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.

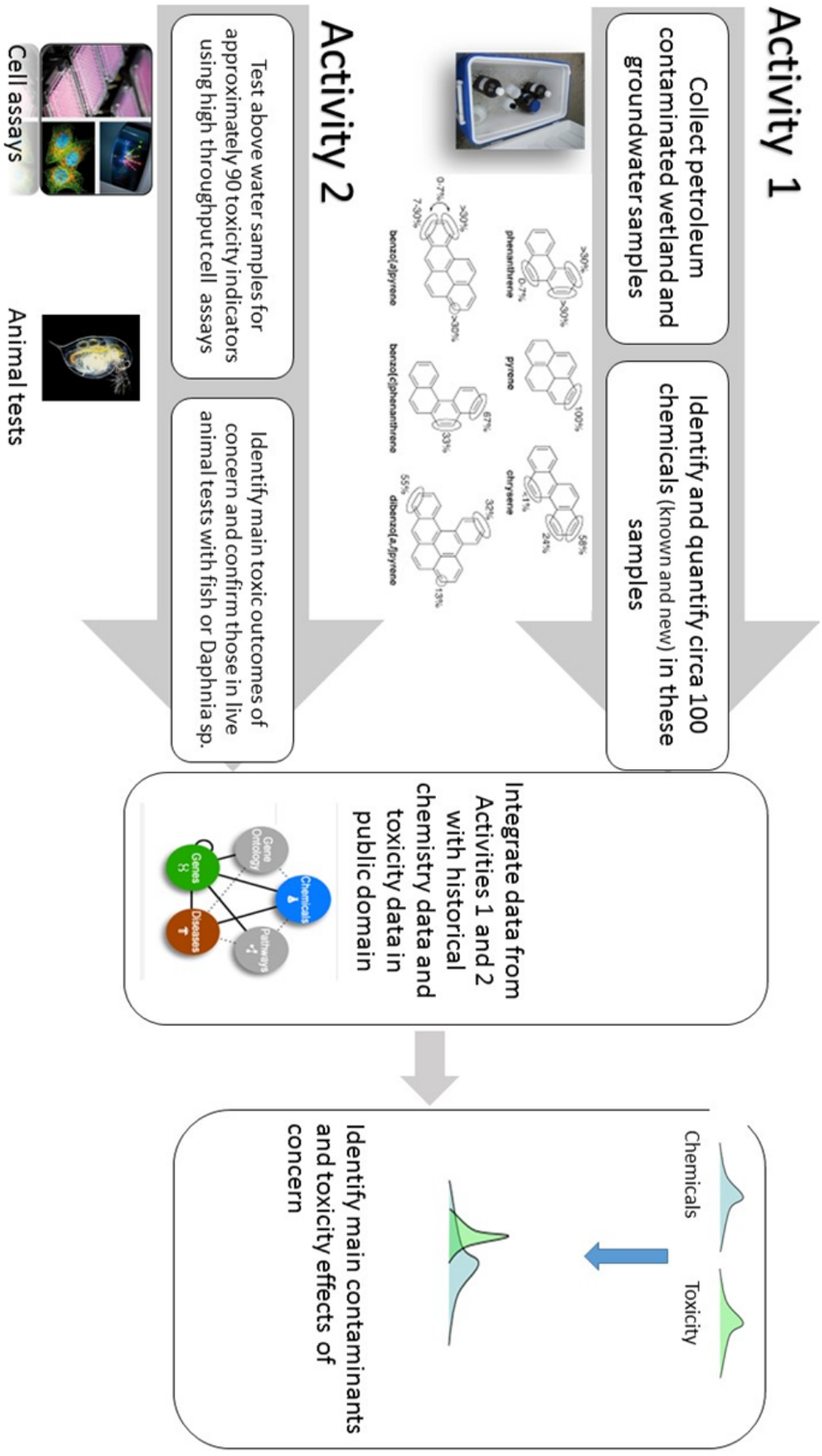
C. Funding History:

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**

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Article

Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment?

Barbara A. Bekins,* Jennifer C. Brennan, Donald E. Tillitt, Isabelle M. Cozzarelli, Jennifer McGuire Illig, and Dalma Martinović-Weigelt

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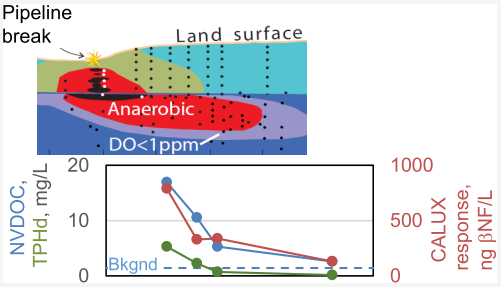
ABSTRACT: In crude oil contaminant plumes, the dissolved organic carbon (DOC) is mainly hydrocarbon degradation intermediates only partly quantified by the diesel range total petroleum hydrocarbon (TPHd) method. To understand potential biological effects of degradation intermediates, we tested three fractions of DOC: (1) solid-phase extract (HLB); (2) dichloromethane (DCM-total) extract used in TPHd; and (3) DCM extract with hydrocarbons isolated by silica gel cleanup (DCM-SGC). Bioactivity of extracts from five wells spanning a range of DOC was tested using an *in vitro* multiplex reporter system that evaluates modulation of the activity of 46 transcription factors; extracts were evaluated at concentrations equivalent to the well water samples. The aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) transcription factors showed the greatest upregulation, with HLB exceeding DCM-total, and no upregulation in the hydrocarbon fraction (DCM-SGC). The HLB extracts were further studied with HepG2 chemically activated luciferase expression (CALUX) *in vitro* assays at nine concentrations ranging from 40 to 0.01 times the well water concentrations. Responses decreased with distance from the source but were still present at two wells without detectable hydrocarbons. Thus, our *in vitro* assay results indicate that risks associated with degradation intermediates of hydrocarbons in groundwater will be underestimated when protocols that remove these chemicals are employed.



INTRODUCTION

Monitored natural attenuation (MNA) has been accepted as a groundwater clean-up strategy for spills of petroleum hydrocarbons for the past 25 years.[1] The effectiveness of MNA for petroleum hydrocarbons was established using laboratory studies of single compounds, field observations, and database compilations of plume lengths.[1] Despite widespread evidence of biodegradation, legacy hydrocarbon groundwater contamination was still present at 126 000 sites in the United States in 2013.[2] Groundwater plumes at hydrocarbon spill sites contain both hydrocarbons and intermediate degradation products of hydrocarbons.[3−13] Many of the degradation intermediates are polar compounds comprising different chemical classes than the parent hydrocarbons and consequently they have different fate, transport, and toxicity properties.[14]

Potential hazards and/or risks posed by degradation intermediates of petroleum hydrocarbons to human health and ecological receptor communities have been the focus of several studies.[15−20] Some conclude that the risk is low;[15−17] but a number of publications present evidence that the risks of petroleum hydrocarbon intermediate degradation products to humans or ecological receptors may be greater than anticipated and in need of further characterization.[20−23] For example, two studies of bioremediation of polyaromatic hydrocarbons (PAHs) found greater toxicity associated with hydroxylated and carboxylated transformation products than with the parent PAHs.[18,19] One aspect of the issue concerns regulatory policy on the appropriate use of analytical methods to quantify total petroleum hydrocarbons (TPH) in water.[14,24] Some methods include degradation intermediates and other methods exclude these classes from quantifications and exposure assessments.

The analytical method for the aqueous concentration of TPH in the diesel range (TPHd) involves the extraction of water with a liquid solvent, typically dichloromethane (DCM) or hexane.[25] The extract is then analyzed by gas chromatography with flame ionization detection to quantify compounds that elute between the *n*-decane (*n*-C10) peak and the conclusion of the *n*-octacosane (*n*-C28) peak. Measured concentrations of TPHd reflect not only hydrocarbons but also polar compounds including some hydrocarbon degradation intermediates and natural organic matter.[8] Degradation intermediates and dissolved natural organic matter can be

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removed from the TPHd extract using silica gel cleanup (SGC), thereby isolating the hydrocarbon fraction. Currently, state regulators generally agree on risk calculation methods and approaches for aromatic and aliphatic hydrocarbons.[24,26] In contrast, approaches for risk calculations of degradation intermediates on human health are poorly constrained and controversial.[24] About half the states in the USA polled in a recent state survey responded that they do not allow removal of degradation intermediates using SGC prior to TPHd analyses.[24] The remaining states varied in their responses but typically require both SGC and non-SGC TPHd concentrations for exposure and risk evaluations.

