DISSERTATION

ASSESSMENT OF WATER QUALITY, TOXICITY AND TREATMENT STRATEGIES DOWNSTREAM OF NPDES OIL AND GAS PRODUCED WATER DISCHARGES INTENDED FOR BENEFICIAL REUSE

Submitted by

Molly Cook McLaughlin

Department of Civil and Environmental Engineering

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2019

Doctoral Committee:

Advisor: Thomas Borch
Co-Advisor: Jens Blotevogel

Juan Lucas Argueso
Paula Mouser
Tom Sale

ABSTRACT

ASSESSMENT OF WATER QUALITY, TOXICITY AND TREATMENT STRATEGIES DOWNSTREAM OF NPDES OIL AND GAS PRODUCED WATER DISCHARGES INTENDED FOR BENEFICIAL REUSE

Produced water is the largest waste stream associated with oil and gas operations. This complex fluid contains petroleum hydrocarbons, heavy metals, salts, naturally occurring radioactive materials (NORMs) and any remaining chemical additives. In the United States, west of the 98th meridian, the federal National Pollutant Discharge Elimination System (NPDES) exemption allows release of produced water for agricultural beneficial reuse if it is of "good enough quality." Due to the complex and variable composition of produced water as well as the variations in permit effluent limits and treatment approaches, the downstream impacts of NPDES produced water releases are not fully understood.

The goal of this dissertation was to determine if the current NPDES produced water permit effluent limits are adequate and if not, to identify additional steps that can be taken to improve water quality. As a first step towards this goal, a detailed chemical and toxicological analysis was conducted on a stream composed of produced water released for agricultural beneficial reuse. Over 50 geogenic and anthropogenic organic chemicals not specified in the effluent limits were detected at the discharge including hydrocarbons, halogenated compounds, and surfactants. Most were removed within 15 km of the discharge due to volatilization, biodegradation, and sorption to sediment. Additionally, the attenuation rate increased substantially in a wetland downstream of the discharge point. Tens of inorganic species were also detected in the watershed, including many sourced from produced water. In contrast to organic chemicals, the concentrations of most inorganic species increased downstream

due to water evaporation. This included contaminants of concern such as boron, selenium and total dissolved solids (TDS).

An assessment of regulatory health thresholds revealed that eight of the organic species detected at the discharge were listed by the U.S. Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) to be known, probable or possible carcinogens. Mutagenicity of this water was assessed using a yeast mutation assay that analyzed copy number variation (CNV) duplications, CNV deletions, forward point mutations and reversion point mutations. These mutations are established as having a role in human disease, including cancer. Higher rates of mutation were observed at the discharge point and decreased with distance downstream. This correlated with the concentrations of known carcinogens detected in the stream including benzene and radium. Mutation rate increases were most prominent for CNV duplications and were higher than mutation rates observed in mixtures of known composition containing all detected organic carcinogens in the discharge. In addition, samples were evaluated for acute toxicity in *Daphnia magna* and developmental toxicity in zebrafish (*Danio rerio*). Acute toxicity was minimal, and no developmental toxicity was observed.

Finally, in response to the observation that attenuation of organic chemicals increased in wetlands, constructed wetlands downstream of three different NPDES produced water discharges, including the discharge of focus in the chemical and toxicological analysis, were evaluated for their viability to polish produced water. The results showed that wetlands are effective at attenuating commonly used non-ionic surfactants, as well as a commonly used biocide. Attenuation was not only due to degradation, but also accumulation in sediments. Sediment accumulation has the potential to limit the lifetime of the wetlands or increase the frequency with which sediment must be excavated.

The results of this dissertation identified multiple improvements that can be made to NPDES produced water regulations. Current regulations apply to the discharge site only. This dissertation

shows that downstream changes in water quality must be considered to adequately evaluate potential impacts of produced water discharges, as exemplified by the increasing concentrations of inorganic species downstream. Secondly, toxicological results showed that chemical analysis alone is insufficient to assess impacts of these releases and that a thorough assessment of chronic toxicity is necessary to fully assess produced water for beneficial reuse. Current regulations require acute toxicity testing, but no assessment of chronic toxicity. Finally, prior to widespread implementation of constructed wetlands for produced water treatment, additional research is needed to assess the impact of oil and gas chemical additives on the maintenance schedules of these systems, as well as the long-term impact to soil health. If these waters can be reused safely and economically, many stakeholders stand to benefit. If this practice is expanded prematurely, the quality and health of water, soil, crops and downstream users could be negatively impacted. The research contained in this dissertation is one step in a life-cycle analysis of the costs, impacts and benefits associated with oil and gas extraction.

ACKNOWLEDGEMENTS

I would like to thank my advisors, Thomas Borch and Jens Blotevogel, for their support and guidance throughout my graduate school experience. They both worked extremely hard to find funding and opportunities for me to conduct research on a topic I am extremely passionate about and I am very grateful for that. I would also like to thank my committee members for their support throughout my degree. Tom Sale provided long-term financial support for my research by supporting me as a GRA. Lucas Argueso provided me with funding, lab space, and materials, for which I am also extremely grateful. I would also like to thank Paula Mouser for her guidance and perspectives throughout my graduate experience.

My husband, Zach Taylor, was a constant source of support and encouragement throughout this experience. I am lucky to have him as a partner. I would like to thank Ranger, the best dog in the world, who was always there for a walk or a cuddle when I needed it. My parents were extremely helpful throughout this process – always picking up the phone when I needed someone to talk to and always making sure to acknowledge even my smaller milestones. I would also like to thank my brother Paul, sister-in-law Helen and niece Julia who supported me and always seemed to Facetime just when I needed a break in the lab. Additionally, my friend Ashley Taylor, has been extremely supportive and helped me find ways to grow and improve my mental health throughout this process.

I would also like to thank other people who helped in my technical and professional growth during graduate school. I am extremely grateful to both Treasure Bailley and Tricia Pfieffer who helped throughout my degree, especially with field work, experimental design and networking opportunities. Bill Schroeder and Amy Clark also helped with field work and navigating EPA regulations. Bonnie McDevitt also helped with experimental design and was a great colleague and friend to brainstorm with throughout this project. Ruthie Watson and Baylee Schell contributed heavily to the lab work in

Chapter 3 and Ruthie was extremely valuable in data interpretation and statistical analysis. Both Brian Cranmer and Greg Dooley were extremely helpful in LC-MS method development and data interpretation throughout my degree. I would also like to thank the numerous collaborators and researchers that helped me in my research and allowed me to learn more by involving me in their research including Andrea Hanson, Nathanial Warner, Tzahi Cath, Karl Oetjen, Cloelle Danforth, Linsey Shariq, Bill Burgos, Benay Akyon, and many others, some of whom who are listed in Chapter 1 of this dissertation. I would also like to thank the landowners I worked with at our field site who assisted me in many ways – with sampling, driving me around the field site and also showing me how complicated these issues are and how important it is to work with and talk with those who are directly impacted by the water-energy nexus. I would also like to thank the operators at our field site who took the time to show me the site and answer my questions. Finally, I would like to thank the members of all three labs I was involved in during my graduate experience – the Borch Lab, the Center for Contaminant Hydrology and the Argueso Lab. I learned so much about science and life from everyone in these labs and am grateful to have them as colleagues and friends.

Finally, I am incredibly grateful for the community I have found in Fort Collins. The members of Graduate Women in Science have always been extremely supportive of me and show me how to best support myself and others. I have made numerous lifelong friends during my time in Fort Collins – many of whom have cooked me dinners and bought me beers on days where I felt too busy or overwhelmed; and many others who have joined me in outdoor pursuits that allowed me to see beautiful places and remind me of the importance of work-life balance. On the days when things were especially hard, I always knew this experience was worth it because of the community I have found in Colorado.

This research was funded by the Colorado Water Center, the Colorado State University School of Global and Environmental Sustainability, the Environmental Defense Fund, and the National Institute of Health grant awarded to Lucas Argueso (R35GM119788).

This dissertation often uses "I" and "my" to describe this work. This is solely a formality, and the credit for this work is rightfully shared amongst me, Thomas Borch, Jens Blotevogel and our collaborators.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1 Natural Gas and Oil Production in the United States

Production of natural gas and oil in the United States has increased drastically over the past decade as a result of the hydraulic fracturing "boom", which began in the late 2000s. Hydraulic fracturing has been used in the oil and gas industry since the 1950s; however, the surge in production began when hydraulic fracturing was combined with horizontal (i.e., directional) drilling.[1] By combining these two technologies, many hydrocarbon formations that were previously too expensive to produce became economically viable. The number of hydraulically fractured wells in the U.S. increased from 36,000 in 2010 to more than 300,000 in 2015.[2] Production of natural gas increased from 1.5 billion cubic meters (Bcm) (52 billion cubic feet (Bcf)) per day in 2005 to almost 2.3 Bcm (80 Bcf) per day in 2015 (Figure 1). In 2005, 25% of this production was from hydraulically fractured wells and by 2015, 67% of natural gas production was from hydraulically fractured wells (Figure 1).[2]

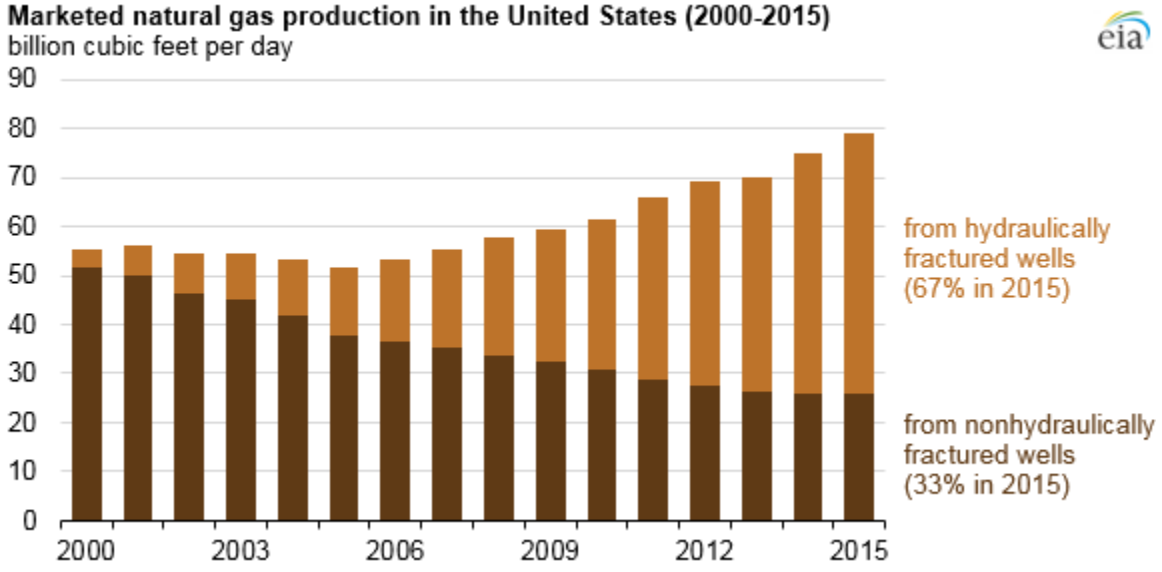

Figure 1. Production of natural gas in the United States between 2000 and 2015. Natural gas production in the U.S. begins to increase in 2006, as does the percentage of natural gas produced from hydraulically fractured wells. Adopted from, U.S. Energy Information Administration artide "Hydraulically fractured wells provide two-thirds of U.S. natural gas production."[2]

A similar trend occurred for oil production in the U.S., which was just below 795 million liters (5 million barrels) a day in 2008 and increased to more than 1.4 billion liters (9 million barrels) per day in 2015 (Figure 2). In 2008, nearly 10% of oil production was from hydraulically fractured wells and by 2015, 51% of oil production was from hydraulically fractured wells (Figure 2).[3] Production of natural gas and oil from hydraulically fractured wells is expected to increase through 2050.[4]

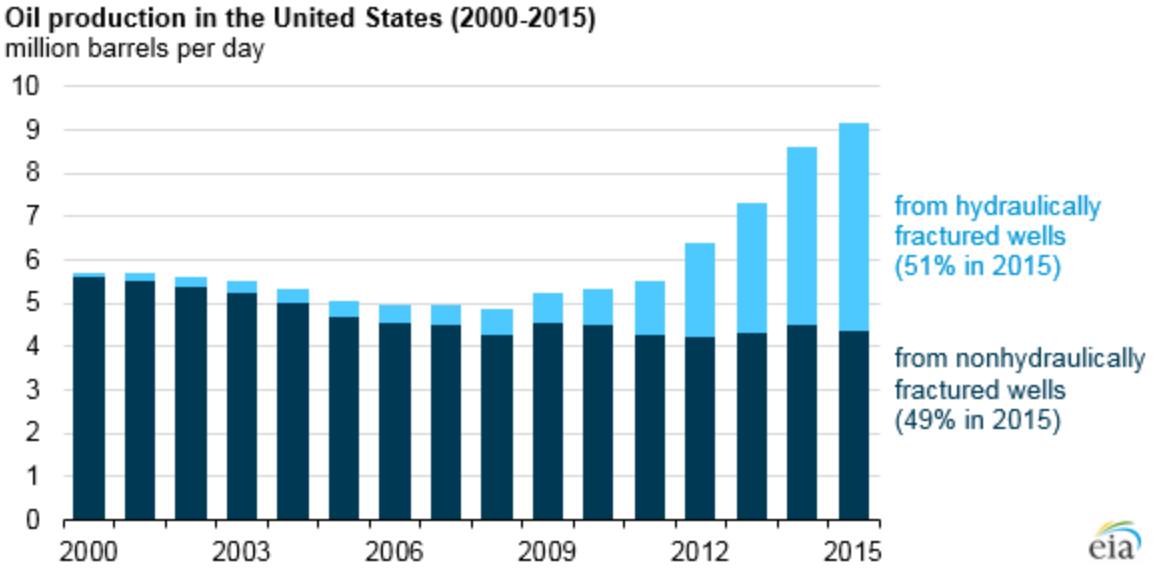

Figure 2. Production of oil in the United States between 2000 and 2015. Oil production in the U.S. begins to increase in 2009, as does the percentage of oil produced from hydraulically fractured wells. Adopted from, U.S. Energy Information Administration artide "Hydraulically fracturing accounts for about half of current U.S. crude oil production."[3]

1.2 Produced Water

1.2.1. Composition and Volume

There are a variety of waste streams generated in oil and gas production including spent drilling fluids, used drilling muds, drill cuttings and produced water (PW), all of which are generated during conventional and unconventional (e.g., hydraulic fracturing) oil and gas extraction. Of these waste streams, PW is the largest by volume, with more than three trillion liters generated each year in the United States.[5]

PW is generated from the hydrocarbon-bearing formation. During the extraction process, PW is brought to the surface simultaneously along with oil and gas. In most cases, the oil-gas-PW mixture is sent to a three-phase separator (oil, gas, water) which uses heat, gravity, and emulsion-breaking chemicals to separate the different fluids. PW can include both formation water, which is the water naturally present in the oil and gas formation, and injection water, which may be added for purposes such as hydraulic fracturing. Because of its geogenic origins, PW contains elevated levels of species associated with the oil and gas depositional environment. The major classes of chemicals include hydrocarbons, salts, metals and naturally occurring radioactive materials (NORMs).[5] It also contains any remaining drilling, stimulation or well maintenance chemicals as well as their transformation products.[6-7] Additionally, PW may contain a variety of microorganisms.[8-10]

The composition of PW varies by geologic formation, over time and with the type and quantity of chemical additives used. In the U.S., total dissolved solids (TDS) can range from as low as 100 mg/L to more than 400,000 mg/L.[5, 11-12] In general, TDS is lower in Colorado, Wyoming and California (e.g., Niobrara and Monterey formations) and higher in Texas, Pennsylvania and North Dakota (e.g., Haynesville, Marcellus and Bakken formations).[11] In the Marcellus shale, total radium (^{226}Ra + ^{228}Ra) concentrations can be as high as 670 Bq/L and 26 Bq/L in unconventional and conventional operations, respectively.[13] In the Niobrara formation, however, total radium concentrations are low compared to other parts of the U.S. (~3 Bq/L).[8] In addition to radium, other commonly found NORMs include uranium, thorium and radon.[5] Metals found at elevated levels in PW include, but are not limited to, arsenic, barium, cadmium, lead and strontium. Abundance and presence of these species also vary by location. Organic matter in PW includes petroleum-derived hydrocarbons and natural organic matter. Total organic carbon (TOC) concentrations range as high as 2,000 mg/L.[5] Total hydrocarbon concentrations have been reported between 40 mg/L to 2000 mg/L.[12] Finally, PW composition is impacted by the drilling, stimulation and well maintenance

chemicals in use.[8, 14-15] On average, 10s of different chemicals are used per well, however, there are hundreds to choose from. As a result of variations in chemicals additives, there are different transformation by-products as well. Periodic fluctuations in composition occur as the result of well maintenance and stimulation activities, which may occur every few months or years.

The volume of PW also varies by geologic formation and with the age of the well. Operators have reported PW-to-oil ratios of less than 1:1 and as high as 1000:1.[1] On average, 7-10 barrels (1000 – 1600 liters) of PW are generated per barrel of crude oil in the U.S.[5] In general, for hydraulically fractured wells, PW and hydrocarbon generation are highest initially and decrease over time.[16-17] For conventional wells, however, PW generation usually increases with the age of the well.[5] As a result of the surge in U.S. oil and gas production, PW volumes have increased substantially and are expected to increase in the future.[1]

1.2.2. Produced Water Management

Management of PW is a considerable cost for oil and gas operations. When the cost of managing PW exceeds profits, the well is temporarily or permanently closed. In some cases, if oil prices rise, the well may start producing again.[18] Disposal into Class I and II underground injection (UIC) wells is the most common PW management practice because it is the least expensive in many areas.[1, 19] Re-injection for enhanced oil recovery is common throughout the U.S. and reuse for hydraulic fracturing is also common in some areas.[20] In total, nearly 85% of PW is disposed of in UIC wells or re-injected for enhanced oil recovery. Underground injection for disposal is only possible in locations with underground geology capable of receiving the water.[5] This practice is common in states such as Colorado, Oklahoma, and Ohio, but absent in others, like Pennsylvania.[18] This practice is limited by the fact that high rates of underground injection have been linked with earthquakes.[21] Additionally, water for disposal is generally not treated and the consequences of contamination due

to unintended releases is high.[1, 22-24] In remote areas and in areas with high PW volumes, trucking costs may make underground injection too expensive.[25]

Nearly 13% of PW is managed via reuse or recycling.[19] This includes disposal at centralized wastewater treatment plants (CWTPs) and beneficial reuse outside of the oil and gas sector (e.g., agricultural uses, road spreading, etc.). As a result of limitations associated with UIC wells and water scarcity, government agencies and oil and gas producers are increasingly looking for treatment options and ways to reuse PW. This is exemplified by the ongoing U.S. Environmental Protection Agency "Study of Oil and Gas Extraction Wastewater Management" which aims "to understand any potential need for, and any concerns over, additional discharge options for onshore oil and gas wastewater" and the U.S. Department of Energy's Water Security Grand Challenge which aims to find a cost-effective PW treatment approach for both agricultural and industrial reuse.[1, 26] Additionally, the state of New Mexico recently entered into a memorandum of understanding (MOU) with the U.S. EPA in 2018 related to re-use, recycling and beneficial reuse of PW in the state.[27]

1.2.3. NPDES Permits for Produced Water Management

Under the Clean Water Act it is illegal to discharge pollutants from a point source into a water of the United States unless the entity obtains a National Pollutant Discharge Elimination System (NPDES) permit. The aim of the NPDES program is to regulate pollution from point sources to ensure the discharge is safe for human and ecosystem health. Pollutants include any industrial, municipal or agricultural waste that is discharged into water. NPDES permits may be required for discharges from a variety of entities including concentrated animal feeding operations (CAFOs), landfills, hospitals, CWTPs and oil and gas facilities.

Management and discharge of PW at CWTPs occurs primarily in Pennsylvania, Ohio and West Virginia in the Marcellus and Utica shale regions.[1, 28] NPDES permits are required for these facilities to discharge PW or other wastewaters. These plants are often not properly designed to remove PW

contaminants, resulting in environmental and ecological issues downstream.[28-30] These issues will be discussed in depth later in this chapter.

Beneficial reuse of PW for agricultural purposes also requires a NPDES permit if the water is released to surface water. The Clean Water Act (CWA) states that "there shall be no discharge of waste water pollutants into navigable waters from any source associated with production, field exploration, drilling, well completion or well treatment (i.e., PW, drilling muds, drill cuttings, and produced sand)." For onshore wells located west of the 98th meridian, however, Subpart E – Agricultural and Wildlife Water Use Subcategory regulates the discharge of PW for agricultural or wildlife propagation. This rule requires that the PW (1) "is of good enough quality to be used for wildlife or livestock water or other agricultural uses", (2) "is actually put to use during period of discharge", and (3) does not exceed the effluent limitation of 35 mg/L oil and grease. Besides the oil and grease limitation, "of good enough quality" is not defined through any other federal regulatory limits. State and federal regulators, however, generally include additional effluent limits when writing NPDES O&G PW permits.

Nearly 80% of PW in the United States is generated in the arid West, where annual precipitation rates are substantially lower than in other areas (Figure 3).[5] The amount of PW varies by location and can be substantial in some areas. Discharge of oil and gas PW under the NPDES permit agricultural and wildlife water exemption occurs primarily in Wyoming and has been occurring for decades.[1] This option is currently only economically viable in areas where TDS is below a few thousand parts per million.[1] PW reuse for irrigation, which occurs primarily in California, is another management approach where there are many unknowns and where more research is needed. This practice does not require a NPDES permit, however, because it does not involve discharge to surface waters.[1] Under the NPDES exemption, however, PW released to surface water could be used for irrigation. The studies presented in the main chapters of this dissertation focus on a well field in

Wyoming where oil and gas PW is released to surface water under the NPDES exemption for beneficial reuse.

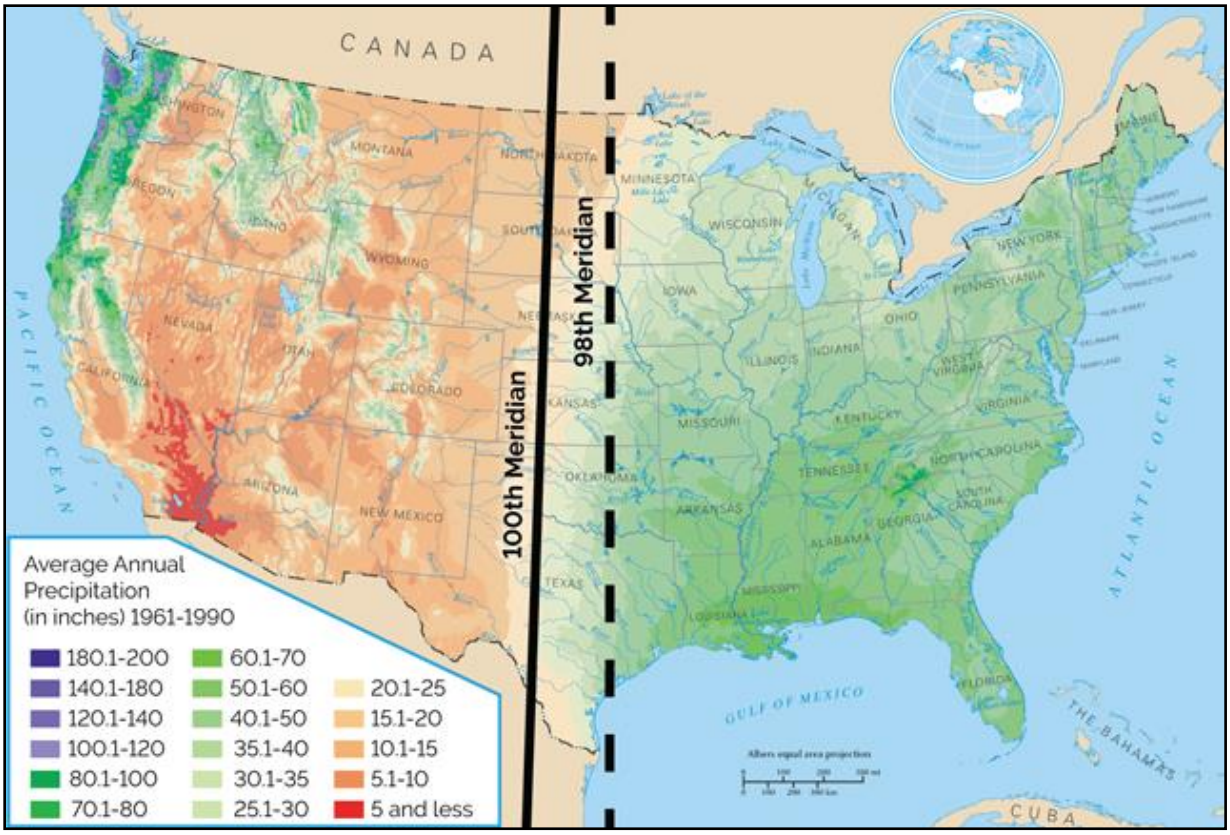

Figure 3. Map of the United States showing the location of the 98th meridian and also the average annual precipitation.[31]

1.3 Impacts Downstream of NPDES Oil and Gas Produced Water Releases

To date, little research has been conducted on the impacts of oil and gas PW released for beneficial reuse under the NPDES agricultural and wildlife exemption. A study that I contributed to as co-author studied the field site that will be discussed in this dissertation. This study found that 3 billion Bq of radium (^{226}Ra + ^{228}Ra) were released at this site annually and that 95% of that radium was transported farther than 100 m from the discharge. Radium activity in sediments downstream of NPDES PW releases was elevated as compared to control sites and increased levels of radium were found as deep as 30 cm below ground surface (bgs). Additionally, in areas where PW was released

directly into an ephemeral stream bed, increases in TDS were observed downstream and attributed to evaporation.[32]

Previous studies on coalbed methane PW discharges in Wyoming have attributed increases in selenium and other inorganic chemicals downstream to both evaporation and increased leaching of naturally present species in the soil and rock, as a result of the PW.[33-34] Irrigation with coalbed methane PW has been linked to chloride accumulation and an increased sodium adsorption ratio (SAR).[35-36] Coalbed methane PW is generally lower in salinity and other contaminants than PW from oil and gas so these results only serve as guidelines for oil and gas PW releases. A recent greenhouse study showed that irrigating wheat with diluted PW (10% and 50%) resulted in decreased physiological characteristics including grain yield, biomass, photosynthetic efficiency and reproductive growth as compared to crops irrigated with tap water.[37] Additionally, this study showed that in addition to salt, other constituents in PW negatively impacted plant growth and health.[37] Another greenhouse study irrigated rapeseed and switchgrass plants with synthetic oil and gas PW and found that as TDS and TOC increased, plant health and growth were negatively impacted.[38]

More research has been conducted on PW releases from CWTPs. Because this practice occurs primarily in the Marcellus and Utica formations, the PW managed at CWTPs is generally higher in TDS and radioactivity (by 1-2 orders of magnitude) than PW released for beneficial reuse. Treatment at CWTPs involves skimming of residual oil off the surface of the water and removing solids via settling ponds. Na_2SO_4 is added to precipitate salts and metals. Flocculation, aerobic digestion and clarification are used to remove organic species.[20, 30, 39] Due to the higher concentrations of TDS and radioactive species, such as radium, treatment at CWTPs targets both organic and inorganic species. This is in contrast to most treatment systems prior to beneficial reuse, which only target organic chemicals. As a result, the findings from studies on CWTPs are not directly applicable to PW releases for beneficial reuse, however, they can serve as guidelines.

One study, which I served as co-author on, analyzed lake sediments downstream of five CWTPs treating oil and gas wastewater. Contaminant signatures associated with PW were present in lake sediment 19 km downstream and persisted in the sediment for at least 10 years. Contaminants included NORMs, salts, metals and nonylphenol ethoxylates, an organic chemical additive used by the oil and gas industry.[28] Other studies have shown increased formation of disinfection by-products (DBPs) downstream of CWTPs treating PW and linked this increase to the high concentration of salt in PW. DBPs are most often formed when oxidizing disinfectants, such as chlorine, ozone and chlorine dioxide, react with natural organic matter or anthropogenic contaminants and the salts, bromide and iodide.[40] Many DBPs are neurotoxic, cytotoxic, mutagenic, genotoxic, carcinogenic and teratogenic.[41] Another study analyzed Sr/Ca and $^{87}Sr/^{86}Sr$ ratios in mussels collected upstream and downstream of CWTPs treating oil and gas PW and showed that oil and gas contaminants can bioaccumulate in mussels, and likely other organisms, downstream of CWTPs used for treatment.[29]

Multiple studies have investigated accidental releases of PW to the environment. Many of these studies have found increased estrogenic and other toxic activities in waters impacted by PW. For example, surface and groundwater samples collected in a drilling-dense region in western Colorado exhibited estrogenic, antiestrogenic, androgenic and antiandrogenic activities at elevated levels as compared to background samples collected from locations with little to no drilling activity. Moderately elevated levels of estrogenic, antiestrogenic, androgenic and antiandrogenic activities were also observed in the much larger Colorado River, which serves as the drainage basin for the sample area.[42] In another study, increased endocrine disrupting activity as compared to background sites collected upstream was observed in surface waters collected near a PW UIC well disposal site in West Virginia.[23] Additional studies at this site found elevated concentrations of organic and inorganic contaminants associated with PW.[22, 24] Increased endocrine and progesterone receptor activities were observed in groundwater collected from areas in Wyoming with a high frequency of oil and gas wells. No major

spills were reported at this site.[43] At a site in North Dakota where 11 million liters of PW were inadvertently released into a stream, increased levels of salts, metals and hydrocarbons were observed more than 20 km downstream of the spill. Concentrations of NORMs, including radium, were 15 times greater at the spill site than background levels. Additionally, fish bioassays revealed substantially decreased fish survival (from 89% upstream, to 2.5% at 7.1 km downstream). Increased estrogenic effects were observed downstream as well.[44] The size of these releases varies, but for all studies, the releases to surface water were unintentional.

1.4 Challenges Associated with Toxicological Analysis of Produced Water

There are many benefits associated with the use of bioassays for toxicological assessment. First, the toxicological impact of a sample can be quantified without determining the detailed chemical composition of the sample. Additionally, a variety of toxicological endpoints can be analyzed including, but not limited to, mutagenicity, endocrine disruption and developmental toxicity. There are also challenges associated with toxicological analysis of PW, many of which are due to the complexity and high TDS content of this waste stream. TDS in PW can range over multiple orders of magnitude (~500 ppm to 400,000 ppm). Increased levels of TDS can cause osmotic stress in organisms used in bioassays, causing acute toxicity and overwhelming bioassays that are designed to analyze chronic effects. Additionally, while TDS is a major contributor to toxicity in many PWs, it is not the only source of toxicity. Thus, non-saline toxicity must be considered as well.[45] In PW where TDS is high, determining the toxicity of the non-saline component is challenging. In many approaches, such as effect-directed analysis (EDA) and the toxicity identification evaluation (TIE), complex waste streams such as PW are often diluted or fractionated in order to determine the toxicity of different groups of chemicals.[46] The components of TDS, however, may have synergistic or antagonistic effects on the toxicity of other chemicals within the mixture. Thus, dilution and fractionation would skew the toxicological results. The approach outlined in Danforth et al, 2019 suggests using the toxicity

identification evaluation (TIE) approach to fractionate and dilute PW samples into a salt and organic fraction.[45] Once the toxicity of the individual mixtures is determined, the salt mixture can be titrated into the organic mixture to further understand mixture effects.

It is also important to consider which types of organisms and which types of assays are in use. *In vitro* bioassays, such as the *Salmonella* Ames test and reporter gene assays conducted in human cell lines, are generally less expensive and/or less time consuming than *in vivo* assays and are therefore used in more studies. In addition to this approach, however, *in vivo* methods are necessary as well since these assays allow for evaluation of complex endpoints that are more difficult to test without whole organism testing.[45] In many cases, the results of *in vivo* tests can be verified and expanded upon by the use of *in vitro* assays. Additionally, differences in organism type, assay protocol and data analysis may result in differing results from toxicological assays.[47] Relatedly, there are challenges associated with relating the results of *in vivo* and *in vitro* tests conducted in organisms such as yeast, bacteria or fish to the expected outcome in mammals, such as humans. Thus, there is a need for standard bioanalytical tools for use in PW toxicity testing. Until that point, however, results from different studies should be viewed with this lens.

1.5 Research Objectives

Previous studies clearly show that insufficient treatment of PW is occurring at CWTPs, resulting in environmental and ecological issues downstream. Beneficial reuse of PW for agricultural purposes is becoming more common in the U.S. American West; however, the impacts of this practice are not well understood. If these waters can be reused safely and economically, many stakeholders stand to benefit. If this practice is expanded prematurely, the quality and health of water, soil, crops and downstream users could be negatively impacted. This would result in thousands of legacy sites that must be remediated, and oil and gas operators may be subject to liability and clean-up costs. The **overarching objective of this study** is to determine if the current NPDES permit effluent limits are

adequate and if not, to identify additional steps that can be taken to improve water quality. This includes additional permit effluent limits, monitoring requirements and treatment options.

In order to achieve this ambitious goal, PW intended for beneficial reuse must be characterized so that treatment methods can be developed and properly assessed. The **first objective of this study** is to conduct a thorough chemical characterization of PW released for beneficial reuse, including an analysis of the environmental fate and transport of chemicals downstream. The **second objective of this study** is to quantify the toxicity of PW released for beneficial reuse. These two objectives are closely related and will be addressed in Chapter 2 and Chapter 3, respectively.

The major chemical classes of PW are well known. The exact composition of PW remains unknown, however, despite the numerous studies which aimed to characterize this fluid. Determining the composition of PW is challenging because 1) there is a lack of analytical methods for numerous chemicals in PW, 2) matrix effects from chemicals (e.g., salts) in this complex solution make detection of other chemicals more challenging, 3) transformation products for many of the chemical additives are unknown, and 4) the composition is highly variable.[8, 14, 48-49] As a result of these unknowns and complexities, an extensive chemical analysis of this water will likely be insufficient for determining the environmental and health risks of this water; however, a detailed chemical characterization is necessary to test if this is true. Firstly, the potential to induce toxic effects may not be known for some of the detected chemicals. Secondly, many analytes - including potentially more toxic transformation intermediates - may go undetected as their concentrations are below instrumental detection limits; yet, these compounds may still be toxic at low concentrations.

Another aim of this dissertation was to determine if using bioassays to quantify toxicity, in addition to chemical characterization, is a more effective method for characterizing the environmental and ecological risks associated with PW releases. The behavior of chemicals in complex mixtures strongly depends on their mode of toxic action. While mixtures of chemicals with a common target

site and the same mode of action act according to concentration/dose additivity, antagonistic or synergistic effects may arise if mixture components interact with each other. Thus, a more integrative chemical and toxicological assessment of these waters is urgently needed to evaluate the risks and impacts associated with current PW beneficial reuse treatment and regulatory practices. By combining the results of the chemical and toxicological studies, best practices can be developed to improve the effluent limits and optimize current treatment strategies, if necessary.

The **third objective of this study** is to assess the viability of constructed wetlands for PW treatment downstream of NPDES releases, with a focus on the environmental fate, transport and removal mechanisms of oil and gas additives. This objective will be addressed in Chapter 4 of this dissertation. Extensive research is being conducted on treatment methods for PW. A variety of approaches have been proposed including membrane separation, membrane distillation, forward osmosis, electrocoagulation, advanced oxidation processes, adsorption, and biological treatment.[7, 50-51] For many oil and gas operations, especially those in rural areas, these treatment methods remain financially and technologically infeasible.[52] Constructed wetlands are a relatively cheap and low-maintenance treatment option that may be viable in some areas; however, more research is needed to understand mechanisms of attenuation in these systems. A better understanding of attenuation mechanisms will allow for improved design parameters and more complete risk assessment of these systems. It is hypothesized that organic oil and gas chemical additives will be attenuated in wetlands as a result of biodegradation and sorption. This hypothesis will be tested by determining the distribution and fate of oil and gas additives in water and sediments samples. In addition, a microbial analysis on sediment and water samples will be conducted. This analysis will aim to determine which organisms are present and if populations change downstream of NPDES releases, with a specific focus on locations immediately upstream, downstream and within wetlands.

1.6 Publications

As a result of my PhD research, I expect to publish 3 first author and 8 co-author peer-reviewed papers. Most of this dissertation work is planned for submission into peer-reviewed journals. Chapters 2 and 3 are in preparation for the Society of Environmental Toxicity and Chemistry (SETAC) journal Environmental Toxicity and Chemistry (ET&C). These manuscripts were submitted for publication in August 2019. Chapter 4 will be submitted for review by the end of 2019. Parts of this dissertation have also been presented at several national and international conferences including the 253rd and 255th American Chemical Society National Meetings in San Francisco, CA (2017) and New Orleans, LA (2018), the University Consortium for Field-Focused Groundwater Contamination Research Annual Progress Meeting in Guelph, ON, Canada (2018), the Remediation Technology Summit (RemTEC) in Denver, CO (2019) and the American Geophysical Union Hydrology Days meeting in Fort Collins, CO (2017).

In addition to the main chapters in this dissertation, I've contributed as a co-author to Oetjen, K.; Giddings, C. G. S.; McLaughlin, M.; Nell, M.; Blotevogel, J.; Helbling, D. E.; Mueller, D.; Higgins, C. P., Emerging analytical methods for the characterization and quantification of organic contaminants in flowback and produced water. *Trends in Environmental Analytical Chemistry* 2017, *15*, 12-23 which addresses analytical methods and challenges for organic chemicals in oil and gas PW.[48] I also contributed to Burgos, W. D.; Castillo-Meza, L.; Tasker, T. L.; Geeza, T. J.; Drohan, P. J.; Liu, X.; Landis, J. D.; Blotevogel, J.; McLaughlin, M.; Borch, T.; Warner, N. R., Watershed-Scale Impacts from Surface Water Disposal of Oil and Gas Wastewater in Western Pennsylvania. *Environmental Science & Technology* 2017, *51* (15), 8851-8860. As mentioned in an earlier section of this Chapter, this manuscript addresses impacts to lake sediments downstream of CWTPs treating oil and gas PW by quantifying both organic and inorganic chemicals versus depth in sediment cores.[28]

Additionally, in conjunction with the studies presented in this dissertation, I contributed to McDevitt, B.; McLaughlin, M.; Cravotta, C. A.; Ajemigbitse, M. A.; Van Sice, K. J.; Blotevogel, J.; Borch, T.; Warner, N. R., Emerging investigator series: radium accumulation in carbonate river sediments at oil and gas produced water discharges: implications for beneficial use as disposal management. *Environmental Science: Processes & Impacts* 2019, 21, 324-338. This manuscript focused on the field site that is the focus of this dissertation and analyzed radium accumulation downstream of the NPDES PW discharges on site.[32] I have also contributed to McDevitt, B.; McLaughlin, M.; Geeza, T.; Vinson, D.; Coyte, R.; Blotevogel, J; Borch, T.; Warner, N.R., Fingerprinting Salinization from Beneficial Use of Oil and Gas Produced Water in the Western U.S., which is currently in preparation and also focused on the site in this dissertation.

I also contributed as co-author to Akyon, B.; McLaughlin, M.; Hernández, F.; Blotevogel, J.; Bibby, K., Characterization and biological removal of organic compounds from hydraulic fracturing produced water. *Environmental Science: Processes & Impacts* 2019, 21, 279-290 which assessed the biological treatment of organic chemicals in PW;[7] Hanson, A. J.; Luek, J. L.; Tummings, S. S.; McLaughlin, M. C.; Blotevogel, J.; Mouser, P. J., High total dissolved solids in shale gas wastewater inhibit biodegradation of alkyl and nonylphenol ethoxylate surfactants. *Science of The Total Environment* 2019, *668*, 1094-1103 which addresses the impact of TDS on the biodegradation of alkyl and nonylphenol ethoxylate surfactants[6]; and, Evans, M. V.; Getzinger, G.; Luek, J. L.; Hanson, A. J.; McLaughlin, M. C.; Blotevogel, J.; Welch, S. A.; Nicora, C. D.; Purvine, S. O.; Xu, C.; Cole, D. R.; Darrah, T. H.; Hoyt, D. W.; Metz, T. O.; Lee Ferguson, P.; Lipton, M. S.; Wilkins, M. J.; Mouser, P. J., In situ transformation of ethoxylate and glycol surfactants by shale-colonizing microorganisms during hydraulic fracturing. *The ISME Journal* 2019 which assessed the transformation of surfactants by shale-colonizing microorganisms.[53] Finally, I also contributed to Shariq, L.; McLaughlin, M.; Rehberg, R.; Blotevogel, J.; Borch, T. Impacts of Hydraulic Fracturing Chemicals on Wheat Plants:

Uptake, Morphological Impacts, and Associated Health Risks which is currently in preparation and assesses plant uptake of organic hydraulic fracturing fluid chemicals in wheat.

CHAPTER 2: ASSESSMENT OF WATER QUALITY DOWNSTREAM OF NPDES OIL AND GAS PRODUCED WATER DISCHARGE: CHEMICAL IMPACTS

2.1 Introduction

Produced water (PW) originating from hydrocarbon reservoirs is extracted concurrently with oil and gas (O&G). This fluid contains elevated levels of chemicals naturally present in the formation, including hydrocarbons and their derivatives, salts, metals and naturally occurring radioactive materials (NORM).[5] It also contains any remaining drilling, hydraulic fracturing, or well maintenance chemicals as well as their transformation products. Composition of this complex fluid varies with time, geologic formation, and variations in chemical use.[8, 14, 18, 54] In the United States, total dissolved solids (TDS) in PW ranges between 100 and 400,000 mg/L;[11-12] radium concentrations range between 3 Bq/L and 67 Bq/L;[8, 13] and total organic carbon (TOC) ranges from below detection limit to 2,000 mg/L.[12]

PW is generated in both conventional and unconventional O&G operations and is the largest upstream waste stream (by volume) associated with O&G. On average in the U.S., each well generates seven to ten times more PW than crude oil, resulting in over three trillion liters of PW per year.[5] Management practices for this waste stream vary by region. Underground injection into Class II disposal wells is the most common management technique in the U.S.; however, high injection rates have been linked to induced seismicity.[21] Treatment at wastewater treatment plants (WWTPs) is another common management approach, but has been shown to increase concentrations of salts, disinfection by-products, and radioactivity downstream.[28, 30, 55]

Economic viability of O&G extraction is partly driven by the high costs of PW management practices at around one to fifteen U.S. Dollars ($) per m^3 of water.[25] This is especially true for older wells, which can generate 50 to 1000 times more PW than oil.[56] Thus, operators are increasingly considering alternative options, including discharging PW for agricultural beneficial reuse.[25]

Municipalities are also interested in this practice because many O&G producing formations in the U.S. are located in the arid West and in dire need of more water to meet demands from citizens, industry, and agriculture.[5]

Under the Clean Water Act it is illegal to discharge pollutants from a point source into a water of the United States unless the entity obtains a National Pollutant Discharge Elimination System (NPDES) permit. The aim of the NPDES program is to regulate pollution from point sources to ensure the discharge is safe for human and ecosystem health. Permits contain limits on both quality and quantity of the discharge(s), and dischargers are required to submit regular reports characterizing the discharge. Pollutants include any type of industrial, municipal or agricultural waste that is discharged into water. NPDES permits are typically required for discharges from a variety of entities including WWTPs, concentrated animal feeding operations (CAFOs), fish hatcheries, landfills, hospitals and industrial mining and O&G facilities.

U.S. Code of Federal Regulations Title 40, Part 435, Oil and Gas Extraction Point Source Category states that "there shall be no discharge of waste water pollutants into navigable waters from any source associated with production, field exploration, drilling, well completion or well treatment (i.e., produced water, drilling muds, drill cuttings, and produced sand)." For onshore wells located west of the 98th meridian, however, Subpart E – Agricultural and Wildlife Water Use Subcategory regulates the discharge of PW for agricultural or wildlife propagation. This rule requires that the PW (1) "is of good enough quality to be used for wildlife or livestock water or other agricultural uses", (2) "is actually put to use during period of discharge", and (3) does not exceed the effluent limitation of 35 mg/L oil and grease. Besides the oil and grease limitation, "of good enough quality" is not defined through any other federal regulatory limits. State and federal regulators, however, generally include additional effluent limits when writing NPDES O&G PW permits.

The lack of both legal definition and available data on the quality of PW discharged under the NPDES program motivated us to investigate the watershed around an O&G extraction site and NPDES PW release in Wyoming. The overarching goal of this study was to increase our understanding of potential impacts of PW beneficial reuse on downstream users and ecosystem services. Our specific objectives were to 1) characterize the chemical composition of the discharge that is being used for beneficial reuse, 2) assess the environmental fate of chemicals in the discharge stream along the flow path, and 3) conduct a systematic evaluation for potential health impacts to humans, livestock and aquatic life based on previously established thresholds and screening levels. Various analytical techniques were used to this end, with the goal of identifying potential contaminants of concern. The health thresholds used in this study are from sources used by regulators when drafting NPDES permits and by farmers when determining safety for their livestock.

2.2 Materials and Methods

2.2.1 Site Description

This study was conducted at an undisclosed well field in Wyoming where over 10 NPDES PW discharges are located. At this site, O&G operations occur in a relatively remote location and there are few other sources of contamination. Analysis focused on one NPDES discharge and the surrounding watershed (Figure 4). Multiple wells contribute PW to this NPDES release, one of which is 100 years old. The operator stated that the PW to oil ratio from this well is 1000:1 and that operation would be economically infeasible if beneficial reuse were not an option.

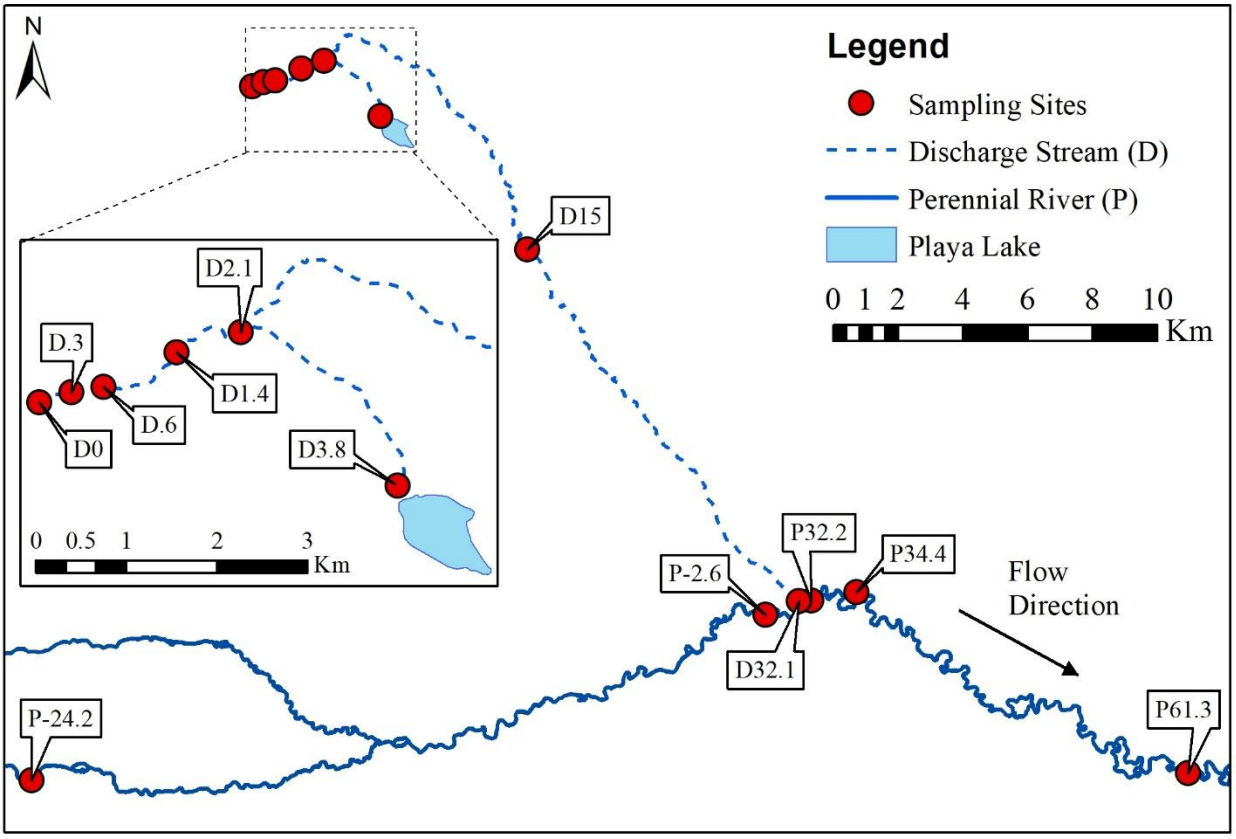

Figure 4. Map of sampling locations at an undisdosed well field in Wyoming. Surface water samples were collected in October 2016 and February 2018 from the discharge (ephemeral) stream (D) and perennial river (P). Site D0 was collected directly from the discharge culvert before entering the stream. All other sites were collected at the indicated distance from the discharge (e.g., D.3 was collected 0.3 km downstream). Sites prefaced with a P were collected on the perennial river, with positive values indicating samples collected downstream of the confluence between the two water bodies (e.g., P32.2 is collected on the perennial river, 32.2 km downstream of the discharge) and negative values indicating samples collected upstream of the confluence (e.g., P-2.6 is collected on the perennial river, 2.6 km upstream of the confluence).

After extraction from the wells, the oil-gas-PW mixture is combined and sent to the treatment system. Treatment includes a three-phase separator (oil, gas, water) which uses heat, gravity, and emulsion-breaking chemicals. Once separated, half of the PW is reinjected into the O&G wells for enhanced oil recovery. The other half is sent to a series of settling ponds where additional oil is removed via flotation and skimming. On average, 4.5 million liters per day of treated PW is released into an ephemeral stream bed from this NPDES discharge. This volume has remained relatively steady since 2005, ranging between 3.6 and 5.5 million liters per day. There is little precipitation in the region

(average 230 mm/year)[57] and no additional tributaries to this stream, resulting in a stream that is composed entirely of O&G PW unless there has been a recent precipitation event. A wetland is located about 2 km from the discharge, followed by a dam that separates the discharge into two equal streams. One continues southeast for about 2 km before emptying into a playa lake that is used by cattle, horses, waterfowl and other wildlife for drinking. Playa lakes are shallow, ephemeral lakes, commonly found in the U.S. High Plains region.[58] The other stream continues another 30 km until connecting with a larger perennial river. Along this 30 km stretch are a series of wetlands that contain fish and serve as watering holes for cattle and other wildlife. The perennial river is used as the drinking water intake for thousands of people downstream. In October 2016 the flow rate of the discharge stream and perennial river were 0.03 m^3s^{-1} (at site D1.4) and 8.5 m^3s^{-1}, respectively.[59] The flow rate in the perennial river was an estimated 6.7 m^3s^{-1} in February 2018.[59] Flow measurements were not taken in the discharge stream in 2018.

Table 1. NPDES permit effluent limits specific to the discharge in this study.

