Using clinical growth-based antimicrobial susceptibility tests as a sensitive indicator of oil sand process-affected water toxicity

by

Kareem Frederick Moghrabi

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in
Physiology, Cell, and Developmental Biology

Department of Biological Sciences
University of Alberta

Abstract

Current approaches for petroleum extraction in northern Alberta oil sands use heat, agitation, chemicals, and water to separate useable bitumen from oil sand deposits. During this process, caustic chemicals and sequestered compounds of environmental concern become concentrated in these waters. These waters are referred to as oil sands process-affected waters (OSPW), and are alkaline, slightly brackish waters that contain several organic and inorganic compounds that have been shown to be detrimental to organism physiology and normal biological function using both *in vivo* and *in vitro* approaches. The deleterious effects of OSPW are often contributed to recalcitrant contaminants such as naphthenic acids (NAs), but there are no clear guidelines regarding acceptable limits for NAs in the environment. As a result, these waters are currently held under a zero-discharge policy, resulting in the accumulation of 1.44 billion m^3 of OSPW as of 2022. As required by provincial legislation, these waters must be returned to a pre-industrial state within 10 years of site closure, prompting significant efforts to remediate OSPW. A variety of treatment regimens that target NAs for removal are currently being tested and will require intensive toxicity monitoring to inform water quality status. As these waters are complex mixtures, the tools used to assess their toxicity and treatment efficacy must be equally varied and sensitive. While *in vivo* assays provide biologically relevant data for affected organisms, *in vitro* approaches allow for rapid, high-throughput screening of water samples to inform treatment decisions and further toxicity testing. Despite their apparent value in guiding both treatment and toxicity testing, relatively few approved *in vitro* methods exist for use in aquatic toxicity assessments. As government and industry seek new toxicity assessment tools to help achieve time sensitive OSPW remediation targets, new approaches must be considered to fill these technical gaps. Bacterial toxicity assays are a candidate for this role, as these

approaches are inexpensive, robust, reproduceable, and high throughput, making them a potentially valuable tool in informing rapid decisions on OSPW treatment, release, and monitoring. In clinical settings, growth inhibition as determined by microdilution minimum inhibitory concentration (MIC) assays have long stood as the gold standard for the assessment of antimicrobial activity against indicator organisms. These approaches are well standardized, widely accepted, and are supported by data showing their effectiveness and sensitivity. As such, I investigated the viability of adapting a microdilution MIC assay for determining the detrimental effects of OSPW and NAs on bacterial growth. Through preliminary optimization, a modified microdilution MIC assay was determined to be stable when exposed to physiochemical parameters seen in freshwater and OSPW. Additionally, this assay demonstrated dose-dependent inhibitory effects on growth as a result of commercial naphthenic acid (cNA) and OSPW exposures, with values comparable to what is observed in the most sensitive *in vivo* and *in vitro* aquatic toxicity assays. To further establish the sensitivity of this approach, a cNA standard and several OSPW samples were treated to remove organic contaminants. Removal of organic compounds was tracked using fluorescence spectroscopy, with chemical analysis data revealing efficient removal of potential contaminants. When assessed using a modified microdilution MIC approach, the reduction in organic compounds was associated with a decrease of inhibitory effects on growth. These approaches were then compared to a standardized bacterial luminescence inhibition assay currently approved for use in aquatic toxicity testing. My data showed that the modified microdilution MIC approach had greater sensitivity to untreated cNA and OSPW, with similar trends observed in terms of monitoring removal and persistence of toxicity in response to treatment. Furthermore, these approaches were resilient against optical interference and several other technical and substrate specific limitations that can perturb

bioluminescence assays. Overall, my thesis data suggests that a modified microdilution MIC assay is a robust, high throughput, cost effective method for monitoring OSPW toxicity. These approaches display sensitivity equivalent to or greater than what is observed in bacterial luminescence inhibition assays but require less sample modification and have exposure times and toxicity endpoints of greater ecological and technical relevance.

Preface

Some of the research conducted for this thesis was done in collaboration with Dr. Mohamed Gamal El-Din at the University of Alberta, Faculty of Engineering - Civil and Environmental Engineering. Treatment procedures, as well as chemical analysis and fluorescence spectroscopy techniques described in chapter 3, 5, and 6 were performed by members of this lab. All other procedures and analysis described in this thesis is the original work of Kareem Frederick Moghrabi. No part of this thesis has been previously published.

This thesis is dedicated to those who will not accept things as they are and strive to make things as they should be. To those who would work to make a sunrise they know they may never see. To those who would dream of a better tomorrow.

Acknowledgments

The work described in this thesis would not be possible without the unconditional support of my family, friends, faculty and lab mates.

I would first like to thank my supervisor, Dr. James Stafford. I am truly fortunate to have you as my supervisor. Your mentorship and guidance have allowed me to excel in this program, but your empathy and understanding saw me through to the end. Through all the challenges, setbacks, and failures experienced during this process, you have shown remarkable support and patience. I'm sure this skill comes naturally to an Oilers fan, but it is appreciated all the same.

I would like to thank my committee member Dr. Patrick Hanington, who has stuck with this project as it bounced through multiple model systems in its infancy. I would also like to thank Dr. Lisa Stein who agreed to provide her wealth of microbiology knowledge as part of my examining committee. A special thanks to several members of Dr. Gamal El-Din's research group. Several aspects of this project would not exist without your contributions, and I thank you for your cooperation.

A sincere thanks to all my lab mates and fellow TAs who made this degree a wonderful experience. All of you have enriched this process in ways I could not have expected and have helped me grow as an individual and a researcher. From the bottom of my heart, thank you.

To my friends who I have shared many great memories with over the last 3 years, I feel forever fortunate to have all of you in my life. My thesis may have been completed faster without you, but I would not have it any other way. Last but certainly not least, I would like to thank my family. Through the highs and lows of this journey, as with all parts of my life, you have loved and supported me. To my family and friends, my gratitude to you is eternal.

Table of Contents

Contents

List of Tables

List of Figures

Chapter I

General Introduction

1.1 Introduction

The introduction of contaminants into the environment as a result of human industrial activity is an ongoing challenge that has consequences that will reach into the distant future. Currently, industries and government prioritize monitoring the presence and impact of these contaminants while preventing their accidental release. As perspectives and priorities shift, efforts are underway to remediate and eventually release previously contaminated substrates upon their successful restoration to a pre-industrial state. A crucial part of this process will involve extensive toxicity monitoring with a wide range of endpoints to ensure the efficacy of remediation and minimize the impact on natural ecosystems.

One such industry that has had a significant impact on the Canadian environment is the petroleum industry. Canada has a proven reserve of 171 billion barrels of oil, with 97% of these located in the Alberta oil sands (Government of Canada 2020). The Alberta oil sands represent the third largest known oil reserve in the world, and the extraction of bitumen from these oil sands has made Canada the fourth largest global producer of crude oil as of 2023 (CAPP 2024a). The petroleum extraction industry built around mining the Alberta oil sands produced an average of ~2.8 million barrels/day of total crude oil in 2023 (AER 2023a) and generated $14.2 billion dollars in royalties, 56% of Alberta's non-renewable resource revenue (Government of Alberta 2023a).

The Alberta oil sands encompass 142,000 km^2 in the Athabasca, Cold Lake, and Peace River boreal forest area (Government of Alberta 2023b). These oil sands are unique from other bitumen deposits as 20% of the reserves exist within 75m of the surface, allowing them to be mined without advanced extraction techniques such as steam assisted gravity drainage (SAGD) or cyclic steam stimulation (CSS) (CAPP 2024b). The viscous petroleum embedded in these mined sand deposits requires careful extraction and treatment before it can be further refined to a variety of useable petroleum products. The most common bitumen extraction processes are derivative of the Clark Hot Water Extraction (CHWE) process, which involves using a combination of steam, heat, agitation, and water flow to separate oil sands into their component parts (Masliyah et al. 2011).

From 2013 to 2022, every barrel of useable crude oil extracted by oil sands mining required on average 2.47 barrels of freshwater. This process has slowly become more efficient, and as of 2022, 80% or 830 million m^3 of the total water used in bitumen extraction from mining was recycled from previous processes (AER 2023c). Despite this, these waters eventually accumulate enough contaminants that they are no longer useable in bitumen extraction. This water is moved to tailings ponds to allow for dense contaminants to settle out of the water and may be chemically or physically treated before or after this process to enhance remediation (Masliyah et al. 2011). The layer of water that forms on top of these settling basins is considered oil sands process-affected water (OSPW), a term which refers to all waters used in the oil sands and may refer to OSPW at various points of remediation and treatment.

While the physiochemistry varies greatly depending on source, both whole OSPW and its specific components have consistently shown detrimental effects on organism physiology *in vivo* as well as interference with normal biological function *in vitro* (Li et al. 2017; Mahaffey and

Dube 2017; Allen 2008a). Due to a lack of provincial regulation and approved remediation options, these waters are held on industrial sites under a zero-discharge policy, leading to the accumulation of a total of 1.44 billion m^3 of fluid tailings as of 2022 (AER 2023b). As production continues to increase to meet global demand, the volume of affected waters will only continue to rise, seeing a 100.3 million m^3 increase in 2022 alone. In response, the Government of Alberta set in place the *Lower Athabasca Region: Tailings Management Framework for Mineable Oil Sands* (TMF) to dictate the reduction, management, and remediation of tailings ponds. Supported under the *Environmental Protection and Enhancement Act* (EPEA)*, Water Act,* and *Oil Sands Conservation Act* (OSCA), this legally binding framework requires sustainable remediation of fluid tailings and mining sites to pre-industrial status within 10 years of closure, as well as monitoring and mitigation of any environmental effects both during and after operation (AER 2023b). As operators attempt to achieve these objectives for closing and current mining projects, extensive efforts have been made to develop new OSPW treatment techniques as well as improve current tools for monitoring OSPW toxicity. *In vitro* approaches have been identified as serving a crucial role in initial determination of treatment efficacy. These models allow for cost-effective determination of treatment success, allowing for investigation with more technically demanding *in vivo* models. When treatment is not determined to be effective as per *in vitro* assays, they can rapidly guide remediation protocols to optimize technologies for toxicant removal (Hatfield Consultants 2019).

1.2 Research Objectives

As these waters are complex mixtures, the tools used to assess their toxicity and treatment efficacy must be equally varied and sensitive. While the Government of Canada has approved approaches for assessing aquatic whole effluent toxicity (WET), both industry and

government have identified a need for more rapid, sensitive methodology to identify and prioritize contaminants of concern (Government of Canada 2024; Viegas et al. 2021). *In vivo* toxicity approaches are, and will likely remain, the most ecologically significant methods that provide insight on biologically relevant organisms and systems. Alternatively, *in vitro* approaches allow for rapid, high-throughput, cost-effective testing that can have strong predictive accuracy for whole organism toxicity and allows for rapid decision making in treatment and monitoring scenarios.

Despite the strengths of *in vitro* testing, only two of the nine aquatic toxicity approaches approved by the Government of Canada for WET testing are *in vitro* approaches. Microalgae have shown high sensitivity to whole OSPW but are technically demanding to culture and enumerate, making their use limited. Alternatively, the bacterial luminescence inhibition approaches are the most common *in vitro* aquatic toxicity approach used to assess OSPW and its constituents. Despite a low technical burden, this assay relies on outdated protocols that have several technical limitations associated with whole effluents and OSPW in particular. Though these assays have limitations, bacteria still have several technical advantages for toxicity testing as these approaches are inexpensive, reproducible, and high throughput. Outside of environmental settings, distinct guidelines exist for determining the susceptibility of bacteria to antimicrobial compounds. With this considered, the objective of this thesis was to explore the possibility of adapting clinical antimicrobial assessment tools for use in measuring OSPW toxicity. To achieve this, I sought to; 1) establish a modified minimum inhibitory concentration (MIC)/broth microdilution assay for use in freshwater and OSPW toxicity settings; 2) perform treatment of OSPW to remove compounds of concern and compare chemical analysis data with

toxicity endpoints in a modified MIC assay; and 3) compare chemical analysis data and MIC data to a traditional bacterial luminescence inhibition assay.

Chapter II

Literature Review

2.1 OSPW Composition

Oil sands process-affected waters can vary greatly in origin and treatment, and this variability is reflected in their water chemistry. Overall, OSPW is an alkaline, moderately brackish water that contains several key components that contribute to both acute and chronic toxicity. Typically, these waters are comprised of 70-80% water, 20-30% solids such as sand, silt, and clay, and 1-3% residual bitumen (Allen 2008a). When analyzing OSPW composition, it is common to separate the complex mixture into organic and inorganic fractions (OF and IF, respectively). The OF includes a broad range of organic compounds including, benzene, toluene, ethylbenzene, xylene (BTEX), polycyclic aromatic compounds (PACs), phenols, and naphthenic acids (NAs) (Hussain and Stafford 2023). A generally less understood but better characterized component of OSPW, the IF is mostly comprised of inorganic ions, namely calcium, magnesium, sodium, chloride, sulfate, and bicarbonate, but also includes a selection of trace metals of environmental and toxicological significance (Allen 2008a).

2.1.1 Organic Fraction - BTEX

A group of volatile organic compounds, BTEX refers to benzene, a monoaromatic hydrocarbon, as well as its alkyl derivatives. In OSPW, BTEX compounds are introduced with industrial solvents used to improve bitumen yield by reducing oil viscosity (Government of Canda 2023). BTEX compounds are often assessed as a group producing lethal concentration 50% (LC_{50}) values ranging from 15 – 37 mg/L, 21 mg/L, 37.8 mg/L in *Daphnia*, *Artemia*, and *Chironomus* sp., respectively (EPA 2024; Li et al. 2013). In *Pimephales promelas*, LC_{50} values

were observed as low as 5 mg/L, with endocrine and cardiac developmental defects being seen in several teleost fish as low as 4 µg/L (Headley et al. 2001; Xu et al. 2020). Notably, the combined effect of these compounds increases toxicity significantly when compared to their effects in isolation. The proposed mechanism of action for BTEX toxicity is believed to be non-selective membrane disruption, or narcosis, due to the highly lipophilic nature of its chemical structure, with effects decreasing with alkylation and molecular weight within this group (Sikkema et al. 1995; Xu et al. 2020).

Though not as extensively evaluated as other parts of the OF, BTEX compounds have been observed at concentrations ranging from 10-21 mg/L in OSPW, concentrations that far exceed Federal water quality guidelines (Stasik et al. 2015). Though much lower in concentration, BTEX compounds have also been detected in surface waters of the Athabasca region impacted by the oil sands in concentrations ranging from 3-20µg/L, suggesting their potential spread from industrial activity (ECCC 2023). Chemical properties of BTEX often lead to it persisting in the environment for long periods of time, also being able to outgas from surface water and spread to the air and neighboring environments (Weelink et al. 2010).

2.1.2 – Organic Fraction - PAC

Another important component of the OF are polycyclic aromatic compounds. This large group includes thousands of compounds from two major groups: heterocyclic aromatic compounds (NSO-PAC) and polycyclic aromatic hydrocarbons (PAH). The NSO-PAC compounds contain nitrogen, sulfur, and oxygen substitutes in the ring structures, while PAHs are comprised of only carbon and hydrogen (Achten and Andersson 2015). Though distinct, these groups of compounds are similar in both behaviour and analysis, with PAHs being significantly better researched due to their association with anthropogenic activity (Blumer 1976). Some PACs

may be produced naturally though pyrogenic events such as volcanic activity and forest fires, but large volumes of these compounds are petrogenic, existing in complex mixtures of crude oil and coal. During bitumen extraction from the oil sands, large volumes of PACs, predominantly PAHs, are released into process waters and can be found in OSPW (Ahad et al. 2020).

Like BTEX, PACs have been established as potentially carcinogenic to humans and have several adverse outcomes for exposed aquatic organisms (Menzie et al. 1992). While specific outcomes and concentrations are dependent on the specific PAC tested, high molecular weight compounds tend to exert greater effects than low molecular weight PACs (Geier et al. 2018). In teleost fish, PAHs compounds showed detrimental effects on cardiac and morphological development in concentrations as low as 0.7 µg/L, with further negative effects on liver and bone metabolism, as well as endocrine disruption observed in adult fish (Honda and Suzuki 2020). In aquatic invertebrates, LC_{50} values were dependentdependent on specific PAH compound, ranging from 0.004 – 41 mg/L, 8 – 20 µg/L, and 250 – 490 µg/L for *Daphnia*, *Artemia* *Chironomus* sp., respectively (Leblanc 1980, Kagan et al 1985, Suedel and Rogers 1996; Millemann et al. 1984). The proposed mechanism of action for toxicity of PACs is narcosis (Di Toro et al. 2020), with observed toxicity increasing with the molecular weight, alkylation, and lipophilic characteristics of each compound (Achten and Andersson 2015; Sikkema et al. 1995).

Concentrations of PACs in OSPW can range from 1.1 – 280 µg/L (Madill et al. 1999; Zhang et al. 2015), with several specific compounds exceeding Federal water quality guidelines (CCME 1999). Regionally, PACs have been detected at significantly higher concentrations in the Athabasca River near oil sands extraction and tailings facilities. Some of these values exceed Federal water quality guidelines but can range greatly with seasonality (Kelly et al. 2009). PACs are significantly less volatile than BTEX compounds, and thus present a lower risk for

mobilization in the environment. Despite this, PACs are a persistent contaminant that is resistant to efficient degradation under ambient conditions and contribute to OSPW toxicity (Wallace et al. 2020).

2.1.3 – Organic Fraction - NA

A major contributor to the proposed toxicity of the OSPW organic fraction, NAs are a large family of acyclic and cyclic carboxylic acids with the general classical formula $C_nH_{2n+Z}O_2$ where *n* indicates the number of carbon and *Z* indicates the presence and number of ring structures. Outside of the classical formula, atypical NAs have also been described with the formula $C_nH_{2n+Z}O_x$, where *x* can range from 2 – 6 (Vander Meulen 2021). Both classical and atypical NAs have a high degree of variation in their alkyl chains, which in combination with variable ring structures, produce a wide range of molecular weights and conformations (Brown and Ulrich 2015). Naturally existing in petroleum deposits, NAs become solubilized during extraction of bitumen and contaminate OSPW (Mahaffey and Dube 2017). NAs are an undesirable by-product of bitumen extraction, reducing efficiency and potentially damaging equipment, ultimately requiring increased cycling of water (Scott 2007). While BTEX and PACs may be a product of several other industrial processes and activity, NAs are unique to petroleum extraction and refinement as they can be found naturally within bituminous deposits in the oil sands.

NAs were originally identified as a crucial component of OSPW mixtures that contributed to acute and chronic toxicity outcomes for aquatic organisms (MacKinnon and Boerger 1986, Mahaffey and Dube 2017). NAs extracted from OSPW have been shown to be acutely toxic to fish, resulting in an effective concentration 50% (EC_{50}) of 5.0 – 12.4 mg/L for *Pimephales promelas* embryos, and concentrations of 5.0 mg/L and lower producing several

developmental deformities related to cardiovascular and spinal structure (Marentette et al. 2015). In aquatic invertebrates, LC_{50} values have been observed at 1.9 – 3.8 mg/L, 2.8 – 6.1 mg/L, and 2.3 –10.9 mg/L for *Daphnia, Hyalella,* and *Chironomus* sp., respectively (Kinley et al. 2016).

Like BTEX and PAC compounds, narcosis is proposed to be the mechanism of toxicity for NAs due to their lipophilic characteristics. Compared to other compounds discussed, the relationship between structure and toxicity for NAs is complicated. Generally, low molecular weight NAs have a stronger association with toxicity than high molecular weight NAs (Frank et al. 2008; Holowenko et al. 2002). However, for similarly structured NAs, as molecular weight increases toxicity increases, but structural differences such as alkyl groups may have a greater influence on toxicity overall (Frank et al. 2008; Brown and Ulrich 2015). A large degree of the observed toxicity in OSPW has been associated specifically with O_2 NAs, making this group a key focal point for treatment and chemical analysis (Hughes et al. 2017).

Despite large degrees of structural variation, concentrations of NAs in OSPW are well documented. Total NA concentrations in OSPW often exist within the range of 2.9 – 100 mg/L (Allen 2008a, Mahaffey and Dube 2017; Hussain and Stafford 2023), though unlike BTEX and PACs, no guidelines currently exist to detail acceptable environmental limits of NAs. This is partially due to challenges associated with characterization of the large group of compounds, as well as high variation in structure specific toxicity (Brown and Ulrich 2015; Vander Meulen 2021). In fact, for NAs, concentration is often a poor predictor of toxicity, with structure having a greater impact on toxicity outcomes (Bauer et al. 2019a, Brown and Ulrich 2015). In regions surrounding the oil sands, NA concentrations were measured at values up to 10 mg/L, values which far exceed the 4 ng/L-2 mg/L measured in areas not affected by bitumen extraction (Ross et al. 2012; Sun et al. 2017; ECCC 2024a). As with other components of the OF, their toxicity is

complicated by their persistence in the environment and resistance to timely degradation via natural processes, posing a major risk to surrounding aquatic environments and groundwater (Ross et al. 2012).

2.1.4 – Organic Fraction – Summary

Overall, the OSPW OF represents a large group of compounds that are challenging to remediate and significantly contribute to OSPW toxicity. The OF presents a major barrier for remediation for two major reasons: persistence and analysis. Components of the OF often represent compounds that are not easily removed by natural process under ambient conditions and can readily spread to surrounding environments (Zhang et al. 2015; Weelink et al. 2010). The OF also encompasses a massive number of compounds that require advanced analysis techniques to track, each with their own advantages and trade-offs. The variation of analysis techniques employed makes it difficult to consistently quantify and evaluate the presence of these problematic compounds, not to mention the cost required for thorough analysis (Han et al. 2008). These combined factors have made it difficult for regulators to provide clear guidelines on OF, and specifically NA management and removal, resulting in a lack of policy.

Narcosis remains the mode of toxic action associated with all components of the OF, largely due to the strong relationship between molecule hydrophobicity/lipophilicity and toxicity (Frank et al. 2009). Despite this relationship, complex interactions with whole organism endocrine, cardiac, and immune systems suggests this may be an oversimplification (Wallace et al. 2020; Incardona 2017). For many components of the OF, concentration can be a poor predictor of toxicity, with individual chemical structural motifs contributing more to toxic outcomes than concentration alone (Jones et al. 2011). To fully understand the toxicity mediated

by these fractions, it will likely require analysis of individual chemical species and the results of their exposure both *in vitro* and *in vivo* (Brown and Ulrich 2015; Frank et al. 2008).

2.1.5 – Inorganic Fraction – Inorganic Ions

The inorganic ions present in OSPW contribute to the water being moderately hard, with average calcium and magnesium concentrations of 2 – 25 mg/L and 5 – 11 mg/L, respectively. OSPW also has concentrations of total carbonate species that range between 575 – 1025 mg/L, resulting in pH values of 7.5 – 8.6 (Celsie et al. 2016). In extreme cases, these values may exceed what is traditionally observed in the Athabasca River and regional lakes, though on average are similar (Allen 2008a, Mahaffey and Dube 2017; ECCC 2024a).