Both methods described above (with and without SGC) are typically based on DCM extracts of groundwater samples. However, recent publications have demonstrated that the DCM extract used to quantify TPHd recovered less than half of the nonvolatile dissolved organic carbon (NVDOC) present in a crude oil plume[22,27] and a refined fuel spill.[28] The NVDOC contains the degradation intermediates as well as background DOC. Solid-phase extraction methods recover greater than 65% of the NVDOC across the range of concentrations studied at a crude oil site,[27] with the highest extraction efficiency of 92% obtained using Oasis hydrophilic−lipophilic balance (HLB) cartridges. Thus, HLB extractions are used in this study. McGuire et al.[20] performed comprehensive, *in vitro*-based bioeffects screening of HLB extracts collected from a crude oil plume. They found upregulation of molecular targets, including human nuclear receptors, that have been associated with adverse effects on development, and endocrine and liver function. However, McGuire et al.[20] did not attempt to distinguish the contributions of the hydrocarbons from the degradation intermediates in the mixture of compounds present in the groundwater plume.

The goal of this study was to establish the relative biological activity of degradation intermediates compared to C_{10}−C_{28} hydrocarbons present in a 40-year-old crude oil plume. The mixtures of compounds tested were obtained from three groundwater extraction/clean-up protocols: (1) the fraction obtained with HLB solid-phase extraction targeting a range of polar and nonpolar compounds (termed HLB); (2) the combination of hydrocarbons and degradation intermediates extracted with DCM (termed DCM-total); and (3) the hydrocarbon-only fraction obtained with DCM extraction followed by silica gel cleanup to remove degradation intermediates (termed DCM-SGC). We sampled a transect of wells in the crude oil groundwater plume spanning a range of NVDOC concentrations and degradation intermediate compounds[12] plus an unaffected reference well. Two types of *in vitro* evaluations of biological effects were performed on the extracts from the groundwater samples. The first, Attagene, measured modulation of 46 transcription factors (TF) by the mixtures of compounds in the three organic extractions.[20] Additionally, we used a human aryl hydrocarbon receptor (AhR)-based chemically activated luciferase expression (CALUX) cell bioassay to quantify activation of the AhR by HLB extracts of well water from across the range of plume NVDOC concentrations.[29]

MATERIALS AND METHODS

Site Description. The study was conducted with groundwater samples from the U.S. Geological Survey's National Crude Oil Spill Fate and Natural Attenuation Research site located near Bemidji, Minnesota (Figure 1). The site became

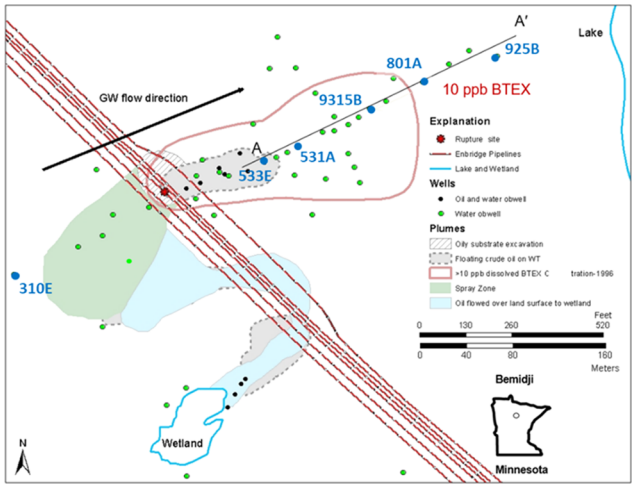

Figure 1. Site map showing locations of a crude oil source, groundwater plume, five wells sampled in the plume, and the background well unaffected by the spill (blue circles). Other features are noted in the legend.

contaminated over 40 years ago by a spill from a ruptured crude oil pipeline. Approximately, 1.7 million liters of light aliphatic crude oil was spilled, but about 75% was removed during the immediate remedial response. The remaining 25% infiltrated the glacial outwash sand and gravel aquifer within days and migrated down to the water table in three locations. Essaid et al.[30] summarized past research publications covering oil distributions, plume chemistry, microbiology, and modeling efforts at the site.

The focus of this study was the groundwater contaminant plume from the north oil body (Figure 1). The north oil body contains oil trapped in the vadose zone at saturations of 10−20% with higher oil saturations of 30−65% at the water table, located 6−8 m below the surface. The dominant microbial population carrying out biodegradation within the oil body is a methanogenic consortium[31−33] dominated by the syntrophic *d-proteobacterium Smithella* and the hydrogenotrophic *Methanoregula*.[33] A plume of nonvolatile dissolved organic carbon (NVDOC) originates in the source zone with concentrations over 30 mg NVDOC/L measured in 2016 (Figure 2). The NVDOC is mainly comprised of nonvolatile organic acids that are degradation intermediates of the crude oil compounds.[4,5,11] The NVDOC concentration migrating from the source has been documented to be over 10 times the highest concentration of benzene[22,34] and comprises the largest proportion of organic carbon in the plume. NVDOC

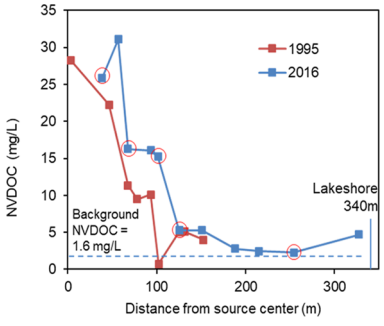

Figure 2. NVDOC concentration versus distance 1995 and 2016. The red circles denote wells sampled in 2018 for this study.

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concentrations decrease with distance from the source (north oil body) and the pool of organic carbon changes in optical character as the organic compounds are transformed in the aquifer.[12] The biotransformation in the groundwater plume is primarily coupled to iron reduction.[30] Between 1995 and 2016, the NVDOC front advanced about a meter per year as iron oxyhydroxides became depleted from the aquifer.[22,35,36] The concentration of NVDOC declines between the source and 188 m, then concentrations appear to level off between 188 m and the lakeshore (Figure 2). The concentration data and optical data suggest that there is a persistent component of the NVDOC plume that migrates to the lake.[12,22]

Sampling and Analyses. Groundwater samples were collected in June 2018 from a background (reference) well located 200 m upgradient from the source and five wells along a flowline in the plume at 39, 68, 102, 125, and 254 m downgradient from the source (Figures 1 and 2). Before sampling, at least three times the water volume in the well casing was purged and field parameters (temperature, dissolved oxygen, specific conductance, and pH) were stable.

Samples for NVDOC analyses were filtered through 0.20 μm Supor in-line filters into baked glass bottles, preserved with hydrochloric acid to a pH of <2, stored on wet ice in the field and then refrigerated at 4 °C in the laboratory until analyzed within 29 days. Concentrations of NVDOC were measured after purging with N_2 to remove inorganic carbon and volatile organics. Data presented from samples collected in 1995 were based on analysis by the persulfate wet-oxidation technique using a carbon analyzer.[37] Samples collected in 2016 and 2018 were analyzed by the high-temperature combustion technique using a Shimadzu TOC Vcsn analyzer (Shimadzu Corporation, Kyoto, Japan) as described by ref 34.

Samples for total petroleum hydrocarbons in the gasoline range (TPHg) were collected in 40 mL volatile organic analysis (VOA) vials and shipped on ice to a commercial lab. Samples were analyzed for TPHg by the EPA method SW-846 8015 using purge-and-trap gas chromatography (GC)/flame ionization detection (FID) analysis to obtain the total concentration of organics in the C_6−C_{10} range.

For TPHd analyses, two samples from each well were collected into unpreserved 1 L amber bottles and shipped on ice overnight to a commercial lab. The two samples were extracted using dichloromethane (DCM; EPA Method 3510). One sample extract was analyzed for TPHd (USEPA method 8015B). The other sample extract was treated with a silica gel cleanup (SGC) column (USEPA method 3630C) and then analyzed for TPHd (TPHd-SGC). Aliquots of the above extracts were used for the high-throughput bioassays (Attagene Inc. Morrisville, NC). To ensure compatibility with the bioassays, DCM-total and DCM-SGC extracts were dried under nitrogen gas and, once dry, reconstituted in 1 mL of dimethysulfoxide (DMSO) resulting in 1000× concentration.

A third water sample was collected from each well to perform Attagene bioassays on the organics obtained with the HLB solid-phase extraction. These samples were kept on dry ice in the field and stored at −20 °C. The samples were processed before Aug 17, 2018 (within 22 days). They were filtered using a GF/F filter (1.0 μm); 250 mL of each filtrate was concentrated using Oasis hydrophilic−lipophilic balance (HLB) 5 cm^3 200 mg cartridges (Waters, Milford, MA). The cartridges were eluted with 6 mL of methanol, followed by 6 mL of a 50:50 mixture of methanol and DCM, and brought to dryness under nitrogen gas at 20 °C. The extracts were reconstituted with 0.5 mL of dimethysulfoxide (DMSO) resulting in 500× concentration. The preparation method removes the volatile fraction. As a result, the toxicity assays performed in this study did not assess the effects of the volatile components in the plume as measured by the TPHg analyses.

