2017 Project Abstract
For the Period Ending June 30, 2021

PROJECT TITLE: Reassessing Toxicity of Petrochemical Spills on Groundwater and Surface Waters
PROJECT MANAGER: Dalma Martinovic-Weigelt
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2017, Chp. 96, Sec. 2, Subd. 04e

APPROPRIATION AMOUNT: $300,000
AMOUNT SPENT: $290,613
AMOUNT REMAINING: $9,387

Sound bite of Project Outcomes and Results

The groundwaters contaminated with chemicals from the decades-old crude oil spill and/or their breakdown products can adversely affect development and hormone and liver functioning if vertebrates were to be exposed to them sufficiently. This project advanced understanding of oil spill remediation and will help protect Minnesota's natural resources/drinking water sources.

Overall Project Outcome and Results

A fundamental issue in protecting ecosystem health in Minnesota is the degree to which waters impacted by, relatively common, petroleum releases (e.g., oil or gasoline spills) are toxic, both initially and over time as the oil breaks down into new chemicals. This study was the first to comprehensively screen the toxicity of groundwater from an aged crude oil spill site. The National Crude Oil Spill Fate and Natural Attenuation Research Site near Bemidji, MN is the site of a 1979 pipeline rupture that released 10,000 barrels of crude oil. This site has been extensively studied for over 40 years offering a unique opportunity to study the toxicity of groundwaters impacted by crude oil. Groundwater samples (collected 2016-2019) were analyzed for over 90 different chemical and toxicity parameters using cutting-edge techniques where living cells were exposed to water samples and screened for potential toxic effects. Analysis of the molecular/toxicity targets that were activated in cells indicated that (even 40+ years after the spill) the groundwaters contaminated with chemicals from the original spill and/or chemicals resulting from the breakdown of the oil compounds have the potential to cause adverse impacts on development, endocrine, and liver functioning if vertebrates (fish, turtles, birds, mammals) were to be exposed to them sufficiently. This work clearly shows the need to improve understanding of the identity and toxicity of oil breakdown products. Furthermore, this work shows that commonly used sampling and analysis methods (including sample extraction and clean-up protocols) can exclude or under-represent oil breakdown products and thus may underestimate risks from these chemicals. This finding is of importance to remediation managers and regulators in Minnesota and nationally because there is an active debate as to which methods and protocols are most suitable for hazard and risk assessment at petroleum spill sites.

Project Results Use and Dissemination

We published three research manuscripts, presented at numerous research conferences, and raised awareness of the issue with Minnesotans statewide (reached circa 1200 individuals at the State Fair exhibits). We introduced oil industry, and managers and regulators in MN and nationally to a new toolbox of novel cell and

artificial intelligence approaches that can streamline hazard assessment and facilitate identification of chemicals/hazards of concern and enhance oil spill remediation monitoring. Results of our work are relevant to Minnesotans as the analyses conducted herein advance an understanding of oil spill remediation and will help protect Minnesota's natural resources/drinking water sources.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2017 LCCMR Work Plan – Final Report

Date of Submission: Aug 15, 2021

Final Report

Date of Work Plan Approval: 06/07/2017

Project Completion Date: June 30 2021

PROJECT TITLE: Reassessing Toxicity of Petrochemical Spills on Groundwater and Surface Waters

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Location: Bemidji, MN

Total ENRTF Project Budget:	**ENRTF Appropriation:**	**$300,000**
	Amount Spent:	**$290,613**
	Balance:	**$9,387**

Legal Citation: M.L. 2017, Chp. 96, Sec. 2, Subd. 04e

Appropriation Language:
$300,000 the first year is from the trust fund to the commissioner of natural resources for an agreement with the University of St. Thomas to reassess long-term effects of oil spills through the analysis of chemical parameters related to oil degradation and evaluate the impacts on aquatic species, groundwater, and surface waters. This appropriation is available until June 30, 2021, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Reassessing Toxicity of Petrochemical Spills on Groundwater and Surface Waters

II. PROJECT STATEMENT:

Threat: Minnesota's water resources (and wildlife and fish that use those) are threatened by petroleum spills from leaking underground storage tanks, oil refineries, and spills from the transnational pipeline that crosses our state. For example, at this time MN Pollution Control Agency-Petroleum Remediation Program (MPCA-PRP) is monitoring more than 19,000 leaking tank sites. Many of the known chemical constituents of petroleum are very toxic to the fish and wildlife, and the toxicity of many of these constituents has not been evaluated.

Major Knowledge Gaps:

We do not know the chemical identity, quantity and toxicity of many chemicals present in petroleum-impacted groundwater and surface water, particularly the chemicals that result as the petroleum degrades over time (i.e., degradation products). **Past toxicity assessments of petroleum-impacted surface and groundwater are: 1. Incomplete** – because only a small subset of known chemicals have been assessed for toxicity, and **2. Inadequate** for identification of many sublethal effects (including those on endocrine, immune and nervous systems) – which are important determinants of organism's survival and population health.

Below we frame our contribution in a well-known risk assessment framework that relies on identification of knowns and unknowns**:**

1. *There are known knowns (these are things we know that we know)* - **this is what we already regulate for and what we monitor in MN.**
2. *There are known unknowns (there are things that we know we don't know)* – **we know that past toxicity assessment omitted analyses of many biologically important effects and propose to evaluate those** (e.g., endocrine, immune, neurotoxic effects).
3. *But there are also unknown unknowns (there are things we don't know we don't know)* – **new technologies allow us to look for unknown toxic chemicals and to detect toxicity in the whole samples that we could not have predicted based on our past knowledge.**

Opportunity: Over the past two years exciting new technologies emerged that will allow us to investigate the toxicity of petroleum-impacted waters **faster, cheaper, and far more completely.** We now have access to new technologies that allow us to analyze whole-water samples (waters containing both original petroleum compounds and degradation products) for over 90 toxicity indicators in a time- and cost-effective manner. Extensive toxicity data can be integrated with existing and new and cutting-edge water chemistry analyses to help us identify unknown pollutants of concern. Furthermore, integration of toxicity and chemistry data with indicators of natural attenuation processes can lead to **better understanding of effectiveness of natural attenuation.**

The work proposed here would improve our understanding of the lasting effects of oil spills on groundwater and associated surface water, and would allow regulators to better prioritize clean-up efforts to mitigate risk to ecological health given limited funds.

III. OVERALL PROJECT STATUS UPDATES:

Project Status as of *[Dec 31 2017]***:**

Historical chemistry data for the groundwater from the national crude oil research site located near Bemidji, MN were compiled and integrated with the hydrologic and redox condition data and analyzed. Based on these analyses sampling strategy was developed. Approximately thirty samples from the reference location and oil-impacted wells were collected, processed, and stored for the characterization of physical and biogeochemical properties, including dominant redox zonation and organic chemistry. Groundwater sample preparation method for high-throughput toxicity analyses was developed and optimized. Twenty five archived groundwater samples

from the Bemidji oil-spill site were prepared for the high-throughput toxicity analyses. Historical chemistry data for semi-volatile hydrocarbons (SVHCs) was integrated with the publically available toxicity data to predict biological targets of SVHCs, and to rank SVHCs based on their toxicity potential. Nuclear receptors (i.e., androgen, estrogen, retinoid X receptor b) were predicted to be the most sensitive biological targets. Four SVHCs were found in the oil-impacted groundwater at concentrations sufficient to activate above mentioned receptors. To facilitate knowledge exchange amongst industry, regulators, peer-researchers and consultants the investigators organized and led project-related scientific sessions, and presented project plans and preliminary findings at two North American scientific conferences. To facilitate idea exchange and to leverage resources, project findings and plans were also discussed in a research meeting (in Oct 2017) with the USGS, Chevron Energy Technology Company, and University of New Orleans representatives who are researching related topics at the Bemidji location.

Project Status as of *[June 30 2018]***:**
Chemistry of five groundwater samples was characterized for a variety of parameters including those used by the regulatory agencies (e.g., total petroleum hydrocarbons in the diesel range - TPHd). Ability of the groundwater samples (oil-impacted and unimpacted) to activate 48 human nuclear receptors and circa 40 biological pathways was also measured. Groundwater samples caused upregulation of several biological targets, including aryl hydrocarbon and estrogen receptor associated pathways. High levels of activation of molecular targets associated with toxicity pathways were observed in the oil-impacted samples. Activation of these biological targets is of concern because it has a potential to lead to adverse effects on endocrine and liver functioning. These findings were presented in April 2018 at the international Conference on Remediation of Chlorinated and Recalcitrant Compounds that is attended by industry, academia and regulators. Manuscript describing these data was submitted to peer-reviewed research journal, and is currently under review.