Another important measurement of inorganic water chemistry is total dissolved solids (TDS), which measures inorganic salts and small organic mater present in water. In OSPW, TDS values can range greatly depending on source but are generally between 400 – 2539mg/L, far more than what is observed in regional freshwater and rivers (Allen 2008a, Mahaffey and Dube 2017; ECCC 2024a). In OSPW the contribution of salts to TDS is generally quite high, with combined sodium, chloride, and sulfate values totalling ~2600mg/L in some samples, resulting in OSPW being considered brackish (Celsie et al. 2016). Conductivity and TDS increase as salinity increases, making these a good indicator of salinity in the absence of specific ion analysis (Rusydi et al. 2018). The conductivity, salinity, and TDS of OSPW greatly exceed what is observed in regional freshwater, posing a risk to several freshwater organism such as *Daphnia, Chironomus,* and *Pimephales* sp. (Bright and Meier 2007; Hassel et al. 2006; Schuytema et al. 1996). Analysis of groundwater and surface water in oil sands affected regions suggest potential infiltration from OSPW sources increasing parameters such as TDS and salinity (Roy et al. 2016). In addition to environmental concerns, the accumulation of inorganic ions and salinity

reduces the viability of waters for use in further bitumen extraction, increasing freshwater consumption (Allen 2008a).

2.1.6 – Inorganic Fraction – Trace metals

Several trace metals of toxicological concern are frequently observed in OSPW as part of the inorganic fraction. These trace metals are often naturally enriched in bitumen deposits and concentrated in OSPW, similar to what occurs for NAs (Hussain and Stafford 2023). Nickel, boron, copper, lead, and arsenic can be found in concentrations over 2.8 mg/L, 3.8 mg/L, 0.9 mg/L, 0.19 mg/L, and 0.015 mg/L, respectively (Allen 2008a), far exceeding water quality guidelines for protection of aquatic life (CCME 1999). In addition to acute and chronic toxicity in exposed organisms, trace metals are unique from components of the OF as they have a high propensity for bioaccumulation (Scott et al. 2020). This means lower-trophic organisms may uptake these compounds and concentrate them, as well as spreading them to other environments and higher-trophic organisms in greater quantities (Rainbow 2007). Trace metals are also generally resistant to removal through natural chemical processes, and pose a strong potential to infiltrate groundwater in areas surrounding tailings ponds (Holden et al. 2013).

2.1.7 – Inorganic Fraction – Summary

Overall, the IF is an important though significantly less understood component of whole OSPW. When tested in isolation, the IF had metabolic and immune modulating effects *in vitro*, but little toxicity testing has been done beyond this (Phillips et al 2020; Qin et al. 2019a). Interestingly, components of the IF may modulate and enhance the toxicity of one another, suggesting the importance of analyzing this fraction as a whole, rather than only investigating its component parts (Celise et al. 2016; Puttaswamy et al. 2012). Despite this, the IF is rarely

analyzed as a fraction and rather attention is given to its component parts. This is likely due to easier chemical analysis of a smaller total range of components in the IF, as well as more challenging extraction techniques compared to acid extraction of the OF (Bauer et al. 2019b). In addition to complex interactions within the fraction, the IF has been shown to contribute to increased toxicity in whole OSPW, suggesting that these fractions have independent, but cumulative effects on toxicity (Qin et al. 2019a, Phillips et al. 2020).

2.1.8 – OSPW Composition Summary

OSPW is a complex mixture with several components that make it of ecotoxicological concern. Often divided into OF and IF to better analyze constituents of concern, each fraction of OSPW has unique environmental considerations. The OF encompasses a massive body of compounds that, while difficult to characterize, are well studied and have strong associations with toxicity outcomes. Though analytical techniques allow for precise characterization, the IF contribution to toxicity remains less clear. Both fractions contain individual components that exceed environmental guidelines, where they exist, and are of significant ecological and health concerns. In whole OSPW, the effects of both fractions could interact chemically and biologically in complex ways that make toxicity difficult to predict. It is crucial to understand OSPW composition to both choose effective tools to monitor toxicity, as well as effectively plan remediation strategies to address components of concern. Understanding individual component toxicity emphasizes the need for precise chemical analysis approaches that allow for a better understanding of resulting whole OSPW toxicity.

2.2 OSPW Toxicity

While the previous section indicates specific components of concern in both the IF and OF, it is important to evaluate toxicity of whole OSPW in addition to individual extracted constituents. As with composition, toxicity for whole OSPW is highly variable and source dependentdependent. Several methods have been implemented to understand the toxicity of whole OSPW and can be generally divided into *in vivo* and *in vitro* testing. From a regulatory perspective, 7 of the methods approved by the Federal government for measuring OSPW toxicity are *in vivo*, while only 2 are *in vitro* (Government of Canada 2024). Both methodologies provide unique insights into OSPW toxicity, as well as having different sensitivities.

2.2.1 – In vivo Tests

Though expensive and more technically demanding, *in vivo* models stand to provide the most ecologically relevant understanding of the effects of OSPW exposure. These assays have numerous endpoints that provide valuable insight on the effects of OSPW exposure on organism physiology and use a broad range of approaches in mammals, birds, fish, and invertebrates. From designing environmental guidelines to approving treatment approaches, *in vivo* outcomes often have, and likely will, serve as the final checkpoint for regulation.

2.2.2 – In vivo Testing – Mammals

Testing of OSPW exposure in mammals has generally been limited to mice and rats and have shown a variety of effects on organism physiology. In rats, oral high dosage exposures for 14 days produced severe complications such as brain hemorrhaging and cardiac defects while longer sub-chronic exposures at lower doses had effects on liver weight and function (Rogers et al. 2002). Oral exposure of mice for 7 – 14 days resulted in minimal effects on body weight, but downregulated pro-inflammatory gene expression (Garcia-Garcia et al. 2011a; Garcia-Garcia et

al. 2012). Longer exposures between 28 – 56 days had significant effects on pro-inflammatory gene expression and immune function, but this could be suppressive or stimulatory depending on the cytokine and function investigated (Garcia-Garcia et al. 2011b; Garcia-Garcia et al. 2012). Exposure of pregnant mice for 14 – 42 days produced no significant affects on pregnancy or lactation, though exposure doses were lower at 55 mg/kg/week compared to some of the higher doses discussed above (1500-100 mg/kg/week) (Li et al. 2019). Notably, most *in vivo* exposures in mammals are done using the acid extracted OF from OSPW as opposed to whole OSPW.

2.2.3 – In vivo Testing – Birds

Much of the research surrounding the effects of OSPW on birds has been done *in situ*, exploring the effects of OSPW being added to artificial wetlands on mine sites. This work is done on tree swallows (*Tachycineta bicolour*) that naturally inhabit the region and consume mostly aquatic insects (Li et al. 2017). Reproductive performance, fledgling size, and hatching success were all negatively impacted on birds nesting in the region, but effects on metabolism and immune function appeared limited (Gentes et al. 2006; Gentes et al. 2007a). It was noted that environmental conditions such as rain and wind may have influenced these outcomes as well as exposure to compounds of concern. Notably, exposure of tree swallow hatchlings to low concentrations of NAs for 7 – 13 days produced no measurable effect on growth or biochemistry, further emphasizing the variability in fractional toxicity compared to whole OSPW (Gentes et al. 2007b).

2.2.4 – In vivo Testing – Fish

The effects of OSPW in fish have been extensively explored due to their ecological relevance in the environment and their defined guidelines as important toxicity risk assessment

checkpoints. Rainbow trout and fathead minnows (*Oncorhynchus mykiss* and *Pimephales promelas*, respectively) comprise the majority of OSPW exposure research in fish, likely due to their establishment as standardized approaches for assessing WET as per the Federal government (Government of Canada 2024). In *Oncorhynchus mykiss*, exposure to whole OSPW for 96 hours produced LC_{50} values ranging from 3-35% (v:v) (MacKinnon 1981, MacKinnon and Boerger 1986, Zubot et al. 2012). In addition to lethality, olfactory sensitivity and associated behaviour responses were impaired at 2% (v:v) OSPW exposure, with concentrations below this threshold resulting in avoidance behaviour from the fish (Lari et al. 2019; Lari and Pyle 2017). For *Pimephales promelas*, 96-hour exposure resulted in low mortality but had an inhibitory effect on immune activity, with longer 21-day exposures negatively affecting fecundity, spawn rates, and endocrine function (Bauer et al. 2019a, Farrell et al. 2004; Kavanagh et al. 2011). Similarly, mortality was low in goldfish and walleye (*Carassius auratus* and *Sander vitreus*, respectively), though significant upregulation of organism immune function and oxidative stress was observed (Marentette et al. 2017; Hagen et al. 2013).

2.2.5 – In vivo Testing – Invertebrates

Like fish, a selection of invertebrates that serve as sentinel species are frequently used to assess OSPW toxicity, as indicated in Federal WET guidelines (Government of Canada 2024). Water fleas, non-biting midges, and scuds (*Daphnia spp.*, *Chironomus spp*., and *Hyalella azteca*, respectively) all serve as highly sensitive organisms that are primarily aquatic and easier to test than larger organisms. A 96-hour exposure of whole OSPW produced LC_{50} values ranging from 2 – 100% (v:v) for *Daphnia pulex* and *Daphnia magna*, with effects on feeding, movement, and chemosensory function being inhibited with exposures as low as 1% (v:v) (MacKinnon and Boerger 1986, Zubot et al. 2012; Lari et al. 2016; Bauer et al. 2019a, Bauer et al. 2019b).

In *Chironomus spp.*, survival and growth of the organism ranged from 100 – 65% and 100 – 80%, respectively, depending on the length of whole OSPW exposure (Anderson et al. 2012). Additional effects on reproductive success were also observed on exposed organisms, as well as reductions in oxidative stress response (Wiseman et al. 2013; Anderson et al. 2012).

Potentially the most sensitive of the three invertebrates to OSPW, *Hyalella azteca* is very sensitive to inorganic and organic fractions, as well as whole OSPW (Bauer et al. 2022). *Hyalella azteca* saw a 66% loss in viability when exposed to whole OSPW that similarly produced no response in *Daphnia, Chironomus* and *Pimephales spp.* when tested under Federal WET guidelines (Bauer et al. 2019a, Bauer et al. 2022). When exposed to whole OSPW, *Hyalella* showed LC_{50} values 4 times lower than *Aliivibrio fischeri*, a bacterium that is largely considered one of the most sensitive *in vitro* indicator assays available. To NAs specifically, LC_{50} values were as low 0.7%, suggesting this organism has strong potential to sensitively detect toxicity from both the OF and IF (Bartlett et al. 2017). *Hyalella* has also served as a valuable tool to investigate the IF of OSPW, allowing for monitoring of uptake and metabolism of trace metals due to a high level of sensitivity and ease of analysis (Jensen-Fontaine et al. 2014).

2.2.6 – In vivo Tests – Summary

While most *in vivo* testing is done with the organisms detailed above, the use of additional genus and species only serves to further our understanding OSPW toxicity. Exposures in amphibians, mussels, and plants mimic what is seen in other organisms, with effects ranging from mortality to physiological and developmental defects (Robinson et al. 2023; Bartlett et al. 2017; Pouliot et al. 2012). Recently, the northern leopard frog (*Lithobates pipiens*) was approved by the Federal government for use in WET testing. This represents a larger effort to understand the complex effects of OSPW exposure on whole organism physiology, as well as understand the

impact of these substrates on organisms of regional ecological relevance (ECCC 2024b). Though effective for providing high resolution toxicity data, protocols for *in vivo* assessment have high technical burdens, long exposure times, and are generally quite costly (Borghesan et al. 2007). Challenges with *in vivo* approaches are amplified when tested *in situ*, where confounding variables and stochastic factors can greatly influence outcomes (Gentes et al. 2007b; Li et al. 2017).

2.2.7 – In vitro Tests

In comparison to *in vivo* testing, *in vitro* testing is generally less technically demanding, inexpensive, and has high reproducibility (Li et al. 2017; Lillico et al. 2023). In addition to these advantages, *in vitro* methods fulfill the criteria of the 3Rs. Established in 1959 and refined in 1992, these principles by Russel and Burch seek to refine, reduce, and eventually replace animal testing for ethical reasons (Archibald 2018; NRC 2011; Russell et al. 1992). The development of new *in vitro* approach methodology and predictive modeling tools has allowed for a significant reduction in the use of animal models (Scarlett et al. 2012; Frank et al. 2009). Given the number of potential compounds of concern in OSPW, *in vivo* testing will likely not be feasible for rapid screening or point detection of contaminants (Islam et al. 2014; De et al. 2022) As such, *in vitro* testing and predictive toxicity will be an important element of plans for effective remediation and monitoring of OSPW (Redman et al. 2018).

2.2.8 – In vitro Testing – Eukaryotic Approaches

Generally, commonly employed *in vitro* methods for assessing OSPW toxicity can be divided by those that use eukaryotic or prokaryotic cells and organisms. A common eukaryotic approach is to use immortalized cell lines from mammals and fish to interrogate the effects of

exposure on biological function (Li et al. 2017). Beyond immortalized cells, eukaryotic organisms such as algae and yeast are also used, with algae being of ecological relevance depending on species chosen (Barrow et al 2023; Debenest et al. 2012).

Exposure of immune cells to OSPW allows for multiple levels of resolution by evaluating effects ranging from cytotoxicity to transcriptional changes and production of functional proteins such as cytokines (Lillico et al. 2023; Qin et al. 2019a). When measured using flow cytometry, extracted OF from OSPW caused a 40% reduction in viable cells at a concentration of 14 mg/L O_2 NAs. Interestingly, whole OSPW diluted to produce the same concentration of O_2 NAs did not have a significant effect on cell viability, suggesting complex and potentially antagonist effects of fractions on whole OSPW toxicity (Qin et al. 2019a). Viability and cellular metabolism as measured by the 3-(4,5-Dimethylthiazolyl-2)-2,5 Diphenyltetrazolium bromide (MTT) assay showed greater sensitivity as low as 2 mg/L for acid extracted O_2 NAs from the OF from OSPW, though similar sensitivity was not observed in whole OSPW (Qin et al. 2019a).

Additional sensitivity can be achieved from immune cells by measuring the release of functional proteins and identifying transcriptional changes in response to OSPW exposure. Sensitivity to both fractionated and whole OSPW can be determined by measuring the production of inducible nitric oxide synthase (iNOS), a potent pro-inflammatory protein. Sensitivity as measured by iNOS production allowed for detection of whole OSPW at concentrations as low as 0.4% (v:v), as well as differentiation between OSPW sources and fractions (Lillico et al. 2023). Similar, if not greater sensitivity is achieved by analyzing transcriptional changes and cytokine release in immune cells upon exposure to fractionated and whole OSPW. Pro-inflammatory cytokines such as Interleukin-1 beta (IL-1β) and Tumour Necrosis Factor alpha (TNF-α) serve as strong indicators of immune cell activation, and

significantly increase upon cell exposure to OSPW (Paul et al. 2023). This sensitivity is further increased when investigating the transcriptome, as OSPW exposure causes increases in numerous pro-inflammatory and stress associated genes (Paul et al. 2023).

Rainbow trout gill and liver cells are also frequently used to examine the effects of OSPW exposure on biological function, though cells from fathead minnows and goldfish are also used. Like what is described for mammalian cells, exposure to both whole OSPW and the OF significantly reduced cell viability in gill and liver cells, though exposure to the OF has a larger impact (Sansom et al. 2013). Similarly, sublethal exposures in liver cells showed significant impacts of OSPW and OF exposure on metabolic and endocrine transcriptional profiles (Gagne et al. 2013). The collective findings of immortalized cell work suggest that narcosis may not be the only process that mediates OSPW toxicity, as many of these exposures are performed at sub-lethal concentrations and may potentially be receptor mediated (Sansom et al. 2012; Gagne et al. 2013; Lillico et al. 2023; Paul et al. 2023). Though not an approved approach, these assays are sensitive, rapid, and highly reproducible, providing critical insight into OSPW toxicity.

The freshwater microalga *Raphidocelis subcapitata* is commonly used and has ecological relevance as a major primary producer in aquatic ecosystems (Debenest et al 2012). Due to its relevance in aquatic ecosystems, the use of *R. subcapitata* in toxicity testing was established as one of two approved *in* vitro WET models. These assays rely on growth inhibition, and have a 72-hour exposure time, longer than many *in vitro* approaches (Government of Canada 2024). *Raphidocelis subcapitata* has shown sensitivity to whole OSPW at concentrations as low as 1.9% (v:v), and are sensitive to OSPW extracted NAs at concentrations of 15 mg/L (Debenest et al. 2012; Swigert et al 2015). Numerous other algae species have been used, though sensitivity to NAs ranged from 14 mg/L – 1000 mg/L depending on the species tested (Beddow et al. 2016;

Woodworth et al. 2012). Some species were entirely resistant to the NAs even with 14 – day exposures at concentrations exceeding 1000 mg/L, suggesting a potential for these organisms in remediation uses rather than toxicity screening (Woodworth et al. 2012). Despite these advantages, algae stocks respond poorly to freezing, requiring persistent culturing with slow growth, making these approaches technically demanding (Government of Canada 2004b).

With *in vivo* testing suggesting potential endocrine disruptive properties of OSPW, the yeast androgen/estrogen screening (YA/ES) tests using *Saccharomyces cerevisiae* have been used for OSPW screening. These approaches use genetically modified yeast with human estrogen and androgen receptors and a reporter gene, the product of its expression leading to a colour change in medium. When used to test OF extracted from whole OSPW, the YE/AS assay showed effective concentration 50% (EC_{50}) as low as 0.5 – 16mg/L (Leclair et al. 2015). Despite this, when used to assess whole OSPW, receptor activation is similar to what is seen from regional freshwater, though some seasonal peaks were noted (Barrow et al. 2023; Gault et al. 2023). Though useful, the YE/AS assay is not frequently used for OSPW toxicity testing, and is susceptible to receptor binding interference, a major concern given the complexity of OSPW as a substrate (Barrow et al. 2023).

2.2.9 – In vitro Testing – Prokaryotic Approaches

While prokaryotes represent the smallest organism discussed so far for testing, they also represent one of the most commonly used approaches for freshwater toxicity. Bacterial luminescence inhibition approaches use the symbiotic marine bacterium *A. fischeri*, which produces light when present in sufficient density under normal metabolic conditions. Briefly, the production of light is ultimately regulated by quorum sensing, a form of chemical intercellular communication used to coordinate bacterial population and community behaviours such as

biofilm formation, pathogenesis, or the production of antimicrobial compounds (Miyashiro and Ruby 2012). As the bacterium grows, the *luxI* gene regulates synthesis of an autoinducer called *N*-3-oxohexanoyl-homoserine lactone (3-oxo-C6 HSL), which accumulates as part of normal metabolic activity. This compound can freely diffuse from the cell, requiring sufficient concentration of this compound to accumulate to reach critical concentrations required to bind to LuxR (Schaefer et al. 1996). Once LuxR has been bound, it activates transcription of the *luxICDABEG* operon that encodes for various components of the bioluminescence reaction. The *luxAB* gene encodes for luciferase, the enzyme required to produce luminescence while *luxCDE* synthesizes a crucial aldehyde substrate used in this reaction (Miyamoto et al. 2000). In the presence of oxygen, luciferase releases light as part of the reaction between aldehyde compounds and reduced flavin mononucleotide ($FMNH_2$) (Brodl et al. 2018; Miyamoto et al. 2012). Disruption of metabolism in the bacteria, or reduction in density of active cells prevents autoinducer accumulation and thus inhibits luminescence emission. For the purposes of toxicity testing this allows for sensitive, though indirect detection of bacterial density and metabolic function using photometry. The use of lyophilized organisms facilitates easy on-site testing of toxicity, with the short 30-minute exposure time allowing for rapid screening (Environment Canada 1992).

Aliivibrio fischeri has been used extensively for the assessment of both fractionated and whole OSPW. For whole OSPW, inhibitory concentrations 50% (IC_{50}) ranged from 32% – 100% depending on OSPW, with extracted OFs having IC_{50} values ranging from 1.2% – 83.9% (Frank et al. 2008; Scarlett et al. 2012; Jones et al. 2011; Scott et al. 2008; Holowenko et al. 2002). When exposed alongside *Daphnia, Chironomus, Hyalella* and *Pimephales spp*. to the same substrate, sensitivity of *A. fischeri* was at best equivalent, if not lower (Bartlett et al. 2017; Frank

et al. 2008; Bauer et al. 2019). The relatively low technical burden makes this organism an ideal choice when doing high-throughput screening, and as such, it is often used to characterize toxicity of large groups of chemicals such as NAs and PACs. When paired with chemical analysis, this organism has accounted for most of the known relationships between toxicity and structure, molecular weight, and lipophilicity for NA species (Holowenko et al. 2002; Jones et al. 2011). In toxicity assessment of OSPW and its treatment, most experiments use *A. fischeri* as the only toxicity endpoint, severely limiting the biological and ecological relevance of these findings (Mahaffey and Dube 2017). While still useful as an initial screening tool, the short exposure time and poor correlative performance suggests a need for increased toxicity endpoints when assessing OSPW (Bartlett et al. 2017).

2.2.10 – In vitro Tests – Summary

In vitro testing remains a popular choice for toxicity testing due to the low technical burden and relatively high reproducibility. Eukaryotic approaches provide high sensitivity at a range of endpoints including cytotoxicity, metabolism, protein secretion, and transcriptional changes. This resolution makes these tools invaluable in exploring the mechanisms of toxicity of OSPW, though only microalgae are currently approved for WET testing as per the Federal government. Inhibition of luminescence in *A. fischeri* is one of the most popular choices for assessing OSPW toxicity in general, and boasts low technical burden, high reproducibility, and rapid results. Despite these advantages, this organism is of no ecological relevance in freshwater settings and has consistently shown to be less sensitive when compared to other *in vivo and in vitro* tests, with poor predictive accuracy for whole organism toxicity outcomes when dealing with effluents.

2.2.11 – OSPW Toxicity – Summary

The toxicity of OSPW has been extensively researched using a wide range of organisms in highly standardized approaches. *In vivo* approaches provide insight into the effects of exposure on whole organisms, and as such are of significant biological and ecological relevance. Beyond this, these assays represent the majority of Federally approved WET tests, but their execution is generally technically demanding and expensive, limiting their practicality as high-throughput screening tools. *In vitro* approaches are cheaper, highly reproduceable, and have a lower technical burden, though can lack ecological relevance. Recent efforts to expand *in vitro* approaches beyond measuring cytotoxicity have greatly increased the resolution these tests provide with respect to the mechanism of OSPW toxicity. Despite their value, there are few Federally approved *in vitro* tests for WET assessment, suggesting an area for investigation given the demand for rapid screening approaches that can reduce the need for animal testing.

2.3 OSPW Remediation

Significant effort has gone into investigating how to remove problematic components in OSPW to potentially reduce toxicity. While petroleum operators have several active extraction sites that meet regulatory requirements for tailings management, only a single tailings facility has been considered reclaimed (AER 2023b; Suncor Energy Inc. 2024). Outside of this successful example, the Alberta Energy Regulator has consistently been required to issue notices of noncompliance to operators for issues ranging from data quality and reporting to failure to contain industrial wastewaters (AER 2024b). As of 2022, one of the largest operators in the region has only managed to treat 31 million m^3 of OSPW, less than 4% of the total tailings this operator has accumulated, and only 11% of the total freshwater used that year (AER 2024b).

Given that the TMF requires a return of water and land quality to pre-industrial state within 10 years of mine closure, the need for effective treatment approaches is critical (AER

2024b). While there is not currently a standardised approach to treatment, numerous broad strategies exist. These approaches prioritize the OF in OSPW for degradation due to its strong association with toxicity and can be divided into active and passive (Allen 2008b). Active treatments are engineered approaches that use chemical or physical stimulation to break down compounds of concern, while passive treatments intend to rely on natural biological process and native organisms to remove or sequester compounds of concern from OSPW (Saborimanesh 2018).

