2018 Project Abstract
For the Period Ending June 30, 2022

PROJECT TITLE: Protect Water Quality with Efficient Removal of Contaminants in Treatment Ponds for Storm Water
PROJECT MANAGER: Heiko L. Schoenfuss
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2018, Chp. 214, Art. 4, Sec. 02, Subd. 04d as extended by M.L. 2021, First Special Session, Chp. 6, Art. 6, Sec. 2, Subd. 18

APPROPRIATION AMOUNT: $325,000
AMOUNT SPENT: $269,063
AMOUNT REMAINING: $55,937

Sound bite of Project Outcomes and Results
Our study demonstrates that pharmaceuticals and pesticides are commonly found in urban stormwater and can impact aquatic life. Stormwater ponds, especially when augmented with iron-enhanced sand filtration, can often reduce these pollutants, and lessen their impact on Minnesota aquatic environments.

Overall Project Outcome and Results
Urban stormwaters carry pollutants, including pharmaceuticals and pesticides, into Minnesota streams, rivers, and lakes. Stormwater ponds have not been studied to determine whether they are effective in removing these pollutants. The goal of this study was, therefore, to assess stormwater composition and treatment to inform natural resource managers to the best options for reducing urban stormwater related pollution to Minnesota waters. Our approach combined water chemistry analysis and assessment of biological toxicity in a range of species living in Minnesota waters. We sampled inflow and outflow of seven urban stormwater ponds across seasons and included traditional ponds and those augmented with additional iron-enhanced sand filters. Each water sample was analyzed for a range of pollutants and was also used to expose cells of animals living in Minnesota waters to assess the samples' toxic potential. Pharmaceuticals and pesticides were commonly found in stormwater. In nearly three-quarters of paired water samples (pond inflow and pond outflow), pharmaceutical concentrations were reduced in the outflow when compared to the inflow. Similarly, in about half of paired samples, pesticide concentrations were lower in the outflow sample. The measured reduction in pollutants was also reflected in improved cell health, but this effect was neither as pronounced nor as widespread as predicted by the water chemistry results. In some instances, exposed cells from some, but not all species did better in inflow water than outflow water and in some instances no changes in cell health were observed. The inconsistency in observed biological improvement may be the result of seasonal differences and/or conditions in specific stormwater ponds. This study demonstrates for the first time that stormwater ponds are effective treatment options to reduce the impact of pharmaceuticals and pesticides on urban aquatic environments. Adding additional filtration, such as iron-enhanced sand filtration can further reduce stormwater pollutants.

Project Results Use and Dissemination
Despite the challenges associated with the Covid-19 pandemic, our team was able to give seven presentations related to this study. These include presentations to natural resource managers in Minnesota and to toxicologists at national and international scientific meetings. A St. Cloud State University graduate student completed a thesis on this project in 2020 which is currently being developed into a manuscript. Water

chemistry data were integrated into a national USGS data base. Additional manuscripts are being prepared for future publication.

Environment and Natural Resources Trust Fund (ENRTF)

M.L. 2018 ENRTF Work Plan Final Report (Main Document)

Today's Date: August 12, 2022

Final Report

Date of Work Plan Approval: 06/05/2018

Project Completion Date: June 30, 2022

PROJECT TITLE: Protect Water Quality with Efficient Removal of Contaminants in Treatment Ponds for Storm Water

Project Manager: Heiko Schoenfuss

Organization: St. Cloud State University

College/Department/Division: Aquatic Toxicology Laboratory

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Location: State-wide

Total Project Budget: $325,000

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Appropriation Language: $325,000 the second year is from the trust fund to the Board of Trustees of the Minnesota State Colleges and Universities system for St. Cloud State University to evaluate the effectiveness of best management practices in removing contaminants from storm water to safeguard aquatic habitats. This appropriation is available until June 30, 2021, by which time the project must be completed and final products delivered.

M.L. 2021, First Special Session, Chp. 6, Art. 6, Sec. 2, Subd. 18. ENVIRONMENT AND NATURAL RESOURCES TRUST FUND; EXTENSIONS. [to June 30, 2022]

I. PROJECT STATEMENT:

Recent stormwater monitoring studies in Minnesota have determined that **urban stormwater is a significant source of contaminants of emerging concern** containing a broad suite of pharmaceuticals (including prescription drugs), current-use pesticides, personal care products, and other organic wastewater chemicals. These pollutants are currently discharged into major streams, rivers, and lakes at concentrations that may exceed those of treated wastewater effluent and are harmful to organisms in receiving waters. Understanding how existing stormwater treatment systems process these contaminants of emerging concern and how aquatic biota respond to stormwater discharges is central to safe-guarding Minnesota's aquatic environment.

The level of exposure of aquatic organisms to stormwater contaminants of emerging concern discharge will depend upon the ability of best management practices (BMPs) to abate pollutant loading to surface waters. A recent pilot study by the MN Pollution Control Agency, St. Cloud State University, and US Geological Survey monitored three different stormwater BMP systems and found evidence of reductions in the number and concentrations of contaminants of emerging concern in some BMP outflow samples. One third of the 48 most frequently detected contaminants of emerging concern showed significant reductions between BMP inlets and outlets suggesting that properly designed BMP treatment systems can reduce some pollutant concentrations depending upon season and stormwater composition. However, biological activity was not always reduced at the same rate as pollutant reduction suggesting differential efficiency in contaminant removal by BMP systems with room for improvement.

An assessment of stormwater composition and treatment in urban centers in Minnesota is needed to inform natural resource managers to the best options for reducing urban stormwater related pollution to Minnesota waters.

Stormwater outfalls and stormwater BMPs provide important wetland habitats for many aquatic species, especially in urban environments. Given the complexity of stormwater and the diversity of species utilizing stormwater impacted habitats, a whole animal study is inappropriate and premature given the many unknowns of stormwater. Instead, we will focus on key species at multiple taxonomic levels (invertebrate, fish, amphibian, reptilian) using a common cell-based assay to identify the most vulnerable species in impacted wetlands and to identifying the most effective stormwater treatment technologies to remove harmful biological effects.

II. OVERALL PROJECT STATUS UPDATES:

First Update January 31, 2019

We have identified seven stormwater ponds in the greater Minneapolis/ St. Paul metropolitan area that matched our selection criteria for this study. In each pond, we are be able to sample stormwater inflow and outflow during precipitation events. Of the seven ponds, five utilize iron-enhanced sand filtration (IESF) prior to stormwater discharge. The remaining two ponds are standard stormwater retention ponds. We collected a full complement of inflow and outflow samples from all seven ponds during stormwater runoff events in early and late summer 2018 (Activity 1). These samples have been curated and readied for shipment to the USGS National Water Quality Laboratory in the coming months. Concurrent with stormwater collections, we have advanced the cell-based assays (Activity 2) which will be used to assess the biological activity of stormwater runoff.

Second Update June 30, 2019

In congruence with our research plan, we have continued to collect stormwater runoff samples during the 2019 snow melt and during a spring 2019 precipitation event and during a summer 2019 storm event. All samples have been curated and are currently prepared for analysis under Activity 1. We have advanced the development of the cell-based assays proposed under Activity 2 and paired this work with larval fathead minnow exposures to place cell-based results into a greater biological context. To accomplish this, we exposed larval fathead minnows for 21 days to water samples from all stormwater collections and measured their survival, growth and ability to feed efficiently. Another round of stormwater sampling is planned for summer 2019 as weather conditions allow.

Third Update January 31, 2020

Consistent with the goals set for Activity 1, stormwater collections continued with an additional sampling event during the current reporting period. All forty-four samples collected to date have been submitted to the USGS National Water Quality Laboratory for analysis and we have received approximately 40% of the results. Concurrently and in accordance with the goals of Activity 2, we further advanced the cell-based assays and have validated their ability assess the impact of contaminants of emerging concern in stormwater. Viability of cell lines exposed to stormwater extracts have been confirmed for three species. A duplicate of water samples collected for Activity 1 have been filtered and solid-phase extracted in preparation for the cell-based assay analysis. In addition, a graduate student contributing to this project recently defended his Master's thesis adding valuable biological endpoints to our assessment of the ecological impacts of stormwater on aquatic life. We are currently evaluating the impact of the historically wet year 2019 on our ability to draw conclusions from our sampling to date and are considering additional sampling in 2020 to augment and strengthen the data set beyond what was proposed in the original work plan.

Amendment Request as of 01/26/2020

We request an amendment to our current budget to allow for a shift of $65 from the Equipment/ Tools/ Supplies budget to cover an overage in our Travel Expenses category. The overage was a result of additional, unexpected sampling trips from St. Cloud to Minneapolis/St. Paul as we encountered on two occasions precipitation events that did not cover our entire sampling area.

Amendment Approved by LCCMR 2/26/2020

Fourth Update June 30, 2020

Progress in this reporting period has been challenged as a result of the covid-19 stay-at-home order and the associated problems. These include the inaccessibility of our laboratory facilities at St. Cloud State University, severe restriction to conduct any follow-up field work, and delays in sample processing the USGS National Water quality Laboratory. At the time of the shut-down, we had received approximately 70% of data from the National Water Quality Laboratory (Activity 1) and had tested approximately one-third of stormwater samples for

cytotoxicity (Activity 2). During the shut-down, we have begun working on Methods and Introduction for an anticipated manuscript submission. We have also utilized this time to preparing receptor constructs for the estrogenicity assay for freshwater mussel, Northern leopard frog, and painted turtle.

Fifth Update January 31, 2021

As outlined under the goals set for Activity 1, sixty-three environmental samples from seven different treatment ponds have been analyzed by the USGS National Water Quality Laboratory and the results have been loaded into the USGS National Water Quality Information System (NWIS). These samples included thirty-one paired inflow-outflow samples from seven ponds collected during spring, summer, and fall seasons. Total pharmaceutical concentrations were reduced in the outflows in 68% of the paired samples. Total pesticide concentrations were reduced in only 48% of the paired samples. Reductions in the total number of pharmaceuticals or pesticides detected were observed in less than 50% of the paired samples.

Activity 2 progressed rapidly during this reporting period as laboratories at St. Cloud State University reopened. All stormwater samples, which had been filter-sterilized during the previous reporting period were now assessed for cell viability and for estrogenicity. These analyses identified interesting seasonal patterns of cytotoxicity and estrogenicity that will be explored in the coming reporting period through the integration of chemical and biological data.

Project extended to June 30, 2022 by LCCMR 6/30/21 as a result of M.L. 2021, First Special Session, Chp. 6, Art. 6, Sec. 2, Subd. 18, legislative extension criteria being met.

Sixth Update June 30, 2021:

Our project has now moved into the final phase of data analysis, quality assurance and linkage between chemical occurrence and biological effects data. Chemical occurrence data from all stormwater collections have allowed us to compare between inflow and outflow samples from standard ponds or IESF ponds and demonstrated that the IESF treatment provides a beneficial reduction of pharmaceutical contaminants in stormwater. Indeed, nearly three-quarter of samples taken from IESF filtered samples showed reductions compared to 50% of samples from standard ponds. Our analysis also revealed that these differences are correlated with seasonality. Our biological data have undergone further quality assurance analysis in preparation of linking biological and chemical data in an upcoming manuscript draft. Similar to our chemical occurrence data, cell viability, a common measure of sample toxicity, was affected by both IESF treatment and seasonality of sampling. In contrast to chemical occurrence, the relationship between cell viability and stormwater treatment was not always as linear and further analysis will be conducted to examine confounding variables that may explain some counterintuitive results. Chemical occurrence data and biological effects data are being collated to provide a solid foundation for an upcoming manuscript draft.

Seventh Update January 31, 2022:

An error in the previous budget spreadsheet (dated June 30, 2021) was corrected in the current budget spreadsheet (dated December 31, 2021). In the previous budget spreadsheet, encumbered salary was accidentally included while the investigator for whom the salary was budgeted moved to new employment with short notice. Salary expenditure has been corrected accordingly in the December 31, 2021 budget spreadsheet accompanying this report.

Preparations are underway to submit two manuscripts to peer-review prior to the final reporting deadline. The first manuscript details the chemistry of stormwater samples to ascertain sources, fate, and treatment efficacy. This manuscript is based on the large analytical chemistry data matrix that has been developed and quality-assured for all stormwater samples gathered during this study. The second manuscript will detail the biological effects experienced by organisms exposed to stormwater samples gathered during this study. This manuscript also includes summaries of analytical chemistry from stormwater samples to link biological effects to chemical occurrence. In addition to these synthesis efforts, the completion of the remaining two bioassays, for painted turtles and Northern Leopard frogs is ongoing. This effort had been hampered by supply issues related to

biomedical supply needs during the COVID-19 pandemic. We are now in a position to complete these assays and exposed the resultant cell lines to already archived stormwater samples from the current study.