Project Status as of *[Dec 31 2018]***:**
A manuscript titled *"Toxicity Assessment of Groundwater Contaminated by Petroleum Hydrocarbons at a Well-Characterized, Aged, Crude Oil Release Site"* describing chemistry and biology findings for Bemidji site was published in peer-reviewed research journal (Environmental Science & Technology). Results show that contaminated groundwater stimulated several biological entities (including metabolic genes, and endocrine receptors -i.e., estrogen receptor), and that more contaminated samples stimulated biological targets more strongly. Our study of affected groundwater contaminated by a crude-oil release 39 years ago suggests that these types of waters may have the potential to cause adverse impacts on development, endocrine, and liver functioning if animals/humans were to be exposed to them sufficiently. Additionally, our work demonstrated a need for improvement of understanding of the toxicity associated with the unknown transformation products (chemicals formed during natural breakdown of the oil) present in hydrocarbon-impacted waters; they may be biologically active and/or toxic. Drs. Lai (UST) and Martinovic-Weigelt (UST) and students have been working on development of big data/machine learning approaches for predicting toxic effects (based on the chemical composition of the groundwater, and the molecular targets that groundwater affects).

We are particularly pleased to report that we had an opportunity to individually discuss this project and importance of protecting and studying ground water quality with well over 200 members of the public during the MN State Fair. This project was showcased for one whole day at one of the UST booths at the MN State Fair. Martinovic-Weigelt and four students discussed role of MN bogs in water quality (showcased a terrarium with mini bog), and citizens had an opportunity to observe groundwater contaminated with the oil 39 years ago. We also disseminated over 600 bookmarks we designed in collaboration with UST marketing and communication specialists; these bookmarks contained instructions for a fun, educational activity that explains how contaminants from the surface spills can move to the groundwater.

Project Status as of *[June 30 2019]***:**
To leverage existing ENTRF efforts on the present project we conducted a collaborative project with the colleagues from the USGS (Menlo Park, CA; Mounds View, MN and Reston, VA) and University of New Orleans. This collaboration resulted in coordinated collection and processing of the groundwater samples, which were then extracted/prepared for chemical and toxicity analyses using three distinct methodologies (one of which was deployed and published in 2018 as a part of the present ENTRF project). This collaborative project is of high importance to regulators as there is a debate which extraction method is most suitable for hazard and risk assessment. Preliminary data indicates that one of the commonly utilized sample extraction/preparation methods does not retain a subset of toxic chemicals, and thus is likely to underestimate risk associated with oil spills. In June 2019 we conducted additional sampling in Bemidji to test the potential of the portable system to quantify trace metals in complex, crude-oil contaminated samples. To facilitate idea exchange and to leverage resources, project findings and plans were also discussed in a research meeting with the representatives from the oil industry (e.g., Shell, BP, Chevron Energy Technology Company), and others (consultants, government – USEPA, USGS) who are researching related topics.

Project Status as of *[December 31 2019]***:**
Chemical and toxicity characterization of oil-impacted groundwater samples, prepared using three different processing methodologies, was completed. Our findings conclusively indicate that commonly deployed sample processing methods inadvertently remove a subset of toxic chemicals capable of activating biological targets associated with adverse health outcomes. This finding is of high importance to the US and MN regulators as there is a debate in those communities which analytical/extraction method is most suitable for hazard assessment of oil-contaminated samples. A research manuscript describing these data is in the final stages of preparation and will be submitted for review to a peer-reviewed research journal in Spring 2020. Major progress was also made on advancing big data/artificial intelligence approaches that will allow us to identify potential toxic effects for organisms based on the molecular screening data. A portion of this computational approach has been published as a peer-reviewed manuscript by Lai, Martinovic-Weigelt an UST collaborators. To facilitate idea exchange and to disseminate results of this project recent findings and future plans were discussed with professional audiences and MN public. Major dissemination efforts included: 1) McGuire (UST) was a featured speaker at the Technical Talks organized by Minnesota Section of The American Institute of Professional Geologists, 2) Martinovic-Weigelt, McGuire and seven UST students engaged in a daylong interaction with public at the MN State Fair. We distributed over 600 lollipop "water towers" and bookmarks that contained instructions for an educational activity (making a "pollution parfait"), which facilitates understanding of groundwater and effects of contamination on it. To raise awareness of the state support ENTRF logo was prominently featured on the bookmarks that were handed out.

Project Status as of *[June 30 2020]***:**

In vitro toxicity results indicated that risks associated with degradation intermediates of hydrocarbons in groundwater will be underestimated when protocols that remove these chemicals are used. This finding is of high importance to regulators as there is a debate which extraction method is most suitable for hazard and risk assessment. Manuscript (*Title: Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment?*) describing these findings was submitted to a premier research journal and has been accepted for publication (pending major revisions). Analysis of effects on estrogen, androgen, thyroid, PPAR receptors indicated that there are differences in sensitivity to groundwater toxicants (across different species). For estrogen receptors fish and turtles were least sensitive, but for thyroid – turtles were the most sensitive. Petroleum- impacted groundwater activated melanocortin 5, prostaglandin D and adrenergic Receptor – beta. These receptors play an important role in nervous, gastrointestinal and cardiovascular system function indicating a need to assess effects on these systems in fish.

Project extended to June 30, 2021 by LCCMR 7/17/20 due to COVID

AMENDMENT REQUEST July 23, 2020 We are requesting funds be shifted from the: 1) **Travel** budget line and 2) **Other** budget line to **Supplies line**. *Travel budget* line would be reduced by $3,163 to a revised budget of $2,837. *Other* budget line would be reduced by $3,000 to a revised budget of $0. Supplies budget would increase by $6,163.27 to a revised budget of $74,245 (original amount was $68,082). These changes are being requested because COVID-19 disrupted our plans to collect additional field samples (*Travel line*). Furthermore, since being funded, we have secured our own funding to cover computing time on Amazon supercomputer (*Other line*). Because we were able to gain access to additional water samples in April 2020 without going to the field (received archived samples from University of New Orleans collaborators) we will not need to return to the field. We plan to use *Travel* and o*ther* line funds to increase number of samples and fish experiments. Because of COVID-18 impact we requested that our project completion date be extended to June 30 2021, which would allow us to significantly strengthen in vivo data for this project. We have revised Outcome #2 in Activity 2 and Project Completion dates accordingly.

Amendment Approved by LCCMR 8/27/2020.

Overall Project Outcomes and Results:

A fundamental issue in protecting ecosystem health in Minnesota is the degree to which waters impacted by, relatively common, petroleum releases (e.g., oil or gasoline spills) are toxic, both initially and over time as the oil breaks down into new chemicals. This study was the first to comprehensively screen the toxicity of groundwater from an aged crude oil spill site. The National Crude Oil Spill Fate and Natural Attenuation Research Site near Bemidji, MN is the site of a 1979 pipeline rupture that released 10,000 barrels of crude oil. This site has been extensively studied for over 40 years offering a unique opportunity to study the toxicity of groundwaters impacted by crude oil. Groundwater samples (collected 2016-2019) were analyzed for over 90 different chemical and toxicity parameters using cutting-edge techniques where living cells were exposed to water samples and screened for potential toxic effects. Analysis of the molecular/toxicity targets that were activated in cells indicated that (even 40+ years after the spill) the groundwaters contaminated with chemicals from the original spill and/or chemicals resulting from the breakdown of the oil compounds have the potential to cause adverse impacts on development, endocrine, and liver functioning if vertebrates (fish, turtles, birds, mammals) were to be exposed to them sufficiently. This work clearly shows the need to improve understanding of the identity and toxicity of oil breakdown products. Furthermore, this work shows that commonly used sampling and analysis methods (including sample extraction and clean-up protocols) can exclude or under-represent oil breakdown products and thus may underestimate risks from these chemicals. This finding is of importance to remediation managers and regulators in Minnesota and nationally because there is an active debate as to which methods and protocols are most suitable for hazard and risk assessment at petroleum spill sites.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: More Completely Characterize The Chemistry Of Waters Impacted By Petroleum.
Description: Water samples will be collected from environments impacted by petroleum release and will include impacted groundwater, as well as water from a lake and wetland. We plan to analyze at least 12 sites for ~ 90 chemical parameters. We will analyze the current and historical water chemistry of these locations using well established methods within the PI's expertise as well as developing new techniques to quantify the extractable organic compounds within the real, "whole water" samples. To capitalize on more than 30 years of investments in data and infrastructure already made, we propose to complete this study at the national crude oil research site located near Bemidji, MN; however, this novel approach could be applied to any well-characterized site to improve our risk-based assessment and clean-up of petroleum impacted sites. We expect these data will

improve regulators ability to cost-effectively remediate sites by better understanding the use of Monitored Natural Attenuation at these common contaminated sites.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 139,060
Amount Spent: $ 133,235
Balance: $ 5,825

Outcome	Completion Date
1. Characterize the current and historical chemistry of contaminated water samples using existing and new analytical techniques. Water chemistry will include both in-situ field chemistry and laboratory analyses and measurements for circa 90 chemical parameters.	August 2019
2. Identify areas of greatest risk and communicate results to regulators (MPCA, MDH).	June 2020

Activity 1 Status as of *[Dec 31 2017]***:**
Outcome 1 - First, historical chemistry data (including measurements of non-volatile dissolved carbon and petroleum hydrocarbons) for the groundwater from the national crude oil research site located near Bemidji, MN were procured and compiled. Second, chemistry data was integrated with the hydrologic and redox condition data and analyzed to guide our groundwater sampling strategy. Twenty five sampling locations of interest were identified; these represent a range of organic chemistry, hydrologic and redox conditions, and a rich historical record of ancillary data. Locations located upgradient of the oil/contaminant plume were selected as reference samples. Twenty five samples from the oil-impacted wells were collected, processed, and stored for the characterization of physical and biogeochemical properties, including dominant redox zonation and organic chemistry.