2.3.1 – Active Remediation

While fluid tailings are complex source specific mixtures, generally all treatment begins with some form of phase separation. The first step in this process is coagulation, which involves adding chemicals such as metal salts and organic polymers to bind together charged organic and inorganic compounds (Wang et al. 2015). Once aggregated, these compounds can be removed from water using filtration, centrifugation, or can be left to settle out of solution. Notably, coagulation alone tends to increase the toxicity of OSPW and requires separation to be an effective treatment approach (Fort and Stover 1995). These separated solids can be stored in basins such as end pit lakes (EPL) and capped with fluid tailings and freshwater, with the hope that problematic compounds will settle and deposit at the bottom of the basin. This approach can be effective for removal of problematic compounds from tailings water but results in their deposition and concentration into sediment, with a potential for infiltration into regional groundwater (Allam et al. 2021). Additionally, slow release of toxic compounds from dewatered tailings into the water cap is a possibility, resulting in persistent toxicity that is difficult to predict (Allam et al. 2021). Despite these concerns, coagulation and separation approaches have been

shown to marginally decrease the toxicity of OSPW, but effectiveness is further increased by combing approaches (Wang et al. 2015).

In additional to mechanical separation of toxicants, chemical processes may be used to target specific compounds of concern and degrade them into less harmful intermediates. An example of one such approach is oxidation, which involves adding oxidants such as chlorine, peroxide, ozone, or sulfate to OSPW in addition to a catalyst (Abdalrhman and Gamal El-Din 2020). When catalyzed, these compounds produce reactive oxygen species and hydroxyl radicals that can partially or fully degrade compounds of concern. These approaches have already shown strong efficacy in breaking down large compounds such as PAC and NAs and can effectively mineralize trace metals for removal (Quinlan and Tam 2015). A major advantage of these approaches is that they do not require storage or disposal like what is seen in coagulation, as compounds are ideally reduced to harmless products (Andreozzi et al. 1999).

Oxidation alone has been shown to significantly reduce the toxicity of whole OSPW and NAs, but these approaches may be further stimulated by using ultraviolet light (UV), electrical current, and even ultrasound (He et al. 2012; Deng and Zhao 2015). Called advanced oxidation processes (AOP), the energy provided from these stimulants drive additional oxidation for enhanced degradation of persistent organic compounds. Originating in wastewater treatment, AOPs have been extensively investigated for treatment of OSPW, showing strong efficacy in reducing organic contaminants, NAs, and OSPW toxicity (He et al. 2012; Wang et al. 2016; Ganiyu et al. 2022a; Sanchez-Montes et al. 2024; Leshuk et al. 2018). Despite the promising findings for AOPs, these treatments are expensive, technically demanding, and do not currently exist in a scale that could practically be used to perform meaningful remediation of fluid tailings (Ganiyu et al. 2022b; Allen 2008b)

2.3.2 – Passive Remediation

While not as rapid and targeted as active remediation, passive remediation is currently being tested at-scale by several oil sands operators as a potential solution for remediating OSPW (AER 2023b). Passive remediation involves the construction of artificial wetland ecosystems that can facilitate degradation of contaminants through microbial and plant-mediated metabolism and biotransformation, or through physical processes such as sedimentation or photolysis (Cancelli and Gobas 2022; Qin et al. 2019b). Though the kinetics of passive processes are significantly slower than active remediation, they have shown to be effective at removing compounds of concern such as NAs, PACs, BTEX, and trace metals (Saborimanesh 2021; Ajaero et al. 2018; Allen 2008b). Correlating with a reduction in compounds of concern, these systems have shown significant decreases in toxicity for OSPW, though notably effects are still observed years after establishment suggesting the presence of residual toxicity (Allen 2008b; Cancelli and Gobas 2022).

A major contributor to the efficacy of passive remediation, bacteria have a large range of metabolic strategies that allow them to break down contaminants of concern. Though effective, under ambient conditions *in situ*, these processes are quite slow, limiting practicality (Xue et al. 2018; Chegounian et al. 2020). These processes may be further enhanced in bioreactors, which are a semi-passive form of treatment that creates optimal growth conditions for microbial degradation of organic compounds. This process is enhanced by modifying temperature, adding growth factors, or supplementing biomass with organisms of value to further increase biodegradation (Zhang et al. 2018; Liu et al. 2015). Notably, though these compounds may be of lower toxicological significance, NAs of high molecular weight were more resistant to microbial

degradation, and thus a combination of approaches will likely be needed to entirely remediate OSPW (Quagraine et al. 2005).

Microbial degradation, while primarily performed by bacteria, is not exclusively done by this domain of life. The majority of Archaea present are methanogens, performing methanogenesis to create optimal conditions for the degradation of high molecular weight organic compounds (Shahimin et al. 2021). Larger eukaryotic organisms such as algae also have been implicated in reducing OSPW toxicity, but the majority of biodegradation is done by the microbial constituents (Mahdavi et al. 2015)

2.3.3 – OSPW Remediation – Summary

Though no one treatment approach has been fully adopted, numerous effective pilot scale strategies have been developed to remediate OSPW. Most of these approaches seek to target the OF, as it is strongly associated with OSPW toxicity. Active remediation is rapid and allows for targeting of specific compounds of concern, but scales poorly to deal with the volume of OSPW that needs treating. Passive remediation is a more practical approach but can be slow without additional stimulation and may have limits to what compounds can be removed from suspension. Overall, effective remediation will likely need to incorporate a range of approaches to effectively remove or sequester the massive suite of contaminants present in OSPW and will require detailed chemical and toxicological analysis to validate treatment efficacy.

2.4 Bacteria and OSPW

The relationship between bacteria and OSPW is complicated and serves as a testament to the diversity of observed bacterial physiology. On one hand, bacteria are some of the first organisms to colonize OSPW and have metabolic processes which can degrade components that

significantly contribute to OSPW toxicity (Chegounian et al. 2020). At the same time, the most used *in vitro* test for determining OSPW toxicity measures the inhibition of *A. fischeri* bioluminescence, relying on the sensitivity of this test organism to a broad range of toxicants (Mahaffey and Dube 2017). This balance of resilience and sensitivity makes bacteria a dynamic solution to complex environmental problems such as fluid tailings.

In OSPW, a wide range of bacterial genera exist that utilize a broad group of metabolic processes. Bacteria present in OSPW have two major sources; namely the freshwater from the Athabasca River used for extraction, and organisms that resided in the previously buried bitumen deposits. The physical and chemical forces used in this process change the identity of these bacterial communities, and the resulting microbiota can vary greatly depending on water physiochemistry, age, and season (Foght et al. 2017). Much of bacterial community dynamics are dictated by the type of carbon available in OSPW, with major changes occurring as degradation progresses and availability shifts. In settled tailings, anaerobic fermenters and syntrophs such as *Desulfurivibrio*, *Tolumonas,* and *Trichococcus spp.* use hydrocarbons to produce carbon dioxide, hydrogen, nitrate and acetate (Schlegal et al. 2013; Golby et al. 2012). These compounds serve as precursors for methanogenic archaea which can degrade short-chain hydrocarbons and BTEX compounds to produce methane (Siddique et al. 2012). The methane produced can be oxidized by methanotrophic bacteria such as *Methylobacter, Methyloparacoccus*, and *Methylovulum spp.* as part of metabolism that results in the biodegradation of halogenated hydrocarbons (Albakistani et al. 2022; Semrau 2011). This process is cyclical, and involves the contribution of numerous manganese-, iron-, sulfur-, and nitrate-reducing bacteria to stimulate metabolism under anaerobic conditions (Semrau 2011).

Beyond their involvement in biodegradation, several common freshwater bacteria also inhabit OSPW including *Cyanobacteria, Actinomycetota,* and *Pseudomonas,* likely contributing to the intricate metabolic cycles occurring (Yergeau et al. 2012; Foght et al. 2017). These organisms are generally associated in biofilms, which confers an enhanced protection to the antimicrobial activity of compounds present in OSPW, as well as an increased potential to biodegrade these compounds (Demeter et al. 2015; Chakraborty et al. 2012; Frankel et al. 2016). These communities can also increase the proliferation of genes associated with environmental resistance and biodegradation of compounds of concern, allowing for persistence in these hostile environments (Dejonghe et al. 2000). Both in isolation and as a part of microbial communities, these organisms are well suited to inhabit OSPW and have strong tolerance of freshwater conditions and contaminants introduced to OSPW.

In stark contrast, *A. fischeri* is a valuable organism in aquatic risk assessment that has displayed sensitivity to a massive range of compounds of ecotoxicological concern including PACs, BTEX, trace metals, and industrial effluents (Hao et al. 1995). This sensitivity has been extensively supported by correlative data which suggests reasonable predictive accuracy for whole organism outcomes in traditional aquatic toxicity settings (Environment Canada 1992). For these reasons, as well as its low technical burden, this assay has been approved for testing aquatic toxicity and is the most common *in vitro* toxicity test used for assessing OSPW components, fractions, or whole waters (Brown and Ulrich 2015; Clemente and Fedorak 2005; Frank et al. 2008; Jones et al. 2011; El-Din et al. 2011).

Despite its widespread use, as noted earlier, this assay has shown to have poor predictive accuracy and sensitivity compared to other approved approaches used to assess OSPW (Bartlett et al. 2017; Frank et al. 2008; Bauer et al. 2019). These results should not be surprising given the

sensitivity and predictive accuracy established for this assay is with isolated toxicants in saltwater, nearly the opposite of the conditions presented by OSPW and effluents (Hao et al. 1995). In OSPW and freshwater, simple parameters such as salinity and pH exist outside the ranges *A. fischeri* can tolerate, requiring significant modification of samples that reduce exposure concentrations and may change observed toxicity (Environment Canada 1992).

In addition to poor correlative and predictive accuracy, there are several other considerations that may limit bacterial luminescence inhibition assay validity for assessing OSPW toxicity. Firstly, the longest recommended exposure time is 30 minutes, with most literature reporting values after only 15 minutes (Hao et al. 1995; Li et al. 2017; Brown and Ulrich. 2015). This is significantly shorter than the recommended 72-hour exposure for the most rapid currently approved *in vitro* WET method using algae (Environment Canada 2007). Even beyond approved approaches, the shortest standardized bacterial toxicity assessment still has a 90-minute exposure with an endpoint of metabolic inhibition (Viegas et al. 2021). Another limitation of this assay is related to its claims that is can effectively measure genotoxicity. Protocols designed for the assessment of mutagenicity suggest performance at optimal growth temperatures over periods of time (48 hrs) that allow for sufficient accumulation of genetic damage across generations of organisms (Tjes 2008). While it is possible that severe genetic damage could occur in the 30-minute exposure recommended for bacterial luminescence approaches, its performance at low temperatures and short time periods would require acute effects from compounds tested (Hauser et al. 1997).

Another major limitation of this assay is its reliance on luminescence as a reporter for viability. As previously discussed, the production of luminescence by *A. fischeri* is related to both cell density and metabolic activity. As such, it is possible to observe reductions in

luminescence without affecting cell density due to interference with metabolism or autoinducer induction and accumulation, an effect that is amplified in complex mixtures (Miyamoto et al. 2000; Gellert et al. 2000). Though interference with metabolic activity can still be considered a form of toxicity, this interpretation is difficult to differentiate without further testing. From a measurement perspective, photometry is generally effective when sample turbidity is low, though there are scenarios where this can be problematic. Depending on size, many nanoparticles possess high adsorption and light absorption properties, and often can scatter light that this assay measures (Qiu et al. 2017; Kroll et al. 2009). These phenomena have shown to interfere with a variety of photometric and colorimetric assay readouts in a toxicity setting. This can result in inhibition of luminescence that may or may not be associated with toxicity to the test organism (Ong et al. 2014; Monteiro-Riviere 2006). While nanoparticles themselves are a substance of environmental concern, these compounds are also used as part of environmental remediation in removal of metals and organic contaminants, emphasizing the need for effective toxicity monitoring tools (Song et al. 2021; Yue and Economy 2005).

2.5 Summary

Any waters that are used as part of bitumen extraction from oil sands are considered OSPW, and these waters can be at various stages of treatment or storage. Generally divided into the OF and IF for analysis purposes, these complex waters contain several compounds of ecotoxicological concern such as BTEX, PACs, NAs, inorganic ions, and trace metals. When in mixture, these compounds can have synergistic or antagonistic effects on toxicity with concentration being a poor predictor of toxicity in whole OSPW. The toxicity of these components in isolation, fractions, or as a part of whole OSPW has been tested extensively with several organisms from a range of taxa. Toxicity testing has revealed that outcomes are largely

organisms and source dependent, but exposure generally results in acute or chronic toxicity as well as adverse effects on organism physiology.

In vivo approaches have strong biological and ecological relevance, but are generally expensive, technically demanding, and are actively being downscaled in modern toxicity testing to achieve the 3Rs. *In vitro* approaches are rapid, inexpensive, and highly reproduceable, resulting in their popularity for assessing OSPW toxicity. As with *in vivo* testing, a wide range of endpoints allows for high-resolution investigation into the mechanism of OSPW toxicity. Despite this, there is a limited number of approved *in vitro* methods, and current models have several technical limitations and can lack ecological relevance. Regardless of the method chosen, whole OSPW has shown significant toxicity using these models, and as such, remediation is of the utmost importance to achieve regulatory standards for these waters.

Though there are no currently standardized treatment approaches, several OSPW remediation approaches exist that are generally divided into active and passive remediation. Active remediation involves using chemical or physical processes for targeted removal of compounds of concern. Approaches such as AOPs have shown significant reduction in organic contaminants in OSPW, and greatly improved toxicity outcomes for treated waters. Despite this, they are expensive and currently are not practical in scale to reasonably address the volume of fluid tailings that require treatment. Passive remediation is much more cost effective and can be reasonably scaled to begin addressing current OSPW production volumes. Passive techniques rely on using natural biogeochemical processes to degrade compounds of concern. This approach has been reasonably successful under ambient conditions but can be further stimulated by creating optimal conditions for microbial degradation of both organic and inorganic compounds of concern.

From a microbiology perspective, bacteria present unique opportunities for resolving challenges associated with bitumen extraction and the resulting waste waters. In OSPW, native microorganisms are actively working to degrade compounds of concern as part of complex metabolic pathways. These organisms show high resiliency to components of concern in OSPW and are well suited to survive in tailings and freshwater conditions. In the lab, a marine luminescent bacterium has provided the majority of currently exiting *in vitro* toxicity data for OSPW and its constituents. Though this assay has been useful for screening aquatic toxicity, it has several technical limitations that limit its significance for this purpose.

Overall, both industrial operators and government regulatory agencies have indicated a demand for *in vitro* approaches that can be used to rapidly screen toxicity when storing, treating, and eventually releasing OSPW. As such, I sought to leverage some of the strengths of already existing antimicrobial assessment tools to investigate an approach that could overcome some of the challenges the bacterial luminescence inhibition approaches face. Specifically, I aimed to demonstrate that an alternative microbial toxicity assay could be modified to interrogate the effects of NAs and whole OSPW. Furthermore, I explored if this approach would be sensitive to OSPW with differing physiochemistry, and ultimately responsive to treatments that reduce contaminants of concern in OSPW. Lastly, this modified approach was compared to a standardized bacterial luminescence inhibition assay to try and understand how this data relates for waters of varying physiochemistry.

Chapter III

Materials and Methods

3.1 General Notes

All pipettes used were validated quarterly to be within ± 5% of the set value. Culturing and assay reagents are listed in detail in Table 3.1. All solutions were prepared to volume in sterile ultra pure water (UPW) unless otherwise indicated. All glassware and reagents used were sterilized via autoclaving where possible. When autoclaving was not possible, reagents were filtered using 0.22 µm bottle top filters.

Table 3.1. Reagents and materials

Tryptic soy broth (TSB)	
30 g/L	Tryptic soy broth - 22092 - Millipore
Tryptic soy agar (TSA)	
40 g/L	Tryptic soy agar - 22091 - Millipore
Luria-Bertani (Miller) + salt (LBS)	
25 g/L	LB broth (Miller) - 244610 - BD Difco
10 g/L	NaCl -S27110 - Fisher
Luria-Bertani (Miller) + salt agar (LBSA)	
1X	LBS
15 g/L	Agar - 214010 - BD Bacto
Cryopreservation solution	
50% (v:v)	Glycerol - G5516 - Sigma
1/2M sodium hydroxide	
40/80 g/L	NaOH - MFCD00003548 - Fisher
1M hydrochloric acid	
85.8 mL/L	HCl - 2104 - Supelco
0.9% saline	
9 g/L	NaCl -S27110 - Fisher
2.0 % saline	
20 g/L	NaCl -S27110 - Fisher

Commercial naphthenic acid (cNA) solution	
300 mg/L	Naphthenic acid - 70340 - Sigma
2 mL/L	2 M NaOH
	1M HCl
1X OSPW replicant	
1.5 g/L	NaCl -S27110 - Fisher
0.35 g/L	$MgCl_2 \bullet 6H_2O$ - M33 - Fisher
0.006 g/L	$CaCl_2 \bullet 2H_2O$ - SLCK7949 - Sigma
	1 M NaOH
3,5-Dichlorophenol	
100 mg/L	$C_6H_4Cl_2O$ - A1532806 - Thermo;
20 g/L	NaCl -S27110 - Fisher
Broth media 5.7	
30 g/L	NaCl -S27110 - Fisher
6.1 g/L	$NaH_2PO_4 \bullet H2O$ - 1.06346 - Thermo
2.75 g/L	$K_2HPO_4 \bullet 3H2O$ - P5504 - Sigma
0.204 g/L	$MgSO_4 \bullet 7H2O$ - 230391 - Sigma
0.5 g/L	$(NH_4)_2HPO_4$ - 1.01207.0500 - Sigma
3mL/L	Glycerol - G5516 - Sigma
5 g/L	Tryptone - 211705 - Gibco
0.5 g/L	Yeast extract - 212750 - Gibco
Agar media 5.8	
1X	Broth media 5.7
12 g/L	Agar - 214010 - BD Bacto
Protective media 5.9	
660 g/L	$C_6H_{12}O_6 \bullet H_2O$ - 49159 - Sigma
40 g/L	NaCl -S27110 - Fisher
20 g/L	L-Histidine - H800 - Sigma
5 g/L	Bovine serum albumin - A7906 - Sigma
Reconstitution solution 5.5	
8 g/L	$C_6H_{12}O_6 \bullet H_2O$ - 49159 - Sigma
20 g/L	NaCl -S27110 - Fisher
2.035 g/L	$MgCl_2 \bullet 6H_2O$ - M33 - Fisher
0.3 g/L	KCl - P9541 - Sigma
11.9 g/L	$C_8H_{18}N_2O_4S$ (HEPES) - H3375 - Sigma
Osmotic adjustment solution 5.10	
220 g/L	NaCl -S27110 - Fisher

3.2 Preparation of Microorganism Stocks

Organisms used in MIC assays were received from stocks provided by the Microbiology 265: Introduction to Microbiology technical staff at the University of Alberta. The clinical organisms received were *Klebsiella pneumoniae* (no strain reference given), *Pseudomonas aeruginosa* ATCC 10145, *Enterococcus faecalis* ATCC 19433, and *Staphylococcus warneri* NCTC 5955. A stock of *Escherichia coli* K12 MG1655 was available in lab as a freezer stock. All organisms provided were initially cultured on solid agar media. In a 14mL culture tube, 10mL of sterile TSB was inoculated and organisms were incubated on a rotary shaker at 37 °C for 24 hours, 110rpm. Following incubation 1000µL of overnight culture was diluted in 1000µL of cryopreservation solution in a cryovial and subsequently stored at -80 °C. A similarly prepared stock of *A. fischeri* NRRL-B-11177 was available in lab. All primary (1°) cultures used in experiments were inoculated from these freezer stocks.

3.3 Culturing of Microorganisms

Primary cultures for experiments were generated by streaking bacteria from the freezer stocks described in section 3.1 on TSA for clinical organisms and LBSA or agar media 5.8 for *A. fischeri*. Plates were incubated for 24 hrs at 35 °C for all clinical species and 24 hrs at 28 °C for *A. fischeri* using a humidified incubator. Following incubation, these plates were parafilmed and stored at 4 °C and served as 1° cultures for experiments for a maximum of 14 days. Liquid overnight (O/N) and secondary (2°) agar cultures were all inoculated using single colonies from a 1° culture of the respective organisms.

3.3 Commercial naphthenic acid

The cNA solution used in this thesis was generated using a technical grade NA solution available from Sigma Aldrich (70340). This reagent was a mixture of alkylated cyclopentane NAs with the general formula $C_{10}H_{18}O_2$. At pH values lower than 7, NAs have low solubility, making the generation of a homogenous solution challenging (Headley et al. 2002). One approach that increases the solubility of NAs is ionizing these compounds into sodium naphthenate salts. Over 99% of NAs found in aqueous tailings are in this protonated form, making this approach a more consistent representation of how these compounds are found in OSPW (Clemente and Fedorak 2005). NAs can be ionized by adding NAs to sodium hydroxide, which increases the pH and provides sodium for ionization into sodium naphthenate salts (Young et al. 2007). Similar to what is described in Bartlett et al. (2017) and Kinley et al. (2016) for dissolving NAs for aquatic exposure, the technical grade NA was added to NaOH, and was subsequently volume adjusted to achieve a final concentration of 300 mg/L. This solution had the pH adjusted to a final value of 8.4 to reflect the water chemistry of test and environmental samples described in Table 3.2. This solution was filter sterilized as described in section 3.6.

3.4 General MIC Procedure

The protocol used for the microdilution MIC protocol was adapted from what is described in Chapter 3 of M07-A10, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 11th Edition (CLSI 2018). The principle of this assay involves the suspension of an antimicrobial compounds in organism specific media (OSM) to prepare a concentrate. This solution is diluted in OSM to achieve a range of antimicrobial concentrations in a 96-well plate. These are considered the test wells and are used to assess the effects of compound exposures on organism growth. Growth control wells are prepared by adding 100% OSM with no antimicrobial compound present, allowing them to serve as a reference to identify

optimal growth without inhibition. A positive control, typically a known effective antimicrobial compound is also prepared in a dilution series. This serves to monitor the consistency in antimicrobial sensitivity of an organism across experiments. Sterility controls are also generated by filling additional wells with all solutions used in the experiment, including OSM, diluents, and all test samples.

All solutions, both test and control are added at 180 µL/well and are generated in triplicate in a 96-well plate. This plate is considered the challenge plate, and the challenge is initiated by inoculating all test, growth control, and positive control wells with 20 µL of test organism suspended at $5x10^6$ CFU/mL in OSM. This results in a microbial load of approximately $5x10^4$ CFU/well in the challenge plate. Sterility controls are inoculated with 20 µL sterile OSM to encourage the growth of any potential contaminants already present in each solution. The inoculated challenge plate is then incubated for 16-20 hrs at 35±2 °C sealed in a bag to prevent drying. Following incubation, the MIC is determined by viewing the challenge plate with the unaided eye and evaluating what wells have no observable growth.

