on this data set, as well as the temporally integrated data set, to determine if there is a correlation between distance from nearest residence and OWC concentration/frequency.

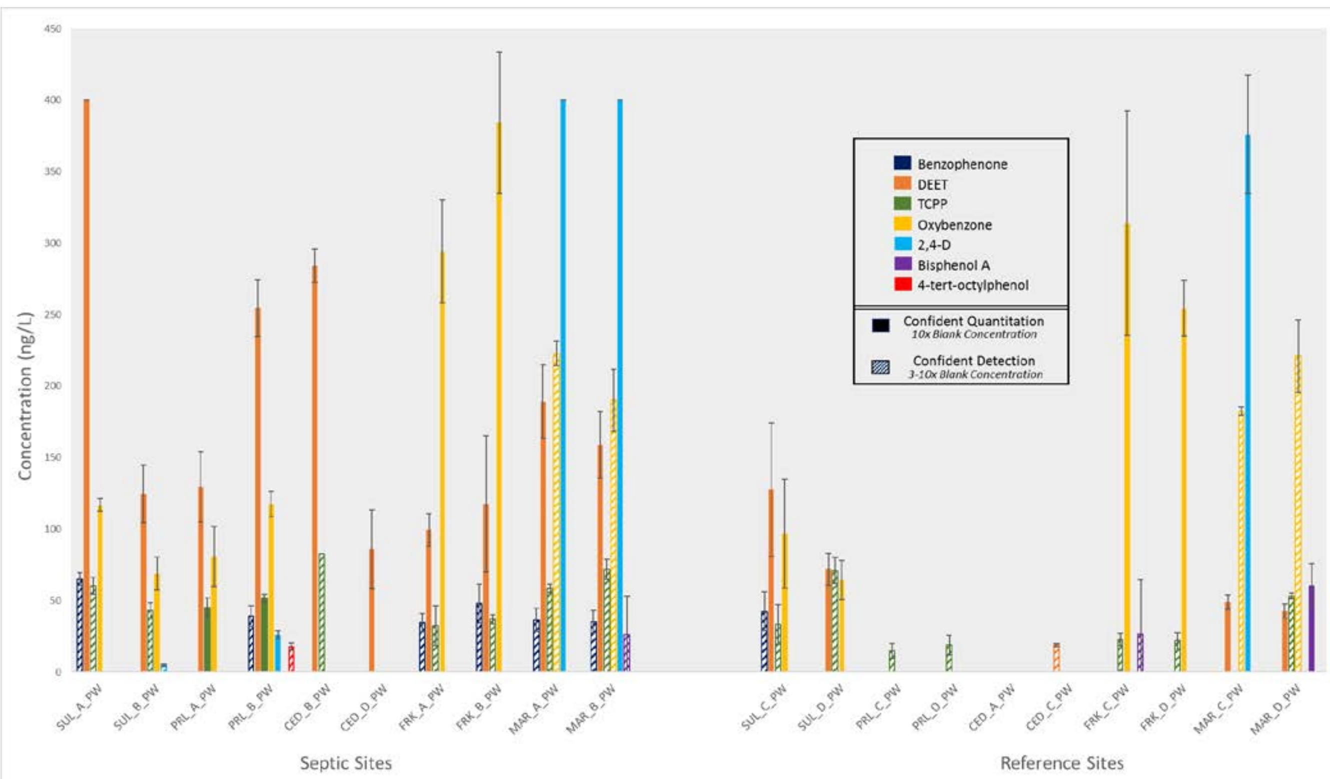

Figure 1: Concentrations of Common Detections in Lake Porewater Grab Samples

Method development for non-target analysis of both grab and temporally integrated samples is currently being completed. Data analysis will be focused on identifying transformation products of the targeted analytes. These data will then be used to calculate relative abundance of transformation products to parent compounds. This information, along with the statistical analysis from the targeted data set, will provide understanding of which OWCs likely originate from nearby residences with OWTSs.

CSM also received initial and final time point samples from the St. Cloud State University lab exposure studies. Samples were prepared by either concentration, dilution, or direct aqueous injection based on anticipated concentrations in each sample. Samples were analyzed using the same LC-MS/MS methods from the field study.

Finally, planning is underway for the summer 2017 sampling campaign, which will focus on OWC concentrations released from surrounding OWTS systems. In addition, we plan to assess their persistence in groundwater plumes feeding into each lake.

Activity Status as of July 1, 2017

Targeted aqueous chemistry results are finalized. Two-group hypotheses tests were conducted on samples attributed to either: a) Residential (RES) sites, those within proximity of residences with on-site wastewater treatment systems (OWTSs), and b) Reference (REF) sites outside proximity of residential OWTSs. Significantly higher ($p < 0.05$) total concentrations of the targeted trace organic chemicals (TOrCs) were detected in the RES porewater grab samples. There was no significant difference in total TOrC concentration or the number of TOrC detections in the temporally integrated samples.

Non-target analysis of lakewater samples will provide a better understanding of differences in aqueous compositions at RES and REF sites. Preliminary analysis of detections in passive samplers show some m/z features have a significantly higher ($p < 0.05$) intensity in RES sites. A principle component analysis (PCA) of the lake compositions show that Cedar Lake and Lake Mary are most similar and their RES and REF sites cluster separately.

Biologic data from resident male fathead minnows in the lakes was compared to grab and temporally integrated porewater chemistries. A positive correlation was found between the number of detections ($r = 0.5749$) and total TOrC concentration ($r = 0.61974$) in grab porewater samples and vitellogenin levels, a marker of endocrine disruption. There was also a negative correlation with glucose. The same correlations were not observed in the temporally integrated samples

Activity Status as of January 1, 2018

Targeted aqueous chemical analyses and resident male biological analyses from the Summer 2016 sampling campaign were synthesized into the manuscript “Trace Organic Contaminant (TOrC) Mixtures in Minnesota Lakes: Effects of On-Site Wastewater Treatment (OWTS) Proximity and Biologic Impact”. The text is currently under review with the journal Science of the Total Environment (STOTEN). Results from the study indicate higher targeted total TOrC concentrations at sites sampled proximal (HOME) versus those distal (REF) to shoreline residences. The applications associated with certain detected TOrCs suggests input from OWTSs (septic systems) along with other residential activities at HOME sites. Additional diffuse sources, such as agricultural operations, are also suspected to impact these lakes, particularly at REF locations. Chemical and biological data from this study prioritize Lake Mary’s HOME sites as having notable endocrine activity and TOrC input from residences.

The non-targeted analysis workflow in this study will use biological results to prioritize features which contribute to the endocrine activity of environmental TOrC mixtures. All grab, temporally integrated, and spatially integrated porewater samples collected in the Summer of 2016 from Cedar Lake, Sullivan Lake, and Lake Mary were analyzed using high resolution mass spectrometry for this analysis. These lakes were chosen for their notable endocrine activity or endocrine inactivity according to the biological analyses associated with the first manuscript. A preliminary principal component analysis (PCA) of the acquired data indicates feature similarity amongst biologically active sites at Lake Mary and Sullivan Lake. This was not observed in the samples from Cedar Lake where all sites were previously determined to be biologically inactive. Future work will focus on characterization of the features at Lake Mary and Sullivan Lake’s biologically active sites.

Additional samples were collected in September of 2017 to address remaining uncertainty regarding the contribution of septic systems to elevated TOrC concentrations near residences. Passive samplers were deployed and gathered 2, 4, and 8 days after initial deployment. Samples were transported to CSM and are currently preserved on cartridges for later analysis.

Confirmatory chemistry from the laboratory exposures has also been addressed. Results from this analysis are currently undergoing quality assurance and quality control procedures.

Final Report Summary:

Results from targeted aqueous chemical analyses of grab porewater samples were published in *Science of the Total Environment* "Trace Organic Contaminant Mixtures in Minnesota Littoral Zones: Effects of On-Site Wastewater Treatment Proximity and Biological Impact" (Guyader et al., 2018). Briefly, elevated total TOrC concentrations were observed at sampling locations (see Figure 1) more proximal to shoreline residences from the five lakes sampled (see Figure 2). Notable detections included 2,4-D and DEET, neither of which are known endocrine disruptors. However, 2,4-D has been linked to promoting the androgen disrupting capabilities of testosterone (Kim et al., 2005). The associated outdoor usage of these chemicals does not unequivocally suggest the presence of on-site wastewater treatment (OWTS) effluent at these locations; however, presence of pharmaceuticals does. It is likely that multiple diffuse pathways transport TOrCs from residential households to nearby littoral zone locations. Elevated concentrations of TOrCs corresponded to elevated blood concentrations of vitellogenin in adult male sunfish, indicating endocrine activity in the sampled porewater (see Figure 3).

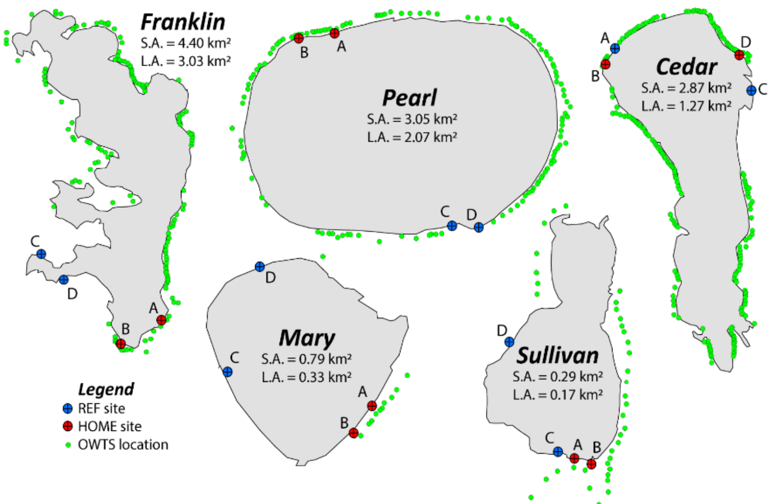

Figure 1: Summary of sampling locations within each of the five lakes involved in the study. Lake characteristics are provided under each lake's name, where S.A. = surface area and L.A. = littoral area.

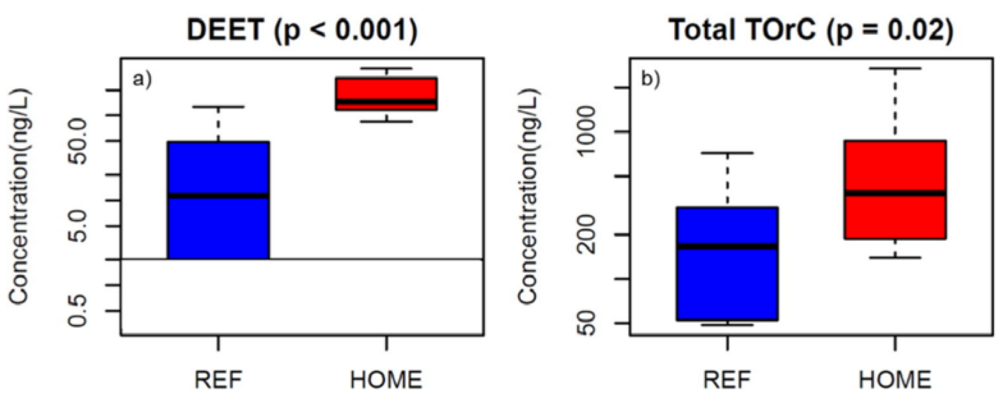

Figure 2: Two-group comparison of average site concentrations across all five lakes of a) DEET and b) total TOrC concentrations of 33 targeted analytes. The detection limit of DEET (2 ng/L) is displayed using a horizontal line. First quartiles estimated below this value are omitted.

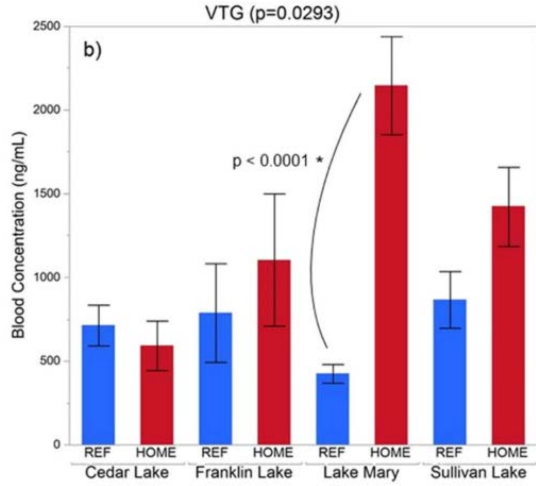

Figure 3: Average blood vitellogenin concentrations of adult male sunfish captured from HOME and REF sites within each lake. Error bars represent the standard deviation of values from the average concentration at each lake. p-values above the plots are comparisons of concentrations infish captured from HOME and REF sites across all lakes. The in-plot p-value is the two group comparison of HOME and REF captured fish from Lake Mary only.