Overall Project Outcome and Results

Urban stormwaters carry pollutants, including pharmaceuticals and pesticides, into Minnesota streams, rivers, and lakes. Stormwater ponds have not been studied to determine whether they are effective in removing these pollutants. The goal of this study was, therefore, to assess stormwater composition and treatment to inform natural resource managers to the best options for reducing urban stormwater related pollution to Minnesota waters. Our approach combined water chemistry analysis and assessment of biological toxicity in a range of species living in Minnesota waters. We sampled inflow and outflow of seven urban stormwater ponds across seasons and included traditional ponds and those augmented with additional iron-enhanced sand filters. Each water sample was analyzed for a range of pollutants and was also used to expose cells of animals living in Minnesota waters to assess the samples' toxic potential. Pharmaceuticals and pesticides were commonly found in stormwater. In nearly three-quarters of paired water samples (pond inflow and pond outflow), pharmaceutical concentrations were reduced in the outflow when compared to the inflow. Similarly, in about half of paired samples, pesticide concentrations were lower in the outflow sample. The measured reduction in pollutants was also reflected in improved cell health, but this effect was neither as pronounced nor as widespread as predicted by the water chemistry results. In some instances, exposed cells from some, but not all species did better in inflow water than outflow water and in some instances no changes in cell health were observed. The inconsistency in observed biological improvement may be the result of seasonal differences and/or conditions in specific stormwater ponds. This study demonstrates for the first time that stormwater ponds are effective treatment options to reduce the impact of pharmaceuticals and pesticides on urban aquatic environments. Adding additional filtration, such as iron-enhanced sand filtration can further reduce stormwater pollutants.

III. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Measure BMP effectiveness of Contaminants of Emerging Concern Removal

Description:

The research goal for this project is to add to our understanding of how aquatic biota responds to CEC discharges into Minnesota's aquatic environment. The primary objective for Activity 1 in this study is to document the role that urban stormwater plays in contributing CEC constituents to high-priority surface water in the large Minneapolis and St. Paul metropolitan area and in the moderate sized St. Cloud metropolitan area. This objective will be accomplished by collecting stormwater runoff at BMPs in St. Cloud and St. Paul during four precipitation events per year for two years (40 samples plus QA/QC samples). The secondary objective is to assess efficacy of emerging stormwater management techniques (BMPs) at reducing emerging contaminant concentrations in stormwater discharge. This objective will be accomplished by collecting pairing inlet and outlet samples from the above objective. All samples will be analyzed for a broad suite of CECs by the USGS National Water Quality Laboratory.

ENRTF BUDGET: $190,000

Outcome	Completion Date
1. Collect 40 stormwater samples (and QA/QC samples)	June 30, 2020
2. Analyze stormwater and QA/QC samples	June 30, 2021

Outcome	Completion Date
3. Estimate of urban storm water contribution to CEC loads in high priority surface waters	June 30, 2021
4. Report on the seasonal efficacy of two iron-enhanced sand filtration BMPs at reducing CECs in storm water discharge	June 30, 2021

First Update January 31, 2019

We evaluated eleven potential sampling sites in the greater Minneapolis/ St. Paul metropolitan area which included seven iron enhanced sand filter ponds and four standard stormwater ponds. After several site visits, seven ponds were found to be suitable for the proposed study (Table 1) including five iron enhanced sand filter (IESF) ponds: Golden Lake Pond (Blain, MN); William Street Pond (Roseville, MN); Trout Brook Sanctuary-Maryland (St. Paul, MN); Trout Brook Sanctuary-Magnolia (St. Paul, MN); Trout Brook Sanctuary-Jenks (St. Paul, MN). And two standard stormwater ponds: Southview Blvd- Anderson Pond (South St. Paul, MN); Birchwood Acres (Lino Lakes, MN). These locations were found to meet the requirements for the study (inflow and outflow can be sampled, appropriate residential land use, iron enhanced sand filtration, accessible location) and will be used as sampling sites for the remainder of the study. Runoff samples were collected at the inflow and outflow of each site during an early and late summer precipitation event. During each event, 30L of stormwater were collected along with general water quality parameters (temperature, pH, conductivity).

All samples have been curated and are currently prepared for analysis (Activity 1 and 2). Samples will be shipped to the USGS National Water Quality Laboratory for analysis.

Table 1. Sampling sites and samples collected during early and late summer stormwater collections.

Sampling site	Early summer sample	Late summer sample
IESF Ponds		
Golden Lake pond	**X**	**X**
William Street pond	**X**	**X**
Trout Brook Sanctuary – Maryland pond	**X**	**X**
Trout Brook Sanctuary – Magnolia pond	**X**	**X**
Trout Brook Sanctuary – Jenks pond	**X**	**X**
Standard stormwater ponds		
Southview Blvd Anderson pond	**X**	**X**
Birchwood Acres pond	**X**	**X**

Second Update June 30, 2019

Twenty-two runoff samples collected from the inflow and outflow of each site during an early and late summer precipitation event in 2018 and a snow-melt sample in 2019 were filtered and processed for shipment to the USGS National Water Quality Laboratory. Preparations for shipment included assigning USGS Station IDs and recording sampling date and times in the USGS NWIS database. All samples have been curated and are currently prepared for analysis under Activity 1.

Continuing the research into the second calendar year, we collected urban stormwater samples from five iron enhanced sand filter ponds: Golden Lake Pond (Blain, MN); William Street Pond (Roseville, MN); Trout Brook

Sanctuary ponds-Maryland, Magnolia, and Jenks (St. Paul, MN).; and two standard ponds: Southview Blvd-Anderson Pond (South St. Paul, MN); Birchwood Acres (Lino Lakes, MN). Runoff samples were again collected in 2019 at the inflow and outflow of each site for snowmelt and during a spring rain precipitation event.

Third Update January 31, 2020

A total of forty-four runoff samples were collected from the inflow and outflow of each pond during three run-off events in 2018 and 2019. Samples were submitted for analysis to the USGS National Water Quality Laboratory for analysis. Data have been returned for 40% of the samples. Additional samples have been archived for analysis if required. All data received from the NWQL have been curated and are currently available online through the USGS NWIS database.

Fourth Update June 30, 2020

Water-quality sampling was completed in May 2020. A total of 41 environmental and QA samples were successfully collected. About 70% of the data have been received from the USGS National Water Quality Laboratory. Turnaround times from the National Water Quality Laboratory have increased because of restrictions due to COVID-19. We anticipate receiving all the water-quality data by the end of September 2020, but if things fall further behind it may be later. This will affect our ability to completely analyze the data and produce a final report within the established timeline. Methods and Introduction for a draft report have been developed while we await the rest of the analytical data from the laboratory.

Fifth Update January 31, 2021

Water-quality sampling was completed in May 2020. All data have been received from the USGS National Water Quality Laboratory and uploaded into the USGS National Water Quality Information System (NWIS). These samples included thirty-one paired inflow-outflow samples from seven ponds collected during spring, summer, and fall seasons as outlined in Table 1. Total pharmaceutical concentrations were reduced in the outflows in 68% of the paired samples. Total pesticide concentrations were reduced in only 48% of the paired samples. Reductions in the total number of pharmaceuticals or pesticides detected were observed in less than 50% of the paired samples.

Comparisons between inflow and outflow samples from standard ponds or IESF ponds showed more frequent reductions in the total concentration of pharmaceuticals from the IESF treatment ponds: 74% of samples from IESF ponds showed reductions compared to 50% of samples from standard ponds. IESF treatment ponds did not perform better than standard ponds in reducing total pesticide concentrations in outflows: 42% of samples from IESF ponds showed reductions compared to 58% of samples from standard ponds. Seasonal patterns in reductions as well as chemical-specific results are being evaluated for the two types of ponds.

Sixth Update June 30, 2021:

Comparisons between inflow and outflow samples from standard ponds or IESF ponds showed more frequent reductions in the total concentration of pharmaceuticals from the IESF treatment ponds: 74% of samples from IESF ponds showed reductions compared to 50% of samples from standard ponds. A summary of paired estimates of reductions by class of compounds shows significant variation between classes. These differences also depend on the seasonal timing of the sampling.

Seventh Update as of January 31, 2022:

All samples have been analyzed, assessed to match quality criteria, and entered into a common data matrix. Data reports have been prepared for an upcoming manuscript on the biological effects of stormwater exposure (Activity 2). Efforts are currently underway to capture the totality of the study in a summary manuscript focused on stormwater chemistry, sources, fate, and treatment efficacy (Activity 1). This manuscript is scheduled to be submitted for peer-review prior to the end of the grant period.

Final Report Summary June 30, 2022

Thirty-six paired inflow/outflow water samples and four quality control samples were collected from seven urban stormwater ponds across seasons. All samples were submitted to chemical analysis for a range of pharmaceuticals and pesticides to document the presence of these pollutants in urban stormwater and to assess their removal by stormwater pond treatment technologies. Duplicate samples for each collection event were used to complete objectives in Activity 2. Although stormwater collection can be problematic as it relies difficult to predict precipitation events, we were successful in capturing strong stormwater runoff events across seasons and treatment technologies (traditional stormwater ponds vs IEAS ponds). Despite substantial delays caused by the lengthy shut-down of federal laboratories during the COVID-19 pandemic, all samples were analyzed, quality controlled, and entered into a common USGS data base. Pharmaceuticals and pesticides were commonly found in stormwater. In nearly three-quarters of paired water samples (pond inflow and pond outflow), pharmaceutical concentrations were reduced in the outflow when compared to the inflow. Similarly, in about half of paired samples, pesticide concentrations were lower in the outflow sample. This study demonstrates for the first time that stormwater ponds are effective treatment options to reduce the impact of pharmaceuticals and pesticides on urban aquatic environments. Adding additional filtration, such as iron-enhanced sand filtration can further reduce stormwater pollutants.

ACTIVITY 2: Measure the reduction in biological activity of stormwater before and after iron-enhanced sand-filtration in BMPs

The research goal for this activity is to determine how urban stormwater runoff affects gene expression across several aquatic species likely exposed to urban stormwater runoff in their natural habitats. To accomplish this research goal, cell-based assays for Leopard frog, and a turtle species will be developed to augment existing cell-based assays for bivalves and fishes. Biological effects of 40 urban stormwater samples (activity 1) will then be assessed using the cell-based assay to evaluate the removal efficiency of iron-enhanced sand filtration.

ENRTF BUDGET: $135,000

Outcome	Completion Date
1. Establish cell-based assay for six aquatic species	December 31, 2018
2. Analyze all stormwater samples (activity 1) for biological activity	December 31, 2020
3. Estimate estrogenicity of stormwater before and after iron-enhanced sand-filtration	June 30, 2021
4. Identify the most effective storm water treatment technology to reduce biological effects associated with CEC exposure.	June 30, 2021

First Update January 31, 2019

To establish the cell-based assays for Leopard frog and painted turtle, their genetic materials were isolated from tissues of Leopard frogs and painted turtles. The quality of the isolates were confirmed using standard molecular biological techniques. To isolate estrogen receptor from both species, genetic tools (PCR primer) were designed. These cell-based assay for Leopard frog and painted turtle will be validated in next two quartiles.

The cell-based assay for estrogen receptor in largemouth bass has been established (Figure 1). Interestingly, estrogen receptor 1-selective agonist, which selectively stimulates one type of estrogen receptor in mammals, did not stimulate estrogen receptor of largemouth bass due to a diversity of receptor affinity among species. The cell-based assay was also modified so that it could evaluate effects of stormwater on cell viability using

respiration specific dye. Biological activity and cell viability of stormwater collected in 20018 through estrogen receptor will be quantified in 2019.

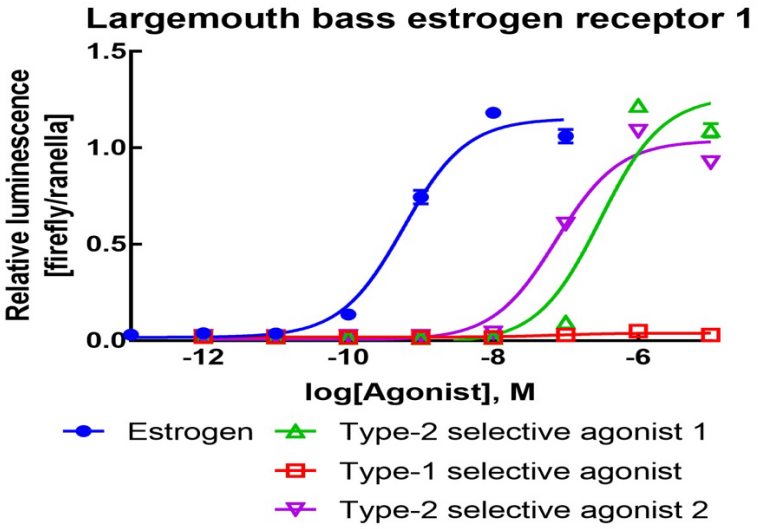


Figure 1. Largemouth bass estrogen receptor binding affinity. The Largemouth bass estrogen receptor will be one of the receptors used to assess the overall biological activity of stormwater samples and to quantify the efficacy of iron enhanced sand filtration in reducing biological activity of stormwater runoff.