From the above protocol, several general and experiment specific modifications were made for the experiments in this study. For the later experiments described in this thesis, the general layout described in Figure 3.1 was followed for challenges. The negative control row in Figure 3.1. shows all growth controls used for the environmental MIC. The growth control (GC) wells were used to describe wells with OSM or an alternative control as described in later experiments. If multiple challenge plates were prepared for the experiments, growth controls were prepared in each plate and averaged to calculate the log reduction. Environmental controls (EC) were also established to confirm the organism's growth under regional freshwater conditions. As the inoculum was prepared in OSM, the OSM also had sterility controls prepared

in addition to the GC media, regional freshwater (EC), and the diluent (DL) used in enumeration. For these experiments a stock of 3,5- dichlorophenol suspended in 2.0% saline was used as a positive control. This is as recommended in Biological Test Method: Toxicity Test Using Luminescent Bacteria (Environment Canada 1992), ISO 11348-1 2nd Edition protocol (2007), and Microtox Acute Toxicity Basic Test Procedure (Microbics 1995).

The target inoculum density was also reduced from the recommended $5x10^6$ CFU/mL to $1x10^6$ CFU/mL and was a deviation from the traditional MIC microdilution protocol. As the modified MIC used does not use OSM as a control, this change was made to account for the reduced concentration of nutrients available to the test organisms, as traditional MIC microdilution assays are performed with test compounds suspended in 100% OSM.

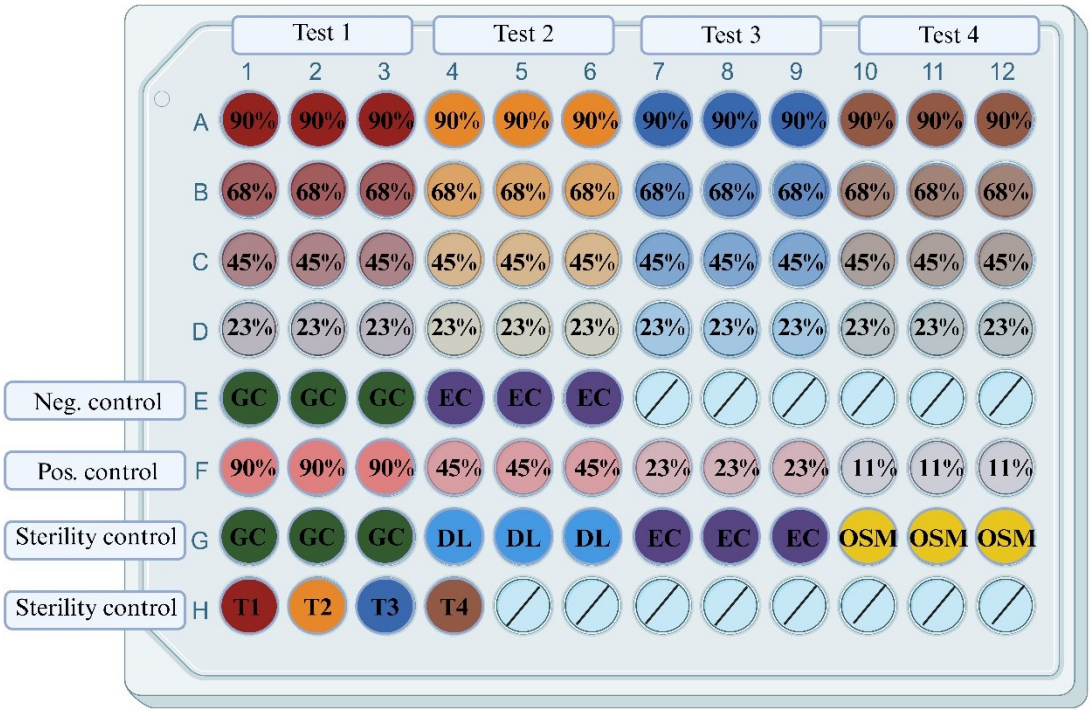

Figure 3.1. Modified MIC plate layout. Section 3.4 describes abbreviations and well

3.5 Bacterial Enumeration

All bacterial enumeration was done using serial dilution and spot plate techniques that are similar to what is described in Thomas et al. (2015). Briefly, LBSA or TSA was poured in 150x15mm petri dishes to achieve an approximate agar depth of ~4 mm. These plates were left to dry for 7 days before being stored at 4 °C until use. For serial dilution, 80-100 µL of the sample to be enumerated was transferred in triplicate to row A of a 96-well challenge plate. Rows B-H had 180 µL of 0.9% saline for clinical organisms and 2.0% saline for *A. fischeri* added to each well. Using a 12-well multi-channel pipette, a serial dilution was performed from row A to row H to generate samples diluted from a factor of 10^0 to 10^8. Following dilution, 10 µL was transferred from each well of the dilution plate and spot platted on LBSA for *A. fischeri* and TSA for all other organisms. Spot plates were incubated for 24 hrs at 28 °C or 35 °C in a humidified incubator for *A. fischeri* and all other organism, respectively. Following incubation, the lowest dilution with countable isolated colony forming units (CFU) was used to determine CFU/10 µL. This value was multiplied by the dilution factor and was multiplied by 20 to determine CFU/well and was then log transformed to determine log growth. Log reduction was determined by calculating the difference in growth between the control and the test sample after the incubation period. A diagram of this workflow is shown in Figure 3.2 calculating the difference between the growth control and the test sample. A diagram of this workflow is shown in Figure 3.2.

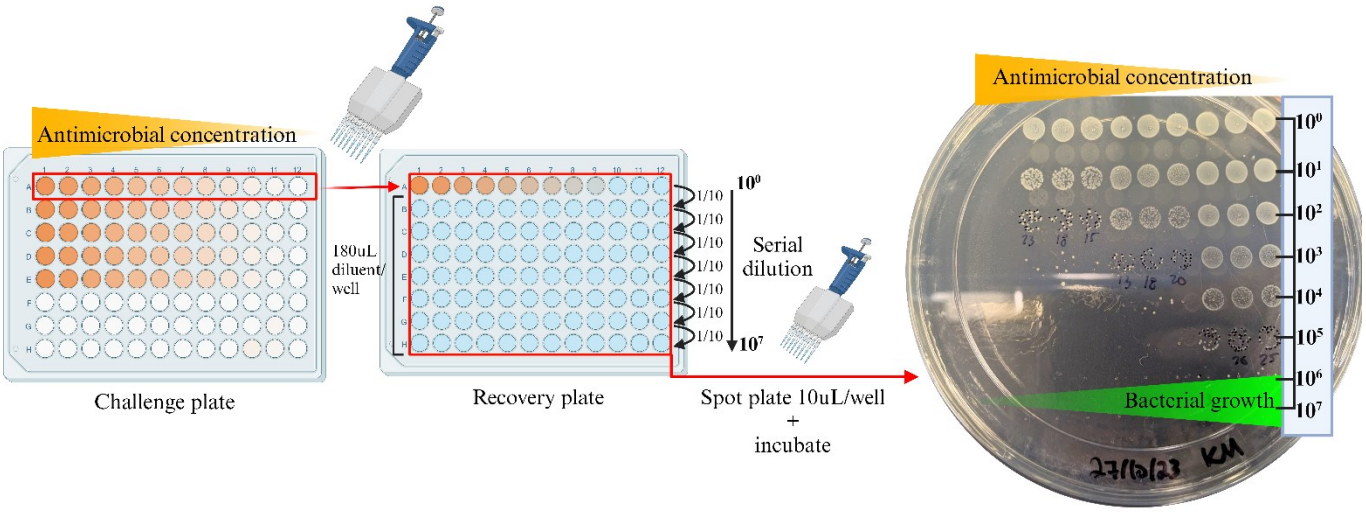

Figure 3.2. Procedure for high throughput enumeration of CFU. This process is repeated 8 times to fully enumerate an entire 96-well challenge plate.

3.6 Filtration

Filter size is important for toxicity assessment, as compounds of concern may be lost due to filtration, affecting the toxicity of the test samples. At the same time, when performing microbial growth and enumeration assays it is crucial that test samples do not introduce additional bacteria, especially those that can be enriched under test conditions. It is acknowledged that bacteria cultured with minimal media and high agitation can result in monodispersed cells that are of small enough body size to pass through a 0.45 µm filter, as observed with *Brevundimonas diminuta* (Lee et al. 2002). Despite this, 0.45 µm filters prove sufficient to exclude most environmental organisms when not used in applications where organisms can colonize the filter, such as long-term continuous flow filtration (Hasegawa et al. 2003; MilliporeSigma 2020).

To ensure the efficacy of the filtering technique used, an unfiltered aliquot of each OSPW was serially diluted and spot platted following the methods described in 3.5. Spot plating was performed on TSA and plates were incubated at 35 °C for 24 hrs to replicate test conditions. The colony forming units CFU/10 µL were averaged and converted to CFU/mL. For OSPW A, B, C, D the average (n=3) CFU/mL was enumerated at $6.7x10^1$, $9.3x10^4$, $1.0x10^2$, and $1.6x10^2$ CFU/mL, respectively. At the same time, an aliquot of each OSPW was filtered using 0.45 µm and 0.22 µm filters, and 200 µL of the filtrate was spread platted on TSA and incubated at 35 °C for 24 hrs. No colonies were observed from either filtrate after 24 hours, so an additional 24-hour incubation was done at 35 °C which did not reveal any colonies. Based on these findings, the decision was made to use 0.45 µm filters due to their exclusion of contaminating organisms while allowing more potential compounds of concern through than 0.22 µm filters. To ensure that no contaminating organisms were introduced, all filtered samples used in experiments had 200 µL spread plated on appropriate agar and were incubated under experimental time and temperature to be monitored for any growth. In addition to this screening, for each replicate, test media was combined with sterile growth control media to enrich for the presence of contaminating organisms.

3.7 Screening of Clinical Indicator Organisms for Sensitivity to NAs

From a 1° culture generated for each of the clinical organism described in section 3.1, a 2° culture was prepared by streaking a single colony on TSA and incubating this plate for 22 hrs at 37 °C. From the 2° cultures, single colonies were picked and used to inoculate 100% TSB. This inoculum had the optical density (OD_{600nm}) measured and an online tool (Agilent Genomics… [accessed 2024]) was used to estimate CFU/mL based on the observed OD. All inoculums were adjusted to an estimated density of $1x10^6$ CFU/mL by further dilution in 100%

TSB. To verify, the initial inoculum and the diluted inoculum were all enumerated following the challenge based on the procedure described in 3.5.

The test wells of the challenge plate were filled with 180 µL of the 300mg/L cNA mixture described in section 3.3. This solution was serially diluted from 300 mg/L to 37.5 mg/L in 0.9% saline using ½ step increments. At the time of this experiment, neither the EC nor positive control had been established yet. The GC used in this experiment was 0.9% saline, as it best represented the tests conditions created in the cNA solution. All test and control wells were prepared in triplicate. The test and GC wells had 20 µL of the inoculum added to them, and the SC wells had 20 µL of sterile 100% TSB added to them. Notably, the addition of inoculum results in the final concentration of test wells being 90% (v:v). The challenge plate was then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.8 Validation of CFU Estimations from Optical Density for *Staphylococcus warneri*

From a 1° culture of *S. warneri,* a 2° culture was prepared by streaking a single colony on TSA and incubating this plate for 22 hrs at 37 °C. From the 2° culture, single colonies were picked and used to inoculate 100% TSB. The inoculated TSB was then diluted in 100% TSB to achieve a range of OD values from 0.440 to 0.020, and the diluted solutions had OD_{600nm} measured in cuvettes using a Thermo Scientific Genesys 30 Visible Spectrophotometer. The diluted TSB solutions were then enumerated in triplicate as described in section 3.5. This experiment was repeated sixteen times for a total of n=16 replicates.

3.9 Assessment of *Staphylococcus warneri* Salt Tolerance

From a 1° culture generated for *S. warneri* a 2° culture was prepared by streaking a single colony on TSA and incubating this plate for 22 hrs at 37°C. From the 2° culture, single colonies were picked and used to inoculate 100% TSB. Using the formula generated by the experiment in section 3.8, CFU/mL was estimated in the initial inoculum based on the measured OD and was diluted to an estimated density of $1x10^6$ CFU/mL in 100% TSB. To verify, the initial inoculum and the diluted inoculum were enumerated following the challenge based on the procedure described in 3.5. All future inocula were generated following this procedure unless indicated otherwise.

The test wells of the challenge plate were filled with 180 µL of 3.9% – 0.0011% NaCl diluted in UPW (v:v). The GC used in this experiment was 100% TSB to assess the growth of the organism under optimal conditions. The TSB used had a NaCl concentration of 0.5% (w:v) when prepared at 30 g/L as per the manufacturer. All test and control wells were prepared in triplicate. The test and GC wells had 20 µL of the inoculum added, and the SC wells had 20 µL of sterile 100% TSB added. Notably, the addition of inoculum results in the final concentration of test wells being 90% (v:v). The challenge plate was then incubated in a humidified incubator for 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.10 Assessment of *Staphylococcus warneri* pH Tolerance

The test wells of the challenge plate were filled with 180 µL of 100% TSB that was pH adjusted using 1 M HCL and 1 M NaOH to achieve a range of pH values from 5.1 – 12.0. The GC used in this experiment was 100% TSB with no pH modification to assess the growth of the organism under optimal conditions. The pH of all solutions was measured using an OHAUS

ST3100-F meter with and OHAUS ST300 pH electrode. This meter was calibrated prior to use using a 3-point slope, and slope values were reported at 97±2%. All test and control wells were prepared in triplicate. The test and GC wells had 20 µL of an inoculum prepared as described in section 3.9 added to them, and the SC wells had 20 µL of 100% TSB added to them. Notably, the addition of inoculum results in the final concentration of test wells being 90% (v:v). The challenge plate was then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.11 Assessment of *Staphylococcus warneri* Nutrient Limitation

An inoculum was generated as described in section 3.9; however, an additional inoculum was created with the bacteria being suspended in 0.9% saline rather than 100% TSB. The test wells of the challenge plate were filled with 180 µL of 100% – 0.08% TSB diluted in 0.9% saline (v:v). The GC used in this experiment was 100% TSB to assess the growth of the organism under optimal conditions. All test and control wells were prepared in triplicate. The wells with 100% TSB were inoculated using the inoculum suspended in 100% TSB. The remaining diluted wells had 20 µL of the inoculum generated in 0.9% saline added to them as to not affect the final intended media concentration. All SC wells had 20 µL of 100% TSB added to them. The challenge plate was then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.12 OSPW Replicant Control Preparation

Traditional MIC assays involve the preparation of an antimicrobial compound in 100% OSM for the test organism. As this is not possible for whole effluent water samples, a negative control was created that replicated major physiochemical properties of whole OSPW. Notably total dissolved solids, calcium/magnesium ion concentrations, and pH were adjusted to target the middle of the ranges described in Table 3.2. The composition of this solution is described in Table 1. The final pH of this solution was adjusted to 8.4 using 1 M NaOH and 1 M HCl. A 100X concentrate of this solution was also generated and was used to adjust the parameters of the previously described cNA solution from section 3.3.

3.13 OSPW Sampling

Whole OSPW samples were labelled OSPW A, B, C, and D and were provided by Suncor Energy Inc. and Imperial Oil Ltd. Samples A and B are treated using a preliminary permanent aquatic storage structure (PASS) process, which dewaters tailings using coagulation and flocculation technologies (Suncor Energy Inc. 2024). Sample A was received in October 2018 and Sample B was received in March 2022. Sample C and D are from a similar dewatering facility and were received in May and September of 2023, respectively. All OSPW samples were stored in sealed polyvinyl chloride containers and stored at 4 °C until used in experiments.

3.14 OSPW Characterization

Basic physiochemical parameters of the OSPW samples used were analyzed in part by members of the Gamal El-Din lab (Department of Civil and Environmental Engineering, University of Alberta). Members of this research group also performed the AOP treatment described in section 3.19 and the synchronous fluorescence spectroscopy (SFS) analysis described in section 3.20. Initial measurements of classical O_2 NAs were performed using ultra-

performance liquid chromatography with quadruple time-of-flight mass spectrometry (UPLC QTof-MS). Specific details on equipment use and settings mirror what is described in Sanchez-Montes et al. (2024) and Huang et al. (2018). Values for pH and TDS were measured in lab, with an overview of all measured OSPW physiochemistry available in Table 3.2. pH values were measured using an OHAUS ST3100-F meter with and OHAUS ST300 pH electrode. This meter was calibrated prior to use using a 3-point slope, and slope values were reported as 97±2%. Values for TDS and conductivity were also measured in lab using a Thermo Scientific Elite CTS Tester. Prior to use, the CTS meter was calibrated using 1413 µS/cm and 12.9mS/cm standards.

Table 3.2. Basic water chemistry of whole OSPW and freshwater

Parameter	OSPW A	OSPW B	OSPW C	OSPW D	OSPW range*	Regional freshwater***
pH	8.5	8.6	7.74	8.04	7.64 - 8.66	8.1± 0.2
Conductivity (mS/cm)	4.35	4.47	1.31	1.42	1.12 - 4.01	0.205± 0.04
DOC (mg/L)	66.0	60.8	33.8	36.2	26 - 67	2.24± 2.65
COD (mg/L)	232	244	147	158	86 - 422	52.2± 18.4
TDS (mg/L)	2784	2860	944	730	400 - 2539	165.2± 45.4
Classical O_2-NAs (mg/L)	17.8	23.9	10.5	10.2	2.9 - 100.0**	0.000004± 0.000001
Mg (mg/L)	10.8	9.4	-	-	4.1 - 30.0	9.42± 1.8
Ca (mg/L)	2.1	1.9	-	-	6.2 - 123	34.4± 5.9
K (mg/L)	18.7	16.4	-	-	9.1 - 18.5	1.36± 0.4

*Ranges compiled from various OSPW samples at various points of processing, as outlined and reviewed in Hussain and Stafford (2023), Mahaffey and Dube (2017), and Allen (2008a)

**Range represents total NA species (O_2-O_6), as these were the reported values

***Data is compiled from the lower Athabasca River between the years of 1998-2024, available from Environment and Climate Change Canada (ECCC 2024) and the Government of Alberta (Government of Alberta 2024). Values were cross referenced with technical reports from the Government of Alberta (Hebben 2009), (Laceby and Emmerton 2021).

3.15 Assessment of Whole OSPW and OSPW Control Using a Modified MIC Assay

The test wells of the challenge plate were filled with 180 µL of 100% OSPW A, 0.9% saline, or 100% OSPW replicant control described in section 3.12. Additional test wells were prepared by diluting OSPW A to 75%, 50%, and 25% (v:v) in OSPW replicant. The GC used in this experiment was 100% TSB to assess the growth of the organism under optimal conditions. All test and control wells were prepared in triplicate. The test and GC wells had 20 µL of an inoculum prepared as described in section 3.9 added to them, and the SC wells had 20 µL of 100% TSB added to them. Notably, the addition of inoculum results in the final concentration of test wells being 90% (v:v). The challenge plate was then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.16 Assessment of *Staphylococcus warneri* cNA MIC Values

The adjusted cNA solution from section 3.3 was initially diluted 1:9 in OSPW replicant to generate a solution at 33.3 mg/L of cNA. Of this diluted solution, 180 µL was added to the challenge plate and serially diluted in OSPW replicant to generate a concentration gradient from 33.3 mg/L – 2.1 mg/L can. The GC used in this experiment was 100% OSPW replicant to assess the growth of the organism under similar physicochemical conditions as seen in the test samples. The EC wells were filled with 180 µL of freshwater from a lake in Northern Alberta. The positive control used was the 3,5- dichlorophenol solution, diluted from 100 mg/L – 12.5 mg/L in OSPW replicant. All test and control wells were prepared in triplicate. The test, GC, EC, and positive control wells had 20 µL of an inoculum prepared as described in section 3.9 added to them, and the SC wells had 20 µL of 100% TSB added to them. Notably, the addition of

inoculum results in the final concentration of test wells being 90% (v:v). The challenge plate was then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.17 Assessment of Evaporation Effects on a Modified MIC Assay

Challenge plates were prepared in duplicate, with both plates having rows A-D filled with 180 µL of 100% OSPW A and rows E – F filled with 100% OSPW replicant. The first challenge plate had the edges of the plate sealed with parafilm, while the second did not. No specific positive or negative controls were implemented given the objective of this experiment. The test and GC wells had 20 µL of an inoculum prepared as described in section 3.9 added to them. Notably, the addition of inoculum results in the final concentration of test wells being 90% (v:v). The challenge plates were then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. All wells in columns 1, 2, 11, and 12 and rows A and H were considered on the edge of the plate. Wells in rows B – G, 3 – 10 were considered in the middle of the plate. In addition to enumeration, the fluid volume was taken from all wells and weighed using a Mettler Toledo New Classic MF ML204 balance to determine the volume lost from evaporation. This experiment was repeated three times for a total of n=3 replicates.

3.18 Assessment of a Modified MIC Freezer Inoculum

In addition to an inoculum generated as described in section 3.9, an O/N culture was generated by inoculating 10 mL of TSB in a 14 mL culture tube and incubating on a rotary shaker at 37 °C for 23 hours, 110rpm. Following incubation, the solution was diluted 1:10 in

TSB and the OD_{600nm} was measured. From the diluted solution and using the formula generated from section 3.8 the CFU of the O/N culture was estimated to be $1x10^9$ CFU/mL. This stock solution was diluted 1:5 in 100% TSB to achieve an estimated density of $2x10^8$ CFU/mL. This solution was then diluted 1:2 in cryopreservation solution (v:v) and placed into cryovials and frozen at -80 °C. The stock solution, diluted freezer stock pre-freezing, and diluted freezer stock post-freezing were enumerated using the procedure described in section 3.5.

Challenge plates were prepared in duplicate, with both plates having wells filled with 180 µL of OSPW A, OSPW replicant, or cNA. OSPW A and cNA were serially diluted in OSPW replicant (v:v) to achieve concentrations ranging from 100% – 12.5%. No additional positive or negative controls were implemented given the objective of this experiment. All wells were prepared in triplicate. One plate was inoculated with 20 µL of the inoculum generated from agar stock. A vial of the freezer inoculum was thawed and was diluted in TSB to an estimated density of $1x10^6$ CFU/mL based on previous enumeration of the freezer inoculum. The second challenge plate was then inoculated with 20 µL of the diluted freezer inoculum. To verify accuracy, the diluted freezer stock and T_0 GC wells from the challenge plate were enumerated following the challenge based on the procedure described in 3.5. Notably, the addition of inoculum results in the final concentration of test wells being 90% (v:v). The challenge plates were then incubated in a humidified incubator at 22.5 hrs at 35 °C on a microplate rotary shaker set to 282 rpm. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.19 Treatment of OSPW and NAs Using Advanced Oxidation Processes

An aliquot of OSPW B and C, as well as a 300 mg/L stock of cNA were treated with a UV-A stimulated AOP. Briefly, samples were placed in a glass beaker and agitated using a

magnetic stirrer. Samples were stirred for 5 minutes prior to applying treatment to ensure homogenous sample mixture. Peroxydisulfate (PDS; S_2O_8 – Fisher Scientific) was added to each sample to achieve a concentration of 1.0 mM in 50 mL. Following addition of PDS, samples were exposed to an LED with a 365 nm main emission line (SOLIS-365C – Thorlabs). Irradiance was adjusted to a value of 0.80 mW/cm^2 using a power controller (DC-20 – Thorlabs). Samples were exposed to UVA conditions for a total of 60 minutes. Following exposure, samples had 1.0 M Na_2SO_3 solution added to prevent residual oxidation activity. Specific details on OSPW treatment mirror what is described in Sanchez-Montes et al. (2024).