Grab samples and temporally integrated samples were also evaluated using a liquid chromatography tandem quadrupole time of flight mass spectrometer (LC-QTOF/MS) to prioritize unknown contaminants in the environmental matrices that may contribute to noticeable difference in endocrine activity in HOME and REF sites from Lake Mary. Features were prioritized using univariate statistics (two-tailed t-test) to note features higher intensities at biologically active sites versus reference sites. Similarly trending features were then grouped using a multivariate statistical method (principal component analysis with principal component variable grouping) to designate them as either ubiquitous at all active sites (AB), or unique to individual active sites (A or B). Identification of prioritized features was then attempted by a suspect search compared to spectral libraries. Features resolved by library spectra were reported in accordance with established confidence metrics. Features unresolved by the spectral library were reported as masses of interest. The list of Level 1(Confirmed Structure with Reference Standard) and Level 2 (Likely Candidate) identified features is provided in Table 1. Shared chemicals across both sites include hydroxyquinoline which functions as a chelating and fungicidal agents in many products, insect repellents and pesticides, and dye agents. Site specific chemicals associated with endocrine disrupting included a steroid, pesticide, commonly manufactured polymers, and plant derivatives in site A. Plant based essential oils, some with traditional medicinal uses, and plant-derived fatty acids were identified exclusively at Site B. Results from this work are currently being assembled into the manuscript "Exploitation of Toxicological Endpoints for the Statistical Prioritization of Liquid Chromatography Tandem High Resolution Mass Spectrometry (LC-HRMS) Features: An Approach to Selecting Candidate Chemicals for Endocrine Disruption Risk Assessment."

A further sampling effort was also executed in September of 2017 to address gaps in understanding of OWTS contribution to elevated TOrC concentrations in lake locations near shoreline residences. Passive samplers (OSORB) and aqueous grab samples extracted using mixed mode solid phase extraction were used to account for temporal variations in single residential wastewater. Samplers were collected from a septic tank, drain field, and porewater from a lake location proximal to the septic tank and a lake reference location distal from the residential property. Final extracts for these samplers were acquired using LC-QTOF/MS in both ESI + and ESI – modes. Preliminary assessments of ESI + modes indicate unique clustering of septic tank and drain field sampling locations in principal component scores plots. Biotransformation products could potentially be identified by pattern finding methodologies for nontarget data processing workflows. Feature similarities amongst septic tank, drain field, and proximal lake site locations versus reference site locations will also be investigated.

Table 1. Confirmed or Likely Structures Identified by Statistical Prioritization of LC-HRMS Features

Dataset	Site(s)	Feature (MW ion/RT)	ID	Confidence Level
grab pos	AB	146.1/6.5	8-hydroxyquinoline	Level 2
	AB	184.1/6.4	Simazine-2-hydroxy	Level 2
	AB	192.1/9.5	N,N-diethyltoluamide (DEET)	Level 1
	AB	146.1/6.9	8-hydroxyquinoline isomer	Level 2
	AB	161.1/8.3	Dihydroxynapthalene	Level 2
	A	176.1/7.3	7-methoxyquin-4-ol	Level 2
	A	273.2/10.3	5-alpha-androst-2-en-17one	Level 2
	A	579.2/7.5	Vitexin-2-O-rhamnoside	Level 2
grab neg	AB	219.0/8.6	2,4-Dichlorophenoxyacetic acid	Level 1
	AB	161.0/8.6	Dichlorophenol	Level 2
	A	153.0/7.0	Dihydroxybenzoic Acid	Level 2
	A	563.1/7.2	Isoschaftoside	Level 2
temp int pos	AB	146.1/6.5	8-hydroxyquinoline	Level 2
	AB	184.1/6.4	Simazine-2-hydroxy	Level 2
	AB	473.4/11.4	Sumaresinolic acid	Level 2
	A	216.1/9.0	Atrazine	Level 2
	A	344.2/7.3	PEG-7-mer ammonium adduct	Level 2
	A	388.3/7.6	PEG_8-mer ammonium adduct	Level 2
	A	399.3/10.3	Tris (2-butoxyethyl) phosphate	Level 2
	A	432.3/7.9	PEG-9-mer ammonium adduct	Level 2
	A	476.3/8.2	PEG-10-mer ammonium adduct	Level 2
	B	211.1/9.3	Jasmonic Acid	Level 2
	B	235.2/9.9	Valerenic Acid	Level 2
	B	251.2/10.2	Sclareolide	Level 2
	B	277.2/9.1	Octadecadiyonic Acid	Level 2
	B	295.2/9.1	9-oxo-10E, 12E-octadecadienoic acid	Level 2
temp int neg	B	179.0/7.2	5-acetyl-2-hydroxybenzoic acid	Level 2
	B	293.2/10.2	13-keto-9Z,11E-octadecadienoic acid	Level 2

ACTIVITY 3: Analysis of Fish for Effects and Causal Linkage to CEC Exposure

Description: CECs are diverse in their presence, concentrations and chemical nature. To establish a causal relationship between CECs measured through chemical analysis and biological effects observed in resident fish, two assumptions have to be tested: (i) fish in lakes with CEC occurrence present pathologies consistent with CEC exposure and (ii) laboratory reared fish exposed to the mixture of CECs measured in surface and ground water will develop similar pathologies. To test these assumption we will:

- We will collect fish from 16 sampling sites in the four study lakes and assess for a comprehensive range of pathological indications consistent with exposure to CECs.
- We will collect approximately 100 egg clutches from the 16 lake study sites to quantify fecundity and health of embryos and juveniles and use the information in population models established as part of previous ENRTF funded research.

- We will expose eggs in the laboratory to ground water from all 16 field sites and expose larvae and adults of both species to four mixtures of CECs based on analytical findings from Activities 1 & 2 and assess similar endpoints.

Taken together, these field and laboratory studies will provide the information needed to establish a causal link between septic system discharge and endocrine disruption in lake fish if such a linkage exists.

Summary Budget Information for Activity 3:

ENRTF Budget:	**$ 96,875**
Amount Spent:	**$ 95,525**
Balance:	**$ 1,350**

Outcome	**Completion Date**
1. Identify four spawning habitats (two septic influenced, two reference) in each of the four study lakes	June 30, 2016
2. Collect and analyze larvae and adults of fathead minnows and sunfish from four sites in each of the four study lakes (Activity 1) for CEC exposure effects	June 30, 2017
3. Expose egg clutches and adult fathead minnows and sunfish to CEC mixtures representing lake exposure conditions (Activity 2)	December 31, 2017
4. Establish causality between lake CEC exposure and biological effects through laboratory fish exposures based on measured environmental concentrations (Activity 2)	June 30, 2018

Activity Status as of January 1, 2016:
Site visits to eight candidate lakes for this study were conducted during the sunfish spawning season in 2015. We evaluated land use characteristics to identify likely septic influenced and reference areas along the lake shore. Thermal probes were used to confirm inflow of groundwater from septic influenced and reference areas. We conducted visual surveys of spawning habitats for sunfish and mapped spawning beds. Based on site visits and previous studies, we identified four putative study lakes (Franklin Lake (Pelican Rapids, MN); Pearl Lake (Kimball, MN); Cedar Lake (Annandale, MN); and Sullivan Lake (Buffalo, MN)). 30L of pore water was collected at four sites each in Pearl, Cedar and Sullivan Lake. Preliminary water analysis suggests that duplicate reference and septic influenced sites in Pearl and Sullivan Lake meet our study criteria. One reference and one septic influenced site in Cedar Lake will require further analysis (currently ongoing) to determine their suitability for this study. A subsample from each collection was shipped to Dr. Higgins (Colorado School of Mines) for analysis of Contaminants of Emerging Concern (Activity 2). The remaining sample was used to expose larval fathead minnows for 21 days post-hatch in a 50% daily static renewal system to evaluate the effects of these waters on predator-avoidance performance. These exposure were recently completed and preliminary results are presented in Figure 1 below. In addition, mature male sunfish were collected from their nest sites in two septic-influences sites in Sullivan Lake and analyzed for a suite of biomarkers commonly associated with exposure to Contaminants of Emerging Concern. Together, these exposure experiments allowed us to establish standard operating procedures for the remainder of the study.

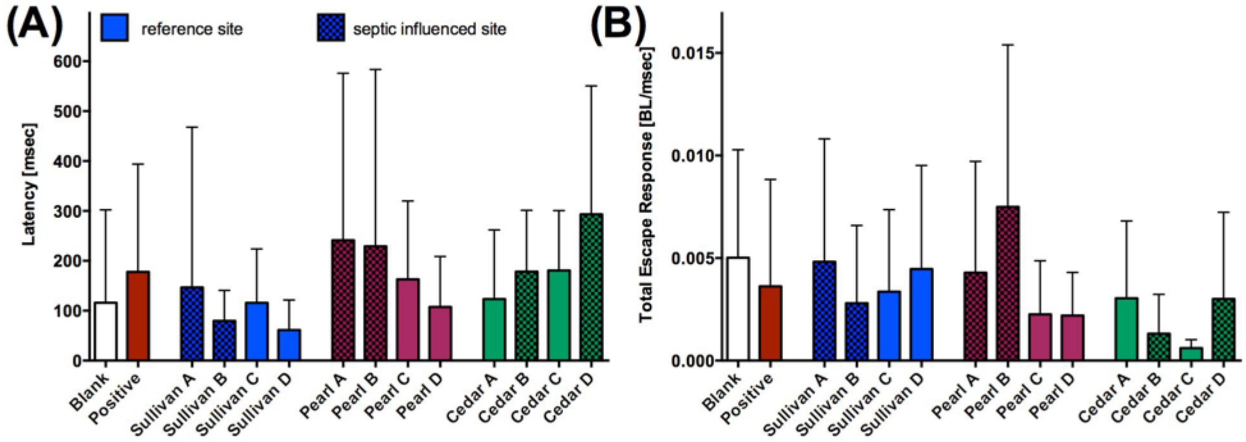

Figure 1. Results of the 21 day static renewal exposure of larval fathead minnows (*Pimephales promelas*) to pore water from twelve lake sites. (A) Reaction time (in msec) of minnows following 21 day exposure. (B) Total escape response (in body length / msec). Blank well water control and positive control (50 ng/L 17b-estradiol) are included with twelve pore water treatments representing three lakes. Septic sites in each lake are indicated by the checkered pattern of the respective columns. Mean +/- standard deviation.

Activity Status as of July 1, 2016

We have completed a first round of pore water collections for analytical chemistry (Activities 1 and 2) from 20 lake sites representing ten putative septic and ten reference sites. We also completed a round of larval fish exposures (Activity 3) that coincided with spawning activity in resident sunfish during May and June 2016. In total we sampled four sites (two putative septic and two putative reference sites) in each of five study lakes (Franklin Lake (Pelican Rapids, MN); Pearl Lake (Kimball, MN); Cedar Lake (Annandale, MN); Lake Mary (Maple Lake, MN), and Sullivan Lake (Buffalo, MN)). One set of water samples was deliver to our collaborators at the Colorado School of Mines (see updates under Activity 2) while 25L of water from each collection site (n=20 unique sites) was frozen for use in larval fish exposure studies (Activity 3).

We have begun collecting adult male fish and eggs from spawning sites in each of the study sites. This work is mostly complete on Sullivan Lake, Lake Mary and Pearl Lake. Cedar Lake and Lake Franklin will be processed as water temperatures and fish spawning activity increases in July 2016.

A first pore water larval fathead minnow 21 day exposure study using water from Lake Mary was completed in June 2016. We anticipate to complete data analysis from this exposure in fall 2016.

Activity Status as of January 1, 2017

With completion of the 2016 summer field season, the focus shifted to completing lab exposure experiments of larval and adult fathead minnows and adult sunfish. We completed all larval fathead minnow pore water exposures in the late fall and preliminary results of these exposures are provided in Figure 2 below. In total, larval fish were exposed to pore water from all 20 sites (10 septic-influenced and 10 reference) from the five study lakes. Analysis of the wild-caught male sunfish is ongoing. Histological analysis of tissues from these fish has begun along with quantification of other physiological biomarkers.