Parameter	Effluent Limitation Daily Maximum
Specific Conductance	7500 µS/cm
Total Dissolved Solids	5,000 mg/L
Chloride	2,000 mg/L
Sulfate	2,500 mg/L
Total Radium 226	60 pCi/L [a]
Oil and Grease	10 mg/L [b]
pH	6.5 - 9.0 [c]

[a] Values taken directly from the permit. 60 pCi/L = 2.22 Bq/L.
[b] Permit also states that there cannot be a "visible sheen in the receiving waters or deposits on the bottom or shoreline of the receiving waters."
[c] pH range given. All other values are maxima.

The daily maximum effluent limits for this specific NPDES permit are shown in Table 1. In addition to these effluent limits, the permit also states that no floating solids or visible foam can be discharged other than in trace amounts. The discharge rate must be reported monthly and sulfide as H_2S must be reported quarterly. A toxic pollutants screen, which includes organic and inorganic

pollutants outlined in U.S. Code of Federal Regulations Title 40, Part 122, Appendix D, must be conducted in the first, third and fifth years of the permit. Permits typically last four to five years. In addition to these chemical limits, acute whole effluent testing (WET) is required quarterly at the site. This involves an acute 48-hour static-renewal toxicity test using *Daphnia magna* and an acute 96-hour static-renewal toxicity test using *Pimephales promelas*. Corrective actions must be taken if mortality of 50% or greater is observed for either species.

2.2.2 Site Sampling

Surface water samples were collected in October 2016 and February 2018 from the discharge stream (D) and perennial river (P). Site D0 was collected directly from the discharge culvert before entering the stream. All other sites were collected at the indicated distance from the discharge (e.g., D.3 was collected 0.3 km downstream, see Table 2). Sites prefaced with a P were collected on the perennial river, with positive values indicating samples collected downstream of the confluence between the two water bodies (e.g., P32.2 is collected on the perennial river, 32.2 km downstream of the discharge) and negative values indicating samples collected upstream of the confluence (e.g., P-2.6 is collected on the perennial river, 2.6 km upstream of the confluence). Field and holding blanks were also collected and processed alongside each analysis. During the 2016 sampling event, the discharge stream and perennial river were not connected via surface water. A direct observation could not be made in 2018 due to unsafe road conditions. The streams have been connected during previous sampling events.

Based on the results of the 2016 sampling event, higher resolution samples were collected between the discharge and the playa lake during the 2018 sampling event. In 2018, one of the downstream samples (D15) was not accessible and was not sampled. Additionally, the control site location was different between the two sampling events. In 2016, the control site was located on the perennial river 2.6 km upstream of the confluence between the two streams (P-2.6). Although this site

was not influenced by surface water from the discharge stream, it was likely influenced by anthropogenic activity from the nearby town. In 2018, a control site was selected 24.2 km upstream of the confluence (P-24.2), in a location that is upstream of most human activity.

Table 2. Field Parameters from October 2016 and February 2018 sampling events. Negative distance values indicate distance upstream of the confluence between the discharge stream and the perennial river. These samples (P-2.6 and P-24.2) were used as the control sites.

Site Name	Distance from Discharge (km)*	Temperature (°C)	pH	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)
		October 2016			
D0	0	39.4	7.87	2150	0.57
D1.4	1.4	31.0	8.14	2070	4.62
D15	15	24.6	8.62	3200	6.73
D32.1	32.1	----------Dry----------			
P-2.6	-2.6	11.8	8.27	420	8.61
P32.2	32.2	6.8	8.09	480	9.60
P61.3	61.3	12.3	8.24	710	8.68
		February 2018			
D0	0	35.6	7.92	2180	0.83
D.3	0.3	33.3	8.38	2050	1.82
D.6	0.6	30.4	8.33	2050	2.69
D1.4	1.4	25.8	8.28	2060	3.44
D2.1	2.1	17.6	7.99	1500	3.58
D3.8	3.8	7.0	7.82	2090	4.99
P-24.2	-24.2	0.3	8.32	180	9.30
P34.4	34.4	0.2	8.19	500	9.00

In the discharge stream, water samples were collected in the center of the stream. In the larger perennial river, samples were collected where the water was flowing freely. Samples were stored on ice in the field and refrigerated at 4°C in the lab until analysis. Duplicate samples were collected from site D0 and P61.3 during the 2016 sampling event and at site D.3 during the 2018 sampling event. The results from these samples are presented as averages in the figures. At each site, a Hanna HI98194 probe was used to measure temperature, pH, dissolved oxygen and specific conductivity of the water.

2.2.3 Organic Analysis

Samples for organic analysis were collected in glass bottles with Teflon-lined caps. Volatile organic compounds (VOCs) were analyzed by gas chromatography/mass spectrometry (GC-MS) following EPA method 8260B. Samples for semi-volatile organic compound (SVOCs) analysis were first liquid-liquid extracted, following EPA method 3520C, and then analyzed by GC-MS following method 8270D. Samples for gasoline range organics (GRO) and diesel range organics (DRO) were acidified in the field using HCl to pH < 2. GRO samples were prepared using purge-and-trap EPA method 5030B followed by gas chromatographic analysis according to EPA method 8021B. Samples for DRO analysis were extracted following EPA method 3520C and analyzed using gas chromatography with flame ionization detector (GC-FID) following EPA method 8015. Samples collected for VOC and GRO analysis were collected without headspace. Some compounds were analyzed via multiple methods (e.g., benzene was analyzed by EPA method 8260B and 8021B). The results for these compounds are presented as averages in the figures.

Solid phase extraction (SPE) was used to concentrate surfactants and reduce the salt concentrations in the samples. Glassware for surfactant analysis was pre-cleaned by washing with deionized water (3x), Milli-Q water (3x) and methanol (1x) followed by baking in a muffle furnace (400°C for 8 hours). Bottles were rinsed three times with sample water prior to collection. Water samples were stored without headspace in amber bottles at 4°C and were extracted within a month. Prior to extraction, high purity hydrochloric acid was added to samples to adjust to pH 3 in order to increase extraction efficiency. Supel Select HLB cartridges (200mg/6mL, Supelco, Bellefonte, PA) were conditioned with methanol (HPLC grade, Fisher) followed by Milli-Q water and Milli-Q water, adjusted to pH 3 using hydrochloric acid. A volume of 1000 mL of sample was applied to the cartridges (5-10 mL min-1). Cartridges were dried under vacuum for 15 minutes. Surfactants were eluted from the cartridge using 10 mL of methanol. Samples were stored at -20°C and analyzed within 24 hours.

Extracts were analyzed using an Agilent 1290 Infinity Series liquid chromatograph coupled with an Agilent 6530 Quadrupole Time-of-Flight mass spectrometer (Q-ToF), using the method described in Thurman et al. (2014) with the following exceptions.[60] Mobile phases were A (0.1% formic acid) and B (acetonitrile). A gradient elution method was developed with 0-2 minutes, 20% B; 2-15 min, 20-95% B; 15-22 min, 95% B; 22-25 min, 20% B. The flow rate was 0.6 mL/min, the injection volume was 20 µL, and the temperature of the drying gas was 325°C. Peaks were identified by accurate mass and potential chemical formulas, which were then verified using surfactant standards. An exact concentration of each surfactant series could not be determined due to a lack of commercial standards with known ethoxymer distribution. Instead, an estimated concentration was determined at the discharge using polyethylene glycol 400, polypropylene glycol (Alfa Aesar, Haverhill, MA) and 4-nonylphenol-polyethylene glycol standards (Sigma Aldrich, Saint Louis, MO). Relative concentrations (C/C_0) were determined for samples downstream since all samples were stored in the same manner and extracted and analyzed at the same time.

2.2.4 Inorganic Analysis

Samples for inorganic analysis were collected in plastic bottles. Samples for cation analysis were filtered in the field using 0.45-µm filters and acidified to pH < 2 with HNO_3. Cations were analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) and by inductively coupled plasma-mass spectrometry (ICP-MS). Samples for major anions were filtered in the field (0.45 µm) and analyzed by ion chromatography. Samples for ammonia as nitrogen were acidified with H_2SO_4 in the field and analyzed colorimetrically, following EPA method 350.1. Alkalinity was analyzed via titration. Radium-228 and Radium-226 were analyzed following EPA NAREL SOP 13 and 14, respectively.

2.3 Results and Discussion

2.3.1 Field Parameters

Field parameters for the sampling sites are shown in Table 2. The water temperature was elevated at the discharge (39.4°C, 2016; 35.6°C, 2018), due to downhole conditions and heat added during separation, and decreased with distance downstream. At D15, the discharge stream sample farthest from the NPDES release and 15 km downstream, water temperature remained elevated as compared to the perennial river (24.6°C vs. 11.8°C). In 2016, pH was 7.87 and increased slightly downstream to 8.62 at D15. In 2018, pH was 7.92 at the discharge and increased until D1.4 (pH = 8.28) but then decreased in the wetland (D2.1, pH = 7.99) and playa lake (D3.8, pH = 7.82). All sampling sites were within the range of the pH permitted at the effluent (pH = 6.5-9). Conductivity was elevated at the discharge (2150 μS/cm, 2016; 2180 μS/cm, 2018) as compared to the perennial river (average ~500 μS/cm) but below the permit effluent limit (7500 μS/cm) and near the minimum value for PW in the U.S.[12] In 2016, conductivity increased nearly 50% between the discharge and site D15, due to water evaporation downstream.[32] In 2018, conductivity remained relatively stable in the discharge stream, except for a decrease of ~500 μS/cm at site D2.1. Dissolved oxygen (DO) was depleted at the discharge (0.57 mg/L, 2016; 0.83 mg/L, 2018) and increased with distance downstream, likely due to aeration in short waterfalls along the flow path, decreasing temperature, and atmospheric equilibration. At D15, DO remained lower than in the perennial stream (6.73 mg/L vs. 8.61 mg/L). In 2016, daytime air temperature ranged between 4.5°C and 13°C. In 2018, daytime air temperature ranged between -12°C and -4°C.

2.3.2 Organic Chemistry of the Discharge

Gas chromatography analysis revealed numerous organic chemicals present at the discharge (Figure 5). The majority of these chemicals were geogenic hydrocarbons (i.e., benzene, DRO, etc.) and many have previously been reported in PW.[12, 54, 61-63] In general, concentrations of individual

organic species were low in comparison to available health thresholds, ranging from 0.29 μg/L (methyl acrylate, 2018) to 49.8 μg/L (acetone, 2018). DRO (C_{10} to C_{28} alkanes; boiling point range ~170°C - 430°C) in the discharge was detected at 1,560 μg/L in 2016 and 1,430 μg/L in 2018. GRO (C_6 to C_{10} alkanes; boiling point range ~ 60°C - 170°C) in the discharge was detected at 156 μg/L in 2016 and 94.2 μg/L in 2018.

Concentrations of VOCs, SVOCs, DRO and GRO at the discharge were relatively consistent between the two sampling events. The discharge is sourced from conventional wells that have been operating for decades and it was expected that concentration of geogenic compounds would remain steady between sampling events. Two chemical species were only observed at one sampling event. This includes 1,2-dichloroethane (0.56 μg/L), which was detected at the discharge in 2016 but not in 2018, and carbazole, which was detected in the discharge only during the 2018 sampling event (3.03 μg/L). Carbazole has many potential sources including crude oil and 1,2-dichloroethane was most likely used as a solvent [64]. Common chemical additives including 2-butoxyethanol and acetone were also detected at the discharge [65]. 2-Butoxyethanol is a product stabilizer, solvent and surfactant. Acetone is a commonly used solvent. It is also a known transformation product of polypropylene glycol surfactants, which were also detected, so may not have been directly used as a well maintenance chemical [66].

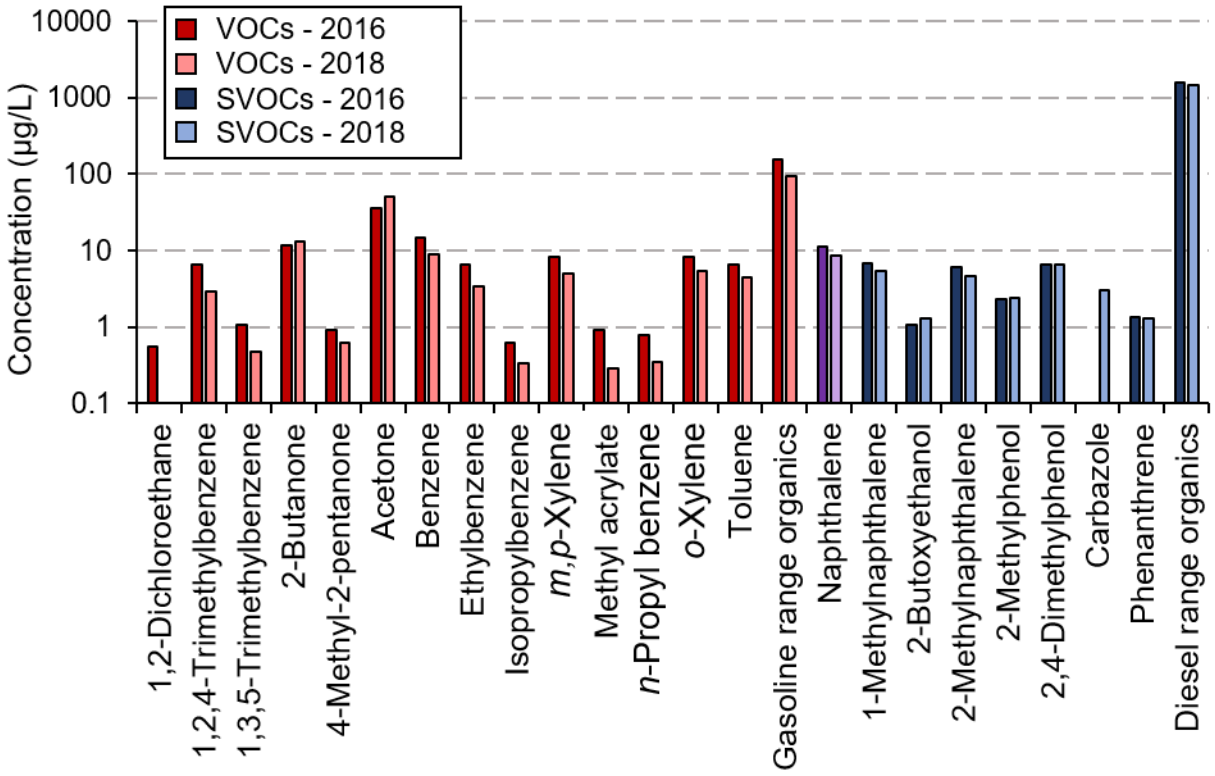

Figure 5. Concentrations of volatile organic chemicals (VOCs), semi-volatile organic chemicals (SVOCs), gasoline range organics (GRO) and diesel range organics (DRO) at the NPDES PW discharge during the October 2016 and February 2018 sampling events. VOCs and GRO are shown in red. SVOCs and DRO are shown in blue. Naphthalene is analyzed by both the VOC and SVOC methods and is therefore shown in purple.

Liquid chromatography analysis was conducted on all samples collected in 2018 and only on the discharge sample in 2016. Analysis revealed the presence of polyethylene glycols (PEGs), polypropylene glycols (PPGs) and nonylphenol ethoxylates (NPEOs). These nonionic surfactant species were present in the discharge at an estimated concentration of 9 µg/L (2016) and 2 µg/L (2018) PEGs; 9 µg/L (2016) and 5 µg/L (2018) PPGs, and 12 µg/L (2016) and 8 µg/L (2018) NPEOs (Figure 6). PEGs, PPGs and NPEOs are surfactants commonly used by the oil and gas industries as emulsifiers, wetting agents and corrosion inhibitors [66]. Despite their widespread use, U.S. regulatory limits for most of these chemical additives do not exist.

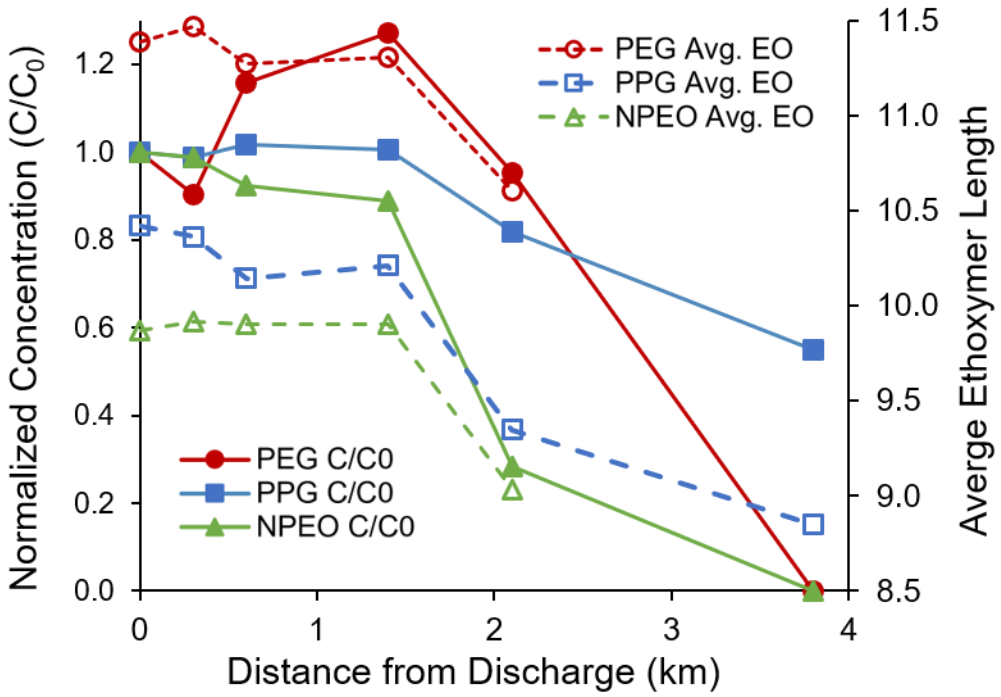

Figure 6. Relative concentration of polyethylene glycol (PEG), polypropylene glycol (PPG) and nonylphenol ethoxylate (NPEO) and average ethoxymer (EO) length for each species versus distance from the NPDES discharge (km) during the February 2018 sampling event. PEG and NPEO were below detection limit 3.8 km downstream and therefore no average ethoxymer length is shown. PEG, PPG and NPEO were all below detection in the control site sample. These species were also detected at the discharge in October 2016 (data not shown). A wetland is located ~1.8 km downstream and may be the source of surfactant removal between 1.5 km and 2.1 km.

2.3.3 Organic Contaminant Changes Downstream

2.3.3.1 Volatile Organic Compounds

Figure 7 shows that benzene, toluene, ethylbenzene and xylenes (BTEX) concentrations decreased with increasing distance downstream. BTEX are a component of crude oil and commonly employed as an indicator of oil and gas releases [67]. In both 2016 and 2018, all BTEX chemicals were detected in the discharge at concentrations of 48.0 µg/L and 31.0 µg/L, respectively. In 2018, BTEX were also detected 0.3 km downstream, albeit at a much lower concentration (1.6 µg/L) than at the discharge. No BTEX chemicals were detected farther than 0.3 km from the discharge or in the perennial river. Benzene at the discharge was 2-3 times greater than the maximum contaminant level (MCL; 5 µg/L) as shown in Figure 7A. The concentrations of the remaining chemicals at the discharge

were well below the MCLs for toluene (700 μg/L), ethylbenzene (1,000 μg/L) and xylenes (10,000 μg/L) (see Table 3 for organic chemical thresholds). At the discharge, both toluene and xylenes were above the thresholds for chronic impacts to aquatic species in fresh surface water (Table 3). Previous studies have reported sublethal effects from flowback water including immobility in *daphnia magna* and oxidative stress in rainbow trout.[68-69] Concentrations of benzene and ethylbenzene were below the chronic aquatic threshold at the discharge but in the same order of magnitude. While these chemicals pose a potential risk at the discharge, all were well below aquatic thresholds by 0.3 km downstream so any impact on aquatic species would be minimal. BTEX concentrations at the discharge were at least three orders of magnitude below risk-based screening levels (RBSLs) for cattle (Table 3). Application of these RBSLs is limited since they have been defined for livestock in the event of an accidental release and not for livestock who consistently drink this water, as is occurring at this site.[70] Additionally, as with all health thresholds available, the RBSLs only pertain to one chemical and do not consider potential synergistic and antagonistic toxicological effects due to mixtures. Finally, based on Wyoming Surface Water Quality standards, the concentration of benzene at the discharge was about 1/3 of the safe level for human consumption of fish, while the concentrations of toluene, ethylbenzene and xylenes were at least 3 orders below this threshold.

The primary removal mechanism for BTEX chemicals from surface water is volatilization as evidenced by the Henry's constants (5.2-6.6 x 10^{-3} atm-m^3/mol, see Table A4 for organic chemical properties).[71-74] Short (0.25-1.0 m) waterfalls have been constructed throughout the discharge stream to increase volatilization of chemicals in the water. Aerobic biodegradation is another possible removal mechanism; however, previous studies have shown that the rate of BTEX biodegradation is likely insignificant as compared to the rate of volatilization (Table A4).[72] Once in the atmosphere, BTEX react readily with hydroxyl radicals and have a half-life on the order of hours to days, depending on the concentration of hydroxyls present.[71-74] Oil and gas production has previously been shown to be a

significant source of BTEX releases in the U.S.[75-76] 1,2,4-Trimethylbenzene was detected only at the discharge and 0.3 km downstream (Figure 8). This chemical has a Henry's constant within the range of the BTEX chemicals and is likely removed from the discharge stream via volatilization.

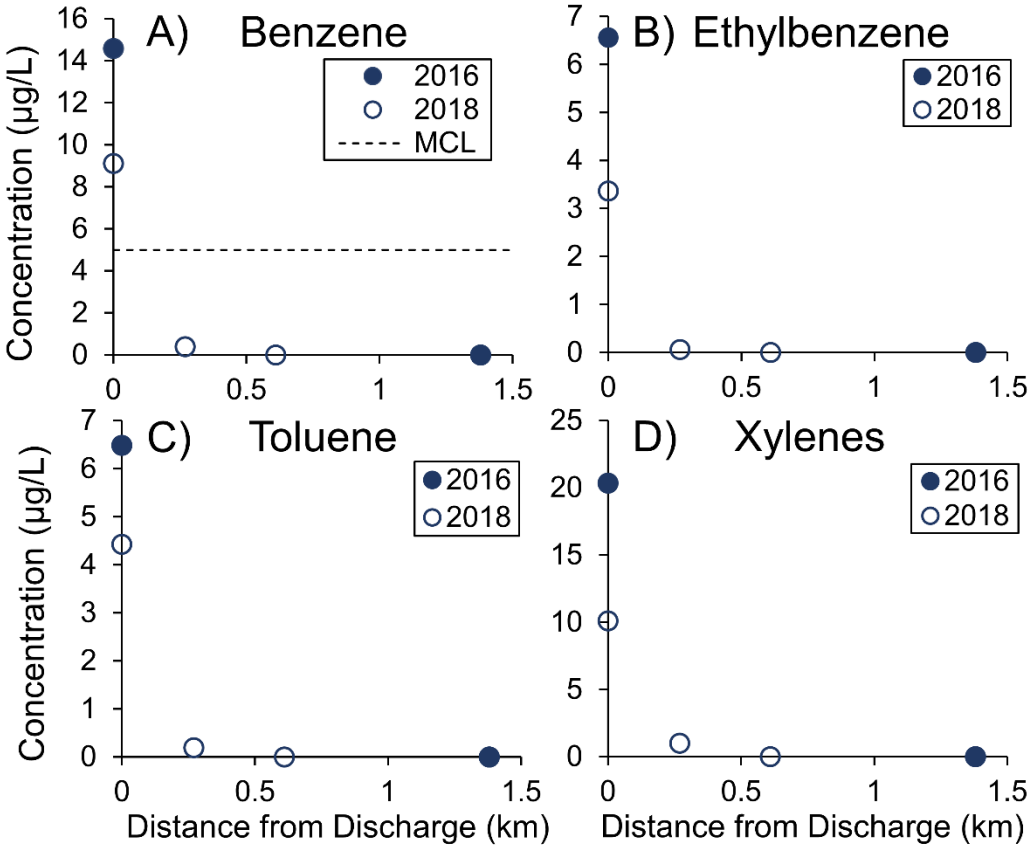

Figure 7. Concentrations of A) benzene, B) toluene, C) ethylbenzene and D) xylenes (BTEX) at the discharge and up to 1.5 km downstream. In both October 2016 and February 2018, all BTEX chemicals were detected in the discharge. The maximum contaminant level (MCL) for benzene (5 µg/L) is shown in plot A. The MCLs for the other chemicals are larger than the observed concentrations by multiple orders of magnitude.

Other VOCs that were detected past the discharge point include 2-butanone and acetone. 2-butanone was highest at the discharge (11.9 µg/L in 2016; 13.4 µg/L in 2018) and detected as far as 3.8 km downstream (1.69 µg/L, 2018) (Figure 8). 2-Butanone is geogenic and has previously been reported in PW.[62] Acetone was the most dominant VOC in the discharge (36.3 µg/L, 2016; 49.8 µg/L, 2018) and one of two organic chemicals detected in both the discharge and perennial streams. As

discussed above, acetone may be a chemical additive or a by-product of PPG or isopropanol degradation.[66] In 2016, acetone concentrations were steady at all locations in the perennial river, ranging from 0.9 to 1.09 µg/L. In 2018, acetone was below detection limit at both sites in the perennial river. Acetone is a by-product of fat metabolism in animals and is also sourced from plants, trees, insects and forest fires.[77] One or all of these are the most likely sources of acetone in the control site samples.

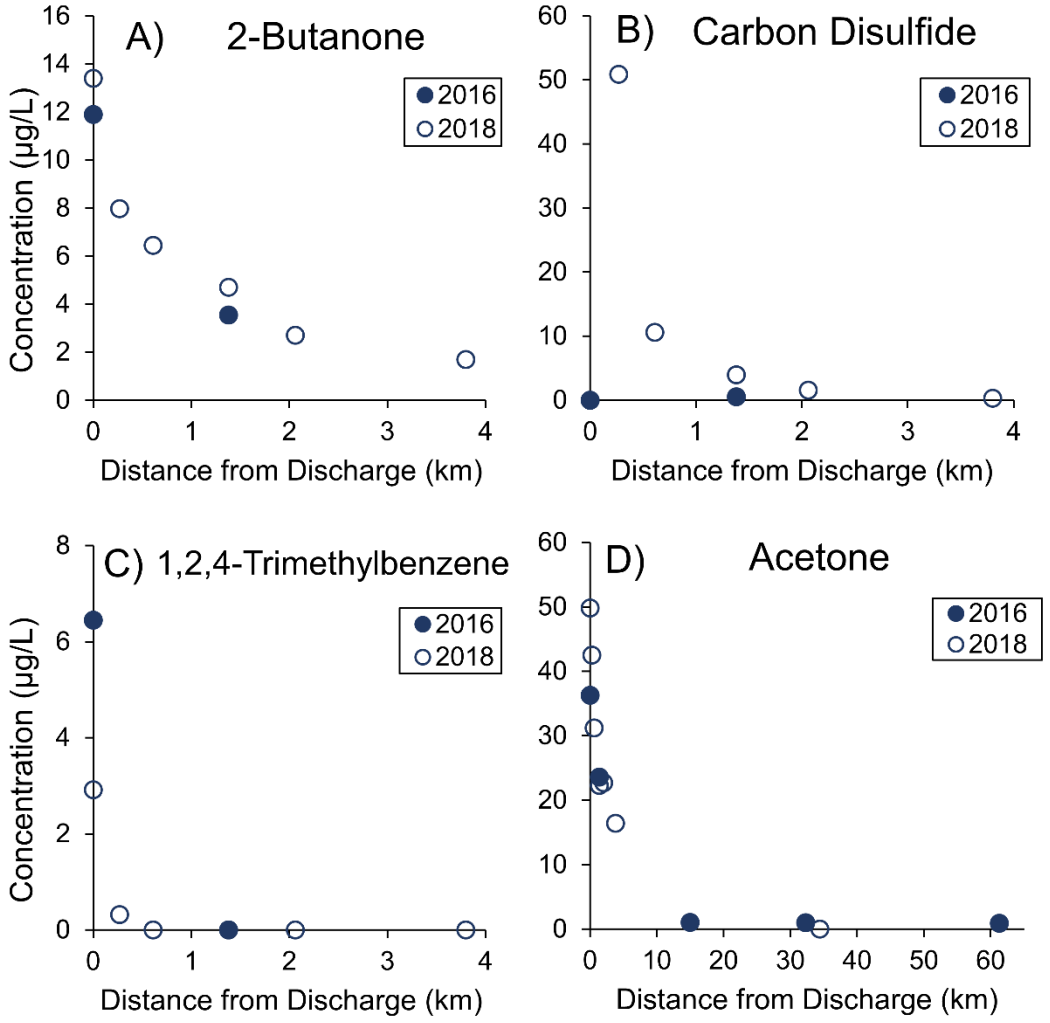

Figure 8. Concentrations of A) 2-butanone, B) carbon disulfide, C) 1,2,4-trimethylbenzene and D) acetone at the discharge and with distance downstream. Note the different x-axis for the acetone data. The y-axes are also different between compounds. These four panels illustrate the volatile organic compounds (VOCs) that were detected past the discharge point that are not BTEX. None of these chemicals were present in the control site samples except acetone (1.09 µg/L, 2016).

Acetone and 2-butanone persisted farther downstream than BTEX. This can be explained partly by their Henry's constants, which are two orders of magnitude less than BTEX (~10^{-5} atm-m^3/mol). Volatilization and biodegradation are the primary removal mechanisms for these species. Previous studies have shown that acetone degradation is slower in saltwater than fresh water and it is possible that the slightly elevated salt concentrations at the discharge contribute to a slower rate of acetone removal.[77] Once in the atmosphere, the residence time of 2-butanone is expected to be less than a day. Acetone has an average residence time of 45 days and therefore has the potential to be transported farther from the NPDES release point.[77] Both species were multiple orders of magnitude below acute and chronic aquatic thresholds.

Carbon disulfide, a VOC that has been detected in PW and in air samples collected near oil and gas activity,[78-79] was not detected at the discharge during either sampling event, but was detected downstream (Figure 8). It can be produced by reaction between methane and hydrogen sulfide, but only at much higher temperature than at the discharge point. Thus, its detection downstream is not due to formation in the stream. More likely, losses of this highly volatile compound occurred during sampling at the discharge since the sample was collected directly from the discharge as the water fell from the culvert into the streambed. In 2016, carbon disulfide was detected at a concentration of 0.54 μg/L at site D1.4 and 0.25 μg/L at site D15. It was not detected in the perennial river. In 2018, carbon disulfide was first detected in D.3 at an average concentration of 50.9 μg/L, which is above the acute threshold for aquatic life in fresh surface water. From this point, concentrations decreased with distance downstream and were below the chronic aquatic threshold by site D1.4. No other thresholds were available for this species. Carbon disulfide was detected at all sites on the discharge stream as well as P34.4, but not in the control site sample. The Henry's constant (1.22 x 10^{-3} atm-m^3/mol) of carbon disulfide indicates that it will quickly partition from water to air via volatilization.[80] Once in the

atmosphere, the half-life of carbon disulfide is 8-12 days, which is long enough for it to be transported and potentially affect air quality in the nearby towns.[78]

2.3.3.2 Semi-Volatile Organic Compounds

Polycyclic aromatic hydrocarbons (PAHs) were detected in the discharge stream and have previously been found in PW.[62] PAHs detected at the discharge included naphthalene, phenanthrene, 1-methylnapthalene and 2-methylnaphthalene (Figure 9). Naphthalene was the most dominant PAH at the discharge (11.4 μg/L, 2016; 8.72 μg/L, 2018) and was detected as far as 1.4 km downstream, making it the most persistent of the PAHs. Phenanthrene was the least concentrated among the detected PAHs at the discharge (1.34 μg/L, 2016; 1.32 μg/L, 2018) and below detection limit (1 μg/L) by 0.6 km downstream. 1- and 2-methylnaphthalene persisted until 0.6 km and 0.3 km downstream, respectively. Both 1-methylnaphthalene and naphthalene were above the chronic aquatic threshold until 0.6 km and 1.4 km downstream, respectively. The drinking water risk-based screening level (RBSL) for cattle for low molecular weight PAHs (defined as PAH with two or three rings) is two orders of magnitude greater than the combined concentration of PAHs present at the discharge. No other thresholds were available for these species.

Like acetone and 2-butanone, volatilization and biodegradation are the most important removal mechanisms for low molecular weight PAHs.[81] As indicated by the atmospheric hydroxylation rate of these compounds, the half-lives of phenanthrene and naphthalene compounds in the atmosphere are less than 1 day. Previous studies have shown that atmospheric concentrations of PAHs are elevated by at least one order of magnitude in areas near oil and gas operations.[82] These compounds exhibit a moderate potential to sorb to sediment and a previous study found PAHs, including naphthalene and phenanthrene, sorbed to sediment in a lake downstream of a wastewater treatment plant processing oil and gas wastewater.[28]

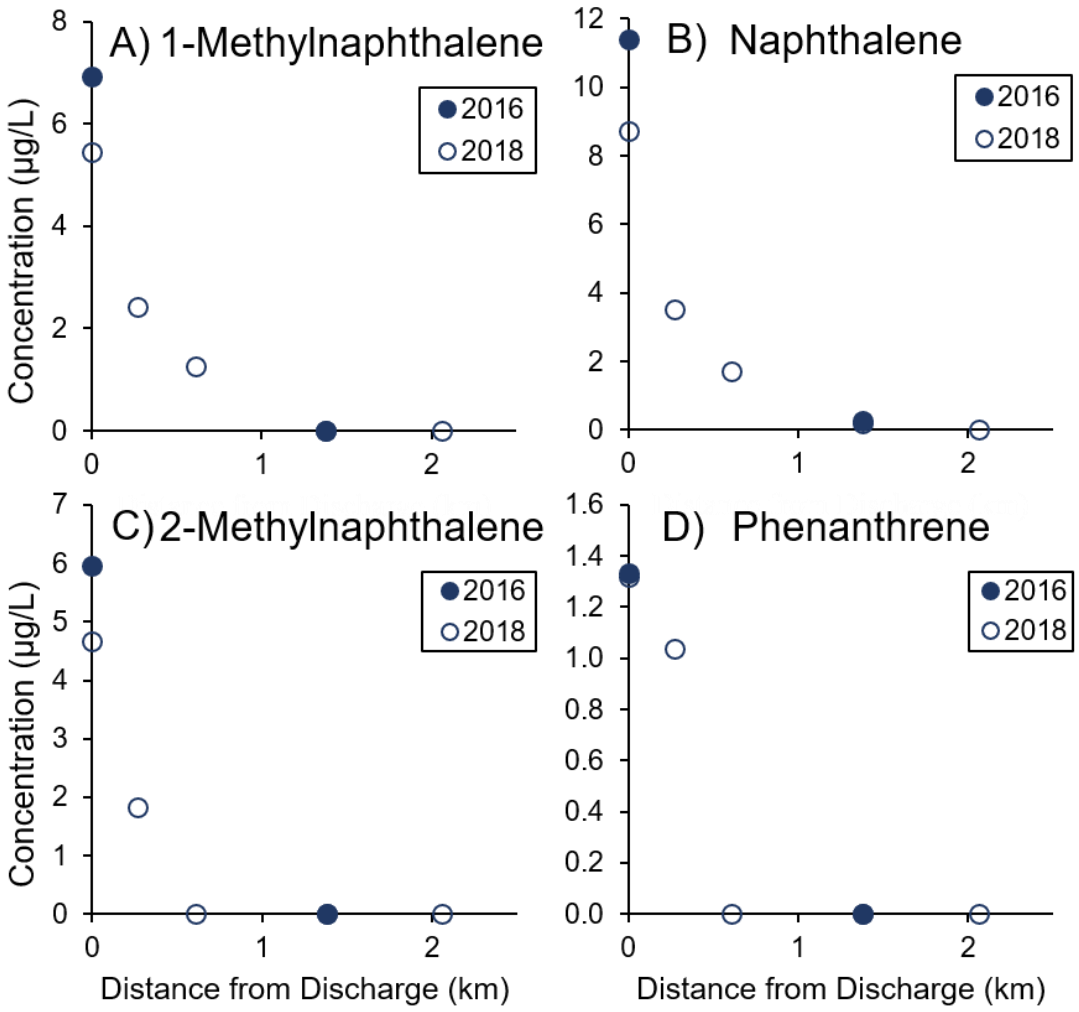

Figure 9. Concentrations of A) 1-methylnaphthalene, B) naphthalene, C) 2-methylnaphthalene and D) phenanthrene at the discharge and up to 2.1 km downstream. Note the different x-axes for each chemical. These four panels illustrate the polycydic aromatic hydrocarbons (PAHs) that were detected at the discharge during the October 2016 and February 2018 sampling events. None of these chemicals were present in the control site samples.

Phenol, 2-methylphenol, and 2,4-dimethylphenol were also detected in the discharge stream (Figure 10). Phenol was not detected at the discharge but was detected in 2018 at site D.3 at a concentration of 1.03 µg/L, indicating it may be an intermediate of benzene biodegradation. Both 2-methylphenol (2.36 µg/L, 2016; 2.41 µg/L, 2018) and 2,4-dimethylphenol (6.57 µg/L, 2016; 6.45 µg/L, 2018) were highest at the discharge. 2,4-Dimethylphenol was present at 2 to 3 times greater concentration than 2-methylphenol and persisted farther in the discharge stream (3.8 km vs. 1.4 km). These species may also be biodegradation intermediates of the respective methylated parents as

xylenes were also present at a 2 to 3 times higher concentration than toluene.[72, 83] All phenol species were at least an order of magnitude below the aquatic chronic thresholds. Phenol concentrations were five orders of magnitude below the criteria for the human health consumption of fish. No other thresholds were available.

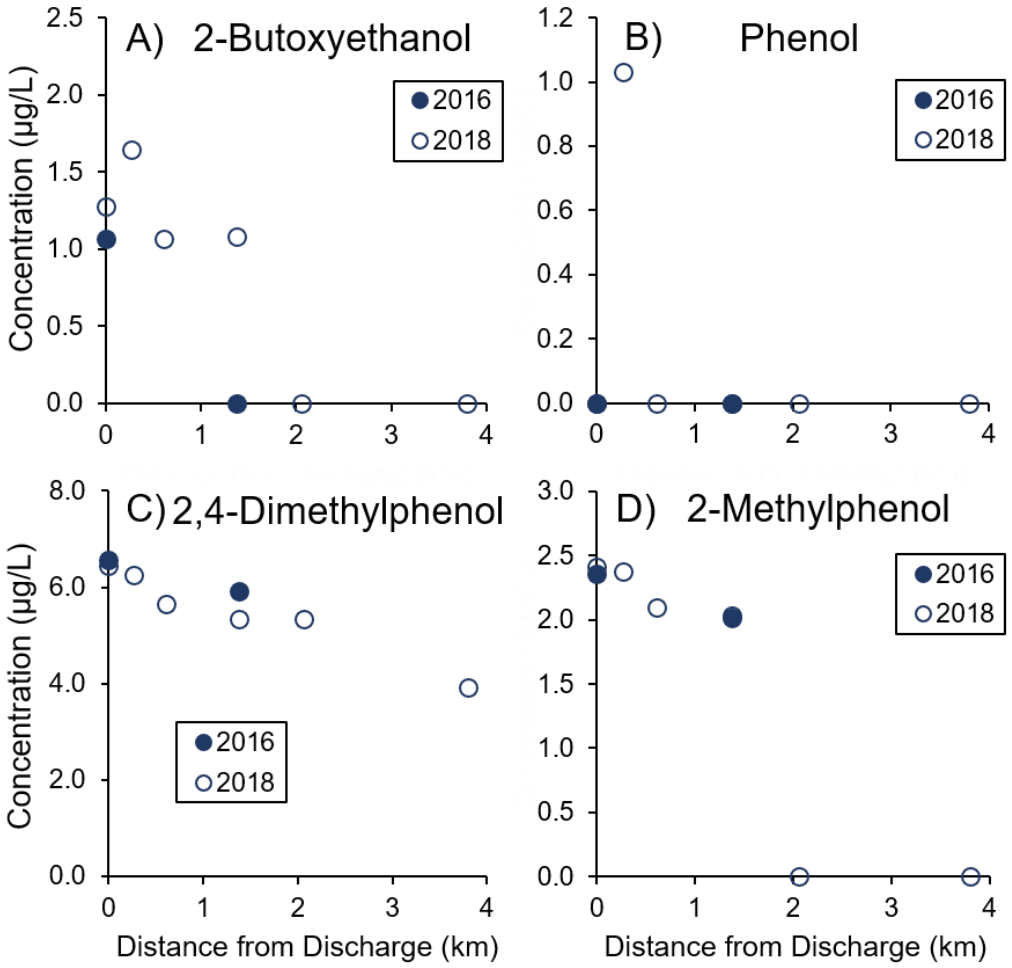

Figure 10. Concentrations of A) 2-butoxyethanol, B) phenol, C) 2,4-dimethylphenol and D) 2-methylphenol at the discharge and up to 4 km downstream. Note the different x-axes for each chemical. These four panels illustrate the alcohols that were detected at the discharge during the October 2016 and February 2018 sampling events. None of these chemicals were present in the control site samples.

The main removal mechanism for phenol and the substituted phenol compounds is biodegradation.[83-84] Volatilization from water will occur but at a slower rate than chemicals previously mentioned in this manuscript as indicated by the lower Henry's constants (10^{-6} – 10^{-7} atm-m^3/mol).

The phenol compounds are also relatively mobile in soil (K_{oc}: 25-175) but degrade quickly in both soils and groundwater, and thus are not expected to persist in the groundwater.[84] For the portion of these compounds that volatilize, atmospheric hydroxylation occurs quickly, and removal will occur within a day.

Two well-maintenance chemicals, 2-butoxyethanol and bis(2-ethylhexyl)phthalate, were also detected in the discharge stream. 2-butoxyethanol is a surfactant and solvent while bis(2-ethylhexyl)phthalate is a diverter and used in PVC piping.[79, 85] It is also possible that bis(2-ethylhexyl)phthalate is a contaminant from a plastic coating used for sample collection, however, this is unlikely since it was only found in two samples and not in the blanks. Both of these compounds have previously been reported in O&G wastewater.[54] 2-Butoxyethanol has also been found in a water well contaminated by oil and gas activity in Pennsylvania.[86] In 2016, 2-butoxyethanol was only detected at the discharge (1.07 μg/L). In 2018, however, it was detected as far as 1.4 km downstream, ranging between 1.07 – 1.65 μg/L over this distance (Figure 10). Few regulatory thresholds were available for 2-butoxyethanol, even though it is commonly used in industry and listed as "possibly carcinogenic to humans" by U.S. EPA. Bis(2-ethylhexyl)phthalate was not detected at the discharge but was detected 15 km downstream in 2016 (4.99 μg/L) and 2.1 km downstream in 2018 (5.13 μg/L). In both samples, bis(2-ethylhexyl) phthalate is above the chronic aquatic threshold and the threshold for human consumption of fish. It is also just below the MCL (6 μg/L). The main removal mechanism for 2-butoxyethanol from water is biodegradation. For bis(2-ethylhexyl) phthalate, the main removal mechanism is sorption to soil, as indicated by its high soil adsorption coefficient. Volatilization of both chemicals occurs very slowly and is not a major removal mechanism for either compound.[87-88]

2.3.3.3 Diesel and Gasoline Range Organics

Both gasoline range organics (GRO) and diesel range organics (DRO) were detected in the discharge stream at a maximum concentration of 156 μg/L (GRO) and 1560 μg/L (DRO) at the

discharge (Figure 11). DRO was detected in the perennial river in 2018 but was not analyzed in 2016. The concentration of DRO at the control site and in the site downstream of the confluence was 37.8 µg/L and 37.3. µg/L, respectively. The perennial river is surrounded by agricultural production, flows through a downstream town and passes under multiple bridges, thus, the control site DRO is likely sourced from cars or other common uses of oil. The combined concentration of GRO and DRO was an order of magnitude below the crude oil drinking water risk-based screening level (RBSL) for calves and also below the effluent limit in the permit for oil and grease (10 mg/L).

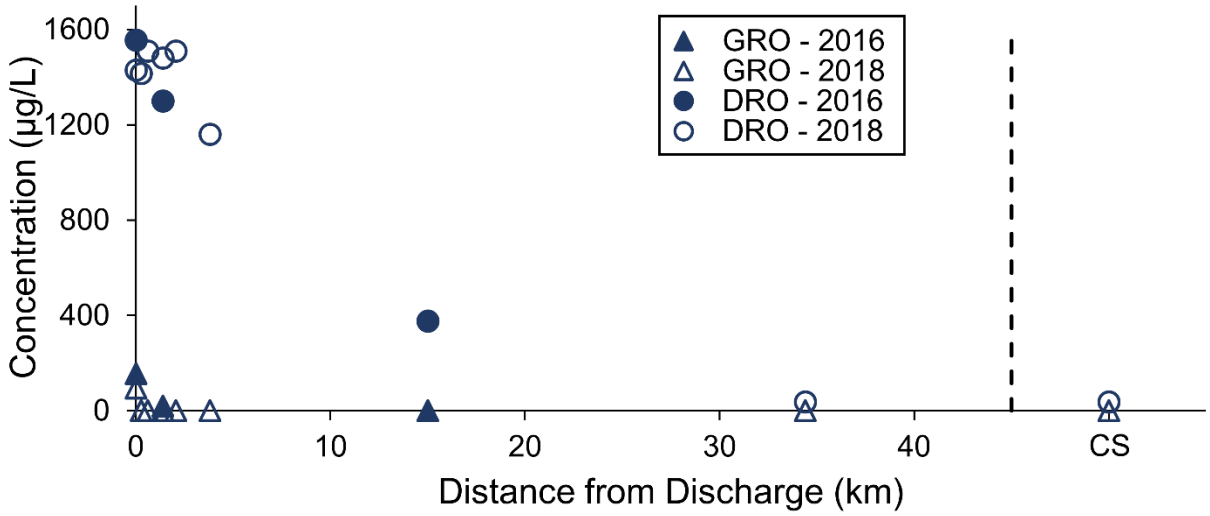

Figure 11. Concentrations of gasoline range organics (GRO) and diesel range organics (DRO) with distance from the NPDES discharge point (km). In October 2016, GRO and DRO were not measured in the perennial river and therefore, control site samples. Data points to the right of the dashed line correspond to the concentration of GRO and DRO in the February 2018 control site (CS) samples.

GRO did not persist as far as DRO. The more volatile and biodegradable compounds were removed first, as indicated by the Henry's constants (K_H: 0.487 to 0.151 atm-m^3/mol for GRO; 0.151 to 7.36 x 10^{-7} atm-m^3/mol for DRO) and the biodegradation half-lives (7-9 days for GRO; 9 to 125 days for DRO).[89] This agrees with the overall trend that more volatile and biodegradable chemicals were removed first, emphasizing the importance of volatilization and biodegradation as the dominant removal pathways for organic chemicals. DRO were likely removed by a combination of

biodegradation, volatilization and sorption as indicated by previous studies and the physiochemical properties listed in Table A4.[90]

2.3.3.4 Surfactants

Downstream samples were analyzed for surfactants only in 2018. Results showed that concentrations of PPGs and NPEOs were highest at the discharge and decreased with distance (Figure 6). The concentration of PEGs decreased initially and then increased between 0.3 and 1.4 km downstream, reaching a maximum normalized concentration of 1.27 (estimated 2.54 μg/L) at site D1.4. The increase in PEGs may be a result of NPEO biodegradation. Previous studies provided evidence for a central fission mechanism for NPEO degradation, which would result in direct generation of PEGs and nonylphenol (NP), an endocrine disruptor.[91-92] A shift in PEGs speciation towards the major NPEOs homologues (EO8 - EO11) was observed (Figure A1-A3) and NPEOs average ethoxymer length remained steady over this distance, indicating that central fission is a potential mechanism for PEGs generation. Concentration of PEGs increased by an estimated 1.4 nM over this distance, while NPEOs only decreased by 1.1 nM. This suggests that other mechanisms, such as variability in discharge composition, are influencing the concentration of PEGs as well. The well maintenance schedule is not provided in the permit for this discharge, however, permits for other NPDES PW discharges in the area report that well-maintenance chemicals are used on a biweekly or bimonthly basis, indicating that discharge composition would vary with time.

As shown in Figure 6, the concentration of surfactant species decreased significantly between 1.4 and 2.1 km downstream, as compared to the overall decrease upstream of this section. The first wetland at the site is located about 1.7 km from the discharge, with site D2.1 located at the end of this wetland. This is most apparent for NPEOs, which were present at 0.88 normalized concentration at site D1.4, decreased to 0.28 by site D2.1 (60% removed), and were fully removed by site D3.8. Removal of PEGs and PPGs increases in the wetland as well, but to a lesser degree than NPEOs.

Decreases in relative concentration were accompanied by decreases in average ethoxymer length (Figure 6). At the discharge, average PEG ethoxymer length was 11.4. This remained steady for the first 1.4 km of the discharge stream and then decreased to an average of 10.6 at site D2.1. Average PPG ethoxymer length at the discharge was 10.4, decreasing to 9.3 at site D2.1 and 8.8 at site D2.8. NPEO average ethoxymer length was 9.9 at the discharge and remained steady until site D2.1 where it dropped to an average of 9.0. These decreases in average ethoxylate number provide evidence that the concentration decreases were due to transformation rather than dilution. All three surfactant species are known to biodegrade via sequential ethoxylate chain shortening, which leads to changes in homolog distribution.[15, 66] In addition, sorption to sediment is an important removal mechanism for NPEOs and may also account for an appreciable portion of PPGs removal.[66] A previous study reported NPEOs sorbed to sediment downstream of an O&G wastewater treatment plant.[28] NPEOs are commonly used by other industries and have been found in sediments around the world.[93] For PPGs, increasing sorption to sediment with an increase in ethoxylate chain length has been reported, which would support the decrease in average ethoxylate number observed in the water samples.[66] Significant sorption or other abiotic removal mechanisms are not expected for PEGs,[15] so their decrease in concentration and average ethoxylate number can likely be linked to biodegradation processes only. A previous study reported that the half-life of NPEOs was shorter than that of PEGs under aerobic conditions in the presence of DRO.[92] While no health thresholds are available for these surfactant species, NPEOs have been banned in Europe, mainly due to their potential to transform into NP, an endocrine disruptor.[94]

2.3.3.5 Organic contaminants in the playa lake

Acetone, 2-butanone, 2,4-dimethylphenol, DRO, PPGs and PEGs all persist past the first wetland and into the playa lake, which is the major watering hole in the area. Herds of cattle, wild horses, birds and pronghorn have all been observed drinking from this lake. Risk-based screening

levels are unavailable for the organic chemicals found in the playa lake except DRO. Thus, a determination on potential health risks of this water for livestock cannot be made. In addition to drinking the water, there could also be negative impacts from contact as bird mortality has been observed at oil and gas wastewater evaporation ponds used for disposal. DRO, surfactants, and high salinity were listed as the causes for 239 bird deaths in these ponds.[95] However, the concentrations of chemicals in the playa lake were likely lower than what would be observed at evaporation ponds since non-treated PW is generally added to these ponds.