OSPW D was treated with a similar AOP process that used biochar with iron oxide nanoparticles (BC-Fe) to stimulate oxidation. A BC-Fe composite catalyst was generated by adding 100 mL of 0.1 M $FeCl_3$ to 5 g of raw flax straw. This mixture was agitated for 60 minutes at 200 rpm on a rotary shaker before being placed on a magnetic stirrer at 80 °C for 2 hrs to evaporate water. Following this, the mixture was placed in a 105 °C oven overnight before being transferred to a 600 °C furnace for 2 hrs. The resulting product was finely ground and then passed through a 425 µm mesh. To perform the treatment, 1 g/L of the BC-Fe catalyst was added to the OSPW D and placed on a rotary shaker and agitated at 200 rpm at 25 °C. Subsequently, peroxymonosulfate ($PDSKHSO_5 \cdot 0.5KHSO_4 \cdot 0.5K_2SO_4$ – Sigma-Aldrich) was added to achieve a concentration of 1.0 mM in OSPW. Following addition of PMS, samples were agitated for 90 minutes. Following treatment, samples had 1.0 M Na_2SO_3 solution added to prevent residual oxidation activity. Treated samples were subsequently filtered using a 0.22 µm filter to remove residual composite catalyst. Specific details on OSPW treatment and BC-Fe preparation are identical to what is described in Vasquez-Aldana (2024).

3.20 Synchronous Fluorescence Spectroscopy Analysis

To monitor the presence of aromatic hydrocarbons, like those seen in NAs and PAHs, SFS was used. This semi-quantitative approach allows for the detection of fluorescent aromatic organic compounds and has been shown to be an effective technique to detect aromatic hydrocarbon compounds in complex mixtures (Sunuwar and Manzanares 2021). This approach has already been used to good effect to measure the presence of organic acids in OSPW due to their signature aromatic structural motifs. When analyzed using SFS, single, double, and triple ringed aromatic compounds such as NAs produce high intensity peaks at wavelengths around ~270 nm, ~310 nm, and ~325 nm, respectively (Kavanagh et al. 2009, Martin et al. 2014). As a result, SFS analysis was performed on cNA, OSPW B, and OSPW C untreated (UT) and 60-minute treated samples with fixed excitation and emission wavelength ranges of 200-600$_{nm}$ and 218-618$_{nm}$, respectively. Specific details on equipment use and settings mirror what is described in Sanchez-Montes et al. (2024).

3.21 Assessment of AOP Treatment on OSPW and cNA Using a Modified MIC Assay

A flow chart of the modified MIC assay workflow is described in Figure 3.3. An inoculum was generated as described in section 3.9; however, in addition to verifying the stock and diluted inoculum density, an additional set of GC wells were prepared in the plates and recovered after the challenge prior in incubation to verify the final inoculum density in the plates.

A sample of the UT cNA solution described in section 3.3 was initially diluted 1:9 in OSPW replicant to generate a solution at 33.3 mg/L of cNA. This same procedure was also followed for the 60-minute treated cNA to ensure consistency between samples. Additional test wells were prepared by diluting the 33.3 mg/L cNA solution to 75%, 50%, and 25% (v:v) in OSPW replicant. For the UT OSPW B, C, and D, a similar dilution series was prepared to

determine the MIC by diluting the sample to 100%, 75%, 50%, and 25% (v:v) in OSPW replicant. The 60-minute and 90-minute treated samples were not diluted.

180 µL of all UT and treated samples, as well as any dilution series prepared were added to the challenge plates. Negative and positive control wells were prepared as described in section 3.16 and added at 180 µL/well. All test and control wells were prepared in triplicate. All test, GC, EC, and positive control wells in the challenge plates had 20 µL of the inoculum added to them. The SC wells had 20 µL of 100% TSB added to them. Notably, the addition of inoculum results in the final concentration of wells being 90% (v:v). The challenge plates were then placed inside a Biotek Synergy H1 plate reader/incubator (Software – Gen 5 2.04). The plate was then incubated for 22.5 hrs at 35 °C with an orbit of 282 rpm. The plate reader measured the OD_{600nm} every 30 minutes during incubation to form a growth curve. Following incubation, wells were enumerated as per the procedure described in 3.5. This experiment was repeated three times for a total of n=3 replicates.

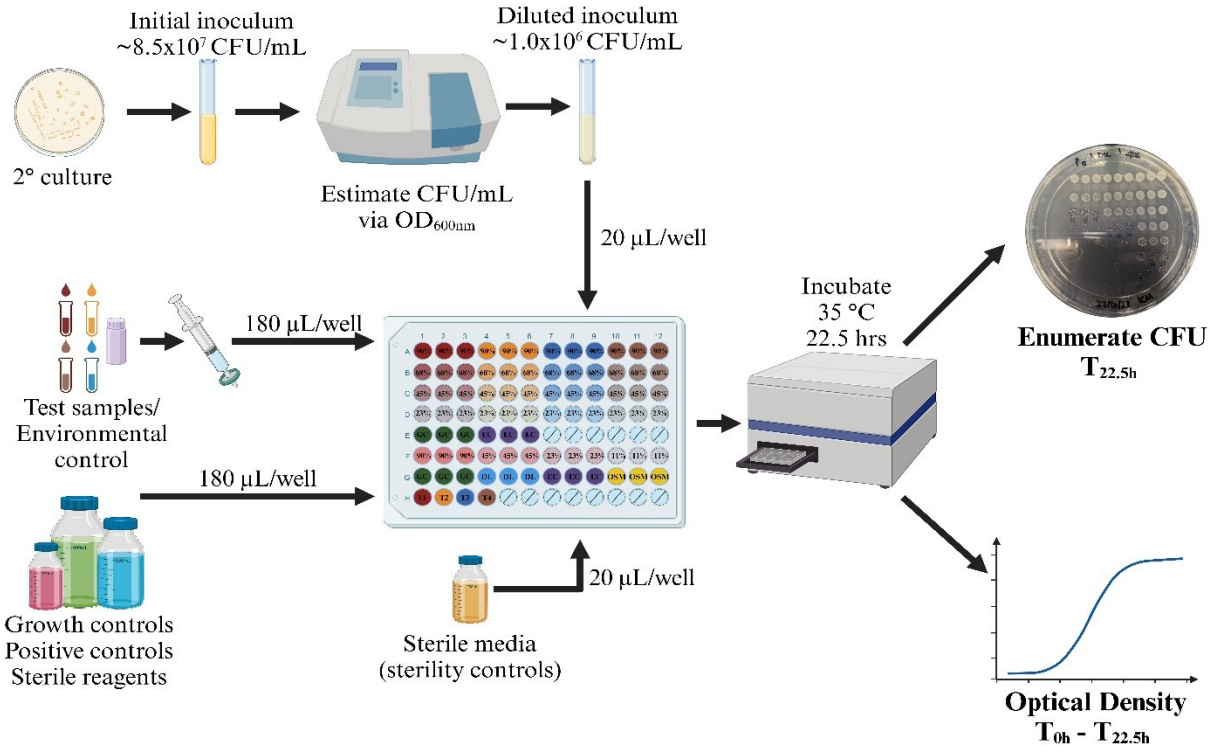

Figure 3.3. General workflow for a modified MIC assay.

3.22 Validation of CFU Estimations from OD for *Aliivibrio fischeri*

As the *A. fischeri* luminescence inhibition protocol used estimated bacterial concentration using optical density, a similar procedure as is described in section 3.8 was performed to validate the relationship between CFU and OD_{600nm}. From a 1° culture of *A. fischeri* a 2° culture was prepared by streaking a single colony on LBSA and incubating this plate for 22 hrs at 20 °C. From the 2° cultures, single colonies were picked and used to inoculate 100% LBS. The inoculated LBS was then diluted in ½ step increments, and the diluted solutions were aliquoted and had OD_{600nm} measured in cuvettes using a Thermo Scientific Genesys 30 Visible

spectrophotometer. The diluted LBS solutions were then enumerated in triplicate as described in section 3.5. This experiment was repeated nine times for a total of n=9 replicates.

3.23 Implementation of a Standardized Bacterial Luminescence Inhibition Assay

The bacterial luminescence inhibition assay used for screening of these OSPW was adapted from the ISO 11348-1 2nd Edition protocol (2007), Biological Test Method: Toxicity Test Using Luminescent Bacteria (Environment Canada 1992), and Microtox Acute Toxicity Basic Test Procedure (1995). While commercially available kits with lyophilized bacteria are available for this assay, this protocol outlined the preparation of freezer stocks and subsequent experiments using these stocks. Solutions used in the protocol are described in Table 3.1. A 1° culture of *A. fischeri* was generated by streaking bacteria from the freezer stocks described in section 3.1 on agar media 5.8 and incubating for 70 hrs at 21 °C. Using visual observation in the dark, luminescent colonies from this plate were marked and used to generate a 2° culture on agar media 5.8 under identical incubation conditions as the 1° culture. From the 2° culture, a single luminescent colony was used to inoculate 50 mL of broth media 5.7 that was incubated at 21 °C for 21 hrs on a rotary microplate shaker set at 300 rpm. Following incubation, the OD_{600nm} of the liquid O/N preculture was measured and based on the estimated cell density from the equation established in 3.22 a main culture of 100% broth media 5.7 was inoculated with the pre culture to reach an estimated cell density of $2.0x10^7$ CFU/mL. Both the preculture and diluted main culture were enumerated based on the protocol in section 3.5, and the main culture was incubated under the same conditions as described for the O/N preculture. Following incubation, the cell density was determined using the enumeration protocol described in section 3.5.

All solutions used in stock preparation were cooled on ice prior to use, and all glassware was precooled in a refrigerator. To generate freezer stocks from the main culture, the main

culture was centrifuged at 5000 *g* in a Thermo Sorvall - Legend XTR refrigerated centrifuge set to 4 °C. Following centrifugation, the supernatant was decanted, and the pellet was resuspended in 10 mL of 2% saline and centrifuged under the same conditions described previously. The supernatant was again decanted, and the pellet was resuspended in 500 µL of 2% saline. The bacterial suspension was then transferred to a beaker in an ice bath on a magnetic stirrer. Under constant agitation, the solution was slowly diluted 1:8 in protective media 5.9 (v:v). This solution had an aliquot diluted 1:100 in 2% saline (v:v), and the OD_{600nm} was measured to estimate cell density. This solution was further diluted with 15 mL of protective media 5.9 to achieve an approximate cell density of $1.5x10^9$ CFU/mL. An aliquot of the suspension was enumerated prior to freezing as per the protocol described in section 3.5 to determine cell density. This solution was stirred for 15 minutes on ice and then aliquoted into cryotubes and stored at -80 °C.

3.24 Comparison of Cell Density and Luminescence in *Aliivibrio fischeri*

All solutions used were placed on ice, and the opaque 96-well plate used was placed on a Echotherm IC22 cooling block set at 15 °C to cool. Initial attempts to use the freezer stock immediately upon rehydration resulted in large fluctuations in luminescence. As such, a freezer stock described in section 3.23 was slowly thawed by placing the cryovial in ice water. After thawing, the solution was diluted 1:5 in reconstitution solution 5.5 (v:v) and placed on ice for 15 minutes. Following this, the solution was further diluted 3:41 in reconstitution solution 5.5 (v:v) and mixed well before resting for 15 minutes on the cooling block set at 15 °C. Following this waiting period, 200 µL of the bacterial suspension was added to row A and B wells 1-3 of a cooled opaque 96-well plate. All remaining wells had 100 µL of 2% saline added to them. The bacterial suspension was then diluted in ½ step increments with 3-wells space being left between rows and columns to prevent sample luminescence from affecting readouts in adjacent wells.

This resulted in a dilution series from 1:1 to 1:32,768 (v:v) across 16 samples. Following dilution, plates were placed in a plate reader and had the luminescence measured in each well as relative light units (RLU) using the blue light filter set to 440-460$_{nm}$. Following measurement of luminescence, the plates were enumerated using the protocol described in section 3.5. This experiment was repeated three times for a total of n=3 replicates.

3.25 Assessment of AOP Treatment on OSPW and cNA using a Bacterial Luminescence Inhibition Assay

A flow chart of the bacterial bioluminescence inhibition assay workflow is described in Figure 3.4. All solutions used were placed on ice, and the opaque 96-well plate used was placed on a Echotherm IC22 cooling block set at 15 °C to cool. A freezer stock was thawed and diluted following the procedure described in section 3.24. Following the second dilution, the bacterial suspension was added to an opaque 96-well plate at a concentration of 20 µL/well for all test wells while sterility control wells had 20 µL/well of 2% saline added to them. Following this addition, the inoculated plate was place on a cooling block set at 15 °C and left for 15 minutes. Upon completion of the final waiting period for the inoculated plate, the plate was placed in a plate reader to measure the initial luminescence (I_0) as RLU using the blue light filter set to 440-460$_{nm}$.

While preparing the inoculum, the challenge plates were prepared similar to what is described in section 3.21 for the MIC assay. The pH of all samples to be used was measured and if below 6.5 or above 7.5, the samples were adjusted. All test samples and the regional freshwater control were adjusted to a pH of 7.0 ± 0.2 using either 1 M HCl or 1 M NaOH. It was ensured that adjustment of pH with NaOH and HCl solution did not increase the total volume by greater than 5%. The cNA sample used in this experiment was diluted 1:8 in 2% saline to produce a

starting concentration of 37.5 mg/L. This same procedure was also followed for the 60-minute treated cNA to ensure consistency between samples. For untreated and treated OSPW B, C and D, as well as the treated samples of each, no initial dilutions were done. A similar dilution series was prepared as is described in section 3.21; with the cNA solution, OSPW B, C, and D being suspended at concentrations of 100%, 75%, 50%, and 25% v:v. All test samples and the regional freshwater control were determined to have salinity values below the recommended 20 g/L. As such, osmotic adjustment solution 5.10 was added 1:10 (v:v) to the regional freshwater control and test samples to adjust the salinity to an approximate target range of 20-30 g/L. The adjustment of pH and salinity for samples reduced the starting concentration of these samples to ~91% (v:v).

As opposed to the replicant OSPW, the GC used was 2% saline (v:v) as recommended by the protocol and required no pH or salinity adjustment. A stock of 100 mg/L 3,5- dichlorophenol (Thermo Scientific - AAA1532806) suspended in 2% saline was used as a positive control and was diluted from 100% - 12.5% (v:v) in 2% saline. In the instance that multiple challenge plates were needed, it was determined that samples would have controls established in each plate and these controls would be used to determine luminescence inhibition. This was done to limit the variability introduced on a per plate basis, as each plate had to be inoculated using a new freezer stock while the MIC used a universal inoculum for multiple challenge plates. As it was intended that these samples were to be enumerated in addition to having luminescence measured, sterility controls were prepared in an identical fashion to what is described for the MIC assay. For all test and control samples, 200 µL of each was added to a 96-well plate in triplicate and placed on a cooling block set at 15 °C to cool while inoculum preparation occurred.

After the I_0 reading, 180 µL of all test and control solutions were added to the inoculated plate after which the plate was moved back to the cooling block set to 15 °C. This 9:1 dilution results in all wells being at a final concentration of 81% (v:v). The luminescence was read again at 5, 15, and 30 minutes (I_5, I_{15}, and I_{30}, respectively). Luminescence inhibition was determined by first standardizing all values to the ratio of change observed between the 2% control at I_0 and I_{30}. Following this standardisation, the difference in luminescence from I_0 to I_x for each well was calculated to determine the precent inhibition. Following the final I_{30} reading, all wells in the plate were enumerated following the procedure described in section 3.5. This experiment was repeated three times for a total of n=3 replicates.

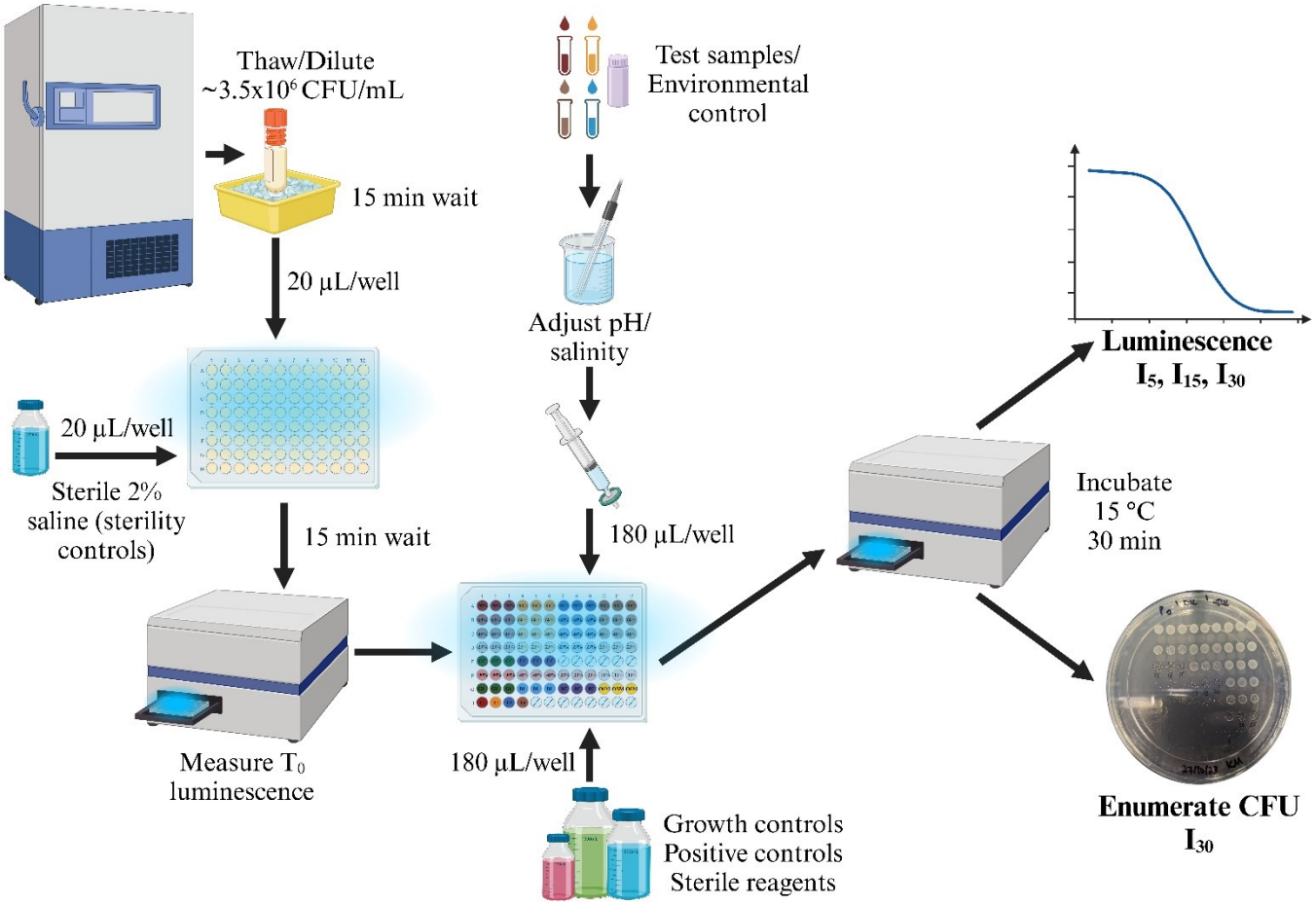

Figure 3.4. General workflow for a bacterial luminescence inhibition assay.

3.26 Statistical Analysis

All data analysis and graphing were done in GraphPad Prism 10.2.3. Datasets from experiments in section 3.9, 3.10, 3.11, 3.15, 3.16, 3.21, and 3.25 were analyzed for normality using a Shapiro-Wilk test. If the dataset was determined to be normally distributed ($\alpha = 0.05$) a Brown-Forsythe and Welch ANOVA test was done, with significance being indicated by $p < 0.001$. Post testing was done using a Dunnett's multiple comparison test with $p < 0.001$ being considered significant. If data was not determined to be normally distributed, a Kruskal-Wallis test with Dunn's posttest was performed, with significance being indicated by $p < 0.001$. Following a Shapiro-Wilk normality test data from experiments in section 3.7, 3.17, and 3.18 was analyzed using a two-way ANOVA with Tukey multiple comparison analysis where significance was indicated by $p < 0.001$. Simple linear regression analysis was done to analyze the relationship between variables in sections 3.8, 3.22, and 3.24 where best fit was determined for 95% confidence intervals. From this analysis, an equation was formed for the line of best fit and the r value was reported. Correlation was also investigated between $\text{Log}_{(\text{CFU/well})}$ reduction data for *S. warneri* and *A. fischeri* and the observed luminescence inhibition for the datasets from section 3.21 and 3.25. This was performed on a per sample basis including controls from the experiment. Similarly, correlation was investigated between $\text{Log}_{(\text{CFU/well})}$ reduction data for *S. warneri* and *A. fischeri* for the datasets from section 3.22 and 3.25. This was performed on a per sample basis including controls from the experiment. The Pearson correlation coefficient was determined for the 95% confidence interval.

Chapter IV

Adapting a modified broth microdilution MIC assay for assessing OSPW toxicity

4.1 Introduction

From a technical perspective, bacterial culturing and assays are generally inexpensive, robust, and highly reproduceable (Viegas et al. 2021). In addition to these advantages, bacteria provide several sub-cytotoxic toxicity endpoints as well as efficient methodologies to determine the impact of toxicant exposures on growth and viability. The generally shorter life cycle of bacteria combined with a smaller scale for testing also allows for a higher throughput approach that requires smaller sample volumes (Viegas et al. 2021). This makes bacteria-based ecotoxicity assessments a potentially valuable tool in making rapid decisions on OSPW treatment, release, and monitoring.

Beyond the technical advantages, bacteria have shown sensitivity to a broad range of compounds of environmental concern including BTEX, hydrocarbons, trace metals, and more recently nanoparticles (Sikkema et al. 1995; Blum and Speece 1991; Ameen et al. 2021). Despite this sensitivity, bacteria also display significant resilience as they occupy and thrive in every possible niche on the planet. Consequently, bacteria must tolerate diverse environmental conditions such as osmolarity, pH, nutrient availability, and even radiation, allowing them to thrive in a dynamic range of conditions that other species would not (Marles-Wright and Lewis 2007). In this study, I assessed the potential to leverage the sensitivity and resiliency of bacteria to develop a system that would be stable under a wide range of freshwater conditions but was sensitive to both whole OSPW and components of concern within these waters. By directly

assessing the effect on growth, I also sought to implement endpoints that were more robust than that of traditional microbial ecotoxicity assessments given the complexity of the waters being tested.

A large component of susceptibility and resistance bacteria display against antimicrobial compounds is associated with the cell wall. The structure of the bacterial cell wall generally divides bacteria, with a few notable exceptions, into Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thick cell wall composed largely of peptidoglycan, but also contains several polymers covalently attached to the cytoplasmic membrane (Salton and Kim 1996). This thick outer layer can confer protection to extreme environmental conditions by maintaining the cell shape against turgor pressure (Garde et al. 2021). Alternatively, the Gram-negative membrane contains a cytoplasmic membrane with a thin layer of peptidoglycan anchored to an outer membrane. This outer membrane contains a vast array of regulatory proteins as well as containing lipopolysaccharides (LPS), which may also provide protection against environmental conditions and antimicrobial activity (Impey et al. 2020). In addition to these structures, bacteria may also possess a capsule and form slime layers and biofilms that further increase their protection against antimicrobial compounds and environmental stress (Mathivanan et al. 2021).