Water chemistry of pore water samples sent to Colorado School of Mines (Activity 1 and 2) were used to create two separate synthetic mixtures to resemble different septic systems (See Activity 2). These two mixtures were used to exposure mature adult fathead minnows and sunfish (n=400 each) at a range of concentrations. Concentrations included the environmentally found concentration along with a 1/10, 10x, and 100x the environmental concentration. Results from these exposures will be used to connect the contaminants with the biological pathologies observed in the wild-caught fish. Larval fish will also be exposed to the synthetic mixtures in the coming months.

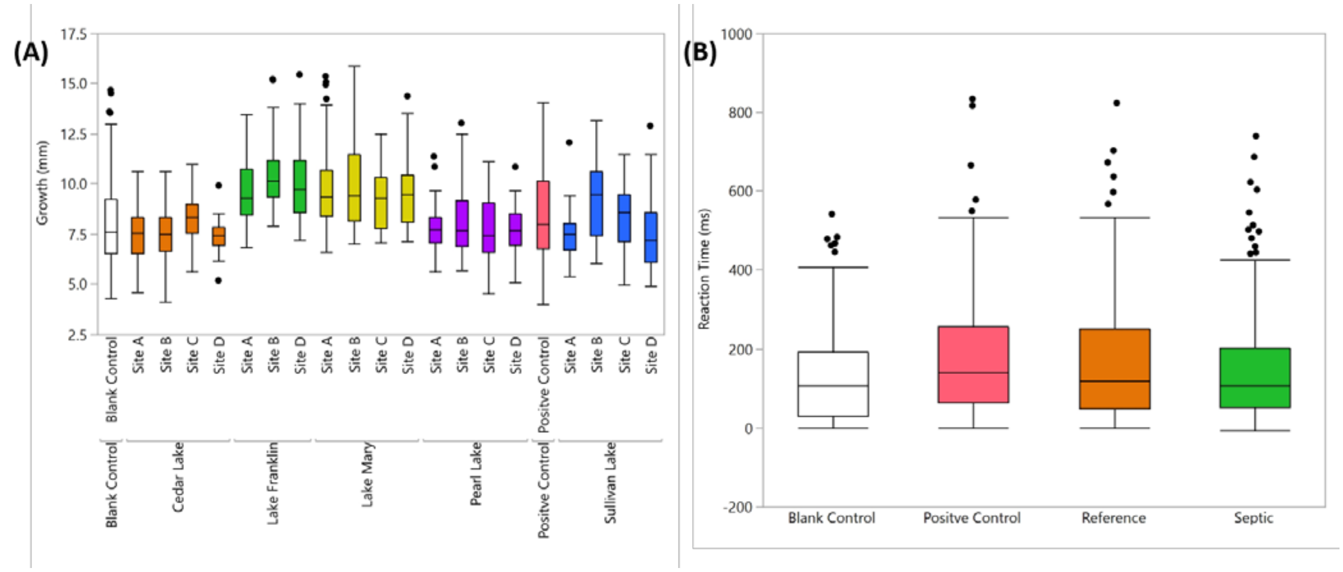

Figure 2. Results from the 21 day static renewal exposure of larval fathead minnows to pore water from twenty lake sites. (A) Growth of larval minnows over the 21 day period (in mm). (B) Reaction time (in msec) of first movement after stimulus. Blank well water controls and positive control (625ng/L Estrone) were performed alongside of the twenty pore water treatments from the five lakes. Mean +/- standard deviation, box and whisker indicated 95% of samples, outliers indicated by dots.

Activity Status as of July 1, 2017

Larval and adult fathead minnow and sunfish exposure experiments to lake pore water and laboratory mixture solutions have been completed. All data have been analyzed and organized for statistical analysis. Statistical analysis is ongoing and attempting to link water chemistry results (Activities 1 and 2) with observed biological effects (Activity 3). This approach allows for a more nuanced assessment of the impacts of CECs on larval and adult fish. A Master's Thesis (Les Warren, Spring 2017, St. Cloud State University) summarizing results to-date has been prepared and defended in May 2017. Results of this effort are currently being composed for publication in a peer-reviewed journal (anticipated fall 2017).

Additional integration of laboratory fish exposure results and confirmatory water chemistry (Higgins Lab at the Colotrado School of Mines) are ongoing and will be incorporated in the manuscript in development and in the next progress report. Particularly noteworthy is the apparent linkage of active ingredients in sunscreen and estrogenic effects in exposed sunfish and fathead minnows. We are conducting follow-up experiments this summer and fall to strengthen these connections prior to manuscript submission.

Activity Status as of January 1, 2018

Fish exposure experiments

Exposure experiments and sample analysis has been completed with only minor outstanding quality assurance/quality control assessments to be completed. Data analysis is ongoing as multiple manuscripts are readied for publication.

Final Report Summary:

This study thoroughly examined the biological effects of septic seepage on native Minnesota fish species using field and laboratory approaches. Native sunfish (*Lepomis macrochirus*) were captured near twenty field sites in which pore- and surface water chemistry was assessed for the presence of Contaminants of Emerging Concern (CECs) (Activities 1 and 2). These fish were subject to extensive analysis of growth and organ morphology, blood parameters and assessment of microscopic changes to liver and reproductive organ. These exhaustive investigations were supplemented with laboratory exposures of hatchery-reared sunfish using mixtures of CECs derived from the pore-water measurements. To extend our interpretive power beyond sunfish to common prey species, we also exposed larval and adult fathead minnows (*Pimephales promelas*) in the laboratory to pore water (larvae only) and CEC mixtures similar in composition to those used to expose hatchery-reared sunfish. Taken together, these experiments revealed subtle, but unmistakable, effects of CECs presence on the biology of both fathead minnows and sunfish. In the resident male sunfish collected from the study lakes, an induction of vitellogenin and the reduction in the hepatosomatic index were observed. Whereas in the laboratory study, the same vitellogenin induction was observed, but no reduction in the hepatosomatic index. Critically, a key indicator of CEC exposure in males, the induction of vitellogenin, was observed in both cohorts of assessed sunfish.

When larval fathead minnows were exposed to either pore water collected from the twenty field sites or to a mixture of CECs based on the analysis of pore water, a reduction in larval survival was observed in both the pore water and laboratory mixture exposures. Lake sites (both septic-influenced and reference sites) had significantly lower survival when compared to the control. This finding is likely related to the persistent presence of CECs at all field sites (albeit at lower concentrations at reference sites). This may indicate that even at lower concentrations, CECs may impact larval survival. This hypothesis is supported by the finding of reduced survival in the medium and high concentration treatments of one of the two CEC mixtures applied to larval fathead minnows in the laboratory. Adult fathead minnow exposures resulted in only subtle changes in the analyzed endpoints, although vitellogenin induction in male fathead minnows was elevated as a result of the CEC mixture exposure.

Overall, the biological analysis of native, hatchery-reared, and laboratory-reared fish exposed to pore-water containing CECs or to laboratory waters spiked with a comparable mixture of CECs provide evidence for biological effects. Although subtle in their expression among individual fish, given the short (21 day) duration of exposure and the CEC concentrations used (parts per trillion – ng/L), these findings never-the-less identify CECs as a source of concern for the health and sustainability of Minnesota fish populations in lakes impacted by septic seepage.

V. DISSEMINATION:

Description: The target audience for results from this research will be professionals in the areas of wastewater treatment and natural resource management. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the DNR and MPCA, and environmental consultants. Results will be disseminated through scholarly publications in peer-reviewed journals such as *Environmental Science and Technology*. Results from the research project will also be presented at regional conferences such as the annual meeting of the *Midwest Chapter of the Society for Environmental Toxicology & Chemistry (SETAC)* and the *Minnesota Water* conference and if possible, at targeted seminars at the DNR and MPCA. Results will be used to determine which whether advanced aeration septic systems provide additional ecological protection.

Status as of January 1, 2016:

A first presentation of this project is planned for the March 2016 Midwest Chapter meeting of the Society for Environmental Toxicology and Chemistry (SETAC) in Madison, WI.

Status as of July 1, 2016

A first presentation was given at the March 2016 Midwest Chapter meeting of the Society for Environmental Toxicology and Chemistry (SETAC) in Madison, WI. An abstract has been submitted for consideration at the North American annual meeting of the Society for Environmental Toxicology & Chemistry (SETAC) to be held in November 2016 (Orlando, FL).

Status as of January 1, 2017

A poster presentation was given at the Society for Environmental Toxicology & Chemistry World Congress (November 2016, Orlando, FL) by Les Warren, the graduate student on this project. Abstracts were submitted (and accepted) to present results of this study at the Fish & Wildlife Conference in Lincoln, NE (February 2017) and at the MN Wastewater Conference (March 2017). A further presentation at the MW SETAC meeting in Minneapolis (March 2017) is planned.

Status as of July 1, 2017
Presentations were given at at the Fish & Wildlife Conference in Lincoln, NE (February 2017), the MN Wastewater Conference (March 2017), and MW SETAC in Minneapolis, MN (March 2017).

Status as of January 1, 2018
A first manuscript describing the presence of contaminants in pore water and linkage to biological effects in lake fish has been accepted by the journal Science of the Total Environment (see reference above under Activity 2). A second manuscript, linking septic proximity to biological effects in lake fish is being readied for submission during the next reporting period. A third manuscript, focusing on the laboratory mixture exposures is currently in the manuscript writing stage with a planned submission later this year.

Final Report Summary:
Results of the current study were disseminated widely in a series of presentations at regional and international scientific conferences:

- March 2016 – Society for Environmental Toxicology & Chemistry chapter meeting in Madison, WI
- November 2016 - Society for Environmental Toxicology & Chemistry world congress in Orlando, FL
- February 2017 - Fish & Wildlife Conference in Lincoln, NE
- March 2017 – MN Wastewater Conference, Brooklyn Park, MN
- March 2017 - Society for Environmental Toxicology & Chemistry chapter meeting in Minneapolis, MN
- April 2017 – Thesis defense (Les Warren) at St. Coud State University
- September 2017 – Seminar (Megan Guyader) at St. Cloud State University
- November 2017 - Society for Environmental Toxicology & Chemistry North America meeting in Minneapolis, MN (two presentations)

In addition, one peer-reviewed manuscript has been published, and two additional manuscripts are in preparation:

- **Guyader M, Warren L, Green E, Proudian A, Kiesling R, Schoenfuss HL, Higgins CP. 2018. Trace Organic Contaminant (TOrC) Mixtures in Minnesota Littoral Zones: Effects of On-Site Wastewater Treatment System (OWTS) Proximity and Biologic Impact. *Science of the Total Environment* 626:1157-1166. DOI: 10.1016/j.scitotenv.2018.01.123.**
- Guyader M, Warren L, Green E, Proudian A, Kiesling R, Schoenfuss HL, Higgins CP. In preparation. Exploitation of Toxicological Endpoints for the Statistical Prioritization of Liquid Chromatography Tandem High Resolution Mass Spectrometry (LC-HRMS) Features: An Approach to Selecting Candidate Chemicals for Endocrine Disruption Risk Assessment.
- Warren L, Guyader M, Kiesling R, Higgins CP, Schoenfuss HL. In preparation. Effects of septic seepage in Minnesota lakes on resident fish species.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$ 76,047	**Heiko Schoenfuss, PI (SCSU)** ($20,180 salary, $7239 fringe, 26% fringe rate; $27,419 total for 3 years; 10% effort per year). **One Graduate Research Assistant (SCSU)** ($27,300 salary,

		$21,328 fringe including tuition; $48,628 total for two years; 50% effort each year)
Professional/Technical/Service Contracts:	$267,125	**Subcontract USGS (Mounds View, MN)** The subcontract amount will include characterization of groundwater flow into lakes; acquisition and deployment of groundwater and surface water passive sampling devices ($89,800), and bi-weekly sample collection and analysis of water samples for common contaminants of emerging concern using ELISA kits ($35,200). The subcontract costs include salary and benefits for a Hydrologist for 3 years at 12% effort ($53,800) and salary and benefits for Co-PI Kiesling for three years at 6% effort ($34,700). **Subcontract Colorado School of Mines** The subcontract amount will include salary and benefits for 50% of a postdoctoral fellow (annual average of $23,412 salary and $9,839 benefits per year for 3 years) and one day of summer salary for Co-PI Higgins ($524 annual average in salary and $237 in benefits per year for three years). In addition, supplies for experiments and measurements (chemicals, analysis time, etc.) will be $13,397 (on average) per year for three years. No travel funds are requested.
Equipment/Tools/Supplies:	$18,600	**Equipment/Tools/Supplies (SCSU)** Fish acquisition and maintenance ($1,000/year for 3 years), gene expression assays ($2,500/year for 3 years), histopathology ($1,200/year for 3 years), exposure experiment setup and execution ($1,500/year for 3 years).
Travel Expenses in MN:	$2,228	Travel to field sites located on four Minnesota lakes for 3 consecutive summers (1,500 miles/year @ $0.495/mile for three years).
TOTAL ENRTF BUDGET:	**$364,000**	

Explanation of Use of Classified Staff: N/A

Explanation of Capital Expenditures Greater Than $5,000: N/A

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 1.3 FTEs

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 2.04

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			

USGS Cooperative Water Program Match Funding	$54,000	$54,000	Support during project period
State			
	$0	$0	
TOTAL OTHER FUNDS:	**$54,000**	**$54,000**	

VII. PROJECT STRATEGY:

A. Project Partners:

The project team consists of the Principal Investigator (PI) Heiko Schoenfuss (St. Cloud State University) and co-PIs Richard W. Kiesling (USGS, Mounds View, MN) and Christopher Higgins (Colorado School of Mines). Dr. Heiko L. Schoenfuss will guide the biological impact research and organize the entire project effort. Dr. Richard Kiesling is a USGS Hydrologist and Limnologist who will guide the lake sampling and characterization effort.