Second Update June 30, 2019

We continued to advance the development of cell-based measures of water estrogenicity using the largemouth bass as model system to inform the development of all proposed cell based assays. To this end, we cloned cDNA for three isotype of the nuclear estrogen receptors (LMB *esr1*, *2a* and *2b)* and characterized their protein using ESR-selective ligands for amniotes. We identified three transcript variants of LMB *esr2a* which missed an exon of either DNA-binding domain or ligand-binding domain. Cloned cDNA in an expression vector was transfected into HEK293T cells with luciferase reporter gene, which would be transcribed via activated ESR. We then tested the products using known estrogenic compounds to assess binding activation and receptor sensitivity. The endogenous estrogen [17β-Estradiol (E_2)] and ESR2-selective ligands [2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN) and 7-Bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY200070)] activated the transcriptions via ESR1 (E_2 > DPN > WAY200070 in the sensitivity), whereas ESR1-selective ligand [4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)] did not activate LMB ESR1. All ligands activated the transcriptions via both LMB ESR2A and 2B within the same order of sensitivity (E_2 > DPN ≥ WAY200070 > PPT) and a different order in the efficacy (ESR2A: WAY200070> DPN ≥ E_2 > PPT; ESR2B: E2 > WAY200070 > PPT). These results suggested the importance of the ESR subtype to assess the estrogenicity of environmental samples. This information will be of crucial importance to guide the development of Leopard frog and turtle cell based assays.

In addition to continuing the development of cell-based assays, we also advanced approaches to connect estrogenicity values with other biological measures of water quality. To accomplish this goal, larval fathead minnows were exposed for 21 days to stormwater runoff samples in addition to control well water. Survival (Figure 2) and feeding ability (Figure 3) of larval fathead minnows exposed to stormwater runoff samples collected in spring and summer 2018 are shown below.

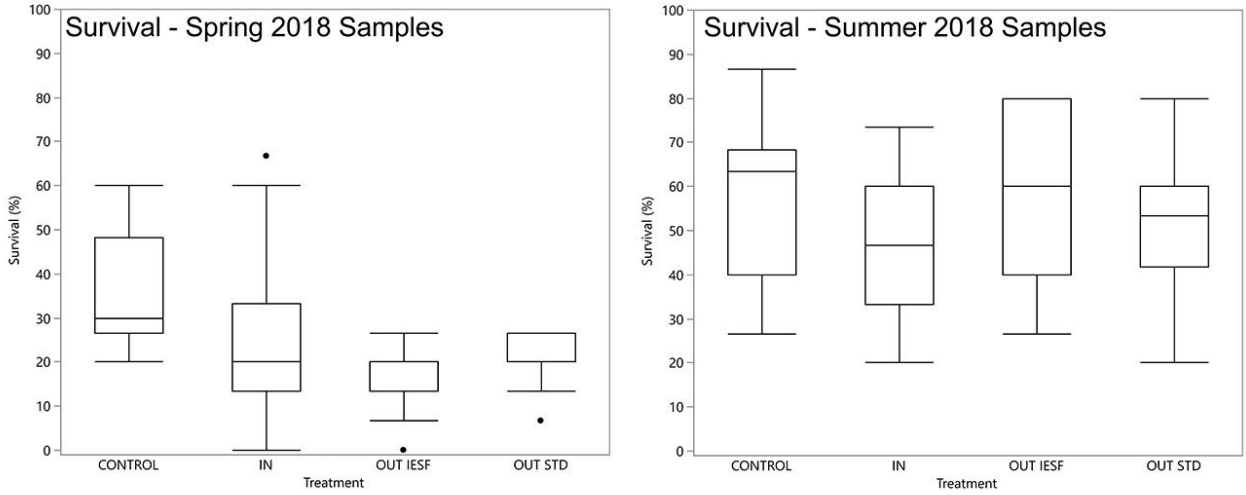


Figure 2. Survival of larval fathead minnows exposed to stormwater runoff for 21 days. Samples are grouped by laboratory control (CONTROL), inflow samples (IN), outflow samples after iron enhanced sand filtration (OUT IESF), and standard pond treatment (OUT STD).

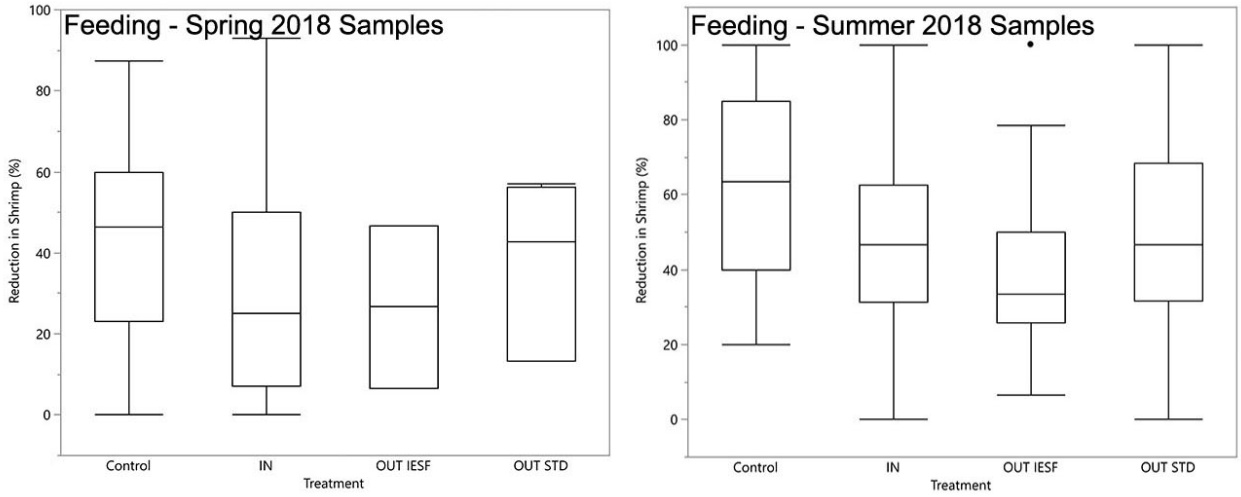


Figure 3. Feeding ability of larval fathead minnows exposed to stormwater runoff for 21 days. Samples are grouped by laboratory control (CONTROL), inflow samples (IN), outflow samples after iron enhanced sand filtration (OUT IESF), and standard pond treatment (OUT STD).

Third Update January 31, 2020

During the current reporting period, we have begun analyzing biological activities of stormwater using the *in vitro* cell culture system. A duplicate set of all water samples collected for Activity 1 has been filtered and solid-phase extracted in preparation for the cell-based assay analysis. In addition, a graduate student (James Gerads) contributing to this project recently defended his Master's thesis adding valuable biological endpoints to our assessment of the ecological impacts of stormwater on aquatic life.

We identified differences in cell-viability among sites with exposure to stormwater (Figure 4 below). To-date we have found that neither site nor treatment affect stormwater estrogenicity in exposed cell lines of fathead minnow, bluegill, and largemouth bass estrogen receptor 1 (Esr1; Figure 5). We are also working on the

molecular cloning of *esr1* gene to establish assays for freshwater mussel, the Northern leopard frog, and the painted turtle.

All collected urban stormwater samples are ready to expose to cells. We have prepared cell culture medium using filter-sterilized all stormwater samples, at a diluted strength of ¾ of the initial stormwater concentration based on our initial viability analysis. We are currently testing the samples at ¾ x strength using Alamar Blue (Bio-Rad) and Dual-Luciferase assay (Promega) for cytotoxicity and estrogenicity, respectively. In addition, we also extracted contaminants from the stormwater samples using a standard solid-phase extraction to have concentrated contaminants in the samples, which would provide the strength at ten times the original sample.

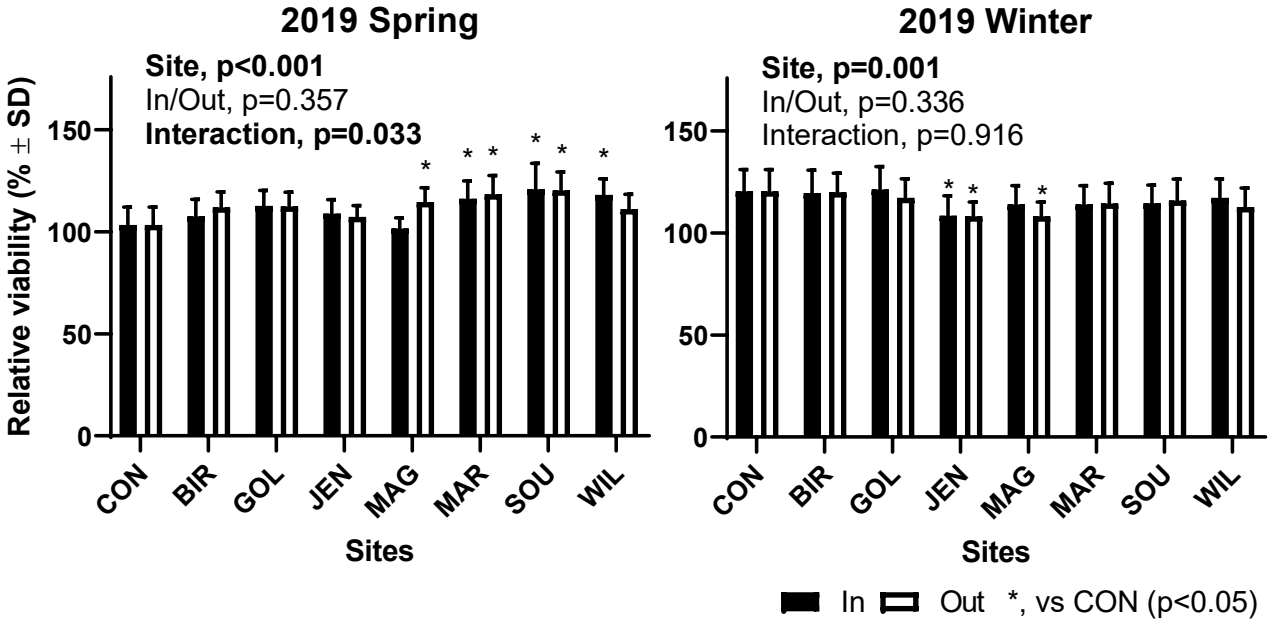


Figure 4. Relative cell viability in cells exposed to stormwater samples from seven stormwater pond inflows and outflow/IESF outflows. A laboratory control (CON) treatment is included. Full names of ponds are listed in Table 1.

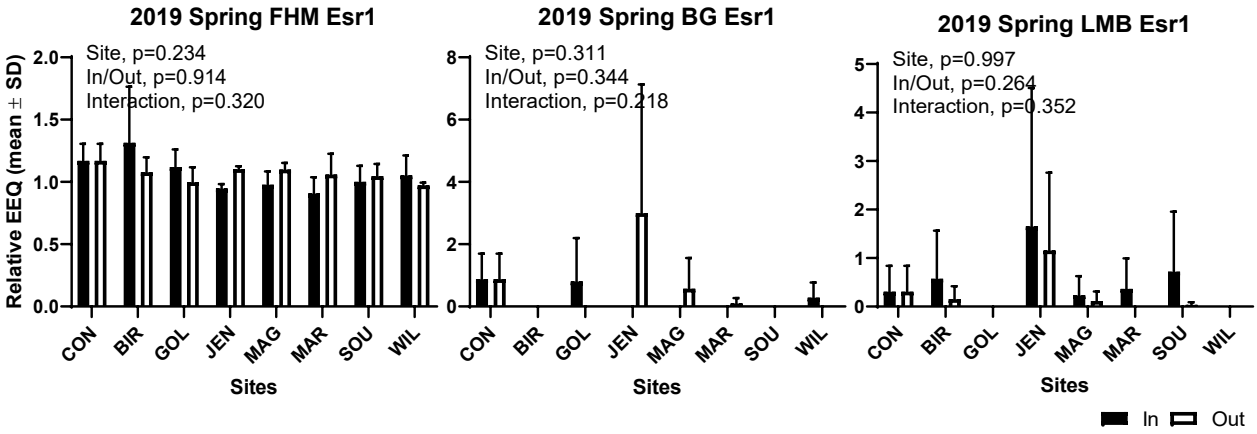


Figure 5. Estrogen Equivalency Values (EEQs) for stormwater samples collected from seven stormwater pond inflows and outflow/IESF outflows across three sampling events in fathead minnows (FHM, left), bluegill sunfish (BG, middle), and largemouth bass (LMB, right). Note the different y-axis scales for the three species.