2.3.4 Inorganic Chemistry of the Discharge and Changes Downstream

The TDS of the discharge was 1200 mg/L and 870 mg/L during 2016 and 2018 (Figure 12), respectively, which is near the minimum of TDS values observed in PWs in the U.S. [11] and below the effluent limit for this NPDES permit (5000 mg/L). Median TDS values in Wyoming O&G basins are 10,000 mg/L or below, which is an order of magnitude lower than values observed in most other Western U.S. basins.[12] Salinity has a major impact on the feasibility of PW treatment and reuse. The relatively low TDS of the PW at this site is a major reason why beneficial reuse of this water is economically viable. TDS concentrations increased downstream, reaching a maximum of 1930 mg/L at site D15. Alkalinity was highest at the discharge (375 mg/L in 2016; 510 mg/L in 2018) and decreased downstream (Figure 12).

Sodium, chloride and sulfate were the most concentrated ions at the discharge site (Figure 13). Previous studies have shown that these are the dominant ions in most Wyoming PW.[12] At the discharge, concentrations of sodium were 273 mg/L in 2016 and 285 mg/L in 2018, which is an order of magnitude lower than the median sodium concentrations for PW in the Western U.S.[12] Compared to the control site, sodium concentrations were elevated by a factor of 15 to 30. This range is due to the fact that ions were generally more concentrated in the 2016 control site sample than the 2018 control site sample. Sodium concentrations increased with distance downstream, reaching a maximum

of 454 mg/L at site D15. Sodium concentrations throughout the discharge were below the chronic aquatic threshold, which is the only threshold available for this species (see Table 4 for inorganic chemical health thresholds).

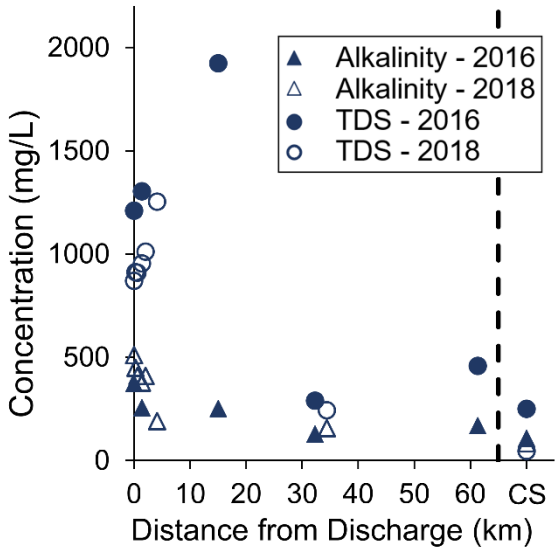

Figure 12. Changes in A) total dissolved solids (TDS) and B) alkalinity versus distance in the discharge and perennial streams. Data points to the right of the dashed line correspond to the concentration of TDS and alkalinity in the control (CS) samples.

Concentrations of chloride at the discharge were 182 mg/L in 2016 and 156 mg/L in 2018, which is two orders of magnitude lower than median values for the Western U.S. and an order of magnitude lower than the permit effluent limit (2000 mg/L).[12] Chloride concentrations were elevated by a factor of 30 to 40 as compared to the control site samples. Similar to sodium and TDS, chloride concentrations increase with distance downstream, reaching a maximum of 251 mg/L at site D15 which is slightly above the MCL (250 mg/L). Sulfate concentrations were near median values for the Western U.S. at the discharge (305 mg/L, 2016; 420 mg/L, 2018) and an order of magnitude below the effluent limit in the permit (2500 mg/L).[12] This element is elevated by a factor of 4 to 22 as compared to the control site. Sulfate concentrations increased with distance downstream. At the

discharge, sulfate concentrations were just above the MCL (250 mg/L) but reached nearly four times greater than the MCL by 15 km downstream (939 mg/L).

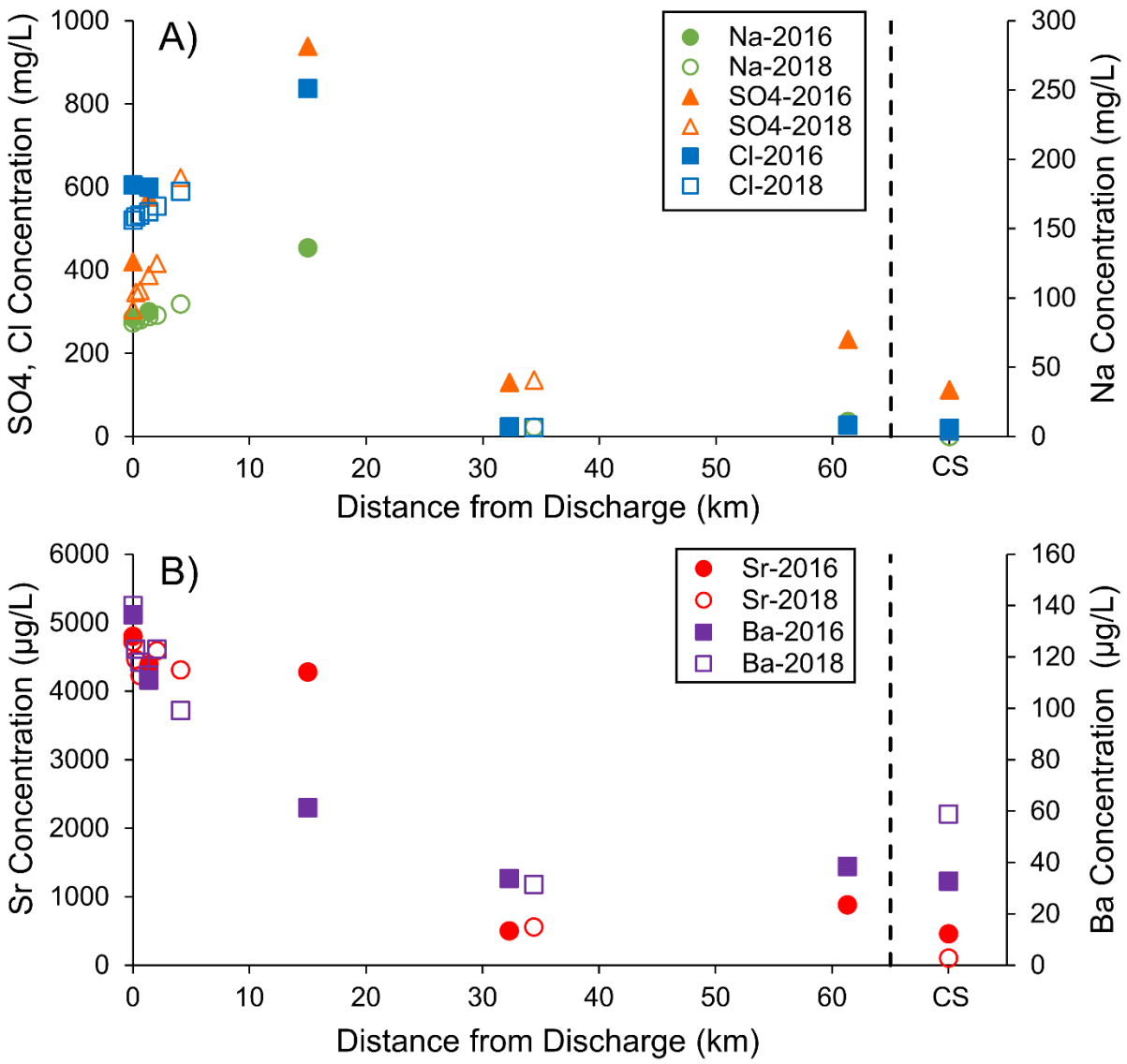

Figure 13. Concentration of A) sodium (Na), sulfate (SO4), chloride (Cl) and B) strontium (Sr), and barium (Ba) versus distance downstream from the NPDES produced water discharge (km) from 2016 (dosed symbols) and 2018 (open symbols). Data points to the right of the dashed line correspond to the concentration of these species in the control site (CS) samples.

Other major ions that were elevated in the discharge as compared to control site include potassium (27.1 mg/L in 2016; 25.7 mg/L in 2018; elevated by a factor of 15 to 25), calcium (74.3

mg/L in 2016; 75.1 mg/L in 2018; elevated by a factor of 2 to 4) and magnesium (32.0 mg/L in 2016; 32.5 mg/L in 2018; elevated by a factor of 2 to 4). Ammonia as N was absent from the control site sample but present at 360 μg N/L in the discharge (only measured in 2016). These species have previously been reported at elevated concentrations in PW.[24, 44] There was no clear trend in calcium concentration downstream (Figure 14). Ammonia concentrations decreased with distance downstream (Figure 14). No thresholds were available for these species. Potassium and magnesium increased with distance downstream, both reaching a maximum at site D15 (30.3 mg/L for potassium; 51.8 mg/L for magnesium; Figure 15). These maxima were below the aquatic thresholds for these ions and are the only available thresholds.

The discharge contained elevated concentrations of minor elements as well, including boron (0.94 mg/L in 2016; 0.95 mg/L in 2018; elevated by a factor of 10 to 20), manganese (4.6 μg/L in 2016; 6.0 μg/L in 2018; elevated by a factor of 3 in 2018) and selenium (4.8 μg/L in 2016; 1.9 μg/L in 2018; elevated by a factor of 1.5 to 5). Concentrations of all three elements increased downstream (Figure 16). Boron reached a maximum of 1,300 μg/L at site D15 and is present above or near the California State Notification Level (1000 μg/L) and above the acute and chronic aquatic thresholds throughout the discharge stream. Manganese reached a maximum concentration of 229 μg/L in the playa lake, which is above the MCL (50 μg/L) and the acute aquatic threshold. Selenium reached a maximum of 12.8 μg/L at site D15, which is just below the acute aquatic threshold and above the chronic threshold. In addition to being sourced from PW, selenium is also naturally elevated in Wyoming soils.[96]

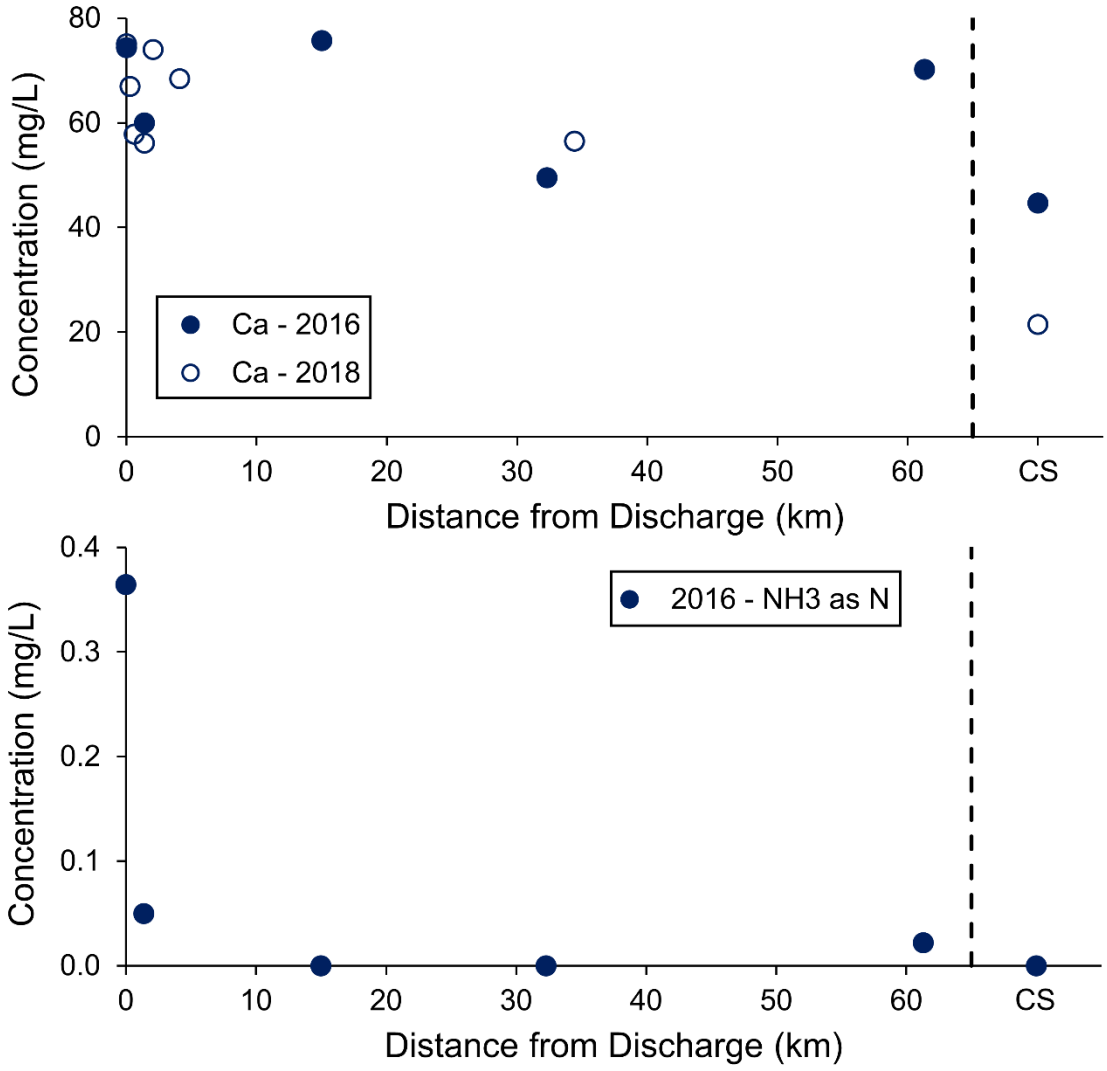

Figure 14. Concentrations of calcium (top) and ammonia as N (bottom) in the discharge and perennial streams. Ammonia was analyzed in October 2016 samples only. Data points to the right of the dashed line correspond to the concentration of calcium and ammonia in the control site (CS) samples.

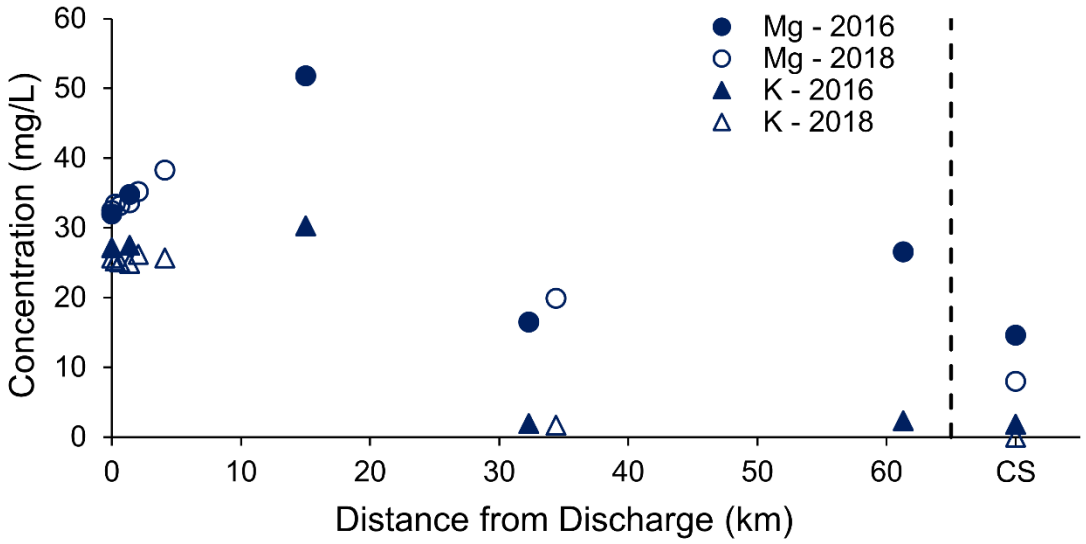

Figure 15. Concentrations of magnesium (Mg) and potassium (K) in the discharge and perennial streams. Data points to the right of the dashed line correspond to the concentration of magnesium and potassium in the control site (CS) samples.

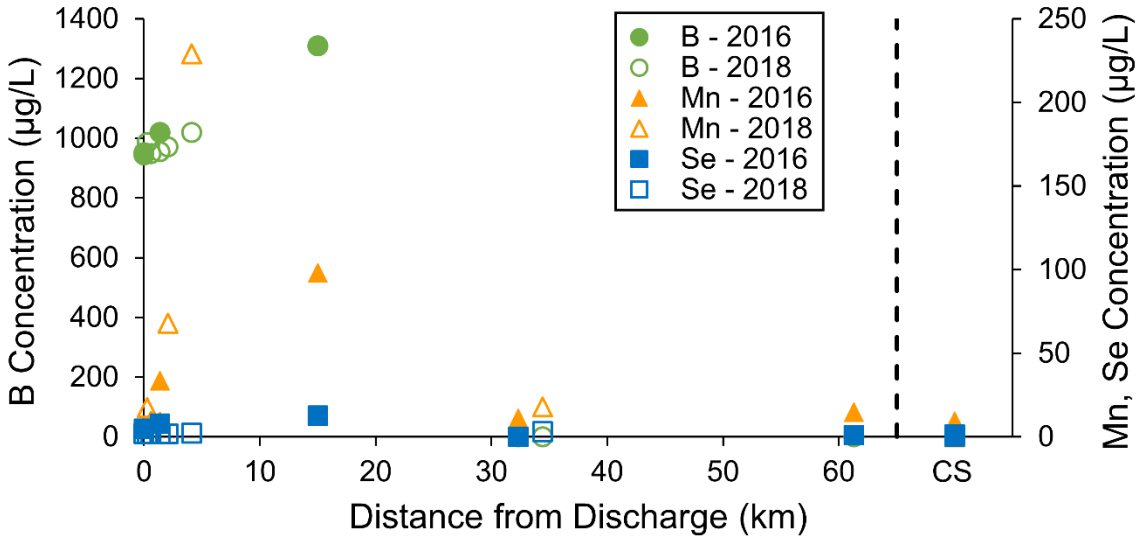

Figure 16. Concentrations of boron (B), manganese (Mn) and selenium (Se) in the discharge and perennial streams. Data points to the right of the dashed line correspond to the concentration of boron, manganese and selenium in the control site (CS) samples.

Strontium, barium and total radium (^{226}Ra + ^{228}Ra) were elevated at the discharge and decreased downstream (Figure 13 and Figure 17). These elements have previously been reported at

elevated levels in PW. [32, 44] Strontium concentrations at the discharge were 4.8 mg/L and 4.7 mg/L in 2016 and 2018, respectively. Strontium is above the chronic aquatic threshold throughout the discharge stream, but never above this level in the perennial river. Barium concentrations were 138 µg/L in 2016 and 143 µg/L in 2018, which is above the acute and chronic aquatic thresholds. Barium was never below the chronic aquatic threshold, even in the perennial river. Total radium (^{226}Ra + ^{228}Ra) concentration was 0.50 Bq/L at the discharge in 2016 which is below the permitted effluent limit of 2.22 Bq/L (60 pCi/L). Increased total radium concentrations are an indicator of PW impacts and a study has been conducted on the fate and transport of radium at this site. This study showed that radium, strontium and barium in the discharge stream were removed via co-precipitation with carbonate, and to a lesser extent, sulfate minerals.[32] Radium was not analyzed in 2018. Other trace inorganic elements including arsenic were analyzed in the water samples.

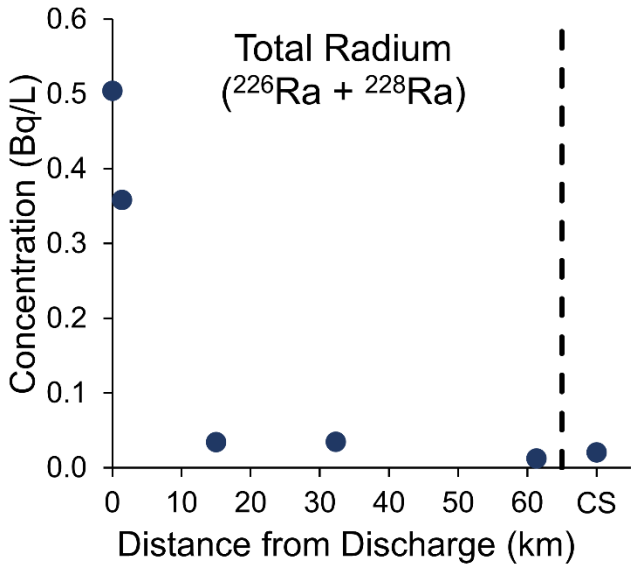

Figure 17. Total Radium concentrations measured in water samples collected during the October 2016 sampling event. Radium was not analyzed in water samples in 2018. Data points to the right of the dashed line correspond to the concentration radium in the control site (CS) sample.

Many of the elements found in the discharge, including chloride, sulfate, sodium, boron, potassium, magnesium, and manganese and selenium, increased in concentration downstream. The

radium study conducted at this site showed that increases in TDS, chloride and sulfate were due to progressive evaporation downstream.[32] Previous studies on coalbed methane PW discharges in Wyoming have attributed increases in selenium and other inorganic chemicals downstream to both evaporation and increased leaching of naturally present species in the soil and rock, as a result of the PW.[33-34]

Multiple inorganic species including barium, boron, fluoride, manganese, molybdenum, strontium, sulfate and uranium were above at least one threshold in the playa lake, which is a major water source for livestock and wildlife in the area. Uranium was not present at the discharge but was present in the playa lake (D0: below limit of detection, D3.8: 2.5 μg/L). Uranium is naturally present in the area and concentrations were similar in the discharge stream and perennial river. Fluoride, which was only analyzed in 2018, was above drinking water risk-based screening level (RBSL) for cattle in the playa lake. All other species listed are above acute and/or chronic aquatic thresholds and/or the MCL. Many of these chemicals reach their maximum value at site D2.1 or D3.8 showing that while the discharge may be safe, changes downstream could result in water that is unsuitable for beneficial reuse.

Table 3. Highest observed values for volatile and semi-volatile organic chemicals detected in the discharge or perennial streams compared to human, aquatic and livestock health thresholds. Values in bold exceed at least one criterion.

Chemical Species	Highest Conc. Observed (µg/L)	SL/MCL [a] (µg/L)	Surface Water Acute [b] (µg/L)	Surface Water Chronic [b] (µg/L)	RBSL (calves/beef cattle) [c] (ug/L)	Human Health Consumption of Fish [d] (µg/L)
1,2,4-Trimethylbenzene	6.45	56/-	-	-	-	-
1,2-Dichloroethane	**0.56**	0.17/5	8,800	100	-	37
1,3,5-Trimethylbenzene	1.07	60/-	-	-	-	-
1-Methylnaphthalene	**6.92**	1.1/-	37	2.1	-	-
2,4-Dimethylphenol	6.57	360/-	212[e]	21[e]	-	850
2-Butanone	13.4	5,600/-	240,000	14,000	-	-
2-Butoxyethanol	1.28	2,000/-	-	-	-	-
2-Methylnaphthalene	5.96	36/-	-	330	-	-
2-Methylphenol	2.41	930/-	230	13	-	-
4-Methyl-2-pentanone	0.93	6,300/-	2,200	170	-	-
Acetone	49.8	14,000/-	28,000	1,500	-	-
Benzene	**14.6**	0.46/5	2,300	46	14,300/31,400	51
Bis(2-ethylhexyl) phthalate	**5.13**[f]	5.6/6	27[e]	0.3	-	2.2
Carbazole	3.03	-/-	-	-	-	-
Carbon disulfide	**50.9**[f]	810/-	17	0.92	-	-
Diesel range organics	1555	-/-	-	-	29,300 [g]/114,000[g]	-
Ethylbenzene	**6.6**	1.5/700	130	7.3	11,700/25,600	2,100
Gasoline range organics	155.5	-/-	-	-	-	-
Isopropylbenzene	0.63	450/-	-	-	-	-
Methyl Acrylate	0.92	42/-	-	-	-	-
Naphthalene	**11.4**	0.17/-	190	1.1	-	-
n-Propyl Benzene	0.8	660/-	-	-	-	-
Phenanthrene	1.34	-/-	30	3.6	-	-
Phenol	1.03	5,800/-	1020[e]	180	-	860,000
Toluene	**6.49**	1,000/1000	120	2	89,500/196,000	15,000
Xylenes	**20.35**	190/10,000	230	13	71,700/157,000	-
LMW PAH[h]	25.62	-/-	-	-	2,010/4,400	-

[a] Screening level (SL) for tap water and Maximum Contaminant Level (MCL) are the EPA CompTox website [89]. SLs are reported with Target Hazard Quotient = 1

[b] Data from NOAA Screening Quick Reference Tables [97] unless noted. If multiple values available, lowest value was selected.

[c] Drinking water risk-based screening levels (RBSLs) for cattle and calves from the American Petroleum Institute [70]

[d] Obtained from State of Wyoming Surface Water Quality Standards [98]

[e] Data from the Baseline Ecological Risk Assessment for Non-Asbestos Contaminants [99]

[f] Maximum concentration was observed at a sampling site other than the discharge.

[g] Value for crude oil.

[h] Low molecular weight (LMW) polycyclic aromatic hydrocarbons (PAHs) are defined as PAHs with three or fewer rings.

Table 4. Highest observed values for inorganic chemicals detected in the discharge or perennial streams compared to human, aquatic and livestock health thresholds. Values in bold exceed at least one criterion.

Chemical Species	Highest Concentration Observed (µg/L)	Location of Highest Concentration	SL/MCL [a] (µg/L)	Surface Water Acute [b] (µg/L)	Surface Water Chronic [b] (µg/L)	Livestock Upper Limit [c] (ug/L)	Human Health Consumption of Fish (µg/L)
Aluminum	**1860**	D2.1	20,000/50 [d]	750 (pH 6.5-9 only)	87 (pH 6.5-9 only)	5000	-
Ammonia as N	364.5	D0	-	-	-	-	-
Antimony	0.5	D2.1	7.8/6	88	30	-	640
Arsenic	**3.6**	D3.8	0.052/10	340	150	200	10
Barium	**140**	D0	3,800/2,000	110	3.9	-	-
Boron	**1310**	D15	4,000/-	30	1.6	5000	-
Cadmium	**0.2**	D2.1	9.2/5	1.4 [e]	0.19 [e]	50	hardness dependent
Calcium	75,700	D15	-/-	-	-	-	-
Chloride	**251,000**	D15	-/250,000 [d]	-	-	-	-
Fluoride	**2910**	D2.1	800/2,000 [d]	200 (hardness < 50)	-	2000	-
Iron	**2620**	D2.1	14,000/300 [d]	-	1,000	-	-
Lead	**3.2**	D2.1	15/15	42 [e]	1.6 [e]	50	-
Magnesium	51,800	D15	-/-	-	82,000 [e]	-	hardness dependent
Manganese	**229**	D3.8	430/50 [d]	2,300	80	-	-
Molybdenum	**84.9**	P34.4	100/-	16,000	34	-	-
Potassium	30300	D15	-/-	373,000	53,000 [e]	-	-
Selenium	**12.8**	D15	100/50	13	5	50	4,200
Silica	39,900	D2.1	-/-	-	-	-	-
Sodium	454,000	D15	-/-	-	680,000 [e]	-	-
Strontium	**4805**	D0	12,000/-	15,000	1,500	-	-
Sulfate	**939,000**	D15	-/250,000 [d]	-	-	-	-
Uranium	**5.2**	P61.3	-/-	46	0.5	-	-
Vanadium	**24.3**	D15	86/-	280	19	100	-
Zinc	67.3	P34.4	6,000/5000 [d]	85	85 [e]	24,000	26,000
Radium	**0.50 Bq/L**	D0	0.19 Bq/L	-	-	-	-

[a] Screening level (SL) for tap water and Maximum Contaminant Level (MCL) are the EPA CompTox website [89]. SLs are reported with Target Hazard Quotient = 1
[b] Data from NOAA Screening Quick Reference Tables [97] unless noted. If multiple values available, lowest value was selected.
[c] Drinking water risk-based screening levels for calves and cattle from the Colorado State University Extension Office [100]
[d] Secondary MCL (In MCL column).
[e] Thresholds from the Baseline Ecological Risk Assessment for Non-Asbestos Contaminants [99]

2.4 Conclusions

A comprehensive chemical analysis was conducted on a stream composed of treated PW released for agricultural beneficial reuse under U.S. EPA's NPDES program. This study aimed to characterize the discharge and assess potential health impacts to downstream users. PW released at the discharge point complied with all effluent limitations, however, permit limits were only defined for six chemical species or classes of chemicals and pH (Table 1). It should also be noted that chemical concentrations at this discharge were near minimum values for PW in the U.S. Due to that and the fact that PW composition varies widely throughout the U.S., these results can only be applied to this site and are not representative of all NPDES PW discharges.

Over 20 different semi-volatile and volatile organic compounds, as well as three surfactant series (PEGs, PPGs and NPEOs), were detected in the discharge that were not specified in the effluent limits. Concentrations of organic chemicals generally decreased with distance from the discharge. Between the discharge and 1.4 km downstream, VOCs were removed at a faster rate than SVOCs and non-volatile organic compounds (NVOCs) (Figure 18), indicating that volatilization was a major removal mechanism for organic chemicals detected in the discharge. Between 1.4 and 2.1 km downstream, biodegradation, and potentially sorption, became a more dominant removal mechanism, as indicated by the increased rate of removal of SVOCs and NVOCs and the decrease in average surfactant ethoxymer length (Figure 6). This is likely due to the wetland located 1.8 km downstream, where notable attenuation of organics was observed, indicating that wetlands may be an effective strategy for managing PW discharge quality. However, the long-term fate of PW constituents regarding accumulation in sediments and infiltration to groundwater remains to be investigated.

A wide range of inorganic species were also detected in the watershed, including many from PW and some natural contaminants in the area (i.e., uranium). Concentrations of most inorganic species increased with distance downstream. This finding has major implications because reporting

requirements for NPDES permits pertain to the discharge site only, while downstream changes are not considered. Consequently, our data reveal that changes in water quality downstream must be assessed to fully understand the potential impact of these releases.

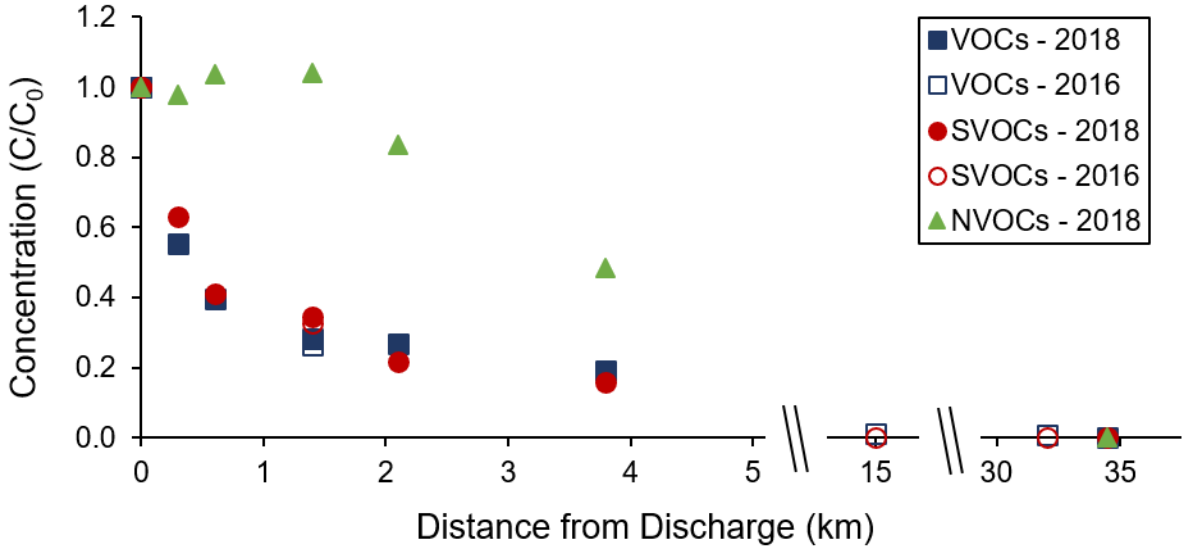

Figure 18. Relative concentration of volatile organic compounds (VOCs), semi-volatile organic compounds (SVOCs) and non-volatile organic compounds (NVOCs) versus distance from the NPDES discharge (km) during the October 2016 (open symbols) and February 2018 (dosed symbols) sampling events. Between the discharge and 1.4 km downstream, volatilization was the dominant removal mechanism and VOCs were removed more quickly than SVOCs. A wetland is located ~1.8 km downstream. At the wetland, biodegradation became dominant and changed the relative distribution of VOCs and SVOCs. Removal of NVOCs also began in the wetland. Only DRO was present at the control site (P-24.2, 37.8 µg/L DRO) and therefore data is not shown.

A complete set of health thresholds reviewed here were only available for 8 chemicals (BTEX, arsenic, cadmium, selenium and zinc) showing that an assessment of potential toxicological impacts is currently limited for most species in this stream. Additionally, while the threshold values cited in this study are helpful guidelines, most of them cannot be directly applied to this site for a multitude of reasons. First, the risk-based screening levels for livestock have not been developed for lifetime consumption by livestock, as occurs at this site. Second, the available thresholds were developed for individual species and do not consider mixture effects. Numerous studies have shown that chemical species may have synergistic or antagonistic effects on the toxicity of other chemicals [101-102]. Finally,

there are likely additional undetected chemicals and transformation products present in the discharge with unknown impacts on livestock and fish. The above limitations also apply to other PW discharge sites and types of beneficial reuse, including crop irrigation, such as in Kern County, California [37].

With the highly variable nature of PW throughout the U.S., widely applicable chemical permit effluent limits are impractical for NPDES PW releases, and standardized analytical methods for many of these chemicals are still lacking. Thus, additional toxicity testing guidelines would add a critical line of evidence to determine if NPDES discharges are "of good enough quality." Currently, NPDES permits in Wyoming only require acute toxicity testing and do not consider chronic toxicity. Many of the chemicals detected at this discharge and in PW throughout the West are known carcinogens (e.g., benzene, radium), endocrine disruptors (e.g., nonylphenol ethoxylates) and developmental toxins [101, 103]. Boron and other chemicals in PW are also toxic to plants at elevated concentrations [104]. Toxicity tests are well suited for complex waste streams with relatively low salinity, such as PW released for beneficial reuse, since they can be used to determine the impact on organisms, even if a detailed chemical analysis of the sample is unavailable.

This study shows that a critical amount of research regarding analytical characterization, environmental distribution, toxicological effects and mechanisms, as well as bioaccumulation and uptake in organisms remains to be conducted before PWs can be deemed "of good enough quality" for environmental release.

CHAPTER 3: ASSESSMENT OF WATER QUALITY DOWNSTREAM OF NPDES OIL AND GAS PRODUCED WATER DISCHARGE: TOXICOLOGICAL IMPACTS

3.1 Introduction

Produced water (PW) is one of the largest waste streams associated with oil and gas (O&G), with trillions of liters generated each year in the United States.[105] Because of its origins in the O&G producing formation, PW contains elevated levels of hydrocarbons, salts, metals and naturally occurring radioactive materials (NORMs).[5] It also contains any remaining drilling, stimulation or well-maintenance chemicals as well as their transformation products. Nearly 80% of PW in the U.S. is generated in the West [5]. In the United States, west of the 98th meridian, the federal National Pollutant Discharge Elimination System (NPDES) exemption in 40 CFR § 435 Subpart E allows release of PW for livestock watering, irrigation and other agricultural purposes if it is "of good enough quality." The only federally defined effluent limit for these permits pertains to oil and grease (< 35 mg/L). All other effluent limits are determined by U.S. Environmental Protection Agency (EPA) regulators in the region. PW contents vary by time, location, geologic formation and variations in chemical use.[8, 14, 18, 61] Treatment of these complex chemical mixtures varies based on composition and by the well operator. As a result, the composition of NPDES O&G PW releases are poorly characterized.

Many of the O&G formations in the western U.S. are located in water-scarce, arid regions.[5] The amount of PW generated varies by location, age of the well, hydrocarbon extracted, and drilling technique. In some areas, the volume of PW is substantial enough to serve as a water resource, given that the PW is of adequate quality.[5, 18, 106] Additionally, due to increased water scarcity stresses and issues associated with other PW management techniques (e.g., earthquakes),[21] operators, municipalities and the federal government are increasingly interested in beneficial reuse of PW. A few studies have assessed environmental impacts associated with reuse of coal bed methane PW, which is usually less

contaminated than O&G PW.[34, 107] Also, a recent study assessed physiological and morphological responses in wheat crops irrigated with diluted O&G PW.[37] The impacts of NPDES O&G PW releases on livestock, plant and human health, however, have not been fully assessed.

As a first step towards this goal, in Chapter 2 of this dissertation, a chemical analysis was conducted on water samples collected from a NDPES PW release point in Wyoming, USA and subsequent sites downstream. Chemical analysis alone, however, is not sufficient to provide an accurate environmental risk assessment of this discharge. Firstly, toxicological information is not known for many of the detected chemicals.[62, 108-109] Secondly, many analytes may go undetected because they are not screened for or their concentrations are below instrumental detection limits.[48] This is especially concerning for transformation intermediates that are potentially more toxic than their parent compounds and therefore remain toxic at low concentrations. Thirdly, the behavior of chemicals in complex mixtures strongly depends on their mode of toxic action.[110] While mixtures of chemicals with a common target site and the same mode of action act according to concentration/dose additivity, antagonistic or synergistic effects may arise if mixture components interact with each other.[111] Thus, an integrated chemical and toxicological assessment of these waters is needed to support a comprehensive evaluation of the risks and impacts associated with current NPDES PW discharge practices.

The acute Whole Effluent Toxicity (WET) test is a requirement for most NPDES PW discharges. While acute toxicity is important, analysis of chronic toxicity is imperative as well. Numerous studies have shown that both acute and chronic toxicity (e.g., endocrine disruption, mutagenicity, etc.) are elevated in surface water downstream of partially treated industrial discharges and wastewater treatment plant (WWTP) effluents.[112-113] Additionally, studies have demonstrated both acute and chronic toxicity in organisms exposed to PW and its constituents. Reporter gene assays conducted in human cell lines and yeast revealed elevated estrogen, androgen, glucocorticoid,

progesterone, and thyroid receptor activities.[23, 42, 101] Reproductive and developmental toxicity was observed in mice exposed to hydraulic fracturing chemicals, many of which are also found in PW.[101] Increased acute toxicity, oxidative stress and endocrine disruption have also been observed in fish exposed to flowback and PW.[44, 69, 114-117] Elliot et al. evaluated available toxicological data and showed that many PW constituents are known or suspected developmental toxins, endocrine disruptors or carcinogens [62, 109]. These studies focused on inadvertent releases of PW, PW prior to treatment or known mixtures of PW constituents. To our knowledge, a toxicological assessment of minimally treated PW for agricultural beneficial reuse (i.e., NPDES release) has not been conducted. Additionally, most laboratory studies have focused on hormonal impacts, with some analysis of developmental outcomes.[23, 42, 68-69, 101, 114, 117-118]

A thorough study on increased cancer risk potential is also needed, including an analysis of increased mutagenesis following PW exposure. The *Salmonella* Ames test is the most widely used assay system to detect mutagenicity in a broad range of samples, including surface waters and complex mixtures.[112, 119] This assay detects primarily point mutations in the DNA nucleotide sequence leading to reversion of a selectable marker. While point mutations have long been established as having a broad role in human disease, particularly in cancer development, studies in the last 10-15 years have uncovered a previously underappreciated role for alterations in chromosome structure leading to gene copy-number variation (CNV) in these same processes. CNVs are now conclusively linked to a wide range of human diseases, including neurodevelopmental disorders (e.g., autism, schizophrenia) and cancer.[120-121] Thus, in order to gain a broad understanding of the health risks associated with environmental mutagenesis, it is important to integrate the use of mutagenicity assays that can detect both nucleotide and structural genetic variation.[122]

In this study we took such an integrated approach through the assessment of mutagenicity in a strain of the *Saccharomyces cerevisiae* budding yeast that was built specifically to support the parallel

measurement of four classes of mutations in a single exposure experiment. These included reversion point mutations directly analogous to those covered by the Ames test, and also forward point mutations, CNV deletions, and disease-relevant low-order gene duplications. The CNV duplication assay used in this study and developed by our group is able to detect a simple doubling in copy number of a reporter cassette, whereas most previous gene amplification assays had lower sensitivity and can detect only high order amplification.[123-124] Due to the complexity of this waste stream, it was hypothesized that increased toxicity may be observed for some endpoints and not others. Thus, in addition to these parallel mutagenicity assays, we also assessed the samples for acute toxicity using *Daphnia magna* and developmental toxicity in *Danio rerio* (zebrafish). The results of these assays were combined to assess chronic and acute toxicity of O&G PW intended for agricultural beneficial reuse. The goal of the present study was to quantify toxicity, with a focus on mutagenic activity, of a NPDES PW release.

3.2 Materials and Methods

3.2.1 Site Description

The field site for this study is an undisclosed well field in Wyoming (Figure 4). On average, 4.5 million liters of minimally treated PW are released from the NPDES discharge per day into an ephemeral stream bed. There is little precipitation in the region (average 230 mm/year)[57] and no additional tributaries, resulting in a stream that is composed entirely of O&G PW discharge unless there has been a recent precipitation event. About 2 km from the NPDES release, a dam separates the discharge into two equal streams. One continues southeast for about 2 km before emptying into a playa lake that is used by cattle, horses, waterfowl and other wildlife for drinking. Playa lakes are shallow, ephemeral lakes, commonly found in the U.S. High Plains region.[58] The other stream flows 30 km until finally connecting with a larger perennial river. During the sampling events discussed in this study, the discharge and perennial streams were not connected via surface water. As recently as

2014, these streams were connected. Details on the treatment and NPDES effluent limits at this site have been previously described in Chapter 2 of this dissertation.

3.3.2 Site Sampling

Surface water samples were collected in October 2016 and February 2018 from the discharge stream (D) and perennial river (P). Site D0 was collected directly from the discharge culvert before entering the stream. All other sites were collected at the indicated distance from the discharge (e.g., D.3 was collected 0.3 km downstream, see Table 2). Sites prefaced with a P were collected from the perennial river, with positive values indicating samples collected downstream of the confluence between the discharge stream and perennial river and negative values indicating samples collected upstream of the confluence. Analysis in 2016 revealed that many carcinogenic organic chemicals were removed by site D1.4. As a result, higher resolution samples were collected between the discharge and the playa lake during the 2018 sampling event. During the 2018 sampling event, one of the downstream samples (D15) was not accessible and was not sampled. Also, the control site was different between the two sampling events (P-2.6 in 2016 vs. P-24.2 in 2018). Both control sites were located upstream of the confluence between the discharge stream and perennial river and were not impacted by PW discharges. In addition to the Wyoming field site, a water sample was collected directly from the discharge of a Fort Collins, CO WWTP for the mutagenicity assays to allow for a comparison between two different types of discharge.

All samples were collected in glass bottles with Teflon-lined caps. Glassware was pre-cleaned by washing with deionized water (3x), Milli-Q water (3x) and methanol (1x) followed by baking in a furnace (400°C for 8 hours). Bottles were rinsed three times with sample water prior to collection. In the PW discharge stream, water samples were collected in the center of the stream. In the larger perennial river, samples were collected where the water was flowing freely. Samples were stored on

ice in the field and refrigerated at 4°C in the lab until analysis. More details on sampling locations and methods can be found in Chapter 2 of this dissertation.

3.3.3 Daphnia magna acute toxicity

Acute toxicity tests were conducted with *Daphnia magna*. This species is also used in the acute WET tests which are required every 6 months at this NPDES site. LC_{50} parameters were performed approximately to standard OECD guidelines with some slight alterations.[125] All lethality assays were static in nature. Four samples collected in both 2016 (D0, D1.4, P-2.6 and P32.2) and 2018 (D0, D1.4, P-24.2, P34.4) were assayed for lethality using a serial dilution exposure regime of concentrations 0 (control, dechlorinated City of Edmonton tap water, moderately hard), 1, 2, 5, 10, 20, and 100 % of raw sample in 30 mL of solution (total). A total of 5 neonate daphnia were used per treatment dilution. In total, 3 replicates per exposure series per sample type were employed to determine neonate daphnid lethality. Daphnia survival/mortality was confirmed by observation under a Leica Zoom 2000 stereomicroscope (Leica Camera CO., GER). All ambient room conditions during exposures were identical to conditions during *Daphnia magna* culturing and housing, details of which can be found in Appendix B.

3.3.4 Yeast Strain

Saccharomyces cerevisiae haploid strain JAY2087 (*MATa ade5-1 his7-2 leu2-3,112 Leu+ ura3-52 trp1-289 CAN1 cup1⊿RSC30 sfa1⊿::hisG PLM2::SFA1-V208I-CUP1-KlURA3-ScURA3-5'SFA1-BglII-KanMX4*) was engineered to measure four different types of mutations in parallel, specifically: 1) CNV amplifications, which are measured via acquisition of resistance to copper plus formaldehyde following amplification (primarily duplication) of the cassette containing the *SFA1-V208I* and *CUP1* genes; 2) CNV deletions, which are measured via acquisition of resistance to 5-fluoroorotic acid (5-FOA) through loss of the cassette containing two diverged but functional copies of the *URA3* gene; 3) forward mutations, measured via acquisition of resistance to canavanine through inactivation of the

CAN1 gene through point mutation or rarely deletion; and 4) reversion point mutations, measured via acquisition of tryptophan prototrophy through reversion of a non-sense mutation present in the *trp1-289* allele, or via acquisition of a non-sense suppressor in a tRNA gene also through point mutation [123-124]. The *trp1-289* reversion assay is analogous to the lysine prototrophy reversion point mutations detected using the Ames *Salmonella* assay. Figure 19 shows a schematic representation of the three chromosomes where the reporters for these assays are present.

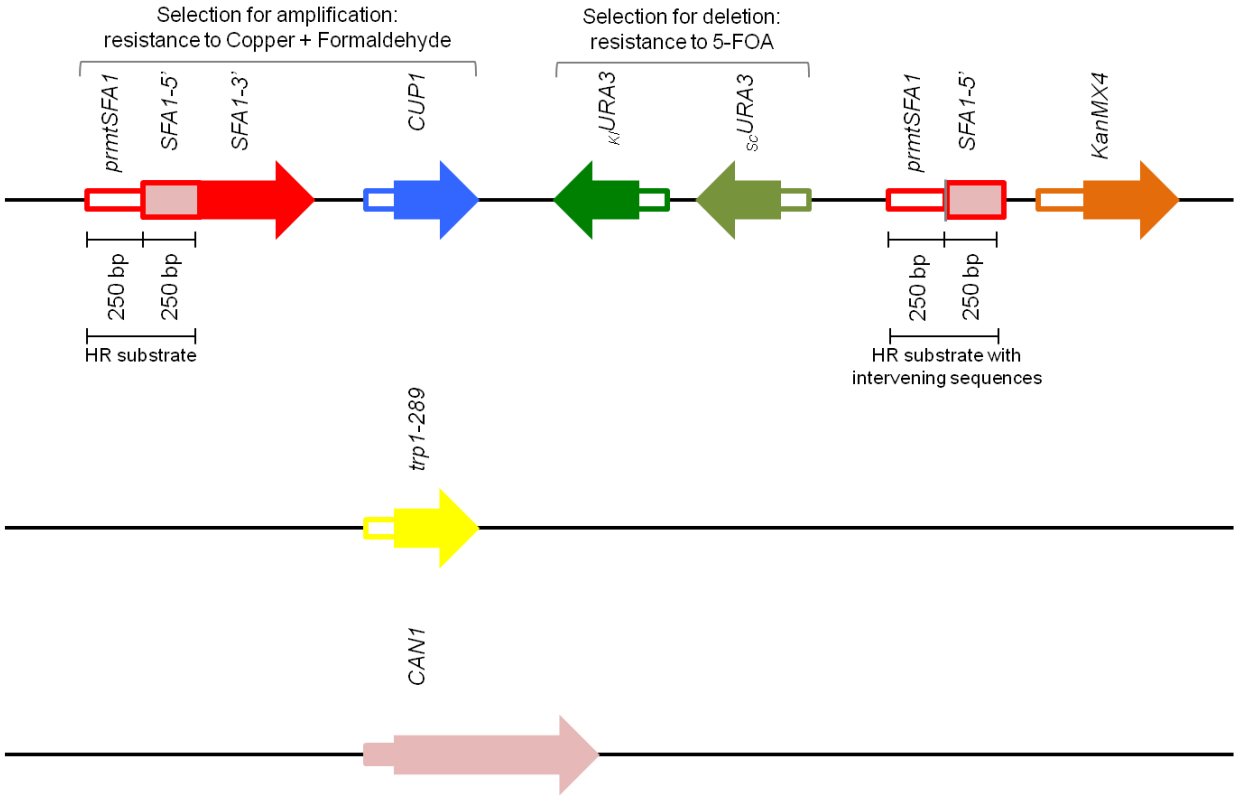

Figure 19. Schematic of a portion of the chromosomal regions from the haploid yeast strain JAY2087. In the top line, a view of the region from chromosome IV where CNV reporters were inserted. The HR substrates represent direct repeats that can mediate homologous recombination leading to amplification and/or deletion of the region containing the copy number reporter genes *SFA1*, *CUP1*, *KlURA3* and *ScURA3*. The intervening sequence present in the HR substrate on the right side is very small (6 bp *Bgl*II site), therefore is not expected to significantly influence recombination between the two substrates. This strain also contained the *trp1-289* mutation, also on chromosome IV, which can revert to a functional Trp+ allele of the *TRP1* gene, and was wild type for the *CAN1* gene on chromosome V, which following a forward inactivating mutation can lead to resistance to canavanine.

3.3.5 Yeast acute toxicity assays

As a first step in the mutation assays, acute toxicity assays were conducted to determine the lowest concentration of water sample, if any, that inhibited yeast growth. Sterile techniques were used throughout the study. Water samples and YPD (yeast-peptone-dextrose media) were filter sterilized using a 0.22 μm polyethersulfone (PES) filter (sterilized, low protein binding, Corning Incorporated, Corning, NY). All other media and materials were purchased sterile and/or autoclaved. Recipes and chemical supplier information for liquid media and agar plates are provided in the Supporting Information. JAY2087 stock was stably maintained long term at -80°C in 30% glycerol. A patch plate was made by streaking frozen JAY2087 cells onto a YPD plate using a sterile toothpick. The plate was incubated at 30°C for 24 hours, at which point yeast from the patch plate was inoculated in 5 mL YPD liquid media and placed on a rotating drum, spinning at 39 rpm, at 30°C for 24 hours to reach saturation. Thirty (30) μL of 10^{-2} dilution of the overnight yeast culture was used to inoculate the cultures for this assay. For each water sample, four different concentrations were initially analyzed (10%, 20%, 40% and 80%) in addition to a control (0%). Duplicate 5 mL solutions were made for each concentration in culture tubes. Cultures contained equal concentrations of YPD. Cultures were placed on the rotating drum at 30°C for 24 hours. After 24 hours, a 10 μL aliquot of four different dilutions of each culture (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were plated on YPD. This plate was incubated for 48 hours at 30°C. Visual observations were used to determine which concentration inhibited yeast growth, if any. If necessary, additional rounds of this assay were completed at lower concentrations to determine the threshold. An example of this assay is shown in the Supporting Information (Figure S1). Yeast mutation assays were conducted with water samples at the highest concentration that did not visibly inhibit yeast growth. In most cases, a second and lower concentration was also used. For 2016 samples, two concentrations were selected for all 2016 samples (50% and 25%). This included the highest concentration that did not visibly inhibit yeast growth (50%) and a lower concentration

(25%). For the 2018 samples, growth inhibition was observed in the discharge sample (D0) at concentrations above 10%. As a result, all 2018 assays were conducted with 10% sample. In downstream samples where growth was not visibly inhibited at any concentration, assays were conducted with 80%.