Extracts generated using DCM-total, DCM-SGC, and HLB were tested in Attagene assays at 1× concentration relative to the groundwater (i.e., 1 μL of 1000× extract or 2 μL of 500× extract were added to 1 mL of growth media). Bioassays that evaluate activation of 46 molecular targets (CIS-FACTORIAL) were performed on these three extracts in duplicate (Attagene Inc. Morrisville, NC). The assay method was described by Romanov et al.[38] and deployed for the identification of molecular targets of interest in oil-contaminated groundwater samples[20] and a variety of surface waters.[39] Briefly, human hepatoma (HepG2) cells transfected with reporter constructs activated by transcription factors (TF) were used. The reporter transcript abundance was measured by isolating the produced RNA, reverse transcription, amplification, labeling, and capillary electrophoresis. Abundance data are reported as the induction by a sample of interest relative to abundance induced by a DMSO solvent control (abundance in the environmental sample was divided by abundance in solvent control). Positive control assays were performed for a subset of molecular targets (Table S2) including AhR (6-formylindolo-[3,2-*b*]carbazole) and PXR (Rifampicin). Because these novel assays are costly and the characterization of their utility is incomplete relative to some of the more traditional technologies,[40] they were primarily used to quickly screen effects on a variety of molecular targets. Once we identified the main targets of interest, we conducted an additional, widely used reporter gene assay to confirm and quantify biological activity (see below).

A fourth large-volume water sample (72−99 L) was extracted from each well with a field extraction column containing solid-phase HLB sorbent. Water samples were pumped from wells into Teflon-lined barrels, then processed through a 30 mm × 50 mm solid-phase extract (SPE), with 15 g Oasis HLB (Waters Corp., Milford, MA), with a peristaltic pump system equipped with a 100 μm fiber prefilter. Samples were either processed onsite or transported to the U.S. Geological Survey Columbia Environmental Research Center (CERC, Columbia, MO) for processing (stored at 4 °C until processed). A procedure blank (100 L Milli-Q water) was also processed through the same SPE system. The HLB sorbent material from the SPE system was transferred to a glass column (2.5 cm inner diameter) containing glass wool and sodium sulfate, the HLB wetted with ethyl acetate (EtOAc), then more sodium sulfate added to the top of the column before being eluted with 100 mL EtOAc followed by 100 mL 80:20 DCM/methyl *tert*-butyl ether (MTBE) and then 100 mL DCM. All rinses were collected in the same flask, evaporated, and brought to a final volume of 25 mL in EtOAc. Bioassay analysis for AhR activity was performed with these extracts using the HG2L7.5c1 chemically activated luciferase expression (CALUX) human hepatoma cell line[29,41] as described in the data release (https://doi.org/10.5066/P9EGYDRJ). Incubation time was 4 h post treatment of the cells to maximize activity. Cells were graciously provided by Dr. Michael S. Denison (University of California, Davis). Relative luminescence values were normalized to protein in cell lysates as described previously.[42] Results for the CALUX assay were expressed in bioanalytical equivalents (BEQ) relative to the

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activity of a well-known polycyclic aromatic hydrocarbon (PAH)-type AhR agonist (β-naphthoflavone (βNF); ng βNF/L) and represent the average of three independent experiments. BEQ values for the large-volume HLB samples were based on the quarter-maximal effective concentration (EC_{25}) value of βNF and the corresponding EC_{25} of the water sample (EC_{25} values were determined from a Hill's 4-parameter curve fit using SigmaPlot (v 14.0)).

RESULTS

Chemistry. Concentrations of NVDOC, TPHd, TPHd-SGC, and TPHg measured in the water samples were plotted versus distance from the source (Figure 3). The NVDOC concentrations in the plume were 22.7 mg/L near the source and decreased with distance but remained above the background value of 1.4 mg/L at all of the plume wells. At the two most downgradient wells (125 and 254 m), the NVDOC was 3.7 and 1.8 times the background value, respectively. TPHd-total concentrations were 31% of NVDOC near the source and decreased to 5% of NVDOC at 254 m downgradient. The concentrations of TPHd-SGC were just 1−3% of NVDOC in each sample (Figure 3). The TPHd-SGC values decreased from 0.56 mg/L near the source to 0.13 mg/L at 102 m and were below detection at 125 and 254 m. Both TPHd-total and TPHd-SGC were below detection at the background well. The volatile components in the plume measured as TPHg were 6.5 mg/L near the source, dropping to 0.4 mg/L at 102 m and below detection at 125 and 154 m.

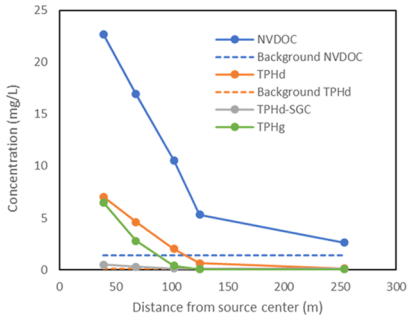

Figure 3. Concentrations of NVDOC (mg/L), diesel range total petroleum hydrocarbons (TPHd), and TPHd after polars were removed by silica gel cleanup (TPHd-SGC) measured at the five plume wells in 2018. The dashed lines show concentrations measured at the unaffected background well.

***In Vitro* Bioeffect Assays.** Biological activities of the HLB extracts from the five wells along a flowline in the plume showed upregulation for five transcription factors, especially PXR and AhR (7-fold upregulation), and exceeded those of both the DCM-total and DCM-SGC treatments (Figures 4A,B and S1). Background well HLB extracts did not upregulate any of the targets (the highest activity was 1.3-fold relative to solvent control). The PXR and AhR activity of HLB extracts increased with increasing NVDOC (Figure 4A,B). DCM-total extracts, which contain hydrocarbons and some degradation intermediates, induced biological activity higher than 2-fold for PXR and AhR, with PXR upregulated by all samples collected from the plume wells (Figures 4A and S1B), but not by the background well sample. AhR activation was the highest near the oil source (2.5-fold relative to solvent control), dropping to the assay background value by the third well (DCM-total

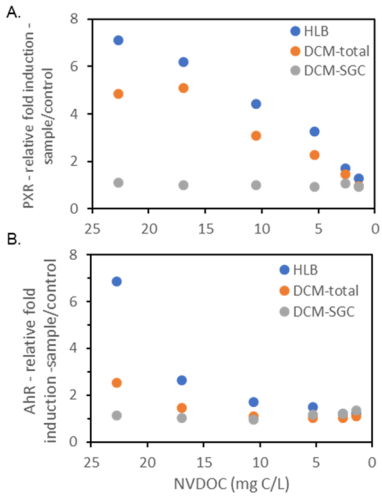

Figure 4. Induction of (A) pregnane xenobiotic receptor (PXR) and (B) aryl hydrocarbon receptor (AhR) for water samples from the five plume wells and background well. Three extracted and cleaned-up fractions were tested at concentrations equivalent to the original water samples: solid-phase extraction with Oasis HLB (HLB), liquid−liquid extraction with dichloromethane (DCM-total), and DCM extraction after removal of polar compounds with silica gel cleanup (DCM-SGC).

samples; Figures 4B and S1B). DCM-SGC extracted samples ("hydrocarbon fraction") were comparable to that of the background well; none of the samples, including the background well, induced biological activity (cutoff of 2-fold relative to solvent control was used). This was the case for all 46 transcription factors evaluated, including PXR and AhR (Figures 4A,B and S1C; see associated data release for all data ref 61). A Kendall-Tau nonparametric correlation analysis shows that there was a statistically significant association between NVDOC and AhR and PXR receptor induction by HLB and DCM-total extracts. In contrast, there was no association between NVDOC and AhR and PXR receptor induction by DCM-SGC extracts (Table S1). Although the number of samples is small, the statistically significant associations provide insight into the importance of NVDOC in driving the responses.

Transcriptional activation of AhR-mediated response in CALUX HG2L7.5c1 cells was chosen for further study because of previously observed AhR activation by HLB extracts from three wells located near the plume source[20] and the widely known responsiveness of this receptor to many hydrocarbons and hydrocarbon degradation products.[43−45] The present study examined HLB extracts from a total of four wells: two with detectable hydrocarbons, and two with degradation intermediates and no detectable hydrocarbons present based on the TPHd-SGC analyses (Figure 3 excluding the well closest to the oil source, well 533E). In Figure 5, the results for CALUX HG2L7.5c1 cells expressed as β-naphthoflavone (βNF) bioanalytical equivalents (BEQ) are plotted together with NVDOC concentrations. The AhR activity yielded average BEQs ranging from 790 ± 140 ng βNF/L near the source to 340 ± 65 and 130 ± 17 ng βNF/L for the two wells where hydrocarbons were below detection (Table 1). The activity decreased with distance and NVDOC correlates with CALUX response at the four sampled wells (n = 4; R^2 = 0.87). A background well (310E) located upgradient of the source zone yielded no activity in the CALUX assay (Table 1).