Fourth Update June 30, 2020

Having completed the collection of stormwater samples through 2019, we proceeded with the preparation of the samples for analysis in our *in vitro* assays. All stormwater samples were filter-sterilized to prepare for the *in vitro* exposures. To purify the contaminants, we conducted solid-phase extraction on all samples and reconstituted samples at 10,000x concentration in DMSO buffer for cell exposure. By the time St. Cloud State University shut down all laboratories due to the covid-19 stay-at home order, we had completed cytotoxicity testing of approximately one-third of samples at 75% strength in fathead minnows, bluegill, and largemouth bass estrogen receptors. During the ongoing shutdown (SCSU is slowly opening laboratories again for research) we have been analyzing our preliminary data and preparing receptor constructs for the estrogenicity assay for freshwater mussel, Northern leopard frog, and painted turtle.

Fifth Update January 31, 2021

We were able to make significant progress in analyzing all collected stormwater samples. The stormwater samples, which had been filter-sterilized and tested for cytotoxicity in the previous reporting period, were now tested for their potential to elicit an estrogenic response in exposed organisms or their cells ("estrogenicity"). We also analyzed the cytotoxicity data that had been gathered previously to assess whether cells remained viable when they were exposed to stormwater extracts.

Cells exposed to stormwater of any season generally showed 10-20% higher cell viability than controls (laboratory ultrapure water). In August 2019, the flow difference between inflow and outflow affected cell viabilities, and IESF treatments significantly reduced cell viability (Fig. 6). In June 2018, regular pond treatment significantly reduced cell viability (Fig. 6). In March and May 2019, significant differences between the type of treatments were identified, whereas there was no difference between flows (Fig. 6). These results indicated that season affected the removal of factors, which altered cell viability in stormwater treatments. Further investigations in the coming reporting period will elucidate potential reasons for the observed effects (for example chemical and nutritional stressors).

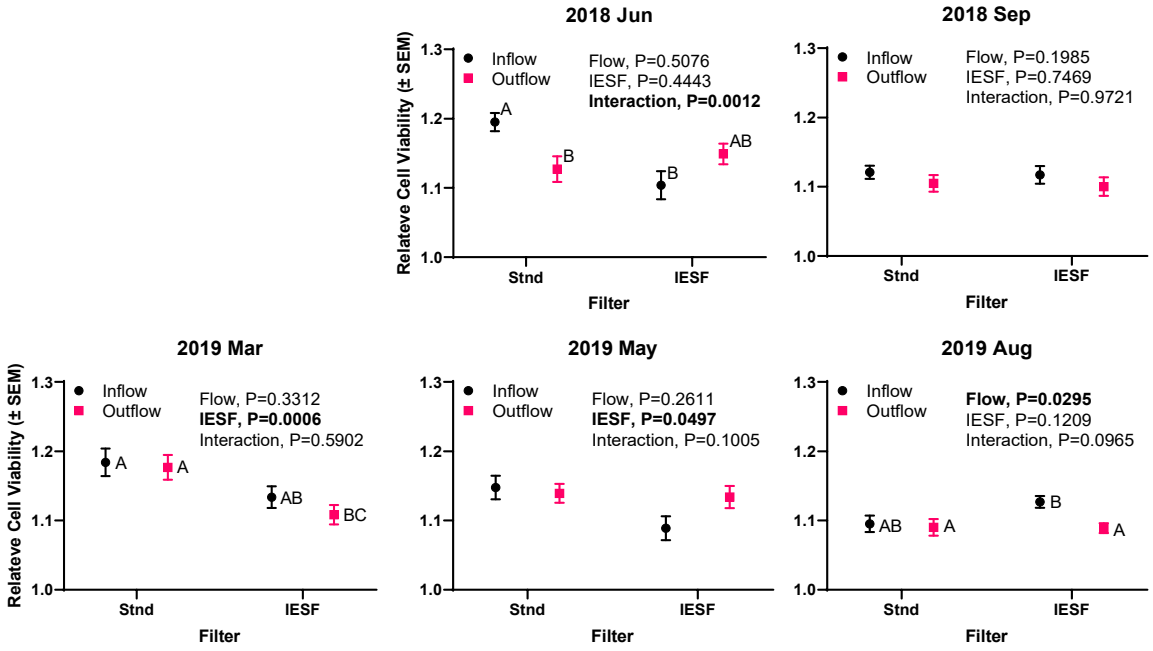


Figure 6. Effects of stormwater on estrogenicity *in vitro*. Human Embryonic Kidney cell 293T (HEK293T) was exposed to medium prepared with stormwater for 6 hours. Cell viability assay was conducted with alamar Blue (Bio-Rad) according to the manufacture's protocol. Assays were repeated at least three times in triplicate. Fold-inductions were also normalized by the bottom and top values of each sigmoid curve and presented as relative a percent activation of Esr ± sem. Effects of flows and type of ponds were evaluated by Two-way ANOVA followed by Tukey's post-hoc test. Different letters indicate significant differences between groups ($p<0.05$).

Estrogenicity

Stormwater slightly activated estrogen receptor 1 (Esr1) of fathead minnow (FHM), bluegill sunfish (BG), and largemouth bass (LMB) at less than 10% activation with species and seasonal variations (Figs. 7-9). General patterns of estrogenicities via BG and LMB were similar despite the percentage of Esr1 activations (Figs. 8 and 9).

Flows and pond types significantly affected estrogenicities via FHM Esr1 in exposures to stormwater collected in August 2019 (Fig. 7). Outflow in regular ponds was more estrogenic than it in IESF ponds, whereas these effects were not identified in other seasons (Fig. 7).

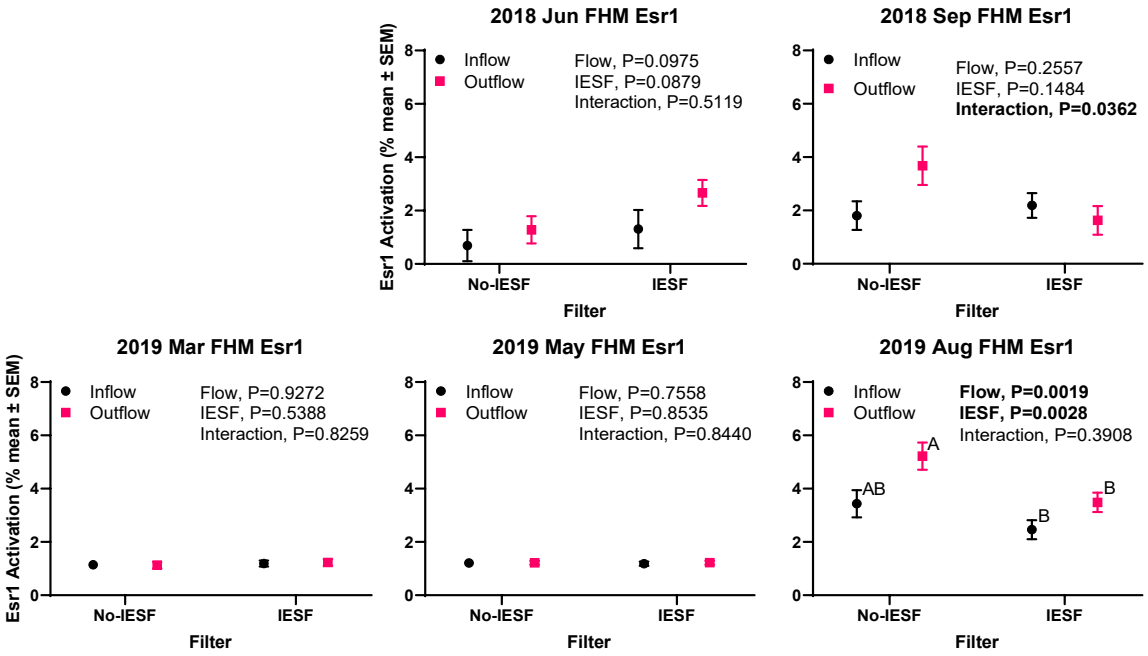


Figure 7. Effects of stormwater on cell viability. Human Embryonic Kidney 293T (HEK293T) cells were transfected with FHM Esr1, and exposed to medium prepared with stormwater for 48 hours. Estrogenic activities were measured using dual luciferase assay according to the manufacture's protocol. Assays were repeated three times in triplicate. Data were shown as relative viability to DMEM prepared with the ultrapure water in the laboratory. Effects of flows and type of ponds were evaluated by Two-way ANOVA followed by Tukey's post-hoc test. Different letters indicate significant differences between groups ($p<0.05$).

Flows and pond types significantly affected estrogenicities via BG Esr1 in exposures to stormwater collected in June 2018 (Fig. 8). Inflow in regular ponds was more estrogenic than other samples, whereas these effects were not identified in other seasons (Fig. 8).

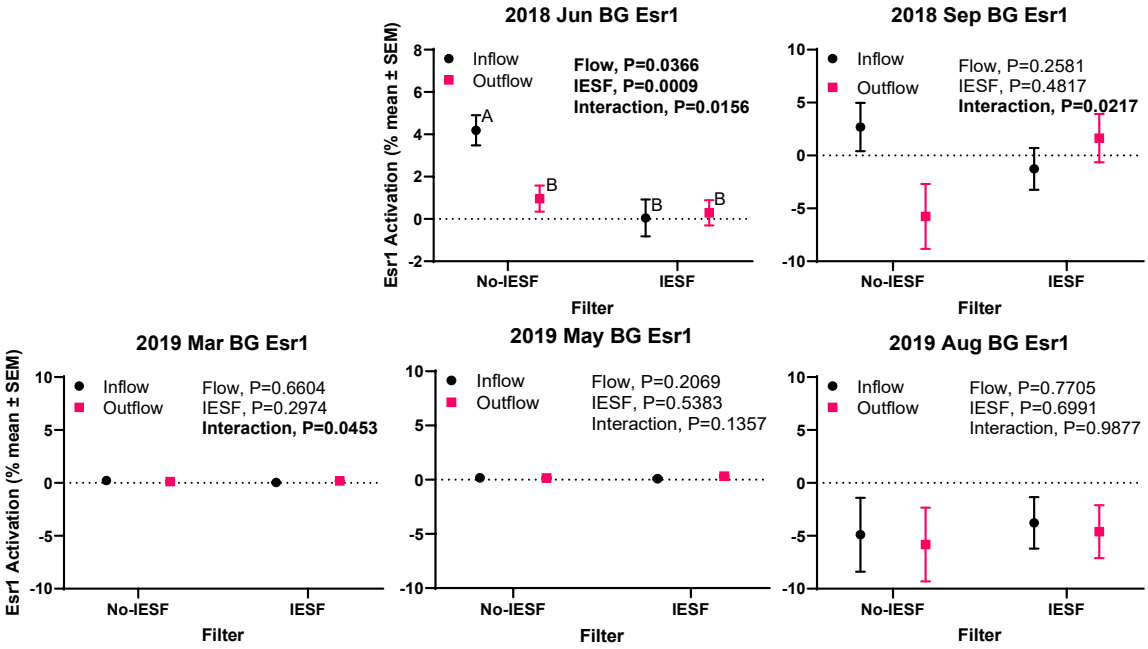


Figure 8. Effects of stormwater on cell viability. Human Embryonic Kidney 293T (HEK293T) cells were transfected with BG Esr1, and exposed to medium prepared with stormwater for 48 hours. Estrogenic activities were measured using dual luciferase assay according to the manufacture's protocol. Assays were repeated three times in triplicate. Data were shown as relative viability to DMEM prepared with the ultrapure water in the laboratory. Effects of flows and type of ponds were evaluated by Two-way ANOVA followed by Tukey's post-hoc test. Different letters indicate significant differences between groups ($p<0.05$).

Pond types significantly affected estrogenicities via LMB Esr1 in exposures to stormwater collected in June 2018 (Fig. 9). Inflow in regular ponds was more estrogenic than other samples, whereas these effects were not identified in other seasons (Fig. 9). This pattern of estrogenicities via LMB was similar to its via BG (Figs. 8 and 9).