3.3.6 Copy number variation and point mutation assays

For the yeast mutation assays, a patch JAY2087 YPD plate was made as described in the acute toxicity assays and allowed to incubate for 1-2 days. Yeast was streaked from the patch plate to single colonies on a YPD plate and incubated at 30°C for 18 hrs. Following the incubation period, single colonies from the YPD plate were inoculated into 4 mL liquid YPD cultures with two different concentrations of each water sample, if possible. The culture tubes were placed on a rotating drum at 30°C, until saturation was reached (~22 hours). Once the cultures reached saturation, a 1 mL aliquot was removed from each culture and washed twice with sterile double-distilled water. Aliquots of the washed yeast were then plated on non-selective (YPD) and each of selective plates required for the individual mutation assays using autoclaved glass beads at the volumes and dilutions shown in Table 5. Plates were incubated at 30°C for the time listed in Table 5. In total, there were 11 independent culture replicates of each sample at each concentration. Negative control (YPD and double-distilled water) and positive control cultures (YPD, double-distilled water and 10 mg/L methyl methanesulfonate (MMS)) were also included. The concentration of MMS (10 mg/L) was determined following the yeast acute toxicity assay procedure.

In addition to samples collected from the field site, a municipal wastewater treatment plant (WWTP) effluent sample from Fort Collins, CO was also analyzed. This sample was included because WWTP effluent is a discharge that people are more familiar with and therefore will help put these findings in perspective. Additionally, similar to NPDES PW discharges, the exact composition of the WWTP effluent is unknown. Previous studies have shown that treatment methods are often

ineffective at removing emerging contaminants from these discharges (e.g., pharmaceuticals) and WWTP effluents have been linked with increased mutagenicity downstream, along with other chronic toxicity endpoints.[112, 126] Mutation assays are commonly used to assess chronic toxicity in WWTP and therefore are a reasonable way to compare these two effluents.

Table 5. Volume, dilution and incubation time for non-selective (YPD) and selective plates used in yeast mutation bioassay.

Plate	Volume Plated (μL)	Dilution	Incubation Time (d)
YPD	150	10^{-5}	2
Copper Formaldehyde	150	10^{-1}	5
5-FOA	150	10^{-1}	3
Canavanine	200	undiluted	3
Tryptophan Drop Out	600	undiluted	4

3.3.6.1 Mixtures of known composition

Mixtures of known composition were analyzed with the yeast mutation assay to investigate the specific source of any mutation rate changes at the NPDES discharge. Chemical mixtures included a benzene, toluene, ethylbenzene and xylenes (BTEX) mixture; a mixture that contained all organic chemicals defined by International Agency for Research on Cancer (IARC) and U.S. EPA to be known, probable and possible carcinogens (Table 7); and a salt control. These mixtures were chosen because negative health effects associated with PW have been attributed to both the organic fraction and salts [114-116]. Chemical concentrations were equivalent to concentrations measured at the NPDES discharge in 2016. Chemical analysis of the water samples was conducted in Chapter 2 of this dissertation. The BTEX mixture contained 15.5 μg/L benzene, 6.3 μg/L toluene, 6.6 μg/L ethylbenzene, 8.4 μg/L *m,p*-xylene, and 8.2 μg/L *o*-xylene. The organic carcinogen mixture contained 0.56 μg/L 1,2-dichloroethane, 2.4 μg/L 2-methylphenol, 0.93 μg/L 4-methyl-2-pentanone, 15.5 μg/L benzene, 6.6 μg/L ethylbenzene, 0.63 μg/L isopropylbenzene, and 11.4 μg/L naphthalene. At the time of analysis, methyl acrylate was listed by the IARC as "not classifiable as to its carcinogenicity to

humans" and was not included in the mixture. It has since been designated "possibly carcinogenic to humans." The salt control was made by mixing a 2016 NPDES discharge sample with 5 g/L activated carbon overnight. Benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene, *p*-xylene, 1,2-dichloroethane and 2-methylphenol were purchased from Fisher Scientific. Isopropylbenzene and 2-methyl-2-pentanone were purchased from Sigma Aldrich. All chemicals were greater than 98% purity. Assays were conducted in cultures containing 25% and 50% concentration of these mixtures to reflect 2016 assay procedures.

3.3.6.2 Estimation of mutation rates

An Interscience Scan 300 was used for automated colony counting to ensure consistency between plates. The total number of viable cells (N_t) in each culture was calculated from the number of colonies that grew on the nutrient rich, permissive YPD plates, with a correction for plating volume and dilution (Table 5). The number of mutant cells (r) was calculated from the number of colonies on each of the four different selective plates, with appropriate correction for the respective plating volume and dilution for each mutation (Table 5). Median mutation rates and 95% confidence intervals were calculated using the Lea-Coulson (LC) method of the median, with minor modifications detailed in Hall et al. (2009) [127-128]. The 95% confidence intervals around the median are displayed in the figures to indicate the width of the distribution of mutation rates for all cultures for each condition, but not for determination of statistical significance between samples and concentrations (see below). Mutation assays are inherently variable because of the stochastic nature of mutation accumulation in cultures over time, therefore for the same treatment, some cultures typically experience few mutations while others experience many. This is normal and explains why the median rate is used to represent the overall results. It is routine and expected to have relatively wide 95% confidence intervals for these types of measurements. Finally, a relative mutation rate was calculated for each mutation type at each site by dividing the median mutation rate (M) at each site by the median mutation rate for the

respective negative control (e.g., (relative median mutation rate for CNV duplications at site D0) = (median mutation rate for CNV duplications at site D0)/(median mutation rate for CNV duplications in the negative controls)). Relative mutation rate analyses permit convenient comparison between concentrations and sampling distances for each the four types of mutation classes measured. The absolute rates vary by orders of magnitude between assays. The mutation rate changes are only comparable within assays, not between.

Statistical analysis for mutation rates was conducted using the Kruskal-Wallis test, a non-parametric test, with the Benjamini-Hochberg adjustment for multiple comparisons. All data for each sampling event were analyzed at once (2016: sampling sites, negative control, mixtures and WWTP; 2018: sampling sites). With the Benjamini-Hochberg adjustment, samples with $p < 0.025$ are considered statistically different. Results of the statistical analysis are presented in the figures as letters above each bar. In these figures, samples with different letters are statistically different from each other. When comparing the mutation rates associated with two different treatments, the statistical call is made based on the differences in the distribution of all measurements (Kruskal-Wallis test), not based on overlap between 95% confidence intervals. It is entirely possible (and typical for mild mutagenic stimulations of 2-3 fold, like those found here) to conclude that there is a significant difference in mutation induction between treatments, even though the 95% CI distributions may overlap.

3.3.7 Zebrafish Developmental Assays

A zebrafish developmental assay was used to evaluate the four samples collected in 2016 (D0, D15, P-2.6 and P32.2) to assess potential adverse neuro-behavioral impacts of the water samples.[129] Analyses included mortality and morphology (from 6 to 120 hours post fertilization; hpf), a 24 hpf embryo photometer response behavior (EPR), 120 hpf larval photometer response behavior (LPR) and responses.[130-131] Detailed methods for these analyses can be found in Appendix B.

3.3 Results and Discussion

3.3.1 Daphnia magna acute toxicity studies

The NPDES permit requires acute WET tests to be conducted with *Daphnia magna* and *Pimephales promelas* every 6 months at this site. Four of the samples collected in 2016 (D0, D1.4, P-2.6, P32.2) and 2018 (D0, D1.4, P-24.2, P34.4) were assayed for lethality using *Daphnia magna* and a serial dilution exposure regime of concentrations 0, 1, 2, 5, 10, 20, and 100 % of each water sample in 30 mL of solution (total). In the analyses conducted with samples from 2016, all daphnia were alive after the 48 hour exposure and no lethality could be determined. For the samples collected in 2018, lethality was low and calculations of a LC_{50} for each individual field-collected sample were unable to be performed. Sample D1.4 (1.4 km downstream of the NPDES discharge, located on the discharge stream) had on average 60% mortality in undiluted conditions, while variable, low-level toxicity was observed across dilutions in sample P34.4 (34.4 km downstream of the NPDES discharge, located on the perennial stream) (Table 6). In general, however, undiluted samples had negligible effects. These results also agree with acute WET tests conducted by the operators, which reported no violations during these sampling events. Previous studies have shown that acute exposure of PW to aquatic organisms, such as rainbow trout and zebrafish, can result in chronic toxicity (e.g., oxidative stress and gill morphology) in organisms where lethality is not observed.[69, 114] Thus, the low lethality observed in this assay does not rule out that *Daphnia magna* may have experienced chronic impacts from PW exposure.

Table 6. *Daphnia magna* neonate lethality results in 2018 discharge stream (D) and perennial river (P) samples following 48 hr exposures.

Site	D0		D1.4		P-24.2		P34.4	
Exposure Dilution	$\overline{x}$ Mortality (%)	σ (%)	$\overline{x}$ Mortality (%)	σ (%)	$\overline{x}$ Mortality (%)	σ (%)	$\overline{x}$ Mortality (%)	σ (%)
0%	0	0	0	0	0	0	0	0
1%	0	0	0	0	0	0	6.66	11.55
2%	0	0	0	0	0	0	0	0
5%	0	0	0	0	0	0	6.66	11.55
10%	0	0	0	0	0	0	0	0
20%	0	0	0	0	0	0	6.66	11.55
100%	0	0	60	0	0	0	6.66	11.55

3.3.2 Yeast copy number variation and point mutation assays

3.3.2.1 October 2016 samples

Mutagenicity assays were conducted in yeast cells exposed to samples collected at the NPDES discharge and downstream. For samples collected in October 2016, growth inhibition was observed in cultures containing greater than 50% concentration of the discharge sample (D0). Mutation assays for all sites were conducted with both 50% and 25% sample concentration and the results of the assays conducted with 50% water samples are presented (Figure 20). Additional data for the 25% cultures are presented in Appendix B. Similar trends were observed in experiments conducted with 25% sample (Figure B2). Mutation rates and differences between sites were generally lower in 25% cultures, most likely due to lower chemical concentrations.

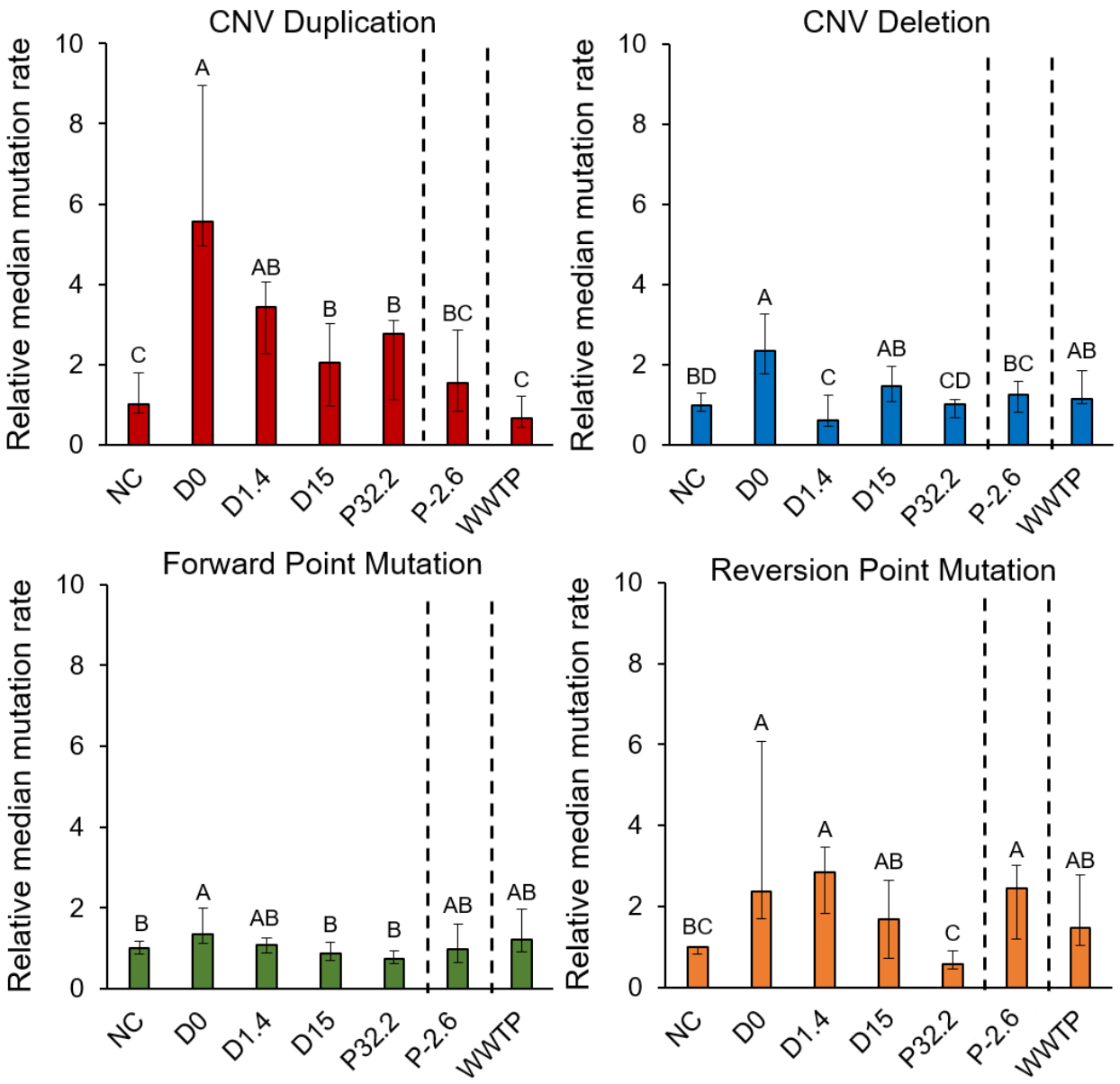

Figure 20. Relative median mutation rate for Copy Number Variation (CNV) duplications, CNV deletions, forward point mutations, and reversion point mutations in 2016 discharge stream (D), perennial river (P) samples and wastewater treatment plant (WWTP) sample. Experiments were conducted with 50% water sample. Median mutation rates are displayed relative to the negative control (NC) set as a 1x reference. Error bars show 95% confidence intervals. Letters show the statistical groupings. Samples that do not share letters are significantly different ($p < 0.025$) from the Kruskal-Wallis test.

The results of the CNV duplication, CNV deletion assay, forward point mutation and reversion point mutation assays conducted at 50% sample concentration are shown in Figure 20. In assays conducted with sample from the NPDES discharge, a robust increase in CNV duplication rate

was observed, which was significantly increased as compared to the negative control (NC) (5.6-fold, $p < 0.0000$). Mutation rate decreased with distance downstream but remained elevated in samples D1.4 and D15 as compared to the negative control (3.4- and 2.0-fold; $p = 0.0001$ and 0.0162, respectively). CNV duplication rate for the positive control (10 mg/L MMS) was 3.3-fold increase as compared to the NC (data not shown).

In the remaining three assays (CNV deletions, forward point mutation and reversion point mutation), a significant increase in mutation rate was observed at the discharge, however, this increase was more modest (CNV deletion: 2.3-fold, $p = 0.0015$; forward point mutation: 1.3-fold, $p = 0.0102$; reversion point mutations: 2.4-fold, $p = 0.0024$). For all three assays, statistical analysis and median mutation rates suggest a trend of mutation rate decreasing with distance from the discharge point. However, we are more cautious about interpretation of results at D1.4 and D15 for these assays since the range of mutagenicity stimulation between D0 and NC was small. For the positive control (10 mg/L MMS), CNV deletion rate was 1.8-fold, forward point mutation rate was 2.1-fold and, reversion point mutation rate was 2.3-fold increase as compared to the NC (data not shown). Preliminary tests were also conducted using the *Salmonella* Ames test, which is analogous to the *trp1-289* reversion point mutation yeast assay conducted in this study. A minimal increase in mutation rate was observed at the discharge point (D0: 1.2-fold increase), agreeing with the results of the yeast assay.

In all four mutation assays, no significant difference in mutation rate was observed between the upstream (control site) and downstream sites on the perennial river (P-2.6 and P32.2). This was expected since the two streams were not connected via surface water at the time of sampling and suggests that the discharge stream did not significantly impact mutation rate in the perennial river. It was important to assess, however, since the two streams connect during times of increased discharge and decreased evaporation. Chemicals found in the discharge can sorb to sediments (e.g., nonylphenol ethoxylates), which may increase toxicity even when flow rates are lower.[28]

Mutation rates generally followed the trend observed for organic chemicals in the discharge stream. As shown in Chapter 2 of this dissertation, concentrations of organic chemicals were highest at the NPDES discharge and decreased with distance downstream. Table 7 lists organic chemicals detected in the discharge stream, including 8 that have been determined by the U.S. EPA and IARC to be known, probable or possible carcinogens. Since most mutagens are likely carcinogens, it is reasonable to compare mutation rate trends over distance to the environmental fate of carcinogens detected at the discharge. Of the 8 carcinogens in Table 7, 6 were present at the NPDES discharge in 2016 but below detection limit by site D1.4, including 1,2-dichloroethane, 4-methyl-2-pentanone, benzene, ethylbenzene, isopropylbenzene and methyl acrylate. Both 2-methylphenol and naphthalene were present at site D1.4 but below detection limit by site D15. At the discharge (D0), four of the carcinogenic organic chemicals were present above the maximum contaminant level (MCL) and/or screening level for tap water including 1,2-dichloroethane, benzene, ethylbenzene and naphthalene.

Five of the inorganic chemicals detected in the NPDES discharge are IARC or EPA known, probable or possible carcinogens including arsenic, cadmium, lead, silica (inhalation route) and radium (Table 8). Only radium concentrations decreased with distance downstream, thereby following the trend observed in mutation rates. Radium was also present above the MCL at the discharge [32]. Arsenic, cadmium and lead were not detected at the NPDES discharge but were detected downstream, suggesting that these species were already present in the soil or were concentrated due to evaporation downstream.

In an effort to put the PW discharge results into perspective, a WWTP effluent sample from Fort Collins, CO was analyzed. In all four mutation assays, the rate of mutation in the WWTP sample was not significantly increased as compared to the NC. This contrasts with the PW discharge, which showed a significant increase in all four mutation rates as compared to the NC.

Table 7. Toxicological data for organic chemicals detected in the discharge stream.

Chemical Species	Highest Conc. Observed (µg/L)	SL/MCL (µg/L)[b]	RfD[c] (mg/kg-day)	Evidence for Toxicity[d]	Carcinogenic Evaluation		CCL[g]
					IARC[e]	EPA[f]	
1,2,4-Trimethylbenzene	6.45	56/-	0.01	RT, CT, SCT, DT, AT	-	-	1, 2
1,2-Dichloroethane	0.56	0.17/5	0.006	RT, CT, SCT, AT, NT	2B	B2	-
1,3,5-Trimethylbenzene	1.07	60/-	0.01	RT, CT, SCT, DT, AT, NT	-	-	-
1-Methylnaphthalene	6.92	1.1/-	0.07	RT, CT, SCT, AT, NT	-	-	-
2,4-Dimethylphenol	6.57	360/-	0.02	RT, CT, SCT, AT	-	-	-
2-Butanone	13.4	5600/-	0.6	RT, CT, SCT, AT	-	-	-
2-Butoxyethanol	1.28	2000/-	0.1	RT, CT, SCT, DT, AT, SAT	3	-	-
2-Methylnaphthalene	5.96	36/-	0.004	RT, CT, SCT, AT, NT	-	-	-
2-Methylphenol	2.41	930/-	0.05	RT, CT, SCT, DT, AT	-	C	1,2
4-Methyl-2-pentanone	0.93	6300/-	-	RT, CT, SCT, AT	2B	-	-
Acetone	49.8	14000/-	0.9	RT, CT, SCT, AT	-	-	-
Benzene	14.6	0.46/5	0.004	RT, CT, SCT, AT	1	A	-
Bis(2-ethylhexyl) phthalate	5.13[a]	5.6/6	0.02	RT, CT, SCT, DT, AT, SAT	2B	B2	-
Carbazole	3.03	-/-	-	CT, SCT, AT	2B	-	-
Carbon disulfide	50.9[a]	810/-	0.1	RT, CT, SCT, AT	-	-	-
Diesel range organics	1555	-/-	-	-	-	-	-
Ethylbenzene	6.6	1.5/700	0.1	RT, CT, SCT, AT, SAT, NT	2B	D	-
Isopropylbenzene	0.63	450/-	0.1	CT, SCT, DT, AT	2B	D	-
Methyl Acrylate	0.92	42/-	-	CT, SCT, AT	2B	D	-
Naphthalene	11.4	0.17/-	0.02	RT, CT, SCT, DT, AT, SAT	2B	C	1
n-Propyl Benzene	0.8	660/-	0.1	CT, SCT, AT, NT	-	-	3,4
Phenanthrene	1.34	-/-	-	RT, CT, SCT, AT	3	D	-
Phenol	1.03	5800/-	0.3	RT, CT, SCT, DT, AT, NT	3	-	-
Toluene	6.49	1100/1000	0.08	RT, CT, SCT, AT, NT	3	-	-
TPH as Gasoline	155.5	-/-	-	-	-	-	-
Xylenes	20.35	190/10,000	0.2	RT, CT, SCT, AT	3	-	-

Notes: [a]Maximum concentration was observed at a sampling site other than the discharge. [b] Screening level (SL) for tap water and Maximum Contaminant Level (MCL) are from comptox.epa.gov. SLs are reported with Target Hazard Quotient (THQ) = 1. [c]RfDo = Reference Dose for Oral Exposure obtained from EPA Comptox Website and EPA Integrated Risk Information System (IRIS) Reports. [d]Evidence for toxicity obtained from EPA CompTox, (RT = Reproductive Toxicology; CT = Chronic Toxicology; SCT = Subchronic Toxicology; DT = Developmental Toxicology; AT = Acute Toxicology; SAT = Subacute Toxicology; NT = neurotoxicology). [e]IARC = International Agency for Research on Cancer (1=Carcinogenic to humans; 2A = Probably carcinogenic to humans; 2B = Possibly carcinogenic to humans; 3 = Not classifiable as to its carcinogenicity to humans; 4 = Probably not carcinogenic to humans); [f]EPA (A = Carcinogenic to humans; B = Probably carcinogenic to humans; C = Possibly carcinogenic to humans; D = Not classifiable as to human carcinogenicity; E = Evidence of non-carcinogenicity); [g]CCL = Contaminant Candidate List.

Table 8. Toxicological data for inorganic chemicals detected in the discharge stream.

Chemical Species	Highest Conc. Observed (µg/L) (location)[a]	MCL[b] (ug/L)	RFD[d] (mg/kg-day)	Evidence for Toxicity[e]	Carcinogenic Evaluation		CCL[h]
					IARC[f]	EPA[g]	
Aluminum	1860 (D2.1)	50[c]	1	RT, CT, SCT, AT	-	-	1,2
Ammonia as N	364.5	-	-	RT, CT, SCT, AT	-	-	-
Antimony	0.5 (D2.1)	6	0.0004	CT, SCT, AT	-	-	-
Arsenic	3.6 (D3.8)	10	0.0003	RT, CT, SCT, AT	1	A	-
Barium	140	2000	0.2	CT, SCT, AT	-	D, E	-
Boron	1310 (D15)	-	0.2	RT, CT, SCT, AT, SAT	-	-	1,2
Cadmium	0.2 (D2.1)	5	0.0005	RT, CT, SCT, AT	1	B1	-
Calcium	75,700 (D15)	-	-	CT, SCT, AT	-	-	-
Chloride	251,000 (D15)	250,000[c]	-	CT, SCT, AT	-	-	-
Fluoride	2910 (D2.1)	2000[c]	0.04	CT, SCT, AT	3	-	-
Iron	2620 (D2.1)	300[c]	0.7	CT, SCT, AT	-	-	-
Lead	3.2 (D2.1)	15	-	RT, CT, SCT, AT	2B, 2A	B2	-
Magnesium	51,800 (D15)	-	-	CT, SCT, AT	-	-	-
Manganese	229 (D3.8)	50[c]	0.14	RT, CT, SCT, AT	-	D	1,4
Molybdenum	84.9 (P34.4)	-	0.005	CT, SCT, AT	-	-	3,4
Potassium	30300 (D15)	-	-	CT, SCT, AT	-	-	-
Selenium	12.8 (D15)	50	0.005	CT, SCT, AT	3	D	-
Silica	39,900 (D2.1)	-	-	RT, CT, SCT, DT, AT, SAT	1, 3	-	-
Sodium	454,000 (D15)	-	-	RT, CT, SCT, AT	-	-	1
Strontium	4805	-	0.6	CT, SCT, AT	-	-	3
Sulfate	939,000 (D15)	250,000[c]	-	CT, SCT, AT	-	-	1
Total Dissolved Solids		500,000	-	-	-	-	
Uranium	5.2 (P61.3)	30	-	CT, SCT, AT	-	-	-
Vanadium	24.3 (D15)	-	0.005	CT, SCT, AT	-	-	1,2,3,4
Zinc	67.3 (P34.4)	5000[c]	0.3	RT, CT, SCT, AT	-	-	-
Radium-226 & Radium-228	0.503 Bq/L	0.185 Bq/L		CT	1	-	-

Notes: [a]Location if other than discharge. [b]Maximum Contaminant Level (MCL) is from comptox.epa.gov. [c]National Secondary Drinking Water Regulation. From EPA. [d]RfDo = Reference Dose for Oral Exposure obtained from EPA Comptox Website and EPA Integrated Risk Information System (IRIS) Reports. [e]Evidence for toxicity obtained from EPA CompTox, (RT = Reproductive Toxicology; CT = Chronic Toxicology; SCT = Subchronic Toxicology; DT = Developmental Toxicology; AT = Acute Toxicology; SAT = Subacute Toxicology; NT = neurotoxicology). [f]IARC = International Agency for Research on Cancer (1=Carcinogenic to humans; 2A = Probably carcinogenic to humans; 2B = Possibly carcinogenic to humans; 3 = Not dassifiable as to its carcinogenicity to humans; 4 = Probably not carcinogenic to humans); [g]EPA (A = Carcinogenic to humans; B = Probably carcinogenic to humans; C = Possibly carcinogenic to humans; D = Not classifiable as to human carcinogenicity; E = Evidence of non-carcinogenicity); [h]CCL = Contaminant Candidate List.

3.3.2.2 Mixtures of known composition

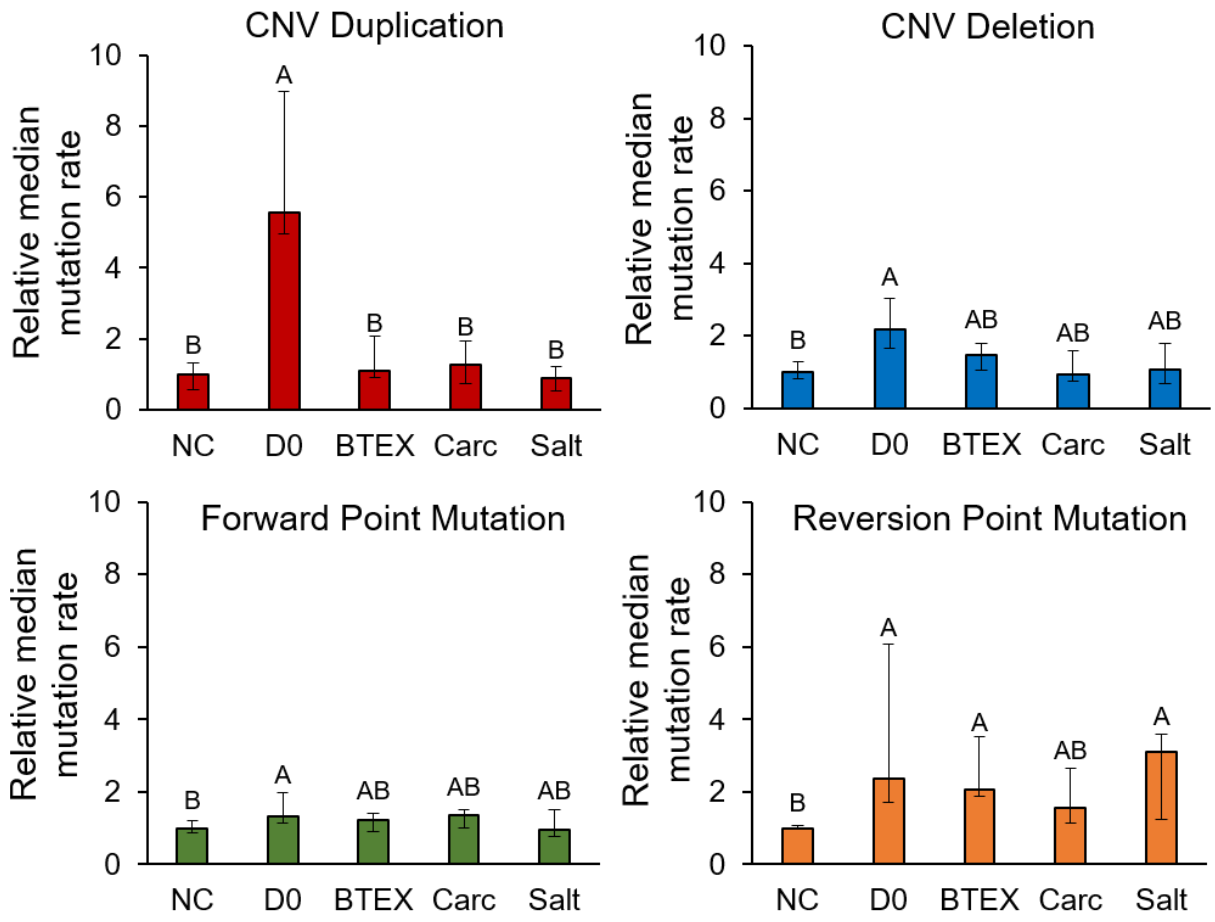

Figure 21. Relative mutation rate for copy number variation (CNV) duplications, CNV deletions, forward point mutations, and reversion point mutations of discharge sample (D0), BTEX mixture, carcinogen mixture (Carc) and salt control relative to the negative control (NC). Experiments were conducted with 50% water sample. Bar height represent median mutation rates and error bars represent 95% confidence intervals. Letters show the statistical groupings for the bioassays. Samples that do not share letters are significantly different.

Assays were conducted with mixtures of known composition to investigate the specific compounds responsible for the detected increase in mutation rates, focusing particularly on the CNV duplications, due to the robust increase in this mutation in the discharge samples. Results for the other three assays were also collected in parallel given the design of this yeast strain. The three know mixtures tested included a BTEX mixture; IARC and U.S. EPA organic carcinogens (Table 7); and a salt control. The discussion below focuses on assays conducted with chemical concentrations equal to

50% of that detected at the discharge in 2016. Experiments were also conducted at 25% concentration (Figure B4) and showed similar trends.

The rates of CNV duplication in cells exposed to the known mixtures were not significantly altered relative to the negative control, while mutation rate in the discharge sample was elevated by 5.6-fold (Figure 21). These results suggested that either the chemical(s) responsible for the increase CNV duplication rate was absent in the mixtures, or that these chemical groups must act through a synergistic mechanism. Similar to the results from the CNV assays, mutation rates in the mixtures were low and generally showed no significant difference as compared to the NC.

The toxicity of many organic chemicals in PW are unknown and therefore, it is possible that some of the chemicals detected at the discharge are mutagens but were not included in the carcinogen mixture. Additionally, due to the complex nature of PW, mutagenic chemicals may be present in the water, but not identified during analysis. This includes chemicals that are below detection limit due to low concentration or matrix effects in the fluid.[48] It also includes chemical additives that are not disclosed in the permits and therefore are more challenging to identify. These issues are further compounded by mixture effects. Studies have shown synergistic toxic effects in mixtures of PW chemicals.[101] Synergistic toxicity has also been observed in mixtures containing more than two PAHs, which is true for the discharge sample.[132] It is also possible that a mixture containing the carcinogens and salts would have resulted in higher mutation rates.

3.3.2.3 February 2018 samples

Yeast mutation assays were conducted with samples collected in 2018 to determine if toxicity changed over time (Figure B3). In 2018, growth inhibition at the NPDES discharge (D0) was observed at concentrations above 10%. As a result, all studies were conducted with 10% water sample in each yeast culture. In all four assays, there were no major trends in mutation rate over distance. For some sites, mutation rate was significantly increased as compared to the negative control only, however,

even in these instances the mutation rate increases were mild. Due to a lack of trend in the mutation data, there is no clear relationship that can be made to the chemical data.

Increases in mutation rate were lower in 2018 than in the 2016 samples conducted at 50% and 25% concentration. This was expected since a lower concentration of sample (10%) was used in the 2018 yeast cultures. For example, in the CNV duplication assays conducted with 10% sample, mutation rate increases ranged between 1.6-fold (D0) and 2.7-fold (D1.4) as compared to the negative control. This is lower than what was observed at the NPDES discharge in assays conducted with 50% sample (5.6-fold increase) and 25% sample (4.5-fold increase) in 2016. It is possible that similar trends and rates of mutation would have been observed in the 2018 samples if acute growth inhibition did not prevent assays from being conducted at higher concentrations. It also suggests that growth inhibition and mutagenicity are likely caused by different agents in the complex mixtures.

No major differences in chemical composition were observed that could explain this difference in acute toxicity (see Chapter 2). It is possible that the differences are seasonal and therefore temperature dependent. Daytime air temperatures in October 2016 ranged from 4.5°C to 13°C, while daytime air temperatures in February 2018 ranged between -12°C to -4°C. Solubility of hydrogen sulfide and other gases increases at lower temperatures. Although it was not quantified, H_2S was detected in the air by meters worn during sampling. Due to the lower temperatures, it was likely present at higher concentrations in the 2018 water samples. H_2S is a known yeast growth inhibitor, but has not been defined as a carcinogen by either EPA or IARC [133-134]. Therefore, it is possible that in order to dilute the H_2S below acute toxicity levels, the chronic toxins were no longer present at concentrations high enough to result in significant increases in mutation rate. Another explanation for this result is that the holding time for the 2016 samples was longer than for the 2018 samples (6 months vs. 1.5 months). All chemical analyses were conducted within standard EPA holding times, so it is possible that concentrations of chemicals, many of which can exhibit acute toxicity (Table 7

and Table 8), were lower in the 2016 samples at the time of analysis. This is true for H_2S as well as other volatile organic compounds. Finally, it is possible that the standard chemical analysis does not reveal all toxic chemicals present at the discharge. No conclusions can be made on how consistent concentrations of undetected chemicals were over time. Overall, however, this shows that acute toxicity of the NPDES discharge and stream can change with time.

3.3.3 Zebrafish developmental toxicity assays

Zebrafish assays were conducted on these water samples to assess developmental toxicity. These assays analyzed for early stage developmental toxicity by quantifying a 24-hour post fertilization (hpf) Embryo Photomotor Response (EPR) behavior and 120 hpf Larval Photomotor Response (LPR) behavior as well as mortality and morphology responses. No significant impacts were observed in the developmental studies. Additionally, there were no significant incidences of mortality or malformation observed in any of the water samples (Figure B5 and Table B3). Holding time before this analysis was 10 months. Due to the extended holding time, it is likely that concentrations of organic chemicals decreased during this time. Previous studies have observed significant changes in LPR when zebrafish are exposed to polycyclic aromatic hydrocarbons (PAHs), including carbazole, naphthalene, 1-methylnaphthalene and 2-methylnaphthalene, which were present in PW discharge.[135] Thus, it is possible that different results would have been observed if holding time was decreased.

3.4 Conclusions

A toxicological analysis was conducted on a NPDES PW discharge and stream released for agricultural beneficial reuse. In addition to permit effluent limits based on chemical concentrations, NPDES PW releases in Wyoming require acute toxicity testing once every 6 months, but no chronic toxicity testing. In this study, acute toxicity was assessed using *Daphnia magna*. Lethality was low in all samples, however, it appeared higher in 2018 versus 2016 samples. A yeast-based mutation assay was used to analyze four different types of mutation – CNV duplications, CNV deletions, forward point

mutations and reversion point mutations. In all mutation assays, higher rates of mutation were observed at the discharge (D0) and decreased with distance downstream. This was most prominent for CNV duplications. A similar trend was observed for the concentrations of IARC and EPA known, probable and possible carcinogens detected in the stream (e.g., benzene, naphthalene, radium). Mixture studies showed that untested chemical(s) or chemical mixtures were responsible for increased rates of CNV duplication; thus, the best treatment strategy to decrease this toxicity remains unknown. Finally, zebrafish assays revealed no increase in developmental toxicity in the water samples.

This study is the first attempt to evaluate the toxicity of NPDES PW releases for beneficial reuse. It is important to note that most chemicals at this site were present at relatively low concentrations compared to other PW in the western U.S. Therefore, the results of this study cannot be applied to all NPDES PW discharges. NPDES PW releases in other geologic formations need to be analyzed to understand toxicological differences. During preparation of this manuscript, access was granted to two additional NPDES PW discharges in the area. One of these sites has failed the acute WET test multiple times in the past 3 years. Preliminary chemical analysis at the site reveals that volatile organic compounds (VOCs) are present at concentrations 2 orders of magnitude higher than at the focus discharge in this study. It is expected that increased concentrations of VOCs would increase both acute and chronic toxicity.

Federal agencies, including the U.S. Department of Energy, are interested in developing treatment strategies to increase beneficial reuse. To assess the efficacy of these treatment strategies, however, the composition and downstream impacts of PW must be better understood. This study lays out a framework for what is needed to properly characterize NPDES PW releases. Chemical analysis alone is insufficient, and a thorough assessment of chronic toxicity is necessary. The state of Colorado requires chronic toxicity testing of NPDES PW releases and Wyoming, as well as other states, should follow suit. Furthermore, a range of toxicological endpoints must be assayed including some, such as

endocrine disruption, that remain to be analyzed at this site.[112] This could be achieved with a high-throughput assay that assesses a range of toxicological endpoints.[45] Many PW treatment studies have focused on a "treat for use" approach. As such, assay trigger values should be defined for different downstream users.[113]

Bioassays are just a first step. Additional research at this site should focus on the health of livestock, aquatic species, crops and humans who consume these products. If these waters can be reused safely and economically, many stakeholders stand to benefit. If this practice is expanded prematurely, the quality and health of water, soil, crops and downstream users could be negatively impacted. This would result in thousands of legacy sites that must be remediated, and oil and gas operators may be subject to liability and clean-up costs

CHAPTER 4: VIABILITY OF CONSTRUCTED WETLANDS FOR PRODUCED WATER POLISHING DOWNSTREAM OF NPDES RELEASES

4.1 Introduction

Due to increasing demand for water and issues associated with other produced water (PW) management strategies (e.g., earthquakes), operators and governments are increasingly interested in finding ways to reuse PW, either in the oilfield or outside. Prior to reuse for any of these purposes, however, PW must be treated. Many approaches for PW treatment have been studied including membrane separation and distillation, forward osmosis, electrocoagulation, advanced oxidation processes, adsorption, and biological treatment.[7, 50-51] Treatment cost can range from a few cents to multiple dollars per barrel depending on treatment type, location, quality of the inlet water and more.[25] Treatment facilities often require skilled staff, high start-up and maintenance costs and external power.[52] These factors can make treatment and reuse financially infeasible, especially in rural areas.

Chemical analysis in Chapter 2 indicated that attenuation of oil and gas chemical additives (i.e., surfactants) increased within a constructed wetland (CW) downstream of a NPDES PW discharge. CWs have been used for decades to treat domestic, agricultural and industrial wastewaters. More recently, CWs have been used to polish PW, generally after treatment with separators and/or chemical additives.[52, 136-137] Guerra et al. 2011 suggested that CWs are not effective for PW treatment because they are not effective at reducing salinity and have even been shown to increase salinity of this brackish waste stream.[5, 138] In areas where salinity is relatively low for PW, however, CWs are likely more appropriate. Additionally, CWs may be the only economically viable option for treatment in remote areas. The composition of PW is unique to other wastewaters that have been managed by CWs due to the unique mixture of chemicals and the addition of oil and gas chemical additives. Thus, further investigation into the viability of CWs for PW management is warranted.

Wetlands are currently used for PW polishing in both Wyoming and California, USA. Operators at these remote sites have selected CWs as a treatment approach because they are less expensive and require less maintenance than other available methods. Additionally, in both Wyoming and California, the local communities are interested in reusing the water for agricultural purposes (e.g., irrigation) and to provide water sources for livestock, migratory birds, and other wildlife.[52, 137] Despite the fact that these CWs have been in operation for decades (some since the 1980s or earlier), minimal analysis has been conducted on PW treated at these sites.

Previous studies, many of which were conducted in the lab, have shown CWs are effective at reducing a range of bulk contaminant parameters including chemical oxygen demand (COD), biological oxygen demand (BOD), oil, trace organics, and in some cases, total dissolved solids (TDS).[136, 139-141] Additional studies have reported attenuation of metals in PW treated via CWs and specifically analyzed cadmium, copper, nickel and zinc.[139-140] Finally, one study showed decreased acute toxicity in *Daphnia magna* after treatment of PW with reverse osmosis followed by CWs.[139] To date, no studies on CWs used for complex PW treatment have addressed the fate of organic chemical additives. Many oil and gas chemical additives are biodegradable and readily sorb to sediment (e.g., nonylphenol ethoxylates) and therefore are likely ideal for treatment in CWs.[28] The impact of sorption of these chemicals on the lifetime and maintenance schedule of CWs, however, is unknown. Additionally, biocides are a commonly used class of chemical in oil and gas well-maintenance. These chemicals are designed to suppress microbial activity. Biodegradation, along with sorption, plant uptake and photodegradation, are the major attenuation mechanisms in CWs. Thus, the addition of biocides may negatively impact biodegradation rates in CWs.

The goals of this study are to 1) assess the viability of CWs for PW polishing, 2) determine the environmental fate and transport of oil and gas organic chemical additives, and 3) assess if microbial communities are impacted by PW discharges. To achieve these goals, this study will focus on three

surface flow CW systems in Wyoming used to polish PW downstream of three different NPDES releases. Surface flow wetlands are generally less expensive than subsurface flow wetlands and can more easily be built in remote locations.[140, 142] Salt concentrations at these discharges are low (TDS: 1000 - 3,500 mg/L) compared to most PWs in the U.S. and therefore organics are the main focus for removal. By determining major mechanisms of attenuation in PW, this study will provide valuable information for the design of additional CWs for PW treatment.

4.2 Materials and Methods

4.2.1 Site description

This study was conducted at an undisclosed field site in Wyoming where over 10 NPDES PW discharges are located. At this site, O&G operations occur in a relatively remote location and there are few other sources of contamination. Analysis focused on three NPDES PW discharges and the wetland(s) used to polish the PW downstream (Figure 22). For the remainder of this study, these sites will be referred to as Discharge A (DA), Discharge B (DB), and Discharge C (DC). Previous studies conducted in Chapter 2 and 3 of this dissertation analyzed the chemistry and toxicity of water samples collected from DC and the surrounding watershed.

Treatment at all three sites is relatively similar. After extraction from the wells, the oil-gas-PW mixture is combined and sent to the treatment system. Treatment includes a three-phase separator (oil, gas, water) which uses heat, gravity, and emulsion-breaking chemicals. Once separated, a portion of the PW is reinjected underground either for enhanced oil recovery or for disposal in cases where TDS exceeds effluent limits. At DA only, sulfide is removed via oxidation and biological methods. Permits for DB provided detailed information on well maintenance chemicals used onsite including scale inhibitors, corrosion inhibitors, and a water clarifier (Table C1). Additionally, the permit stated

that hydraulic fracturing occurs every other year at this site. Details regarding well maintenance chemicals and stimulation schedule were not available for the other two discharges.

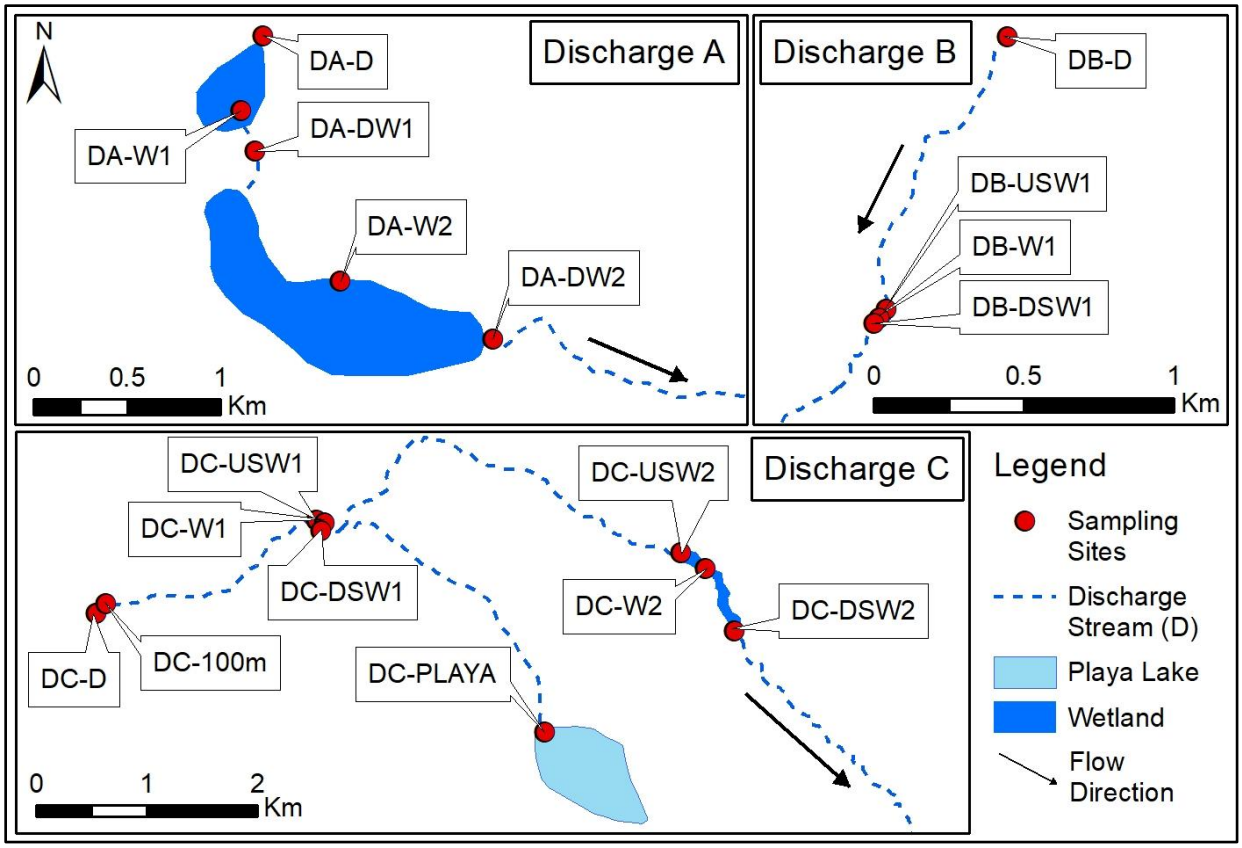

Figure 22. Map of sampling locations at three undisdosed NPDES produced water discharges in Wyoming, Discharge A (DA), Discharge B (DB) and Discharge C (DC). Surface water and sediment grab samples were collected in November 2018. Sites DA-D, DB-D and DC-D were collected directly from the discharge culvert. All other sites were collected upstream (US), downstream (DS) or within the wetlands. The first wetland on each discharge is indicated by W1 and the second by W2. When large enough, wetlands are indicated on the map in dark blue. In some instances (DB-W1 and DC-W1) the wetlands are smaller and hidden beneath the sampling site indicators. Site DC-100m was collected 100 m downstream of DC-D.

At DA, discharge rates average 1.5 million liters per day and range between 0.4 and 6.5 million liters per day. This discharge is released directly into a large (~40,000 m^2) wetland. Water then flows approximately 0.3 km into a ~200,000 m^2 wetland that is less vegetated than the first. After leaving the second wetland, water flows nearly 15 km and passes through additional CWs before it connects with a much larger perennial stream that is used as a drinking water intake downstream. There is little precipitation in the region (average 230 mm/year)[57] and no additional tributaries to the wetlands and

streams discussed at these sites. As a result, the wetlands and streams downstream of all three discharges are composed entirely of O&G PW unless there has been a recent precipitation event.

At DB, PW from 13 wells is combined, treated and released, resulting in a PW to oil ratio exceeding 20:1. On average, DB releases 4.0 million liters per day directly into an ephemeral stream bed. The stream bed contains some vegetation, including many reeds. A 350 m^2 wetland is located 0.8 km downstream of DA and the reeds are more concentrated in this area. Water exits the wetland through a culvert and flows another 0.3 km to a pond, which was dry during the sampling event. At the time of sampling, multiple wells at this site were not operating due to low oil prices. This resulted in a lower than average discharge rate.

DC was the focus of an extensive chemical and toxicological evaluation presented in Chapters 2 and 3 of this dissertation. At DC, an average of 4.5 million liters of PW are released per day into an ephemeral stream bed. A 450 m^2 CW is located 1.8 km from the discharge, followed by a dam that separates the discharge into two equal streams. One continues southeast for about 2 km before emptying into a playa lake which is a shallow, ephemeral lake, commonly found in the U.S. High Plains region.[58] The other stream continues another 30 km until connecting with a larger perennial river that is used as the drinking water intake for thousands of people downstream. Along this 30 km stretch are a series of CWs, the first of which is located 5.2 km downstream of the discharge. This wetland is approximately 2500 m^2. Similarly to DB, a portion of the wells at this site were not in operation during sampling due to low oil prices.

Daily maximum effluent limits for these NPDES discharges are provided in Table 9. Effluent limits are the same between all permits except DA has effluent limits for sulfide (as H_2S) and selenium, while the other two discharges do not. In addition to these effluent limits, the permit also states that no floating solids or visible foam can be discharged other than in trace amounts. Discharge rate must be reported monthly for DA and DC and every six months for DB. For DB and DC, sulfide as H_2S

must be reported quarterly. A toxic pollutants screen, which includes organic and inorganic pollutants outlined in U.S. Code of Federal Regulations Title 40, Part 122, Appendix D, must be conducted in the first, third and fifth years of the permit. Permits typically last four to five years. In addition to these chemical limits, acute whole effluent testing (WET) is required quarterly at the site. This involves an acute 48-hour static-renewal toxicity test using *Daphnia magna* and an acute 96-hour static-renewal toxicity test using *Pimephales promelas*. Over the past three years, violations to these permits have been identified at both DA and DB. At DA, the oil and grease effluent limits were exceeded. Violations at DB included failed acute toxicity tests and exceedances for sulfate. No violations were reported at DC in this time period.

Table 9. NPDES permit effluent limit daily maximums specific to the discharges in this study. If no limit listed, that parameter was not specified for that discharge.

Parameter	Discharge A	Discharge B	Discharge C
Specific Conductance	7500 µS/cm	7500 µS/cm	7500 µS/cm
Total Dissolved Solids	5,000 mg/L	5,000 mg/L	5,000 mg/L
Chloride	2,000 mg/L	2,000 mg/L	2,000 mg/L
Sulfate	2,500 mg/L	2,500 mg/L	2,500 mg/L
Total Radium 226	60 pCi/L [a]	60 pCi/L [a]	60 pCi/L [a]
Oil and Grease	10 mg/L [b]	10 mg/L [b]	10 mg/L [b]
pH	6.5 - 9.0 [c]	6.5 - 9.0 [c]	6.5 - 9.0 [c]
Sulfide (as H2S)	200 mg/L	-	-
Selenium	5.0 µg/L	-	-

[a] Values taken directly from the permit. 60 pCi/L = 2.22 Bq/L.
[b] Permit also states that there cannot be a "visible sheen in the receiving waters or deposits on the bottom or shoreline of the receiving waters."
[c] pH range given. All other values are maxima.