When encountering traditional antimicrobial compounds such as antibiotics, the cell wall plays a major role in toxicity outcomes for organisms. Generally, the Gram-negative membrane is more selective and less permeable, conferring greater resistance to these antimicrobial challenges (Reygaert 2018). The outer membrane requires that even antibiotics that target the cell wall must first pass this outer barrier, while those that target intracellular machinery must pass through both barriers (Miller 2016). Alternatively, the Gram-positive membrane is

significantly less regulated, with less selectivity and a thick layer of peptidoglycan on the exterior of the cell presenting a major target for antibiotics. These factors result in greater antibiotic susceptibility in Gram-positive bacteria when compared to Gram-negative bacteria (Garde et al. 2021, Kohanski et al. 2010). While the relationship between bacterial cell wall and traditional antibiotic susceptibility is well understood, this relationship is not as predictable when investigating other antimicrobial compounds.

Many compounds of environmental concern, especially those found in OSPW, are predicted to exert toxicity through narcosis. This process is by-definition non-selective, forgoing the need for specific target sites that are utilized by antibiotics. Highly lipophilic compounds such as BTEX, PACs, and NAs may deposit in the membrane and thus disrupt structural integrity (Di Toro et al. 2020; Xu et al. 2020; Holowenko et al. 2002). The LPS on the Gram-negative membrane is lipophilic, and while it prevents the access of many hydrophobic compounds, it has been shown to allow highly lipophilic compounds to penetrate the cell wall (Sikkema et al. 1995). Alternatively, hydrophilic compounds, depending on molecular weight, may enter through regulatory proteins called porins embedded in the outer membrane. Despite these possibilities, Gram-negative bacteria generally remain more resilient due to the gross protection afforded by possessing multiple membrane barriers to maintain structural integrity (Sikkema et al. 1995; Bitton et al. 1988).

Similar trends are observed when investigating the effects of trace metals on bacteria. Generally, Gram-negative bacteria have greater tolerance for heavy metal exposure due to the outer membrane preventing easy absorption of metals into the cell (Sterritt and Lester 1980). Metal exposure in bacteria can have a range of detrimental effects from non-specific ion imbalance and membrane disruption to interference with specific systems such as redox reactions

in aerobic respiration (Igiri et al. 2018). While overt cytotoxicity is an outcome for metal exposure in bacteria, a trend which is also described for hydrocarbon compounds, several sublethal effects can also be observed such as inhibition of growth and structural abnormalities (Sterritt and Lester 1980). It must be noted that the effects are heavily species and contaminant dependent, and for almost all the previously discussed sensitivities and susceptibility, there are exceptions and bacteria with specific systems for resolving encounters with these toxicants (Wani et al. 2023).

Given these findings, it is apparent that components of concern in OSPW may have deleterious effects on bacterial physiology. Yet effective assessment of the toxicity of compounds require that organisms are resilient to changes in substrate conditions that these compounds are suspended in. Eukaryotic cells generally require strict regulation of pH in the external environment to maintain the optimal pH of 7.3 inside the cell (Casey et al. 2010). Though bacteria only have a slightly larger observed optimal internal pH range of 6.3 – 8.5, many bacteria persist in hostile environments with external pH conditions ranging from 3.0 – 12.0 (Dhakar and Pandey 2016). To maintain internal pH conditions, bacteria implement both passive and active approaches in concert to regulate pH. Active strategies include uptake and efflux of protons or generation and consumption of protons via enzymatic activity while passive strategies involve structural organization to repel or attract protons (Krulwich et al. 2011). Beyond extremophiles, most neutrophilic bacteria can tolerate a wide range of pH conditions between 4.8 – 9.0 without requiring significant pH regulation strategies or seeing impacts on growth and viability (Slonnczewski et al. 2009).

Another major factor in bacterial survival is the osmolarity of the environment. Both high and low salt environments present potential challenges to microbial growth, with bacteria

possessing a range of passive and active solutions. Halophiles can tolerate salt concentrations in excess of 150 g/L, environments that require significant adaptation to maintain structural integrity under osmotic pressure (Dhakar and Pandey 2016). Conversely, numerous halotolerant bacteria can survive low salt concentrations that similarly threaten structural integrity of the cell. In these settings, compounds such as sugars, alcohols, and amino acids may be used as compatible solutes to maintain turgor pressure (Margesin and Schinner 2001). Bacteria may employ symport or antiport through membrane proteins to maintain cell osmolarity, in addition sequestration or accumulation of ions or compatible solutes through metabolic processes (Wood et al. 2001).

Another important consideration for bacteria growth and survival is the availability of nutrients. Bacterial metabolic diversity is truly staggering and can produce chemical energy via a large range of carbon sources under both oxic and anoxic conditions. Some bacterial species can even generate energy without oxidation of organic compounds using sunlight or inorganic ions as energy sources to drive the production of chemical energy (Jurtshuk 1996). In addition to a carbon and energy sources, bacteria also require crucial compounds such as nitrogen, sulfur and phosphates to grow and perform basic physiological functions (Shimizu 2013). When present in sufficient concentration, as seen in OSM and non-selective media, bacteria may replicate freely. If any one of these factors begins to become limited, bacteria may implement a range of scavenging or sequestration strategies, in addition to activating alternative metabolic pathways to continue to grow (Harder and Dijkhuizen 1983). When facing severe nutrient limitation bacterial growth may be limited, but bacterial populations can persist for extended periods in the absence of nutrients without seeing significant reduction in viable populations (Ferenci 2001; Kjelleberg et al. 1993).

When nutrient, pH, osmolarity, and temperature conditions are optimal for a species, normal metabolic processes and bacterial growth can occur. It is worth noting that like what is mentioned for antimicrobial effects, for all the previously described resiliency, there are examples of organisms that experience significant reductions in growth, metabolic activity, and even survival if any one of these factors deviates from the optimal conditions (Aertsen and Michiels 2008). As such, the establishment of any bacterial toxicity assay requires the validation of a model organism that is relatively stable under changing substrate conditions but displays sensitivity to potential contaminants of concern (ECHA 2017).

Based on these principles numerous bacteria have been investigated and established for l use in toxicity assessment. Disruption of metabolic activity and genotoxicity of bacteria such as *Escherichia coli* or *Salmonella typhimurium* can be investigated using reporter genes being fused to genes associated with hydrolytic enzymes or DNA repair, respectively (EBPI 2024a; EBPI 2024b). Colorimetric assays can also be used to determine the production of metabolic byproducts or membrane damage (Hach 2024; Padilla-Martinez 2015; Viegas et al. 2021). *Aliivibrio fischeri* bioluminescence inhibition assays are the most common bacterial aquatic toxicity test, and while this test will be discussed in detail in Chapter VI, it too uses luminescence as a measure of normal bacterial metabolic activity (Miyamoto et al. 2000; Environment Canada 1992). While using a proxy for normal metabolism or physiological integrity allows for faster interpretation of data, it can be complicated by assay interference common in environmental settings (Ong et al. 2014; Qiu et al. 2017). Though current bacterial luminescence inhibition assays have several challenges that complicate their use in aquatic toxicity, bacteria still have the potential to serve a critical role in ecotoxicity assessment and screening.

While assessing the antimicrobial effects of compounds on bacteria is a valuable tool in an environmental setting, it can be a matter of life and death in a clinical setting. Antimicrobial efficacy testing is crucial in evaluating new antibiotics, monitoring the development of antibiotic resistance in organisms, and developing treatment strategies in a clinical setting (Radlinski and Conlon 2018). In this setting, a select list of approaches has been stringently reviewed and long used to suit this purpose. Even as advanced tools have been developed to discover new antibiotics and monitor antibiotic resistance in the environment, traditional antimicrobial efficacy testing is still relied upon to validate these findings (Zisanur et al. 2022; Ishii 2020). These approaches are primarily broth and agar dilution MIC assays, highly standardized assays where antimicrobial endpoints can be precisely quantified. Broth microdilution, specifically, is the most widely accepted approach for determining antimicrobial activity, delivering quantitative data that is precise, high throughput, and rapid, as is demanded in clinical settings (Balouiri et al. 2016). Broth microdilution assays have strong correlation with other antimicrobial efficacy tests such as the Etest and agar dilution approaches, and have well defined guidelines as per CLSI, a testament to their reliability and effectiveness (Balouiri et al. 2016).

In this thesis chapter I worked to establish and optimize a modified microdilution MIC protocol to investigate the toxicity of NAs and whole OSPW. To do this I also sought to establish relative stability of the assay under similar physiochemical conditions to that observed in whole OSPW and regional freshwater. The specific aims of this chapter were as follows: 1) to screen traditional clinically significant bacteria for sensitivity to NAs, 2) to assess the growth of sensitive species under physiochemical conditions like those observed in freshwater and OSPW, 3) to determine the MIC of sensitive species to NAs and a whole OSPW, and 4) to further optimize the MIC protocol to ensure accuracy and reduce technical burden.

4.2 Results

4.2.1 The effect of naphthenic acid exposure on the growth of clinically significant bacteria

Five clinically significant bacteria were diluted to an estimated density of ~$1x10^6$ CFU/mL in TSB. The inoculum was then added 1:9 (v:v) towels containing cNA solution at concentrations ranging from 300 mg/L to 37.5 mg/L diluted in 0.9% saline. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which all wells were enumerated. Log reduction was calculated by determining the difference in growth between the cNA exposed wells and the 0.9% saline control for each respective organism. Significance was determined by a p-value < 0.001.

Table 4.2 details the average stock and diluted inoculum densities for all organisms used. All the inoculums were within ± $0.3x10^6$ CFU/mL of the targeted $1x10^6$ CFU/mL. No significant reduction in growth was observed for *K. pneumoniae* and *P. aeruginosa* after 22.5 hrs at any of the tested cNA concentrations (Fig. 4.2.1). Alternatively, *E. coli* saw a significant reduction at both 270 mg/L and 135 mg/L with an average log reduction of 1.23 ± 0.22 and 0.42 ± 0.12 Log_{10}(CFU/well), respectively. No significant reduction was observed at 67.5 or 33.75 mg/L (Fig. 4.2.1). *Enterococcus faecalis* saw a significant reduction in growth after exposure to cNA at concentrations of 270, 135, and 75 mg/L with average log reduction values of 2.66 ± 0.16, 1.71 ± 0.34, and 1.05 ± 0.22 Log_{10}(CFU/well), respectively (Fig. 4.2.1). Notably, the growth of *E. faecalis* in the 0.9% saline control was significantly lower at 6.34 ± 0.21 Log_{10}(CFU/well) as opposed to the 8.39 ± 0.10, 8.26 ± 0.11, 8.05 ± 0.22, and 7.12 ± 0.20 Log_{10}(CFU/well) growth observed for *P. aeruginosa, K. pneumoniae, E. coli,* and *S. warneri*.

Of all the organisms assessed, *S. warneri* was the most sensitive to cNA exposure under experimental conditions. At concentrations of 270, 135, 67.5, and 33.75 mg/L cNA, growth was significantly reduced by 3.94 ± 0.22, 3.14 ± 0.26, 2.59 ± 0.25, and 2.01 ± 0.28 Log_{10}(CFU/well), respectively, when compared to the control (Fig. 4.2.1). The growth in the 0.9% saline control for *S. warneri* was ~1 Log_{10}(CFU/well) lower than what was observed for *P. aeruginosa, K. pneumoniae,* and *E. coli* but greater than what was observed for *E. faecalis*.

4.2.2 The relationship between optical density and colony forming units for Staphylococcus warneri

A tube of sterile TSB was inoculated with colonies from a 2° culture of *S. warneri* and diluted in TSB. Diluted cultures had OD_{600nm} measured and were subsequently enumerated to determine CFU/mL. The enumerated CFU/mL was graphed as a function of OD to determine the linear relationship between the variables. Simple linear regression analysis was performed to determine the goodness of fit and equation for the line of best fit with 95% confidence intervals. The slope was determined to be significantly non-zero with a p value of < 0.000001. The equation for the line of best fit was calculated to be $y = 6.57x10^8 x + 1.42x10^6$ with an R^2 value of 0.8415 (Fig. 4.2.2).

4.2.3 The effect of salt concentration on Staphylococcus warneri growth

An inoculum with an estimated density of ~$1x10^6$ CFU/mL was generated in TSB. The inoculum was added 1:9 (v:v) to wells containing NaCl solutions ranging from 3.9% to 0.0011% diluted in UPW or 100% TSB. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which all wells were enumerated. Log growth was compared between all salt

concentrations as well as each concentration and the control. Significance was determined by a p-value < 0.001.

The stock inoculum was determined to have a concentration of 6.67×10^7 CFU/mL and was diluted to a final concentration of 1.10×10^6 CFU/mL. No significant difference in log growth was observed between any of the NaCl concentrations ranging from 3.5% to 0.001% (Fig. 4.2.3). The highest growth observed was the wells with 0.2% NaCl, which had 7.45 ± 0.13 Log_{10}(CFU/well). The wells with 0.001% NaCl had the lowest growth at 7.25 ± 0.07 Log_{10}(CFU/well). While no significant difference was observed between any of the test wells, the growth in all the NaCl test wells was significantly lower than the 8.24 ± 0.11 Log_{10}(CFU/well) in the TSB control by an average of 0.86 Log_{10}(CFU/well) (Fig. 4.2.3).

4.2.4 The effect of pH on Staphylococcus warneri growth

An inoculum with an estimated density of ~1×10^6 CFU/mL was generated in TSB. The inoculum was added 1:9 (v:v) to wells containing 100% TSB that was pH adjusted to values ranging from 5.1 to 12.0 or unmodified 100% TSB. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which all wells were enumerated. Log growth was compared between all adjusted samples as well as each adjusted sample and the control. Significance was determined by a p-value < 0.001.

The initial stock inoculum was determined to have a concentration of 1.08×10^7 CFU/mL and was diluted to a final concentration of 9.44×10^5 CFU/mL. No significant difference in log growth was observed between pH 5.1-10.0 when compared to the unmodified control or any of the other adjusted samples in this range (Fig 4.2.4). At pH 11.2, a significant 1.11 Log_{10}(CFU/well) reduction in growth was observed compared to the unmodified control. This

reduction was increased to 7.48 Log_{10}(CFU/well) at pH 12.0 when compared to the unmodified control. Similarly, at pH 11.2 and 12.0, log growth was significantly less than what was observed in the pH ranges of 5.1-10.0 (Fig 4.2.4).

4.2.5 The effect of media concentration on Staphylococcus warneri growth

An inoculum diluted in either 100% TSB or 0.9% saline was generated with an estimated density of ~$1x10^6$ CFU/mL. The inoculum generated in 100% TSB was added 1:9 (v:v) to wells containing 100% TSB, while the inoculum diluted in 0.9% saline was used to inoculate wells containing TSB diluted in 0.9% saline at concentrations of 50% to 0.08%. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Log growth was compared between all concentrations and significance was determined by a *p*-value < 0.001.

The initial stock inoculum was determined to have a concentration of $2.20x10^7$ CFU/mL and was diluted to a final concentration of $1.08x10^6$ CFU/mL for the TSB inoculum and $1.03x10^6$ CFU/mL for the saline inoculum. There was no significant difference between growth observed at 100% and 50% TSB concentrations (Fig. 4.2.5). Growth at TSB concentrations from 10% to 0.008% were all significantly lower than the 8.07 ± 12 Log_{10}(CFU/well) growth observed in 50% TSB and reduced sequentially from 7.66 ± 0.09 to 2.83 ± 0.21 Log_{10}(CFU/well). Notably, the largest reductions in growth between dilutions were observed at 50% to 10%, 0.63 to 0.31%, and 0.31% to 0.16% with differences of 0.41, 1.51 and 2.03 Log_{10}(CFU/well), respectively (Fig 4.2.5).

4.2.6 The effect of whole OSPW and an OSPW control on Staphylococcus warneri growth

An inoculum with an estimated density of ~$1x10^6$ CFU/mL was generated in TSB. The inoculum was added 1:9 (v:v) to wells containing OSPW A at concentrations of 100%, 75%, 50% and 25% diluted in OSPW replicant, 0.9% saline, 100% OSPW replicant, or 100% TSB. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Log reduction was calculated by determining the difference in growth between the TSB control wells and the wells with saline, OSPW replicant, OSPW A. Log reduction was also calculated by assessing the difference between the OSPW replicant and OSPW A at all concentrations to determine the MIC. Significance was determined by a *p*-value < 0.001.

The initial stock inoculum was determined to have a concentration of $1.52x10^7$ CFU/mL and was diluted to a final concentration of $1.00x10^6$ CFU/mL. There was a significant 0.48 ± 0.14 Log_{10}(CFU/well) reduction in growth for the wells with OSPW replicant when compared to the 100% TSB (Fig. 4.2.6). This reduction was significantly smaller than the 1.10 ± 0.13 Log_{10}(CFU/well) reduction observed for wells with 0.9% saline. At 90% concentration, OSPW A produced a significant 3.09 ± 0.16 Log_{10}(CFU/well) reduction in growth compared to the 100% TSB control, a value that was also significantly higher than what was observed for the OSPW replicant and 0.9% saline (Fig. 4.2.6). When Log reduction was calculated using the 90% OSPW replicant as a reference control, a MIC of 45 - 22.5% was determined with a 0.60 ± 0.13 and -0.09 ± 0.28 Log_{10}(CFU/well) reduction in growth, respectively.

4.2.7 Determination of the MIC of commercial naphthenic acids for Staphylococcus warneri

An inoculum with an estimated density of ~$1x10^6$ CFU/mL was generated in TSB. The inoculum was added 1:9 (v:v) to wells containing cNA solution at concentrations of 33.3 to 2.1 mg/L in OSPW replicant, 100% regional freshwater, dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in OSPW replicant, or 100% OSPW replicant. Challenge plates were incubated for

22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Log reduction was calculated by determining the difference in growth between all wells and the OSPW replicant wells. Significance was determined by a *p*-value < 0.001.

The initial stock inoculum was determined to have a concentration of $1.73x10^8$ CFU/mL and was diluted to a final concentration of $1.04x10^6$ CFU/mL. A significant dose dependent log reduction was observed for cNA concentrations from 30.0 mg/L to 3.8 mg/L with values ranging from 3.81 to 2.26 Log_{10}(CFU/well) reduction, respectively. This resulted in a MIC of 3.8 mg/L - 1.9 mg/L (Table 6.2). No significant log reduction was observed for the regional freshwater control, with a value of 0.23 ± 0.20 Log_{10}(CFU/well) (Fig 4.2.7). The dichlorophenol positive control produced a significant dose-dependent log reduction in growth that ranged from 7.99 ± 0.40 to 1.86 ± 0.70 Log_{10}(CFU/well) at 90.0 mg/L and 11.3 mg/L, respectively. Notably, a 3.43 ± 0.19 Log_{10}(CFU/well) reduction in growth was observed at 22.5 mg/L dichlorophenol concentration (Fig 4.2.7).

4.2.8 The effect of evaporation in microplates on Staphylococcus warneri growth

An inoculum with an estimated density of ~$1x10^6$ CFU/mL was generated in TSB. The inoculum was added 1:9 (v:v) to wells containing 100% OSPW A or 100% OSPW replicant. Prior to incubation, one plate was sealed with parafilm. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Log growth was compared between samples in the parafilmed and non-parafilmed plate. Additionally, Log growth was compared between wells at the edge of the plate and in the middle of the plate. Liquid was also extracted from wells in the middle and edge of the plate and compared in weight to estimate the volume evaporated. Significance was determined by a *p*-value < 0.001.

The initial stock inoculum was determined to have a concentration of $5.13x10^7$ CFU/mL and was diluted to a final concentration of $9.56x10^5$ CFU/mL. There was no significant difference in log growth observed between the parafilmed and non-parafilmed plate for both the replicant control and OSPW A wells (Fig. 4.2.8). Furthermore, no significant difference in log growth was observed between wells in the middle and edge of the plate for both the OSPW A and replicant control wells in the parafilmed and non-parafilmed plate. No significant difference was observed in the weight of wells between the parafilmed and non-parafilmed plates. There was an average ~5.32 mg less weighed fluid in the wells at the edge of the plate when compared to those in the middle for both parafilmed and non-parafilmed plates, however this value was not determined to be significant.

4.2.9 Generation of a Staphylococcus warneri freezer inoculum for an environmental MIC

An O/N culture was prepared and diluted in TSB before being diluted again in cryopreservation solution and frozen at -80 °C. The initial diluted O/N culture and the cryopreservation diluted O/N culture pre- and post-freezing were all enumerated to verify cell density. A diluted freezer inoculum and traditional inoculum both at ~$1x10^6$ CFU/mL were added 1:9 (v:v) to wells containing 100% OSPW A and 100% OSPW replicant. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which all wells were enumerated. Log reduction was calculated by determining the difference in growth between all difference between the OSPW A and the OSPW replicant wells for each inoculum approach. Additionally, the log growth was compared for both OSPW A and the OSPW replicant for each inoculum approaches. Significance was determined by a p-value < 0.001.

It was determined that the initial O/N culture contained 1.30 ± 0.04 $x10^9$ CFU/mL. Following dilution in cryopreservation media and prior to freezing it was determined that the

freezer inoculum had a density of 1.17 ± 0.04 x10^8 CFU/mL. Following freezing, the thawed freezer inoculums were determined to have a density of 8.89 ± 0.015 x10^6 CFU/mL. The freezer inoculum was adjusted to a final density of 1.29x10^6 CFU/mL while the agar inoculum was adjusted to a density of 1.00x10^6 CFU/mL. When inoculated using the freezer inoculum a log reduction of 2.06 ± 26 Log_{10}(CFU/well) was observed after growth in 90% OSPW A. This was significantly lower and had far greater variability that the 2.56 ± 0.06 Log_{10}(CFU/well) reduction in growth observed for the agar inoculum. Both the 90% OSPW A and 90% OSPW replicant wells experienced significantly better growth when inoculated using the agar inoculum compared to the freezer inoculum (Fig. 4.2.8).

4.3 Discussion

In this chapter, I investigated the use of a modified microdilution MIC assay to test OSPW samples and doses of cNAs for toxicity. This was performed by screening a selection of bacteria for potential sensitivity to NAs. Upon determining sensitive species, the stability of the organism was established under freshwater test conditions before determining sensitivity to whole OSPW and cNA. Overall, a modified microdilution MIC assay using *S. warneri* experienced consistent growth that was not inhibited under freshwater and simulated OSPW conditions. In addition to this stability, the assay showed sensitivity to cNA and whole OSPW. As performed, this modified microdilution MIC approach leverages the precision of culture-based enumeration techniques to reliably screen samples for toxicity.

4.3.1 Species screening

Traditional microdilution MIC approaches can be further enhanced by directly enumerating bacteria following exposures. Despite impressive advancements in genetics and

proteomics technologies used to identify the presence of bacteria, culture-based techniques remain a major driver in microbiological research and biotechnology (Prakash et al. 2013). More specifically, the use of serial dilution and spot platting to estimate microbial load, is the most widely implemented techniques to quantify bacteria across microbiology. Even in the face of accurate modern techniques such as flow cytometry and quantitative reverse transcription polymerase chain reaction (RT-qPCR), serial dilution and spot platting remains a precise and cost-effective method to enumerate culturable bacteria, especially when initial concentrations are not known (Davis 2014). This approach has the additional advantage of confirming culture purity and identifying potential sublethal effects on colony morphology. As such, serial dilution and spot plate approaches were implemented to precisely quantify bacterial growth under exposure conditions.