Dr. Chris Higgins will lead the analytical characterization of selected samples. The proposed study requires a specific blend of advanced instrumentation and expertise. It is the unique blend of analytical chemical instrumentation and experience with onsite wastewater treatment systems that can only be brought to this study by CSM and Dr. Higgins. The CSM lead, Associate Professor Christopher P. Higgins, has a significant track record of publications in which his laboratory has measured wastewater-derived organic contaminants such as CECs that may result in endocrine disruption activity. Moreover, several of these publications have specifically examined the occurrence and removal of CECs in onsite wastewater systems. Dr. Higgins' environmental chemistry analytical laboratory is one of the best equipped in the nation. Dr. Higgins has two liquid chromatography tandem mass spectrometry (LC-MS/MS) systems (ABSCIEX 3200 and ABSCIEX 3200 QTrap) for use in the targeted analysis of compounds. Moreover, Dr. Higgins will also use an ABSCIEX 5600+ quadropole time of flight (TripleTOF) MS system for the analysis of "known unknowns" and "unknown unknowns." Dr. Higgins' substantial expertise in targeted analysis of CECs will be supplemented by an on-going collaboration with ABSCIEX to further the use of non-targeted screening of chemicals (i.e., the "unknown unknowns). Further justification for Dr. Higgins collaboration on this project has been provided in a separate attachment.

B. Project Impact and Long-term Strategy:

The proposed research fits into a larger research agenda centered at St. Cloud State University and the USGS focused on contaminants of emerging concern and protection of lake ecosystems. We have previously determined that fish exposed to estrogens (a known class of potent CECs) in small pond-like settings will delay spawning which may have detrimental effects on fish populations (ML 2009, Chp. 142, Sec. 2, Subd. 5b). These effects were found to be of environmental relevance when we assessed in the context of estrogen concentrations in point-source (municipal treatment plants and industrial discharge) (M.L. 2010, Chp. 362, Sec. 2, Subd. 5c). Furthermore, in addition to point-source discharge, our recent studies also determined that estrogenic compounds are found in lake habitats near onsite septic systems (M.L. 2010, Chp. 362, Sec. 2, Subd. 5e). These findings, mostly related to the potent estrogens associated with human and animal excretions lead to a recently funded proposal to assess how already scheduled changes in wastewater treatment technology to reduce effluent nitrogen loads may further benefit the environment through reduction in estrogens (M.L. 2014, Chp. 226, Sec. 2, Subd. 03d). The current proposal builds on these findings and other information in the published literature to examine how non-point sources such as septic systems affect fish reproducing in the areas of lakes most impacted by septic seepage. The addition of Dr. Higgins at the Colorado School of Mines to this project will vastly expand the analytical component of this study to encompass all major groups of known and suspected CECs and to include their metabolites. This capability is critical as it is becoming increasingly apparent that the overall adverse biological effect observed in lake fish (for example intersex) cannot be the result solely of estrogenic exposure. The proposed research, therefore, builds upon and complements current and prior research in this area. When taken together, this research will provide a more complete picture of how to assess the environmental impact of onsite septic system, improve treatment, and safeguard our fish populations.

C. Funding History:

Funding Source and Use of Funds	Funding Timeframe	$ Amount
ML 2009, Chp. 142, Sec. 2, Subd. 5b "Vulnerability of Lakes to Endocrine Disruption"	July 1, 2009 - June 30, 2013	$297,000
M.L. 2010, Chp. 362, Sec. 2, Subd. 5c "Ecological Impacts of Effluent in Surface Waters and Fish"	July 1, 2010 – June 30, 2014	$340,000
M.L. 2010, Chp. 362, Sec. 2, Subd. 5e "Assessing Septic System Discharge to Lakes"	July 1, 2010 – June 30, 2014	$594,500
M.L. 2014, Chp. 226, Sec. 2, Subd. 03d "Cost-benefit Analysis of Wastewater Treatment and Fish Abundance" (Novak, PI)	July 1, 2014 – June 30, 2017	$500,000

VIII. FEE TITLE ACQUISITION/CONSERVATION EASEMENT/RESTORATION REQUIREMENTS: N/A

IX. VISUAL COMPONENT or MAP(S): attached

X. RESEARCH ADDENDUM: See attached Research Addendum subject to peer review by the U.S. Geological Survey

XI. REPORTING REQUIREMENTS:
Periodic work plan status update reports will be submitted no later than January 1, 2016; July 1, 2016; January 1, 2017; July 1, 2017; January 1, 2018. A final report and associated products will be submitted between June 30 and August 15, 2018.

Environment and Natural Resources Trust Fund
M.L. 2015 Project Budget

Project Title: Biological Consequences of Septic Pollution in Minnesota Lakes
Legal Citation: M.L. 2015, Chp. 76, Sec. 2, Subd. 04c
Project Manager: *Heiko L. Schoenfuss*
Organization: St. Cloud State University
M.L. 2015 ENRTF Appropriation: $364,000
Project Length and Completion Date: 3 years, June 30, 2018
Date of Report: 06/30/2018

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 Budget	Amount Spent	Activity 1 Balance	Activity 2 Budget	Amount Spent	Activity 2 Balance	Activity 3 Budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM	**Integrate surface and groundwater sampling for CECs**			**Analysis of samples for known and unknown CECs**			**Determining the causal linkage between CEC exposure and biological effects observed in lake fishes.**				
Overall Personnel (Wages and Benefits)							$76,047	$76,047	$0	$76,047	$0
Heiko Schoenfuss, PI (SCSU) ($20,180 salary, $7239 fringe, 26% fringe rate; $27,419 total for 3 years; 10% effort per year)											
One Graduate Research Assistant (SCSU) ($27,300 salary, $21,328 fringe including tuition; $ 48,628 total for two years; 50% effort each year)											
Professional/Technical/Service Contracts											
Subcontract USGS (Mounds View, MN) The subcontract amount will include characterization of groundwater flow into lakes; acquisition and deployment of groundwater and surface water passive sampling devices ($89,800), and bi-weekly sample collection and analysis of water samples for common contaminants of emerging concern using ELISA kits ($35,200). The subcontract costs include salary and benefits for a Hydrologist for 3 years at 12% effort ($53,800) and salary and benefits for Co-PI Kiesling for three years at 6% effort ($34,700).	$125,000	$125,000	$0								
Subcontract Colorado School of Mines The subcontract amount will include salary and benefits for 50% of a postdoctoral fellow (annual average of $23,412 salary and $9,839 benefits per year for 3 years) and one day of summer salary for Co-PI Higgins ($524 annual average in salary and $237 in benefits per year for three years). In addition, supplies for experiments and measurements (chemicals, analysis time, etc.) will be $13,397 (on average) per year for three years. No travel funds are requested.				$142,125	$142,125	$0				$142,125	$0
Equipment/Tools/Supplies											
Fish acquisition and maintenance ($1,000/year for 3 years), gene expression assays ($2,500/year for 3 years), histopathology ($1,200/year for 3 years), exposure experiment setup and execution ($1,500/year for 3 years).							$18,600	$18,600	$0	$18,600	$0
Travel expenses in Minnesota											
Travel to field sites located on four Minnesota lakes for 3 consecutive summers (1,500 miles/year @ $0.495/mile for three years).							$2,228	$878	$1,350	$2,228	$1,350
COLUMN TOTAL	**$125,000**	**$125,000**	**$0**	**$142,125**	**$142,125**	**$0**	**$96,875**	**$95,525**	**$1,350**	**$364,000**	**$1,350**

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Trace organic contaminant (TOrC) mixtures in Minnesota littoral zones: Effects of on-site wastewater treatment system (OWTS) proximity and biological impact

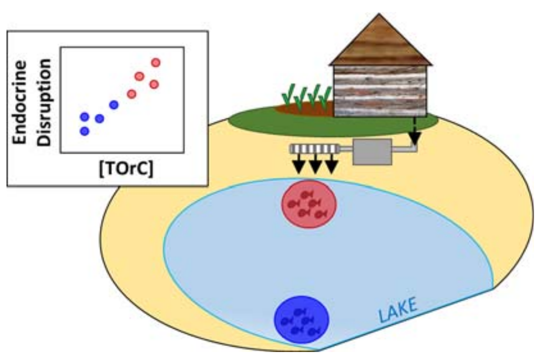
Meaghan E. Guyader [a], Les D. Warren [b], Emily Green [c], Riley Bertram [b], Andrew P. Proudian [d], Richard L. Kiesling [e], Heiko L. Schoenfuss [b], Christopher P. Higgins [a,*]

[a] Department of Civil and Environmental Engineering, Colorado School of Mines, 1500 Illinois Street, Golden, CO 80401, United States
[b] Aquatic Toxicology Laboratory, St. Cloud State University, WSB-273, 720 Fourth Avenue South, St. Cloud, MN 56301, United States
[c] Chemical Engineering Department, Colorado School of Mines, 1500 Illinois St, Golden, CO 80401, United States
[d] Department of Applied Physics, Colorado School of Mines, 1500 Illinois St, Golden, CO 80401, United States
[e] U.S. Geological Survey, 2280 Woodale Drive, Mounds View, MN 55112, United States

HIGHLIGHTS

- OWTSs are a potential diffuse source of TOrCs in Minnesota Lakes.
- TOrC concentrations increase with residential proximity.
- Increased endpoints of endocrine disruption in fish near residences
- Targeted analysis may limit understanding of mixture bioactivity.

GRAPHICAL ABSTRACT



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ABSTRACT

On-site wastewater treatment systems (OWTSs) are an international wastewater management strategy for rural and semi-rural communities without access to centralized sewage treatment. These systems are a suspected source of trace organic contaminants (TOrCs) that may be responsible for endocrine disrupting effects to resident fish species in Minnesota Lakes. This study assessed localized porewater concentrations of TOrCs in near-shore environments across five Minnesota Lakes. Sampling sites were designated as either likely (HOME) or unlikely (REF) to receive OWTS discharges based on their proximity to shoreline households. Sampling sites also served as sunfish spawning habitats concurrently studied for biological impacts to resident adult males. Two-group hypothesis tests demonstrated significantly ($p = .02$) higher total TOrC concentrations in HOME (Mean = 841 ng/L) versus REF (Mean = 222 ng/L) sites. HOME sites also contained a wider suite of TOrC detections relative to REF sites. The distance to the nearest household (most proximal distance; MPD) negatively correlated ($r = -0.62$) with total TOrC concentrations. However, 2,4-D and DEET were major contributors to these total concentrations, suggesting that anthropogenic influence from households may not be exclusively attributed to OWTS discharges. Further, TOrC presence and elevated nitrogen concentrations in REF site porewater suggest additional, non-household TOrC discharges to these lakes. Significantly higher

Abbreviations: HOME, site impacted by domestic wastewater; REF, reference site; MPD, most proximal distance; TOrC, trace organic contaminant; OWTS, on-site wastewater treatment system.