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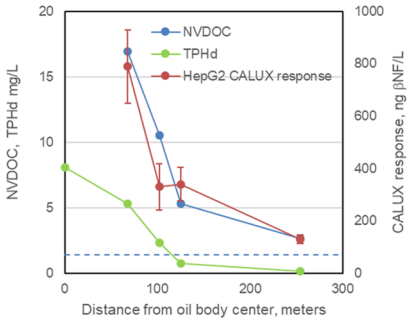

Figure 5. Values of β-naphthoflavone (βNF) bioanalytical equivalents (BEQ) as determined by CALUX human hepatoma cells (HG2L7.5c1) for four plume wells and the background well together with NVDOC and TPHd concentrations in the same wells. The dashed line is background NVDOC concentration.

Table 1. Transcriptional Activation of AhR-Mediated Response in CALUX Human Hepatoma HG2L7.5c1 Cells Expressed as β-Naphthoflavone (βNF) Bioanalytical Equivalents (BEQ)

well ID	location (m from source)	NVDOC (mg/L)	BEQ average (± se)[a], ng βNF/L water	BEQ range (min–max), ng βNF/L water
310E	background	1.42	/	/
531A	hydrocarbon plume (68 m)	16.9	790 ± 140	520–990
9315B	hydrocarbon plume (102 m)	10.5	330 ± 89	210–500
801A	oxidation product plume (125 m)	5.32	340 ± 65	270–470
925D	oxidation product plume (254 m)	2.63	130 ± 17	100–160

[a]Average of three replicate experiments; / denotes results that did not exceed 25% of the maximum response for βNF (see Figure S2).

DISCUSSION

Chemistry. NVDOC concentrations were 3–20 times the TPHd concentrations at the five wells in the plume (Figure 3). These ratios were consistent with 2010 and 2016 results from the site,[20,22] indicating they are characteristics of this crude oil plume. Thus, the TPHd analyses provided a confusing picture because they quantified both hydrocarbons and a variable fraction of the degradation intermediates. The cause was likely the use of DCM extraction for the water samples prior to TPHd analyses. Zito et al.[27] found that the mass of organic carbon obtained with DCM extractions as a percentage of NVDOC ranged from 25% near the source to 17% at 254 m downgradient. When SGC was used prior to the TPHd analyses, the reduction in mass was 97%. Moreover, beyond 125 m, the TPHd-SGC concentrations were below detection. Thus, risk calculations based on hydrocarbon concentrations obtained with TPHd and especially TPHd-SGC only account for a small portion of the NVDOC mass in this plume. Beyond about 125 m downgradient, the NVDOC concentrations in the plume leveled off at 3–5 mg/L, suggesting limited further biodegradation of NVDOC before the plume reaches the lake (Figure 2). These results are consistent with the observations in Cozzarelli et al.[34] that showed NVDOC concentrations in 2013 were 2.5–4.3 mg/L at the two furthest downgradient wells.

The very small mass of hydrocarbons remaining after SGC, compared to the total NVDOC pool, shows that the plume is dominated by degradation intermediates. Some authors have suggested that the NVDOC in the plume is comprised of a mixture of degradation intermediates and products of biosynthesis such as proteins, carbohydrates, lipid, and nucleic acids released by the aquifer microbial population.[46,47] However, data from a combination of five quantitative and qualitative analytical techniques demonstrate that the NVDOC near the source is composed of highly reduced compounds like those in the crude oil source.[48] As the components of crude oil biodegrade downgradient from the source, the NVDOC becomes more oxygenated and composition changes continuously. Principle component analysis of the combined data set shows a compositional gap between the plume wells and the unaffected background well, consistent with NVDOC originating in the oil body and differing from the background NVDOC. Accounting for the background NVDOC that migrates into the source zone, the percentage of degradation intermediates in the plume NVDOC ranges from 94% in the well closest to the source to 46% in the most downgradient well (Table 1).

Biological Effects. Previously, McGuire et al.[20] presented 2016 data for HLB extracts showing six transcriptional pathways upregulated more than 2-fold (CIS-factorial assays). The highest upregulation of biological activity was observed for PXR and AhR; biological activity decreased with distance from the source. The findings of the present study are congruent with that of McGuire et al.;[20] HLB extracts have similar profiles of biological activity and exhibited similar spatial trends—PXR decreased from 7- to 1.7-fold and AhR from 7- to 1.2-fold between 39 and 254 m downgradient. For the DCM-total extracts, this study found upregulation of only PXR and AhR with lower values compared to those for HLB (Figure 4). The use of HLB has been shown to extract an average of 92.4% of the NVDOC in the plume, the highest extraction efficiency of any methods tested.[27] Thus, the observed effects for HLB were based on the highest possible extracted concentrations of degradation intermediates. The results from this study showed that the use of DCM liquid–liquid extraction protocols missed a portion of organic carbon compounds that were associated with increased biological effects. Moreover, the use of DCM-SGC to isolate the hydrocarbons resulted in extracts with negligible activity in the Attagene assays, demonstrating that the measured biological effects in the plume were associated with the degradation intermediates. The negligible activity of the hydrocarbons in the diesel range was likely due to their low concentrations (Figure 3).

The results from the HepG2 CALUX assay showed responses throughout the plume (Figure 5). The BEQ values decreased with distance from the oil source but persisted beyond 125 m downgradient, where NVDOC values from 2016 (Figure 2) appeared to level off before the plume reached the lake. The activity was present at two wells with no detectable hydrocarbons (Figure 3), underscoring the importance of using an extraction method that captures a large fraction of the NVDOC when testing for biological effects. The downgradient AhR activity where the NVDOC concentrations were relatively constant with distance indicated that the most refractory intermediate degradation products of the petroleum hydrocarbons were agonists of the AhR and, as such, may pose risks to exposed human or ecological receptors.

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Studies are needed to assess whether the *in vitro* activity observed here translates to adverse outcomes *in vivo*. Additionally, studies are also needed to characterize the nature of the chemicals in the persistent fraction of NVDOC capable of activation of AhR pathways.

Our results can be compared with comparable assays that used βNF as the positive control to estimate BEQs. Yeast cells transfected with human AhR and its dimerization partner, the human aryl hydrocarbon receptor nuclear translocator (ARNT), have found BEQ values for river water ranging from ∼10 to 950 ng/L BEQ,[40,49] 390 to 740 ng/L in wastewater treatment plant effluent,[50] and 2 000 ng/L to 1 500 μg/L in untreated wastewater.[50−52] In one of the studies measuring BEQ of river water, the sites with the highest BEQ values (200−950 ng/L) were located within an industrial textile region of Japan,[49] an area known for containing AhR agonists due to contributions from dye industry effluents.[53] The second study examining BEQ values in river water similarly found the highest BEQ values (approximately 50−130 ng/L) to be located within an industrialized region.[40] The literature BEQ values from waters subject to industrial influence were like those obtained in this study.

Others have suggested degradation intermediates continue to biodegrade[54] and are unlikely to pose a health risk.[16,17] The results presented here suggest that the intermediate degradation products are responsible for the observed effects near the source and persist well beyond where hydrocarbons were detected. In the most downgradient well, the AhR activity was 130 ± 17 ng βNF/L (925D; Table 1), where concentrations of NVDOC were less than twice those in the background aquifer. Humic substances are known to activate AhR pathways at concentrations of 5 mg/L or greater,[55] and AhR activity was documented in surface water draining a wetland with high DOC (15.9 mg/L).[39] Thus, we examined the effect of the native NVDOC extracted from the background well at equivalent concentrations to those measured in the plume. The AhR responses at the range of tested concentrations are illustrated in Figure S2A,B. The activity of the background NVDOC was much lower than that for similar concentrations as in the plume (Figure S2B). Furthermore, there was no response of the background NVDOC at the concentration found in the background well (1.42 mg/L). This tells us that AhR ligands capable of transcriptional activation of AhR pathways were present in the downgradient portions of the plume and were caused by the intermediate degradation products and not the native aquifer NVDOC.