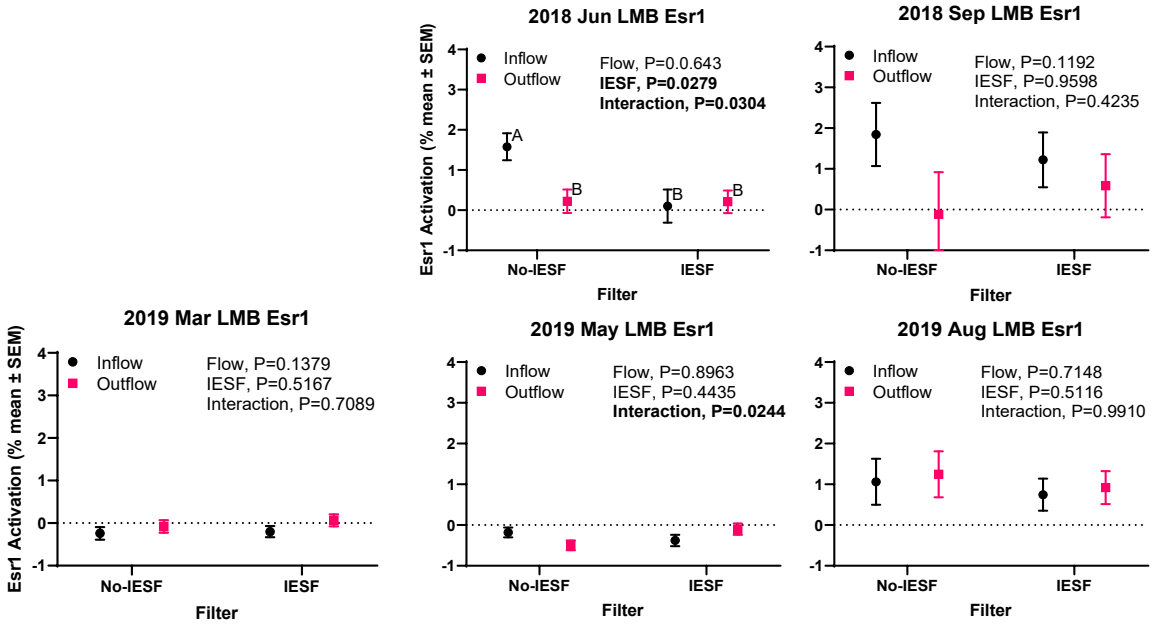


Figure 9. Effects of stormwater on cell viability. Human Embryonic Kidney 293T (HEK293T) cells were transfected with LMB Esr1, and exposed to medium prepared with stormwater for 48 hours. Estrogenic activities were measured using dual luciferase assay according to the manufacture's protocol. Assays were repeated three times in triplicate. Data were shown as relative viability to DMEM prepared with the ultrapure water in the laboratory. Effects of flows and type of ponds were evaluated by Two-way ANOVA followed by Tukey's post-hoc test. Different letters indicate significant differences between groups ($p<0.05$).

These results indicated that season affected the removal of estrogenic contaminants in stormwater. Further investigations in the coming reporting period will integrate these findings with the ongoing chemical analyses of stormwater to understand the origin of the observed effects.

Sixth Update June 30, 2021:

During the reporting period, we conducted additional quality assurance analysis in preparation for data use in a manuscript submission. We especially examined cell viability as a function of different experimental factors. Data gathered in the previous reporting periods have been linked to the now completed water chemistry for an analysis of co-variance. Filter types, sites, and treatments had different effects on cell viability depending on the season (Fig. 10). In June 2018, all filter types, sites, and treatments significantly affected cell viability, with standard filters significantly reducing cell viability, whereas IESF did not affect (Fig. 10). The cell viability of WIL was significantly higher than that of GOL and SOU in June 2018 (Fig.10). The treatments reduced cell viability in BIR and GOL in June 2018, although viability was still higher than in controls (Fig. 10). Similarly, in September 2018, sites and treatments significantly impacted cell viability, and IESF filters significantly increased and decreased cell viability in JEN and MAG, respectively (Fig. 10).

In March and May 2019, only sites, but not treatment, significantly impacted cell viability (Fig. 10). The cell viability of JEN in March 2018 was significantly higher than that of SOU and MAG (Fig.10). The cell viability of SOU was significantly higher than that of MAG in June 2018, and the standard filter reduced the cell viability at WIL (Fig.10). In August 2019, all filter types, sites, and treatments significantly affected cell viability, whereas neither standard nor IESF filter did not affect cell viability (Fig. 10). The cell viability of MAR was significantly higher than that of BIR, SOU, and GOL in August 2019. IESF filters significantly increased and decreased cell viability in JEN and WIL, respectively (Fig. 10).

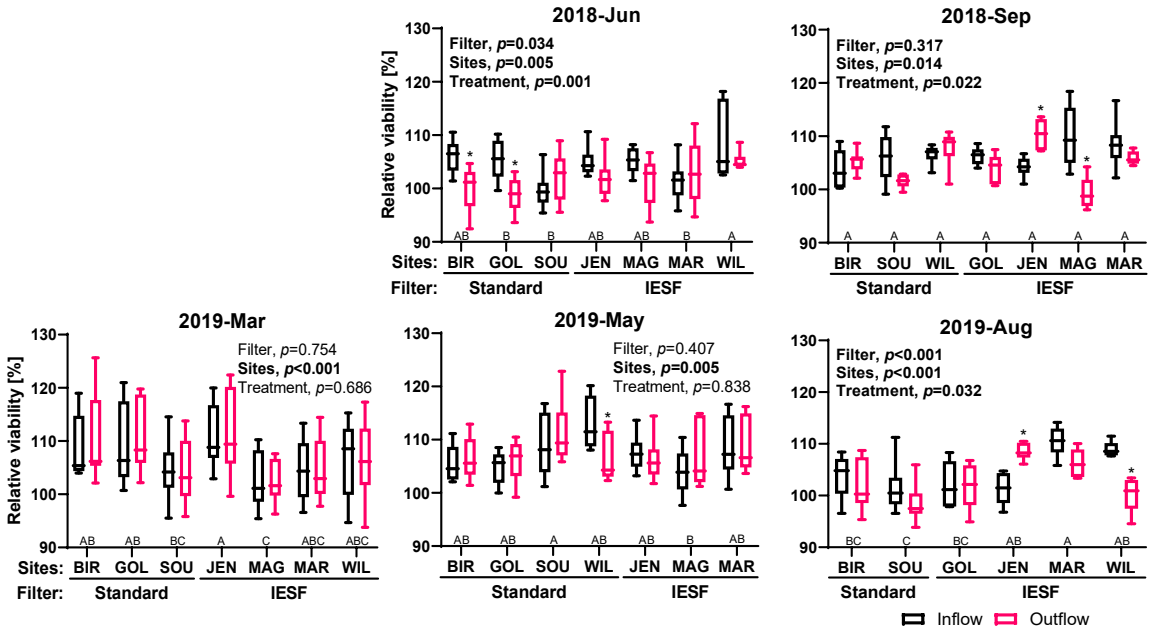


Figure 10. Relative cell viability in vitro. HEK293T cells were exposed to urban stormwater extract for 6 hours. Cell viability was measured as oxidation using a cell viability assay reagent, alamarBlue. Data were expressed as relative to vehicle control, 0.1% DMSO. ANOVA results indicating effects of filter type, sites, and treatments were shown on each chart. The letters indicate significant differences among sites (p<0.05); Asterisks indicate a significant difference between inflows and outflows.

Cell viability at 10-fold concentration of urban stormwater was differed significantly among season and sites due to different contaminant concentrations and types in inflow. To elucidate the effectiveness of IESF urban stormwater treatment, it is better to focus on treatment reduction / induction rate in cell viability. Further comparison of fish survival and estrogenicity with chemical occurrence data will be used to directly link organismal response to urban stormwater exposures and the potential for mitigation through treatment technologies. This approach will be used in a manuscript now under development.

Seventh Update as of January 31, 2022:

A manuscript outlining the biological effects of stormwater exposure (Activity 2) is currently finalized for peer-review submission. The manuscript incorporates summaries of the analytical chemistry (Activity 1) and a detailed assessment of biological effects observed in whole organism exposures to stormwater samples collected in the current study.

To complete the multi-species bioassays, we have been able to isolate RNA from painted turtles (*Chrysemys picta*) and Northern Leopard frogs (*Lithobates pipiens*), the remaining two proposed study species and the two species with the least prior data available. RNA was extracted from tissues using the single-step acid guanidinium thiocyanate-phenol-chloroform method followed by a clean-up with the SV total RNA isolation system including DNase-I treatment (Promega, Madison). RNA quantity and purity was evaluated by measuring optical density using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific), and quality of the RNA was assessed using denaturing agarose gel electrophoresis.

For cDNA cloning, first-strand cDNA was synthesized from 5 µg total RNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific) and oligo(dT) primer in 20 µL of reaction. Each estrogen receptor (ESR) cDNA was amplified by PCR using PrimeStar DNA polymerase (Takara Bio, Otsu, Japan). Gene-specific primers

were designed according to *Painted turtle* and *Northern leopard frog* ESR1 and ESR2 sequences in NCBI database (Table 2).

Table 2. PCR Primers

Name	Sequence	Position	GenBank Accession #
Cpic_ESR1-F1	GTCATGTGACCCTCTCAGCC	106	NM_001282246
Cpic_ESR1-R1	CACTGGTGCCTATTGGAGTGT	2126	
Cpic_ESR1-F2	TCCAGAAGTAATGCCAACAGC	137	
Cpic_ESR1-R2	TGTTGGGATTCTCAGAACCTTGT	2055	
Cpic_ESR2-F1	TAGCTGCAGTCAGTCTCCTCT	487	XM_005285890
Cpic_ESR2-F2	GCCCATGGTGTGATGCAAGA	753	
Cpic_ESR2-R1	GCCCATGTAAAGCTTTCTGCT	2448	
Cpic_ESR2-R2	TCCAATTGCCAAGGTGATGTG	2287	
Rpip_esr1-5F	TGGTGTCTGGTCTTGTGAGGGCTGT	5	DQ398027
Rpip_esr1-75F	TGCCCCGCAACCAATCAGTGTACCA	75	

The resulting PCR products/fragments were run on 1% agarose gel in TAE buffer, and were purified using a Wizard SV Gel and PCR Clean-Up System (Promega). Amplified cDNA was cloned into the sequencing vector using TOPO XL-2 Complete PCR Cloning Kit (Thermo Fisher Scientific). Cloned cDNA was analyzed using Sanger sequencing (Eurofins Genomics).

Painted turtle

Cloned cDNA for the Painted turtle ESR1 and ESR2 were 1,223 base and 1,535 base encoding 388 and 415 amino acid, respectively (Fig. 11).