* Corresponding author.
E-mail address: chiggins@mines.edu (C.P. Higgins).

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blood concentrations of vitellogenin (p = .03) and 11-ketotestosterone (p = .01) were observed in adult male sunfish captured from HOME versus REF sites. Comparisons between chemical and biological data indicate enhanced bioactive effects of co-contaminants. The findings from this study demonstrate multiple diffuse transport pathways contribute to the presence of biologically active TOrC mixtures in Minnesota Lakes, and mitigation efforts should consider minimizing residential inputs of chemicals associated with both outdoor and OWTS activity.

1. Introduction

Trace organic contaminants (TOrCs) represent many emerging contaminants prioritized in current environmental monitoring efforts. TOrCs encompass pharmaceuticals, herbicides, pesticides, hormones, steroids, personal care products, cleaning agents, and food preservatives detected at low (ng/L) concentrations throughout the environment (Fatta-Kassinos et al., 2011; Kolpin et al., 2002; Lapworth et al., 2012). Many of these chemicals are not currently regulated despite the association of several TOrCs with adverse biological impacts, particularly endocrine disrupting effects (Ortiz de García et al., 2014). Endocrine disrupting chemicals mimic or inhibit normal androgen or estrogen receptor function, resulting in abnormal masculinization or feminization of affected species, respectively (Diamanti-Kandarakis et al., 2009; Söffker et al., 2015). The endocrine disruption capabilities of individual TOrCs are defined using laboratory exposure experiments through biochemical, histological, and behavioral endpoints (Blair et al., 2000; Elliott et al., 2014; Han et al., 2010; Oropesa et al., 2016). Still, the endocrine activity of environmental TOrC mixtures are poorly understood, particularly in light of the likely co-occurrence of unknown TOrCs (with unknown biological activities) and the potential for co-contaminants to enhance biological impacts (McCarty and Borgert, 2006).

Minnesota littoral zones are a prime field environment for studying diffuse sources of TOrCs and subsequent effects to aquatic life. Previous assessments of nutrient loadings in the United States (US) have led to the consensus that diffuse sources, such as agricultural runoff, groundwater infiltration, and atmospheric deposition, are responsible for most water quality degradation (U.S. Environmental Protection Agency, 1996). The lack of discrete inputs within proximity of surveyed Minnesota water resources indicates diffuse sources are also responsible for widespread TOrC occurrence in these waters (Erickson et al., 2014; Ferrey et al., 2015; Writer et al., 2010). In addition, many lakes in the state (90% of those surveyed by Writer et al.) also contain adult male fish with elevated vitellogenin concentrations, a biomarker of fish feminization (Writer et al., 2010). The spatial and seasonal heterogeneity of TOrC presence, both across lakes and within the same lake, impedes alignment of current chemical and biological observations (Baker et al., 2014). More strategic sampling methods, specifically sampling porewater in littoral zones (near-shore environments with depth < 5 m) during the spring and summer months should enable better characterization of biologically active TOrC mixtures that affect Minnesota fish species. Littoral zones serve as spawning habitats for fish species, such as the commonly studied bluegill sunfish *Lepomis macrochirus*. Spawning season is a critical time of TOrC exposure for larvae and the adult male sunfish that guard them (Becker, 1983). On-site wastewater treatment systems (OWTSs) are one of the proposed diffuse sources affecting the health of these fish (Baker et al., 2014; Writer et al., 2010). Analysis of sediment porewater in these locations advantageously provides insight into the TOrC concentrations of inflowing, potentially OWTS-impacted shallow groundwater and relevant exposure concentrations to fish interacting with lake sediments while spawning.

OWTSs are a documented diffuse source of wastewater-derived contaminants in groundwater, drinking water wells, and surface waters around the world (Gago-Ferrero et al., 2017; Godfrey et al., 2007; Phillips et al., 2015; Schaider et al., 2014; Subedi et al., 2015). This method of treatment typically serves rural and semi-rural populations without access to centralized sewage, around 25% of the population in the United States and 20% of Minnesotans (U.S. Environmental Protection Agency, 2014; West, 2008). Removal of nutrients, suspended solids, and pathogens is achieved by percolating pre-treated wastewater through unsaturated native soils (Crites and Tchobanoglous, 1998; Stanford et al., 2010). TOrCs readily sorbed or biotransformed in these subsurface conditions are also effectively attenuated, even though their removal is not considered in OWTS design (Conn et al., 2006, 2010; Teerlink et al., 2012b). Nevertheless, certain TOrCs remain recalcitrant to modern wastewater treatment technologies (Du et al., 2014; Wode et al., 2015). For this reason, several TOrCs, such as carbamazepine, are now designated as environmental domestic wastewater indicators (Kahle et al., 2009). Furthermore, the ability of OWTSs to effectively treat heterogeneous inputs of TOrCs at their small sewershed scale is highly variable (Teerlink et al., 2012a). Out-of-compliance systems, attributed to either improper installation or maintenance, allow insufficiently treated wastewater to reach the water table and enter shallow groundwater flow paths (Bremer and Harter, 2012; Yates, 1985). In addition, cesspool and leach pit OWTS designs have a decreased ability to remove TOrCs compared to a conventional two-stage system (Schaider et al., 2017). Transport through the subsurface poses an environmental health risk, and can lead to diarrhea outbreaks in children consuming water from OWTS-impacted drinking wells (Borchardt et al., 2003). An estimated 21% of OWTSs in Minnesota are operated out of compliance (Robinson and Schultz, 2015), but subsurface clay moraines with low hydraulic conductivity may also compromise soil driven treatment in the region (Engelking and Kovacevic, 2016). While advanced treatment options, such as aerated biofilters, are available, current OWTS regulation does not require their implementation (Jantrania and Gross, 2006). In light of the potential for OWTSs to serve as sources of TOrCs to Minnesota waters, it seems prudent to evaluate their occurrence in relation to OWTSs and their potential biological impacts before additional steps are taken to reduce these potential impacts.

The objectives of this study were to characterize targeted TOrC mixtures in littoral zones affected by discharges from OWTSs and evaluate potential associations between these TOrCs and biological impacts to adult fish. We hypothesized that locations more proximal to shoreline households would have more TOrC detections at higher concentrations and these locations would contain sunfish with elevated biomarkers of endocrine disruption. The following research questions were addressed: (1) what TOrC mixtures are present at near-shore environments in Minnesota Lakes, (2) are there significant compositional differences between sites likely impacted by OWTSs versus those which likely are not, (3) how are localized environmental TOrC mixtures related to biological responses in fish species? To address these questions, targeted aqueous analysis of porewater grab samples from spawning habitats in five Minnesota Lakes were compared to endpoints of biological impact in captured adult male sunfish.

2. Materials and methods

2.1. Site selection

Five lakes were selected for this study: Cedar Lake (Wright County, MN), Franklin Lake (Otter Tail County, MN), Lake Mary (Wright County, MN), Pearl Lake (Stearns County, MN), and Sullivan Lake (Wright

County, MN) (Fig. 1). Lakes were chosen based on the following criteria: influence of groundwater, presence of suitable bluegill nesting habitats, shoreline development >30%, and the use of OWTSs for wastewater treatment of domestic wastewater (regardless of OWTS functionality). The lakes are from the North Central Hardwood Forest Ecoregion from the EPA's EcoRegion III classifications (U.S. Environmental Protection Agency, 2013). Lakes were surrounded by residences, agricultural croplands, and several municipal buildings including churches and a summer camp. Each lake is also associated with recreational activities, including a public access boat ramp and regular stocking for recreational fishing (Minnesota Department of Natural Resources, 2017). Likelihood of groundwater influx was examined using historical water tables, groundwater flow, and stable isotope data collected from U.S. Geological Survey (USGS) Hydrologic Atlases and Minnesota Department of Natural Resources County Geologic Atlases (Adams, 2016; Lujan Jr. and Peck, 1992). A preliminary survey in the summer of 2015 verified groundwater influx at the candidate lakes. Areas with persistent temperature and specific conductance differences between sediment porewater and overlying surface water were deemed as "gaining" due to groundwater input and selected as sites for the study. Sediment porewater was pumped to the surface with a mini piezometer to monitor basic water quality measurements, such as temperature and specific conductance for verification of the influence of groundwater. The use of bed sediment temperatures as an indicator of groundwater inputs is relatively accurate in determining the influence of groundwater on surface water in riverine systems (Conant, 2004) and lakes (Constantz et al., 2007; Jones, 2006).

Four active sunfish spawning habitats per lake were chosen according to their proximity to shoreline households: two sites which likely received discharges from household OWTSs (HOME) and two reference sites (REF) likely unaffected by OWTS inputs. This study design assumes that the littoral zone in each lake sampled was not well mixed and discrete inputs would result in localized chemical and biological signatures of impact from OWTSs. Explicitly, REF sites are used as a control group in this study rather than reference lakes. As undeveloped lakes are anomalous with respect to land use characteristics in the Upper Midwest where lakeshore properties are highly desirable, the use of reference lakes would not have strengthened the experimental design of the study. HOME and REF were distinguished in the field as sites with or without a household in eyesight of the spawning habitat, respectively.

Site distinctions for two group analysis were re-examined after sampling using aerial imagery analysis. OWTS locations are extremely difficult to obtain; even with access to their public records, designs are usually detailed by hand-drawn representations with minimal geographic information. Therefore, household locations were used as a proxy. Household locations were recorded based on Google Maps imagery (Maps, 2017). Latitude and longitude direct decimal (DD) coordinates were recorded as the center of visible households or the center of household plots covered by trees after verifying addresses with Google Map streetview. Sampling location coordinates were obtained in the field using a global positioning system (GPS). The distance between each sampling location and all shoreline households within 100 m of lakeshore were determined using the Euclidean distance technique (Gower, 1982). The nearest household at each sampling location was determined by calculating the minimum of the set of distances attributed to each sampling location. The minimum distance at each site is herein referred to as the most proximal distance (MPD).

2.2. Sample collection

2.2.1. Aqueous samples

Porewater grab samples were collected between the months of May and July 2016. Samples were collected by pumping porewater through piezometers to the surface and accumulating water in appropriate sampling vessels. Piezometers were driven into the sediment until the screened terminal end of the probe reached saturated conditions determined to contain inflowing groundwater (~0.5–1 m depth), as verified by the methods described in the site selection section. Once placed, a peristaltic pump (Geotech Environmental Supply, Denver, CO, U.S.)