4.2.2 Site Sampling

Surface water and sediment grab samples were collected at all three field sites in November 2018. Samples DA-D, DB-D and DC-D were collected directly from the NPDES discharge point (D), immediately before the water entered the streams. All other sampling sites were located immediately upstream, downstream or within a wetland. The naming conventions for these sites indicates their location. For example, DC-USW1 is located upstream (US) of the first wetlands (W1). DC-W1 is

located within the first wetland (W1) and DC-DSW1 is located downstream (DS) of the first wetland (W1). Exceptions to these rules include DC-100m, which is located 100 m downstream of DC-D, and DC-PLAYA, which is located near the inlet to the playa lake downstream of DC-D. In addition, a control site wetland (CSW) that was unimpacted by PW releases was also sampled. A complete list of site names, site descriptions and distances from the discharge are provided in Table 10.

Table 10. Sampling site names, descriptions and distance from discharge point.

Site Name	Distance from Discharge (km)	Site Description
CSW	-	Control Site Wetland
Discharge A (DA)		
DA-D	0.00	NPDES Discharge Point
DA-W1	0.33	Wetland 1
DA-DW1	0.53	Downstream of Wetland 1
DA-W2	1.41	Wetland 2
DA-DW2	2.06	Downstream of Wetland 2
Discharge B (DB)		
DB-D	0.00	NPDES Discharge Point
DB-USW1	0.79	Upstream of Wetland 1
DB-W1	0.82	Wetland 1
DB-DSW1	0.84	Downstream of Wetland 1
Discharge C (DC)		
DC-D	0.00	NPDES Discharge Point
DC-100m	0.10	100 m downstream of discharge
DC-USW1	1.79	Upstream of Wetland 1
DC-W1	1.85	Wetland 1
DC-DSW1	1.90	Downstream of Wetland 1
DC-USW2	5.24	Upstream of Wetland 2
DC-W2	5.40	Wetland 2
DC-DSW2	6.00	Downstream of Wetland 2
DC-PLAYA	4.10	Playa Lake Inlet

Water samples were collected in the center of the streams and as close to the center of the wetlands as possible. Water samples for microbial analysis were collected using Sterivex filters (0.22 μm, polyethersulfone, Millipore). Sediment samples in the streams were collected near the shore in an area of sediment accumulation. In the wetlands, sediment samples were collected as close to the water sample as possible, also in an area of sediment accumulation. With the exception of water samples for

NPOC analysis, samples for organic analysis were collected in glass bottles with Teflon-lined caps. Water samples for NPOC analysis were collected in plastic bottles and acidified in the field. Prior to collection, all glassware was cleaned with Milli-Q water and methanol and baked in a muffle furnace for 6 hours at 450°C. Samples for microbial analysis were collected in sterile plastic bags. Field and lab blanks were also collected and processed alongside each analysis. At each site, a Hanna HI98194 probe was used to measure temperature, pH, and specific conductance of the water.

4.2.3 Non-purgeable Organic Carbon and Total Nitrogen Analyses of Water

Water samples for non-purgeable organic carbon (NPOC) and total nitrogen (TN) were collected in plastic bottles, acidified using HCl (pH < 2) in the field and immediately placed on ice. Samples were stored at -20°C in the lab and analyzed within 4 weeks of collection. NPOC and TN of water samples was determined using a Shimadzu TOC-L equipped with a platinum catalyst. Triplicate injections were performed at 720°C. Standardization was based on a 6-point calibration curve using aqueous potassium hydrogen phthalate (KHP) and potassium nitrate (KNO_3) stock standards. Dilutions were performed in the instrument and the limit of detection was approximately 0.2 mg/L. Each sample was analyzed three times to ensure data repeatability. Check standards were run every 10 samples.

4.2.4 Total Organic and Inorganic Carbon of Sediments

Sediments for total carbon and total nitrogen were collected in glass bottles, stored on ice in the field and then stored at -20°C in the lab until analysis. Analysis was conducted using a LECO TruSpec CN. Wet sediments were dried in glass containers in the muffle furnace at 105°C, ground using a mortar and pestle and then sieved through 2 mm sieve. Samples ranging from 0.05 g to 0.2 g were weighed into tin sampling cups and placed in the autosampler for analysis. A Sidney High soil standard was used for calibration. A blank and check standard were run every ten samples. Blanks

consisted of empty tin cups for TC and TN. Results are reported as percent of sediment mass on a dry weight basis.

Inorganic carbon (i.e., carbonate) content of soils was analyzed using a calcimeter, pressure transducer and voltage meter following methods in Sherrod, 2002.17. Samples ranging from 0.25 to 1.0 g were weighed into amber glass vials, depending on expected inorganic carbon concentration. Next, 2 mL of a 6N HCl + 3% ferrous chloride solution was added to a 0.5 dram (1.84 mL) vial, which was carefully placed into each amber vial to avoid spilling. Vials were then capped using a rubber stopper and aluminum seal. Capped vials were shaken vigorously for one minute to ensure that the HCl solution had wet the entire sample. Vials were then allowed to rest for two hours while the reaction continued. After two hours, the voltage from each vial was measured using a voltage meter. A needle attached to the voltage meter was quickly inserted into the septa of each vial and the voltage was recorded. This needle was rinsed after each sample. Concentrations were determined using a 7-point CaCO3 standard curve. Blanks consisted of empty headspace vials containing 2 mL of a 6N HCl + 3% ferrous chloride solution. Organic carbon concentrations for sediments were determined by subtracting the inorganic carbon value from the total organic carbon value.

4.2.5 Volatile Organic Compounds Analysis

Samples for volatile organic compound (VOC) analysis were collected without headspace, stored on ice in the field and stored at 4°C until analysis. Water samples were prepared following EPA Method 5021A using a Tekmar 7000 Headspace Autosampler and analyzed for volatile organics following EPA Method 8015 using an Agilent 6890N Network Gas Chromatography (GC) System with a Flame Ionization Detector (FID). Analysis parameters for the headspace analyzer are shown in Table 11. For GC-FID analysis, a Rtx-5 column (30 m length, 0.32 mm internal diameter, 0.25 μm film thickness, Restek) and the following temperature program were used: 40 °C (held for 2 min), then increased at 12°C min^{-1} to 150°C, then increased at 30°C min^{-1} to 250°C (held for 3 min). Ultra-high

purity helium was used as a carrier gas at a constant flow rate of 3 mL/min. Sample injection volumes were 1 mL. Compound identification was achieved using retention times of analytical standards, including Gasoline Range Organics (Restek, Bellefonte, PA) and naphthalene (Alfa Aesar, Ward Hill, MA).

Table 11. Headspace analyzer parameters.

Variable	Value
Platen/Sample Temp	75°C
Valve Oven Temp	150°C
Transfer Line Temp	150°C
Standby Flow Rate	
Sample Equilibration Time	15 min.
Pressurize	10 psig
Pressurize Time	1 min.
Pressurize Equilibration Time	0.2 min.
Loop Fill Time	0.2 min.
Inject Time	1 min.
Mixer	ON
Mixing Time	2 min.
Mixer Level	3
Mixer Stabilize Time	0.1 min.
Constant Heat Time	ON

4.2.6 Semi-Volatile Organic Compound Analysis

Both water and sediment samples analyzed for semi-volatile organic compounds were stored on ice in the field and kept at -20°C in the lab until analysis. Water samples were filtered through glass microfiber filters (Whatman, Grade 934-AH; cleaned in the muffle furnace) and then extracted. Samples (500 mL) were adjusted to pH = 11 using 6M sodium hydroxide and then extracted 3 times with dichloromethane (DCM) (60 mL) using an Erlenmeyer flask. Each DCM portion was collected into a glass flask. After 3 washes, the water sample was then adjusted to pH 2 and three additional extractions were performed with 60 mL of DCM each. The DCM portions were collected in the same flask as the pH 11 extracts. The DCM extract was then filtered through 30 g of $NaSO_4$ (pre-combusted at 450°C for 4 hours) to remove any remaining water in the DCM. Subsequently, DCM was evaporated

using a rotary evaporator at 35°C, 130 rpm until ~5 mL of the sample remained. Extracts were then transferred to pre-weighed glass sample vials and evaporated under a gentle stream of N_2 gas until ~1 mL of extract remained. At this point, the vials were weighed again and the internal standard (Restek, SV Internal Standard Mix) was added. Extracts were stored at -20°C until analysis.

Sediments for SVOC analysis were freeze-dried in glass containers, ground using a mortar and pestle and sieved through 2 mm sieve. For each site, 5 g of sediment was combined with 5-11 mL of DCM, depending on sample density. Vials were shaken by hand for 60 seconds, allowed to vent and then placed on a vibration shaker table for 60 minutes. After settling for 1 hour, a 1 mL aliquot of DCM was removed from each vial. The internal standard (Restek, SV Internal Standard Mix) was added and extracts were stored at -20°C until analysis.

DCM extracts for water and sediment samples were analyzed for (semi-)volatile organics by an Agilent 6890 gas chromatograph equipped with an Agilent 5973N Mass Selective Detector using a VF-5MS column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, Agilent) and the following oven temperature program: 50°C (held for 2 min), then increased at 7°C min^{-1} to 215°C, then increased 15°C min-1 to 315 and held for 5 min. Ultra-high purity helium was used as a carrier gas at a constant flow rate of 1 mL/min. Sample injections were 2 μL. Injector temperature was set at 285°C. The GC-MS transfer line temperature was maintained at 320°C and the ion source temperature was held at 230°C. The mass spectrometer was operated in electron ionization mode (70 eV). Mass spectra were recorded in full scan mode (m/z 45-600). Compound identification was achieved using mass spectra and retention time of analytical standards, including Gasoline Range Organics, Diesel Range Organics (Restek, Bellefonte, PA), EPA 625 Semivolatiles Calibration Mix (Supelco, Bellefonte, PA), 1-methylnapthalene , and 2-butoxyethanol (Sigma Aldrich, Saint Louis, MO).

4.2.7 Non-Volatile Organic Compound Analysis

Both water and sediment samples were analyzed for non-volatile organic compounds. Water samples were collected without headspace, stored on ice in the field and stored at 4°C in the lab until analysis. Sediment samples were stored on ice in the field and at -20°C in the lab. Water samples were filtered through glass microfiber filters (Whatman, Grade 934-AH) and then extracted. Solid phase extraction (SPE) was used to concentrate surfactants and reduce the salt concentrations in the samples. Glassware for surfactant analysis was pre-cleaned by washing with deionized water (3x), Milli-Q water (3x) and methanol (1x) followed by baking in a muffle furnace (400°C for 8 hours). Bottles were rinsed three times with sample water prior to collection. Prior to extraction, high purity hydrochloric acid was added to water samples to adjust to pH 3 in order to increase extraction efficiency. Supel Select HLB cartridges (200mg/6mL, Supelco, Bellefonte, PA) were conditioned with methanol (HPLC grade, Fisher) followed by Milli-Q water and Milli-Q water, adjusted to pH 3 using hydrochloric acid. A volume of 1000 mL of sample was applied to the cartridges (5-10 mL min^{-1}). Cartridges were washed with 50 mL of 5% methanol solution and then dried under vacuum for 15 minutes. Surfactants were eluted from the cartridge using 10 mL of methanol. Samples were stored at -20°C and analyzed within 24 hours.

Sediment extracts were prepared following methods described in Lara-Martin et al., 2011[143]. Sediment was freeze-dried, milled and sieved following the procedures described for SVOCs. Extraction was performed using three 30-minute cycles in a sonicator bath at 50°C. Methanol was used as the solvent. After each sonicator cycle, samples were centrifuged for 5 minutes at 8,000 rpm and the solvent was decanted. All three extracts were combined and then filtered through a glass microfiber filter (Whatman, Grade 934-AH). Samples were evaporated to 2 mL using a gentle stream of nitrogen and then reconstituted to 100 mL using Milli-Q water. Samples were extracted using the SPE method described for the water samples, with the wash volume reduced to 10 mL, and then

evaporated down to 1 mL using a gentle stream of nitrogen. For both water and sediment extracts, octaethylene glycol monodecylether (Sigma Aldrich, Saint Louis, MO) was added as an internal standard.

Water and sediment methanol extracts were analyzed for NVOCs using a Quadrupole Time-of-Flight mass spectrometer (Q-ToF-MS). Extracts were analyzed using an Agilent 1290 Infinity Series liquid chromatograph coupled with an Agilent 6530 Quadrupole Time-of-Flight mass spectrometer (Q-ToF), using the method described in Thurman et al. (2014)[144] with the following exceptions. Mobile phases were A (0.1% formic acid) and B (acetonitrile). A gradient elution method was developed with 0-2 minutes, 20% B; 2-15 min, 20-95% B; 15-22 min, 95% B; 22-25 min, 20% B. The flow rate was 0.6 mL/min, the injection volume was 20 µL, and the temperature of the drying gas was 325°C. Peaks were identified by accurate mass and potential chemical formulas, which were then verified using surfactant standards. An exact concentration of each surfactant series could not be determined due to a lack of commercial standards with known ethoxymer distribution. Instead, an estimated concentration was determined at the discharge using polyethylene glycol 400, polypropylene glycol (Alfa Aesar, Haverhill, MA), and 4-nonylphenol-polyethylene glycol (Sigma Aldrich, Saint Louis, MO) standards. For alkyldimethylbenzylammonium chloride (ADBAC), three different alkyl lengths (C10, C12, C14) were detected and a dodecyldimethyl-*n*-benzylammonium chloride (Alfa Aesar, Haverhill, MA) standard was used to estimate concentration. Relative concentrations (C/C_0) were determined for samples downstream since all samples were stored in the same manner and extracted and analyzed at the same time.

4.2.8 Microbial Analysis (16S rRNA gene Sequence Analysis)

Sterivex filters and sediments for microbial analysis were stored in sterile plastic bags on dry ice in the field and at -70°C in the lab until analysis. Total nucleic acids were extracted from 0.4 g of sediment using the DNeasy PowerSoil Ki (Qiagen) and eluted with 10 µL of elution buffer, then stored

at -20°C. Extracted DNA purity and quantity were measured on a Qubit Fluorometer (Thermofisher Scientific). DNA was sequenced at the Colorado State University next-generation sequencing facility. Bacterial 16s rRNA gene libraries were prepared according to the two-step PCR workflow in the *Illumina 16s Metagenomic Sequencing Library Preparation Protocol (Part 15044223 Revision B)*. Round one primers were modified to include n=0 to n=3 base pair heterogeneity spacers according to Galan et al., 2018. Round two primers included two eight base pair barcodes for sample multiplexing (Frank et al., 2009). HiFi HotStart ReadyMix (Roche Ltd.) was used to amplify libraries. Individual libraries were pooled at approximately equimolar ratios and library QC included visualization on with Tapestation HS D1000 reagents (Agilent, Inc.) and qPCR using Library Quantification Master Mix and Standards (Roche Ltd.). The pooled libraries were sequenced at 10pM on the MiSeq instrument (Illumina Inc.) using the 500 cycle (2 x 250 base pair) V2 Reagent Kit with 15% PhiX spike-in to increase base-call heterogeneity during the run.

Data processing was conducted with QIIME2 following the protocol of Borton et al. 2017.[145] Samples with less than 5000 reads were discarded due to low data quality. Statistical analysis was performed primarily using the R statistical package "vegan". Alpha diversity was calculated with the diversity function to investigated both richness and Shannon's diversity. Beta diversity was calculated by analyzing Bray-Curtis dissimilarities using the relative abundance of samples, and then plotting these values with non-parametric multi-dimensional scaling (NMDS) plots in R. Both a multi response permutation procedure and mean dissimilarity matrix (mrpp) function and an analysis of similarities (anosim) function were calculated to determine the significance of differences between sample groups.

4.3 Results and Discussion

4.3.1 Field Parameters

Field parameters for the sampling sites are shown in Table 12. For all three discharges, temperature was highest at the discharge point (DA-D: 35.4°C; DB-D: 10.6°C; DC-D: 40.4°C) and decreased with distance downstream. Temperature decrease was more rapid in DA and DB than in DC. At DA and DB, pH was lowest at the discharge (DA-D: 7.07; DB-D: 7.40) and trended upward in the wetlands and with distance downstream. At DC, pH was 7.90 at the discharge, increased slightly through the first wetland and then decreased through the second wetland. Previous studies have observed increases in pH downstream of both oil and gas and coalbed methane (CBM) PW discharges in Wyoming and attributed the increases to evaporation and carbonate precipitation.[32, 146] All sampling sites were within the range of the pH permitted at the effluent (pH 6.5-9).

In both DA and DB, specific conductance was lowest at the discharge (DA-D: 6,400 μS/cm; DB-D: 5,080 μS/cm) and increased with distance downstream. This is likely due to evaporation. In Discharge A, specific conductance was above the permit effluent limits (7,500 μS/cm) at site DA-W2 (7,830 μS/cm) and DA-DSW2 (11,400 μS/cm). Specific conductance at DC was lower than in DA and DB and remained relatively steady at all sampling sites, ranging between 1910 and 2290 μS/cm. In the control site wetland (CSW), temperature was 4.9°C, pH was slightly higher than in the impacted wetlands (8.80), and specific conductance was substantially lower than in the impacted wetlands (900 μS/cm).

Table 12. Field parameters for all sites during November 2018 sampling event.

Site Name	Temperature (°C)	pH	Specific Conductance (µS/cm)
CSW	4.9	8.80	900
Discharge A			
DA-D	35.4	7.07	6400
DA-W1	1.9	8.41	5790
DA-DW1	3.6	8.05	6330
DA-W2	2.1	8.30	7830
DA-DW2	2.4	8.56	11400
Discharge B			
DB-D	10.6	7.40	5080
DB-USW1	----------Water too low for sampling----------		
DB-W1	1.0	7.62	6420
DB-DSW1	1.9	7.77	6430
Discharge C			
DC-D	40.4	7.90	2270
DC-100m	41.1	8.11	2290
DC-USW1	29.8	8.40	2090
DC-W1	27.1	8.50	2080
DC-DSW1	24.4	8.36	2010
DC-USW2	16.5	7.73	2000
DC-W2	15.7	7.76	1910
DC-DSW2	2.4	7.81	1990
DC-PLAYA	11.8	7.72	2090

4.3.2 Total Organic Carbon and Total Nitrogen in Water

Non-purgeable organic carbon (NPOC) and total nitrogen (TN) were analyzed in water samples to understand changes in bulk contaminant parameters due to treatment with CWs. NPOC ranged from 2.4 to 8.7 mg/L in water samples. In both Discharge B and C, NPOC concentrations generally decreased with distance downstream and rates of removal increased in the wetlands (Figure 23). In contrast to the other two discharges, NPOC in Discharge A increased steadily with distance and the rate of attenuation was not altered in the wetlands. NPOC in the unimpacted wetland was 6.25 mg/L. TN in water samples ranged from 0.0 to 3.0 mg/L in all sites. In all three discharges, TN concentrations trended downward with distance downstream. Previous studies have reported that

CWs are effective at reducing NPOC and TN in PW, as well as other wastewaters.[136, 139] The increase in NPOC in DA was unexpected and the cause is currently unknown.

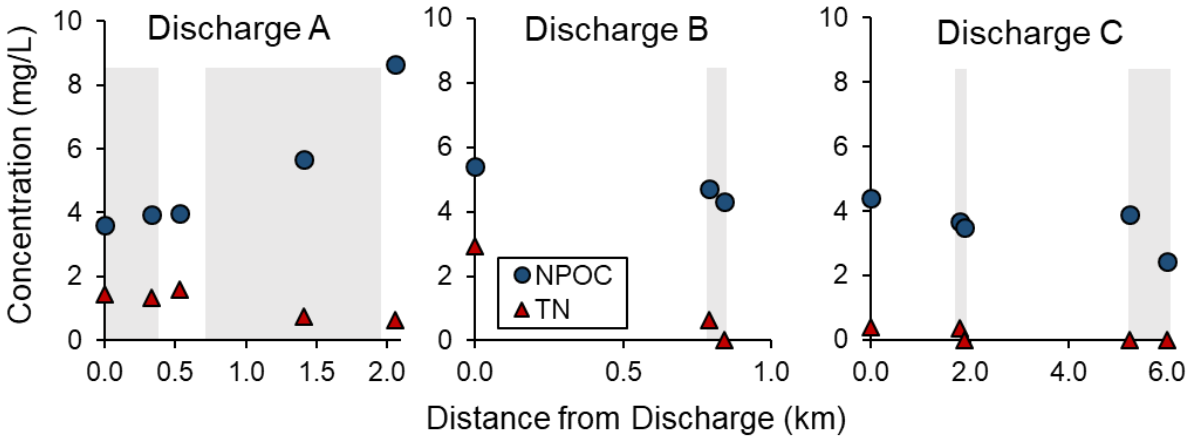

Figure 23. Non-purgeable organic carbon (NPOC) and total nitrogen (TN) in water samples collected at the three discharges. Wetlands are represented by grey boxes.

4.3.3 Total Carbon, Organic Carbon, Inorganic Carbon and Total Nitrogen in Sediment

Total carbon, organic carbon, inorganic carbon and total nitrogen were analyzed in the sediment samples because it was hypothesized that these sediment characteristics may impact attenuation rates and sorption of well maintenance chemicals. Sediments in the three discharges ranged from 0.5 to 12.9 % (dry wt.) total carbon (TC). In general, TC trended downward with distance from the discharge (Figure 24). In both Discharge A and Discharge C, however, TC was lower at the discharge site (DA-D, DC-D) than at the site immediately downstream. Additionally, TC increased slightly within the first wetland on Discharge B (DB-W1) and within the second wetland on Discharge C (DC-W2).

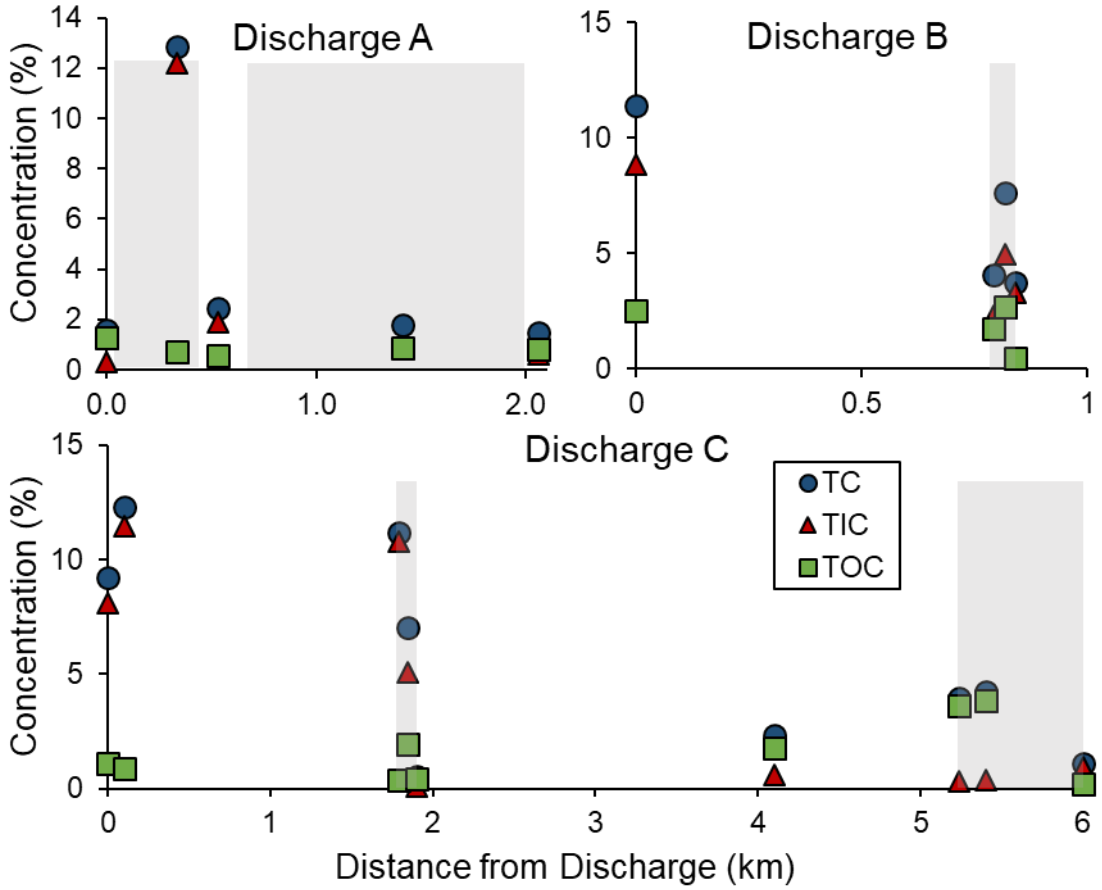

Figure 24. Total Carbon (TC), total inorganic carbon (TIC) and total organic carbon (TOC) concentrations (% dry wt.) versus distance downstream (km) from each of the discharges. Grey areas indicate locations of wetlands.

For all three discharges, total inorganic carbon (TIC) was the dominant form of carbon through the first wetland and therefore followed the same trend as TC. In sediments collected from the second wetland in Discharge A and Discharge C, organic carbon (OC) was the dominant form of carbon, as shown by the increasing TOC:TIC ratios downstream (Figure C1). An increase in organic carbon content was observed in most of the wetlands including DA-W2, DB-W1, DC-W1 and DC-W2. This is likely due to decomposition from plants. In the control site wetland, total carbon was 5.5%, TIC was 2.0%, TOC was 3.5% and the TOC to TIC ratio was 1.8. Finally, in all three discharges, the carbon to nitrogen ratio of sediments generally decreased with distance from the discharge (Figure C2).

4.3.4 Volatile Organic Compounds Analysis

Volatile organic compounds (VOCs) detected at the discharge included 1,2,4-trimethylbenzene, benzene, toluene, ethylbenzene, xylenes (BTEX) and acetone (Figure 25). VOC concentrations were highest at DB-D, with benzene (848 μg/L) and toluene (1,070 μg/L) being the most prominent. Total BTEX released at this site was 2,640 μg/L. Concentrations of VOCs were lower at DA-D (BTEX: 880 μg/L), by as much as an order of magnitude, and were lowest at DC-D by another order of magnitude (BTEX: 70.0 μg/L). Both benzene and toluene were detected downstream in DA and DB, but below the limit of quantification (LOQ). All other VOCs were below the detection limit in downstream samples. As a result, changes in VOC chemical concentration cannot be used to assess the efficacy of these wetlands for PW polishing. Previous studies have shown, however, that benzene removal in both wetlands and streams is dominated by volatilization.[147] This was also observed and discussed in Chapter 2 of this dissertation.

The VOCs detected at these discharges are geogenic chemicals and therefore naturally present in PW. Many are also components of well maintenance chemicals identified in the NPDES permits (1,2,4-trimethylbenzene, toluene, xylenes and ethylbenzene; Table C1). Additionally, acetone is a commonly used solvent and a known by-product of polypropylene glycol (PPG) biodegradation, another well-maintenance chemical detected at these sites.[66] Permit effluent limits are not defined for these species, however, benzene, toluene and ethylbenzene are monitored as part of the toxic pollutant screening (DA: every three months; DB and DC: every two years).

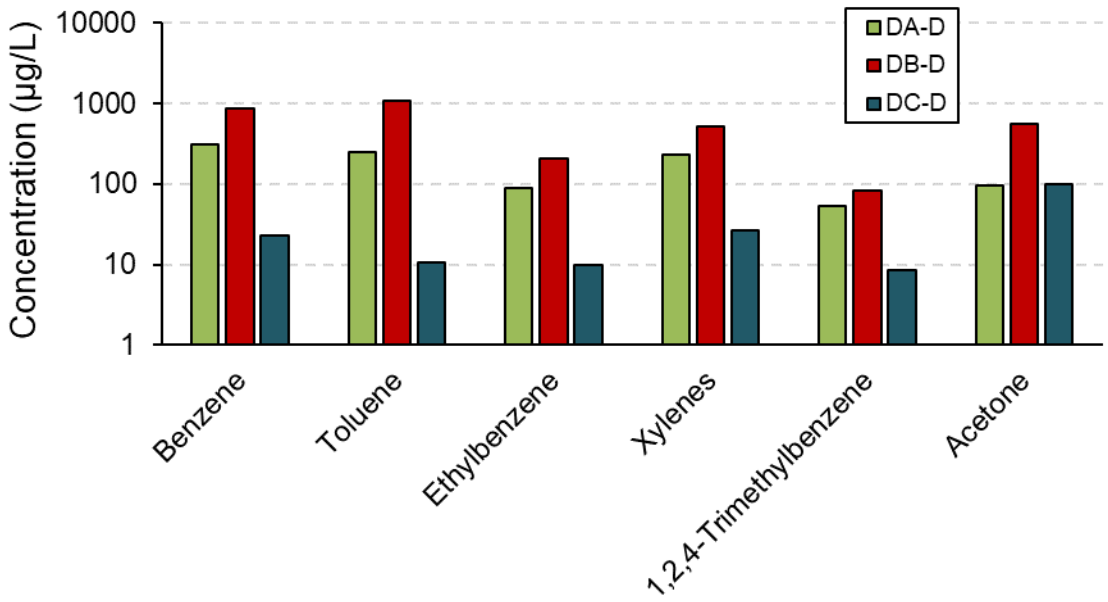

Figure 25. Volatile organic compound (VOC) concentrations detected at each of the discharges.

In January 2019, Discharge B failed both the *Daphnia magna* and *Pimephales promelas* acute toxicity tests. As a result of this failure, the operators are required to test the toxicity again and conduct a toxicity identification evaluation if the issue isn't resolved. The permit does not indicate if discharge could be halted due to continued noncompliance on this test. Toluene, ethylbenzene and xylene concentrations at DB-D all exceeded the surface water acute toxicity values for aquatic species (Toluene: 120 µg/L; Ethylbenzene: 130 µg/L; Xylenes: 230 µg/L) and likely contributed to the failed toxicity assays (Table 3). It should also be noted that benzene is a known carcinogen and was released at concentrations 170 times greater than the maximum contaminant level (MCL) for drinking water (Table 7).

At DA-D, toluene and xylenes were also above the acute toxicity threshold for aquatic species; however, no violations of the acute toxicity test have been reported at this site. This may be due to the WET testing methods which state that "aeration may be used to bring the [dissolved oxygen] and other gases into equilibrium with air" and would therefore reduce VOC concentrations. Concentrations of VOCs at DC-D were not above acute aquatic toxicity thresholds. At all discharge

points, BTEX were above chronic toxicity thresholds for aquatic species (Table 3). Thus, chronic impacts to downstream users (e.g., fish, livestock, waterfowl) are possible including endocrine disruption, and increased potential for cancer, etc.

4.3.5 Non-Volatile Organic Compounds: Water Analyses

Water samples were analyzed for non-volatile organic compounds (NVOCs) using liquid chromatography. Analysis revealed the presence of polyethylene glycols (PEGs), polypropylene glycols (PPGs), nonylphenol ethoxylates (NPEOs), and alkyldimethylbenzylammonium chlorides (ADBACs) in all three discharges and in many samples downstream (Figure 26, Figure 27, Figure C3). Concentrations of all four surfactants were below detection limit in the control site wetland (CSW). PEGs, PPGs and NPEOs are non-ionic surfactants commonly used by the oil and gas industries as emulsifiers, wetting agents and corrosion inhibitors.[66] These surfactants are not listed as components of the well maintenance chemicals reported at DB; however, ethylene glycol and propylene glycol, the monomer of PEGs and PPGs, respectively, are both listed (Table C1). These chemicals are more likely to be used in hydraulic fracturing at the sites, which occurs every other year at DB and possibly on a similar schedule at the other two discharges. ADBACs are a cationic surfactant and quaternary ammonium compound mixture commonly used as a biocide in the oil and gas industry.[49] This chemical is also listed as a component of the well maintenance chemicals used at DB (Benzyl-Dimethyl-Dodecyl-Ammonium Chloride; Table C1).

4.3.5.1 Discharge A

In DA-D, ADBACs were the most prominent surfactant and were detected at a concentration two orders of magnitude greater than the other three surfactants (PEGs: 4.4 μg/L; PPGs: 2.1 μg/L; NPEOs: 2.7 μg/L; ADBACs: 347 μg/L). Concentrations of all surfactants decreased with distance downstream in Discharge A (Figure 26). After the first wetland, 9% of PEGs, 66% of PPGs, 16% of NPEOs and <0.1% of ADBACs remained. After the second wetland, 5% of PEGs and 6% of PPGs

remained and both NPEOs and ADBACs were below detection limit. Wetlands are indicated by the grey boxes in Figure 26. The rate of attenuation was fastest for ADBACs, which were almost completely removed in the first wetland. ADBACs were likely attenuated faster than the other surfactants due to the fact that it is positively charged and therefore strongly attracted to sediments, which are largely negatively charged.[148] Sorption also occurs due to the large hydrophobic moieties on ADBACs and interactions with soil organic matter.[49, 149] Three different alkyl lengths of ADBAC were detected including C10, C12 and C14 (decyl, dodecyl and tetradecyl) and it is expected that preferential sorption due to hydrophobic interactions would increase with increasing chain length.[149] The starting composition of the ADBAC mixture is unknown, however, so this hypothesis cannot be tested. Additionally, concentration of ADBACs were below the minimum inhibitory concentration, which have been reported on the order of 100 mg/L.[150-152] Aqueous biodegradation of ADBACs have been reported in some instances; however, due to its high soil sorption coefficient (log K_{oc} = 5.5-7), it is expected that sorption is the dominant attenuation mechanism for this species.[49, 148, 153]

PEGs, PPGs and NPEOs all persisted past the first wetland in Discharge A. Within the first wetland, PEGs and NPEOs attenuation rates were greater than observed for PPGs. Within the second wetland PPGs attenuation rates remained steady, however, rates of attenuation for PEGs and NPEOs decreased. Previous soil microcosm studies have shown that PPGs are more recalcitrant than PEGs and NPEOs, which agrees with observations in the first wetland.[66, 154] The reason for different attenuation rates in the second wetland in unknown; however, there were some clear differences between the wetlands that are likely to impact attenuation rates. Most importantly, the second wetland is less vegetated and more saline than the first.

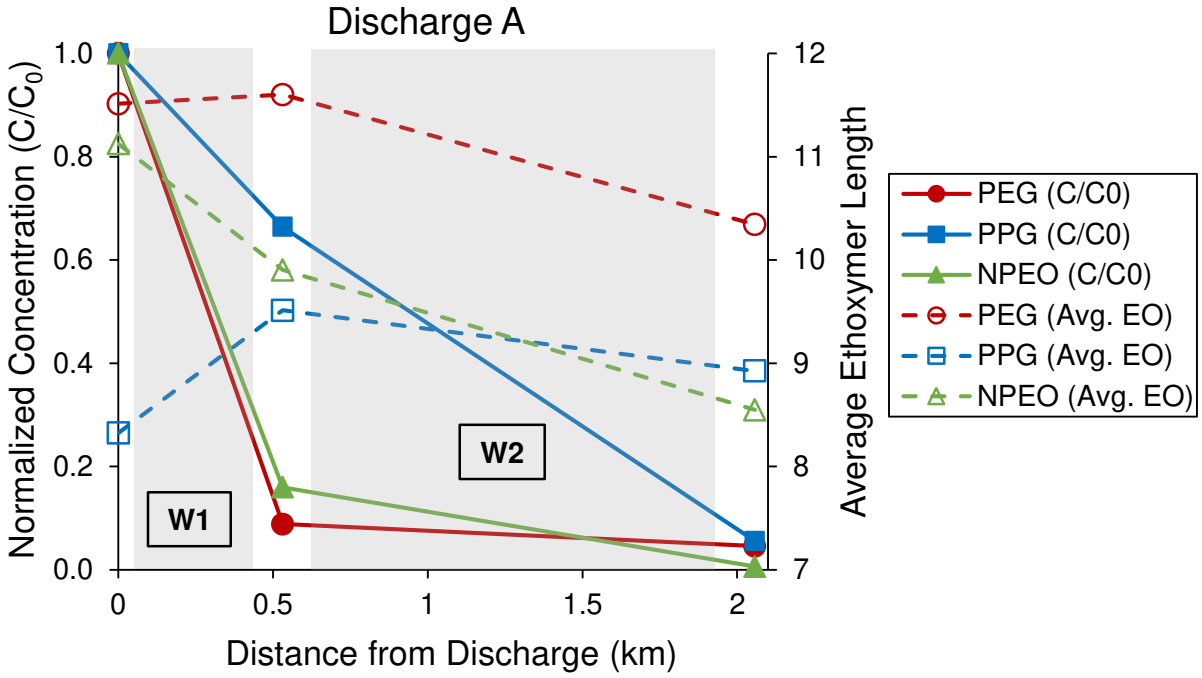

Figure 26. Relative concentration of polyethylene glycol (PEG), polypropylene glycol (PPG) and nonylphenol ethoxylate (NPEO) and average ethoxymer (EO) length for each species versus distance from the NPDES discharge (km) at Discharge A. PEG, PPG and NPEO were all below detection in the control wetland. Grey areas indicate locations of wetlands.

Additionally, changes in average ethoxymer length were observed for all three non-ionic surfactant species with distance downstream (Figure 26), providing another line of evidence that concentrations of these species decreased due to transformation and not dilution. Within the first wetland, average PEGs ethoxymer length increased slightly (11.5 to 11.6), average PPGs ethoxymer length increased (8.3 to 9.5) and average NPEOs ethoxymer length decreased (11.1 to 9.9). In the second wetland, average ethoxymer length for all three non-ionic surfactants decreased (PEGs: 10.3; PPGs: 8.9; NPEOs: 8.5). All three surfactant species are known to biodegrade via sequential ethoxylate chain shortening, which leads to changes in homolog distribution depending on preferences in ethoxymer chain length.[15, 66] This is most clear for average ethoxymer length of NPEOs, which decrease steadily throughout both wetlands. A decrease in NPEOs average ethoxymer length, however, is also indicative of hydrophilic interactions between NPEOs and mineral components in

sediment.[155] These interactions are relatively weak, and consequently biodegradation is likely the dominant mechanism.[155] A similar attenuation mechanism is likely for PEGs and PPGs in the second wetland due to the combined decrease in concentration and average ethoxymer length. The increase in PEGs and PPGs average ethoxymer length within the first wetland may be due to preferential biodegradation of shorter ethoxymer lengths, which has been observed previously for PEGs.[15]

4.3.5.2 Discharge B

In DB-D, ADBACs were once again the most prominent surfactant and was detected at a concentration one order of magnitude greater than the other three surfactants (PEGs: 7.0 μg/L; PPGs: 4.2 μg/L; NPEOs: 1.7 μg/L; ADBACs: 62 μg/L). The total concentration of detected surfactants was one order of magnitude lower than that in DA-D (75 μg/L in DB-D vs. 356 μg/L in DA-D) as a result of the lower ADBACs concentration at this site. All non-ionic surfactants were within the same order of magnitude as detected at DA-D. All four surfactants detected at DB-D were below detection limit in the first wetland (DB-W1), which was the first sample collected downstream. Due to low water levels and ice in this system, a water sample could not be collected upstream of the first wetland. As mentioned previously, PW discharge rate was below average at this site due to decreased oil prices at the time of sampling. As a result of increased interaction between the sediment and water, lower water volumes would likely increase sorption rates, which is a major mechanism of surfactant attenuation. Biodegradation could also be increased due to increased interaction with microbial soil communities.[15, 156] Thus, surfactants may persist farther downstream when discharge rates are increased.

4.3.5.3 Discharge C

In DC-D, all non-ionic surfactants were present at concentrations one order of magnitude greater than ADBACs (PEGs: 2.5 μg/L; PPGs: 6.5 μg/L; NPEOs: 3.8 μg/L; ADBACs: 0.1 μg/L). The total concentration of surfactants detected in DC-D (12.9 μg/L) was lower than in DA-D and DB-D. Additionally, DC-D was the only discharge where ADBACs were not the most prominent

chemical additive. Concentrations of PPGs, NPEOs and ADBACs decreased with distance downstream. After the first wetland, 81% of PPGs and 65% of NPEOs remained and ADBACs were below detection limit. After the second wetland, 4% of PPGs remained and NPEOs were below detection limit. As shown in Figure 27, attenuation rates for PPGs and NPEOs increased within the wetlands (indicated by grey boxes) as compared to the streams.

A slightly different trend was observed for the concentration of PEGs in Discharge C. Initially, the concentration of PEGs decreased with distance and 96% remained upstream of the first wetland. Within the first wetland, however, relative concentration of PEGs increased to 102%. An increase in surfactant concentration was not observed for any other surfactant or at any other discharge, however, analysis conducted in February 2018 showed an increase in concentration of PEGs upstream of this wetland (see Chapter 2). This increase may be due to NPEO biodegradation. Previous studies have provided evidence for a central fission mechanism for NPEO degradation, which would generate PEGs and nonylphenol (NP).[91] This is supported by the fact that a shift towards the major NPEO homologues EO8-EO11 was observed in PEGs (Figure C5 and Figure C9) and that reduction of NPEOs (1.0 nM) was greater than generation of PEGs (0.3 nM). Other mechanisms, including variability in discharge composition, are also possible. Downstream of the first wetland, concentration of PEGs decreased and were below detection limit downstream of the second wetland. Similar to PPGs and NPEOs, attenuation rate for PEGs increased within the second wetland as compared to the streams.

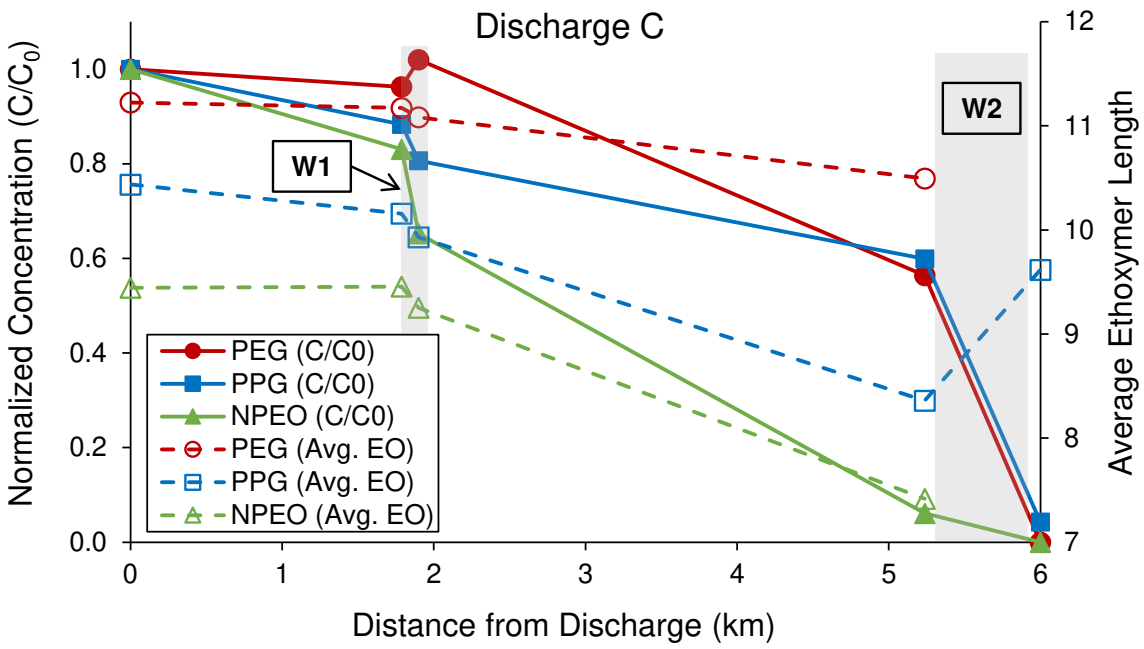

Figure 27. Relative concentration of polyethylene glycol (PEG), polypropylene glycol (PPG) and nonylphenol ethoxylate (NPEO) and average ethoxymer (EO) length for each species versus distance from the NPDES discharge (km) at Discharge C. PEG and NPEO were below detection limit at DC-W2 (6 km) and at DC-PLAYA (4.1 km). Grey areas indicate locations of wetlands.

Similar to Discharge A, decreases in surfactant concentration were accompanied by changes in average ethoxymer length. In general, average ethoxymer length decreased with distance, with the exception of PPGs between the upstream and downstream site on the second wetland (DC-USW2 vs. DC-DSW2) (Figure 27). PEGs and NPEOs were below detection limit downstream of the second wetland (DC-DSW2) so an average ethoxymer length could not be calculated for those species at that site. Similar to attenuation rates, the rate of ethoxymer decrease was generally higher within the wetlands than within the stream. As mentioned previously, this decrease in ethoxymer length is indicative of sequential ethoxylate chain shortening, which is a well-known biodegradation mechanism for PEGs, PPGs and NPEOs. Thus, the increased rate of ethoxymer decrease combined with increased attenuation rates suggests that biodegradation of surfactants is increased within the wetlands. As discussed in Discharge A, sorption is another important attenuation mechanism for these

surfactants. Previous studies have shown that sorption of NPEOs is greater than PEGs and PPGs and therefore this is likely why NPEOs were attenuated faster than the other non-ionic surfactants.[66]

4.3.6 Non-volatile Organic Compound: Sediment Analyses

Sediment sample extracts were also analyzed for non-volatile organic compounds (NVOCs) using liquid chromatography. PEGs, PPGs, NPEOs and ADBACs were detected at all sites downstream of the three discharges (Table 13), indicating that these species sorbed to sediments. Surfactants were below detection limit in many of the water samples, suggesting that these species accumulated over time in the sediment and/or that the chemicals were previously used at higher concentrations. PEGs and PPGs were also detected in the control site wetland (CSW: PEGs: 6.9 μg/kg; PPGs: 2.0 μg/kg) at concentrations lower than in the PW impacted samples. NPEOs and ADBACs were below detection limit in the control site wetland (CSW).

4.3.6.1 Discharge A

At DA-D, ADBACs were the most prominent surfactant and were present two orders of magnitude higher than any of the other surfactants (PEGs: 15.5 μg/kg; PPGs: 19.2 μg/kg; NPEOs: 30.9 μg/kg; ADBACs: 1430 μg/kg). Concentrations of all surfactants increased in the first wetland (DA-W1). This was most apparent for ADBACs which increased by two orders of magnitude to 455,000 μg/kg, thereby accounting for 99.9% of the mass of identified surfactant species at DA-W1. This increase is likely due to a variety of factors most importantly electrostatic sorption of ADBACs to the negatively charged sediment. The cationic portion of ADBAC sorbs to negatively charged clay and can exceed the cationic exchange capacity (CEC) if the alkyl chain length is large enough to sufficiently increase hydrophobic effects.[49, 149] ADBAC with alkyl lengths of 10-14 carbons were detected at these sites and a previous study has observed that ADBAC with more than 8 carbons in the alkyl chain can sorb beyond the CEC.[149] Studies have also shown that this can lead to extensive clay aggregation, which has the potential to impact the lifetime of the wetland. Sorption above the

CEC may decrease surface area of the clays and reduce the ability to attenuate contaminants; however, additional studies are needed to further elucidate the impact of this process. Additionally, concentration of ADBACs may be elevated at this site because it is less biodegradable than the other surfactants, although some studies have shown that degradation rates of ADBACs, NPEOs and PEGs in sediments are similar.[157]

Table 13. Concentration of polyethylene glycols (PEGs), polypropylene glycols (PPGs), nonylphenol ethoxylates (NPEOs) and alkyldimethylbenzylammonium chlorides (ADBACs) in sediment grab samples collected from the three discharges and the control site wetland (CSW).

Site Name	PEGs (µg/kg)	PPGs (µg/kg)	NPEOs (µg/kg)	ADBACs (µg/kg)
CSW	6.9	2.0	Below LOD	Below LOD
Discharge A				
DA-D	15.5	19.2	30.9	1430
DA-W1	196	22.8	70.0	455000
DA-DSW1	202	10.2	29.2	154
DA-W2	85.1	7.8	43.1	45.0
DA-DSW2	15.7	5.4	23.6	5.7
Discharge B				
DB-D	66.8	1150	132	6110
DB-USW1	33.9	16.1	11.6	1.4
DB-W1	24.8	28.3	44.5	1.8
DB-DSW1	50.0	38.0	28.0	2.8
Discharge C				
DC-D	59.3	76.5	122	83.5
DC-100m	44.7	38.7	11.6	11.3
DC-USW1	25.8	35.7	138	6.4
DC-W1	18.9	11.8	23.3	3.5
DC-DSW1	14.0	7.3	14.6	1.4
DC-USW2	47.8	184	167	3.7
DC-W2	44.4	382	194	0.7
DC-DSW2	28.6	3.4	11.0	3.9
DC-PLAYA	17.7	76.9	136	1.9

Surfactant concentrations may also be elevated at this site as a result of ADBACs suppressing microbial activity, resulting in increased surfactant accumulation in the sediments. Quaternary ammonium compounds may retain their biocidal properties when sorbed.[158] Biodegradation is a

dominant removal mechanisms for all four surfactants in sediment and subsequently removal rates would be influenced by a decrease in microbial activity.[157] At downstream sampling locations, ADBAC concentrations decreased substantially (DA-DSW1: 154 μg/kg) and concentrations of all non-ionic surfactants trended downward. Decreased accumulation is likely due to the fact that these sediments are exposed to lower concentrations of surfactants and potentially because total carbon decreases downstream as well.

4.3.6.2 Discharge B

At DB-D, ADBACs were the most prominent surfactant, accounting for 81.9% of the mass of sediments detected at the site (PEGs: 66.8 μg/kg; PPGs: 1150 μg/kg; NPEOs: 132 μg/kg; ADBACs: 6110 μg/kg). In downstream samples, concentrations of surfactants were lower than detected at DB-D. Over the distance of the wetland, total concentration of surfactants increased slightly downstream (DA-USW1: 63.0 μg/kg; DA-W1: 99.3 μg/kg; DA-DSW1: 119 μg/kg). Concentrations of NPEOs increased within the wetland (DB-USW1: 11.6 ng/g; DB-W1: 44.5 ng/g), which also corresponded with a 2-fold increase in both inorganic and organic carbon. A previous study found that sorption of NPEOs increased by an order of magnitude when sediment organic carbon increased 3-fold.[155] Additionally, in samples downstream of the discharge, ADBACs were the least prominent surfactant as compared to the other surfactants detected in those samples, indicating that this surfactant was relatively immobile as compared to the other surfactants detected.