Activity 1 Status as of *[June 30 2018]***:**
Outcome 1 – Five groundwater samples were analyzed for nonvolatile dissolved carbon, total petroleum hydrocarbons in the diesel range (TPHd), methane, and dissolved arsenic. In June 2018 team traveled to Bemidji and completed additional groundwater sampling. The samples were collected from the set of wells that several other groups (government, academia and industry) were investigating in order to leverage our research efforts with additional datasets, and thus maximize the completeness and impact of our findings.

Outcome 2 – During the field campaign our team exchanged information about our research plan with the representatives from the state and federal agencies and private sector (Chevron).

Activity 1 Status as of *[Dec 31 2018]***:**
Outcome 1 – Groundwater samples collected in summer 2018 have been extracted and prepared for chemistry analyses. A collaboration was established with U of New Orleans and USGS researchers (local office and Menlo Park, CA) in order to compare performance of different groundwater preparation/extraction methods (those mandated by current regulation vs. research based ones). Groundwater samples are being analyzed for nonvolatile dissolved carbon, total petroleum hydrocarbons in the diesel range (TPHd), methane, and dissolved arsenic. A manuscript titled *"Toxicity Assessment of Groundwater Contaminated by Petroleum Hydrocarbons at a Well-Characterized, Aged, Crude Oil Release Site"* describing chemistry and biology findings for Bemidji site was published in October 2018 in the selective and highly ranked peer-reviewed research journal (Environmental Science & Technology, 2018, 52, 21, 12172-12178).

Outcome 2 – During the field campaign, and in a series of follow-up calls and e-mail exchanges our team exchanged information about our research plan and results with the representatives from the county, state and federal agencies.

Activity 1 Status as of *[June 30 2019]***:**

Outcome 1 – Preliminary data indicates that one of the commonly utilized sample extraction/preparation methods does not retain a subset of toxic chemicals, and thus is likely to underestimate risk associate with oil spills. In June 2019 we conducted additional sampling in Bemidji to test the potential of a portable system (that utilizes voltammetry) to quantify trace metals in complex, crude-oil contaminated samples. Preliminary findings indicate that oil is interfering with the analyses and that sample pre-processing may be needed to get reliable quantitative estimates of trace metals. Groundwater samples from 2018 were analyzed for nonvolatile dissolved carbon and total petroleum hydrocarbons in the diesel range (TPHd).

Outcome 2 – During the field campaign, and in a series of follow-up calls and e-mail exchanges our team exchanged information about our research plan and results with the representatives from the county, state and federal agencies.

Activity 1 Status as of *[Dec 31 2019]***:**

Outcome 1: Concentration of non-volatile dissolved carbon and total petroleum hydrocarbon analyses were completed for all samples collected in 2018. McGuire lab is continuing development of methods for evaluation of metals in situ on 2019 samples.

Outcome 2: In December 2019 McGuire was a featured speaker at the Technical Talks organized by Minnesota Section of The American Institute of Professional Geologists. These talks are regularly attended by professional Geologists, licensed in the State of Minnesota, and attendance of these is a part licensing requirement. Dr. McGuire presented results of this ENTRF funded research project as a part of the technical talk.

Activity 1 Status as of *[June 30 2020]***:**

Outcome 1: Analyses of following chemicals were completed for all samples of interest: Cyclohexane, Benzene, Methylcyclohexane, Toluene, Ethylbenzene, p/m-xylene, o-xylene, 1,3,5-Trimethylbenzene; 1,2,3-trimethylbenzene; 1,2,4-trimethylbenzene; 1,2,4,5-tetramethylbenzene; 1,2,3,5-tetramethylbenzene; 1,2,3,4-tetramethylbenzene; Naphthalene; 2-methylnaphthalene; 1-methylnaphthalene; biphenyl; 2-ethylnaphthalene; 1-ethylnaphthalene; 1,5-dimethylnaphthalene; 2,6-dimethylnaphthalene and Acenaphthene. Reference groundwater samples did not have detectable levels of any of the above 22 analytes. Groundwater samples that had above average values for the above analytes were also some of the most toxic samples based on the Microtox test which evaluates general toxicity. This indicates that some of these chemicals may be responsible for general toxicity.

Outcome 2: No major outcomes to report. We submitted abstracts for presentations at two national and two regional conferences, but due to COVID-19 pandemic we could not travel and present the results of the work.

Activity 1 Status as of *[December 31, 2020]***:**

Outcome 1: No major outcomes to report.

Travel budget has been credited $487.71 since the last report. This change is due to covering a subset of the travel expenses with internal University of St. Thomas funds instead of ENTRF funds.

Outcome 2: A subset of chemical analyses (non-volatile dissolved organic carbon) published as a part of a research manuscript in Aug 2020. Further details about manuscript available under Dissemination. No other major outcomes to report.

Final Report Summary: [*August 15, 2021]***:**

Groundwater samples were collected at the National Crude Oil Spill Fate and Natural Attenuation Research Site near Bemidji, MN, U.S.A. In 1979 a pipeline rupture released 10,000 barrels of crude petroleum to the land surface and shallow subsurface and had been the site of active research on crude oil fate and transport ever since. This project focused on investigation of the groundwater contaminated by the "north oil body" that contains oil trapped in the vadose zone (a.k.a. underground water above the water table; saturations of 10−20%), and the zones with higher oil saturations of 30−65% at the water table, located 6−8 m below the surface. Due to the timing of the spill (circa 40 years ago) and extensive existing infrastructure, this site offered unique opportunity to study toxicity of groundwaters impacted by both, petroleum hydrocarbons and their breakdown products.

During the ENTRF project period we conducted comprehensive chemical and biogeochemical characterization of 25 groundwater samples (circa 90 (geo)chemical analyses). A subset of groundwater samples that represented different areas of the plume (including areas near oil source vs. those that are away, and those from the areas of differing biogeochemical conditions) were analyzed for nonvolatile dissolved carbon (NVDOC), total petroleum hydrocarbons in the diesel range (TPHd), methane, and dissolved arsenic on multiple occasions and using multiple sample preparation/extraction methods. Our chemical analyses indicate that despite widespread evidence of petroleum breakdown/degradation, legacy hydrocarbon groundwater contamination is still present at Bemidji site. Groundwater plume contains both hydrocarbons and degradation/breakdown products of hydrocarbons. We repeatedly established that the concentrations of both NVDOC and TPHd were highest near the oil source and decreased with distance from the oil source and in the direction of flow; the concentrations of NVDOCs stayed above background/reference site levels. It is notable that NVDOC concentrations were greater than three times the TPHd concentrations near the oil source and over 20 times higher beyond 150 m (towards the leading edge of the plume). These findings indicate that NVDOC analyses capture breakdown/degradation products of crude petroleum that are not measured in the TPHd analyses often used for risk assessment. Furthermore, our work indicates that certain water sample preparation methods (i.e., silica gel cleanup) reduce and/or remove transformation/breakdown products/chemicals and as such might underestimate risk. Analyses of 22 specific chemicals (semi-volatile hydrocarbons "SVHCs that included variety of chemicals indicative of petroleum spills) showed that reference samples ("control" groundwater samples collected within Bemidji site that were not impacted by the petroleum spill) did not have detectable levels of any of the above 22 analytes. Groundwater samples that had above average values for these analytes were also some of the most toxic samples based on the Microtox test which evaluates general toxicity.

ACTIVITY 2: Determine the Toxicity of Petroleum-Impacted Waters
Description: Samples will be analyzed for approximately 90 different toxicity types (including carcinogenesis, DNA damage, endocrine disruption, neurotoxicity) using cutting-edge techniques where living cells/proteins are exposed to "whole" water samples of interest and screened for changes in biological activity that are indicative of potential toxic effects. Unlike past approaches, these novel methods can quickly and efficiently screen samples for many toxicity responses and evaluate the potential of the complex environmental mixtures to pose health hazards. In addition, assays with aquatic organisms important to ecosystem function (e.g., bacteria/ *Daphnia sp*. and/or native fish) will be conducted. Direct assessments of impacts on aquatic organisms is important as petroleum products are a common water pollutant. The effects on the health of exposed organisms will be evaluated by measuring gene/metabolic responses that are important for maintenance of normal reproductive and metabolic function. Data will be disseminated to peer researchers, managers and entities involved in education (see Section V. *Dissemination* for details).