PXR plays a role in the regulation of hepatic glucose and lipid metabolism, but its role in xenobiotic sensing and metabolism and disposition was discovered first and is thus better characterized.[56,57] The normal physiological function of the AhR is not fully elucidated, but there is growing evidence that it plays a role in neurogenesis, hematopoiesis, and cardyomyogenesis.[58] The best understood role of AhR is in the metabolism of xenobiotic compounds; its activation can be indicative of an exposure to a foreign chemical. Activation of AhR-mediated pathways does not always lead to adverse health outcomes. However, it has been demonstrated that activation of these pathways over a period of time and over a threshold amount leads to adverse health outcomes in vertebrates.[52] Risk is proportional to activation of the AhR pathways, when that activation occurs over a threshold. Activation of PXR and AhR responses in the cell bioassays was indicative of chemicals being present in the groundwater samples at great enough concentrations to initiate the cascade of events known to be associated with these receptors. However, it is not known if the chemicals capable of activating these pathways were present at amounts significant enough to cause adverse outcomes in whole animals. Further studies are required to characterize the exact nature of the chemicals responsible for PXR and AhR activation and to determine if those chemicals are present at concentrations sufficient to cause adverse effects in organisms that might be exposed. These studies should evaluate the potential for groundwater samples along the plume, to cause adverse outcomes consistent with AhR mechanisms. Developing fish embryos would be an appropriate model for future studies, owing to their known sensitivities to both PAHs and their hydroxylated metabolites.[53]

Implications. The finding that TPHd analyses capture only a fraction of degradation products has been replicated at a second crude oil spill.[22] In a study of five fuel terminal spills, Zemo et al.[16] reported TPHd and TPHd with SGC concentrations for 22 groundwater samples. An analysis of these data reveals that polar compounds comprised an average of 77% of TPHd concentrations in the source zones and 71% in downgradient wells where hydrocarbons were below detection (see Table 4 in Zemo et al.[16]). The authors did not address or discuss the possibility that additional polar compounds might be present in these plumes that were not captured with the TPHd method.

Two alternative approaches for evaluating the risk of degradation intermediates on human health were included in the recently published "Total Petroleum Hydrocarbons (TPH) Risk Evaluation at Petroleum-Contaminated Sites."[24] The first approach assumes degradation intermediates are present in the same proportions as aromatics and aliphatics in the spilled fuel or oil, and those degradation intermediates have equal toxicity.[24] The second approach is based on a series of studies examining the polar compounds in DCM extracts.[15−17] Over 760 polar compounds were identified but not quantified.[15] The identified polar compounds were grouped into structural classes, and toxicity factors for representative compounds in each class were used to estimate the potency for the entire class.[24] The first method is problematic because it assumes that degradation intermediates have the same toxicity as parent compounds and are present at the same concentrations, even though they have different properties. Moreover, recent studies have found greater toxicity in PAH degradation products than for the parent hydrocarbons.[18,19] The second method is limited because it assumes toxicity factors for single compounds are adequate to represent a class of compounds.[21] More importantly, it is based on the fraction that is extracted with DCM (DCM-total), which recovers one-third or less of the degradation intermediates present at this site.

More information is needed on the composition of oil spill degradation intermediates and their potential risks to disrupt biological processes in both human and ecological receptors. For example, oxygenated PAHs cause acute toxic stress in a variety of aquatic organisms and both cytotoxicity and oxidative stress in mammalian cells.[59] A range of adverse effects on neurobehavior and development were observed in zebrafish embryos exposed to approximately 95 different PAH derivatives, and many of these derivatives induced CYP1A in the embryos.[60] Additionally, these oil spill degradation intermediates are often more polar than the parent compounds and move more readily through soil and water, which can increase potential for transport to a greater number of

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receptors. Our findings indicated that the intermediate degradation 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 intermediate degradation products of oil may underestimate risks from these chemicals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02220.

Results of Kendall-Tau nonparametric correlation analyses between NVDOC and AhR and PXR induction; positive control compounds used for a subset of molecular targets; results of bioassays; molecular targets with greater than 1.5-fold induction relative to abundance induced by a DMSO solvent control; the response of CALUX human hepatoma HG2L7.5c1 cells to dilutions of HLB extracts presented as water equivalents and nonvolatile dissolved organic carbon (PDF)

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Notes

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Toxicity Assessment of Groundwater Contaminated by Petroleum Hydrocarbons at a Well-Characterized, Aged, Crude Oil Release Site

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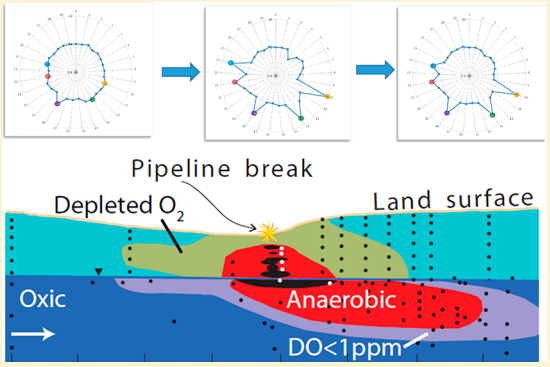
Supporting Information

ABSTRACT: Management of petroleum-impacted waters by monitored natural attenuation requires an understanding of the toxicology of both the original compounds released and the transformation products formed during natural breakdown. Here, we report data from a groundwater plume consisting of a mixture of crude oil compounds and transformation products in an effort to bridge the gap between groundwater quality information and potential biological effects of human exposures. Groundwater samples were characterized for redox processes, concentrations of nonvolatile dissolved organic carbon (NVDOC) and total petroleum hydrocarbons in the diesel range, as well as for activation of human nuclear receptors (hNR) and toxicologically relevant transcriptional pathways. Results show upregulation of several biological pathways, including peroxisome proliferator-activated receptor gamma and alpha, estrogen receptor alpha, and pregnane X receptor (PXR) with higher levels of hNR activity observed in more contaminated samples. Our study of affected groundwater contaminated by a crude-oil release 39 years ago shows these types of waters may have the potential to cause adverse impacts on development, endocrine, and liver functioning in exposed populations. Additionally, positive trends in activation of some of the molecular targets (e.g., PXR) with increasing NVDOC concentrations (including polar transformation products) demonstrate the importance of improving our understanding of the toxicity associated with the unknown transformation products present in hydrocarbon-impacted waters. Our results begin to provide insight into the potential toxicity of petroleum-impacted waters, which is particularly timely given the ubiquitous nature of waters impacted by petroleum contamination not only recently but also in the past and the need to protect drinking-water quality.



INTRODUCTION

Comprehensive characterization of the exposure and effects associated with petroleum- and fuel-impacted waters is essential for protection of human health and ecosystem integrity. At sites where water is contaminated with petroleum hydrocarbons, the analyses required by the U.S. Environmental Protection Agency include dissolved total petroleum hydrocarbons in the gasoline, diesel, or oil range (TPHg, TPHd, or TPHo). At old spill sites, much of the dissolved organic matter consist of polar compounds from the partial degradation of hydrocarbons in situ.[1] Recent results based on nonvolatile dissolved organic carbon (NVDOC) analyses show that polar transformation products quantified with TPHd analyses represent only a fraction of the polar transformation products present at a refined fuel site[2] and a crude-oil spill site.[3] Due to the vast number of residual oil and fuel spills that remain in the subsurface[4] characterization of the concentrations, toxicity, and natural attenuation of transformation products (metabolites) is essential to protect water quality.

As groundwater impacted by crude oil or petroleum hydrocarbon fuel spills ages and undergoes natural attenuation, both the original compounds and the more soluble breakdown products migrate away from the spill, forming a groundwater plume. In the 1990s, considerable evidence suggested that the spatial extent of such plumes was limited (i.e., plume length of indicator compounds such as benzene would "stabilize" then shrink) due to natural attenuation processes, principally biodegradation by in situ bacterial populations, at rates controlled by availability of electron acceptors such as oxygen, nitrate, iron oxyhydroxides, and sulfate.[5] However, recent studies suggest that this assumption may not be sufficiently protective of water resources. For example, a study of 10 closed hydrocarbon sites in Wisconsin found that benzene concen-

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Table 1. Site Characteristics—Biogeochemistry and Chemistry

well number	310B	956	533E	531A	9315B
sample type	reference	spray zone	below oil body	plume	plume
distance from center of the oil body (m)	−200.5	−125	38.8	67.6	101.6
biogeochemical characterization	suboxic	aerobic	methanogenic	methanogenic	iron reducing
nonvolatile dissolved organic carbon (mg/L of C)	1.66	14.7	25.9	16.3	15.2
total petroleum hydrocarbons diesel range (mg/L)	BDL	0.92	6.6	3.5	2.8
CH_4 (mg/L)	0.07	<0.01	7.93	3.16	0.63
arsenic (μg/L)	3.60	0.620	57.0	50.1	13.7

trations exceeded those measured at the time of closure.[6] In addition, because the breakdown products of petroleum hydrocarbons can have lower volatility and greater polarity (and thus greater solubility), their fate and transport properties are quite different than those of the parent compounds[7] and would be expected to form different plumes lengths and create different toxicity profiles upon exposure. Thus, comprehensive studies of older plumes are critical to understand the potential effects of exposure to petroleum-impacted waters.