To determine the relative sensitivity of these approaches to one of the major compounds of concern in OSPW, a stock solution of commercial grade naphthenic acid was generated. Commercially available NA solutions do not reflect the complexity of the NAs present in OSPW, as these solutions tend to be formed of primarily classical NA species as opposed to the mixture observed in real whole OSPW (Marentette et al 2015). These compounds produce different toxicity responses when compared to fractionated or whole OSPW, which have complex interactions between compounds of concern that can greatly impact toxicity (Bartlett et al. 2017; Brown and Ulrich 2015; Tollefsen et al. 2012). Despite this, these compounds can serve as valuable tools to investigate the mechanisms of toxicity of NAs or as an indicator of NA sensitivity in systems. This is partially due to their well characterized chemical properties and composition compared to extracted NAs, allowing for easier interpretation of results from a toxicity perspective (Garcia-Garcia er al. 2011; West et al. 2011).

The five organisms tested for sensitivity represent a relatively diverse range of genera with unique physiology. In general, the Gram-positive organisms were more sensitive to the cNA exposure than the Gram-negative bacteria. These findings are not entirely unexpected, as based on molecular mass alone, many NA compounds would be too large to easily pass through the outer membrane. Highly lipophilic compounds have been shown to penetrate the membrane through interaction with LPS, but this phenomenon is more commonly observed with compound such as steroids (Plesiat and Nikaido 1992). With respect to cyclic hydrocarbons, toxicity requires integration and disruption of the membrane, a process that is more likely to occur in Gram-positive bacteria which lack the additional permeability barrier of the outer membrane. This results in greater observed toxicity of hydrocarbons and highly lipophilic compounds for Gram-positive bacteria (Lazaroaie 2010; Sikkema 1995).

The observed resistance in *P. aeruginosa* and *K. pneumonia* aligns with research which shows that numerous *Klebsiella* and *Pseudomonas* spp. have high tolerance to aromatic hydrocarbon compounds. When exposed to hydrocarbons, these organisms can implement a range of mechanisms including adaptations in the phospholipid and fatty acid composition of the membrane, energy-dependent efflux, and vesicle formation (Ramos et al. 2002). In addition to these specific hydrocarbon tolerance mechanisms, both *K. pneumoniae* and *P. aeruginosa* show high levels of resistance to a range of antimicrobial compounds due to low membrane permeability and strong biofilm formation (Lordelo et al. 2024). As a result, *K. pneumoniae* and *P. aeruginosa* strains are frequently isolated from petroleum extraction settings. These strains show high levels of tolerance to aromatic hydrocarbons and can facilitate the degradation of these compounds (You et al. 2018). Combined, these factors make these organisms better suited

to remediation uses, though the high levels of tolerance to aromatic hydrocarbons and NAs can allow for sub-cytotoxic assessments of the effects of these compounds (Bookout et al. 2024).

Based on these findings, it was determined that the modified microdilution MIC would use *S. warneri* NCTC 5955 due to its high sensitivity to cNA. While *E. faecalis* also showed similar sensitivity, it had significantly weaker growth in the 0.9% saline control than *S. warneri*. This may be in part due to the organism being grown in TSB as opposed to brain heart infusion (BHI) agar/broth that is recommended by ATCC (ATTC 2022). Though TSB and BHI are both common choices for growing *E. faecalis* that result in similar planktonic growth kinetics and density, it is noted that TSB does favour the formation of biofilms. The use of TSB and low media concentration can stimulate biofilm formation, potentially resulting in a lower proportion of recovered planktonic CFU (Seneviratne et al. 2023; Gelinas et al. 2021). Under these test conditions it is difficult to determine if this phenomenon accounts for the lower growth observed, or the if the low media concentration (10% v:v) in isolation limited the observed growth.

Staphylococcus warneri is a Gram-positive, non-motile, coagulase-negative staphylococci (CoNS) commonly found as part of the human skin microbiome. As a facultative anaerobe, *S. warneri* can utilize a range of carbon sources through both fermentation and respiration pathways (Schleifer and Bell 2015). Genomic analysis suggests the closest relative to *S. warneri* is *Staphylococcus pasteuri*, but it is also closely related to both *Staphylococcus aureus* and *Staphylococcus epidermidis* (Liu et al. 2020). This organism is one of the most abundant members of the healthy skin microbiota, though it has been isolated from nosocomial infections suggesting it may be an opportunistic pathogen (Kanuparthy et al. 2020). There is relatively little information on this organism's tolerance to compounds of ecotoxicological concern, though isolates have been shown to be resistant to organic solvents when cultured in the presence of

these compounds. This organism is also a strong biofilm former compared to other *Staphylococcus* spp., with similar tolerance to harsh environmental conditions such as salinity and pH (Liu et al. 2020; Fu et al. 2013). Limited research exists to inform the suitability of this organism for environmental toxicity testing beyond a predicted tolerance for environmental stress, though the initial susceptibility observed to cNA appeared promising.

4.3.2 Optimization of a modified microdilution MIC assay for OSPW toxicity assessment

The use of OD is a rapid, effective technique to estimate bacterial density that is precise in most substrates and often is well correlated with other enumeration approaches. Though accurate, this approach must be validated for each organism, as factors such as cell arrangement and body size can influence the relationship between OD and CFU (Szermer-Olearnik et al. 2014). Preliminary data revealed that the digital tool used overestimated the cell density present in the stock inoculum based on OD. This result was not expected, as the tool was developed using *E. coli*, which has a larger cell size than staphylococci, likely leading to it underestimating the cell density of the smaller cell (Schleifer and Bell 2015; Scheutz and Stockbine 2015).

It is possible that the arrangement of staphylococci in clusters leads to the overestimation of cell density via OD, as multiple cells can contribute to photometric interference at levels that align with what is seen for a single cell of the larger *E. coli*. This possibility is unlikely however, as a similar effect would be observed for CFU enumeration data where multiple clustered cells grow into a single colony (Beal et al. 2020). This has been investigated for *S. aureus* where it was determined that cell aggregation can increase variation but overall does not significantly impact the accuracy of OD for estimating cell density (Haaber et al. 2016). Another possibility is the presence of dead or quiescent cells that contribute to OD, but do not produce countable colonies via culture dependent enumeration techniques (Beal et al. 2020). Regardless of the

cause, the relationship as defined by simple linear regression proved accurate and precise in estimating the CFU from OD for inoculum preparation.

As previously discussed, for validation of this assay under OSPW and freshwater conditions it was important to ensure stability of organism growth under a range of physiochemical conditions that may be encountered. One of the first parameters that was investigated was salinity. In luminescence inhibition protocols using *A. fischeri* samples must be adjusted to a minimum 2% (w:v) salinity to prevent inhibitory effects associated with osmotic stress. This adjustment is not ideal in whole effluent assessment as osmotic adjustment results in sample dilution and potential changes in toxicity as it transitions from freshwater to brackish conditions (Environment Canada 1992). As members of the skin microbiota, many *Staphylococci* species display halotolerance with good growth between 7.5% – 10% salinity, and the potential to tolerate concentrations greater than 15%. *Staphylococcus warneri* specifically has been demonstrated to have optimal growth at salinity concentrations as high as 10% (Schleifer and Bell 2015). While there is a large body of literature that analyzes the halotolerance of *Staphylococci* spp., there is limited discussion on its survival in freshwater and low salt conditions. As OSPW and freshwater samples have salinity values between 0.25% – 0.01% (w:v), it was imperative to investigate this organism's tolerance to low salt conditions like those seen in freshwater environments (Table 3.2).

Based on the observed growth, concentrations of NaCl ranging from 3.5% – 0.001% (w:v) produced no differential growth between each other, however, growth in this range was lower than the 100% TSB control. This disparity likely has less to do with salinity and more to do with the optimal nutrient conditions provided by the 100% growth media as opposed to the 10% media conditions of the test samples. It is worth noting that under exposure conditions of

this MIC protocol the minimum salinity exposure would be 0.05% (w:v). The TSB used for inoculum preparation had a salinity of 0.5% NaCl (w:v) when prepared as per manufacturer instructions (Table 3.1). The organism was suspended in 100% TSB to generate the inoculum and then diluted 1:9 (v:v) in the test sample. Even if it is assumed that the test sample provides no additional salinity, this would still result in a concentration of 0.05% NaCl. The data shows that at this salinity and greater, there is no significant impact on growth, suggesting stability under freshwater and OSPW conditions. This data aligns with research showing *Staphylococcus* spp. can survive and readily transition between freshwater and marine conditions (Levin-Edens et al. 2011; Silva et al. 2020). This tolerance provides *S. warneri* with a unique advantage to toxicity approaches that use freshwater organisms such as *Ceriodaphnia,* which can be perturbed by the salinity of OSPW (Zubot et al. 2021).

A similar investigation was done to determine the tolerance of *S. warneri* under a range of pH conditions. As with salinity, performance of luminescence inhibition assays using *A. fischeri* require adjustment of pH to a range of 6.0 – 8.5 to prevent off-target effects of sample pH on luminescence. Depending on the buffering capacity of the sample, this generally does not result in significant dilution of the sample but can still have effects on sample chemistry and toxicity as acid or base is added to the sample (Environment Canda 1992). As commensal skin bacteria, *Staphylococci* are well adapted to survive the relatively low pH (5.0 – 5.5) of the skin. The tolerance of these organisms at higher pH can be species dependent, with frequently cited values for *S. epidermidis* and *S. aureus* being around 7.5 and 9.3, respectively (Le loir et al. 2003; Iyer et al. 2021).

My data shows optimal growth at pH values ranging from 5.1 – 10.0, with significant reductions in growth observed outside this range. This is consistent with findings that

demonstrate *Staphylococci* pH tolerance at values exceeding 9.5 under low salt conditions (< 5.8%) (Vaish er al. 2019). Beyond pH tolerance, it must also be noted that the TSB growth media used does contain K_2HPO_4, a known buffering agent (Becton Dickinson 2022). As the inoculum is suspended in TSB and then diluted into the sample 1:9 (v:v), this may introduce some buffering capacity into the sample in addition to any tolerance the organism may have for alkalinity. The OSPW and freshwater used tended to be alkaline with values around 7.6 – 8.7 (Table 3.2), which are well within the range of optimal growth for this organism (Iyer et al. 2021). Optimization of AOP treatment can involve pH adjustment prior to treatment and can result in pH changes due to the production of radicals. Treatment with PMS and PDS oxidants can produce pH values ranging from 5.7 – 10.0, making the observed tolerance crucial for assessing AOP treated OSPW (Fernandes et al. 2018). Resident microbial communities that have been isolated from OSPW have been identified as having high tolerance for salinity and alkaline pH values (Miles et al. 2019). As I am attempting to isolate the effects of whole OSPW toxicity to constituent compounds such as NAs and trace metals, high tolerance for fluctuations in basic physiochemical parameters such as pH and salinity can help prevent off target inhibitory effects.

In toxicity assessment, it has been previously demonstrated that decreasing nutrient concentration can increases sensitivity of compounds tested (Lenz et al. 1986). Traditional broth microdilution approaches have the advantage of suspending test compounds in OSM, allowing for antimicrobial effects to be isolated to the addition of the compound tested. For environmental sampling, this is challenging to do, requiring inoculation of the plate at the desired CFU/well, centrifugation and subsequent aspiration of the supernatant, and then resuspension of the bacteria in test solutions. This would generate an exposure concentration of ~100% but would introduce several drawbacks in addition to increasing the technical burden. In some settings centrifugation

has been shown to affect bacteria physiology, changing cell structure and adherence behaviour (Peterson et al. 2012). Beyond this, the only nutrients available to the bacteria in each well would be introduced by the test sample. As the OSPW samples used are environmental samples, they have their own community of microbial constituents (Hussain and Stafford 2023), as partially demonstrated by the previous filtration experiment in section 3.6. While filtration was shown to effectively remove these constituents, a range of metabolic and cellular byproducts would remain in solution that could be used as growth factors for the test organism. This would be challenging to standardize and may lead to variation in growth between test samples that is not related to the effects of any toxic constituents.

To overcome the challenges mentioned previously, it was determined that an effective approach would be to suspend the inoculum in TSB. This ensures that each test sample has been supplemented with optimal growth media as to standardize the 'potential' for growth. As such, I investigated the minimum concentration of TSB the organism could be provided while still expecting growth. My data showed that there was no significant reduction in growth at 50% TSB, but this concentration would not be an improvement on the ~81% exposure concentration of luminescence inhibition approaches (Environment Canada 1992). The growth observed at 10% TSB marked the first significant decrease in growth compared to the 100% TSB sample. It was determined that this would be the minimum concentration of TSB provided to the organism to allow for the highest exposure concentration of test samples while still ensuring near optimal growth.

From the previous three experiments, *S. warneri* showed stability under a range of physicochemical parameters at and beyond what would exist in freshwater, untreated OSPW, and treated OSPW. However, these findings must be tempered by two major considerations. The first

is that the endpoint of these experiments is enumeration, which provides insight into the number of cells present at the end of the experiment. Significant deviation in pH, salinity, and nutrient concentration can all result in similar growth at the end of incubation, but dramatically different kinetics of growth. This phenomenon has been demonstrated for *Staphylococcus* spp. where pH values outside of optimum will affect growth kinetics while still resulting in the same final microbial density (Iyer et al. 2021). Similarly, as salt concentrations increase, *S. aureus* can take longer to reach the same terminal growth density (Toshiro 1999). These affects can also be observed in compounds of environmental concern such as metals and hydrocarbons, where sub-cytotoxic doses often lead to disruption of normal growth kinetics (Sikkema et al. 1995; Sterritt and Lester 1980). The implementation of a 22.5 hr endpoint that measures terminal growth would not be able to assess these effects unless inhibition was severe enough to limit the final bacterial density. The most effective way to determine these effects is by monitoring growth kinetics via OD, though this approach in isolation has several caveats that will be discussed in detail in the next chapter.

The second consideration is that the effects of environmental and antimicrobial stress on bacteria is often cumulative. This has been displayed for *Staphylococcus* spp. where increasing concentration of salt can decrease pH tolerance (Vaish et al. 2019). These interactions extend to factors such as nutrient concentration and temperature, which can cause cumulative stress that decreases bacterial growth (Whiting et al. 1996). The increased stress incurred by factors such as salinity can also increase the sensitivity of bacteria to antimicrobial compounds, making sensitivity unpredictable under stress conditions (Ganjian et al. 2012). Conversely, stress can induce biofilm formation, greatly increasing resistance to both environmental factors and antimicrobial activity (Mack et al. 2004). If stress conditions are severe enough, it can induce a

viable but non-culturable (VBNC) state, which greatly increases bacterial survivability to environmental and antimicrobial stresses (Li et al. 2020). Based on the data in Table 3.2, many of the physiochemical parameters of the samples tested are well within optimal ranges for *Staphylococcus* spp. and would be unlikely to induce significant stress responses. However, it must be acknowledged that it is possible that these factors may have synergistic effects that could increases or decrease the observed sensitivity of the organism to toxicants of concern such as NAs.

With the stability of the organism established under treated OSPW, untreated OSPW, and freshwater conditions, the next effort was to determine the sensitivity of this assay to a whole OSPW sample. To assist with this process, a new control was prepared to better represent OSPW conditions. This control had lower salinity than the 0.9% saline previously used but was supplemented with additional physiologically important ions such as magnesium and calcium, as well as adjusting the pH to 8.4. In retrospect, supplementation with environmentally relevant concentrations of potassium should have been considered due to its crucial role in pH regulation, proton motive force generation, and metabolism (Gries et al. 2016). The TSB media used for the inoculum does contain potassium, however; this would be diluted significantly in the control media compared to what is seen in the OSPW and environmental samples (Table 3.2). Ultimately, this would lead to more favourable growth conditions in the test sample, potentially underestimating of the effects of toxicants in the sample.

Despite this potential limitation, the OSPW replicant showed significantly better growth than the 0.9% saline that was previously used. While this growth was still lower than 100% TSB, the sub-log reduction demonstrated the stability of the organism under these control conditions. The assay showed dose dependent effects of OSPW exposure on growth with a MIC of 45% –

22.5%, comparable to the 2 – 100% that has been previously described for whole OSPW for several *in vivo* and *in vitro* approaches, including bacterial luminescence inhibition (Allen 2008a; Li et al. 2017). However, these comparisons have limited value due to the source dependent physiochemistry that makes OSPW samples distinct from one another. In addition to this variation, researchers often implement inconsistent sampling and chemical analysis approaches, making comparisons between OSPW samples challenging (Mahaffey and Dube 2017).

To implement a more standardized measurement of NA sensitivity, a cNA solution was prepared. As previously discussed, these solutions are not representative of the complexities of OSPW, but are chemically well defined, allowing for easier interpretation of toxicity data than what is seen for extracted NAs or whole OSPW. The MIC approach showed a cutoff value of 3.8 – 1.9 mg/L. This value is comparable to some of the most sensitive model organisms such as *P. promelas* and numerous aquatic invertebrates which often have LC_{50}/IC_{50} values below 5 mg/L for similar commercial solutions (Marentette et al. 2015; Swigert et al. 2015; Kinley et al. 2016). These values are also significantly lower than what has been seen for bacterial luminescence approaches, with EC_{50} values that often range from 15 mg/L – 50 mg/L (Bartlett et al. 2017; Swigert et al. 2015; Tollefson et al. 2015). These values are more closely comparable to sub-lethal experiments that investigate transcriptional changes induced by NA exposure, with sensitivities often below 2.5 mg/L (Garcia-Garcia et al. 2011a; Bookout et al. 2024). While cNA solutions are considerably more standardized than whole OSPW samples, similar variation in solution preparation, NA supplier, and protocol implementation can lead to variation in results (West et al. 2011; Marentette et al. 2015).

This experiment also introduced two controls to help standardize the results. The first was a 3, 5-dichlorophenol positive control, a known antimicrobial compound used as a toxicity

standard in bacterial luminescence inhibition protocols. This compound showed a significant dose dependent bactericidal effect, consistent with what is expected for phenolic compounds (Bennett 1959). This specific compound was chosen as it is used as a toxicity standard in bacterial luminescence inhibition protocols, with these protocols citing an expected I_5 IC_{50} of 9 – 26 mg/L (Environment Canada 1992; ISO 2007; Microbics 1995). Given the large difference in exposure time between these protocols (22.5 hrs compared to 5 mins), no specific result was expected, however, this data allowed for comparison between experiments to ensure consistency in sensitivity.

In additional to this positive control, a sample of water was taken from a recreation lake located in Northern Alberta. While the replicant control does replicate OSPW conditions, this control is generated in lab using pure reagents and under sterile conditions. Though the freshwater sample was filtered to remove microbial contaminants, it served as a negative control that exposed the organism to freshwater conditions that have been in contact with both abiotic and biotic factors from the environment. This control did not produce a significant reduction in growth, an expected result given that *Staphylococcus* spp. have been shown to survive under freshwater conditions (Levin-Edens et al. 2011; Silva et al. 2020).

With the relative sensitivity of the modified microdilution MIC approach to OSPW and cNA established, additional efforts were undertaken to optimize the protocol. Edge effects are well characterized phenomena in assays that use microplates. Wells at the edge of the plate are subject to higher levels of evaporation than those in the middle, resulting in regional variation for bioassays (Lau et al. 2015). To prevent this regional variation, traditional MIC assays recommend parafilming plates or incubating them in a sealed bag (CLSI 2018), though these actions can introduce unwanted condensation and can even effect organism growth (Shinn-

Thomas et al. 2019). Overall, these experiments showed that wells at the edge of the plate had less volume than those in the middle, but this difference was similar whether parafilmed or not. In terms of growth, no significant difference was observed between the middle and edge of the plate, or between parafilmed and non-parafilmed plates for either control or test wells. This data suggests that the use of a humidified incubator was sufficient to prevent significant evaporation and edge effects in these experiments.

To attempt to reduce the technical burden, the preparation of freezer inoculums was investigated to try and remove the need for 1° and 2° culturing. While not necessarily technically demanding, the generation of 1° and 2° cultures does require that culturing begins at least 48 hrs prior to the start of experiments (this time is shortened to 24 hrs if the 1° has already been prepared). Protocols for bacterial luminescence inhibition use either frozen inoculums or freeze-dried cultures to allow for rapid test initiation (Environment Canada 1992; ISO 2007; Microbics 1995). While these procedures reduce assay start time and technical burden, they must be used responsibly as both freeze-drying and simply freezing bacteria has shown to have deleterious effects on microbial growth, antimicrobial sensitivity, and stress response (Ray and Speck 1973; Sinskey and Silverman 1969; Humphrey and Cruickshank 1985). From my experiment it was shown that freezer inoculums did not increase sensitivity to OSPW but rather decreased growth in both control and test samples in similar proportions. As a result of these findings, it was determined that a freezer inoculum would not be used to directly perform experiments due to the detrimental effects on growth.

4.4 Future Directions

Overall, my findings suggest that a modified microdilution MIC assay using *S. warneri* is stable under basic physiochemical parameters found in freshwater and OSPW, but sensitive to

NAs, the main toxicant of concern present in OSPW. Given that numerous stress conditions result in changes on organism growth kinetics, I determined that OD would be monitored in future experiments to further explore how the control solutions and test samples are affecting microbial growth. These approaches would also allow us to detect inhibitory effects on growth kinetics that may not be observed by a single enumeration endpoint. Additionally, as toxicity to compounds can be difficult to compare using literature due to inter-lab variation, additional toxicity models should be used to interrogate the same samples to allow for a more direct comparison of sensitivity, as was done in later chapters. Lastly, genotypic analysis could allow for investigation of cumulative stress on the bacteria. Investigation of genes associated with pH and osmotolerance, as well as those associated with membrane structure and DNA repair may allow for determination of physiological systems that are affected by OSPW exposure.

Table 4.2. Average inoculum density.

Inoculum Density (CFU/mL)	*E. coli*	*S. warneri*	*P. aeruginosa*	*K. pneumoniae*	*E. faecalis*
Stock	$9.89x10^8$	$4.56x10^8$	$1.76x10^9$	$1.08x10^9$	$5.29x10^8$
Diluted	$1.18x10^6$	$1.26x10^6$	$1.19x10^6$	$1.11x10^6$	$2.93x10^6$

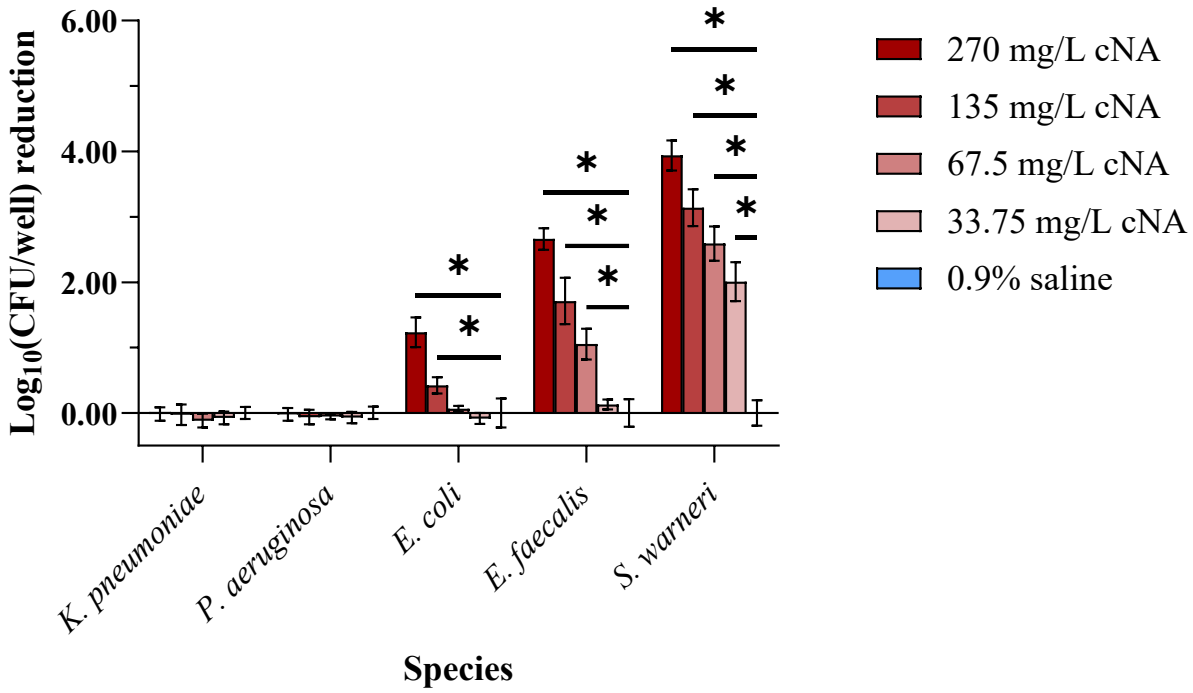

Figure 4.2.1. Effect of naphthenic acid exposure on the growth of various bacteria. Bacteria were suspended at an average density of $1.11x10^6$ CFU/mL in TSB. The inoculum was then diluted 1:9 (v:v) in 0.9% saline or cNA solution diluted from 300 mg/L to 37.5 mg/L in 0.9% saline. Plates were incubated at 35 °C for 22.5 hrs, 282 rpm after which the wells were enumerated. Bars represent mean Log reduction ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a two-way ANOVA was performed with Tukey post test. * represent significance ($p < 0.001$).