Summary Budget Information for Activity 2:

ENRTF Budget: $ 160,940
Amount Spent: $ 157,378
Balance: $ 3,562

Outcome	Completion Date
1. Analyze whole waters for 90 toxicity outcomes using high-throughput techniques.	August 2019
2. Characterize the resulting water chemistry, toxicity and hazard to native aquatic species (e.g., invertebrates/fish) using adverse outcome pathway framework and in vivo experiments with fish.	June 2021
3. Communicate findings to regulators (MPCA, MDH), peer researchers and consultants.	June 2020
4. Outreach activities via UST courses and extracurricular venues.	May 2020

Activity 2 Status as of *[Dec 31 2017]***:**

Outcome 1 - Groundwater sample preparation method for high-throughput analyses was developed and optimized; 25 archived groundwater samples from the Bemidji oil-spill site were processed for the high-throughput toxicity analyses, and will be analyzed for 90 toxicity outcomes. Groundwater samples were filtered, concentrated using solid-phase extraction, dried and re-suspended in a solvent that is suitable for the high-throughput toxicity analyses. In addition to this, a portion of each raw groundwater sample was frozen immediately to avoid a loss of volatile contaminants that could also be toxic. These raw samples will also be evaluated for toxicity using analyses that target nuclear receptors predicted to be affected by the volatile contaminants.

Outcome 2 - Historical chemistry data for 22 semi-volatile hydrocarbons ("SVHCs"; generated and provided by the USGS subcontractor) for the 25 groundwater sites selected in Activity 1 was integrated with the publically available toxicity data (ToxCast Dashboard; https://actor.epa.gov/dashboard/). Analysis of the integrated data was conducted to predict biological targets of SVHCs, and to rank SVHCs based on their toxicity potential. Nuclear receptors (i.e., androgen, estrogen, retinoid X receptor b) were predicted to be the most sensitive and the most likely biological targets of the SVHCs found in the oil-impacted groundwater at Bemidji site. Excessive activation of these nuclear receptors has been linked to a variety of adverse outcomes including endocrine disruption and reproductive impairment in fish, humans and wildlife. Of the 22 measured SVHC chemicals, the ones most likely to affect nuclear receptors were: 1,2,4,5-tetramethylbenzene, 1,2,4-trimethylbenzene, naphthalene, and 1,3,5 trimethylbenzene. These four chemicals were found in the oil-impacted groundwater at concentrations sufficient to activate above mentioned nuclear receptors.

Outcome 3 - To facilitate knowledge exchange amongst industry, regulators, peer-researchers and consultants investigators organized and co-chaired a session "Lingering Impacts of Oil and Fuel Spills – Fate and Toxicity of Persistent Hydrocarbons and Polar Metabolites" at Society of Environmental Toxicology and Chemistry (SETAC) – North America annual meeting. Our project plans and initial findings were also presented at this conference ("Evidence of potential toxicity of groundwater contaminated by a 1979 crude oil pipeline release"). SETAC is an international organization of professionals that promotes a tri-partite (academia, business and government) approach to solving environmental problems. Because 2017 conference was held in Minneapolis, it was accessible to state regulators and stakeholders, and was attended by a number of representatives from MN Department of Health and MN Pollution Control Agency. To facilitate idea exchange and to leverage resources, project findings and plans were also discussed in a research meeting (in Oct 2017) with the USGS, Chevron Energy Technology Company, and University of New Orleans representatives who are researching related topics at the Bemidji location.

Outcome 4 - A classroom lab activity about Bemidji oil-impacted groundwater has been developed and integrated in the undergraduate classroom curriculum at University of St. Thomas.

Activity 2 Status as of *[June 30 2018]***:**

Outcome 1 - Ability of the groundwater samples (collected from the oil-impacted and unimpacted sites) to impact 48 human nuclear receptors and circa 40 biological pathways was measured. The most highly impacted human nuclear receptors included estrogen (alpha and beta), peroxisome proliferator, and retinoic acid receptors. Aryl hydrocarbon receptor (AhR) associated pathway was also highly upregulated in the set of samples collected below the oil body and within the plume. AhR is typically involved in, but not limited to, regulation of biological responses to aromatic hydrocarbons and dioxins, and its activation can cause adverse effects in developing vertebrates. The highest effects were observed in the groundwater collected from beneath the oil body, then in those collected "downstream" from the oil body, and the lowest in the groundwater collected from the reference site (a nearby site not impacted by the oil spill). Activation of the above biological targets is of concern because it has a potential to lead to adverse effects on endocrine and liver functioning.

Outcome 2 – no major progress; effort was focused on the outcomes 1, 3 and 4.

Outcome 3 - The chemistry and biology findings for five groundwater samples were presented at the Battelle's Chlorinated Conference - one of the world's largest meetings on the application of innovative technologies and approaches for characterization, monitoring and management of chlorinated and complex sites. A manuscript describing these data was submitted to peer-reviewed research journal, and is currently under review.

Outcome 4 - A classroom lab activity about Bemidji oil-impacted groundwater has been developed and executed in the undergraduate classroom curriculum at University of St. Thomas. Students analyzed acute toxicity of samples and were made aware of this research project. We reached 26 undergraduate students via this activity. In addition, four undergraduate students participated in a variety of research activities associated with this project (sample preparation, chemical analyses, toxicity evaluation), and two attended the sampling campaign conducted in June 2018. Students had an opportunity to engage in the field research, and to interact with circa 25 researchers from all over USA working at this site.

Activity 2 Status as of *[Dec 31 2018]***:**

Outcome 1 - Groundwater samples collected in June 2018 have been extracted and prepared for toxicity analyses. We will evaluate how different sample preparation/extraction practices affect toxicity.

Outcome 2 – In order to predict toxicity and hazard to native aquatic species (e.g., invertebrates/fish) Drs. Lai (UST) and Martinovic-Weigelt (UST) and students have been working on development of big data/machine learning approaches that would allow us to enter identity of molecular targets (those affected by the groundwater samples), and use the publically available toxicity data (e.g., Comparative Toxicogenomics Database, National Library of Medicine databases and abstracts) to quickly identify potential toxic effects at organismal level. Major progress was made in development of these computational techniques, and the approach we developed was presented at the North American Meeting of the Society of Environmental Toxicology and Chemistry, Sacramento, CA.

Outcome 3 - A manuscript titled *"Toxicity Assessment of Groundwater Contaminated by Petroleum Hydrocarbons at a Well-Characterized, Aged, Crude Oil Release Site"* describing chemistry and biology findings for Bemidji site was published in October 2018 in the selective and highly ranked peer-reviewed research journal

(Environmental Science & Technology, 2018, 52, 21, 12172-12178). Our results expand the understanding of the potential toxicity of petroleum-impacted waters and demonstrate the need for additional data (not captured by current regulatory requirements for TPHd analyses) both in terms of the water chemistry (i.e., to include components of NVDOC not captured) as well as additional toxicological end points to evaluate the effectiveness of monitored natural attenuation as a remediation strategy for waters impacted by petroleum contamination.

Outcome 4 – We discussed this project and importance of protecting and studying ground water quality individually with well over 200 citizens during the MN State Fair. UST marketing team assisted with design of the bookmarks that contained instruction for an educational activity (making a "pollution parfait"), which facilitates understanding of groundwater and effects of contamination on it. These bookmarks were distributed to circa 600 attendees during the STEM Day at the annual MN State Fair.

Activity 2 Status as of *[June 30 2019]***:**

Outcome 1 - Groundwater samples collected in June 2018 have been extracted and preliminary toxicity analyses (identical to those conducted on 2016 samples) were conducted. Preliminary analyses confirmed that the types of the molecular targets affected (by the crude oil-impacted groundwater samples) were similar to those observed in 2016 samples; no new molecular targets were identified in 2018 samples. One of the three extraction methods tested yielded sample extracts that had very low/no toxicity; extraction method removed chemicals responsible for toxicity. Quantitative analyses of extraction method performance, and evaluation of the effects on the additional molecular targets are underway.

Outcome 2 – no major progress; effort was focused on the outcomes 1, 3 and 4.

Outcome 3 - To facilitate idea exchange and to leverage resources, project findings and plans were discussed on Feb 20th 2019 in a research meeting with the representatives from the oil industry (Chevron Energy Technology Company, Shell, BP, Exxon Mobil), consulting (Exponent), government (USGS, US EPA, USGS) and academia (U of New Orleans) who are researching related topics at the Bemidji location and elsewhere. Furthermore, the researchers and collaborators attended annual meeting in Bemidji (June 2019) where representatives of MN Pollution Control Agency and other parties involved in the research at the Bemidji site were present.

Outcome 4 – A classroom lab activity about Bemidji oil-impacted groundwater was integrated in the undergraduate classroom curriculum at University of St. Thomas (24 students were reached). One undergraduate student attended Bemidji field research in June 2019. Six St. Thomas undergraduate students conducted a variety of research activities associated with this project.