4.3.6.3 Discharge C

At DC-D, NPEOs were the most prominent surfactant, although all surfactants were present within a similar range (PEGs: 59.3 μg/kg; PPGs: 76.5 μg/kg; NPEOs: 122 μg/kg; ADBACs: 83.5 μg/kg). Concentrations of ADBACs generally decreased with distance downstream. Sediment concentrations of the non-ionic surfactants generally decreased with distance, until the site upstream of the second wetland (DC-USW2) where all three increased. The highest total concentration of

surfactants was observed within the second wetland (621 μg/kg) and then decreased downstream. The TOC concentration is also highest at this site, suggesting that sorption of PPGs and NPEOs increases with increased organic carbon. Sorption of NPEOs increases with increasing organic carbon, however, a similar study on sorption of PPGs is not available.[155]

4.3.7 Microbial Community Analysis

A microbial community analysis was conducted with 16S rRNA gene sequences of the water and sediment samples to determine which organisms were present and if microbial communities changed with distance downstream of the PW discharges. Figure 28 shows the relative abundance of microorganisms present in each water sample at the class taxonomic rank and Figure 29 shows relative abundance for sediment samples. Samples are arranged by discharge and presented versus distance, with the most upstream sample on the left. To simplify presentation, classes are only shown if they account for at least 3% relative abundance in at least one sample. All taxa below this threshold are reported as "Other." Samples with less than 5000 reads were not included in this analysis. Thus, some samples are not shown in these results including DA-D, water; DA-D, sediment; DC-W1, sediment; and DC-DSW1, sediment. Both DA-D (water) and DC-W1 (sediment) are currently being rerun and will be included in additional analyses if reads are high enough.

In water samples, there were some clear trends in microbial community composition based on relative abundance of 16S rRNA gene sequences versus distance in each discharge. In general, it was clear that the microbial community was influenced by PW closer to the discharge and that as the water became more polished (with distance downstream), the communities became more similar to the communities within the CSW. In the CSW water sample, the microbial community was dominated by Actinobacteria (21%), Bacteroidia (21%), Gammaproteobacteria (16%) and Oxyphotobacteria (11%). In general, Bacteroidia was less prominent closer to the PW discharges than it was in the CSW and increased with distance downstream. Gammaproteobacteria was generally more prominent closer to

the PW discharges than it was in the CSW, and decreased with distance downstream. Additionally, the relative abundance of Actinobacteria was less than 2% in all water samples collected from the NPDES PW discharges, which is an order of magnitude less than that observed in the CSW. Similarly, Oxyphotobacteria were always less dominant in the samples collected downstream of the NPDES PW discharges than in the CSW.

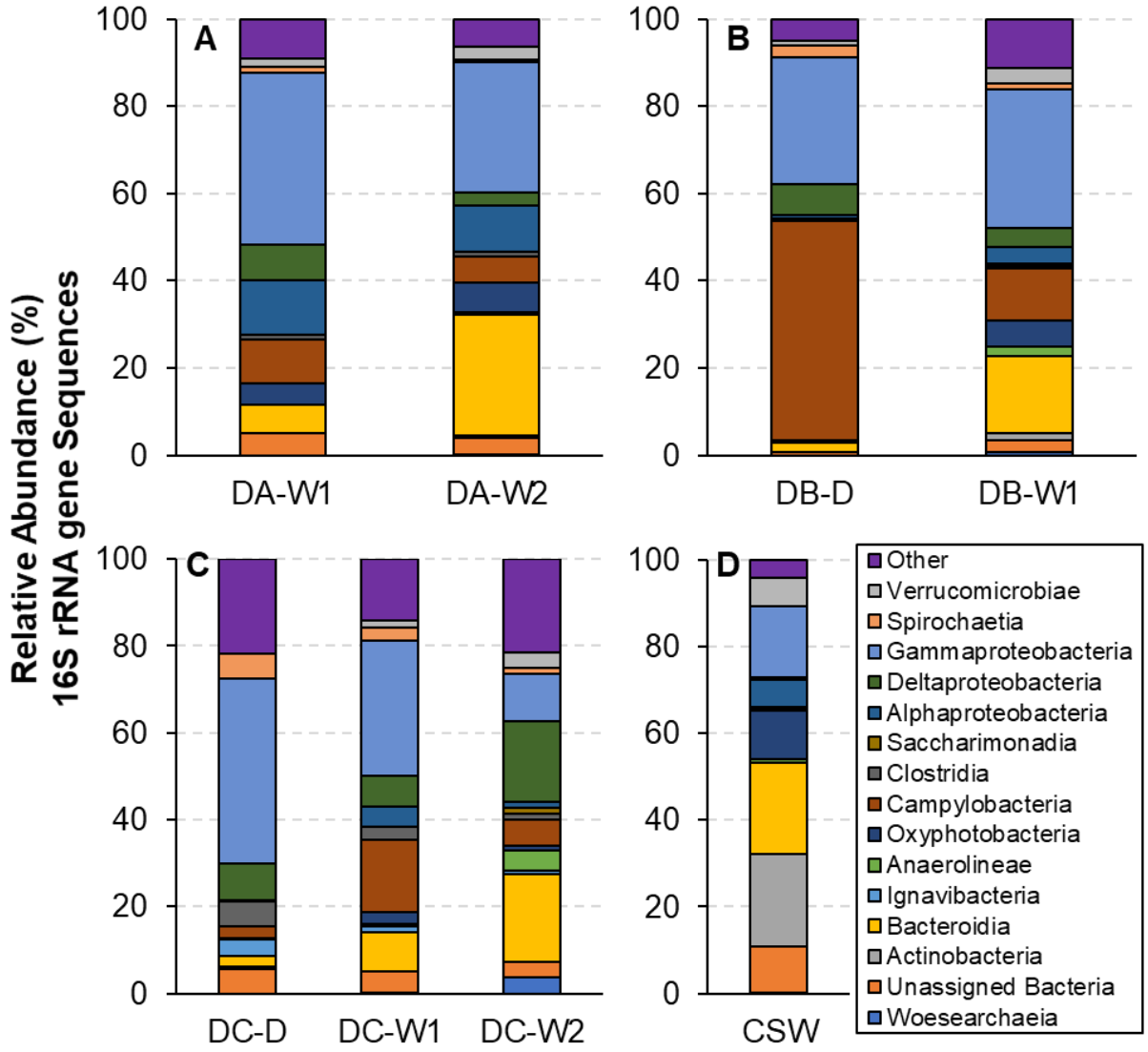

Figure 28. Microbial community composition (based on relative abundance of 16S rRNA gene sequences) for water extracts by class for A) Discharge A, B) Discharge B, C) Discharge C and D) Control Site Wetland. Sites are arranged from closest to the discharge (left) to farthest from the discharge (right). The water extract for DA-D is currently being re-run due to evaporation issues during the first round of analysis.

The results from the abundance plots are further supported by the beta diversity results from the NMDS analysis (Figure 30) conducted on the water samples. NMDS analysis showed that the control site wetland microbial community was significantly different from the microbial communities in the impacted wetlands ($p<0.0000$). The difference between the CSW and the discharges was also significantly different ($p = 0.0001$). There was no significant difference between the impacted wetlands and discharge samples, likely because the two discharge samples were very different from each other.

Differences in microbial community were not as prominent in the sediment samples as they were in the water samples, however, in both Discharge A and Discharge C differences were observed versus distance downstream. In the sediment collected from the CSW, the most dominant taxa were Bacteroidia (18%), Deltaproteobacteria (14%), Gammaproteobacteria (14%), Anaerolineae (11%) and Other (20%). In Discharge A, the microbial community in the most upstream sediment sample (DA-W1), was different from that observed at the CSW. While Deltaproteobacteria (13%) and Other (24%) were dominant at this site, similar to CSW, Phycisphaerae (16%) and Unassigned Bacteria (8%) were also prominent. In samples downstream, however, the microbial community became more similar to the CSW, with Bacteroidia, Deltaproteobacteria, Gammaproteobacteria, Anaerolineae, and Other being the most dominant taxa in the three sediment samples collected downstream. Thus, it appears that the most upstream sample (DA-W1) was impacted by the PW discharge.

In Discharge B, an impact of the PW on the microbial community was not observed and the microbial communities at all sites were dominated by Bacteroidia, Deltaproteobacteria, Gammaproteobacteria, Anaerolineae, and Other, similar to at the CSW. In Discharge C, the PW appeared to impact the microbial community at the most upstream samples (DC-D, DC-100m). In both DC-D and DC-100m, Chloroflexia is the most dominant taxa (DC-D: 18%; DC-100m: 35%). In samples downstream of these sites, however, the microbial community is similar to that observed at the CSW. Chloroflexia are known to grow well at high temperatures and are likely present at DC-

D and DC-100m due to the fact that temperatures are elevated at these sites (DC-D: 40.4°C; DC-100m: 41.1°C). NMDS analysis was conducted on the sediment samples as well, however, no significant groupings or trends were observed in that analysis. This suggests that microbial communities in the sediment may be more resistant to changes from PW than the water.

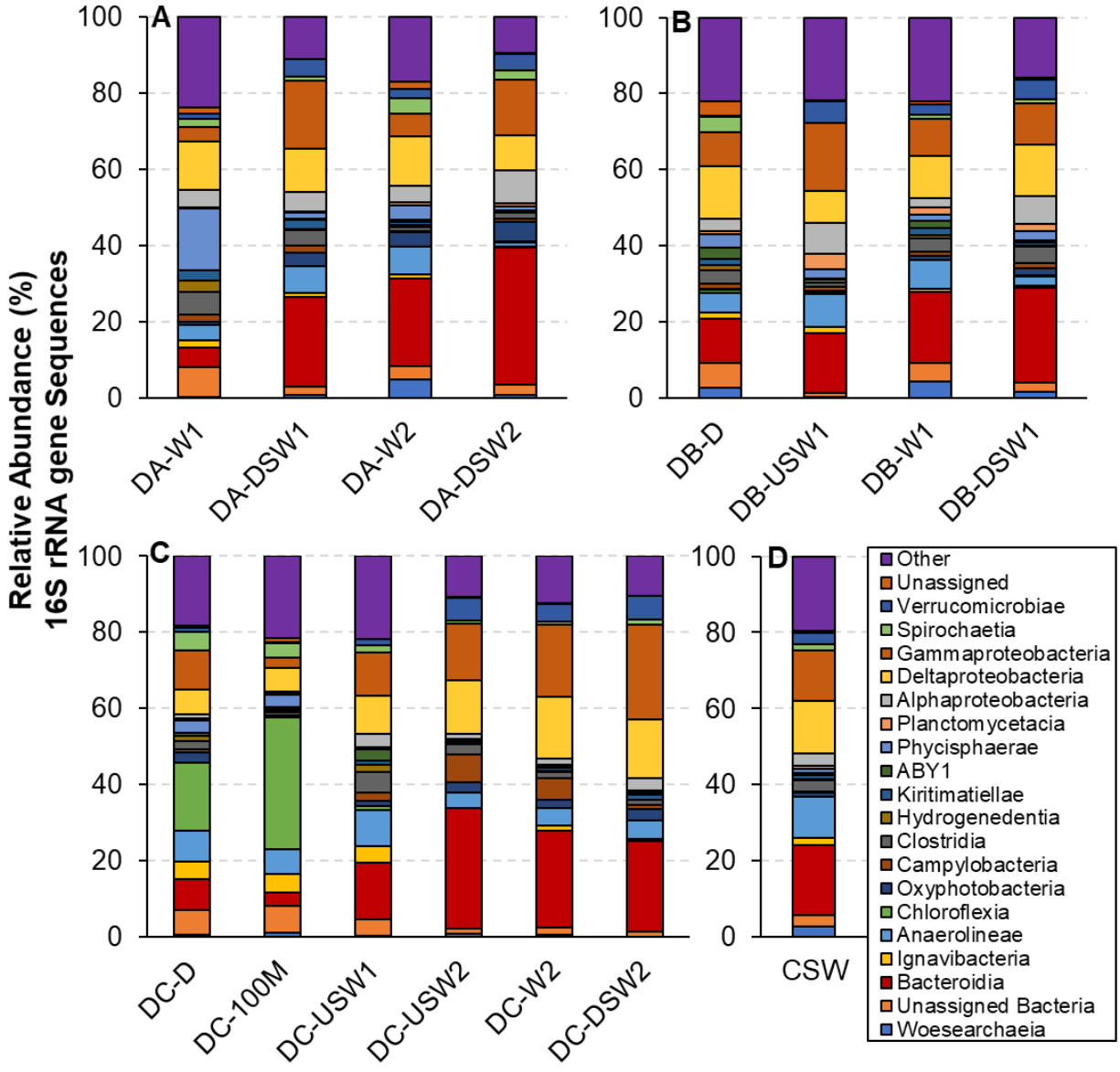

Figure 29. Relative abundance plots for 16S rRNA gene sequencing sediment extracts by class for A) Discharge A, B) Discharge B, C) Discharge C and D) Control Site Wetland. Sites are arranged from closest to the discharge (left) to farthest from the discharge (right). Due to low amplification, some samples could not be included in this analysis.

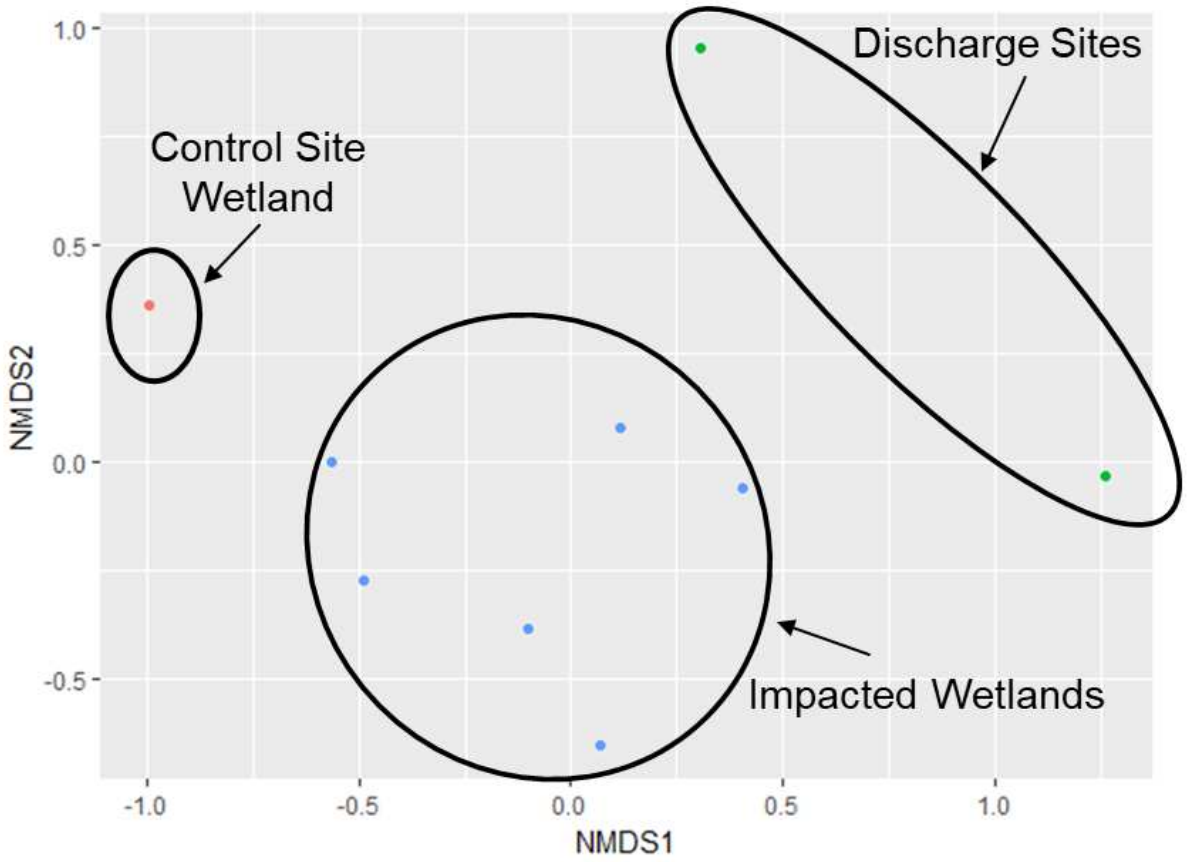

Figure 30. NMDS analysis of microbial community in water samples collected from the control site wetland, impacted wetlands and the produced water discharge sites. The impacted wetlands include any wetland downstream of the Discharge A, Discharge B or Discharge C NPDES produced water discharges.

4.4 Conclusions

In this study, a chemical analysis was conducted on the distribution and fate of organic chemicals in CWs downstream of three different NPDES PW discharges, with a focus on four oil and gas chemical additives. Additionally, an exploratory microbial community analysis was conducted on water and sediment samples collected at these sites.

Organic chemicals detected at these sites included the non-ionic surfactants polyethylene glycols (PEGs), polypropylene glycols (PPGs) and nonylphenol ethoxylates (NPEOs), as well as the cationic surfactant and biocide, alkyldimethylbenzylammonium chloride (ADBAC). Results show that the wetlands at all three discharges are effective at reducing concentrations of PEGs, PPGs, NPEOs and ADBACs in PW discharges. As shown in Discharge C, attenuation rates for non-ionic surfactants

are increased in wetlands as compared to streams. In regards to ADBAC, attenuation is achieved over similar distances in both Discharge A and Discharge B, which are discharged into a wetland and stream, respectively. Thus, there is no quantifiable advantage of a wetland over a stream for attenuating this chemical. For all four chemicals, biodegradation and sorption were determined to be the most important attenuation mechanisms within the CWs.

Sediment analyses revealed that all four surfactants accumulated in sediments over time and were detected at μg/kg levels even at sites where water concentrations were below detection limit. This was most prominent for ADBAC, which was present at concentrations exceeding 450 mg/kg in the wetland downstream of one of the NPDES PW discharges (Discharge A). This concentration is two orders of magnitude higher than ADBAC concentrations reported in sediments collected downstream of the Jamaica Bay Sewage Treatment Plant, a site heavily sewage-impacted site near New York City that is impacted by significantly larger volumes of wastewater.[153] Concentrations of other quaternary ammonium compounds detected at Jamaica Bay are slightly higher, but all are at least an order of magnitude lower than the highest ADBAC concentration observed in this study. Quaternary ammonium compounds (QACs), such as ADBAC, have been shown to retain their biocidal activity after sorption and therefore may adversely impact the biodegradation mechanisms of the wetland. Additionally, ADBAC has the potential to sorb beyond the cation exchange capacity of a sediment and could therefore impact sorption capacity for additional contaminants. Treatment of PW with CWs is critical at these remote sites and their performance in contaminant removal should be regularly checked. If deteriorating, the sediment may have to be dredged and removed.

Additionally, it is important to understand the toxicity of these sediment to benthic organisms. Microbial community analysis revealed that microbial communities in both water and sediment samples were influenced by the PW discharges and that as the water was polished with CWs, the

microbial communities became more similar to microbial communities at the control site wetland, which was not impacted by PW.

Wetlands are a low-cost solution for treatment of low-saline PW. Prior to mass implementation, however, more information is needed on mechanisms of attenuation within these systems. Results of this study can be used to determine best practices for CWs designed to polish PW.

CHAPTER 5: SUMMARY

The research performed in this dissertation was designed to determine if the current NPDES PW beneficial reuse permit effluent limits are adequate and if not, identify additional steps that can be taken to improve water quality and environmental health downstream of these discharges. To achieve this goal, three different studies have been completed at a field site in a remote location in Wyoming, where beneficial reuse of PW has been occurring for decades. The studies detailed here aimed to 1) characterize the chemical composition of PW released from a NPDES PW discharge and analyze the environmental fate and transport of chemicals downstream (Chapter 2), 2) quantify the toxicological impacts downstream (Chapter 3) and 3) assess constructed wetlands as an onsite treatment and polishing option for NPDES PW discharges (Chapter 4).

Based on the results of this study, it is clear that current NPDES PW effluent limits for the watersheds investigated here are inadequate and that general improvements are needed in the regulatory approach. Specifically, the results of the chemical characterizations in Chapters 2 and 4 showed that chemical changes downstream must be considered to adequately assess the impact of NPDES PW discharges. At the NPDES PW discharge which was the focus of Chapter 2, the concentration of most inorganic species increased with distance downstream as a result of evaporation. This included four of the six chemicals or classes of chemicals with defined permit effluent limits at this site including TDS, specific conductance, sulfate and chloride (Table 1). Currently, effluent limits for NPDES PW permits pertain to the discharge site only. Changes in water quality or toxicity downstream are not regulated. Downstream of the NPDES PW discharge in Chapter 2, all regulated chemicals remained below the permit effluent limits throughout the discharge stream, however, that may not be the case at all NPDES PW discharges and was not the case for one of the discharges studied in Chapter 4. Chapter 4 showed that, downstream of Discharge A, specific

conductance increased to above the permissible discharge limit. Additionally, downstream of the NPDES PW discharge in Chapter 2, boron, sodium, potassium, magnesium, manganese, and selenium also increased in concentration. Increased concentrations of boron are of particular concern in PW released for agricultural purposes since boron can negatively impact both crop and livestock health.[5] Individual inorganic species in water samples collected for Chapter 4 will be analyzed in the future by our collaborators as Penn State, Bonnie McDevitt and Nathanial Warner.

In addition to increased concentrations of inorganic species, increased concentrations of carbon disulfide were also observed downstream of the discharge studied in Chapter 2. During both the October 2016 and February 2018 sampling events, carbon disulfide was below detection limit in samples collected at the discharge but detected in samples collected downstream. In February 2018, carbon disulfide was detected above the surface water acute toxicity threshold for aquatic species 0.3 km downstream of the discharge. This NPDES discharge consistently passes acute whole effluent toxicity (WET) tests, which are conducted with water collected from the discharge. The reason for increased carbon disulfide concentration downstream is unknown. This result, however, provides another line of evidence for why additional analysis downstream is necessary to determine impacts associated with these releases and adequately determine permit effluent limits.

In Chapter 1 of this dissertation, it was hypothesized that an extensive chemical analysis of PW would be insufficient for determining the environmental and health risks of this water. As one way to test this hypothesis, I conducted a systematic evaluation for potential health impacts to humans, livestock and aquatic life based on previously established thresholds and screening levels. A complete set of health thresholds reviewed were only available for 8 chemicals (BTEX, arsenic, cadmium, selenium and zinc) showing that an assessment of potential toxicological impacts is currently limited for most species in this stream. In addition, the available thresholds are limited by the fact that they have not been developed for complex mixtures, such as PW. Additionally, this approach is limited by

the fact that there were likely numerous chemicals present in the PW discharge that were not detected with chemical analysis and therefore cannot be compared to available thresholds. Thus, based on the current health thresholds available for chemicals detected in the PW discharge, chemical analysis alone is not sufficient for determining the environmental and health risks associated with this water.

Due to the lack of regulatory thresholds and the determination that a complete chemical characterization of PW was infeasible, a toxicological analysis was conducted on the water samples. It was hypothesized that a combined chemical and toxicological assessment would be more effective to characterize environmental and health impacts associated with PW releases. Chapter 3 showed that mutation rates were elevated at the discharge and decreased with distance downstream. Mutation rate increases in the discharge stream were most prominent for CNV mutations, as compared to the other three mutations that were analyzed. In order to assess which chemical groups were responsible for this increase, mutation assays were conducted with mixtures of known composition and regulatory importance including a BTEX mixture, a mixture of organic carcinogens and a salt control. This analysis revealed that the chemicals contained in the mixtures were insufficient to increase CNV mutation rates to levels observed at the discharge. This result showed that even with a detailed chemical analysis, unidentified chemicals were resulting in increased toxicity; thus, chemical analysis alone is insufficient for determining health impacts. This result also confirmed the hypothesis that a combined chemical and toxicological assessment is a more effective approach for characterizing environmental and health impacts associated with PW releases.

Chapter 3 also showed that that a thorough assessment of toxicity (acute and chronic) is necessary to understand impacts associated with PW releases. Currently, acute whole effluent toxicity (WET) testing is required quarterly at the sites discussed in this dissertation. In addition to the mutagenicity assays conducted in Chapter 3, samples were evaluated for acute toxicity in *Daphnia magna* and developmental toxicity in zebrafish. Acute toxicity was minimal, and no developmental toxicity

was observed. It should be noted that these analyses were only conducted at one discharge (Discharge C; the focus of Chapter 2 and 3) and that different results may have been observed for the other two discharges sampled in Chapter 4 (Discharges A and B). The results from Chapter 3 not only showed that assessment of chronic toxicity is important but also that assessing a range of toxicological endpoints is necessary because increased toxicity may only be observed for some endpoints but not others. In this study, developmental toxicity was not observed, however absence of developmental toxicity does not rule out the possibility of other chronic effects, such as mutagenicity, which was observed at elevated rates. Additionally, it should be noted that one of the discharges discussed in Chapter 4 failed WET tests twice in 2018, yet continued to discharge PW.

Results from Chapter 4 showed that constructed wetlands are effective at attenuating commonly used non-ionic surfactants, as well as a commonly used biocide. It was hypothesized that biodegradation and sorption would be the main attenuation mechanisms in these wetlands and that was proven correct. All four chemicals accumulated in sediments and were even observed in sediments at sites where water concentrations were below detection limit. At upstream sites, alkyldimethylbenzylammonium chloride (ADBAC) was the most prominent species detected, with concentrations as high as 455 mg/kg in one sample. Quaternary ammonium compounds (QACs), such as ADBAC, are commonly used as oil and gas additives. Studies have shown that they can sorb beyond the cation exchange capacity of soil and lead to extensive clay aggregation, both of which could impact soil structure and therefore impact sorption capacity of the wetland. Additionally, QACs may retain their biocidal activity when sorbed and therefore could limit biodegradation in sediments. Biodegradation and microbial community health are important aspects of attenuation in constructed wetlands. Thus, more research is needed to determine the impact of sorption to sediments and wetland viability in order to determine optimum design parameters and maintenance schedules (e.g., sediment

excavation) for constructed wetlands treating PW. Additionally, since these biocides are inherently toxic, it is important to understand the toxicity of these sediment to benthic organisms.

Overall, it is clear that more research is needed to improve regulations and permit effluent limits for NPDES PW released under the agricultural and wildlife exemption. This is especially important right now since multiple government agencies (U.S. EPA and DOE) are interested in expanding reuse of PW in the western U.S. and since many states and communities are exploring this option as well.[26-27] Currently, the impact of these releases on downstream users (soil, water, crops, livestock, fish and humans) are poorly understood. Previous PW management strategies have failed because practices were implemented without properly understanding the impacts first. In Pennsylvania for instance, PW "evaporation ponds" resulted in increased salt concentrations in groundwater, and treatment at CWTPs resulted in increased concentrations of carcinogens (radium and DBPs) downstream.[159] Another management strategy is road-spreading which released 4 times as much radium to the environment as is released when PW is treated at CWTPs.[160-161] In more western states, most notably Oklahoma, management of PW via injection into Class II underground injection wells has resulted in manmade earthquakes. Earthquakes as a result of PW UIC wells have also occurred in other areas, including Greeley, Colorado.

Finally, before widespread implementation of another PW management strategy, such as beneficial reuse, clear guidelines and procedures must be in place to determine the life cycle impacts and benefits of the approach. A holistic life cycle analysis is necessary to compare the potential benefits of reusing PW versus the negative impacts associated with our continued extraction and use of fossil fuels. Many of the environmental issues that motivate communities to reuse PW (e.g., water scarcity and drought) are further exacerbated by climate change, which is caused by continued burning of fossil fuels. We should strongly consider if it makes economic and moral sense to continue investing in technologies to extract and burn more fossil fuels and treat the waste products created. The research

contained in this dissertation is only one part in a life cycle analysis of oil and gas impacts. It is important that we let science lead the policy. Our water and soil resources are too precious not to.

REFERENCES

1. Study of Oil and Gas Extraction Wastewater Management Under the Clean Water Act. Agency, U. S. E. P., Ed. Washington, D.C., 2019.
2. Energy, U. S. D. o. Today in Energy: Hydraulically fractured wells provide two-thirds of U.S. natural gas production. https://www.eia.gov/todayinenergy/detail.php?id=26112.
3. Energy, U. S. D. o. Today in Energy: Hydraulic fracturing accounts for about half of current U.S. crude oil production. https://www.eia.gov/todayinenergy/detail.php?id=25372 (accessed July 2, 2019).
4. Administration, U. S. E. I., Annual Engery Outlook 2018 with projections to 2050. Agency, U. S. E. I., Ed. 2018.
5. Guerra, K.; Dahm, K.; Dundorf, S. *Oil and gas produced water management and beneficial use in the western United States*; Science and Technology Program Report No. 157; U.S. Department of the Interior Bureau of Reclamation: Denver, CO, 2011.
6. Hanson, A. J.; Luek, J. L.; Tummings, S. S.; McLaughlin, M. C.; Blotevogel, J.; Mouser, P. J., High total dissolved solids in shale gas wastewater inhibit biodegradation of alkyl and nonylphenol ethoxylate surfactants. *Science of The Total Environment* **2019,** *668*, 1094-1103.
7. Akyon, B.; McLaughlin, M.; Hernández, F.; Blotevogel, J.; Bibby, K., Characterization and biological removal of organic compounds from hydraulic fracturing produced water. *Environmental Science: Processes & Impacts* **2019,** *21*, 279-290.
8. Rosenblum, J.; Nelson, A. W.; Ruyle, B.; Schultz, M. K.; Ryan, J. N.; Linden, K. G., Temporal characterization of flowback and produced water quality from a hydraulically fractured oil and gas well. *Science of The Total Environment* **2017,** *596-597*, 369-377.
9. Riley, S. M.; Ahoor, D. C.; Regnery, J.; Cath, T. Y., Tracking oil and gas wastewater-derived organic matter in a hybrid biofilter membrane treatment system: A multi-analytical approach. *Science of The Total Environment* **2018,** *613-614*, 208-217.
10. Mouser, P. J.; Borton, M.; Darrah, T. H.; Hartsock, A.; Wrighton, K. C., Hydraulic fracturing offers view of microbial life in the deep terrestrial subsurface. *FEMS Microbiology Ecology* **2016,** *92* (11), fiw166-fiw166.
11. Kahrilas, G. A.; Blotevogel, J.; Corrin, E. R.; Borch, T., Downhole Transformation of the Hydraulic Fracturing Fluid Biocide Glutaraldehyde: Implications for Flowback and Produced Water Quality. *Environmental science & technology* **2016,** *50* (20), 11414–11423.
12. Benko, K. L.; Drewes, J. E., Produced Water in the Western United States: Geographical Distribution, Occurrence, and Composition. *Environmental Engineering Science* **2008,** *25* (2), 239-246.
13. Rowan, E.; Engle, M.; Kirby, C.; Kraemer, T., *Radium Content of Oil-and Gas-Field Produced Waters in the Northern Appalachian Basin (USA): Summary and Discussion of Data*. 2011.
14. Oetjen, K.; Chan, K. E.; Gulmark, K.; Christensen, J. H.; Blotevogel, J.; Borch, T.; Spear, J. R.; Cath, T. Y.; Higgins, C. P., Temporal characterization and statistical analysis of flowback and produced waters and their potential for reuse. *Science of The Total Environment* **2018,** *619-620*, 654-664.
15. McLaughlin, M. C.; Borch, T.; Blotevogel, J., Spills of Hydraulic Fracturing Chemicals on Agricultural Topsoil: Biodegradation, Sorption, and Co-contaminant Interactions. *Environmental science & technology* **2016,** *50* (11), 6071–6078.
16. Kondash, A.; Vengosh, A., Water Footprint of Hydraulic Fracturing. *Environmental Science & Technology Letters* **2015,** *2* (10), 276-280.
17. Jackson, R. B.; Lowry, E. R.; Pickle, A.; Kang, M.; DiGiulio, D.; Zhao, K., The Depths of Hydraulic Fracturing and Accompanying Water Use Across the United States. *Environmental science & technology* **2015,** *49* (15), 8969-8976.

18. Clark, C. E.; Veil, J. A. *Produced water volumes and management practices in the United States*; ANL/EVS/R-09/1; Argonne National Laboratory: Argonne, IL, 2009.
19. Veil, J. A. *U.S. Produced Water Volumes and Management Practices in 2012* 2015.
20. Lauer, N. E.; Warner, N. R.; Vengosh, A., Sources of Radium Accumulation in Stream Sediments near Disposal Sites in Pennsylvania: Implications for Disposal of Conventional Oil and Gas Wastewater. *Environmental science & technology* **2018,** *52* (3), 955-962.
21. Weingarten, M.; Ge, S.; Godt, J. W.; Bekins, B. A.; Rubinstein, J. L., High-rate injection is associated with the increase in U.S. mid-continent seismicity. *Science* **2015,** *348* (6241), 1336-1340.
22. Orem, W.; Varonka, M.; Crosby, L.; Haase, K.; Loftin, K.; Hladik, M.; Akob, D. M.; Tatu, C.; Mumford, A.; Jaeschke, J.; Bates, A.; Schell, T.; Cozzarelli, I., Organic geochemistry and toxicology of a stream impacted by unconventional oil and gas wastewater disposal operations. *Applied Geochemistry* **2017,** *80*, 155-167.
23. Kassotis, C. D.; Iwanowicz, L. R.; Akob, D. M.; Cozzarelli, I. M.; Mumford, A. C.; Orem, W. H.; Nagel, S. C., Endocrine disrupting activities of surface water associated with a West Virginia oil and gas industry wastewater disposal site. *Science of The Total Environment* **2016,** *557–558*, 901-910.
24. Akob, D. M.; Mumford, A. C.; Orem, W. H.; Engle, M. A.; Klinges, J. G.; Kent, D. B.; Cozzarelli, I. M., Wastewater Disposal from Unconventional Oil and Gas Development Degrades Stream Quality at a West Virginia Injection Facility. *Environmental science & technology* **2016**.
25. Dolan, F. C.; Cath, T. Y.; Hogue, T. S., Assessing the feasibility of using produced water for irrigation in Colorado. *Science of The Total Environment* **2018,** *640-641*, 619-628.
26. Department of Energy, O. o. E. E. a. R. E. Water Security Grand Challenge. https://www.energy.gov/eere/water-security-grand-challenge (accessed July 22).
27. Mexico, S. o. N.; Agency, U. S. E. P., Oil and Natural Gas Produced Water Governance in the State of New Mexico—Draft White Paper. Agency, U. S. E. P., Ed. 2018.
28. Burgos, W. D.; Castillo-Meza, L.; Tasker, T. L.; Geeza, T. J.; Drohan, P. J.; Liu, X.; Landis, J. D.; Blotevogel, J.; McLaughlin, M.; Borch, T.; Warner, N. R., Watershed-Scale Impacts from Surface Water Disposal of Oil and Gas Wastewater in Western Pennsylvania. *Environmental science & technology* **2017,** *51* (15), 8851-8860.
29. Geeza, T. J.; Gillikin, D. P.; McDevitt, B.; Van Sice, K.; Warner, N. R., Accumulation of Marcellus Formation Oil and Gas Wastewater Metals in Freshwater Mussel Shells. *Environmental science & technology* **2018,** *52* (18), 10883-10892.
30. Warner, N. R.; Christie, C. A.; Jackson, R. B.; Vengosh, A., Impacts of Shale Gas Wastewater Disposal on Water Quality in Western Pennsylvania. *Environmental science & technology* **2013,** *47* (20), 11849-11857.
31. Leifert, H. Dividing line: The past, present and future of the 100th Meridian. https://www.earthmagazine.org/article/dividing-line-past-present-and-future-100th-meridian (accessed July 22).
32. McDevitt, B.; McLaughlin, M.; Cravotta, C. A.; Ajemigbitse, M. A.; Van Sice, K. J.; Blotevogel, J.; Borch, T.; Warner, N. R., Emerging investigator series: radium accumulation in carbonate river sediments at oil and gas produced water discharges: implications for beneficial use as disposal management. *Environmental Science: Processes & Impacts* **2019,** *21*, 324-338.
33. Ramirez, P., Mobilization of Selenium from Coal Bed Methane (CBM) Produced Water Evaporation/Infiltration Impoundments in the Powder River Basin, Wyoming. Service, U. S. F. W., Ed. Cheyenne, Wyoming, 2013.
34. Jackson, R. E.; Reddy, K. J., Geochemistry of Coalbed Natural Gas (CBNG) Produced Water in Powder River Basin, Wyoming: Salinity and Sodicity. *Water, Air, and Soil Pollution* **2007,** *184* (1), 49-61.

35. Bern, C. R.; Breit, G. N.; Healy, R. W.; Zupancic, J. W.; Hammack, R., Deep subsurface drip irrigation using coal-bed sodic water: Part I. Water and solute movement. *Agricultural Water Management* **2013,** *118*, 122-134.
36. Bern, C. R.; Breit, G. N.; Healy, R. W.; Zupancic, J. W., Deep subsurface drip irrigation using coal-bed sodic water: Part II. Geochemistry. *Agricultural Water Management* **2013,** *118*, 135-149.
37. Sedlacko, E. M.; Jahn, C. E.; Heuberger, A. L.; Sindt, N. M.; Miller, H. M.; Borch, T.; Blaine, A. C.; Cath, T. Y.; Higgins, C. P., Potential for beneficial reuse of oil-and-gas-derived produced water in agriculture: physiological and morphological responses in spring wheat (Triticum aestivum). *Environmental Toxicology and Chemistry* **2019,** *0* (ja).
38. Pica, N. E.; Carlson, K.; Steiner, J. J.; Waskom, R., Produced water reuse for irrigation of non-food biofuel crops: Effects on switchgrass and rapeseed germination, physiology and biomass yield. *Industrial Crops and Products* **2017,** *100*, 65-76.
39. Ferrar, K. J.; Michanowicz, D. R.; Christen, C. L.; Mulcahy, N.; Malone, S. L.; Sharma, R. K., Assessment of Effluent Contaminants from Three Facilities Discharging Marcellus Shale Wastewater to Surface Waters in Pennsylvania. *Environmental science & technology* **2013,** *47* (7), 3472-3481.
40. Richardson, S. D.; Plewa, M. J.; Wagner, E. D.; Schoeny, R.; DeMarini, D. M., Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: A review and roadmap for research. *Mutation Research/Reviews in Mutation Research* **2007,** *636* (1–3), 178-242.
41. Wagner, E. D.; Plewa, M. J., CHO cell cytotoxicity and genotoxicity analyses of disinfection by-products: An updated review. *Journal of Environmental Sciences* **2017,** *58*, 64-76.
42. Kassotis, C. D.; Tillitt, D. E.; Davis, J. W.; Hormann, A. M.; Nagel, S. C., Estrogen and Androgen Receptor Activities of Hydraulic Fracturing Chemicals and Surface and Ground Water in a Drilling-Dense Region. *Endocrinology* **2014,** *155* (3), 897-907.
43. Kassotis, C. D.; Vu, D. C.; Vo, P. H.; Lin, C.-H.; Cornelius-Green, J. N.; Patton, S.; Nagel, S. C., Endocrine-Disrupting Activities and Organic Contaminants Associated with Oil and Gas Operations in Wyoming Groundwater. *Archives of Environmental Contamination and Toxicology* **2018**.
44. Cozzarelli, I. M.; Skalak, K. J.; Kent, D. B.; Engle, M. A.; Benthem, A.; Mumford, A. C.; Haase, K.; Farag, A.; Harper, D.; Nagel, S. C.; Iwanowicz, L. R.; Orem, W. H.; Akob, D. M.; Jaeschke, J. B.; Galloway, J.; Kohler, M.; Stoliker, D. L.; Jolly, G. D., Environmental signatures and effects of an oil and gas wastewater spill in the Williston Basin, North Dakota. *Science of The Total Environment* **2017**.
45. Danforth, C.; McPartland, J.; Blotevogel, J.; Coleman, N.; Devlin, D.; Olsgard, M.; Parkerton, T.; Saunders, N., Alternative Management of Oil and Gas Produced Water Requires More Research on its Hazards and Risks. *Integrated Environmental Assessment and Management* **2019,** *0* (ja).
46. Brack, W.; Ait-Aissa, S.; Burgess, R. M.; Busch, W.; Creusot, N.; Di Paolo, C.; Escher, B. I.; Mark Hewitt, L.; Hilscherova, K.; Hollender, J.; Hollert, H.; Jonker, W.; Kool, J.; Lamoree, M.; Muschket, M.; Neumann, S.; Rostkowski, P.; Ruttkies, C.; Schollee, J.; Schymanski, E. L.; Schulze, T.; Seiler, T.-B.; Tindall, A. J.; De Aragão Umbuzeiro, G.; Vrana, B.; Krauss, M., Effect-directed analysis supporting monitoring of aquatic environments — An in-depth overview. *Science of The Total Environment* **2016,** *544*, 1073-1118.
47. Di Paolo, C.; Ottermanns, R.; Keiter, S.; Ait-Aissa, S.; Bluhm, K.; Brack, W.; Breitholtz, M.; Buchinger, S.; Carere, M.; Chalon, C.; Cousin, X.; Dulio, V.; Escher, B. I.; Hamers, T.; Hilscherová, K.; Jarque, S.; Jonas, A.; Maillot-Marechal, E.; Marneffe, Y.; Nguyen, M. T.; Pandard, P.; Schifferli, A.; Schulze, T.; Seidensticker, S.; Seiler, T.-B.; Tang, J.; van der Oost, R.; Vermeirssen, E.; Zounková, R.; Zwart, N.; Hollert, H., Bioassay battery interlaboratory investigation of emerging contaminants in spiked water extracts – Towards the implementation of bioanalytical monitoring tools in water quality assessment and monitoring. *Water Research* **2016,** *104*, 473-484.

48. Oetjen, K.; Giddings, C. G. S.; McLaughlin, M.; Nell, M.; Blotevogel, J.; Helbling, D. E.; Mueller, D.; Higgins, C. P., Emerging analytical methods for the characterization and quantification of organic contaminants in flowback and produced water. *Trends in Environmental Analytical Chemistry* **2017,** *15*, 12-23.
49. Kahrilas, G. A.; Blotevogel, J.; Stewart, P. S.; Borch, T., Biocides in Hydraulic Fracturing Fluids: A Critical Review of Their Usage, Mobility, Degradation, and Toxicity. *Environ. Sci. Technol.* **2015,** *49* (1), 16-32.
50. Sun, Y.; Wang, D.; Tsang, D. C. W.; Wang, L.; Ok, Y. S.; Feng, Y., A critical review of risks, characteristics, and treatment strategies for potentially toxic elements in wastewater from shale gas extraction. *Environment International* **2019,** *125*, 452-469.
51. Freedman, D. E.; Riley, S. M.; Jones, Z. L.; Rosenblum, J. S.; Sharp, J. O.; Spear, J. R.; Cath, T. Y., Biologically active filtration for fracturing flowback and produced water treatment. *Journal of Water Process Engineering* **2017,** *18*, 29-40.
52. Stefanakis, A. I., Constructed Wetlands Case Studies for the Treatment of Water Polluted with Fuel and Oil Hydrocarbons. In *Phytoremediation: Management of Environmental Contaminants, Volume 6*, Ansari, A. A.; Gill, S. S.; Gill, R.; R. Lanza, G.; Newman, L., Eds. Springer International Publishing: Cham, 2018; pp 151-167.
53. Evans, M. V.; Getzinger, G.; Luek, J. L.; Hanson, A. J.; McLaughlin, M. C.; Blotevogel, J.; Welch, S. A.; Nicora, C. D.; Purvine, S. O.; Xu, C.; Cole, D. R.; Darrah, T. H.; Hoyt, D. W.; Metz, T. O.; Lee Ferguson, P.; Lipton, M. S.; Wilkins, M. J.; Mouser, P. J., In situ transformation of ethoxylate and glycol surfactants by shale-colonizing microorganisms during hydraulic fracturing. *The ISME Journal* **2019**.
54. Orem, W.; Tatu, C.; Varonka, M.; Lerch, H.; Bates, A.; Engle, M.; Crosby, L.; McIntosh, J., Organic substances in produced and formation water from unconventional natural gas extraction in coal and shale. *International Journal of Coal Geology* **2014,** *126*, 20-31.
55. Parker, K. M.; Zeng, T.; Harkness, J.; Vengosh, A.; Mitch, W. A., Enhanced Formation of Disinfection Byproducts in Shale Gas Wastewater-Impacted Drinking Water Supplies. *Environmental science & technology* **2014,** *48* (19), 11161-11169.
56. Scanlon, B. R.; Reedy, R. C.; Nicot, J. P., Comparison of Water Use for Hydraulic Fracturing for Unconventional Oil and Gas versus Conventional Oil. *Environmental science & technology* **2014,** *48* (20), 12386-12393.
57. NOAA Past Weather by Zip Code - Data Table. https://www.climate.gov/ (accessed Sept. 22, 2018).
58. Osterkamp, W. R.; Wood, W. W., Playa-lake basins on the Southern High Plains of Texas and New Mexico: Part I. Hydrologic, geomorphic, and geologic evidence for their development. *GSA Bulletin* **1987,** *99* (2), 215-223.
59. USGS National Water Information System: Web Interface. waterdata.usgs.gov (accessed Sept. 22, 2018).
60. Thurman, E. M.; Ferrer, I.; Rosenblum, J.; Linden, K.; Ryan, J. N., Identification of Polypropylene Glycols and Polyethylene Glycol Carboxylates in Flowback and Produced Water from Hydraulic Fracturing. *Journal of Hazardous Materials* **2017**.
61. Orem, W. H.; Tatu, C. A.; Lerch, H. E.; Rice, C. A.; Bartos, T. T.; Bates, A. L.; Tewalt, S.; Corum, M. D., Organic compounds in produced waters from coalbed natural gas wells in the Powder River Basin, Wyoming, USA. *Applied Geochemistry* **2007,** *22* (10), 2240-2256.
62. Elliott, E. G.; Ettinger, A. S.; Leaderer, B. P.; Bracken, M. B.; Deziel, N. C., A systematic evaluation of chemicals in hydraulic-fracturing fluids and wastewater for reproductive and developmental toxicity. *J Expos Sci Environ Epidemiol* **2016**.

63. Luek, J. L.; Gonsior, M., Organic compounds in hydraulic fracturing fluids and wastewaters: A review. *Water Research* **2017,** *123*, 536-548.
64. Frolov, Y. B.; Smirnov, M. B.; Vanyukova, N. A.; Sanin, P. I., Carbazoles of crude oil. *Petroleum Chemistry U.S.S.R.* **1989,** *29* (2), 87-102.
65. Drollette, B. D.; Hoelzer, K.; Warner, N. R.; Darrah, T. H.; Karatum, O.; O'Connor, M. P.; Nelson, R. K.; Fernandez, L. A.; Reddy, C. M.; Vengosh, A.; Jackson, R. B.; Elsner, M.; Plata, D. L., Elevated levels of diesel range organic compounds in groundwater near Marcellus gas operations are derived from surface activities. *Proceedings of the National Academy of Sciences of the United States of America* **2015,** *112* (43), 13184-13189.
66. Heyob, K. M.; Blotevogel, J.; Brooker, M.; Evans, M. V.; Lenhart, J. J.; Wright, J.; Lamendella, R.; Borch, T.; Mouser, P. J., Natural Attenuation of Nonionic Surfactants Used in Hydraulic Fracturing Fluids: Degradation Rates, Pathways, and Mechanisms. *Environmental science & technology* **2017,** *51* (23), 13985-13994.
67. Gross, S. A.; Avens, H. J.; Banducci, A. M.; Sahmel, J.; Panko, J. M.; Tvermoes, B. E., Analysis of BTEX Groundwater Concentrations from Surface Spills Associated with Hydraulic Fracturing Operations. *J Air Waste Manag. Assoc.* **2013,** *63* (4), 424-432.
68. Blewett, T. A.; Delompré, P. L. M.; Glover, C. N.; Goss, G. G., Physical immobility as a sensitive indicator of hydraulic fracturing fluid toxicity towards Daphnia magna. *Science of The Total Environment* **2018,** *635*, 639-643.
69. Blewett, T. A.; Weinrauch, A. M.; Delompré, P. L. M.; Goss, G. G., The effect of hydraulic flowback and produced water on gill morphology, oxidative stress and antioxidant response in rainbow trout (Oncorhynchus mykiss). *Scientific Reports* **2017,** *7*, 46582.
70. Pattanayek, M.; DeShields, B. *Risk-Based Screening Levels for the Protection of Livestock Exposed to Petroleum Hydrocarbons*; American Petroleum Institute: 2004.
71. Toxicological profile for Toluene. (ATSDR), A. f. T. S. a. D. R., Ed. 2017.
72. Toxicological profile for Benzene. (ATSDR), A. f. T. S. a. D. R., Ed. 2007.
73. Hosseini, H.; Javadi, M.; Moghiman, M.; Rad, M. H. G., Carbon Disulfide Production via Hydrogen Sulfide Methane Reformation *World Academy of Science, Engineering and Technology International Journal of Chemical and Molecular Engineering* **2010,** *4*.
74. Toxicological profile for Xylenes. (ATSDR), A. f. T. S. a. D. R., Ed. 2007.
75. Hildenbrand, Z. L.; Mach, P. M.; McBride, E. M.; Dorreyatim, M. N.; Taylor, J. T.; Carlton, D. D.; Meik, J. M.; Fontenot, B. E.; Wright, K. C.; Schug, K. A.; Verbeck, G. F., Point source attribution of ambient contamination events near unconventional oil and gas development. *Science of The Total Environment* **2016,** *573*, 382-388.
76. Marrero, J. E.; Townsend-Small, A.; Lyon, D. R.; Tsai, T. R.; Meinardi, S.; Blake, D. R., Estimating Emissions of Toxic Hydrocarbons from Natural Gas Production Sites in the Barnett Shale Region of Northern Texas. *Environmental science & technology* **2016,** *50* (19), 10756-10764.
77. Toxicological profile for acetone. (ATSDR), A. f. T. S. a. D. R., Ed. Atlanta, GA, 1994.
78. Rich, A. L.; Patel, J. T.; Al-Angari, S. S., Carbon Disulfide (CS2) Interference in Glucose Metabolism from Unconventional Oil and Gas Extraction and Processing Emissions. *Environmental Health Insights* **2016,** *10*, EHI.S31906.
79. Butkovskyi, A.; Bruning, H.; Kools, S. A. E.; Rijnaarts, H. H. M.; Van Wezel, A. P., Organic Pollutants in Shale Gas Flowback and Produced Waters: Identification, Potential Ecological Impact, and Implications for Treatment Strategies. *Environmental science & technology* **2017,** *51* (9), 4740-4754.
80. Toxicological profile for Carbon Disulfide. (ATSDR), A. f. T. S. a. D. R., Ed. 1996.
81. Toxicological profile for Polycyclic Aromatic Hydrocarbons (PAHs). (ATSDR), A. f. T. S. a. D. R., Ed. 1995.