Here, we report data from groundwater collected from The National Crude Oil Spill Fate and Natural Attenuation Research Site near Bemidji, MN, U.S.A. This site is ideal for investigating the toxicity of waters impacted by both petroleum hydrocarbons and their breakdown products, as the hydrological, microbiological, and geochemical controls on biodegradation and contaminant transport have been studied for nearly 40 years https://mn.water.usgs.gov/projects/bemidji/. Prior work has shown that the 50 ug/L contour of benzene is relatively stable, while the plume of breakdown products, as measured as nonvolatile dissolved organic carbon (NVDOC), extends well beyond the benzene plume. Modeling indicates that concentrations of NVDOC decrease under anaerobic conditions at a first-order rate of 0.13% per day,[8] but the position of the 5 mg/L (as carbon) contour has expanded from 125 m downgradient in 1988 to over 200 m in 2010.[3] In addition, concentrations of NVDOC from the crude oil are 10−20 times higher than the concentrations of benzene and 3−23 times higher than TPHd.[3] Thus, the required analyses do not include many of the organic compounds migrating in groundwater from residual petroleum hydrocarbon sources. In addition, the possible biological effects of exposure to waters that contain a complex mixture of known and unknown transformation products are unknown.

Risk assessment of petroleum- and fuel-impacted waters has mostly focused on the evaluation of the acute and baseline toxicity (a.k.a. narcosis—a nonspecific toxicity due to the disruption of the lipid membrane[9,10]). Podgorski et al. found a positive correlation between acute toxicity and NVDOC data[11] on groundwater samples collected from this study site. Emerging evidence suggests that petroleum-impacted waters also have a potential to initiate adverse effects by specific interactions with molecular targets (e.g., aryl hydrocarbon, estrogen and androgen receptor[12−14]). A more complete characterization of specific mechanisms of toxicity of petroleum-impacted waters is needed. Given the complex composition of dissolved organic matter in aged plumes (ca. 15 000 chemicals at the site of the present study[15]) and a lack of knowledge of identities and toxicity of the individual chemicals, the characterization of specific toxicity based on chemical composition alone is not feasible. Bioeffects-based assays can be used instead, as they allow for time- and cost-effective screening of activation of specific molecular targets by complex mixtures.[16]

Here we present results of comprehensive in vitro biological activity screening combined with measurements of TPHd and NVDOC to identify potential mechanisms of toxicity of a mixture of crude-oil compounds and their transformation products. Results indicate the potential of petroleum-impacted waters to activate human nuclear receptors (hNR) and several transcription factors, including those for which disruption may lead to developmental toxicity, endocrine disruption, and metabolic dysfunction.

MATERIALS AND METHODS

Study Site. This study was conducted at the National Crude Oil Spill Fate and Natural Attenuation Research Site near Bemidji, MN, U.S.A., because it offers an opportunity to characterize toxicity in the context of monitored natural attenuation (MNA). The site was contaminated in 1979 when a buried pipeline ruptured spraying an estimated 1.7 million liters of light crude oil on the land surface. Sprayed oil coated the surface soil (referred to here as the spray zone) and flowed toward local depressions where it infiltrated the aquifer forming three subsurface oil bodies (Figure S1 of the Supporting Information, SI). Groundwater is contaminated below the spray zone and downgradient from the oil bodies. The spray zone and contaminant plume movement,[8] redox zones,[17] biogeochemistry, and microbiology are well characterized.[18]

In August 2016, water samples were collected from 30 groundwater locations at the site, representing a range of redox conditions and organic chemical composition.[11] A location upgradient from the contaminant plume was used as a reference sample for the uncontaminated aquifer.[17] Water samples were characterized for biogeochemical properties, including dominant redox zonation and organic chemistry. A subset of samples collected from five wells, representing the different biogeochemical zones, (310B, 956, 533E, 531A, 9315B; Table 1) were also screened for biological activity using a battery of in vitro assays.

Water Chemistry. Groundwater samples were collected after the well was purged to at least three times the water in the well casing and field parameters (temperature, dissolved oxygen, specific conductance, and pH) were stabilized. All water chemistry samples were stored on wet ice in the field and then refrigerated at 4 °C in the laboratory until analyzed. Samples for NVDOC were filtered through 0.20-μm Supor in-line filters into baked glass bottles, preserved with hydrochloric acid to a pH of <2, and analyzed using a Shimadzu TOC Vcsn analyzer (Shimadzu Corporation).

Samples for total petroleum hydrocarbons in the diesel range (TPHd) were collected into 1-L amber bottles with Teflon lined caps and preserved with 5 mL of 50% HCl at the time of collection; these samples were extracted within 7 days of sample collection. The samples were analyzed by Pace Analytical, St. Paul, MN, using the Wisconsin modified DRO method.[19] Briefly, organic constituents were extracted with hexane,

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analyzed with a gas chromatograph and a flame ionization detector (FID), and quantified based on a diesel component standard. The reporting limit was 0.1−0.54 mg/L.

Samples for methane (CH_4) concentrations were collected in Glaspak syringes connected directly to the sample-pump outlet and transferred into 25 mL serum bottles containing TSP (trisodium phosphate dodecahydrate). Dissolved CH_4 concentrations were measured by headspace analysis using a 5890 Series II HP Gas Chromatograph split/splitless inlet FID with a fused silica capillary column.

Samples for total dissolved arsenic (As_T) were filtered in-line through 0.2 μm Nuclepore membranes and preserved to pH < 2 with double distilled nitric acid. Dissolved As_T was analyzed using a PerkinElmer ELAN 9000 Inductively Coupled Plasma Mass Spectrometry (ICP-MS), with a detection limit for dissolved As_T of 0.1 μg/L, and reported as total concentration. Arsenic species were not identified, but Cozzarelli and colleagues[17] showed As(III) accounts for 80−100% of As species present in the anoxic groundwater.

Biological Activity. Water samples (500 mL) from 5 sites, shown in Table 1, were kept on dry ice in the field and frozen at −20 °C until processed. Samples were filtered using a GF/F filter (1.0 μm). Filtrates were concentrated using OASIS HLB 5 cm^3 200 mg cartridges (Waters, Milford, MA), eluted with 50:50 methanol: dichloromethane, and brought to dryness under nitrogen gas. Once dry, each sample was suspended in 0.5 mL dimethyl sulfoxide (DMSO). This preparation method removed the volatile fraction, including benzene, toluene, ethylbenzene and xylenes (BTEX), and is expected to remove the inorganic arsenic.[20] Each sample was tested in triplicate at three concentrations including in situ concentration (1×) and concentrated (3× and 10×).

Commercially available, well-characterized TRANS- and CIS-FACTORIAL assays (Attagene Inc. Morrisville, NC) were used[21] to assess biological activities of water samples. TRANS-FACTORIAL can measure water-sample activities against 48 human nuclear receptors (hNR). CIS-FACTORIAL measures the effects on the activity of more than 40 toxicologically relevant transcriptional pathways. (SI Tables S1 and S2). FACTORIAL assays use a library of reporter constructs ("reporter transcription units"—RTUs) similar to conventional reporter gene constructs where a transcription factor (TF) responsive promoter is linked to a downstream reporter sequence. FACTORIAL assays do not rely on translation of RTU transcripts into proteins; the reporter transcript abundance is measured (total RNA is isolated, reverse-transcribed, amplified, labeled, and quantified by capillary electrophoresis).[21] The methodology is distinct from conventional approaches, because the RTUs have identical reporter sequences. Distinction between reporter transcripts is achieved by tagging of the reporter sequences with "processing tags" that identify a unique cleavage position for each of the reporter cDNAs. The main difference between the two types of assays is that CIS- measures activities of endogenous TFs, whereas the TRANS- evaluates changes in activities of exogenous, chimeric NR-Gal4 proteins.[21] Because the HepG2 cell line does not express all nuclear receptors the TRANS assay is used to complement the CIS assay's hNR suite.

Molecular target activation data for individual samples at 10× concentration were expressed relative to induction by 10 uL/L DMSO (solvent control), log_2 transformed and median centered. Euclidean distance average linkage hierarchical clustering (Heatmapper software[22]) was conducted on the transformed data (SI Figure S2).