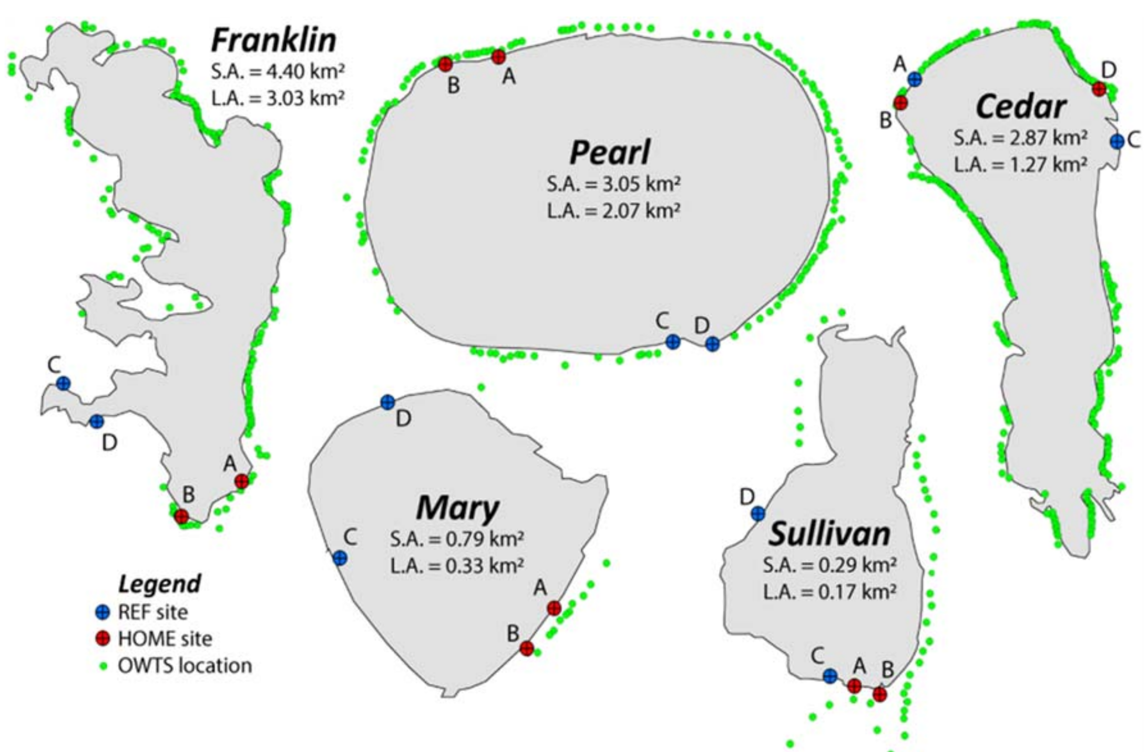

Fig. 1. Summary of sampling locations within each of the five lakes. Lake characteristics are provided under each lake's name, where S.A. = surface area, L.A. = littoral area, and bars represent 0.25 or 0.5 km distances.

was connected to the piezometer and porewater was pumped (~35 mL/min) for collection at the surface. This pumping rate was chosen so that the rate of groundwater replenishment at the sampling point was not exceeded. Pumping equipment was rinsed thoroughly with filtered water (Omni Water; Thermo Fisher Scientific, Walthman, MA, U.S.) before collection at each site to prevent cross contamination. Samples intended for TOrC analysis were collected in 1 L amber glass bottles pre-cleaned by scrubbing with liquinox, rinsing with DI water, and triple rinsing with reagent grade methanol. Samples for inorganic analysis were collected in 50 mL polypropylene tubes. All samples were stored and shipped at 4 °C to the Colorado School of Mines (CSM; Golden, CO, U.S.) for further analysis.

2.2.2. Biological samples

Resident sunfish samples were collected concurrently with the aqueous grab samples. Male fish were collected directly off spawning beds by rod and reel (permitted by Minnesota Department of Natural Resources). During the spawning season, male sunfish will continuously defend their small nest site (approximately 0.5 m in diameter). Consequently, the fish are unable to forage for food and will readily accept baited hooks. This behavior ensures that only nest defending males, who have likely been site-bound for days or weeks, were captured as non-nest holding sneaker males are able to feed between forays into the spawning grounds. A total of 124 male fish were collected from OWTS-influenced sites and 116 from reference sites. On a lake basis, 98 sunfish were collected from Sullivan Lake, 83 from Lake Mary, 35 from Cedar Lake, and 24 from Lake Franklin. Fish were not collected at Pearl Lake because early ice-off in the spring of 2016 disrupted spawning activity (i.e. $n_{bio} = 8$ rather than 10). Males captured were immediately euthanized using a buffered MS-222 solution approved by the St. Cloud State University Institutional Animal Care and Use Committee (IACUC # 8-77). A whole blood sample from the caudal vein was taken using a 22-gauge needle, stored on ice, and transferred to St. Cloud State University (SCSU; St. Cloud, MN, U.S.) for centrifugation. Fish carcasses were placed on ice and transferred to SCSU for dissection and further analysis.

2.3. Sample analysis

2.3.1. Chemical analysis

Basic porewater characteristics at each site, including temperature, pH, specific conductance, and dissolved oxygen (DO) concentrations were collected in the field using a Yellow Spring Instrument (YSI; Yellow Springs, OH, U.S.) probe. Surface water at each site was also measured for DO concentrations, specific conductance, and temperature to confirm adequate environmental conditions to support fish spawning.

Samples received at CSM intended for TOrC analysis were filtered using Whatman GF/F filters and spiked with surrogate standards (20 ng each of 48 unique stable isotope standards; Sigma Aldrich, St. Louis, MO, U.S.) within 48 h of sampling. Samples were then enriched using solid phase extraction (SPE) within two weeks of spiking with surrogate. SPE was executed using an AutoTrace 280 as follows: 6cm^3 500 mg Oasis HLB cartridges were pre-conditioned with 5 mL methyl tert-butyl ether (MTBE), followed by 5 mL methanol, and then 5 mL HPLC water. The 1 L sample was then loaded onto the cartridge. The cartridge was washed with 10 mL HPLC water, and then dried for 60 min using nitrogen gas. Cartridges were eluted with 5 mL methanol followed by 5 mL of 90/10 Methanol/MTBE (% v/v). The extract was blown down to 500 μL using an N-Evap system, then reconstituted in 2 mL of methanol. Methanol extracts were diluted 10:1 in ultra-pure water (Thermo Fisher Scientific, Walthman MA, U.S) and analyzed using a AB Sciex 3200 QTRAP liquid chromatograph tandem mass spectrometer (LC–MS/MS). Sample data was acquired with two 1 mL injections: one positive electrospray ionization method (ESI +) and one negative electrospray ionization (ESI -) method. Targeted analytes were selected to represent common indicators of domestic wastewater and TOrCs with known endocrine disrupting capabilities (Table S1). For ESI + runs, a Phenomenex Luna C18 column (Phenomenex, Torrance, CA, U.S) was used with water and methanol eluents buffered with 10 mM ammonium formate and 1 mL formic acid (Thermo Fisher Scientific, Walthman MA, U.S). The ESI – method used a Phenomenex Gemini C18 column (Phenomenex, Torrance, CA, U.S) with water and methanol eluents buffered with 5 mM ammonium fluoride (Thermo Fisher Scientific, Walthman, MA, U.S).

Inorganic samples were prepped and preserved at CSM in accordance with their intended instrument analysis. All inorganic samples were filtered using 0.4 μm syringe filter within 48 h of sampling. Major nutrients were analyzed using ion chromatography (Dionex Thermo Fisher ICS-900) using unacidifed aliquots of the filtered sample. Total nitrogen and total organic carbon were assessed with sample acidified using hydrochloric acid on a Schimadzu TOC system (Shimadzu TOCV-TNM-LCSH). Trace metals were quantified using inductively coupled plasma atomic emission electroscopy (ICP-AES) with filtered aliquots acidified with nitric acid.

2.3.2. Biological analysis

Glucose concentrations in whole blood samples were measured using a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT, U.S.). Blood samples were then centrifuged (8000 ×g) for 12 min at 4 °C, plasma was pipetted into separate vials and frozen at − 80 °C until analysis. Plasma vitellogenin concentration was determined through an enzyme linked immunosorbent assay (ELISA) using purified sunfish vitellogenin and sunfish validated vitellogenin antibodies. The protocol followed parameters as used in Schultz et al. (2013). 11-ketotestosterone concentrations were determined using an ELISA (Cayman Chemical Company, Kit #582751) following the manufacturer's guidelines. Wet weight of fish carcasses was determined upon return to SCSU (within 6 h of fish capture), and liver and gonad were excised and weight. From these values, body condition factor (weight / (total length)^3 × 100,000), hepatosomatic index (liver weight / mass fish × 100), and gonadal somatic index (gonad weight / mass fish × 100) were calculated (Bolger and Connolly, 1989; Fulton, 1904).

2.3.3. Quality assurance and quality control

Reported aqueous chemistry data were subject to various field and laboratory quality control measures. All grab samples were collected in triplicate along with field blanks and equipment blanks collected at each lake. Field blanks were collected at each lake by passing purified water (OmniWater, Thermo Fisher Scientific, Walthman, MA, U.S.) through the piezometer pumping set up. Laboratory blanks and instrument blanks were also included to ensure contamination introduced during sample handling could not skew reported results. Raw data from targeted LC–MS/MS data acquisition was initially processed using Sciex's MultiQuant software. Quantitation limits for each analyte were set as the concentration of the lowest calibration standard included in a valid calibration curve. Valid calibration curves needed to include at least four points, exclude points with possible instrument or laboratory contamination (defined as containing analyte peak area greater than three times analyte peak area in the blank run before the calibration standards), and have a (1/x)- or (1/x^2)-fit trendline with a Pearson's r value >0.99. Analyte signals influenced by field contamination were redacted by setting reporting limits above the quantitation limit. Reporting limits were set as the value of the average field and equipment blank concentrations plus three times the standard deviation. For analytes with only one field blank or equipment blank with quantifiable contamination, this value was set as three times the field blank concentration (Table S2). Average concentrations were reported if at least two out of three replicates had quantified results. Sites with detections in zero or only one of the three replicates were reported as below the quantitation limit. Below reporting limit and below quantitation limit values were handled as censored values during statistical analysis according to Helsel (2012). Censored data analysis methods are explained in the Statistical analysis section. Instrument performance was verified with regular calibration standards and calibration verification standards. TOrC results were also constrained by matrix spike and surrogate recoveries (Table S1). Furthermore, analytes were redacted if their matrix spike recoveries were outside the 70–130% range or if surrogate recoveries were <10%. Thirty-seven of the TOrCs analyzed met all QA/QC requirements and were used for statistical comparisons between HOME and REF sites.

Biological data were also subject to quality assurance and quality control procedures. For plasma vitellogenin measurements, all samples were analyzed at three dilutions (1:50, 1:250, and 1:1000). An eight-point standard curve was then used to reference absorbance readings of samples. Four replicate samples were added to each plate and replicate samples were added across plates. All samples were randomized across plates.

2.3.4. Statistical analysis

Non-parametric statistical comparisons of biological and chemical data were executed as two-group sample hypothesis tests between HOME ($n_{bio} = 8$, $n_{chem} = 10$) and REF ($n_{bio} = 8$, $n_{chem} = 10$) sites. Rejection of the null hypothesis was considered valid for p-values < .05. Tests on aqueous chemistry data were conducted using the “NADA” package in RStudio to ensure proper handling of the numerous left-censored data in both organic and inorganic targeted datasets (Lee, 2017; Rstudio Team, 2015). Average concentrations of all inorganic and organic analytes, as well as the sum of the average targeted TOrC concentrations, referred to hereafter as total TOrC concentration, in HOME and REF groups were compared using the cendiff( ) function, a Mann-Whitney-Wilcoxon test of the empirical cumulative distribution functions within each group. This non-parametric statistical test does not assume a normal distribution of values within each group, which is appropriate for comparing concentrations across lakes from different geographical regions, unique OWTS owners, and different resultant baseline TOrCs in the respective lake systems. The larger number of biological samples collected allowed for HOME and REF two group tests to be executed at both inter- and intra-lake levels. Intra-lake comparisons were assumed to have a normal distribution in biological endpoints. Mean comparisons from biological data were conducted with Tukey's honest significant difference (HSD) test and two-sided t-tests.

A bivariate analysis of the transformed variables ΔMPD and ΔTOrC provide a parametric assessment of OWTS proximity on TOrC concentrations. The variables are defined as:

$$\Delta MPD = MPD_{site} - MPD_{median} \quad (1a)$$

$$\Delta TOrC = TOrC_{site} - TOrC_{median} \quad (1b)$$

where MPD_{site} is the MPD value specific to a sampling location and MPD_{median} is the calculated median MPD value attributed to the four sampling locations at each lake. Similarly, $TOrC_{site}$ is the total TOrC concentration specific to a sampling location and $TOrC_{median}$ is the calculated median total TOrC concentration attributed to the four sampling locations at each lake. These transformed variables were used to allow better comparison across lakes. Explicitly, MPD values were modified to ΔMPD to better compare HOME/REF site selection across lakes with varied surface areas, and TOrC values were modified to $\Delta TOrC$ to better compare across lakes with varied background concentrations.

3. Results and discussion

3.1. Site distinction analysis

MPDs were significantly (p = .005) lower at HOME sites relative to REF sites (Fig. S1). This corroborates the two-group distinctions used for non-parametric hypothesis testing of the chemical and biological results. Importantly, the MPD metric of proximity does not consider pronounced chemical and biological effects in regions that could be impacted by OWTS leachate from more than one system. Assessing impact from density, such as the approach used in Bremer and Harter, was also considered (Bremer and Harter, 2012); however, obtaining the exact location and compliance status of all relevant OWTSs would require a level of cooperation from homeowners unattainable at this time. Analysis of OWTS density in this study was, therefore, determined to be unreliably speculative.