>Painted turtle ESR1

```
TCCAGAAGTAATGCCAACAGCCTTCTTGGATAAATGGCACAACGACTACATGTGTCCCGC    60
          M  P  T  A  F  L  D  K  W  H  N  D  Y  M  C  P  A     17
TACTAACCAGTGCACCATCGACAAGAACAGGAGAAAGAGCTGTCAGGCCTGCCGGCTGCG   120
  T  N  Q  C  T  I  D  K  N  R  R  K  S  C  Q  A  C  R  L  R     37
AAAGTGCTATGAAGTGGGAATGATGAAAGGTGGGATCCGAAAAGACCGCAGAGGCGGACG   180
  K  C  Y  E  V  G  M  M  K  G  G  I  R  K  D  R  R  G  G  R     57
TATGCTGAAACACAAACGTCAAAGAGAGGAACAGGATGCCAGGATTGCAGGGACTTCTTC   240
  M  L  K  H  K  R  Q  R  E  E  Q  D  A  R  I  A  G  T  S  S     77
TACTGAGATGAGAACCCCCACCCTCTGGACAAGTCCTCTTGTGATTAAGCATAGCAAGAA   300
  T  E  M  R  T  P  T  L  W  T  S  P  L  V  I  K  H  S  K  K     97
GAACAGTCCAGCTTTGTCCCTTACAGCAGAGCAGATGGTCAGTGCCTTACTGGAAGCTGA   360
  N  S  P  A  L  S  L  T  A  E  Q  M  V  S  A  L  L  E  A  E     117
ACCACCCATAGTCTATTCAGAGTATGATCCTAACAGACCTTTCAGTGAAGCCTCCATGAT   420
  P  P  I  V  Y  S  E  Y  D  P  N  R  P  F  S  E  A  S  M  M     137
GACCCTGTTGACCAACCTTGCAGACAGGGAACTGGTGCACATGATCAACTGGGCAAAAAG   480
  T  L  L  T  N  L  A  D  R  E  L  V  H  M  I  N  W  A  K  R     157
GGTCCCAGGGTTTGTGGATTTAACACTCCATGATCAGGTACATCTACTGGAATGTGCCTG   540
  V  P  G  F  V  D  L  T  L  H  D  Q  V  H  L  L  E  C  A  W     177
GTTAGAGATACTGATGATTGGCTTAGTCTGGCGTTCAATGGAACATCCGGGAAAACTCTC   600
  L  E  I  L  M  I  G  L  V  W  R  S  M  E  H  P  G  K  L  S     197
ATTTGCACCTAATCTATTACTGGACAGGAATCAAGGGAAGTGCGTAGAGGGCATGGTGGA   660
  F  A  P  N  L  L  L  D  R  N  Q  G  K  C  V  E  G  M  V  E     217
GATCTTTGACATGTTGCTGGCCACTGCTGCTCGGTTCCGTGTGATGAATCTCCAGGGGGA   720
  I  F  D  M  L  L  A  T  A  A  R  F  R  V  M  N  L  Q  G  E     237
GGAATTTGTGTGCCTTAAGTCAATCATCCTGCTCAATTCAGGTGTGTATACATTTCTTTC   780
  E  F  V  C  L  K  S  I  I  L  L  N  S  G  V  Y  T  F  L  S     257
CAGCACCTTGAAATCTTTGGAAGAGAAGGAGCATATTCATCGTGTTCTGGACAAAATCAC   840
  S  T  L  K  S  L  E  E  K  E  H  I  H  R  V  L  D  K  I  T     277
AGATACATTGATTCATTTAATGGCCAAGTCGGGTCTCTCTCCAGATCAGCAACACAGACG   900
  D  T  L  I  H  L  M  A  K  S  G  L  S  P  D  Q  Q  H  R  R     297
ACTGGCTCAGCTCCTCCTCATACTCTCACACATCAGGCACATGGGCAATAAAGGGATGGA   960
  L  A  Q  L  L  L  I  L  S  H  I  R  H  M  G  N  K  G  M  E     317
GCACCTATACAACATGAAGTGCAAAAATGTAGTCCCGCTTTATGATTTATTGCTGGAAAT  1020
  H  L  Y  N  M  K  C  K  N  V  V  P  L  Y  D  L  L  L  E  M     337
GCTAGATGCTCACCGATTGCGTGCCCCAGCAGCCAAAAATGCTCCCCAGATGGAAGAAGA  1080
  L  D  A  H  R  L  R  A  P  A  A  K  N  A  P  Q  M  E  E  E     357
GAATCGGAGCCAGTTGACAACTGCATCAACTTCATCACATTCCTTGCAGACCTTTTATGT  1140
  N  R  S  Q  L  T  T  A  S  T  S  S  H  S  L  Q  T  F  Y  V     377
AAACAGGGAAGATGAGAATTTGCAAAATACAGTATTGAGCGTTCACATGTTAAAAAAGCCT  1200
  N  R  E  D  E  N  L  Q  N  T  V                               388
ACAAGGTTCTGAGAATCCCAACA                                        1223
```

>Painted turtle ESR2

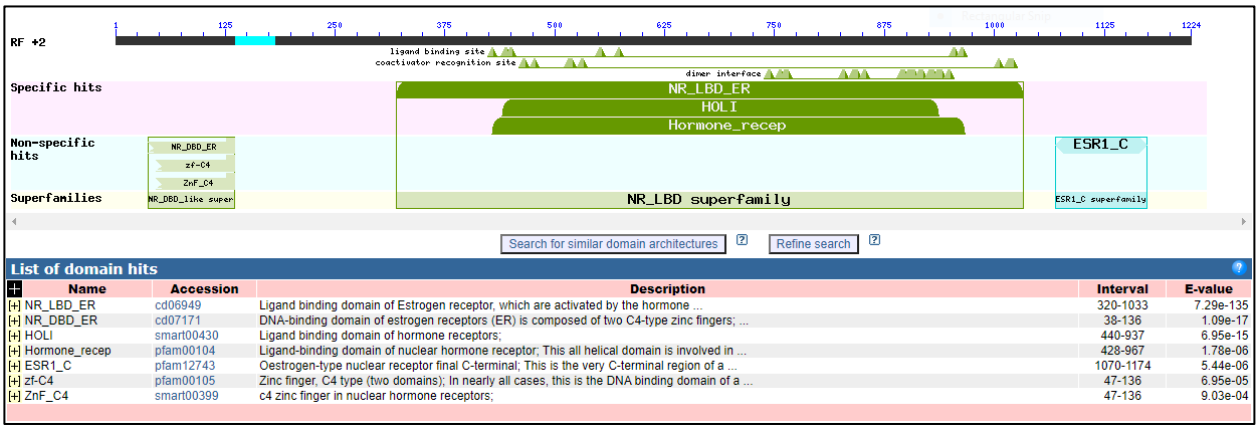
```
GCCCATGGTGTGATGCAAGACCAGTGGACCATGCACTGCCTATGAACAGAGAGACATTAA    60
                                          M  N  R  E  T  L  K     7
AAAGAAAATCTAAAGGCAGTGATTGTACAAGTCCTGCTGTGAATAGTCCTGGTTCAAAAA   120
  R  K  S  K  G  S  D  C  T  S  P  A  V  N  S  P  G  S  K  R     27
GAGATGCACACTTCTGTGCAGTCTGCAGTGATTATGCTTCAGGATATCACTATGGCGTTT   180
  D  A  H  F  C  A  V  C  S  D  Y  A  S  G  Y  H  Y  G  V  W     47
GGTCTTGTGAAGGATGTAAAGCATTCTTTAAAAGAAGTATCCAAGGACACAATGATTACA   240
  S  C  E  G  C  K  A  F  F  K  R  S  I  Q  G  H  N  D  Y  I     67
TCTGTCCAGCTACCAATCAATGCACAATAGATAAAAATAGGCGTAAAAGCTGTCAGGCAT   300
  C  P  A  T  N  Q  C  T  I  D  K  N  R  R  K  S  C  Q  A  C     87
GTAGACTACGGAAATGCTGTGAAGTAGGAATGATGAAATGTGGTTCAAGAAGAGAACGTT   360
  R  L  R  K  C  C  E  V  G  M  M  K  C  G  S  R  R  E  R  C    107
GTGGGTATCGTATCATACGCCGTCATCGTAATGCAGAAGATCAAGTGCATTGCACTGGCA   420
  G  Y  R  I  I  R  R  H  R  N  A  E  D  Q  V  H  C  T  G  K    127
AAGCTAAAAAGAATAGTGAAAATACAACCCGAGTGAAAGAAATCCTTCTTTGTTCACTCA   480
  A  K  K  N  S  E  N  T  T  R  V  K  E  I  L  L  C  S  L  S    147
GTCCTGAGCAGTTTGTTTTAACGTTACTTGAAGCTGAACCTCCTAATGTGTTGCTGGTGA   540
  P  E  Q  F  V  L  T  L  L  E  A  E  P  P  N  V  L  L  V  S    167
GTCGCCCCAGCAAGCCATTTACAGAAGTCTCCATGATGATGTCATTGACAAAACTTGCAG   600
  R  P  S  K  P  F  T  E  V  S  M  M  M  S  L  T  K  L  A  D    187
ACAAAGAATTGGTTCACATGATTGGTTGGGCCAAGAAAATTCCTGGCTTCATAGAGCTCA   660
  K  E  L  V  H  M  I  G  W  A  K  K  I  P  G  F  I  E  L  S    207
GTCTGTATGACCAAGTGAGGCTTTTGGAGAGTTGTTGGATGGAAGTTTTAATGGTGGGTC   720
  L  Y  D  Q  V  R  L  L  E  S  C  W  M  E  V  L  M  V  G  L    227
TGATGTGGAGGTCGATTGATTATCCTGGCAAGCTAATTTTTGCACCAGATCTTGTATTAG   780
  M  W  R  S  I  D  Y  P  G  K  L  I  F  A  P  D  L  V  L  D    247
ACAGGGATGAGGGGAAATGTGTAGAAGGAATTCTGGAAATCTTTGACATGCTCCTGGCCA   840
  R  D  E  G  K  C  V  E  G  I  L  E  I  F  D  M  L  L  A  T    267
CAACATCAAGATTTCGAGAGCTGAAATTACAGCACAAGGAGTATCTGTGTGTCAAGGCTA   900
  T  S  R  F  R  E  L  K  L  Q  H  K  E  Y  L  C  V  K  A  M    287
TGATTCTTCTCAATTCCAATATGTTTCCATTGTCAGCTGCTGCAGAAGAATCTGAAAGCA   960
  I  L  L  N  S  N  M  F  P  L  S  A  A  A  E  E  S  E  S  N    307
ACAGGAAGCTACCTCACTTGCTTAATGCAGTAACTGATGCTTTGGTGTGGGTTATTGCAA  1020
  R  K  L  P  H  L  L  N  A  V  T  D  A  L  V  W  V  I  A  K    327
AAAGTGGAATTCCATCTCAGCAACAGACAACTCGTTTGGCTAACTTGTTAATGTTACTTT  1080
  S  G  I  P  S  Q  Q  Q  T  T  R  L  A  N  L  L  M  L  L  S    347
CTCATGTCAGGCATGCAAGTAACAAAGGTATGGAGCACCTTCTGAGCATGAAGTGTAAAA  1140
  H  V  R  H  A  S  N  K  G  M  E  H  L  L  S  M  K  C  K  N    367
ATGTGGTCCCGGTCTATGACTTGCTGCTGGAAATGCTGAATGCACACACGCTTCGAGGTC  1200
  V  V  P  V  Y  D  L  L  L  E  M  L  N  A  H  T  L  R  G  Q    387
AAAGAAAGTCTGCAGTCACAGATTCTGAATTTGGCACATCAGAACAAACTGATAGTAGAG  1260
  R  K  S  A  V  T  D  S  E  F  G  T  S  E  Q  T  D  S  R  D    407
ATGATACGCGGAATATGGCAGAACAGTAACTTTATAATTACCTGGAAGATCAGCCCAGCC  1320
  D  T  R  N  M  A  E  Q                                        415
CAACCCTAGGAAGTAACCCTACGGTGAAAAATCAGAGGCCAGACTTCAGTATCAAGCTAC  1380
GAAAGGGTTATTGTAATCAACTGAGAGTAAAAATAATTTTGAAGATGTCTGAAAGATAGC  1440
TTGGTAAGGTTCTTTTCCAACTTATTTTTTGGGACATTTTGAGATATGGTAAAAGCTCCT  1500
TGTATATTCTATATCACATCACCTTGGCAATTGGA                             1535
```

Figure 11. Cloned cDNA sequences for the Painted turtle ESR1 (left) and ESR2 (right). Italicized letters indicated deduced amino acid sequence. Start and stop codons were bold and underlined.

Cloned cDNA for the Painted turtle ESR1 and ESR2 contains two essential functional domains such as DNA-binding and ligand-binding domains (Fig. 12).

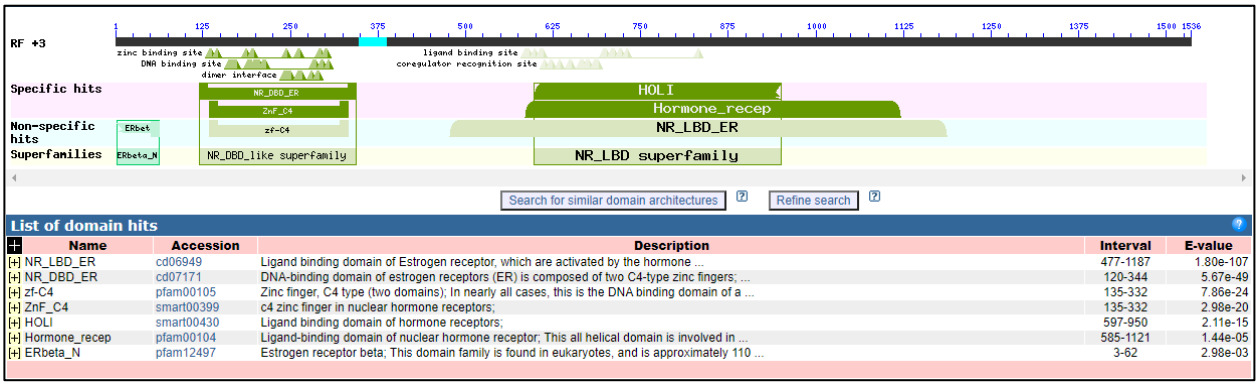
Deduced amino acid sequences of the painted turtle ESR1 and ESR2 were 100% identical to those in the NCBI database, although there was a missing region at the amino terminus of the proteins (Fig. 13).