Activity 2 Status as of *[Dec 31 2019]***:**

Outcome 1 - Toxicity characterization of samples prepared using three different sample extraction methodologies (for chemical and toxicity analyses) was completed. A research manuscript describing the data is in the final stages of preparation and will be submitted for review to a peer-reviewed research journal in Spring 2020. The findings of this collaborative portion of the project indicate that commonly deployed extraction methods can remove a subset of toxic chemicals of concern. These chemicals are capable of inducing biological activity of molecular targets (aryl hydrocarbon receptor, estrogen receptor) and activation of which has been linked to adverse health outcomes in humans and fish. This finding is of high importance to regulators as there is a debate which extraction method is most suitable for hazard and risk assessment.

Outcome 2 - In order to predict toxicity and hazard to organisms Lai (UST) and Martinovic-Weigelt (UST) and other UST colleagues and students have been working on development of big data/machine learning approaches that would allow us to enter identity of molecular targets (those affected by the groundwater samples), and use the publically available toxicity data to quickly identify potential toxic effects at organismal level. Major progress was made in development of these computational techniques, and a portion of the approach Lai and others developed has been published as a peer-reviewed manuscript: Y. He, C. Lai, D. Martinović-Weigelt and Z. Long, "A Pipeline Approach in Identifying Important Input Features from Neural Networks," 2019 14th Annual Conference System of Systems Engineering (SoSE), Anchorage, AK, USA, 2019, pp. 25-30. doi: 10.1109/SYSOSE.2019.8753849

Outcome 3 – Please see the peer-reviewed publication listed under Outcome 2. In addition to that an abstract in collaboration with CA, MN and VA USGS co-authors titled "Comparison of Bioeffect Screening Results for Hydrocarbons and Hydrocarbon Oxidation Products" was submitted for a presentation at the Twelfth International Conference on Remediation of Chlorinated and Recalcitrant Compounds (May 31-June 4, 2020, Portland, Oregon). This conference is one of the world's largest meetings on the application of innovative technologies and approaches for characterization, monitoring and management of contaminated sites.

Outcome 4 – We used our University's regular presence at the MN State Fair to highlight this project. We discussed this project and importance of protecting and studying ground water quality individually with approximately 150 citizens during the MN State Fair. Attendees had an opportunity to learn about groundwater and McGuire (UST) designed a hands-on activity to improve understanding of the geology and hydrology. Martinovic-Weigelt, McGuire and UST students distributed over 600 lollipop "water towers" and bookmarks that contained instructions for an educational activity (making a "pollution parfait"), which facilitates understanding of groundwater and effects of contamination on it. To raise awareness of the funding source ENTRF logo was prominently featured on the bookmarks that were handed out. Six St. Thomas undergraduate students conducted a variety of research activities associated with this project throughout the summer and academic year.

Activity 2 Status as of *[June 30 2020]***:**

Outcome 1 - Toxicity characterization of samples prepared using three different sample extraction methodologies was completed. A research manuscript describing these data (*Title: Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment?*) was submitted for publication in a premier research journal Environmental Science & Technology. It was accepted for publication under condition that major revisions are completed. In vitro toxicity results indicated that risks associated with degradation intermediates of hydrocarbons in groundwater will be underestimated when protocols that remove these chemicals are used. This finding is of high importance to regulators as there is a debate which extraction method is most suitable for hazard and risk assessment. Additional toxicity analyses were performed, for a select subset of molecular targets (estrogen, androgen, thyroid, PPAR receptor), to determine whether toxicity responses differed across vertebrates (fish, frog, turtle, bird, mouse and human). For estrogen receptors fish and turtles were least sensitive. For thyroid – turtles were the most sensitive. None of the tested species had consistent upregulation of androgen receptor. For PPAR fish were less sensitive than mammals. Furthermore, we completed toxicity analyses for additional 24 molecular targets (including neurotransmitter, adrenergic, and prostaglandin receptors) that were not assessed before. We found that petroleum-impacted groundwater activated melanocortin 5, prostaglandin D and adrenergic Receptor – beta. These receptors play an important role in nervous, gastrointestinal and cardiovascular system function.

Outcome 2 - In order to predict hazard to organisms we identified Adverse Outcome Pathways associated with chemicals measured in Activity 1: Outcome 1. Following potential adverse outcomes were indicated: breast cancer, ovulation inhibition and reduced fertility, and hepatic steatosis – condition that can progress to serious

liver disease. Our in vitro toxicity (Activity 2, Outcome 1) analyses indicated that molecular targets involved in these adverse outcomes (estrogen receptor and pregnane X receptor) were routinely stimulated by the petroleum impacted groundwater samples collected at the Bemidji site. We also developed a series of new in vivo methods with fish in order to evaluate organismal-level effects associated with activation of molecular targets identified in Activity 2, Outcome 1. We focused on investigation of effects on fish behavior indicative of nervous system malfunction, and assessment of cardiovascular and gastrointestinal health. Because of COVID-19 interruption we plan to run additional experiments with these assays to strengthen the sample sizes for these organismal experiments.

Outcome 3 – Please see the peer-reviewed publication listed under Activity 2, Outcome 1. We submitted abstracts for presentations at two national and two regional conferences, but due to COVID-19 pandemic we could not travel and present the results of the work.

Outcome 4 – Four St. Thomas undergraduate students conducted a variety of research activities associated with this project, and 25 college students engaged in class-activities associated with this project in Spring semester of 2020.

Activity 2 Status as of *[December 31, 2021]***:**

Outcome 1- A research manuscript describing toxicity characterization of samples prepared using three different sample extraction methodologies was revised, resubmitted for publication and has been published: (Title: Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment) in a premier research journal Environmental Science & Technology. The paper was co-authored by ENTRF project team (Drs. Martinovic-Weigelt, McGuire Illig, Cozzarelli) https://doi.org/10.1021/acs.est.0c02220 in collaboration with other scientists (Drs. Bekins, Tillit, Brennan).

Distribution of spending in supplies category has been revised in the current budget report. The cost associated with sample dilution and preparation for high throughput assays (row 23 Activity 2 -$1,732) is now separated from that of the high throughput cell setup and assay runs (row 22 Activity 2 -$ 55,991).

Outcome 2 - We have made further progress on experiments that investigate effects on fish behavior – we revised equipment and software setup to generate better quality fish images. We generated additional data and will continue to do so to improve sample sizes – critical for endpoints like behavior that have large variation. We focused on investigation of effects indicative of nervous system malfunction during this reporting period.

Outcome 3 -To facilitate idea exchange and to leverage resources, ENTRF project findings and plans were discussed twice in December 2020. The first meeting included 34 scientists who conduct petroleum spill related research at the Bemidji site and included MN PCA representatives from the Remediation Division (Petroleum Remediation Section). Second research meeting was broader in terms of type of audience and discussion scope. It included representatives (and presenters) from the MN PCA, oil industry (including Principal Technical Expert Environmental Soil & Groundwater at Shell Energy Technology Company and scientists from Chevron that conducted research at Bemidji site), as well as consultants who work in the area of petroleum remediation (Exponent), and government (MN PCA, USGS) and academia (U of New Orleans, U of St. Thomas) representatives who are researching related topics nationwide. Martinovic-Weigelt presented overview of the work funded by the ENTRF and participated in a discussion. Present ENTRF funding allowed us to introduce this community to a new toolbox of in vitro and in silico (computational) approaches that can streamline hazard assessment process and facilitate identification of novel chemicals of concern, and remediation monitoring.

Outcome 4 – no major progress on this outcome.

Final Report Summary: *[August 15, 2021]***:**

The present study was the first to comprehensively screen biological activity of groundwater from an aged crude oil spill site. Groundwater samples were analyzed for 90+ different targets indicative of variety of toxicity types (including carcinogenesis, DNA damage, endocrine disruption, neurotoxicity) using cutting-edge techniques where living cells/proteins are exposed to water samples of interest and screened for changes in biological activity that are indicative of potential toxic effects. Our work demonstrates that these novel, cell-based toxicity assessment technologies can be used to: 1) investigate the effectiveness and progression of the natural attenuation in petroleum impacted environments (e.g., compare magnitude of toxicity across different sites and biogeochemical environments), 2) evaluate and compare utility of different sample preparation methods for the risk assessment, and 3) characterize types of toxicity mechanisms faster, cheaper, and more completely than traditional whole organism-based methods. Use of these technologies, combined with the chemistry data and publicly available chemical toxicity data allowed us to identify adverse outcomes that can be initiated by the exposure to waters that contain original petroleum compounds and/or their transformation products. Detailed descriptions and analyses of our findings were published in peer reviewed journals (please see dissemination section). Below we summarize the main findings of interest.