RESULTS AND DISCUSSION

Water Chemistry. Concentrations of NVDOC and TPHd (Figure 1) were highest near the oil source and decreased with distance in the direction of groundwater flow. NVDOC concentrations were greater than three times the TPHd concentrations near the source and over 20 times higher beyond 150 m, consistent with earlier results that NVDOC analyses capture partial transformation products not measured in the TPHd analyses.[3]

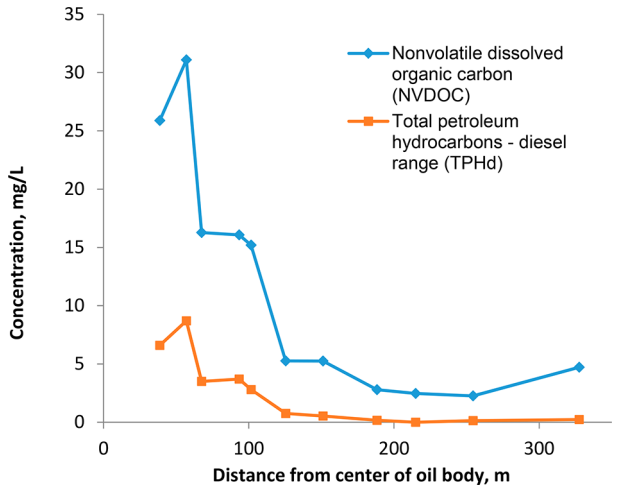

Figure 1. Concentrations of nonvolatile dissolved organic carbon (NVDOC) and total petroleum hydrocarbons in the diesel range (TPHd) measured August, 2016, along the centerline of the dissolved plume at the study site near Bemidji, Minnesota, U.S.A. Well locations and oil spill source location shown in SI Figure S1.

Locations, redox conditions, and chemical data for the five wells screened for biological activity are shown in Table 1, including a background, suboxic well with naturally occurring organic matter (310B), a well below the subsurface oil source (533E), two wells in the plume (531A and 9315B), and a well below the spray zone (956). Contaminants within the spray zone were exposed to sunlight and oxygen and thus subjected to photooxidation as well as aerobic biodegradation. Dissolved arsenic (and other metals) are released into the groundwater system when reduction (and dissolution) of naturally occurring iron oxide minerals coupled to the oxidation of organic matter occurs.[23] The generation of dissolved CH_4 is an additional effect of the hydrocarbon biodegradation in the most contaminated portions under the oil bodies.[18]

Biological Activity. Activation of several human nuclear receptors (hNR) was detected (Figure 2A). The background sample (310B) only activated the pregnane X receptor (PXR). In the spray-zone sample (956), peroxisome proliferator-activated receptor gamma (PPARg), estrogen receptor alpha (ERa) and PXR were upregulated more than 2-fold compared to controls. The most highly activated hNRs in the water collected from beneath the oil body (well 533E) included ERa, ERb, PXR, PPARg, PPARa, and retinoic acid receptor beta (RXRb). Those same nuclear receptors responded to the waters collected from downgradient within the contaminant plume (531A and 9315B), but typically with lower magnitude (Figure 2A). CIS-FACTORIAL activation patterns were similar—the background

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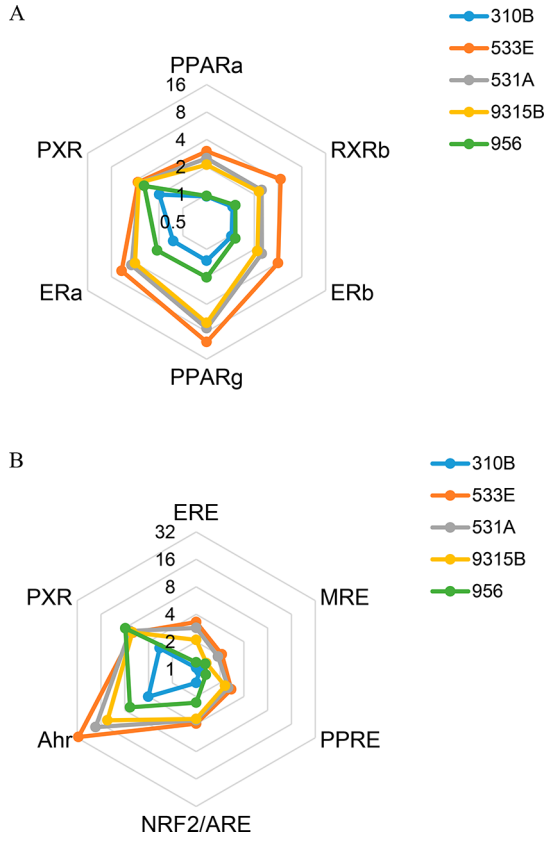

Figure 2. TRANS-FACTORIAL (A) and CIS-FACTORIAL (B) molecular target activation (fold change relative to DMSO control, *y* axis is log_2-scaled) by environmental sample concentrates (10×). Only molecular targets up/downregulated more than 2-fold in at least one of the wells are shown. PPARa: Peroxisome proliferator-activated receptor-alpha, PPARg: Peroxisome proliferator-activated receptor-gamma, PXR: Pregnane X receptor, ERa: Estrogen receptor-alpha, ERb: Estrogen nuclear receptor-beta, RXRb: Retinoid X receptor-beta, PXR: The pregnane X receptor (PXR), Xenobiotic Pathway, Ahr: The Aryl hydrocarbon receptor (AhR)/Xenobiotic Response, ERE: Estrogen Receptor (ER) pathway, MRE: Metal regulatory transcription factor 1 (MTF-1), PPRE: Peroxisome proliferator activating receptor (PPAR)a, d, g, NRF2/ARE: Antioxidant Response Element (ARE)-binding Nuclear factor (erythroid-derived 2)-like 2 (NRF2).

sample (310B) only mildly upregulated the PXR and aryl hydrocarbon receptor (AhR) pathways, whereas sample 533E, collected from beneath the oil body, activated the highest number of targets, and typically with the highest magnitude (Figure 2B).

Hierarchical clustering of biological activity data (SI Figure S2) revealed that the well below the main oil body (533E) was distinct from the others. Waters collected from downgradient, within the plume, clustered together (531A and 9315B). Similarly, background (310B) and spray-impacted wells (956) clustered together. Overall, clustering indicated distinct transcriptional activation profiles for samples collected beneath the oil body versus within the plume versus the background and spray zone wells.

A small number of molecular targets had a strong influence on the clustering of sample 533E (water beneath the oil body) away from the activity profiles of other samples. These included receptors PPARa, ERb, PXR, and pathways associated with peroxisome proliferator activating receptor (PPRE), estrogen receptor (ERE), and nuclear factor erythroid 2-related factor antioxidant (NRF2ARE) (SI Figure S2). Several of those targets are related (i.e., PPARa and PPRE, ER and ERE) and were activated in the two distinct sets of assays (Figure 2). The congruency between CIS- and TRANS- data provides strong evidence that these molecular targets and associated pathways can be activated by the oil-body derived contaminants. 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.

Only six targets per assay type (CIS vs TRANS) were up/downregulated more than 2-fold in at least one of the analyzed groundwater samples. Because of a paucity of comprehensive transcriptomic and/or in vitro TF/hNR data (especially for mammalian models and weathered crude oils) it is difficult to know whether responses from the broader set of hNRs and/or TFs should have been observed in this study or could be expected at other similarly impacted sites. The present study is the first to comprehensively screen biological activity of groundwater from an aged crude oil release site. Below, we provide a brief review of the literature, including mining databases (i.e., Toxcast) to determine whether biological responses observed in the present study have been documented by other studies that investigated the toxicity of petroleum-impacted waters.