A. ESR1



Name	Accession	Description	Interval	E-value
[+] NR_LBD_ER	cd06949	Ligand binding domain of Estrogen receptor, which are activated by the hormone ...	320-1033	7.29e-135
[+] NR_DBD_ER	cd07171	DNA-binding domain of estrogen receptors (ER) is composed of two C4-type zinc fingers; ...	38-136	1.09e-17
[+] HOLI	smart00430	Ligand binding domain of hormone receptors;	440-937	6.95e-15
[+] Hormone_recep	pfam00104	Ligand-binding domain of nuclear hormone receptor; This all helical domain is involved in ...	428-967	1.78e-06
[+] ESR1_C	pfam12743	Oestrogen-type nuclear receptor final C-terminal; This is the very C-terminal region of a ...	1070-1174	5.44e-06
[+] zf-C4	pfam00105	Zinc finger, C4 type (two domains); In nearly all cases, this is the DNA binding domain of a ...	47-136	6.95e-05
[+] ZnF_C4	smart00399	c4 zinc finger in nuclear hormone receptors;	47-136	9.03e-04

B. ESR2



Name	Accession	Description	Interval	E-value
[+] NR_LBD_ER	cd06949	Ligand binding domain of Estrogen receptor, which are activated by the hormone ...	477-1187	1.80e-107
[+] NR_DBD_ER	cd07171	DNA-binding domain of estrogen receptors (ER) is composed of two C4-type zinc fingers; ...	120-344	5.67e-49
[+] zf-C4	pfam00105	Zinc finger, C4 type (two domains); In nearly all cases, this is the DNA binding domain of a ...	135-332	7.86e-24
[+] ZnF_C4	smart00399	c4 zinc finger in nuclear hormone receptors;	135-332	2.98e-20
[+] HOLI	smart00430	Ligand binding domain of hormone receptors;	597-950	2.11e-15
[+] Hormone_recep	pfam00104	Ligand-binding domain of nuclear hormone receptor; This all helical domain is involved in ...	585-1121	1.44e-05
[+] ERbeta_N	pfam12497	Estrogen receptor beta; This domain family is found in eukaryotes, and is approximately 110 ...	3-62	2.98e-03

Figure 12. Conserved domain search results of cloned painted turtle ESRs. Homologous proteins homologous to cloned cDNA was searched on NCBI Blastx search. Conserved domains of cloned cDNA were revealed in concerved domain search. Two representative sequences were analyzed here representing tw estrogen receptors: painted turtle ESR1 (A) and ESR2 (B).

A

estrogen receptor [Chrysemys picta]

Sequence ID: NP_001269175.1 Length: 588 Number of Matches: 1

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 208 to 588 GenPept Graphics ▼ Next Match ▲ Previous

Score	Expect	Method	Identities	Positives	Gaps	Frame
751 bits(1940)	0.0	Compositional matrix adjust.	379/381(99%)	380/381(99%)	0/381(0%)	+2

```
Query  32    KWHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVgmmkggirkdrrggrmLKHKRQREE  211
             + HNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQREE
Sbjct  208   QGHNDYMCPATNQCTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQREE  267

Query  212   QDARIAGTSSTEMRTPTLWTSPLVIKHSKKNSPALSLTAEQMVSALLEAEPPIVYSEYDP  391
             QDARIAGTSSTEMRTPTLWTSPLVIKHSKKNSPALSLTAEQMVSALLEAEPPIVYSEYDP
Sbjct  268   QDARIAGTSSTEMRTPTLWTSPLVIKHSKKNSPALSLTAEQMVSALLEAEPPIVYSEYDP  327

Query  392   NRPFSEASMMTLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVW  571
             NRPFSEASMMTLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVW
Sbjct  328   NRPFSEASMMTLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLEILMIGLVW  387

Query  572   RSMEHPGKLSFAPNLLLDRNQGKCVEGMVEIFDMLLATAARFRVMNLQGEEFVCLKSIIL  751
             RSMEHPGKLSFAPNLLLDRNQGKCVEGMVEIFDMLLATAARFRVMNLQGEEFVCLKSIIL
Sbjct  388   RSMEHPGKLSFAPNLLLDRNQGKCVEGMVEIFDMLLATAARFRVMNLQGEEFVCLKSIIL  447

Query  752   LNSGVYTFLSSTLKSLEEKEHIHRVLDKITDTLIHLMAKSGLSPDQQHRRLAQLLLILSH  931
             LNSGVYTFLSSTLKSLEEKEHIHRVLDKITDTLIHLMAKSGLSPDQQHRRLAQLLLILSH
Sbjct  448   LNSGVYTFLSSTLKSLEEKEHIHRVLDKITDTLIHLMAKSGLSPDQQHRRLAQLLLILSH  507

Query  932   IRHMGNKGMEHLYNMKCKNVVPLYDLLLEMLDAHRLRAPAAKNAPQMEEENRSQLTTAST  1111
             IRHMGNKGMEHLYNMKCKNVVPLYDLLLEMLDAHRLRAPAAKNAPQMEEENRSQLTTAST
Sbjct  508   IRHMGNKGMEHLYNMKCKNVVPLYDLLLEMLDAHRLRAPAAKNAPQMEEENRSQLTTAST  567

Query  1112  SSHSLQTFYVNREDENLQNTV  1174
             SSHSLQTFYVNREDENLQNTV
Sbjct  568   SSHSLQTFYVNREDENLQNTV  588
```

B

estrogen receptor beta [Chrysemys picta bellii]

Sequence ID: XP_005285947.1 Length: 556 Number of Matches: 1

Range 1: 129 to 556 GenPept Graphics ▼ Next Match ▲ Previous Ma

Score	Expect	Method	Identities	Positives	Gaps	Frame
861 bits(2224)	0.0	Compositional matrix adjust.	428/428(100%)	428/428(100%)	0/428(0%)	+3

```
Query  3     PWCDARPVDHALPMNRETLKRKSKGSDCTSPAVNSPGSKRDAHFCAVCSDYASGYHYGVW  182
             PWCDARPVDHALPMNRETLKRKSKGSDCTSPAVNSPGSKRDAHFCAVCSDYASGYHYGVW
Sbjct  129   PWCDARPVDHALPMNRETLKRKSKGSDCTSPAVNSPGSKRDAHFCAVCSDYASGYHYGVW  188

Query  183   SCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCCEVGMMKCGSrrerc  362
             SCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCCEVGMMKCGSRRERC
Sbjct  189   SCEGCKAFFKRSIQGHNDYICPATNQCTIDKNRRKSCQACRLRKCCEVGMMKCGSRRERC  248

Query  363   gyriirrhrNAEDQVHCTGKAKKNSENTTRVKEILLCSLSPEQFVLTLLEAEPPNVLLVS  542
             GYRIIRRHRNAEDQVHCTGKAKKNSENTTRVKEILLCSLSPEQFVLTLLEAEPPNVLLVS
Sbjct  249   GYRIIRRHRNAEDQVHCTGKAKKNSENTTRVKEILLCSLSPEQFVLTLLEAEPPNVLLVS  308

Query  543   RPSKPFTEVSMMMSLTKLADKELVHMIGWAKKIPGFIELSLYDQVRLLESCWMEVLMVGL  722
             RPSKPFTEVSMMMSLTKLADKELVHMIGWAKKIPGFIELSLYDQVRLLESCWMEVLMVGL
Sbjct  309   RPSKPFTEVSMMMSLTKLADKELVHMIGWAKKIPGFIELSLYDQVRLLESCWMEVLMVGL  368

Query  723   MWRSIDYPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAM  902
             MWRSIDYPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAM
Sbjct  369   MWRSIDYPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQHKEYLCVKAM  428

Query  903   ILLNSNMFPLSAAAEESESNRKLPHLLNAVTDALVWVIAKSGIPSQQQTTRLANLLMLLS  1082
             ILLNSNMFPLSAAAEESESNRKLPHLLNAVTDALVWVIAKSGIPSQQQTTRLANLLMLLS
Sbjct  429   ILLNSNMFPLSAAAEESESNRKLPHLLNAVTDALVWVIAKSGIPSQQQTTRLANLLMLLS  488

Query  1083  HVRHASNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRGQRKSAVTDSEFGTSEQTDSRD  1262
             HVRHASNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRGQRKSAVTDSEFGTSEQTDSRD
Sbjct  489   HVRHASNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRGQRKSAVTDSEFGTSEQTDSRD  548

Query  1263  DTRNMAEQ  1286
             DTRNMAEQ
Sbjct  549   DTRNMAEQ  556
```

Figure 13. Results of Blast search on painted turtle ESR1 (A) and ESR2 (B). The results confirmed that cloned cDNAs were painted turtle ESRs.

Northern Leopard Frog

Cloned cDNA for the Northern leopard frog ESR1 and ESR2 were partial sequences with 824 base and 773 bases encoding 144 and 65 amino acids, respectively (Fig. 14). Cloned cDNA for the Painted turtle ESR1 and ESR2 contains two essential functional domains such as DNA-binding and ligand-binding domains (Fig. 4). Although primers were designed for ESR1, we have cloned both isotypes. We need to clone a longer fragment of Northern leopard frog ESR2 because it did not obtain the entire ligand-binding domain and contained the stop-codon in the Ligand-binding domain (Fig. 14).

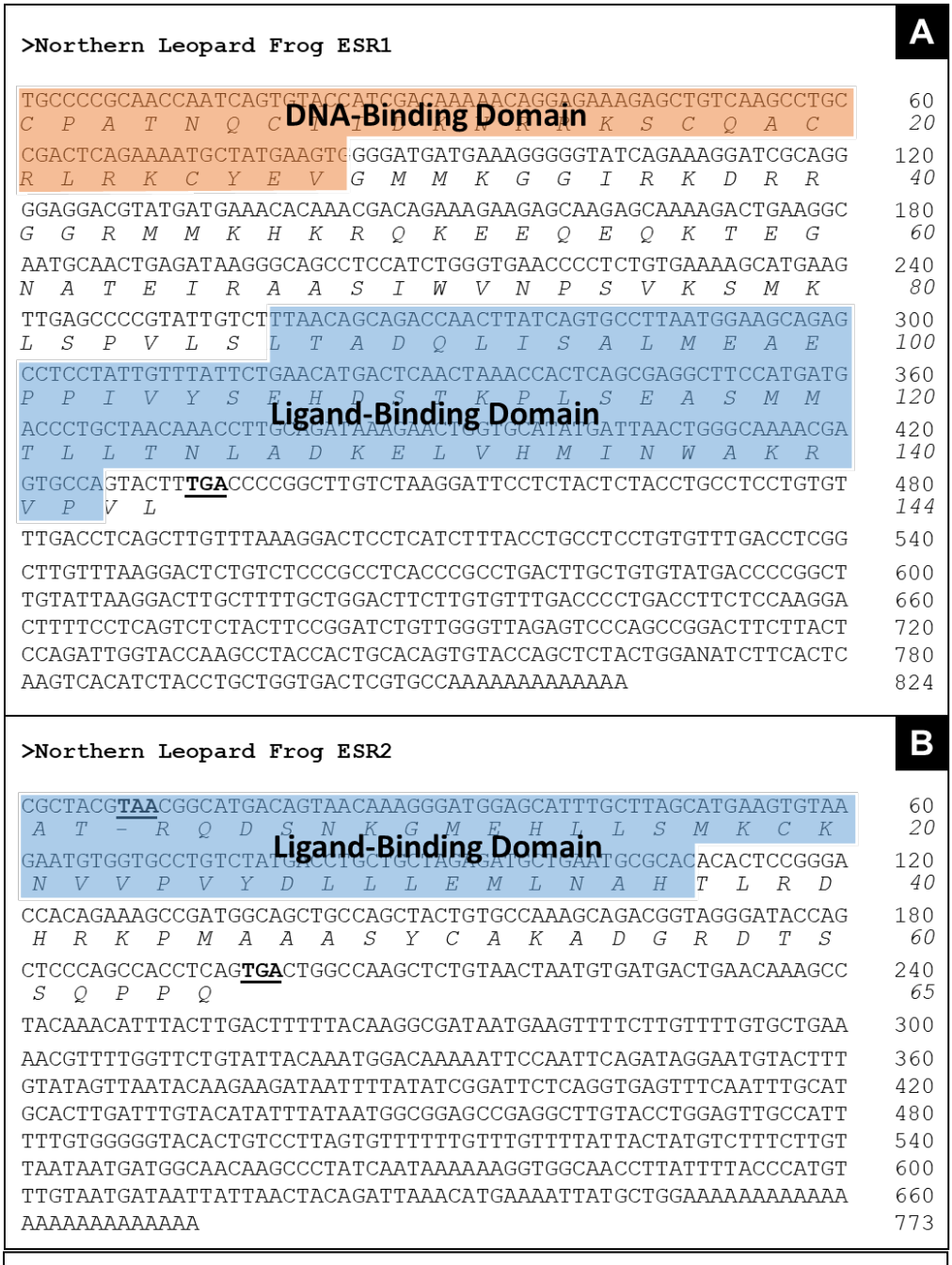


Figure 14. Cloned cDNA sequences for the Northern leopard frog ESR1 (left) and ESR2 (right). Italicized letters indicated deduced amino acid sequence. Start and stop codons are in bold text and underlined.