82. Paulik, L. B.; Donald, C. E.; Smith, B. W.; Tidwell, L. G.; Hobbie, K. A.; Kincl, L.; Haynes, E. N.; Anderson, K. A., Impact of Natural Gas Extraction on PAH Levels in Ambient Air. *Environmental science & technology* **2015**.
83. Toxicological profile for Cresols. (ATSDR), A. f. T. S. a. D. R., Ed. 2008.
84. Toxicological profile for Phenol. (ATSDR), A. f. T. S. a. D. R., Ed. 2008.
85. Elsner, M.; Hoelzer, K., Quantitative Survey and Structural Classification of Hydraulic Fracturing Chemicals Reported in Unconventional Gas Production. *Environmental science & technology* **2016**.
86. Llewellyn, G. T.; Dorman, F.; Westland, J. L.; Yoxtheimer, D.; Grieve, P.; Sowers, T.; Humston-Fulmer, E.; Brantley, S. L., Evaluating a groundwater supply contamination incident attributed to Marcellus Shale gas development. *Proceedings of the National Academy of Sciences* **2015,** *112* (20), 6325-6330.
87. Toxicological profile for Di(2-ethylhexyl)phthalate (DEHP). (ATSDR), A. f. T. S. a. D. R., Ed. 2002.
88. Toxicological profile for 2-butoxyethanol and 2-butoxyethanol acetate. (ATSDR), A. f. T. S. a. D. R., Ed. 1998.
89. Williams, A. J.; Grulke, C. M.; Edwards, J.; McEachran, A. D.; Mansouri, K.; Baker, N. C.; Patlewicz, G.; Shah, I.; Wambaugh, J. F.; Judson, R. S.; Richard, A. M., The CompTox Chemistry Dashboard: a community data resource for environmental chemistry. *Journal of Cheminformatics* **2017,** *9* (1), 61.
90. Balseiro-Romero, M.; Monterroso, C.; Casares, J. J., Environmental Fate of Petroleum Hydrocarbons in Soil: Review of Multiphase Transport, Mass Transfer, and Natural Attenuation Processes. *Pedosphere* **2018,** *28* (6), 833-847.
91. Franska, M.; Franski, R.; Szymanski, A.; Lukaszewski, Z., A central fission pathway in alkylphenol ethoxylate biodegradation. *Water Research* **2003,** *37* (5), 1005-1014.
92. Wyrwas, B.; Chrzanowski, Ł.; Ławniczak, Ł.; Szulc, A.; Cyplik, P.; Białas, W.; Szymański, A.; Hołderna-Odachowska, A., Utilization of Triton X-100 and polyethylene glycols during surfactant-mediated biodegradation of diesel fuel. *Journal of Hazardous Materials* **2011,** *197*, 97-103.
93. Ferguson, L. P.; Brownawell, B. J., Degradation of nonylphenol ethoxylates in estuarine sediment under aerobic and anaerobic conditions. *Environmental Toxicology and Chemistry* **2003,** *22* (6), 1189-1199.
94. Soares, A.; Guieysse, B.; Jefferson, B.; Cartmell, E.; Lester, J. N., Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environment International* **2008,** *34* (7), 1033-1049.
95. Ramirez, P., Bird Mortality in Oil Field Wastewater Disposal Facilities. *Environmental Management* **2010,** *46* (5), 820-826.
96. Raisbeck, M. F.; L. Riker Bs, S.; Jackson, R.; A. Smith, M.; Reddy, K. J.; R. Zygmunt Bs, J., *Water Quality for Wyoming Livestock & Wildlife A Review of the Literature Pertaining to Health Effects of Inorganic Contaminants*. 2008; Vol. 1183.
97. Buchman, M. F. *Screening Quick Reference Tables (SQuiRTs)*; United States, National Ocean Service, Office of Response and Restoration; United States, National Ocean Service, United States, National Oceanic and Atmospheric Administration.: 2008.
98. Quality, W. D. o. E., Wyoming Surface Water Quality Standards. **2017**.
99. Baseline Ecological Risk Assessment for Non-Asbestos Contaminants Agency, U. S. E. P., Ed. Denver, CO, 2013.
100. Soltanpour, P. N.; Raley, W. L. *Livestock Drinking Water Quality Fact Sheet No. 4908*; Colorado State University Cooperative Extension: 1999.

101. Kassotis, C. D.; Klemp, K. C.; Vu, D. C.; Lin, C.-H.; Meng, C.-X.; Besch-Williford, C. L.; Pinatti, L.; Zoeller, R. T.; Drobnis, E. Z.; Balise, V. D.; Isiguzo, C. J.; Williams, M. A.; Tillitt, D. E.; Nagel, S. C., Endocrine-Disrupting Activity of Hydraulic Fracturing Chemicals and Adverse Health Outcomes After Prenatal Exposure in Male Mice. *Endocrinology* **2015,** *156* (12), 4458-4473.
102. Tang, J. Y. M.; McCarty, S.; Glenn, E.; Neale, P. A.; Warne, M. S. J.; Escher, B. I., Mixture effects of organic micropollutants present in water: Towards the development of effect-based water quality trigger values for baseline toxicity. *Water Research* **2013,** *47* (10), 3300-3314.
103. Zoller, U., Estuarine and coastal zone marine pollution by the nonionic alkylphenol ethoxylates endocrine disrupters: Is there a potential ecotoxicological problem? *Environment International* **2006,** *32* (2), 269-272.
104. Hayes, J. E.; Pallotta, M.; Garcia, M.; Öz, M. T.; Rongala, J.; Sutton, T., Diversity in boron toxicity tolerance of Australian barley (Hordeum vulgare L.) genotypes. *BMC Plant Biology* **2015,** *15* (1), 231.
105. Clark, C. E.; Horner, R. M.; Harto, C. B., Life Cycle Water Consumption for Shale Gas and Conventional Natural Gas. *Environmental science & technology* **2013,** *47* (20), 11829-11836.
106. Bai, B.; Goodwin, S.; Carlson, K., Modeling of frac flowback and produced water volume from Wattenberg oil and gas field. *Journal of Petroleum Science and Engineering* **2013,** *108*, 383-392.
107. Bern, C. R.; Boehlke, A. R.; Engle, M. A.; Geboy, N. J.; Schroeder, K. T.; Zupancic, J. W., Shallow groundwater and soil chemistry response to 3 years of subsurface drip irrigation using coalbed-methane-produced water. *Hydrogeology Journal* **2013,** *21* (8), 1803-1820.
108. Colborn, T.; Kwiatkowski, C.; Schultz, K.; Bachran, M., Natural Gas Operations from a Public Health Perspective. *Hum. Ecol. Risk Assess.* **2011,** *17* (5), 1039-1056.
109. Elliott, E. G.; Trinh, P.; Ma, X.; Leaderer, B. P.; Ward, M. H.; Deziel, N. C., Unconventional oil and gas development and risk of childhood leukemia: Assessing the evidence. *Science of The Total Environment* **2017,** *576*, 138-147.
110. Escher, B. I.; Hermens, J. L. M., Modes of Action in Ecotoxicology: Their Role in Body Burdens, Species Sensitivity, QSARs, and Mixture Effects. *Environmental science & technology* **2002,** *36* (20), 4201-4217.
111. Plackett, R. L.; Hewlett, P. S., Quantal Responses to Mixtures of Poisons. *Journal of the Royal Statistical Society. Series B (Methodological)* **1952,** *14* (2), 141-163.
112. Ohe, T.; Watanabe, T.; Wakabayashi, K., Mutagens in surface waters: a review. *Mutation Research/Reviews in Mutation Research* **2004,** *567* (2–3), 109-149.
113. Blackwell, B. R.; Ankley, G. T.; Bradley, P. M.; Houck, K. A.; Makarov, S. S.; Medvedev, A. V.; Swintek, J.; Villeneuve, D. L., Potential Toxicity of Complex Mixtures in Surface Waters from a Nationwide Survey of United States Streams: Identifying in Vitro Bioactivities and Causative Chemicals. *Environmental science & technology* **2019,** *53* (2), 973-983.
114. Folkerts, E. J.; Blewett, T. A.; He, Y.; Goss, G. G., Alterations to Juvenile Zebrafish (Danio rerio) Swim Performance after Acute Embryonic Exposure to Sub-lethal Exposures of Hydraulic Fracturing Flowback and Produced Water. *Aquatic Toxicology* **2017,** *193*, 50-59.
115. He, Y.; Flynn, S. L.; Folkerts, E. J.; Zhang, Y.; Ruan, D.; Alessi, D. S.; Martin, J. W.; Goss, G. G., Chemical and toxicological characterizations of hydraulic fracturing flowback and produced water. *Water Research* **2017,** *114*, 78-87.
116. He, Y.; Folkerts, E. J.; Zhang, Y.; Martin, J. W.; Alessi, D. S.; Goss, G. G., Effects on Biotransformation, Oxidative Stress, and Endocrine Disruption in Rainbow Trout (Oncorhynchus mykiss) Exposed to Hydraulic Fracturing Flowback and Produced Water. *Environmental science & technology* **2017,** *51* (2), 940-947.

117. Folkerts, E. J.; Blewett, T. A.; He, Y.; Goss, G. G., Cardio-respirometry disruption in zebrafish (Danio rerio) embryos exposed to hydraulic fracturing flowback and produced water. *Environmental Pollution* **2017,** *231*, 1477-1487.
118. Kassotis, C. D.; Tillitt, D. E.; Lin, C.-H.; McElroy, J. A.; Nagel, S. C., Endocrine-Disrupting Chemicals and Oil and Natural Gas Operations: Potential Environmental Contamination and Recommendations to Assess Complex Environmental Mixtures. *Environmental Health Perspectives* **2016,** *124* (3), 256-264.
119. Claxton, L. D.; Umbuzeiro, G. d. A.; DeMarini, D. M., The Salmonella Mutagenicity Assay: The Stethoscope of Genetic Toxicology for the 21st Century. *Environmental Health Perspectives* **2010,** *118* (11), 1515-1522.
120. Stratton, M. R.; Campbell, P. J.; Futreal, P. A., The cancer genome. *Nature* **2009,** *458*, 719.
121. Krepischi, A. C. V.; Pearson, P. L.; Rosenberg, C., Germline copy number variations and cancer predisposition. *Future Oncology* **2012,** *8* (4), 441-450.
122. Conover, H. N.; Argueso, J. L., Contrasting mechanisms of de novo copy number mutagenesis suggest the existence of different classes of environmental copy number mutagens. *Environmental and Molecular Mutagenesis* **2016,** *57* (1), 3-9.
123. Zhang, H.; Zeidler, A. F. B.; Song, W.; Puccia, C. M.; Malc, E.; Greenwell, P. W.; Mieczkowski, P. A.; Petes, T. D.; Argueso, J. L., Gene Copy-Number Variation in Haploid and Diploid Strains of the Yeast Saccharomyces cerevisiae. *Genetics* **2013,** *193* (3), 785-801.
124. Klein, H. L.; Bačinskaja, G.; Che, J.; Cheblal, A.; Elango, R.; Epshtein, A.; Fitzgerald, D. M.; Gómez-González, B.; Khan, S. R.; Kumar, S.; Leland, B. A.; Marie, L.; Mei, Q.; Miné-Hattab, J.; Piotrowska, A.; Polleys, E. J.; Putnam, C. D.; Radchenko, E. A.; Saada, A. A.; Sakofsky, C. J.; Shim, E. Y.; Stracy, M.; Xia, J.; Yan, Z.; Yin, Y.; Aguilera, A.; Argueso, J. L.; Freudenreich, C. H.; Gasser, S. M.; Gordenin, D. A.; Haber, J. E.; Ira, G.; Jinks-Robertson, S.; King, M. C.; Kolodner, R. D.; Kuzminov, A.; Lambert, S. A.; Lee, S. E.; Miller, K. M.; Mirkin, S. M.; Petes, T. D.; Rosenberg, S. M.; Rothstein, R.; Symington, L. S.; Zawadzki, P.; Kim, N.; Lisby, M.; Malkova, A., Guidelines for DNA recombination and repair studies: Cellular assays of DNA repair pathways. *Microbial cell (Graz, Austria)* **2019,** *6* (1), 1-64.
125. OECD, *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*. 2013.
126. Buckley, J. A., Quantifying the antiestrogen activity of wastewater treatment plant effluent using the yeast estrogen screen. *Environmental Toxicology and Chemistry* **2010,** *29* (1), 73-78.
127. Lea, D. E.; Coulson, C. A., The distribution of the numbers of mutants in bacterial populations. *Journal of Genetics* **1949,** *49* (3), 264.
128. Hall, B. M.; Ma, C.-X.; Liang, P.; Singh, K. K., Fluctuation AnaLysis CalculatOR: a web tool for the determination of mutation rate using Luria–Delbrück fluctuation analysis. *Bioinformatics* **2009,** *25* (12), 1564-1565.
129. Truong, L.; Reif, D. M.; St Mary, L.; Geier, M. C.; Truong, H. D.; Tanguay, R. L., Multidimensional in vivo hazard assessment using zebrafish. *Toxicol Sci* **2014,** *137* (1), 212-233.
130. Truong, L.; Harper, S. L.; Tanguay, R. L., Evaluation of Embryotoxicity Using the Zebrafish Model. In *Drug Safety Evaluation: Methods and Protocols*, Gautier, J.-C., Ed. Humana Press: Totowa, NJ, 2011; pp 271-279.
131. Reif, D. M.; Truong, L.; Mandrell, D.; Marvel, S.; Zhang, G.; Tanguay, R. L., High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. *Archives of toxicology* **2016,** *90* (6), 1459-1470.
132. Alnafisi, A.; Hughes, J.; Wang, G.; Miller, C. A., Evaluating polycyclic aromatic hydrocarbons using a yeast bioassay. *Environmental Toxicology and Chemistry* **2007,** *26* (7), 1333-1339.
133. ATSDR, TOXICOLOGICAL PROFILE FOR HYDROGEN SULFIDE AND CARBONYL SULFIDE. **2016**.

134. Spiropoulos, A.; Bisson, L. F., MET17 and Hydrogen Sulfide Formation in Saccharomyces cerevisiae. *Applied and Environmental Microbiology* **2000,** *66* (10), 4421-4426.
135. Geier, M. C.; Chlebowski, A. C.; Truong, L.; Massey Simonich, S. L.; Anderson, K. A.; Tanguay, R. L., Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons. *Archives of toxicology* **2018,** *92* (2), 571-586.
136. Ji, G. D.; Sun, T. H.; Ni, J. R., Surface flow constructed wetland for heavy oil-produced water treatment. *Bioresource Technology* **2007,** *98* (2), 436-441.
137. Ramirez, P., Jr., Oil field-produced water discharges into wetlands: Benefits and risks to wildlife. *Environmental Geosciences* **2005,** *12* (2), 65-72.
138. Murray-Gulde, C.; Heatley, J. E.; Karanfil, T.; Rodgers, J. H.; Myers, J. E., Performance of a hybrid reverse osmosis-constructed wetland treatment system for brackish oil field produced water. *Water Research* **2003,** *37* (3), 705-713.
139. Knight, R. L.; Kadlec, R. H.; Ohlendorf, H. M., The Use of Treatment Wetlands for Petroleum Industry Effluents. *Environmental science & technology* **1999,** *33* (7), 973-980.
140. Alley, B. L.; Willis, B.; Rodgers, J.; Castle, J. W., Water depths and treatment performance of pilot-scale free water surface constructed wetland treatment systems for simulated fresh oilfield produced water. *Ecological Engineering* **2013,** *61*, 190-199.
141. Alley, B. L.; Willis, B.; Rodgers, J.; Castle, J. W., Seasonal Performance of a Hybrid Pilot-Scale Constructed Wetland Treatment System for Simulated Fresh Oil Field-Produced Water. *Water, Air, & Soil Pollution* **2013,** *224* (9), 1639.
142. Abed, R. M. M.; Al-Kharusi, S.; Prigent, S.; Headley, T., Diversity, Distribution and Hydrocarbon Biodegradation Capabilities of Microbial Communities in Oil-Contaminated Cyanobacterial Mats from a Constructed Wetland. *PLOS ONE* **2014,** *9* (12), e114570.
143. Lara-Martín, P. A.; González-Mazo, E.; Brownawell, B. J., Multi-residue method for the analysis of synthetic surfactants and their degradation metabolites in aquatic systems by liquid chromatography–time-of-flight-mass spectrometry. *Journal of Chromatography A* **2011,** *1218* (30), 4799-4807.
144. Thurman, E. M.; Ferrer, I.; Blotevogel, J.; Borch, T., Analysis of Hydraulic Fracturing Flowback and Produced Waters Using Accurate Mass: Identification of Ethoxylated Surfactants. *Anal. Chem.* **2014,** *86* (19), 9653-9661.
145. Borton, M. A.; Sabag-Daigle, A.; Wu, J.; Solden, L. M.; O'Banion, B. S.; Daly, R. A.; Wolfe, R. A.; Gonzalez, J. F.; Wysocki, V. H.; Ahmer, B. M. M.; Wrighton, K. C., Chemical and pathogen-induced inflammation disrupt the murine intestinal microbiome. *Microbiome* **2017,** *5* (1), 47-47.
146. McBeth, I. H.; Reddy, K. J.; Skinner, Q. D., COALBED METHANE PRODUCT WATER CHEMISTRY IN THREE WYOMING WATERSHEDS1. *JAWRA Journal of the American Water Resources Association* **2003,** *39* (3), 575-585.
147. Eke, P. E.; Scholz, M., Benzene removal with vertical-flow constructed treatment wetlands. *Journal of Chemical Technology & Biotechnology* **2008,** *83* (1), 55-63.
148. Reregistration Eligibility Decision for Alkyl Dimethyl Benzyl Ammonium Chloride (ADBAC) Agency, U. S. E. P., Ed. 2006.
149. Kwolek, T.; Hodorowicz, M.; Stadnicka, K.; Czapkiewicz, J., Adsorption isotherms of homologous alkyldimethylbenzylammonium bromides on sodium montmorillonite. *Journal of Colloid and Interface Science* **2003,** *264* (1), 14-19.
150. Buffet-Bataillon, S.; Branger, B.; Cormier, M.; Bonnaure-Mallet, M.; Jolivet-Gougeon, A., Effect of higher minimum inhibitory concentrations of quaternary ammonium compounds in clinical E. coli isolates on antibiotic susceptibilities and clinical outcomes. *Journal of Hospital Infection* **2011,** *79* (2), 141-146.

151. Struchtemeyer, C. G.; Morrison, M. D.; Elshahed, M. S., A critical assessment of the efficacy of biocides used during the hydraulic fracturing process in shale natural gas wells. *International Biodeterioration & Biodegradation* **2012,** *71*, 15-21.
152. Mc Cay, P. H.; Ocampo-Sosa, A. A.; Fleming, G. T. A., Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture. *Microbiology* **2010,** *156* (1), 30-38.
153. Li, X.; Doherty, A. C.; Brownawell, B.; Lara-Martin, P. A., Distribution and diagenetic fate of synthetic surfactants and their metabolites in sewage-impacted estuarine sediments. *Environmental Pollution* **2018,** *242*, 209-218.
154. Wagener, S.; Schink, B., Fermentative degradation of nonionic surfactants and polyethylene glycol by enrichment cultures and by pure cultures of homoacetogenic and propionate-forming bacteria. *Applied and environmental microbiology* **1988,** *54* (2), 561-565.
155. John, D. M.; House, W. A.; White, G. F., Environmental fate of nonylphenol ethoxylates: Differential adsorption of homologs to components of river sediment. *Environmental Toxicology and Chemistry* **2000,** *19* (2), 293-300.
156. Wagner, T. V.; Parsons, J. R.; Rijnaarts, H. H. M.; de Voogt, P.; Langenhoff, A. A. M., A review on the removal of conditioning chemicals from cooling tower water in constructed wetlands. *Critical Reviews in Environmental Science and Technology* **2019**, 1-32.
157. Brownawell, B. J.; Chen, H.; Zhang, W.; Westall, J. C., Sorption of Nonionic Surfactants on Sediment Materials. *Environ. Sci. Technol.* **1997,** *31* (6), 1735-1741.
158. Harney, M. B.; Pant, R. R.; Fulmer, P. A.; Wynne, J. H., Surface Self-Concentrating Amphiphilic Quaternary Ammonium Biocides as Coating Additives. *ACS Applied Materials & Interfaces* **2009,** *1* (1), 39-41.
159. Associates, M. a. *Impacts of Land Surface Discharge of Shallow Oil Field Production Water on Groun Water*; 1997.
160. Tasker, T. L.; Burgos, W. D.; Piotrowski, P.; Castillo-Meza, L.; Blewett, T. A.; Ganow, K. B.; Stallworth, A.; Delompré, P. L. M.; Goss, G. G.; Fowler, L. B.; Vanden Heuvel, J. P.; Dorman, F.; Warner, N. R., Environmental and Human Health Impacts of Spreading Oil and Gas Wastewater on Roads. *Environmental science & technology* **2018**.
161. Harkness, J. S.; Dwyer, G. S.; Warner, N. R.; Parker, K. M.; Mitch, W. A.; Vengosh, A., Iodide, Bromide, and Ammonium in Hydraulic Fracturing and Oil and Gas Wastewaters: Environmental Implications. *Environmental science & technology* **2015,** *49* (3), 1955-1963.
162. Kimmel, C. B.; Ballard, W. W.; Kimmel, S. R.; Ullmann, B.; Schilling, T. F., Stages of embryonic development of the zebrafish. *Developmental Dynamics* **1995,** *203* (3), 253-310.
163. Mandrell, D.; Truong, L.; Jephson, C.; Sarker, M. R.; Moore, A.; Lang, C.; Simonich, M. T.; Tanguay, R. L., Automated zebrafish chorion removal and single embryo placement: optimizing throughput of zebrafish developmental toxicity screens. *J Lab Autom* **2012,** *17* (1), 66-74.
164. Knecht, A. L.; Goodale, B. C.; Truong, L.; Simonich, M. T.; Swanson, A. J.; Matzke, M. M.; Anderson, K. A.; Waters, K. M.; Tanguay, R. L., Comparative developmental toxicity of environmentally relevant oxygenated PAHs. *Toxicology and applied pharmacology* **2013,** *271* (2), 266-275.
165. Howe, K.; Clark, M. D.; Torroja, C. F.; Torrance, J.; Berthelot, C.; Muffato, M.; Collins, J. E.; Humphray, S.; McLaren, K.; Matthews, L.; McLaren, S.; Sealy, I.; Caccamo, M.; Churcher, C.; Scott, C.; Barrett, J. C.; Koch, R.; Rauch, G.-J.; White, S.; Chow, W.; Kilian, B.; Quintais, L. T.; Guerra-Assunção, J. A.; Zhou, Y.; Gu, Y.; Yen, J.; Vogel, J.-H.; Eyre, T.; Redmond, S.; Banerjee, R.; Chi, J.; Fu, B.; Langley, E.; Maguire, S. F.; Laird, G. K.; Lloyd, D.; Kenyon, E.; Donaldson, S.; Sehra, H.; Almeida-King, J.; Loveland, J.; Trevanion, S.; Jones, M.; Quail, M.; Willey, D.; Hunt, A.; Burton, J.; Sims, S.; McLay, K.; Plumb, B.; Davis, J.; Clee, C.; Oliver, K.; Clark, R.; Riddle, C.; Elliot, D.; Threadgold, G.; Harden, G.; Ware, D.; Begum, S.; Mortimore, B.; Kerry, G.; Heath, P.; Phillimore, B.;

Tracey, A.; Corby, N.; Dunn, M.; Johnson, C.; Wood, J.; Clark, S.; Pelan, S.; Griffiths, G.; Smith, M.; Glithero, R.; Howden, P.; Barker, N.; Lloyd, C.; Stevens, C.; Harley, J.; Holt, K.; Panagiotidis, G.; Lovell, J.; Beasley, H.; Henderson, C.; Gordon, D.; Auger, K.; Wright, D.; Collins, J.; Raisen, C.; Dyer, L.; Leung, K.; Robertson, L.; Ambridge, K.; Leongamornlert, D.; McGuire, S.; Gilderthorp, R.; Griffiths, C.; Manthravadi, D.; Nichol, S.; Barker, G.; Whitehead, S.; Kay, M.; Brown, J.; Murnane, C.; Gray, E.; Humphries, M.; Sycamore, N.; Barker, D.; Saunders, D.; Wallis, J.; Babbage, A.; Hammond, S.; Mashreghi-Mohammadi, M.; Barr, L.; Martin, S.; Wray, P.; Ellington, A.; Matthews, N.; Ellwood, M.; Woodmansey, R.; Clark, G.; Cooper, J. D.; Tromans, A.; Grafham, D.; Skuce, C.; Pandian, R.; Andrews, R.; Harrison, E.; Kimberley, A.; Garnett, J.; Fosker, N.; Hall, R.; Garner, P.; Kelly, D.; Bird, C.; Palmer, S.; Gehring, I.; Berger, A.; Dooley, C. M.; Ersan-Ürün, Z.; Eser, C.; Geiger, H.; Geisler, M.; Karotki, L.; Kirn, A.; Konantz, J.; Konantz, M.; Oberländer, M.; Rudolph-Geiger, S.; Teucke, M.; Lanz, C.; Raddatz, G.; Osoegawa, K.; Zhu, B.; Rapp, A.; Widaa, S.; Langford, C.; Yang, F.; Schuster, S. C.; Carter, N. P.; Harrow, J.; Ning, Z.; Herrero, J.; Searle, S. M. J.; Enright, A.; Geisler, R.; Plasterk, R. H. A.; Lee, C.; Westerfield, M.; de Jong, P. J.; Zon, L. I.; Postlethwait, J. H.; Nüsslein-Volhard, C.; Hubbard, T. J. P.; Roest Crollius, H.; Rogers, J.; Stemple, D. L., The zebrafish reference genome sequence and its relationship to the human genome. *Nature* **2013,** *496* (7446), 498-503.

166. Tropepe, V.; Sive, H. L., Can zebrafish be used as a model to study the neurodevelopmental causes of autism? *Genes, Brain and Behavior* **2003,** *2* (5), 268-281.

167. Kokel, D.; Dunn, T. W.; Ahrens, M. B.; Alshut, R.; Cheung, C. Y. J.; Saint-Amant, L.; Bruni, G.; Mateus, R.; van Ham, T. J.; Shiraki, T.; Fukada, Y.; Kojima, D.; Yeh, J.-R. J.; Mikut, R.; von Lintig, J.; Engert, F.; Peterson, R. T., Identification of nonvisual photomotor response cells in the vertebrate hindbrain. *J Neurosci* **2013,** *33* (9), 3834-3843.

168. Raftery, T. D.; Isales, G. M.; Yozzo, K. L.; Volz, D. C., High-Content Screening Assay for Identification of Chemicals Impacting Spontaneous Activity in Zebrafish Embryos. *Environmental science & technology* **2014,** *48* (1), 804-810.

APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER 2 – ASSESSMENT OF WATER QUALITY DOWNSTREAM OF NPDES OIL AND GAS PRODUCED WATER DISCHARGE: CHEMICAL IMPACTS

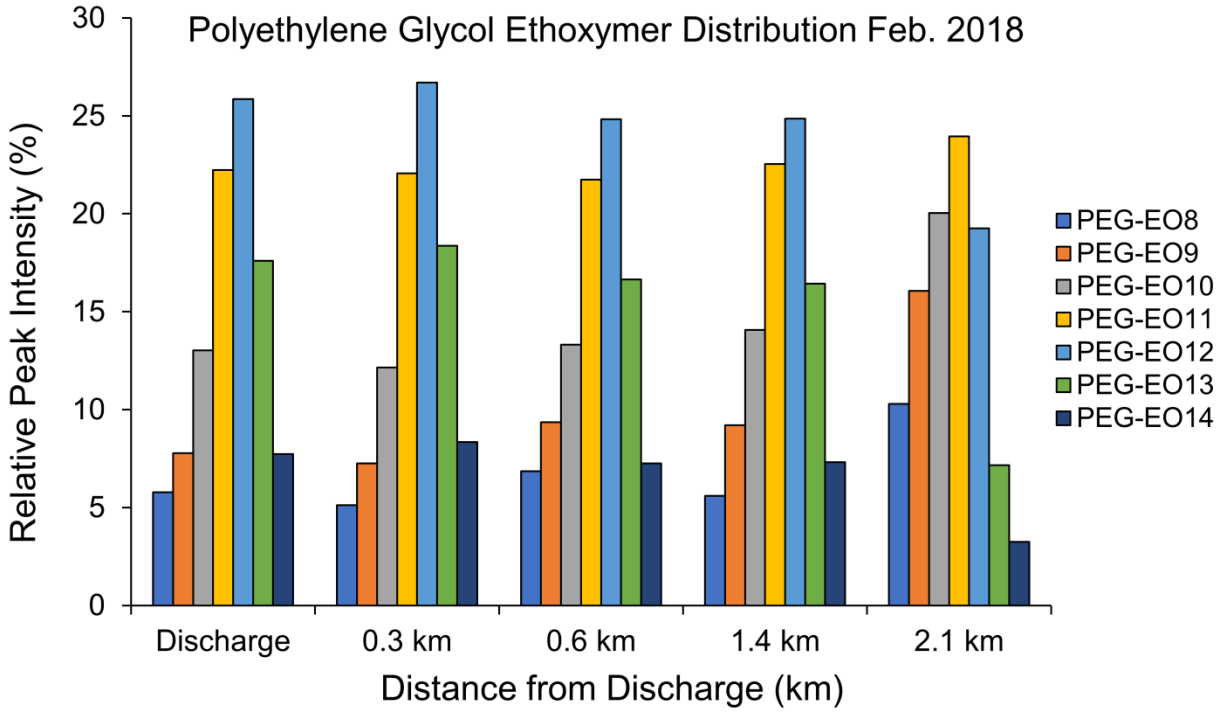

Figure A1. Changes in polyethylene glycol (PEG) relative peak intensity with increasing distance from the discharge. A shift towards lower molecular weight (lower EO) PEGs was observed with distance, providing evidence for transformation of this species, rather than dilution. PEG was below detection limit 3.8 km downstream and therefore no data is provided.

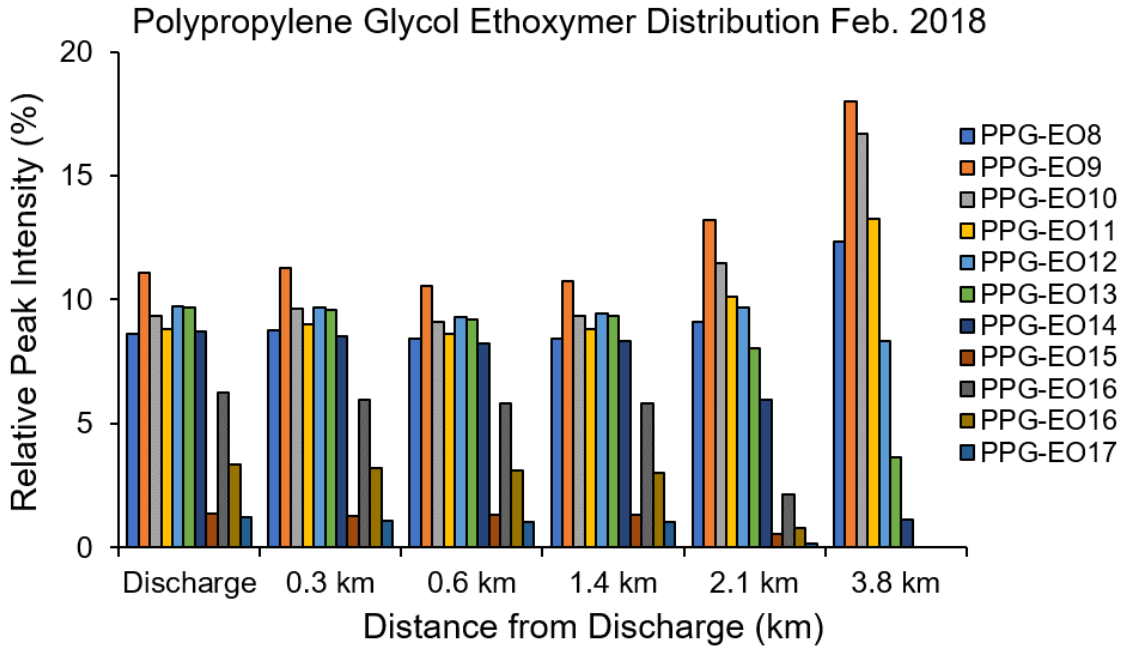

Figure A2. Changes in polypropylene glycol (PPG) relative peak intensity with increasing distance from the discharge. A shift towards lower molecular weight (lower EO) PPGs was observed with distance, providing evidence for transformation of this species, rather than dilution.

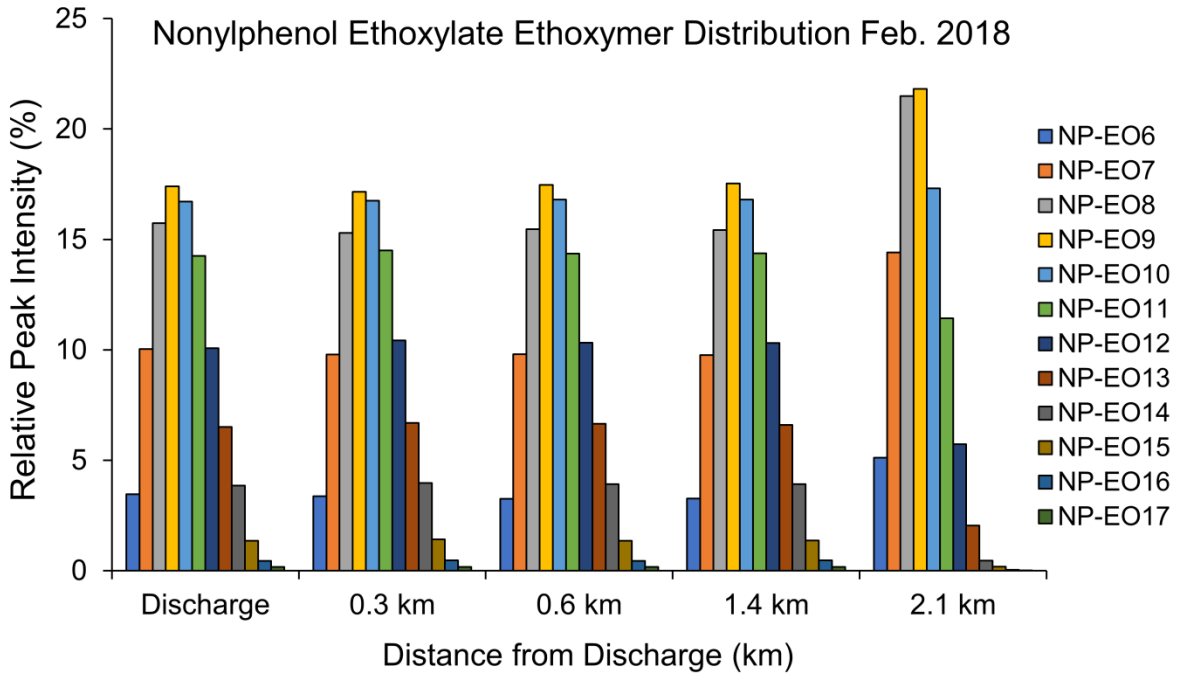

Figure A3. Changes in nonylphenol ethoxylate (NPEO) relative peak intensity with increasing distance from the discharge. A shift towards lower molecular weight (lower EO) NPEOs was observed with distance, providing evidence for transformation of this species, rather than dilution. NPEO was below detection limit 3.8 km downstream and therefore no data is provided.

Table A1. Polyethylene glycol accurate mass table from LC/Q-ToF/MS analysis.

Surfactant Species	Retention Time (min)	Base Peak	Base Peak Formula	Observed *m/z*	Theoretical *m/z*	Error (ppm)
PEG-EO8	4.3	$[M+Na]^+$	$C_{16}H_{34}O_9Na^+$	393.2099	393.2095	-1.0
PEG-EO9	4.5	$[M+NH_4]^+$	$C_{18}H_{38}O_{10}NH_4^+$	432.2803	432.2803	0.0
PEG-EO10	4.8	$[M+NH_4]^+$	$C_{20}H_{42}O_{11}NH_4^+$	476.3069	476.3065	-0.8
PEG-EO11	5.1	$[M+NH_4]^+$	$C_{22}H_{46}O_{12}NH_4^+$	520.3325	520.3328	0.6
PEG-EO12	5.6	$[M+NH_4]^+$	$C_{24}H_{50}O_{13}NH_4^+$	564.3595	564.359	-0.9
PEG-EO13	6.1	$[M+NH_4]^+$	$C_{26}H_{54}O_{14}NH_4^+$	608.3849	608.3852	0.5
PEG-EO14	6.5	$[M+NH_4]^+$	$C_{28}H_{58}O_{15}NH_4^+$	652.4113	652.4114	0.2

Table A2. Polypropylene glycol accurate mass table from LC/Q-ToF/MS analysis.

Surfactant Species	Retention Time (min)	Base Peak	Base Peak Formula	Observed *m/z*	Theoretical *m/z*	Error (ppm)
PPG-EO4	7.6	$[M+Na]^+$	$C_{12}H_{26}O_5Na^+$	273.167	273.1672	0.7
PPG-EO5	8.5	$[M+Na]^+$	$C_{15}H_{32}O_6Na^+$	331.2086	331.2091	1.5
PPG-EO6	9.4	$[M+Na]^+$	$C_{18}H_{38}O_7Na^+$	389.2506	389.251	1.0
PPG-EO7	10.3	$[M+Na]^+$	$C_{21}H_{44}O_8Na^+$	447.2927	447.2928	0.2
PPG-EO8	11.1	$[M+NH_4]^+$	$C_{24}H_{50}O_9NH_4^+$	500.3785	500.3793	1.6
PPG-EO9	12.0	$[M+NH_4]^+$	$C_{27}H_{56}O_{10}NH_4^+$	558.4213	558.4212	-0.2
PPG-EO10	12.8	$[M+NH_4]^+$	$C_{30}H_{62}O_{11}NH_4^+$	616.4632	616.463	-0.3
PPG-EO11	13.6	$[M+NH_4]^+$	$C_{33}H_{68}O_{12}NH_4^+$	674.5053	674.5049	-0.6
PPG-EO12	14.5	$[M+NH_4]^+$	$C_{36}H_{74}O_{13}NH_4^+$	732.5468	732.5468	0.0
PPG-EO13	15.3	$[M+NH_4]^+$	$C_{39}H_{80}O_{14}NH_4^+$	790.5888	790.5886	-0.3
PPG-EO14	16.2	$[M+NH_4]^+$	$C_{42}H_{86}O_{15}NH_4^+$	848.6301	848.6305	0.5
PPG-EO15	17.1	$[M+NH_4]^+$	$C_{45}H_{92}O_{16}NH_4^+$	906.6722	906.6724	0.2
PPG-EO16	18.2	$[M+NH_4]^+$	$C_{48}H_{98}O_{17}NH_4^+$	964.7141	964.7142	0.1
PPG-EO17	18.9	$[M+NH_4]^+$	$C_{51}H_{104}O_{18}NH_4^+$	1022.7564	1022.7561	-0.3
PPG-EO18	20.0	$[M+NH_4]^+$	$C_{54}H_{110}O_{19}NH_4^+$	1080.7977	1080.798	0.3

Table A3. Nonylphenol ethoxylate accurate mass table from LC/Q-ToF/MS analysis

Surfactant Species	Retention Time (min)	Base Peak	Base Peak Formula	Observed *m/z*	Theoretical *m/z*	Error (ppm)
NP-EO17	16.3	$[M+NH_4]^+$	$C_{49}H_{92}O_{18}NH_4^+$	986.6615	986.6622	0.7
NP-EO16	16.4	$[M+NH_4]^+$	$C_{47}H_{88}O_{17}NH_4^+$	942.6354	942.6360	0.6
NP-EO15	16.6	$[M+NH_4]^+$	$C_{45}H_{84}O_{16}NH_4^+$	898.6097	898.6098	0.1
NP-EO14	16.7	$[M+NH_4]^+$	$C_{43}H_{80}O_{15}NH_4^+$	854.5832	854.5835	0.4
NP-EO13	16.9	$[M+NH_4]^+$	$C_{41}H_{76}O_{14}NH_4^+$	810.5575	810.5573	-0.2
NP-EO12	17.0	$[M+NH_4]^+$	$C_{39}H_{72}O_{13}NH_4^+$	766.5312	766.5311	-0.1
NP-EO11	17.2	$[M+NH_4]^+$	$C_{37}H_{68}O_{12}NH_4^+$	722.5049	722.5049	0.0
NP-EO10	17.3	$[M+NH_4]^+$	$C_{35}H_{64}O_{11}NH_4^+$	678.4782	678.4787	0.7
NP-EO9	17.5	$[M+NH_4]^+$	$C_{33}H_{60}O_{10}NH_4^+$	634.4525	634.4525	0.0
NP-EO8	17.7	$[M+NH_4]^+$	$C_{31}H_{56}O_9NH_4^+$	590.4265	590.4263	-0.3
NP-EO7	17.8	$[M+NH_4]^+$	$C_{29}H_{52}O_8NH_4^+$	546.4003	546.4000	-0.5
NP-EO6	18.0	$[M+NH_4]^+$	$C_{27}H_{48}O_7NH_4^+$	502.374	502.3738	-0.4

Table A4. Organic chemical physiochemical properties. [a]

Chemical Name	CASRN [b]	K_H (atm-m^3/mol) [c]	Log K_{ow}	Biodegradation half-life (days)	Water Solubility (mol/L)	Atmospheric Hydroxylation Rate (cm^3/molecule*sec)	K_{oc} (L/kg) [d]
1,2,4-Trimethylbenzene	95-63-6	6.16×10^{-3}	3.63	4	-	-	-
1,2-Dichloroethane	107-06-2	1.1×10^{-3}	1.48	10.3 [e]	-	-	-
1,3,5-Trimethylbenzene	108-67-8	8.77×10^{-3}	3.42	3	-	-	-
1-Methylnaphthalene	90-12-0	3.6×10^{-4}	3.87	9.17	1.81×10^{-4}	5.30×10^{-11}	2290
2,4-Dimethylphenol	105-67-9	9.51×10^{-7}	2.30	4.07 [e]	6.64×10^{-2}	7.15×10^{-11}	174 [e]
2-Butanone	78-93-3	5.77×10^{-5}	0.29	3.67 [e]	3.09	1.15×10^{-12}	3.55 [f]
2-Butoxyethanol	111-76-2	5.44×10^{-6}	0.83	4.45 [e]	8.46	1.86×10^{-11}	67.6
2-Methylnaphthalene	91-57-6	4.99×10^{-4}	3.86	14	1.73×10^{-4}	5.23×10^{-11}	2455 [f]
2-Methylphenol	95-48-7	1.2×10^{-6}	1.95	5.29 [e]	0.239	4.20×10^{-11}	135 [e]
4-Methyl-2-pentanone	108-10-1	1.4×10^{-4}	1.31	4.43 [e]	-	-	-
Acetone	67-64-1	4.26×10^{-5}	-0.24	7.57 [e]	17.2	2.19×10^{-13}	5.37 [f]
Benzene	71-43-2	5.5×10^{-3}	2.13	6	2.29×10^{-2}	1.23×10^{-12}	60 to 83 [f]
Bis(2-ethylhexyl) phthalate	117-81-7	2.6×10^{-7} [e]	7.60	4.82 [e]	1.08×10^{-7}	2.03×10^{-11} [e]	87100 [f]
Carbazole	86-74-8	1.16×10^{-7}	3.72	41.1 [e]	-	-	-
Carbon disulfide	75-15-0	1.22×10^{-2}	1.94	18 [e]	1.55×10^{-2}	1.17×10^{-13} [e]	54 [f]
Diesel range organics [g]	NA	0.151 [e] to 7.36×10^{-7} [e]	5.01 to 11.80 [e]	9 to 125	3.65×10^{-7} to 6.99×10^{-9} [e]	1.16×10^{-11} to 6.15×10^{-12} [e]	11400[e] to 10200[e]
Ethylbenzene	100-41-4	6.6×10^{-3}	3.13	8	1.59×10^{-3}	7.10×10^{-12}	240 [f]
Gasoline range organics [g]	NA	0.487 [e] to 0.151 [e]	3.90 to 5.01	7 to 9	1.10×10^{-4} to 3.65×10^{-7}	5.61×10^{-12} to 1.16×10^{-11}	1300[e] to 11400[e]
Isopropylbenzene	98-82-8	1.15×10^{-2}	3.66	15	-	-	-
Methyl Acrylate	96-33-3	2.0×10^{-4}	0.80	4.12	-	-	-
Naphthalene	91-20-3	4.6×10^{-4}	3.29	3	2.42×10^{-4}	2.16×10^{-11}	912
***n*-Propyl Benzene**	103-65-1	0.0105	3.69	4			
Phenanthrene	85-01-8	4.23×10^{-5}	4.46	42	6.45×10^{-6}	1.30×10^{-11}	22400
Phenol	108-95-2	3.33×10^{-7}	1.46	4.58 [e]	0.88	2.63×10^{-11}	26.9 [f]
Toluene	108-88-3	6.64×10^{-3}	2.72	2	5.71×10^{-3}	5.96×10^{-12}	37 to 178 [f]
Xylenes (total)	1330-20-7	5.18×10^{-3}	3.12	6	-	-	25.4 to 540 [f]

[a] All values are from EPA Comptox Website unless noted.
[b] CASRN = Chemical Abstract Service Registry Number
[c] KH = Henry's constant
[d] Koc = Soil adsorption coefficient
[e] Indicates predicted values. All other values are experimental.
[f] Values are from individual ATSDR reports for each chemical.
[g] Diesel range organics and gasoline range organics values are a range for alkanes in that mixture. All other ranges for chemicals highlight discrepancies amongst reported values.

APPENDIX B: SUPPORTING INFORMATION FOR CHAPTER 3 - ASSESSMENT OF WATER QUALITY DOWNSTREAM OF NPDES OIL AND GAS PRODUCED WATER DISCHARGE: TOXICOLOGICAL IMPACTS

B.1 Recipes for Liquid Media and Agar Plates

YPD media (for liquid cultures exclude the agar)

- 10 g yeast extract (Yeastolate, Molecular Biology Grade, USBiological, Salem, MA)
- 20 g peptone Y (BSE-free, Casein Peptone, Molecular Biology Grade, USBiological, Salem, MA)
- 20 g glucose (anhydrous, granular, lab grade, Ward's science, Rochester, NY)
- 20 g bacteriological agar (Bacteriological, Molecular Biology Grade, USBiological, Salem, MA)
- ddH2O to bring to 1 L

Tryptophan drop out media

- 1.7 g Yeast Nitrogen Base w/o amino acids, carbohydrate and without ammonium sulfate (Powdered, USBiological, Salem, MA)
- 5 g ammonium sulfate (Certified ACS Granular, Fisher Chemical, Fair Lawn, NJ)
- 20 g glucose (anhydrous, granular, lab grade, Ward's science, Rochester, NY)\
- 20 g bacteriological agar (Bacteriological, Molecular Biology Grade, USBiological, Salem, MA)
- 1.4 g Drop-out Mix Synthetic Minus Tryptophan w/o Yeast Nitrogen Base (Powder, USBiological, Salem, MA)
- ddH2O to bring to 1 L

Canavanine media

- 1.7 g Yeast Nitrogen Base w/o amino acids, carbohydrate and without ammonium sulfate (Powdered, USBiological, Salem, MA)
- 5 g ammonium sulfate (Certified ACS Granular, Fisher Chemical, Fair Lawn, NJ)
- 20 g glucose (anhydrous, granular, lab grade, Ward's science, Rochester, NY)\
- 20 g agar (Bacteriological, Molecular Biology Grade, USBiological, Salem, MA)
- 750 mL ddH2O
- Autoclave above materials
- Heat 250 mL of ddH2O to ~40°C and combine with ingredients below. Then filter sterilize.
- 1.4 g Drop-out Mix Synthetic Minus Arginine w/o Yeast Nitrogen Base (Powder, USBiological, Salem, MA)
- 60 mg Canavanine Sulfate (Enzo, Farmingdale, NY)

- Add to 750 mL autoclaved base media when it reaches 60-65°C.

Copper-Formaldehyde media

- 1.7 g Yeast Nitrogen Base w/o amino acids, carbohydrate and without ammonium sulfate (Powdered, USBiological, Salem, MA)
- 5 g ammonium sulfate (Certified ACS Granular, Fisher Chemical, Fair Lawn, NJ)
- 20 g glucose (anhydrous, granular, lab grade, Ward's science, Rochester, NY)\
- 20 g agar (Bacteriological, Molecular Biology Grade, USBiological, Salem, MA)
- 1 g Drop-Out Mix Complete, Adenine Rich, without Yeast Nitrogen Base (Powder, USBiological, Salem, MA)
- 1 g Drop-out Mix Synthetic Minus Lysine w/o Yeast Nitrogen Base (Powder, USBiological, Salem, MA)
- ddH2O to bring to 1 L
- A 100 mM CuSO4 stock solution was made by combining 1.596 g of $CuSO_4$ (anhydrous) into 100 mL ddH2O, filter sterilized
- Each time, a fresh 1000mM formaldehyde mixture was made by combining 406 µL of 37% formaldehyde (Fisher Chemical, Fair Lawn, NJ) with 4594 µL sterile ddH2O
- Add appropriate volumes of 100 mM CuSO4 and 1000mM FA to autoclaved base media when it reaches 60-65°C.

5-FOA recipe

- 1.7 g Yeast Nitrogen Base w/o amino acids, carbohydrate and without ammonium sulfate (Powdered, USBiological, Salem, MA)
- 5 g ammonium sulfate (Certified ACS Granular, Fisher Chemical, Fair Lawn, NJ)
- 20 g glucose (anhydrous, granular, lab grade, Ward's science, Rochester, NY)
- 20 g agar (Bacteriological, Molecular Biology Grade, USBiological, Salem, MA)
- 750 mL ddH2O
- Autoclave above materials
- Heat 250 mL of ddH2O to ~40°C and combine with ingredients below. Then filter sterilize.
- 1 g Drop-Out Mix Complete, Adenine Rich, without Yeast Nitrogen Base (Powder, USBiological, Salem, MA)
- 1 g Drop-out Mix Synthetic Minus Lysine w/o Yeast Nitrogen Base (Powder, USBiological, Salem, MA)
- 5-Fluoroorotic Acid monohydrate (5-FOA) (Goldbio.com, St. Louis, MO)
- Add to 750 mL autoclaved base media when it reaches 60-65°C.

B.2 Daphnia Culturing and Housing

Daphnia magna colonies were obtained in June of 2017 from Aquatic Research Organisms (Aquatic Research Organisms Inc., NH, USA) and transported to the University of Alberta where they were housed and cultured according to Organization for Economic Cooperation and Development (OECD) guidelines[1,2] with some slight adjustments. Briefly, *Daphnia* were maintained at 20 ± 1 °C in 10 L glass aquaria with dechlorinated City of Edmonton tap water (moderately hard: $[Na^+]$ = 14.6 mg/L, $[Ca^{2+}]$ = 55.9 mg/L, $[Mg^{2+}]$ = 15.3 mg/L, $[K^+]$ = 2.5 mg/L, titration alkalinity ~ 119 mg/L as $CaCO_3$, pH ~ 7.6, hardness ~ 180 mg/L as $CaCO_3$, conductivity ~ 385 µS/cm). Water changes occurred 3x weekly with daily feedings of ~ 2 mL Roti-Rich invertebrate diet (VWR, Edmonton, Alberta, Canada) to satiation. Organisms were subjected to a 14 h light/ 10 h dark photo-period for culture and exposure durations. Neonate daphnids (≤ 24 hrs old) were collected immediately prior to exposure commencement and used for median lethal concentration (LC_{50}) analyses.

B.3 Zebrafish Studies

B.3.1 Zebrafish

Tropical 5D wild-type adult zebrafish were housed at an approximate density of 1000 per 100 gallons. Spawning funnels were placed into the tanks the night prior, and embryos were collected and age-staged [162]. Developing zebrafish have a cellular envelope that surrounds them, and can act as a barrier. To increase bioavailability, the chorion was enzymatically removed using pronase (63.6 mg/ml, ≥ 3.5 U/mg) at 4 hpf (hours post fertilization) using a custom automated dechorionator [163].

B.3.2 Chemical Preparation

All sample preparations were provided as 1X aqueous solutions. The samples were tested as fractional dilutions of the 1X samples as shown in Table S2 and, prior to testing, the pH and conductivity (μS) of each dilution was measured with a Hanna HI-9813-6 meter and recorded. Total dissolved solid content was calculated directly from conductivity (scale: NaCl (1EC = 500ppm)). Conductivity and pH parameters were all well within the acceptable range to support normal zebrafish development. Dilutions of the 1X sample were conducted using the appropriate solution (depending on the conductivity of the provided 1X aqueous solution) which was either fish water (high and low conductivity; ~500-1700 μS), or ultrapure water (~4 μS).