Activation of the AhR and ERb observed in the present study is consistent with the findings generated by focused screenings of four crude oils and seven refined fuel products for hNR activity using conventional reporter gene assays. For example, 100% of the 11 tested samples activated ERb[24] and AhR.[12] The maximum potencies of these samples were found to be highly variable—from 40 to a million times lower than the potency of well-known AhR agonists benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin.[12] Both aromatic and polar fractions of the crude oil have been identified as sources of the AhR agonists.[25] AhR induction of CYP enzyme activities, particularly CYP1A1, through the aryl hydrocarbon receptor (AhR) in most vertebrate species is a well-documented response to planar and aromatic organic contaminants.[26] Temkin et al.[27] reported that crude oil did not activate PPARg in vitro, but other studies have shown that exposure to PAHs induced PPAR signaling pathways.[28] In vivo studies with larval fish exposed to weathered crude oil also indicated interaction with PPAR pathways, and noted extensive interaction with the thyroid receptor/RXR and liver X receptor/RXR toxicity pathways.[29] The present study also noted effects on RXRb which is known to form heterodimers with the thyroid (TR) and vitamin D (VDR) receptors. Because nonpermissive heterodimers (i.e., those RXRb forms with TR or VDR) can only be activated by the partner's ligand while RXR is silent, the activation of RXR by samples 531A, 9315B, and 956 indicates potential effects on the thyroid and vitamin D signaling.[30]

In contrast to the above studies of oil and refined fuels, there is a paucity of studies that characterize biological activity of the chemicals that comprise complex mixtures of partial transformation products found at attenuated, aged sites (ca. 15 000 chemicals at our study site[15]). Because 18 low-molecular-weight organic acids were quantified at this site in the past,[31] we used ToxCast data to determine their potential to exert biological activities in the Attagene assays utilized in the present study. Exposure to activity ratios (EARs) were calculated by dividing the concentration of each organic acid by its activity concentration at cutoff (ACC) in Attagene assay.[32] The ACC estimates the chemical concentration at which the activity cutoff

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is achieved within an assay of interest. Because of the size and complexity of the ToxCast data we used *toxEval 1.0.1* to calculate EARs. The results indicate that some of the organic acids, if present in the current samples, could have contributed to PXRE and NRF2-ARE activity in the CIS- assays, and GR and PPARa activity in the TRANS- assays. However, the EARs for the individual organic acids were very low (<0.0048) indicating that additive effects and/or additional chemicals should be considered. It is important to note that some of the TOX21 assays, unlike Attagene, indicated potential of the examined organic acids to agonize ERa (i.e., benzoic acid had EAR of 0.11). This indicates that using multiple suites of assays may be beneficial when conducting initial screenings, as the assays may vary in sensitivity and may be susceptible to interference associated with the complex nature of the sample. The approach identified in this study is suitable for screening of molecular targets and chemicals of interest, but follow-up effects directed analyses that use well-characterized assays, combined with novel, chemometric reduction approaches[25] should be conducted to identify groups of chemicals that might be the drivers of the biological activities.

In the present study, the activation of the molecular targets was benchmarked to well-understood chemicals (i.e., several positive controls were incorporated in the experiments). Maximum activities of the in situ (1X) samples relative to their respective positive controls were as follows: ERa—12% of 0.2 μM 17β-estradiol, PXR—97% of 10 μM rifamicin, PPARg—11% of 1 μM rosiglitazone, and AhR—21% of the 1uM 6-Formylindolo [3,2-*b*] carbazole response. Extrapolating these molecular level, in vitro effects to the in vivo effects is difficult because of the complexity and unknown chemical composition of the tested groundwater samples. Typically, researchers use reverse dosimetry to extrapolate bioactive concentrations for individual chemicals in in vitro test systems to the comparable doses for in vivo exposure to test species or to humans.[33] Furthermore, in vitro assays have limited metabolic activity; understanding of the metabolism is critical to estimating in vivo toxicity of chemicals. Finally, predicting in vivo adverse outcomes without dose- and temporally intensive data brings additional uncertainty; the exposure regimen utilized by our study may not be representative of the likely environmental exposure. Thus, we limit our discussion of human health risks to suggest the direction for future studies.

The effects observed at the molecular level do not necessarily indicate adverse outcomes at the organismal level. The activation of the NRF2ARE pathway was likely initiated to protect cells from oxidative stress-induced cell death.[34] Activation of PXR and AhR might be a sign of chemical exposure and metabolism. PXR is activated by many endogenous and exogenous chemicals and one of the primary targets of PXR activation is the induction of CYP3A4, a phase I oxidative enzyme that aids with metabolism of chemicals.[35] PXR has been shown to be one of the most sensitive targets that is activated in these assays,[36] which is consistent with its well-documented role in xenobiotic sensing and metabolism.[37] Thus, induction of PXR by all groundwater samples (including background) is not surprising, nor should be assumed to lead to adverse outcomes. Similarly, AhR is best known for its role in mediating metabolism of xenobiotics.[38] AhR is typically involved in, though not limited to, regulation of biological responses to aromatic hydrocarbons (PAHs) and dioxins.[13,39] Activation of the AhR by exogenous chemicals has been shown to cause a range of adverse effects in fish, especially during early life stages.[40] Nevertheless, it is important to note that both PXR and AhR also mediate a variety of endogenous, "normophysiological" biological processes.[38,41] For example, AhR signaling has been indicated in mediation of neurogenesis and neuronal cell development, hematopoiesis, cardyomyogenesis, and endocrine regulation.[38] PXR is thought to regulate hepatic glucose and lipid metabolism, and the activation of PXR has been shown to cause glucose intolerance and hepatic lipid accumulation.[41] Thus, we propose that the interaction of the groundwater samples with AhR and PXR remains of interest, and that the future studies should determine whether adverse effects on the above pathways modulated by AhR and PXR are observed in vivo.

Activation of ERs might have implications for human health; dysregulation of estrogen receptor mediated pathways has been associated with endocrine disruption.[42] The query of the Comparative Toxicogenomics Database (ctdbase.org) for estrogen receptor (NCBI ID 2099) disease associations indicated breast, lung, prostatic and liver neoplasms, oligospermia, male and female infertility, hepatocellular carcinoma, and neoplastic cell transformation (those were top 10 disease associations based on the direct evidence query; CTD inference score >113).

Interference with normal PPAR function by contaminants can lead to the alteration of lipid and glucose metabolism and cellular differentiation.[43] PPARs have been identified as modulators of metabolic syndrome, cardiovascular disease, immune impairment and have been associated with the increased risks of cancer.[44]

Relationship of Biological Activity to Water Chemistry. Figure 3 shows concentrations of TPHd and NVDOC versus the upregulation of AhR and PXR at in situ concentrations (1×). A positive relationship between TPHd and AhR is evident, with AhR increasing from 1.6 in the background to 15.4 fold-increase in the most contaminated portion of the plume, as TPHd increases from below detection in the background to 6.6 mg/L in the most contaminated

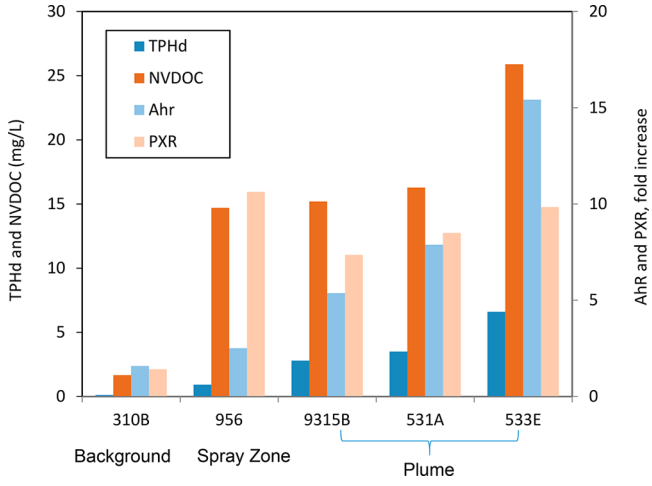

Figure 3. Concentrations of total petroleum hydrocarbons in the diesel range (TPHd), nonvolatile dissolved organic carbon (NVDOC) plotted together with upregulation of AhR and PXR at 1× concentration in the five tested wells. TPHd and AhR increase together. PXR is high at well 956 where TPHd is low but NVDOC is high indicating that the partial transformation products affect the response.

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portion of the plume. In contrast, TPHd and PXR appear less related because PXR activation is high in the spray zone where TPHd is low. PXR is more closely associated with NVDOC, suggesting that the upregulation of PXR may be related to transformation products created by photo-oxidation and aerobic biodegradation. It should be noted that bioeffects assays were conducted on sample extracts. Other potentially toxic components in the groundwater removed during extraction, such as arsenic and BTEX, may have additional biological effects. This underscores the importance of conducting additional studies that explore additive or antagonistic effects of multiple components in complex natural samples.

Although limited in scope, these results show a positive relationship between a complex mixture of nonvolatile hydrocarbons and transformation products, measured as NVDOC, and a subset of molecular targets, thus demonstrating a gap in our current understanding of the nature and effects of hydrocarbon-impacted waters. Though we recognize that correlation is not causation, the stronger relationship between some of the molecular targets (e.g., PXR targets) and NVDOC rather than TPHd demonstrates the importance of improving our understanding of the potential toxicity associated with the unknown transformation products present in hydrocarbon-impacted waters.

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 MNA as a remediation strategy for waters impacted by petroleum contamination. This need is particularly urgent given the ubiquitous nature of both new and residual source contamination and the need to protect drinking water quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b03657.

Site map, hierarchical clustering of the molecular target responses, list of molecular targets for TRANS assays, list of molecular targets for CIS assays, and TRANS-FACTORIAL and CIS-FACTORIAL molecular target activation by environmental sample concentrates (1×) (PDF)

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Notes

The authors declare no competing financial interest.

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