Toxicity analyses of groundwaters collected at Bemidji site - The most commonly activated nuclear receptors were: pregnane X receptor (PXR), peroxisome proliferator activated receptor alpha and gamma (PPARa and PPARg), estrogen receptor alpha and beta (ERa and ERB) and the retinoic acid receptor beta (RXRb). The background (a.k.a control) sample only activated PXR. Water collected from beneath the oil body was most potent/toxic and it stimulated ERa, ERb, PXR, PPARg, PPARa, and RXRb. Those same nuclear receptors were stimulated by the waters collected from downgradient area within the contaminant plume, but stimulation was of lower magnitude. Patterns of stimulation/toxicity were similar when using a different assay (CIS set); background sample mildly stimulated the PXR and aryl hydrocarbon receptor (AhR) pathways, whereas sample collected from beneath the oil body, activated the highest number of targets, and typically with the highest magnitude. Statistical analyses of these biological activity data revealed distinct toxicity profiles for samples collected beneath the oil body versus within the plume versus the background and spray zone wells. Notably, the AhR clustered away from all other genes and was highly upregulated in the set of samples collected below the oil body and within the plume.

Activation of the AhR and ERb observed in the present study is consistent with the findings generated by others who used more conventional assays; most other targets that we evaluated were not assessed by others. In most vertebrate species an induction of liver CYP enzymes (they facilitate metabolism of contaminants) through the AhR is a well-documented response to planar and aromatic organic chemicals found in petroleum. Both aromatic and polar fractions of the crude oil have been identified as sources of the chemicals that stimulate AhR.

We also completed toxicity analyses for additional 24 molecular targets (including neurotransmitter, adrenergic, and prostaglandin receptors). We found that petroleum-impacted groundwater activated melanocortin 5, prostaglandin D and adrenergic receptor beta. Excessive and/or inappropriately timed activation of these receptors could adversely affect nervous, gastrointestinal and cardiovascular system function, but further detailed animal studies are needed to understand whether the activation by Bemidji samples is sufficient to do so.

Additional toxicity analyses were performed, for a select subset of molecular targets (estrogen, androgen, thyroid, PPAR receptor), to determine whether toxicity responses differed across vertebrates (fish, frog, turtle, bird, mouse and human). For estrogen receptors fish and turtles were least sensitive. For thyroid – turtles were the most sensitive. None of the tested species had consistent upregulation of androgen receptor so sensitivity analysis was not possible for that receptor. For PPAR fish were less sensitive than mammals. This work indicates that species-specific sensitivity should be considered when conducting risk assessment.

Once we established which types of molecular biological targets are initiated by the petroleum impacted waters we conducted another series of experiments to determine whether degradation/breakdown products could be contributing to biological activity/toxicity. To do so we prepared three extracts of groundwater samples: 1) solid-phase extract (HLB); 2) dichloromethane (DCM-total) used in TPHd analyses; and 3) DCM extract with hydrocarbons isolated by silica gel cleanup (DCM-SGC). We established that the TPHd based sample preparation (DCM-total) captures only a fraction of degradation products, especially so if it involves silica gel cleanup (DCM-SGC). The aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) transcription factors showed the greatest upregulation by the extracts. HLB extracts were the most potent and exceed effects observed with DCM-total. No upregulation was observed by what is considered by some "hydrocarbon fraction" (DCM-SGC). These results indicate that the degradation/breakdown products from oil spills in groundwater have biological activity and can persist. Thus, sampling and analysis methods (including extraction and clean-up protocols) that exclude or under-represent the contribution of these intermediate degradation/breakdown products of oil may underestimate risks from these chemicals. This finding is of importance to regulators as there is a debate which extraction method is most suitable for hazard and risk assessment.

To further understanding of the hazard that activation of these molecular targets may cause we ran acute in vivo studies with a bacterium (*Vibrio fisheri*), Daphnia magna (waterflea) and fathead minnow (*Pimephales promelas)* larvae. *Vibrio fisheri* toxicity data was negatively correlated with the with the distance from the center of the oil body. Samples from the wells adjacent to the oil body had highest toxicity. Acute toxicity rapidly decreased to zero from 34 to 100 m from the center of the oil body. Daphnia survival assay indicated no significant mortality observed in 12-hour exposure test under 6.25, 12.5, 25, and 50% sample solution conditions. Fathead minnow survival, behavior and physiology experiments did not yield conclusive results because of insufficient survival across treatment groups. We propose that the likely cause of the insufficient survival across sites was ionic imbalance. Future experiments should conduct analyses with the water extracts that eliminate effect of the groundwater ionic composition. Such work was not possible this time as the volumes of the groundwater samples needed for such extractions and experiments would have required additional sampling season – this was not possible due to COVID-19 pandemic.

Biological targets of 22 semi-volatile hydrocarbons "SVHCs" were predicted, and SVHCs were ranked based on their toxicity potential. Nuclear receptors (i.e., androgen, estrogen, retinoid X receptor b) were predicted to be the most sensitive and the most likely biological targets of the SVHCs found in the oil-impacted groundwater at Bemidji site. Excessive activation of these nuclear receptors and/or their activation at inappropriate times has been linked to a variety of adverse outcomes including endocrine disruption and reproductive impairment in fish, humans and wildlife. Of the 22 measured SVHC chemicals, the ones predicted most likely to affect nuclear receptors at Bemidji site were: 1,2,4,5-tetramethylbenzene, 1,2,4-trimethylbenzene, naphthalene, and 1,3,5 trimethylbenzene. These four chemicals were found in the impacted groundwater at concentrations sufficient to activate above mentioned nuclear receptors. Following potential adverse outcomes were associated with SVHCs: reproductive organ cancer, ovulation inhibition and reduced fertility, and hepatic steatosis – condition that can progress to serious liver disease. Our biological, cell-based toxicity analyses indicated that molecular targets involved in these predicted adverse outcomes (estrogen receptor and pregnane X receptor) were routinely stimulated by the petroleum impacted groundwater samples collected at the Bemidji site.

Analysis of the molecular target activation experiments indicate that even after 40 years both, groundwaters sitting below oil and those away from it that are rich in transformation products, may have the potential to cause adverse impacts on development, endocrine, and liver functioning if animals/humans were to be exposed to them sufficiently. Present work demonstrates a need for improvement of understanding of the toxicity associated with the unknown transformation products (chemicals formed during natural breakdown of the oil) present in hydrocarbon-impacted waters; they may be biologically active and/or toxic and should be considered by the managers and regulators.

V. DISSEMINATION:

Description: Outreach and Dissemination of project data will be used 1) to present and publish findings for researchers in this field, 2) to share findings with regulatory state agencies (e.g., MDH, MPCA), and 3) private entities that facilitate/evaluate effectiveness of oil remediation projects (e.g., consultants). We will also use this data to enhance Minnesota's science, technology, engineering, and math (STEM) education programs via a) direct training of undergraduate research students, b) undergraduate classroom activities, and c) dissemination of educational materials through extracurricular routes (e.g., after-school programs etc.).

Status as of *[Dec 31 2017]***:**

We presented project plans and initial findings at two international professional conferences (The Geological Society of America 129th Annual Meeting, and SETAC North America's 38th Annual Meeting), we shared project plans and findings with the government and private entities that facilitate/evaluate effectiveness of oil remediation projects (e.g., USGS, Chevron). The project provided a direct training to four undergraduate research students, and has been integrated in the undergraduate classroom activities (expected reach: 50 students annually).

Status as of *[June 30 2018]***:**

The data for five groundwater samples was presented at the Battelle's Chlorinated Conference – one of the world's largest meetings on the application of innovative technologies and approaches for characterization, monitoring and management of chlorinated and complex sites. Circa 1600 professionals from academia, state and federal government agencies, consulting firms, research organizations, and industries from around the world are represented at this conference. A manuscript describing these data was submitted to peer-reviewed research journal, and is currently under review. Circa 30 undergraduate students were reached via classroom and undergraduate research opportunities.

Status as of *[Dec 31 2018]***:**

A manuscript describing chemistry and biology findings for five groundwater samples these data was published in October 2018 in the selective and highly ranked peer-reviewed research journal (Environmental Science & Technology, 2018, 52, 21, 12172-12178). Computational approach that allows for predicting diseases based on the molecular effects data was presented at the North American Meeting of the Society of Environmental Toxicology and Chemistry (circa 2,200 attendees representing industry, government and academia) in Sacramento, CA. We discussed this project and importance of protecting and studying ground water quality individually with well over 200 citizens during the MN State Fair. Bookmarks that contained instruction for an educational activity (that facilitates understanding of groundwater and effects of contamination on it) were distributed to circa 600 attendees during the STEM Day at the MN State Fair.

Status as of *[June 30 2019]***:**

In February 2019 we shared project plans and findings with the industry, government and private entities that facilitate/evaluate effectiveness of oil remediation projects. The project provided a direct training to six undergraduate research students, and has been integrated in the undergraduate classroom activities (reach- 24 students).