Deduced amino acid sequences of the Northern leopard frog ESR1 and ESR2 were 99% and 98% identical to those of the European common brown frog (*Rana temporaria*) in the NCBI database (Fig. 15). These ESR sequences of the European common brown frog are new, and they did not exist when we designed the PCR primers. This will help to design better PCR primers for cloning ESR2.

Figure 15. Results of Blast search on Northern leopard frog ESR1 (A) and ESR2 (B). The results confirmed that cloned cDNAs were Northern leopard frog ESRs.

In the remaining months of this study, we plan to clone these cDNAs into expression vectors. Once we obtained these clones, we will be able to use already filtered and archived stormwater samples in the bioassay to complete the study.

Final Report Summary June 30, 2022

To complete our study objectives, we developed a common cell line with cloned estrogen receptors for a range of aquatic species commonly found in Minnesota waters. These include three species of fish (fathead minnow, bluegill sunfish, bass), leopard frogs, and painted turtles. Despite the challenges of the COVID-19 pandemic (no, or only restricted access to our laboratories, severe shortage of needed supplies as our study used molecular supplies also needed for pandemic-related testing), we were able to identify and clone estrogen receptors for

these species. The purpose of this activity was to examine in a biological system to what degree waters taken from inflow and outflows from seven stormwater ponds would interact with these cells and affect their health and viability. The cell lines were then exposed to all 36 water samples (and 4 controls) to ascertain the effects of stormwater pollutants on cell viability (survival) and health. The type of stormwater filtration (traditional or iron-enhanced sand filtration [IESF]) affected cell viability in only have of the sampling events. When effects were observed, they were complex and at times counter-intuitive pattern. In spring 2018 and summer 2019, outflows from traditional stormwater ponds had better cell viability than outflows from IESF (Figure 10). In spring 2018, fall 2018, and summer 2019, cell viability was paradoxically better in stormwater inflow samples than in their paired outflows.

Cell health was also affected by treatment (inflow vs. outflow) and type of outflow (traditional vs. IESF). In summer 2019, the outflow of stormwater ponds was more estrogenic than the inflow as measured by the fathead minnow estrogen receptor, but not by any other species tested. Using IESF filtration, this estrogenic effect was removed suggesting that IESF filtration is effective in removing estrogenic pollutants. A similar effect was observed with exposed bluegill and bass cell lines in summer 2019. However, here IESF treatment did not improve estrogen removal over traditional filtration. Overall, fathead minnows were more sensitive to estrogenic pollutants in urban stormwater suggesting that observed negative effects in this important prey species could affect the population health of aquatic predators such as bluegill and bass. Despite the complexity of obtained results, this activity demonstrates the importance of assessing the full biological impact of stormwater in addition to the analysis of selected pollutants in stormwater.

IV. DISSEMINATION:

Description:

The target audience for results from this research will be professionals in the areas of stormwater 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. The regular meetings of the *Contaminant Roundtable* of MN State and federal agencies will provide another ready outlet for results from the current study. 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 whether iron-enhanced sand filtration in stormwater pond systems provide additional ecological protection.

First Update January 31, 2019

James Gerads, the graduate student on this project, presented a poster overviewing the site selection process at the *33rd Conference on the Environment* (November 7, 2018, Minneapolis, MN).

Second Update June 30, 2019

James Gerads gave an oral presentation about the progress of this project at the at the *82nd Annual Wastewater Operations Conference* (March 29, 2019, Brooklyn Park, MN).

Third Update January 31, 2020

James Gerads, a graduate student advancing the goals of this project, gave an oral presentation detailing the biological effects of the collected stormwater samples on larval fathead minnows at the *40th* annual meeting of the *Society for Environmental Toxicology and Chemistry* (November 2019, Toronto, CA).

James Gerads defended his Master's thesis entitled " The Effects of Urban Stormwater Runoff on Fathead Minnows " on October 14, 2019 in a public seminar at St. Cloud State University. In addition, he published his Master's thesis on the same topic in December 2019.

Fourth Update June 30, 2020

The cancellation of the Midwest annual meeting of the Society for Environmental Toxicology & Chemistry prevented a planned presentation of our preliminary data by Dr. Kohno.

Fifth Update January 31, 2021

S. Kohno, J. Gerads, and H.L. Schoenfuss. Iron-Enhanced Sand Filtration on Mitigation of Biological Active Contaminants in Urban Stormwater. SETAC North America 41st Annual Meeting, Fort Worth, TX (online), November 15-19, 2020.

Sixth Update June 30, 2021:

S. Kohno, J.E. Gerads, H.L. Schoenfuss, Mitigation of biological-active contaminants of emerging concern in urban stormwater utilizing Iron-enhanced sand filtration. 2021 Emerging Contaminants in the Environment Conference (ECEC21) Urbana, IL, USA (Online), April 27-28, 2021.

Seventh Update as of January 31, 2022:

S. Kohno, J.E. Gerads, H.L. Schoenfuss, The mitigating potential of retention ponds with iron-enhanced sand filtration (IESF) on urban stormwater. UCOWR/NIWR Virtual Conference, June 8-10, 2021

S. Kohno, J.E. Gerads, H.L. Schoenfuss, Iron-Enhanced Sand Filtration (IESF) on Contaminant Mitigation and Source of Contamination using Environmental DNA in Urban Stormwater. SETAC North America 42nd Annual Meeting, Portland, OR, USA (Virtual Conference), November 14-18, 2021.

Final Update June 30, 2022

No additional presentations occurred during this reporting period

Final Report Summary

Despite the challenges associated with the COVId-19 pandemic, our team was able to give seven presentations related to this study. These include presentations to natural resource managers in Minnesota, and to toxicologists at national and international scientific meetings. A St. Cloud State University graduate student completed a thesis on this project in 2020 which is currently being developed into a manuscript. Water chemistry data were integrated into a national USGS data base. Additional manuscripts are being prepared for future publication.

V. PROJECT BUDGET SUMMARY:

A. Preliminary ENRTF Budget Overview: See attached spreadsheet

Explanation of Use of Classified Staff: N/A

Total Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 1.5

Enter Total Estimated Personnel Hours: 1,716	Divide by 2,080 = TOTAL FTE: 0.83

Total Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 0.5 (USGS subcontract)

Enter Total Estimated Personnel Hours: 1040	Divide by 2,080 = TOTAL FTE: 0.5

B. Other Funds:

SOURCE OF AND USE OF OTHER FUNDS	Amount Proposed	Amount Spent	Status and Timeframe
Other Non-State $ To Be Applied To Project During Project Period:			
USGS Cooperative water fund support	$ 11,933	$0	Secured
USGS Cooperative water fund support	$23,866	$0	Secured
Other State $ To Be Applied To Project During Project Period: *N/A*			
	$	$	
Past and Current ENRTF Appropriation:			
Funding History: ML 2009, Chap.142, Sec. 2, Subd. 5b "Vulnerability of Lakes to Endocrine Disruption"	$297,000	$ 297,00	*completed*
Funding History: M.L. 2010, Chp. 362, Sec. 2, Subd. 5c "Ecological Impacts of Effluent in Surface Waters and Fish"	$340,000	340,00	*completed*
Funding History: M.L. 2010, Chp. 362, Sec. 2, Subd. 5e "Assessing Septic System Discharge to Lakes"	$594,500	594,500	*completed*
Funding History: M.L. 2014, Chp. 226, Sec 2, Subd. 03d "Evaluation of Wastewater Nitrogen and Estrogen Treatment Options" (Novak, PI - Activity 2: Schoenfuss $186,600)	$186,600	186,600	*completed*
Funding History: M.L. 2015, Chp. 76, Sec 2, Subd. 04c " Biological Consequences of Septic Pollution in Minnesota Lakes" (Schoenfuss, PI, Kiesling Co-PI)	$364,000	362,650	*completed*
Other Funding History:			
MN Pollution Control Agency (Pilot Study)	$ 60,689	$ 60,689	*Completed (09/30/2017)*

VI. PROJECT PARTNERS:

A. Partners receiving ENRTF funding

Name	Title	Affiliation	Role
Heiko L. Schoenfuss	Professor	St. Cloud State University	Principal Investigator
Satomi Kohno	Assistant Professor	St. Cloud State University	Oversight Activity 2

Name	Title	Affiliation	Role
Richard W. Kiesling	Hydrologist	US Geological Survey	Co-PI, Oversight Acivity 1

B. Partners NOT receiving ENRTF funding

Name	Title	Affiliation	Role
N/A			

VII. LONG-TERM-IMPLEMENTATION AND FUNDING:

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 MN aquatic 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 and M.L. 2014, Chp. 226, Sec 2, Subd. 03d). 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 identify other sources of CECs and determine their impact on receiving aquatic ecosystems. 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 stormwater discharge, improve treatment through best management practices, and safeguard our aquatic species.

VIII. REPORTING REQUIREMENTS:

- **The project is for 4 years, will begin on 07/01/2018, and end on 06/30/2022.**
- **Periodic project status update reports will be submitted 01/31 and 07/31 of each year.**
- **A final report and associated products will be submitted between June 30 and August 15, 2022.**

IX. SEE ADDITIONAL WORK PLAN COMPONENTS:

A. Budget Spreadsheet

B. Visual Component or Map *(embedded in Research Addendum)*

E. Research Addendum

Attachment A:
Environment and Natural Resources Trust Fund
M.L. 2018 Final Budget Spreadsheet

Project Title: Protect Water Quality with Efficient Removal of Contaminants in Treatment Ponds for Storm Water
Legal Citation: M.L. 2018, Chp. 214, Art. 4, Sec. 02, Subd. 04d
Project Manager: Heiko L. Schoenfuss
Organization: St. Cloud State University
College/Department/Division: Aquatic Toxicology Laboratory
M.L. 2018 ENRTF Appropriation: $325,000
Project Length and Completion Date: 4 years, June 30, 2022
Date of Report: August 12, 2022

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	TOTAL BUDGET 2/26/2020	TOTAL SPENT	TOTAL BALANCE
BUDGET ITEM			
Personnel (Wages and Benefits)	$112,505	$61,518	$50,987
Heiko L. Schoenfuss, Project Manager (74% salary, 26% benefits); 5% FTE per year for 3 years (total: $13,923)			
Satomi Kohno - Activity 2 oversight (62% salary, 30% fringe); 25% FTE year 1, 50% FTE years 2 & 3 (total: $98,582)			
Professional/Technical/Service Contracts			
Subcontract USGS (Mounds View, MN) Stormwater sampling and analysis coordinated by the US Geological Survey (Activity 1) including analytical services from the USGS National Water Quality Lab for the analysis of 40 samples (32 stormwater samples & 8 QA/QC samples) for three analytical schedules of approximately 400 chemicals ($112,000), expendable supplies ($2,300), shipping of samples to Nat'l Quality Lab ($2,800), communication costs with remote equipment ($520), and travel to collection sites ($1,680). Subcontract includes salary and benefits for project staff: (Kiesling - 79% salary, 21% benefits = $32,500; Sarah Elliott - 72% salary, 28% benefits = $12,000); GS-6 Hydrologic Technician - 68% salary, 32% benefits = $5800; Program Manager Mark Brigham- 79% salary, 21% benefits = $13,000; and Administrative Assistance 69% salary, 31% benefits = $7,400).	$190,000	$190,000	$0
Equipment/Tools/Supplies			
Preparation of receptor constructs, sequencing and cloning into expression vactor ($2,400), cloning kit, 5'RACE kit amd 3"RACE kit. Analysing biological activity of contaminants across six species for 40 stormwater samples ($19,595): $80/species x 6 species x 40 stormwaters.	$21,930	$16,982	$4,948
Travel expenses in Minnesota			
For sampling, visiting two stormwater BMPs four times/ year for 2 years according to the Commissioner's Plan = $565	$565	$563	$2
COLUMN TOTAL	**$325,000**	**$269,063**	**$55,937**