3.2. TOrC detections and non-parametric two group comparisons

Fifteen of the reported TOrCs were detected in at least one of the sites sampled in this study (Table S3). Pharmaceuticals, such as carbamazepine, are a preferred indicator of wastewater presence in an environmental matrix (Subedi et al., 2015). While detections of particular pharmaceuticals were not widespread enough to generate meaningful statistics comparing HOME and REF sites, it is noted that the pharmaceuticals carbamazepine, dilantin, and ibuprofen were only detected at HOME sites (Tables S3 and S4). Interestingly, the synthetic estrogen 17α-ethinylestradiol (EE2) was only detected in two REF sites. EE2 is a known endocrine disruptor that was shown to collapse a fish population during a lake dosing study in Canada (Kidd et al., 2007). EE2 is used as both a synthetic birth control hormone and a livestock hormone to improve productivity and treat livestock diseases (Gadd et al., 2010). The REF sites with EE2 detections, SUL_D at 25 ng/L and CED_A at 5 ng/L are suspected to receive shallow groundwater carrying agricultural runoff from fields that use hormone-fed livestock manure as fertilizer (Zaharin Aris et al., 2014). These concentrations match the upper end of previously observed EE2 concentrations in surface waters (Zaharin Aris et al., 2014). In addition, bed sediments usually have higher EE2 concentrations attributed to the contaminants hydrophobic and persistent properties (Zaharin Aris et al., 2014). Therefore, the observed concentrations of EE2 in porewater sampled during this study are consistent with those expected in bed sediments (Zaharin Aris et al., 2014). Other known or suspected endocrine active compounds that were commonly detected include the cosmetic preservative methylparaben and estrone, which were detected in 40% and 20% of the sites sampled, respectively (Bergman et al., 2012). The steroidal hormones androstenedione and testosterone, as well as 4-tert-octylphenol (used in the manufacturing of anionic surfactants, such as detergents or, less commonly, as an emulsifier in personal care products such as insect repellents) were also detected, but less frequently (detection frequency ≤ 10%). Some TOrCs typically detected in environments down-gradient of OWTSs, such as sulfamethoxazole, were not detected in this study. These non-detections are attributed to the heterogeneity of inputs to wastewater treatment systems at such a small sewershed scale (Teerlink et al., 2012a), as well as the variability of subsurface conditions that affect TOrC removal in soil treatment units. Explicitly, these TOrCs may simply not have been used at the households within proximity to sampling locations, or soil regions were anaerobic and suitable for sulfamethoxazole attenuation (Massmann et al., 2008).

The most frequently detected analyte (detection frequency = 85%) was *N*,*N*-diethyltoluamide, more commonly referred to as DEET. DEET is neither a persistent nor bioaccumulative organic pollutant, with a half-life in the order of days to weeks as well as acute and chronic effect concentrations orders of magnitude above observed environmental concentration (Weeks et al., 2012). This insect repellent ingredient has been previously detected in lakes across the state of Minnesota where lake recreation and mosquitoes are very common in the summer months (Ferrey et al., 2015; Writer et al., 2010). This study is the first to note significantly higher concentrations of DEET in Minnesota lake sites more proximal to households across all lakes sampled (Fig. 2a). DEET could enter household wastewater streams through bathing or clothes washing which could then enter OWTS discharges. Gago-Fererro et al. also noted seasonally high concentrations of DEET in surface waters adjacent to OWTSs at concentrations an order of magnitude lower than

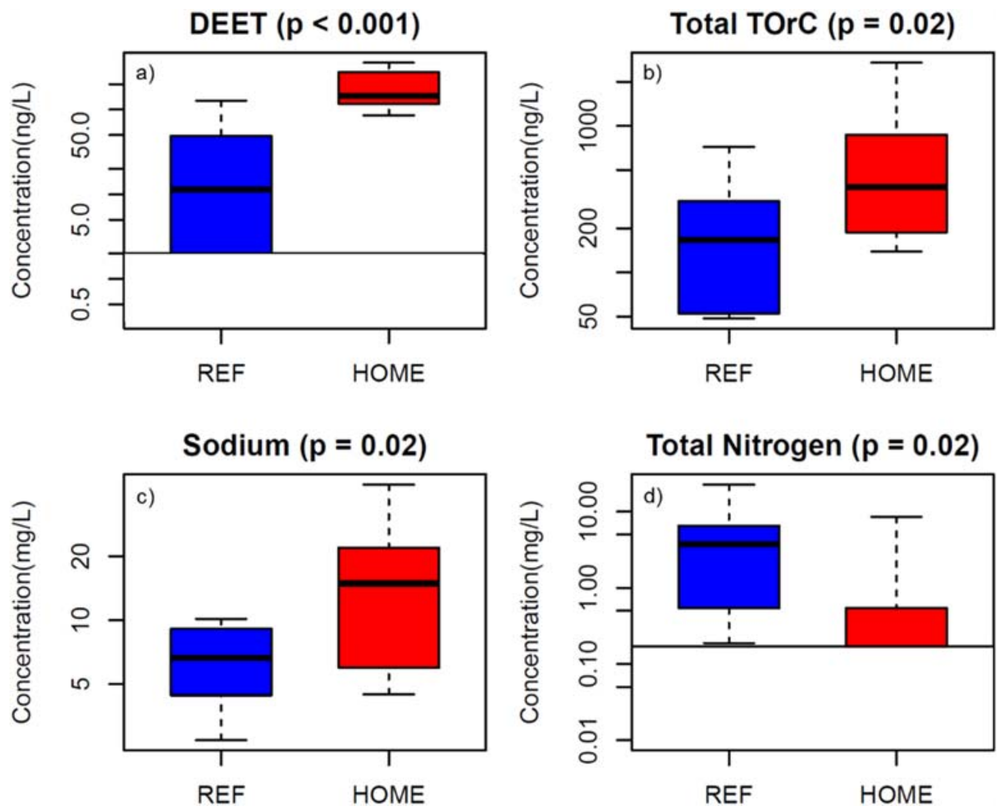

Fig. 2. Two-group comparison of average site concentrations across all five lakes. The analytes a) DEET, b) total targeted TOrCs, c) sodium, and d) total nitrogen had significantly different concentrations in HOME and REF sample groups. Each boxplot displays minimum, first quartile, median, third quartile, and maximum values specific to each group. The detection limits of DEET and Total Nitrogen are displayed using horizontal lines at 2 ng/L and 0.17 mg/L, respectively. First quartiles estimated below these values through censored statistical analysis are omitted from display.

those observed in our study (average summer sampling concentration of 13 ng/L) (Gago-Ferrero et al., 2017). However, designating it as a wastewater indicator in these lake systems is inappropriate (Tran et al., 2014). The associated outdoor usage of DEET suggests that this contaminant may also be entering lake systems from general anthropogenic activity in and around lakes.

The total TOrC concentrations at HOME sites were significantly ($p = .02$) higher compared to those at REF sites (Fig. 2b). A more detailed presentation of the measured analyte concentrations at each lake site is provided in Fig. 3. HOME sites contained a wider suite of targeted analytes (14 out of the 37 reported) as compared to REF sites (7 out of the 37 reported). Fig. 3 also shows the consistently higher concentrations of specific TOrCs detected at the HOME versus REF sites. Lake Mary's HOME sites, MAR_A and MAR_B, had the highest measured total TOrC concentration across all sites assessed in this study. This is mainly attributed to the measured concentrations of the herbicide 2,4-D at both of the lake's HOME sites (mean of 2200 ng/L). 2,4-D is commonly used for outdoor home gardening applications suggesting that, similar to DEET, household activity other than OWTSs are impacting littoral porewater of these lakes (Mnif et al., 2011). 2,4-D is also one of the few measured TOrCs monitored by the EPA, with a maximum contaminant level (MCL) in drinking water of 50 μg/L as a result of its association with blood, kidney, and liver toxicity (EPA, 1998).

Non-household diffuse sources are expected to contribute to the "background" presence of TOrCs, such as DEET and oxybenzone, observed at many of the REF sites (Fig. 3). The only sites DEET was not detected at were the REF sites PRL_C, FRK_C, and FRK_D. As suggested before, agricultural operations could contribute to TOrC occurrence, particularly herbicides, pesticides, and feedlot hormones, in the REF sites sampled. Agricultural fields surround all of the lakes, and the boundaries of these operations are in closer proximity to lake shoreline unoccupied by household lots. Recreational activities, such as boating, may also act as a non-point source of TOrCs in lake locations distant from households. Each lake sampled is a stocked fishery with household and public boat ramp access points (Minnesota Department of Natural Resources, 2017). Most lakeshore households have their own docks and boats (verified with aerial imagery; Google Maps accessed March 2017) (Maps, 2017). Oxybenzone, common in sunscreens, and DEET, common in insect repellents, are both TOrCs integrated into personal care products associated with these lake recreational activities. The transport processes resulting in their presence in groundwater-impacted lake porewaters for these contaminants is not immediately clear. Ferrey et al. proposed atmospheric deposition as a diffuse TOrC transport mechanism to Minnesota Lakes (Ferrey et al., 2015). DEET has been reported to be widely present in atmospheric samples (Balducci et al., 2012; Cheng and Lehmann, 1985). However, the significantly higher concentrations of DEET in sediment porewater near households in this study suggest long range aerial transport is unlikely. TOrCs introduced at the lake surface may enter into shallow groundwater after application near the water's surface. Contaminants may then settle with suspended solids in the lake and accumulate in the sediment where they may then partition into sediment porewater and reenter littoral zones with the influx of shallow groundwater (Winter et al., 1999).

3.3. Patterns in inorganic non-point source indicators

Basic water quality assessments of the sampled porewater confirm inflowing groundwater had "young" or shallow flowpaths (Table S5). The consistently low (<5 mg/L) dissolved oxygen (DO) measurements of the porewater are typical for groundwater (Peterson and Risberg, 2009). There were no significant ($p > .05$) differences between porewater DO concentrations at HOME versus REF sites, but the

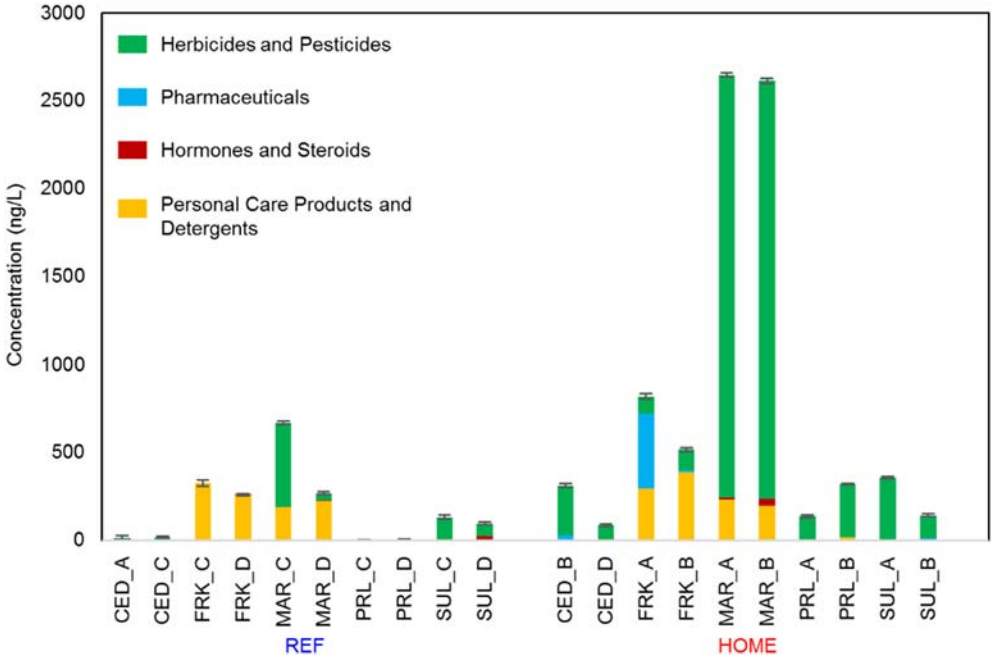

Fig. 3. Average total TOrC concentrations measured at each site. Detected analytes are color coded by compound use. Average analyte-specific concentrations measured at each site are displayed in Table S5. Error bars represent ± average standard deviation across all TOrCs measured at each site.

maximum observed values were present in the REF sites. In addition, conductivity values across all sites' porewater samplers were at the lower end of the typical groundwater values (typical range 50–50,000 μS/cm, (Sanders, 1998)), suggesting influence from shorter groundwater flowpaths (Erickson et al., 2014). Lake Mary notably had the lowest porewater DO concentrations. Previous studies have noted that anoxic regions lead to longer range transport of TOrCs, as these pollutants are generally more effectively attenuated through aerobic degradation (Carrara et al., 2008; Phillips et al., 2015). We speculate anoxic regions are present in the subsurface surrounding Lake Mary and contribute to the higher detected TOrC concentrations in Lake Mary littoral sediment porewaters; however, a more thorough characterization of groundwater flow paths and redox conditions at this lake are required to test this hypothesis.