Status as of *[Dec 31 2019]***:**

Project generated a peer-reviewed manuscript: Y. He, C. Lai, D. Martinović-Weigelt and Z. Long, "A Pipeline Approach in Identifying Important Input Features from Neural Networks," 2019 14th Annual Conference System of Systems Engineering (SoSE), Anchorage, AK, USA, 2019, pp. 25-30. Doi: 10.1109/SYSOSE.2019.8753849. To facilitate idea exchange and to disseminate results of this project recent findings and future plans were also discussed with professional audiences and MN public. Major dissemination efforts included: 1) McGuire (UST) was a featured speaker at the Technical Talks organized by Minnesota Section of The American Institute of

Professional Geologists, 2) Martinovic-Weigelt, McGuire and seven UST students engaged in a daylong interaction with public at the MN State Fair (circa 600 individuals reached).

Status as of *[June 30 2020]***:**
A research manuscript describing these data (*Title: Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment?*) was accepted for publication in a premier research journal Environmental Science & Technology (under condition that major revisions are completed). We submitted abstracts for presentations at two national and two regional conferences, but due to COVID-19 pandemic conferences were canceled and/or we could not travel and present the results of the work.

Status as of *[December 31, 2020]***:**
One research manuscript was revised, resubmitted and has been published in a in a premier research journal Environmental Science & Technology: https://doi.org/10.1021/acs.est.0c02220

In Dec 2020 Martinovic-Weigelt presented overview of the work funded by the ENTRF and participated in a research planning and discussion that included participants from industry (Shell, Chevron scientists), MN Pollution Agency, USGS (regional and National groups present) and other academics. Martinovic-Weigelt introduced this community that typically relies on the chemistry analysis and traditional in vivo testing to a new toolbox of in vitro and in silico (computational) approaches that can streamline hazard assessment process and facilitate identification of novel chemicals and/or hazards of concern, and enhance remediation monitoring.

Final Report Summary: *[August 15, 2021]***:**

Five types of dissemination activities were performed:

Publication of research manuscripts:

A manuscript titled *Toxicity Assessment of Groundwater Contaminated by Petroleum Hydrocarbons at a Well-Characterized, Aged, Crude Oil Release Site* describing chemistry and biology findings for Bemidji site was published in October 2018 in the selective and highly ranked peer-reviewed research journal Environmental Science & Technology.

The computational approach that allows for predicting diseases based on the molecular effects data was presented at the North American Meeting of the Society of Environmental Toxicology and Chemistry (circa 2,200 attendees representing industry, government and academia) in Sacramento, CA. This part of the project also generated a peer-reviewed manuscript titled "A Pipeline Approach in Identifying Important Input Features from Neural Networks," 2019 14th Annual Conference System of Systems Engineering (SoSE), Anchorage, AK, USA, 2019, pp. 25-30. The paper was authored by ENTRF project team (Drs. Martinovic-Weigelt and Lai) in collaboration with other University of St. Thomas scientists.

In 2020 a research manuscript describing toxicity characterization of samples prepared using three different sample extraction methodologies was published in a premier research journal Environmental Science & Technology. *Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment*. The paper was co-authored by ENTRF project team (Drs. Martinovic-Weigelt, McGuire Illig, Cozzarelli) in collaboration with USGS scientists (Drs. Bekins, Tillit, Brennan).

In addition to the above, our work was disseminated by the project investigators and students at regional/national/international research conferences multiple times each year. The notable examples included

presentations at the Geological Society of America Annual meetings, and North American Society of Environmental Toxicology and Chemistry meetings. These societies serve variety of scientists form academia, government and industry and capture a variety of disciplines and interdisciplines (geologists, biologists, chemists, environmental scientists, toxicologists). We also presented ENTRF-funded work at highly regarded specialized conferences such as Battelle's Chlorinated Conference – one of the world's largest meetings on the application of innovative technologies and approaches for characterization, monitoring and management of chlorinated and complex sites. Circa 1600 professionals from academia, state and federal government agencies, consulting firms, research organizations, and industries from around the world are represented at this conference.

We also participated in annual presentations and discussions with stakeholders, regulators who have deep familiarity with this site and petroleum spill remediation and regulation, including MN PCA. We regularly presented overview of the work funded by the ENTRF and participated in a research planning and discussion that included participants (circa 35 individuals) from oil industry (Shell, Chevron scientists and managers), MN Pollution Agency, USGS (regional and national groups present), consultants who work in petroleum remediation (e.g., Exponent), and other academics who do similar research or research Bemidji site. As a result of ENTRF funding, we introduced this community - that typically relies on chemistry analysis and traditional *in vivo* testing - to a new toolbox of *in vitro* (cell-based) and *in silico* (computational) approaches that can streamline hazard assessment process and facilitate identification of novel chemicals and/or hazards of concern and enhance remediation monitoring.

Circa 50 undergraduate students were reached annually via classroom and undergraduate research opportunities. Six undergraduates were deeply involved in this project and attended field research campaigns and assisted with the experiments. One undergraduate student contributed impactful data that was published with that student as a co-author.

We used our University's regular presence at the MN State Fair (in 2018 and 2019) to highlight this project at two day-long events that were a part of the STEM Day at the MN State Fair. Our student-faculty teams discussed this project and importance of protecting and studying ground water quality individually with approximately 400 citizens during the MN State Fair. Attendees had an opportunity to learn about groundwater and engage with a hands-on activity that builds understanding of the geology, hydrology and oil spills. Furthermore, the attendees learned about northern MN bogs and had an opportunity to see a live model of a bog habitat and learn about unique biology and hydrology of bogs. Martinovic-Weigelt, McGuire and UST students also designed and distributed over 1200 lollipop "water towers" and bookmarks that contained instructions for an educational at home activity (making a "pollution parfait"), which facilitates understanding of groundwater and effects of contamination on it. To raise awareness of this project and the funding source, the ENTRF logo was prominently featured on the bookmarks that were handed out.

VI. PROJECT BUDGET SUMMARY:

A. Preliminary ENRTF Budget Overview:

***This section represents an overview of the preliminary budget at the start of the project. It will be reconciled with actual expenditures at the time of the final report.**

- Please see attached budget spreadsheet.

Explanation of Use of Classified Staff: N/A

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

Total Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 2.64 FTE

Total Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 0 FTE

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
US Geological Survey	$54,756	$0	In kind support - Dr. Cozzarelli, US Geological Survey - 1 month per year of her salary for 3 years ($16252 per year, $48,756 total) and $2000 per year in field travel funds (total $6000).
University of St. Thomas	$123,000	$0	Indirect costs to University of St. Thomas not recouped (including field and lab equipment, boats, vehicles and miscellaneous supplies).
State	$0	$0	N/A
TOTAL OTHER FUNDS:	$177,756	$0	

VII. PROJECT STRATEGY:

A. Project Partners:

Partners receiving ENRTF funding:

University of St. Thomas, ENTRF funds: $253,000

Dalma Martinovic-Weigelt, Ph.D., Project Manager – Responsible for managing and coordinating overall project, high throughput assay assessment, analyses and interpretation, compiling reports and disseminating results.

Jennifer McGuire, Ph.D., Biogeochemist - Responsible for coordinating and conducting field experiments & chemistry analyses, and compiling reports and disseminating results.

Chih Lai, Ph.D. - Bioinformatician and Data Analyst – Responsible for acquisition/analyses of data from on-line databases, and for statistical integration of project data (field toxicology and chemistry data) with publically available toxicity data.

Mike Axtell, Ph.D., Mathematician – Responsible for predictive mathematical modeling of chemical mixtures.

Two summer and two academic year undergraduate research assistants (to be determined), assist with field and laboratory data generation.

U.S. Geological Survey, ENTRF funds: $47,000

Isabelle Cozzarelli, Ph.D., Chemist- Responsible for conducting field research and chemistry analyses.

Partners NOT receiving ENRTF funding

N/A

B. Project Impact and Long-term Strategy:

Data collected will improve understanding of the longer term effects of oil spills on ecological and human health. The findings of this project will inform: 1) the decisions about use of natural attenuation for remediation of similar sites, 2) monitoring design, and 3) prioritization of sites, site zones and chemical constituents for remedial action. Overall, the approaches and results presented herein will lead to more focused and informed

remediation planning by regulatory agencies, such as the Minnesota Pollution Control Agency and Minnesota Department of Health, which are tasked with managing contaminated sites safely.