Table B1. Sample nomendature, properties and test conditions.

Sample	Concentration	Conductivity (μS/cm)	pH	TDS (mg/L)
D0	0X	2400	7.1	1200
	0.0625X	2384.1	8.34	1120.7
	0.125X	2382.1	8.28	1141
	0.25X	2400.4	8.16	1185.2
	0.5X	2490.5	8.09	1165
	1X	2519.6	8.12	1159.8
D15	0X	2400	7	1200
	0.0625X	1171.5	7.29	1142.6
	0.125X	2705.2	7.35	1170.6
	0.25X	2764.3	7.45	1239.5
	0.5X	2142	7.75	1380.6
	1X	3203.2	7.95	1400.2
P-2.6	0X	520	7.32	268
	0.0625X	577.1	7.24	291.4
	0.125X	574.3	7.51	286.3
	0.25X	568.4	7.31	280.6
	0.5X	555.8	7.58	274.2
	1X	529	7.56	261.4
P32.2	0X	520	7.32	268
	0.0625X	578.8	7.81	289
	0.125X	577.8	7.76	288.9
	0.25X	575.2	7.72	287.5
	0.5X	570.1	7.7	285
	1X	556.3	7.7	278.2

B.3.3 Chemical exposures

The 6-concentration curve and 96-well plate layout for the 7 samples is shown in Table S3. Plates were run in duplicate to obtain N = 32 animals per concentration; 1 embryo was exposed per well, 16 embryos exposed per concentration per plate. Zebrafish embryos without the chorion were loaded 1 per well at 6 hpf into 100 µl of embryo medium in 96-well plates by an automated embryo placement system (AEPS) which ensured allocation to study groups was random [163]. All testing conditions were identical across plates, chemicals, and testing days.

Table B2. Sample layout over 6 concentrations duplicated in a 96-well format, 1 embryo per well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
B	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
C	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
D	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
E	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
F	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
G	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X
H	1X	0.5X	0.25X	0.125X	0.625X	0X	1X	0.5X	0.25X	0.125X	0.625X	0X

B.3.4 Endpoints assessed

These in vivo assays used the zebrafish embryo (*D. rerio*) as a biological sensor to evaluate a comprehensive battery of developmental endpoints for chemical hazard via multiple mechanisms of action [129, 164]. The developmental zebrafish assays are conducted in physiologically intact organisms, and the embryos develop in a short window in which there is a high probability of detecting adverse outcomes such as developmental delays, morphological abnormalities and behavioral alterations. Zebrafish is a highly prolific, small, complex organism that shares a highly-conserved anatomy and physiology with all vertebrates [165]. Importantly, the critical processes of zebrafish neurodevelopment are homologous to those in humans [166].

Early in zebrafish embryogenesis (roughly 19-29 hours post-fertilization, hpf), spontaneous tail flexions occur as the muscles in this region are innervated [162]. This spontaneous behavior at 22 hpf

is sensitive to light perturbation via photoreceptors in the developing hindbrain and has been designated the embryo photomotor response (EPR) [167]. The EPR is an early, fast and sensitive assay to detect chemical perturbation of development. While the EPR readout is behavioral, later stage developmental defects predicted by an abnormal EPR are not restricted to behavioral outcomes but often include morphological deficits as well [168].

B.3.5 Embryo Photomotor Response behavior (EPR)

At 24 hpf, embryos were assessed, in plate, for photomotor response using a custom photomotor response analysis tool (PRAT) [131]. For every exposure plate, 850 frames of digital video were recorded at 17 frames s-1 from beneath a custom 96-well plate mount, and lighted from above with white LED and infrared lights. The light cycle consisted of 30 seconds of dark background (prior to the first light pulse), a short pulse of light, and 9 seconds later, a 2nd pulse of light, and then 10 more seconds of dark. Animals dead or malformed at the 24 hpf timepoint were excluded from the behavior data sets. The statistical analysis of activity considered only the Background (B), Excitatory (E), and Refractory (R) intervals and performed according to Reif et al [131].

B.3.6 Larval Photomotor Response behavior (LPR)

At 120 hpf (5 days post fertilization) zebrafish are free swimming larvae and the photomotor response assayed total movement (swim distance) in response to multiple light -> dark transitions (Figure S5). Briefly, a Zebrafish behavior chamber (ViewPoint Life Sciences, Montreal, CA) with an infrared backlit stage was used to track total movement in 96 wells during a 24-minute assay. HD video was captured at 15 frames s-1 and processed in real time by the manufacturer's software. The assay consisted of 3 cycles of 3 minutes visible light, 3 minutes dark (IR light). Additional animals dead or malformed at the 120 hpf timepoint were excluded from the larval behavior data analysis. Area under the curve for each treatment was statistically compared to the control movement by t-test. The

LPR at a given exposure concentration was considered valid only when statistical significance ($p < 0.05$) was reached and the percent change in AUC was ≥ 40% above the control group AUC.

B.3.7 Mortality and morphology responses

Embryos were statically exposed until 120 hpf. At 24 hpf, embryos were assessed for 4 developmental toxicity endpoints (MO24: mortality at 24 hpf, DP: developmental progression, SM: spontaneous movement, and NC: notochord distortion)[130]. At 120 hpf, 18 developmental endpoints were assessed [130]. Each well was evaluated for all the listed endpoints and in recorded in a binary manner into a custom program, the Zebrafish acquisition and analysis program (ZAAP). The statistical analysis of the data was conducted using custom R-code and a Fisher's exact test was used to compare each dilution to the control ($p<0.01$).

B.4 Additional Figures and Tables

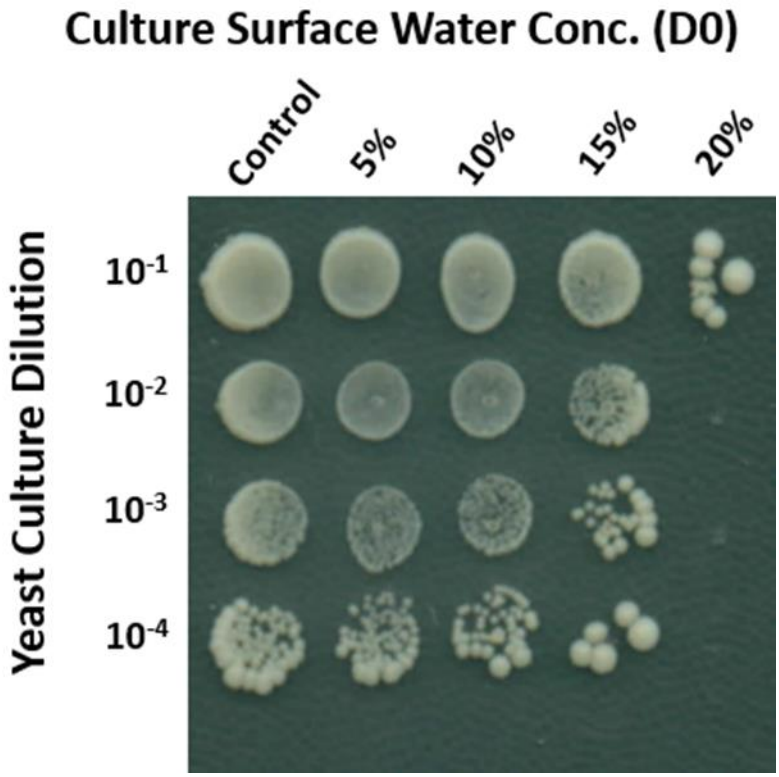

Figure B1. Yeast acute toxicity assay conducted on the discharge sample (D0) collected in February 2018 showing that yeast growth was inhibited above 10% water volume/culture volume.

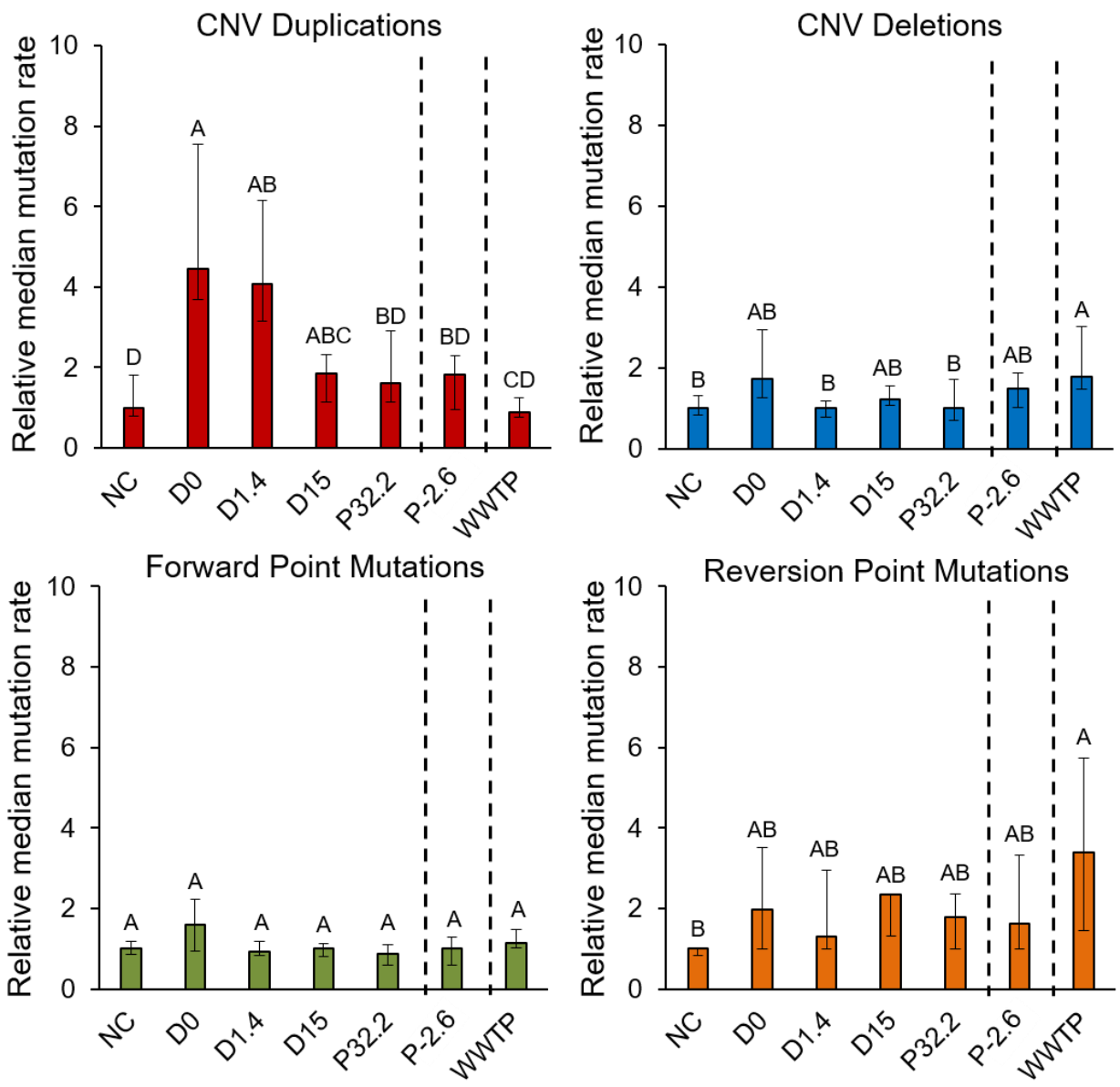

Figure B2. Relative median mutation rate for Copy Number Variation (CNV) duplications, CNV deletions, forward point mutations, and reversion point mutations in 2016 discharge stream (D), perennial river (P) samples and wastewater treatment plant (WWTP) sample. Experiments were conducted with 25% water sample. The background sample (BG) is site P-2.6. Median mutation rates are displayed relative to the negative control (NC). Error bars show 95% confidence intervals. Letters show the statistical groupings. Samples that do not share letters are significantly different ($p < 0.025$) from the Kruskal-Wallis test.

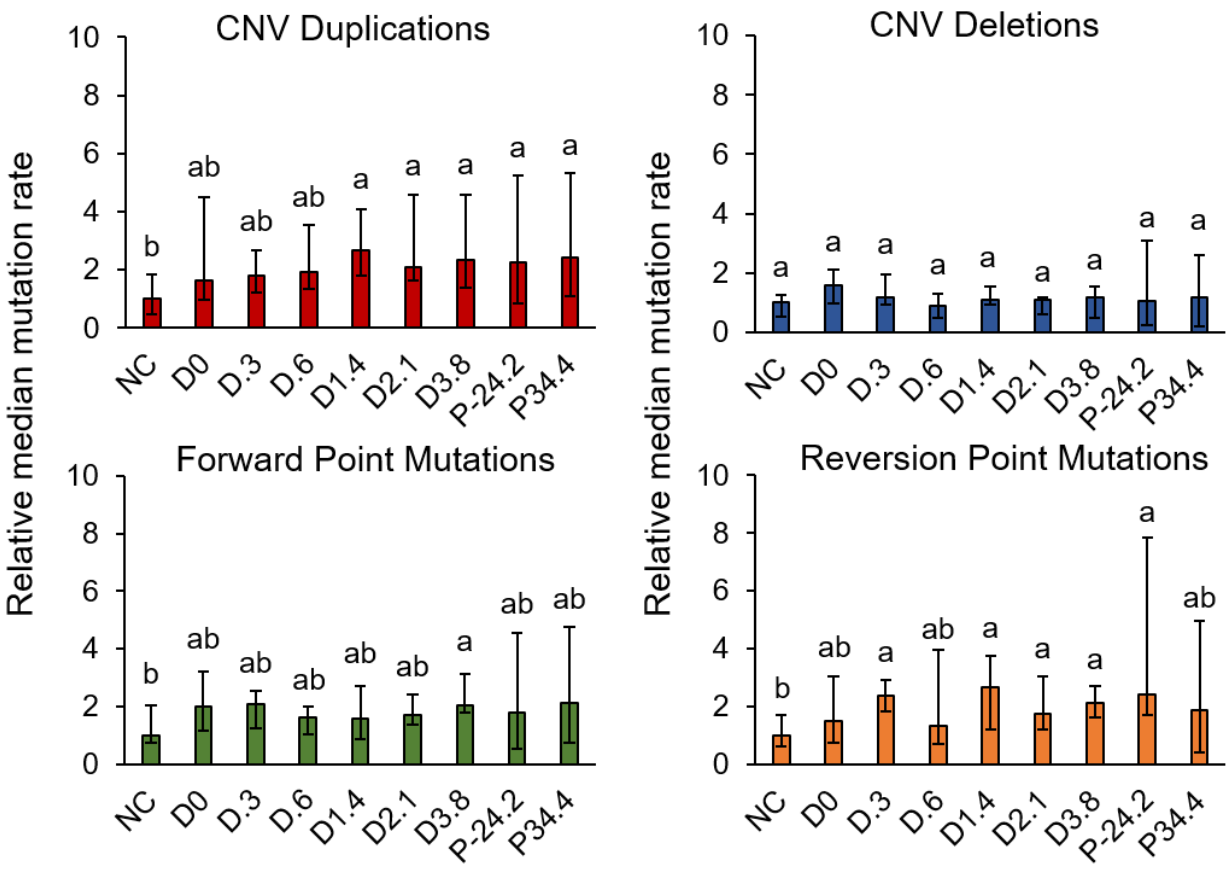

Figure B3. Relative median mutation rate for Copy Number Variation (CNV) duplications, CNV deletions, forward point mutations, and reversion point mutations in 2018 discharge stream (D) and perennial river (P) samples. Experiments were conducted with 10% water sample. Median mutation rates are displayed relative to the negative control (NC). Error bars show 95% confidence intervals. Letters show the statistical groupings. Samples that do not share letters are significantly different ($p < 0.025$) from the Kruskal-Wallis test.

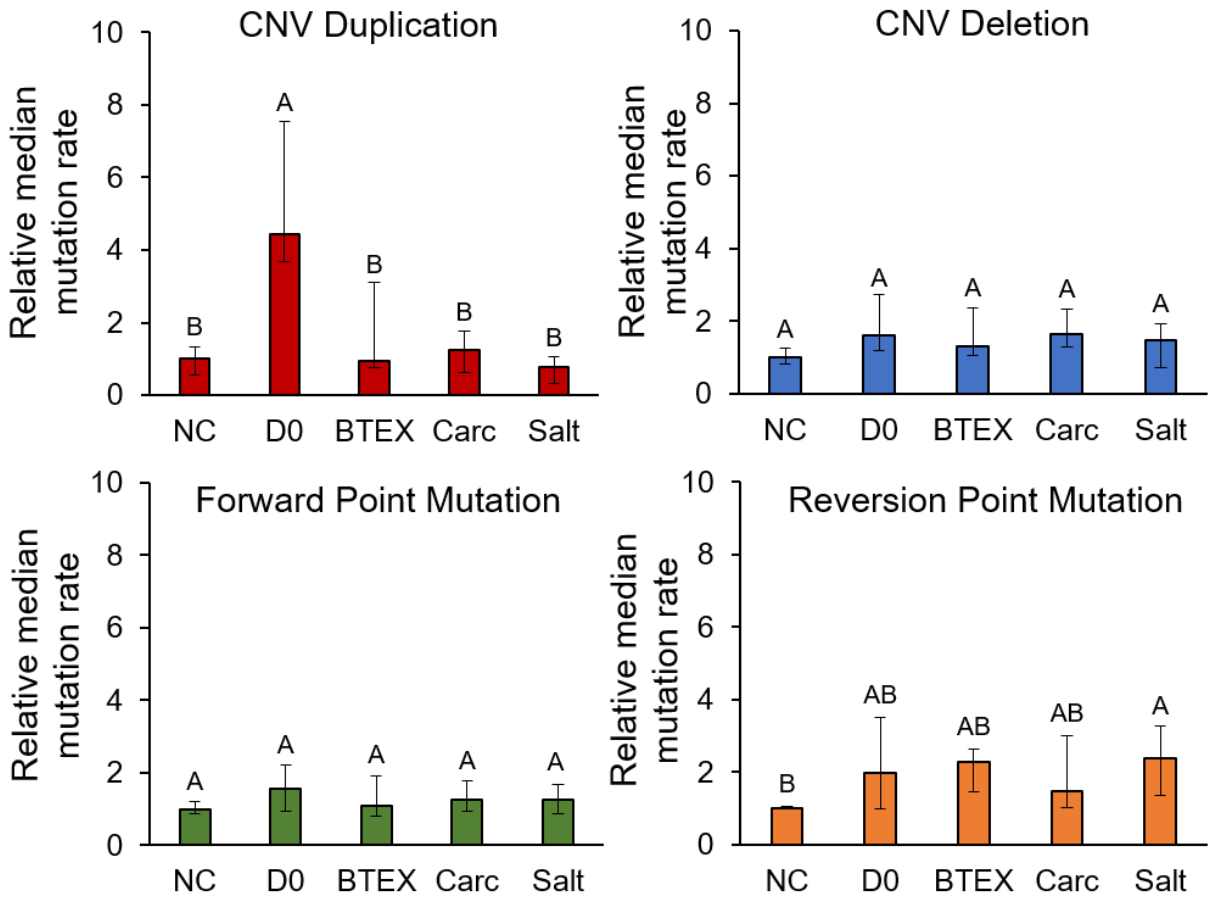

Figure B4. Relative mutation rate for copy number variation (CNV) duplications, CNV deletions, forward point mutations, and reversion point mutations of discharge sample (D0), BTEX mixture, carcinogen mixture (Carc) and salt control relative to the negative control (NC). Experiments were conducted with 25% water sample. Bar height represent median mutation rates and error bars represent 95% confidence intervals. Letters show the statistical groupings for the bioassays. Samples that do not share letters are significantly different.

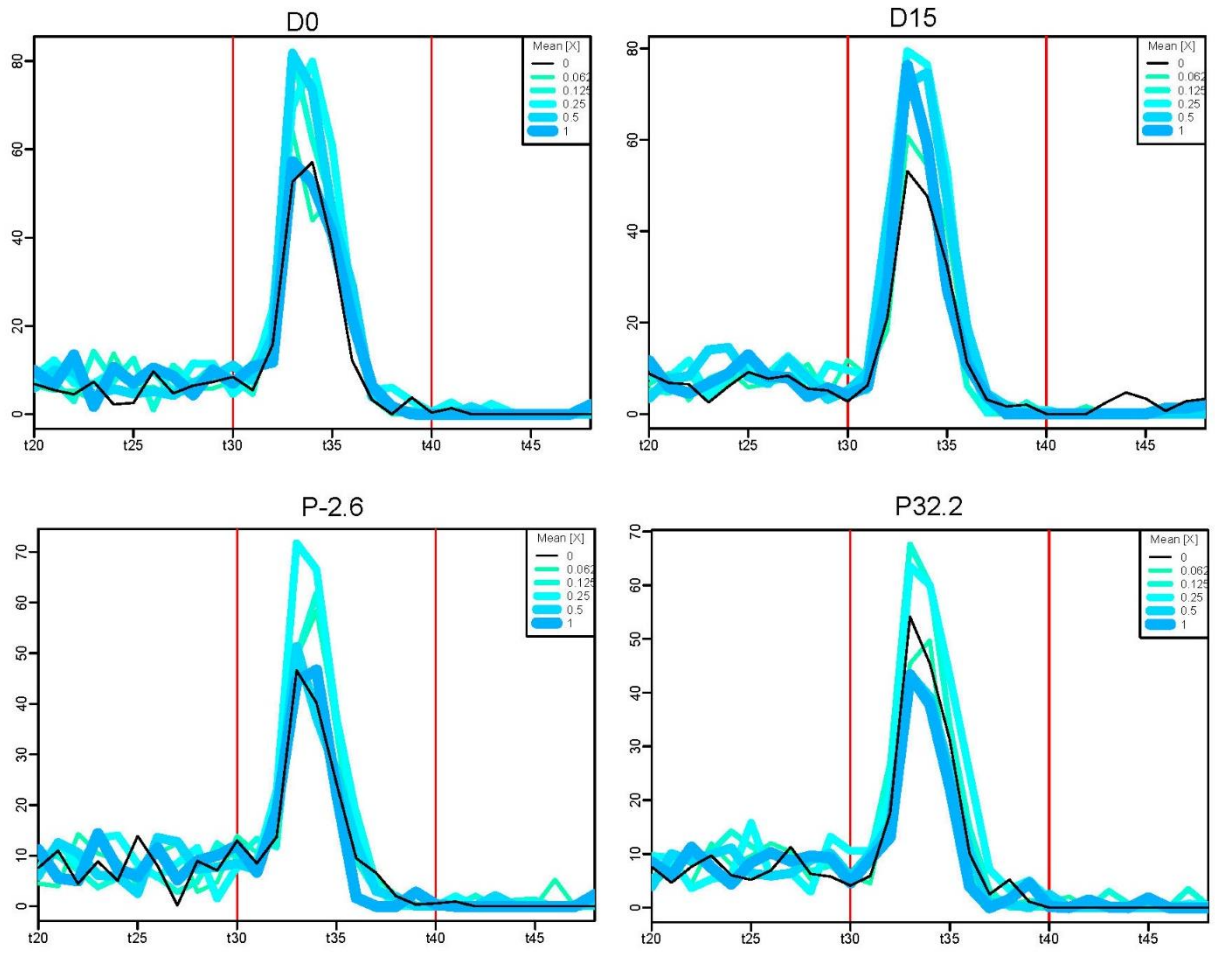

Figure B5. Embryo Photomotor Response behaviors (EPR) at 24 hours post fertilization (hpf) associated with MTPW impacted stream water exposures. The x-axis shows time in seconds. The red vertical lines indicate the times of a bright, 1s flash of visible white light. Before the 1st red line is the background, where no activity is expected, following the flash, (the excitatory phase), a normal embryo exhibits an increase in activity, then a steady decrease until the next flash (the refractory), where the embryo should not move. The samples were diluted to by 2-fold from 100 to 6.25. Each panel represents a sample dilution, and none were identified as exhibiting different responses than the control (in black).

Table B3. Significantly abnormal LPRs in the light and dark phases of the assay.

Chemical	Conc. (X)	Photo Interval	N	% change	Activity
D0	0.0625	LIGHT	29	-45.2	HYPO
D0	0.125	LIGHT	30	-50.3	HYPO
D0	0.5	LIGHT	30	-71.3	HYPO
D0	1	LIGHT	30	-73.9	HYPO
D15	1	LIGHT	32	-51.3	HYPO
P-2.6	0.0625	LIGHT	28	44.1	*HYPER*
P-2.6	0.5	LIGHT	28	-56.1	HYPO
P-2.6	1	LIGHT	30	-42.1	HYPO
P32.2	0.25	LIGHT	31	62.2	*HYPER*

APPENDIX C: SUPPORTING INFORMATION FOR CHAPTER 4 – VIABILITY OF CONSTRUCTED WETLANDS FOR PRODUCED WATER POLISHING DOWNSTREAM OF NPDES RELEASES

C.1 Additional Materials and Methods

Table C1. Well maintenance products and composition reported for Discharge B.

Product Function	Frequency of Use	Chemical Name	CAS Number	Concentration (%)
Emulsion Breaker	Daily			
		Heavy Aromatic Naphtha	64742-94-5	60-100
		Naphthalene	91-20-3	5-10
		Methanol	67-56-1	5-10
		1,2,4-Trimethylbenzene	95-63-6	1-5
		Reaction Product of PPG and EPON	36484-54-5	1-5
		Ethylbenzene	100-41-4	0.1-1
Emulsion Breaker	Daily			
		Heavy Aromatic Distillate	64742-94-5; 64742-48-9	10-30
		Organic sulfonic acid salt	Proprietary	10-30
		Isopropanol	67-63-0	10-30
		2-Ethylhexanol	104-76-7	5-10
		Xylene	1330-20-7	5-10
		Ethylbenzene	100-41-4	1-5
		Naphthalene	91-20-3	1-5
		Propylene Glycol	57-55-6	1-5
		1,2,4-Trimethylbenzene	95-63-6	1-5
		Toluene	108-88-3	0.1-1
Scale Inhibitor	Daily			
		Ethylene Glycol	107-21-1	10-30
		Triethanolamine Tri(Phosphate Ester), Sodium Salt	68171-29-9	10-30
Corrosion Inhibitor	Daily			
		Methanol	67-56-1	10-30
		n-Benzyl-Alkylpyridinium Chloride	68909-18-2	5-10
		Ethoxylated Tallow Alkyl Amine	61791-26-2	5-10
		Organic sulfonic acid	Proprietary	1-5
		Benzyl Chloride	100-44-7	0.1-1
Water Clarifier	Daily			
		Zinc Chloride	7646-85-7	30-60
		Isopropanol	67-63-0	10-30
Corrosion Inhibitor	Bi-monthly			
		Methanol	67-56-1	30-60
		Tall Oil, DETA Imidazoline Acetates	68140-11-4	5-10
		Benzyl-Dimethyl-Dodecyl-Ammonium Chloride	139-07-1	1-5
		Thioglycolic Acid	68-11-1	1-5

C.2 Additional Results and Discussion

C.2.1 Total Carbon, TOC, TIC and Total Nitrogen in Sediment

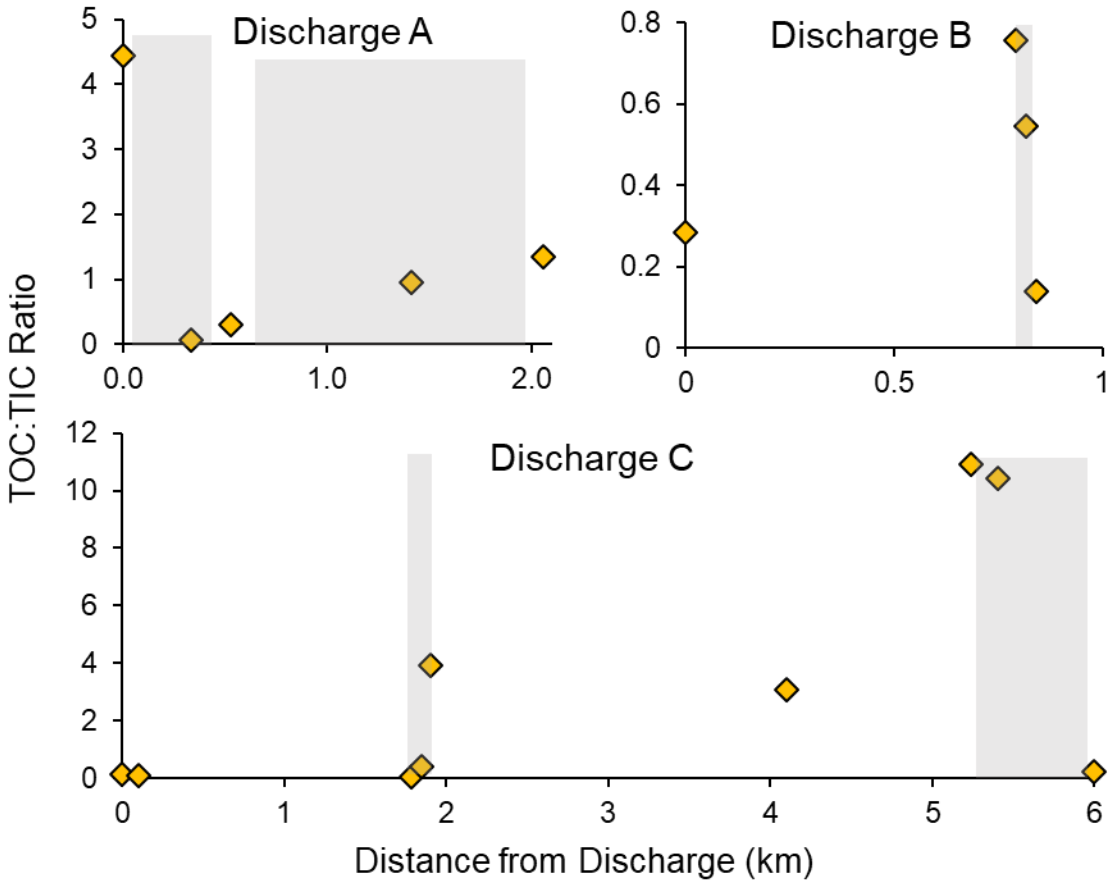

Figure C1. Total organic carbon (TOC) to total inorganic carbon (TIC) ratio in sediments versus distance from the discharge. Grey areas indicate locations of wetlands.

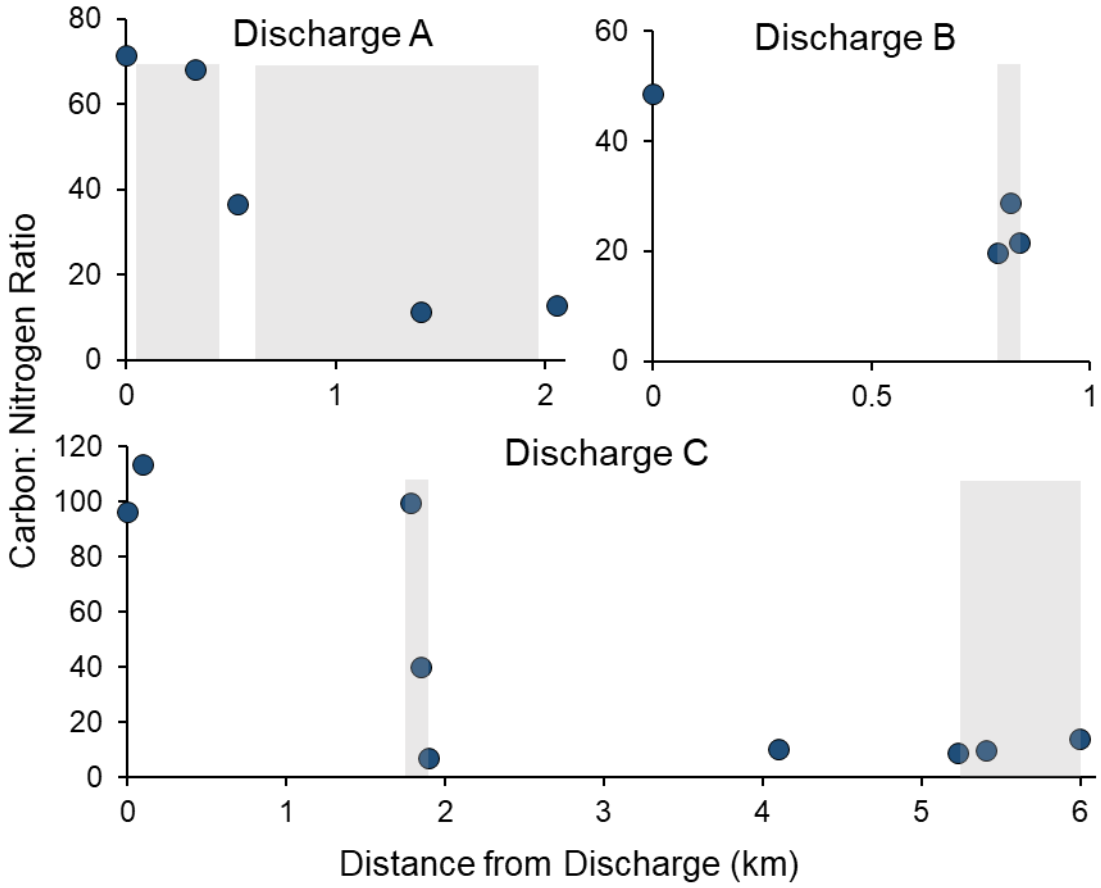

Figure C2. Carbon to nitrogen ratios in sediments versus distance downstream of each discharge. Grey areas indicate locations of wetlands.

C.2.2 Additional Surfactant Distribution Results

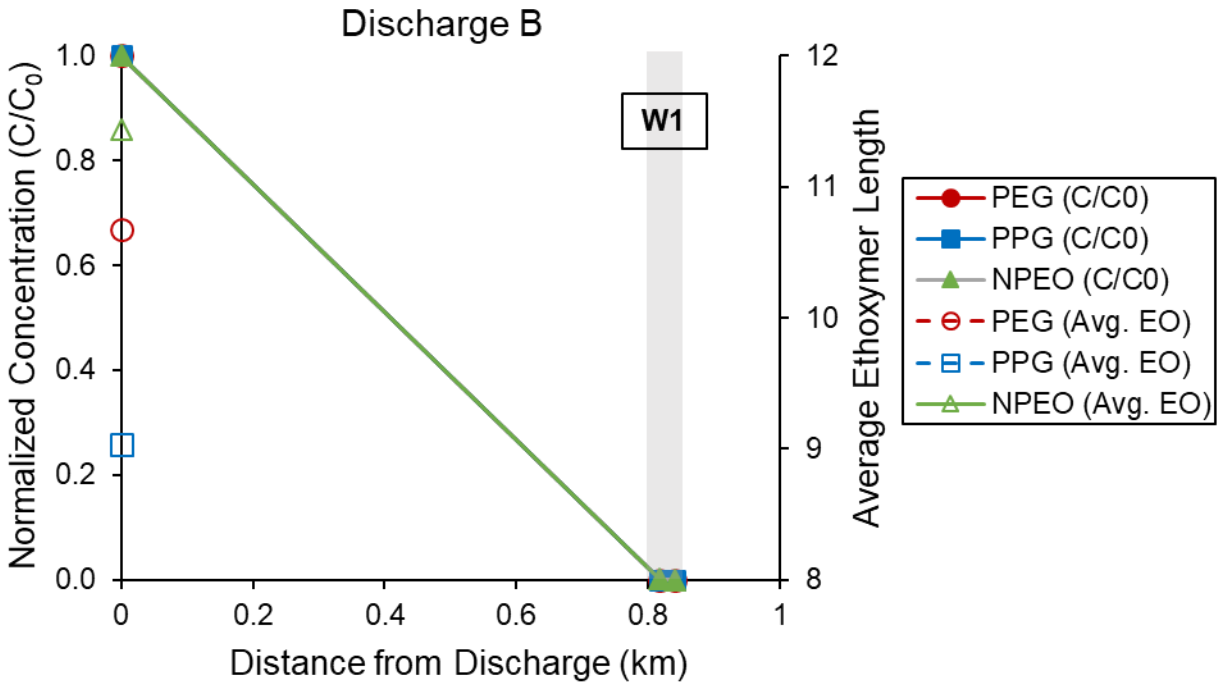

Figure C3. Relative concentration of polyethylene glycol (PEG), polypropylene glycol (PPG) and nonylphenol ethoxylate (NPEO) and average ethoxymer (EO) length for each species versus distance from the NPDES discharge (km) at Discharge B. All three species were below detection limit downstream of discharge so concentrations and average EO length is not reported. Grey areas indicate locations of wetlands.

C.2.2.1 Surfactant Distribution in Water Samples

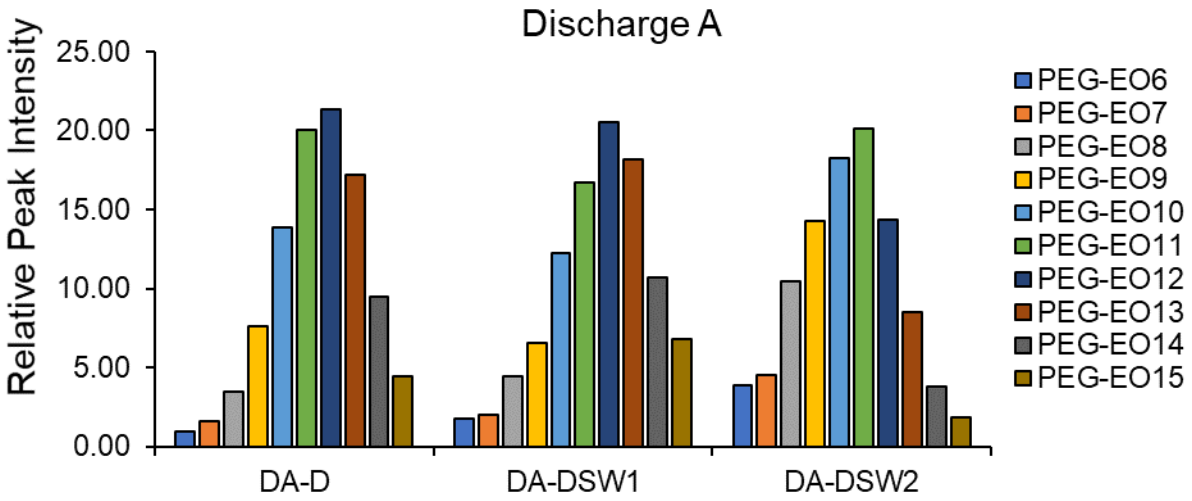

Figure C4. Polyethylene glycol (PEG) relative peak intensity at the discharge (DA-D), downstream of the first wetland (DA-DSW1) and downstream of the second wetland (DA-DSW2) at Discharge A. Shorter homologues (EO 6-10) increase with distance downstream and larger homologues (EO 12-15) decrease with distance downstream, indicating biodegradation as a removal mechanism for PEG in this system.

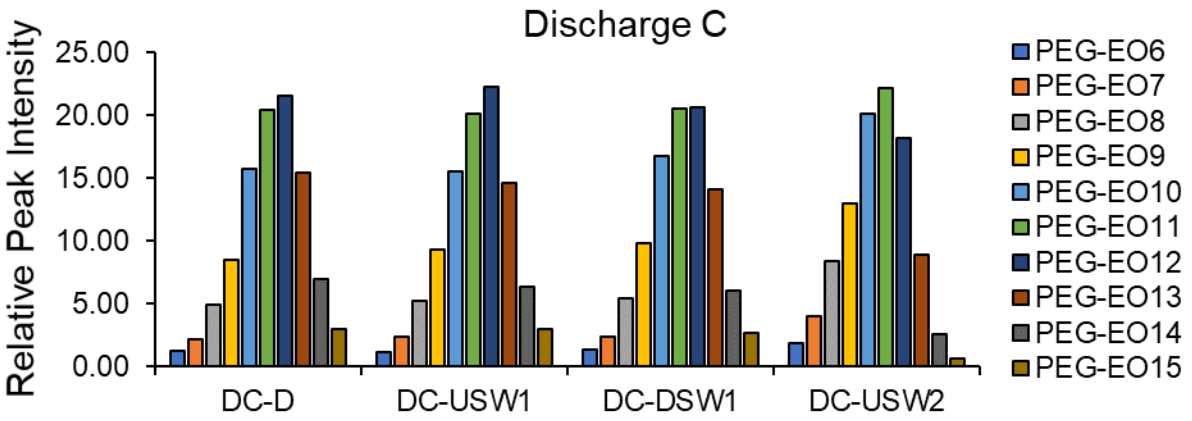

Figure C5. Polyethylene glycol (PEG) relative peak intensity at the discharge (DC-D), upstream of the first wetland (DC-USW1), downstream of the first wetland (DC-DSW1) and upstream of the second wetland (DC-USW2) at Discharge C. Shorter homologues (EO 6-11) increase with distance downstream and larger homologues (EO 12-15) decrease with distance downstream, indicating biodegradation as a removal mechanism for PEG in this system.

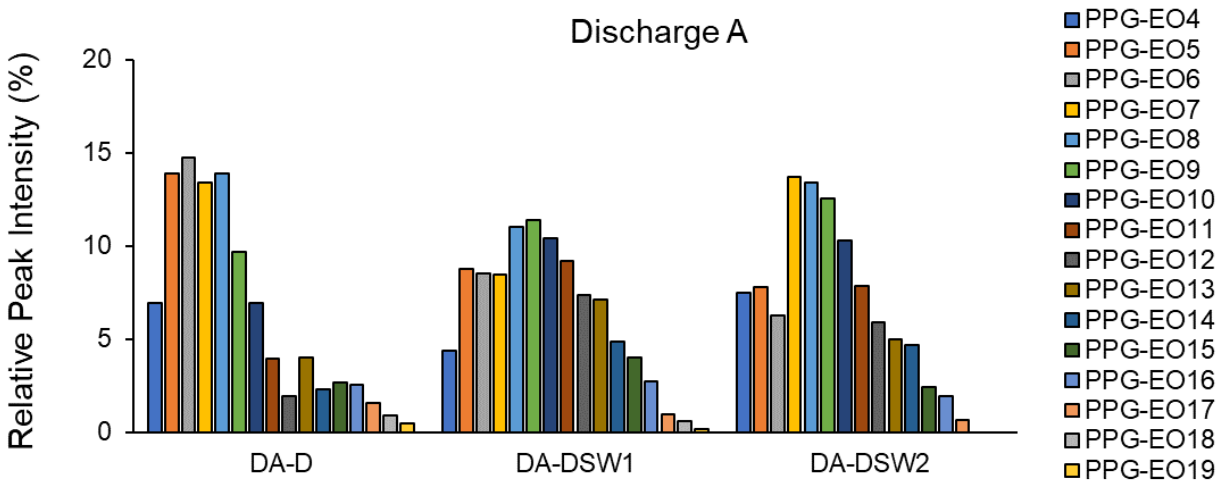

Figure C6. Polypropylene glycol (PPG) relative peak intensity at the discharge (DA-D), downstream of the first wetland (DA-DSW1) and downstream of the second wetland (DA-DSW2) at Discharge A.

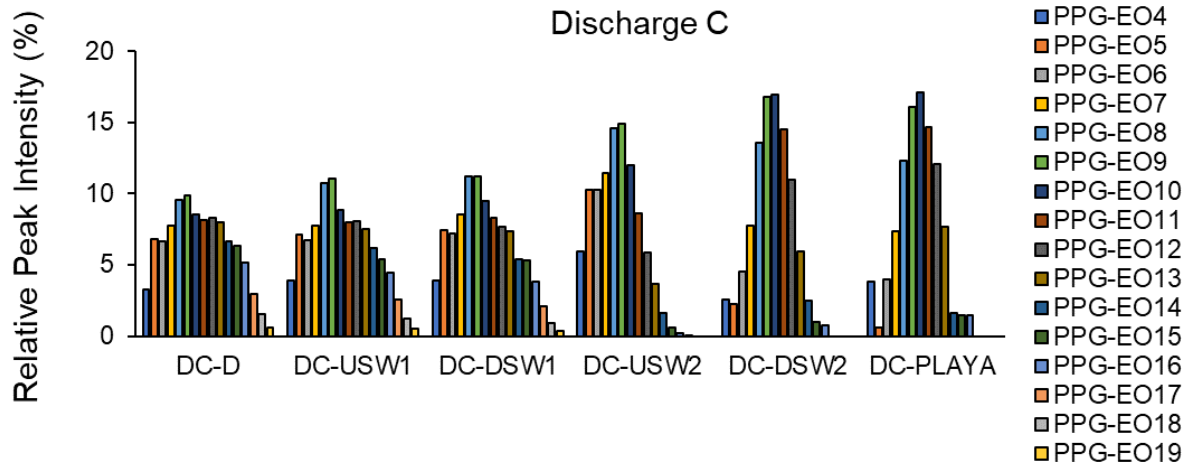

Figure C7. Polypropylene glycol (PPG) relative peak intensity at the discharge (DC-D), upstream of the first wetland (DC-USW1), downstream of the first wetland (DC-DSW1), upstream of the second wetland (DC-USW2), downstream of the second wetland (DC-DSW2), and in the playa lake (DC-PLAYA) at Discharge C.

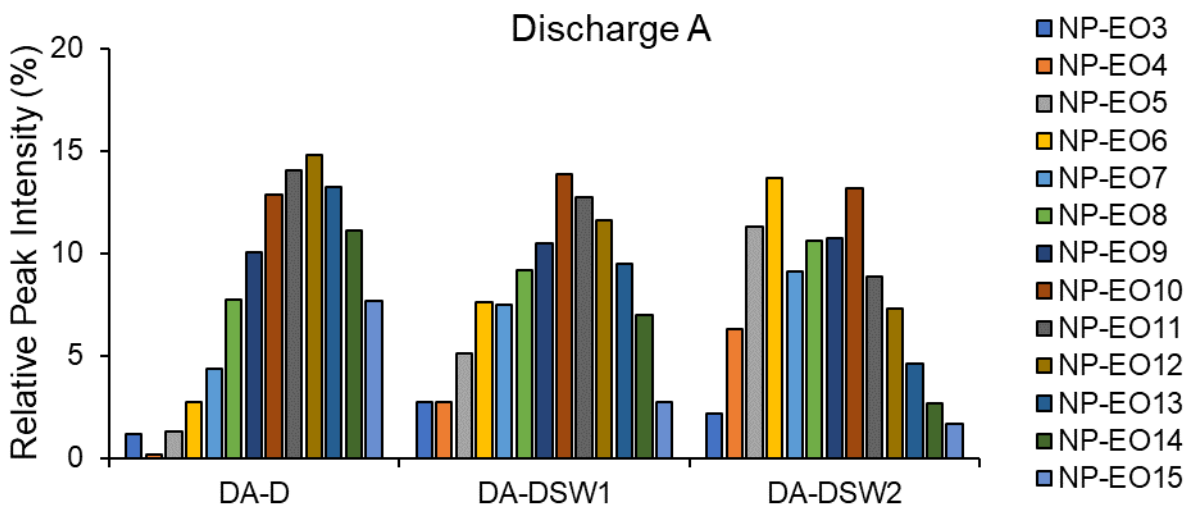

Figure C8. Nonylphenol ethoxylate (NPEO) relative peak intensity at the discharge (DA-D), downstream of the first wetland (DA-DSW1) and downstream of the second wetland (DA-DSW2) at Discharge A. Shorter homologues (EO 3-9) increase with distance downstream and larger homologues (EO 11-15) decrease with distance downstream, indicating biodegradation as a removal mechanism for NPEO in this system.

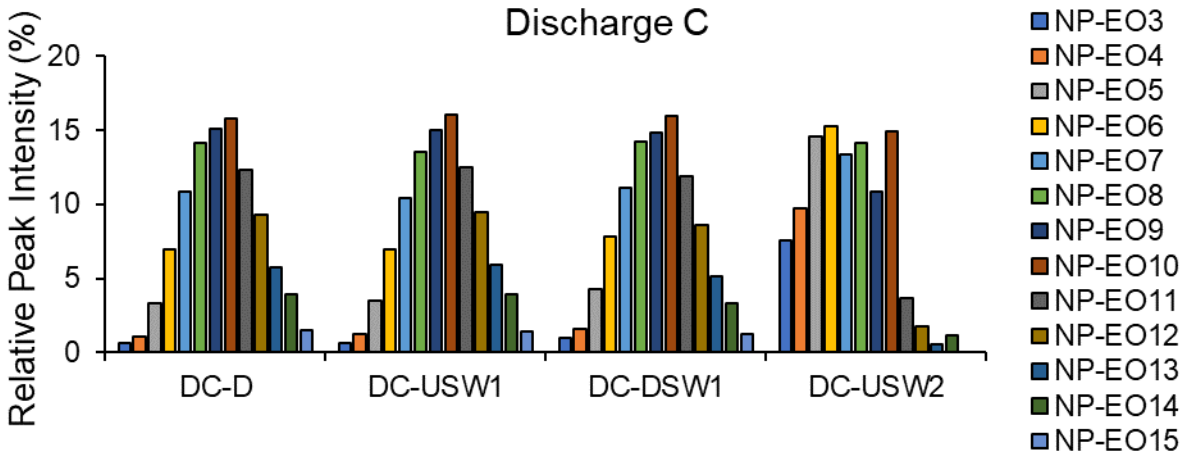

Figure C9. Nonylphenol ethoxylate (NPEO) relative peak intensity at the discharge (DC-D), upstream of the first wetland (DC-USW1), downstream of the first wetland (DC-DSW1), upstream of the second wetland (DC-USW2), downstream of the second wetland (DC-DSW2), and in the playa lake (DC-PLAYA) at Discharge C. Shorter homologues (EO 3-7) increase with distance downstream and larger homologues (EO 11-15) decrease with distance downstream, indicating biodegradation as a removal mechanism for NPEO in this system.

C.2.2.2 Surfactant Distribution in Sediment

Table C2. Average ethoxymer (EO) length for polyethylene glycol (PEG), polypropylene glycol (PPG), nonylphenol ethoxylates (NPEO) and alkyldimethylbenzylammonium chloride (ADBAC) in sediment grab samples collected from the three discharges and the control site wetland (CSW).

Site Name	PEG Avg. EO	PPG Avg. EO	NPEO Avg. EO	ADBAC Avg. EO
CSW	7.8	6.6	Below LOD	Below LOD
Discharge A				
DA-D	9.2	8.5	7.3	11.3
DA-W1	10.0	10.7	10.2	11.4
DA-DW1	9.5	9.0	7.8	10.4
DA-W2	9.5	9.1	8.1	10.7
DA-DW2	7.9	8.5	6.4	10.5
Discharge B				
DB-D	8.5	11.6	10.3	10.2
DB-USW1	8.8	11.0	6.7	11.4
DB-W1	9.0	10.0	8.0	11.2
DB-DSW1	8.8	11.7	8.0	11.2
Discharge C				
DC-D	9.0	11.1	8.1	10.5
DC-100m	9.0	12.5	8.4	10.6
DC-USW1	8.0	9.9	8.1	10.4
DC-W1	8.6	9.0	7.6	10.8
DC-DSW1	8.4	8.5	6.2	11.4
DC-USW2	10.7	9.9	8.2	10.2
DC-W2	9.7	10.0	8.1	10.8
DC-DSW2	9.1	8.7	5.9	11.4
DC-PLAYA	8.9	10.0	8.4	11.4