Nutrient and trace metal data were also compared to assess chemical differences between HOME and REF sites (Tables S6 and S7). Previous studies have shown total nitrogen concentrations to positively correlate with TOrC occurrence, particularly TOrCs derived from OWTSs (Del Rosario et al., 2014; Phillips et al., 2015; Schaider et al., 2017). Surprisingly, there were significantly ($p = .02$) higher total nitrogen concentrations at the REF sites, further indicating the presence of an additional non-point source in the region (Fig. 2d). This was not reflected by significant differences ($p > .05$) in the measured concentrations of nitrite or nitrate. Unmeasured ammonia or organic nitrogen are suspected to be the dominant nitrogen species at these locations, with the exception of Pearl Lake's REF site C which had nitrate as the dominant nitrogen species. Pearl Lake's REF site C also had the highest total nitrogen concentration measured across all sampling locations. Each of the lakes sampled have agricultural fields surrounding them, particularly in non-residential parts of the shoreline, that could contribute to observed elevated nitrogen concentrations at REF versus HOME sites. There was no significant difference in total organic carbon between HOME and REF sites ($p > .05$). Many of the other nutrients analyzed were below detection limits. Chloride and bromide ratios can be used to assess sources of groundwater (Katz et al., 2011), but consistent censoring of bromide concentrations hindered this calculation. Trace metal analysis only showed significantly higher ($p = .019$) sodium concentrations in the HOME sites (Fig. 2a). Sodium salts are common in detergents and other common household products as well as softening systems, which could explain this significant difference in HOME and REF concentrations. The wastewater tracer boron, also common in household products (Woods, 1994), showed no significant ($p > .05$) difference in concentrations between HOME and REF sites.

3.4. MPD effect on total TOrC concentration

Results from the parametric analysis are displayed in Fig. 4. We anticipated HOME sites would have more positive ΔTOrC and negative ΔMPD values, in agreement with the hypothesis that sites more proximal to household OWTSs would have higher total TOrC concentrations. ΔMPD negatively ($r = -0.62$, $p < .001$) correlated with ΔTOrC, supporting this hypothesis. Certain lakes showed more pronounced differences between HOME and REF sites than others. Specifically, Lake Mary's HOME and REF total TOrC concentrations showed the greatest difference, attributed to the high concentrations (~2200 ng/L) of 2,4-D at the lake's HOME sites. Sullivan, Cedar and Pearl lakes clustered around the origin of the plot, demonstrating poorer distinction between HOME and REF sites at these lakes. As expected, these sites with similar MPDs had less differentiation in total measured TOrC concentrations.

The high concentrations measured at Lake Mary may be attributed to the lake's small surface area, along with many households at the south and eastern shorelines where the HOME site samples were collected. This spatial arrangement of households and OWTSs creates the potential for multiple wastewater streams to impact the adjacent littoral environments with minimal effects from dilution and attenuation. In addition, the DO readings for this porewater were very low (~1 mg/L), which has been associated with longer range transport of untransformed TOrC species in OWTS plumes (Carrara et al., 2008).

3.5. Biological data two group hypothesis tests

Field measurements of the lake surface water affirmed that the littoral environments could support healthy spawning at the sites sampled. DO levels were still hospitable for aquatic life (>6 mg/L). Further, all observed aqueous temperatures were suitable for spawning with the exception of Pearl Lake (average = 12 °C), which experienced an early

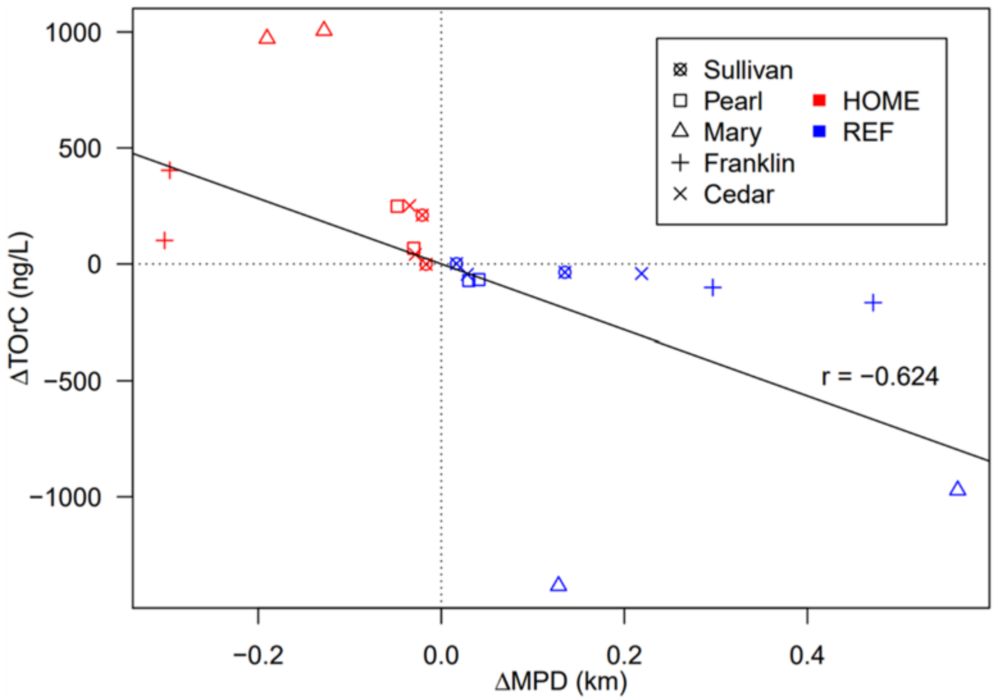

Fig. 4. Bivariate analysis of residential proximity's effect on total TOrC concentration in littoral site porewater. Variables ΔMPD and ΔTOrC are defined in Eqs. (1a) and (1b), respectively. Trendline: $y = a * x$, where $a = -1413.0 \pm 427.8$ ($p = .00374$); $r^2 = 0.3313$.

ice off. No sunfish were captured or analyzed from Pearl Lake; therefore, only chemical data are reported from this lake.

Two group comparison of biological data demonstrated more pronounced biological impacts in HOME site spawning male sunfish with respect to vitellogenin and 11-KT concentrations (Fig. 5). Increased concentrations of vitellogenin in male fish is a Tier 1 indicator of estrogen agonism and elevated concentrations of 11-ketotestosterone in male fish is a Tier 3 indicator of steroidogenesis according to the EPA's Endocrine Disruptor Screening Program (Borgert et al., 2014). All other biological endpoints measured, i.e. gonadal somatic index, glucose concentrations, and body condition factor showed no significant ($p > .05$) difference for captured fish at HOME and REF. Vitellogenin concentrations were significantly ($p = .0108$) higher in HOME versus REF sites, driven by the results from Lake Mary ($p \leq .0001$). Higher vitellogenin concentrations in HOME versus REF fish, particularly those inhabiting Lake Mary, suggest these males are being exposed to mixtures of TOrCs with estrogenic activity. This finding is particularly interesting when considering Lake Mary's HOME sites had the most detections with the highest concentrations. We speculate the notably high concentrations of 2,4-D may be enhancing estrogen agonist effects of known endocrine active co-contaminants measured at these sites. Even though 2,4-D is not itself consider an endocrine disruptor by the EPA's EDSP for the 21st century (EDSP21) dashboard (U.S. Environmental Protection Agency, 2017), a study by Kim et al. demonstrated 2,4-D and its transformation product 2,4-dichlorophenol (DCP) could enhance the androgenic effects of 5-dihydroxytestosterone (Kim et al., 2005). Steroidogenesis effects were also more pronounced at HOME sites as demonstrated by significantly higher concentrations of 11-KT in blood

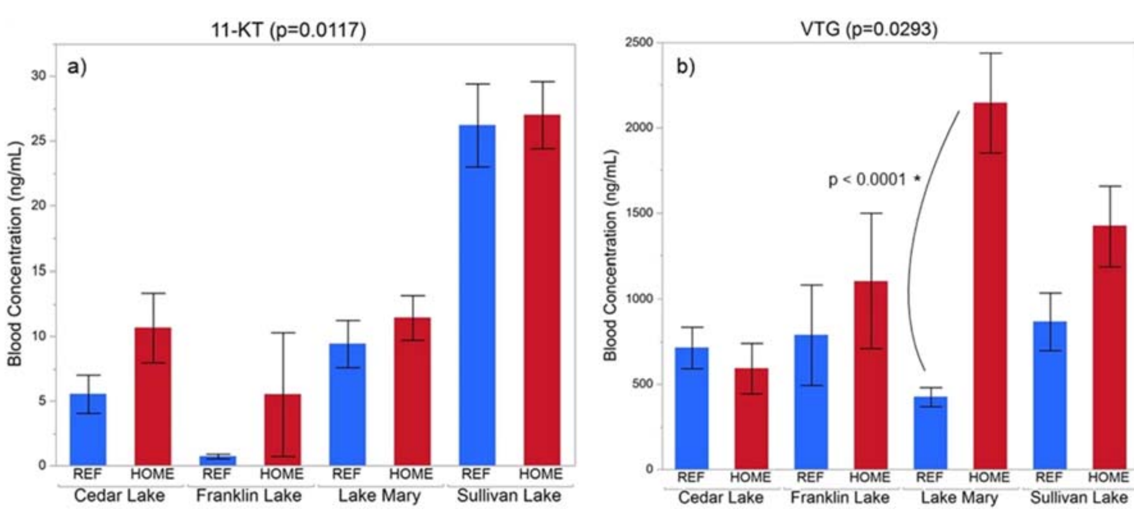

Fig. 5. Average blood concentrations of a) 11-Ketotestostrone and b) vitellogenin in fish captured at HOME and REF within each lake. Error bars represent the standard deviation of values from the average concentration at each lake. p-Values above the plots are comparisons of concentrations in fish captured from HOME and REF sites across all lakes. In panel b, the in-plot p-value is the two group comparison of HOME and REF captured fish from Lake Mary only (color, 2 panel).

samples (p = .0117) when compared across all lakes. Cedar and Franklin Lake showed the most pronounced intralake site differences in 11-KT concentrations (Fig. 5a). Interestingly, all fish sampled from Sullivan Lake, from both HOME and REF sites, had significantly higher (p = .003, two way ANOVA) 11-KT concentrations than fish sampled from other lakes. Previously reported 11-KT blood concentrations for adult male sunfish averaged at 13.8 ng/mL (Knapp and Neff, 2007), suggesting Sullivan Lake's fish were anomalously high and Franklin Lake's REF site was anomalously low. Chemical analyses did not indicate the presence of potent endocrine disruptors at Sullivan Lake, suggesting that the targeted analysis in this study may not sufficiently describe the localized TOrC mixtures present at the Sullivan Lake sites that may be impacting observed 11-KT concentrations.

4. Implications

The findings of this study suggest that TOrC occurrence in sunfish spawning habitats of Minnesota littoral environments are affected by groundwater inflows. Lakeshore households increase concentrations and detection frequency at adjacent lake locations. Minimizing TOrC loadings from households requires consideration of heterogeneous outdoor activity and domestic wastewater chemical compositions at shoreline residential locations. Hydrologic processes, such as stormwater infiltration, are suspected to increase the mobility of TOrCs in the subsurface and encourage transport from residences to littoral zones. Inputs from agricultural operations and recreational activity separate from residential locations must also be considered during mitigation efforts, particularly as this study suggests they may be sources of potent endocrine disruptors. Resultant endocrine disrupting effects are only partially justified by the TOrCs detected, suggesting total concentrations reported are merely a proxy for all components of biologically active mixtures in these environments. Non-targeted analysis with high resolution mass spectrometry could better resolve components of environmental TOrC mixtures that contribute to pronounced biologic activity (Schymanski et al., 2015).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.123.

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