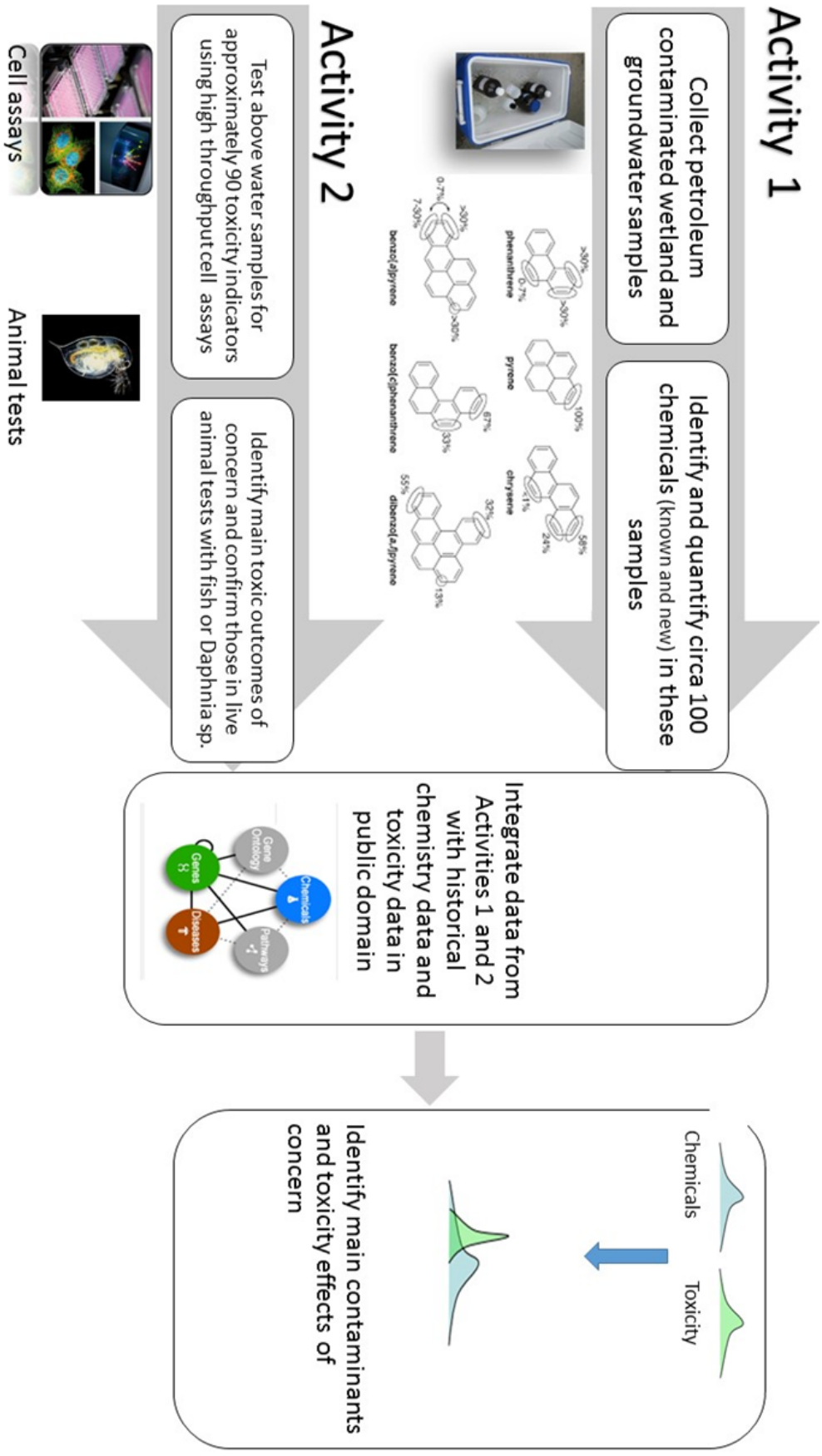
C. Funding History:

Funding Source and Use of Funds	Funding Timeframe	$ Amount
National Crude Oil Spill Fate and Natural Attenuation Research Site, a collaborative venture of the USGS, Enbridge Energy, Limited Partnership, the Minnesota Pollution Control agency, and Beltrami County: Evaluating oil spill toxicity to improve water remediation II. ($11,949). Mc-Guire (PI) Martinovic-Weigelt (co-PI) - project to be completed by July 1 2017.	05/16-08/17	$11,949
National Crude Oil Spill Fate and Natural Attenuation Research Site, a collaborative venture of the USGS, Enbridge Energy, Limited Partnership, the Minnesota Pollution Control agency, and Beltrami County: Evaluating oil spill toxicity to improve water remediation. ($14,517) Mc-Guire (PI) Martinovic-Weigelt (co-PI) - project not active; funds exhausted and project completed.	05/15-08/16	$14,517

VIII. REPORTING REQUIREMENTS:

- **The project is for 4 years, will begin on 07/01/2017, and end on 06/30/2021.**
- **Periodic project status update reports will be submitted *[06/30]* and *[12/31]* of each year.**
- **A final report and associated products will be submitted between June 30 and August 15, 2021**.

IX. VISUAL COMPONENT or MAP(S):



Environment and Natural Resources Trust Fund

M.L. 2017 Project Budget

Project Title: Reassessing Toxicity of Petrochemical Spills on Groundwater and Surface Waters
Legal Citation: M.L. 2017, Chp. 96, Sec. 2, Subd. 04e
Project Manager: *Dalma Martinović-Weigelt*
Organization: *University of St. Thomas (UST)*
M.L. 2017 ENRTF Appropriation: $ *300,000*
Project Length and Completion Date: 4 *Years, June 30, 2021*
Date of Report: Aug 15 2021 - Final Report

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Revised Activity 1 Budget
BUDGET ITEM	***Chemistry of***
Personnel (Wages and Benefits)	$ 78,723
Dr. Dalma Martinovic-Weigelt, Project Manager, Toxicologist, UST: $44,688 (93% salary, 7% benefits); 8.33% FTE each year for two years; 21.41% FTE for one year	
Dr. Jennifer McGuire, Biogeochemist, UST: $63,589 (93% salary, 7% benefits); 16.67% FTE each year for 3 years	
Dr. Chih Lai, Bioinformatician and Data Analyst, UST: $31,847 (93% salary, 7% benefits); 8.33% FTE each year for 2 years, 4.17% FTE for 1 year	
Dr. Mike Axtell, Mathematician, UST: $5,526 (93% salary, 7% benefits); 4.99% FTE for 1 year	
2 undergraduate Academic Year Research Assistants, UST: $9,600 (100% salary, 0% benefits); each student @ 8.83% FTE each year for 3 years	
2 undergraduate Summer Research Assistants, UST: $20,669 (93% salary, 7% benefits); each student @ 16.67% FTE each year for 3 years	
Professional/Technical/Service Contracts	$ 47,000
US Geological Survey - Analytical chemistry service - 1440 chemical analyses at $32.63 each totaling $47,000 (i.e., 60 analytes at a minimum of 12 site locations in duplicate) will be conducted by USGS laboratories supervised by Dr. Isabelle Cozzarelli.	
Equipment/Tools/Supplies	
High throughput toxicity assay supplies, assay runs and assay setup - 9000 analyses at $5.56 each totaling $50,000 (i.e., 50 samples tested in duplicate for 90 toxicity parameters)	
Miscellaneous lab supplies - totaling $18,082 - capillaries, reagents, filters, buffers, sample processing supplies (disposable plastic sampling containers, pipette tips, chemicals, extraction columns), animal microcosm setups, and microbiology supplies.	$ 10,500
Travel expenses in Minnesota	
Travel for project staff from St. Paul, MN to Bemidji, MN to conduct field sampling/experiments, 1 week field campaign - team of 4 x 5 days x $100 (cost of daily lodging and food) x 3 years = $6,000	$ 2,837
Other - Computing time on a supercomputer/server	$ -
COLUMN TOTAL	**$ 139,060**

Amount Spent	Activity 1 Balance	Revised Activity 2 Budget	Amount Spent	Activity 2 Balance	TOTAL BUDGET
petroleum-impacted waters		***Toxicity of petroleum-impacted waters***			
$ 77,512	$ 1,211	$ 97,195	$ 98,324	$ (1,129)	$ 175,918
				$ -	$ -
				$ -	$ -
				$ -	$ -
				$ -	$ -
				$ -	$ -
				$ -	$ -
$ 47,000	$ -	$ -	$ -	$ -	$ 47,000
				$ -	$ -
				$ -	$ -
$ -	$ -	$ 56,163	$ 55,991	$ 172	$ 56,163
$ 6,374	$ 4,126	$ 7,582	$ 3,064	$ 4,518	$ 18,082
				$ -	$ -
$ 2,349	$ 488	$ -	$ -	$ -	$ 2,837
$ -	$ -	$ -	$ -	$ -	$ -
				$ -	$ -
$ 133,235	**$ 5,825**	**$ 160,940**	**$ 157,378**	**$ 3,562**	**$ 300,000**

ENVIRONMENT
AND NATURAL RESOURCES
TRUST FUND

TOTAL SPENT	TOTAL BALANCE
$ 175,835	$ 83
$ -	$ -
$ -	$ -
$ -	$ -
$ -	$ -
$ -	$ -
$ -	$ -
$ 47,000	$ -
$ -	$ -
$ -	$ -
$ 55,991	$ 172
$ 9,438	$ 8,644
$ -	$ -
$ 2,349	$ 488
$ -	$ -
$ -	$ -
$ 290,613	**$ 9,387**