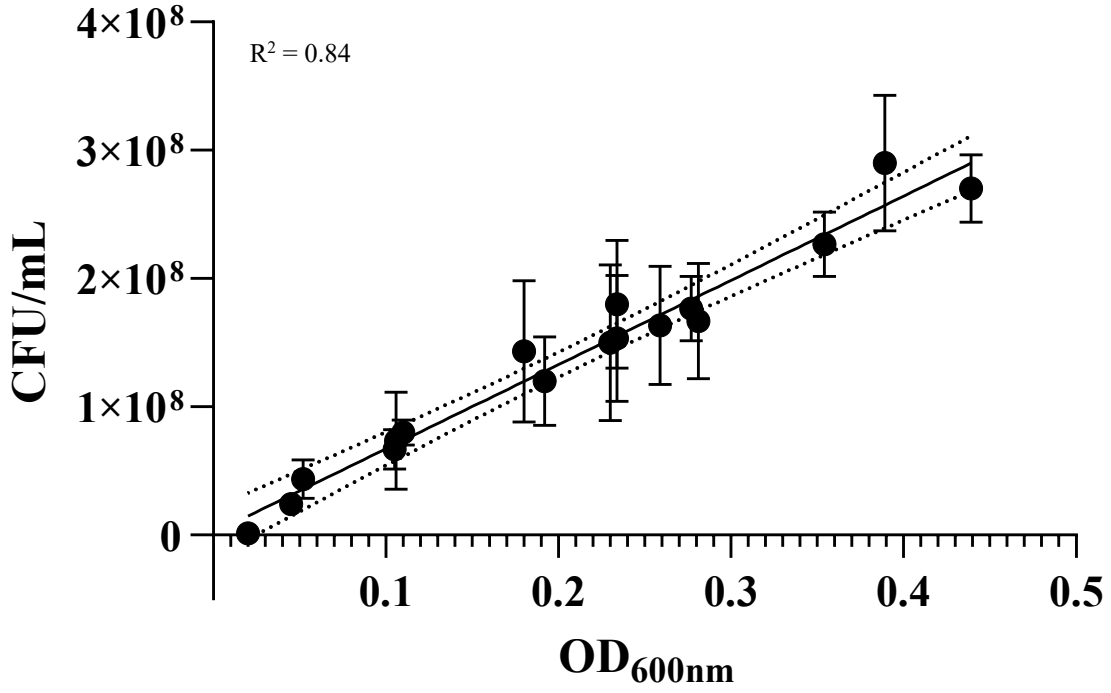

Figure 4.2.2. Relationship between CFU/mL and optical density for *Staphylococcus warneri*. Colonies were suspended from solid agar in TSB and diluted. Following dilution optical density at 600_{nm} was measured. After OD_{600nm} measurement, dilutions were serially diluted and spot plated for enumeration. Simple linear regression analysis was performed. The line represents best-fit with 95% confidence intervals (n=16).

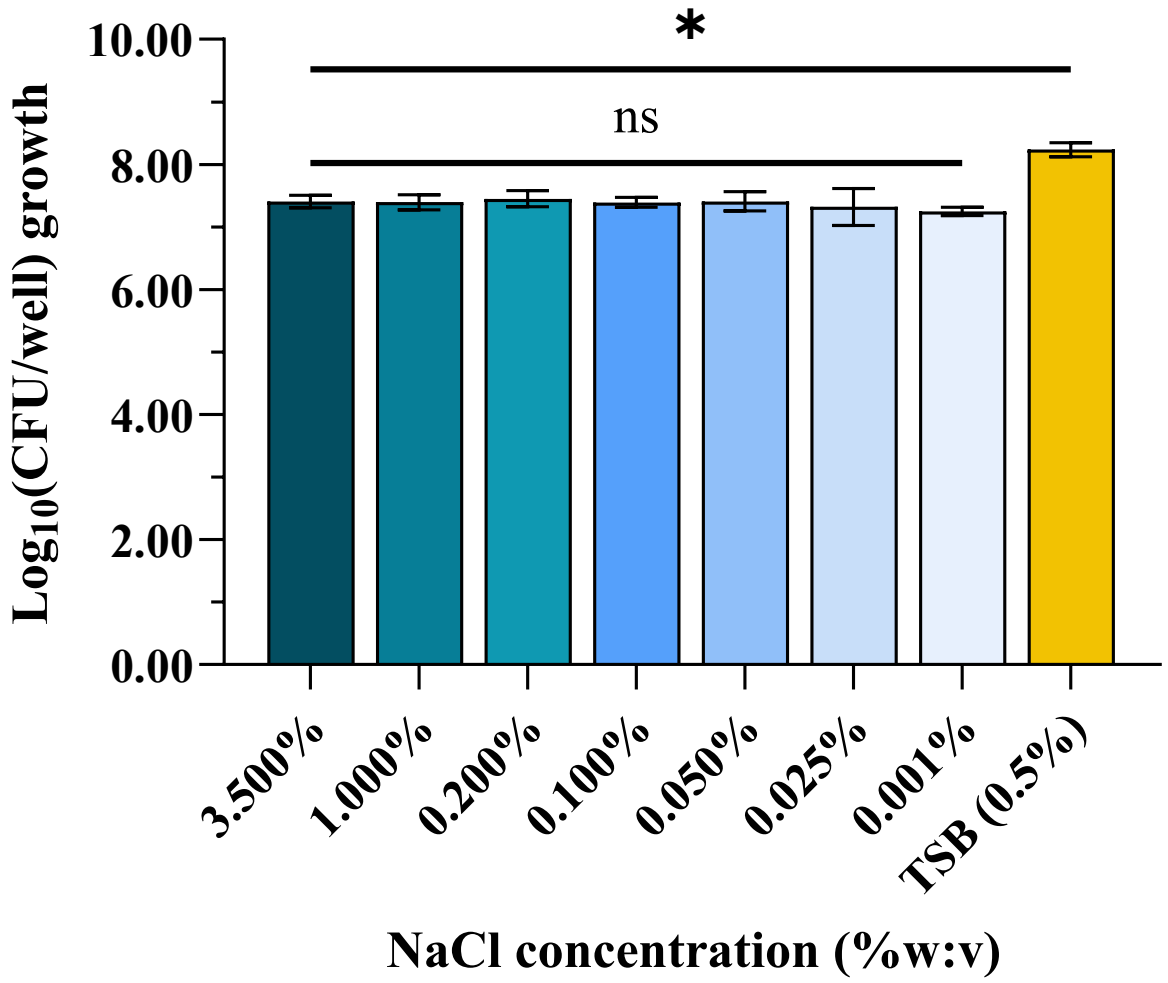

Figure 4.2.3. Effect of NaCl concentration on *Staphylococcus warneri* growth. Bacteria were suspended at an average density of 1.10x10^6 CFU/mL in TSB. The inoculum was added 1:9 (v:v) to 100% TSB or NaCl solutions ranging from 3.9% to 0.0011% diluted in UPW. Plates were incubated at 35 °C for 22.5 hrs, 282 rpm after which the wells were enumerated. Bars represent mean Log growth ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

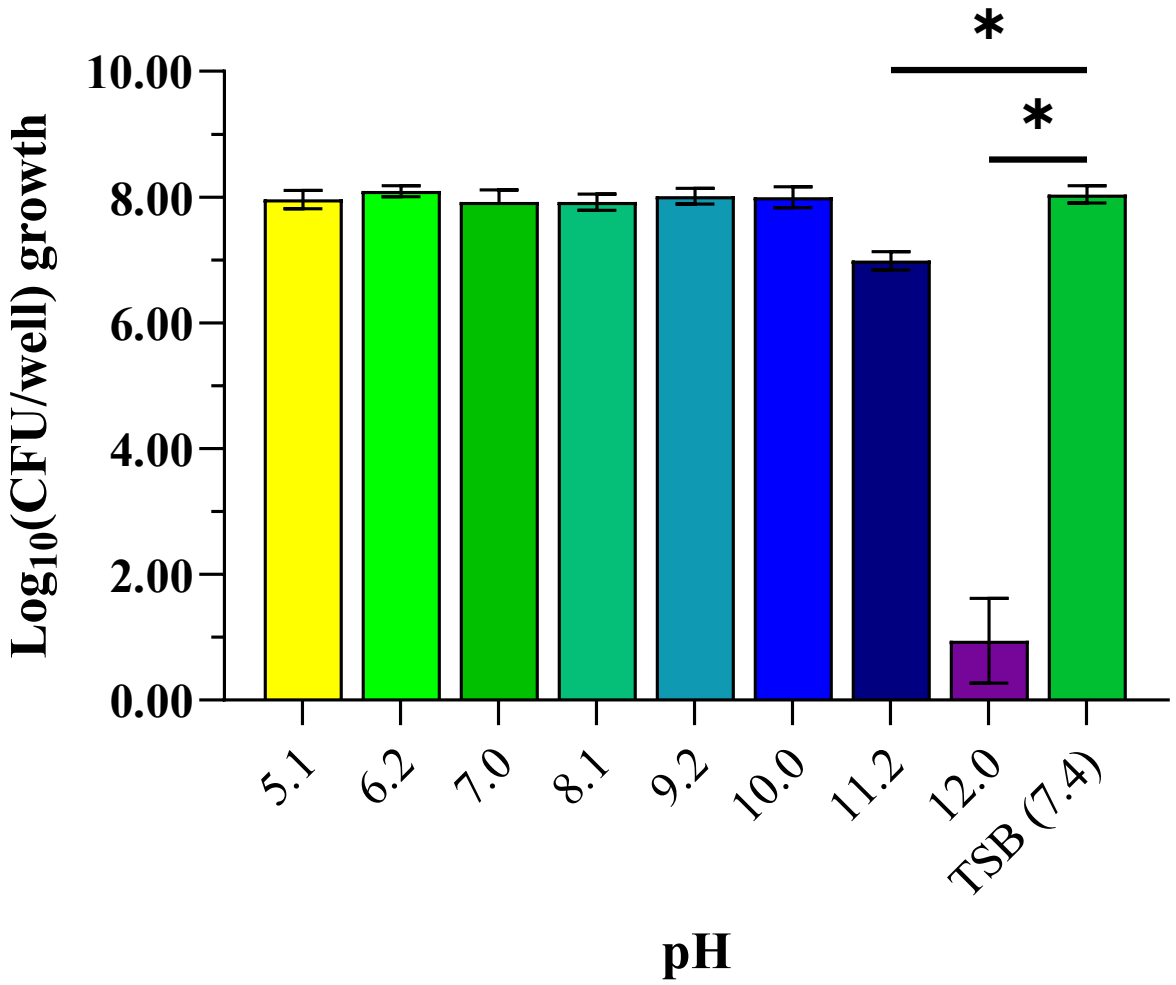

Figure 4.2.4. Effect of pH variation on *Staphylococcus warneri* growth. Bacteria were suspended at an average density of $9.44x10^5$ CFU/mL in TSB. The inoculum was added 1:9 (v:v) to 100% TSB with pH values adjusted to 5.1 to 12.0. Plates were incubated at 35 °C for 22.5 hrs, 282 rpm after which the wells were enumerated. Bars represent mean Log growth ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

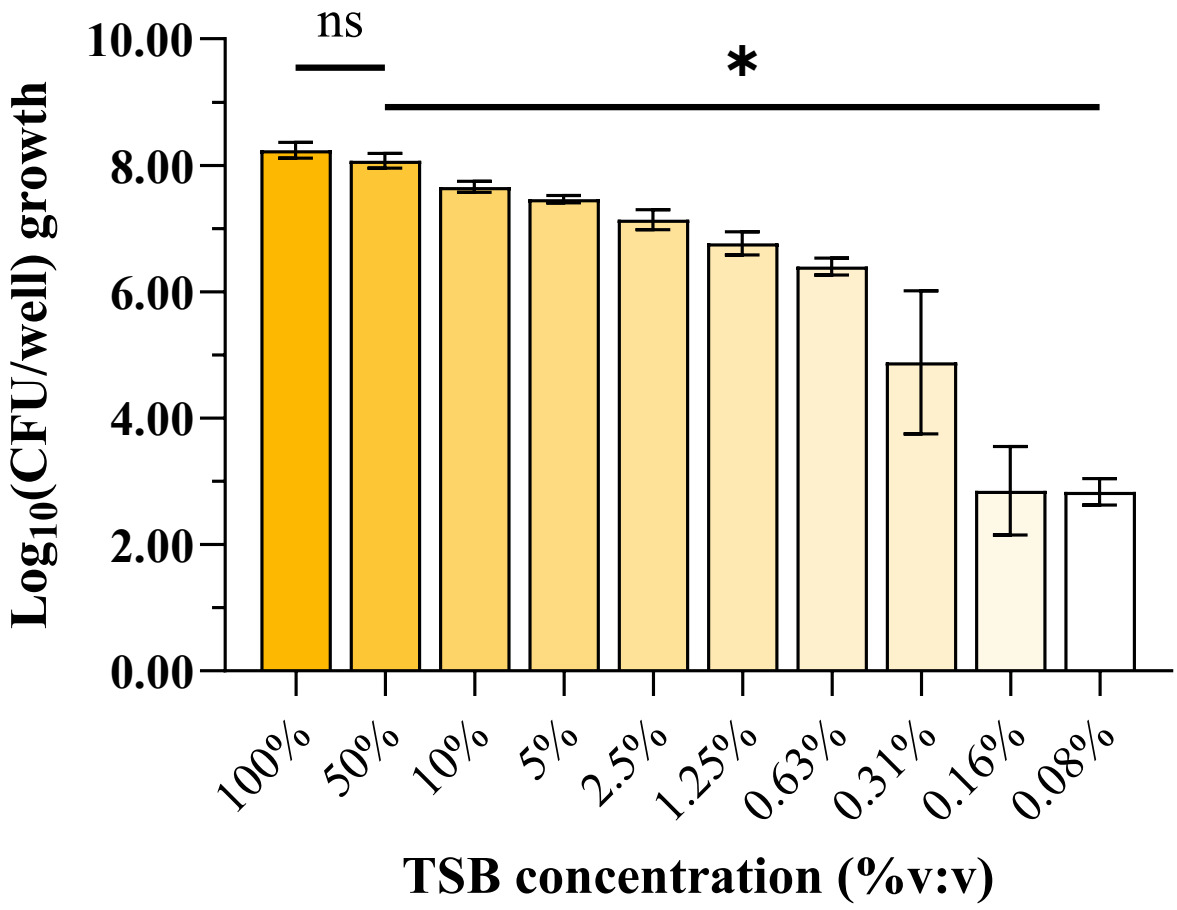

Figure 4.2.5. Effect of media concentration on *Staphylococcus warneri* growth. Bacteria were suspended at an average density of $1.08x10^6$ CFU/mL in TSB and $1.03x10^6$ CFU/mL in saline. The inoculum was added 1:9 (v:v) to 100% TSB with pH values adjusted to 5.1 to 12.0. Plates were incubated at 35 °C for 22.5 hrs, 282 rpm after which the wells were enumerated. Bars represent mean Log growth ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

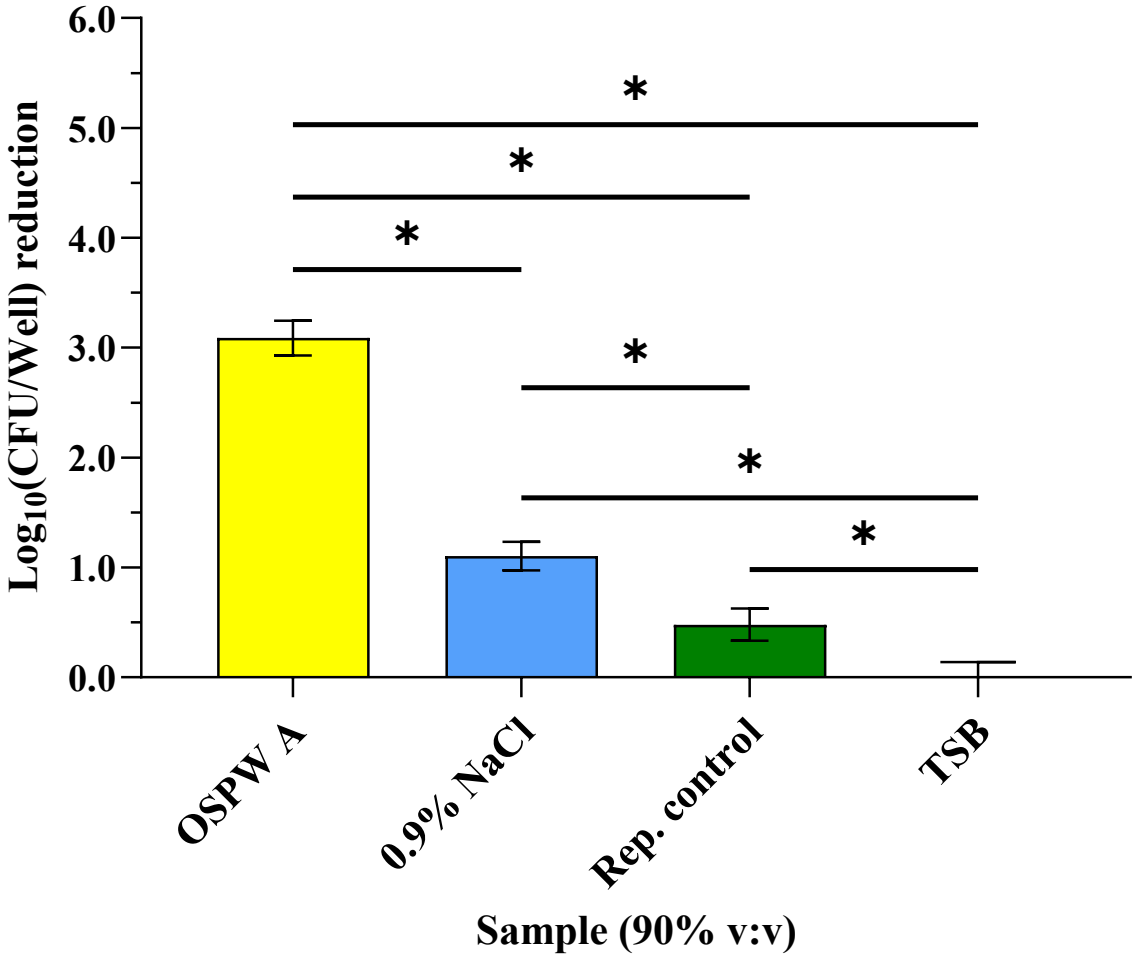

Figure 4.2.6. Effect of whole OSPW and OSPW replicant media exposure on *Staphylococci warneri* growth. Bacteria were suspended at an average density of 1.00×10^6 CFU/mL in TSB. The inoculum was added 1:9 (v:v) to wells containing OSPW A at concentrations of 100%, 0.9% saline, 100% OSPW replicant, or 100% TSB. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Log reductions were determined by calculating the difference between each test sample and the TSB reference control. Bars represent mean Log reduction ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

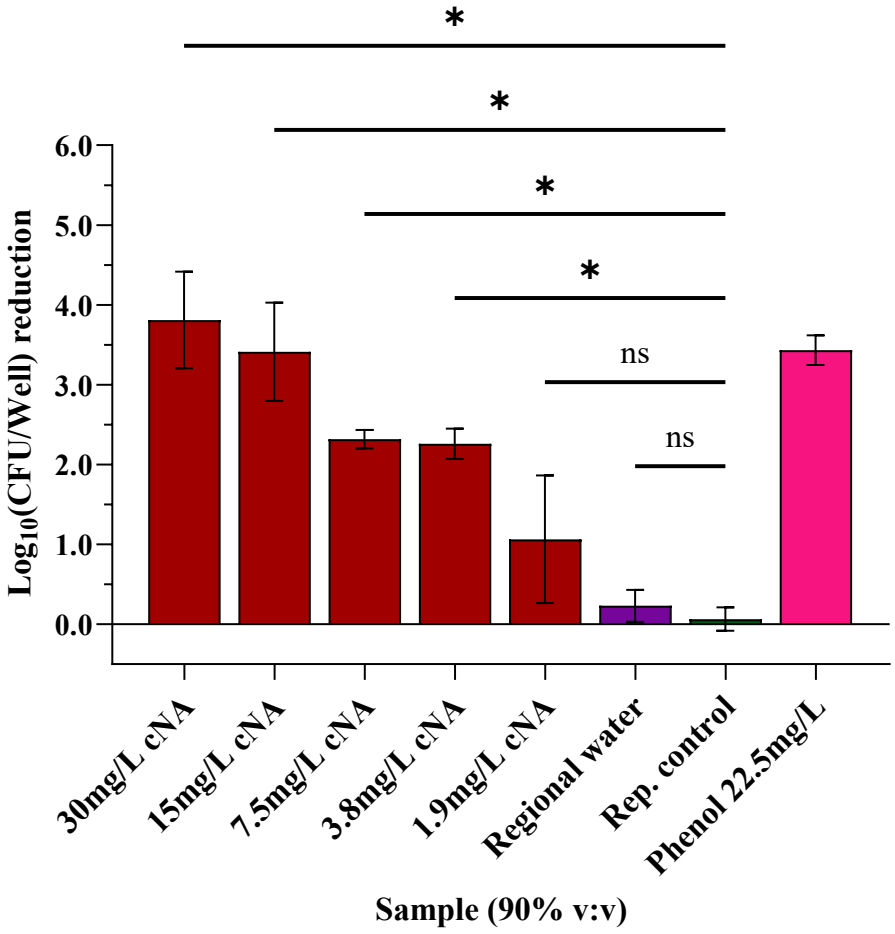

Figure 4.2.7. Effect of NA exposure on *Staphylococcus warneri* growth. Bacteria were suspended at an average density of $1.04x10^6$ CFU/mL in TSB. The inoculum was added 1:9 (v:v) to wells containing cNA solution at concentrations of 33.3 to 2.1 mg/L in OSPW replicant, 100% regional freshwater, dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in OSPW replicant, or 100% OSPW replicant. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Log reductions were determined by calculating the difference between the test sample and the OSPW replicant control. Bars represent mean Log reduction ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

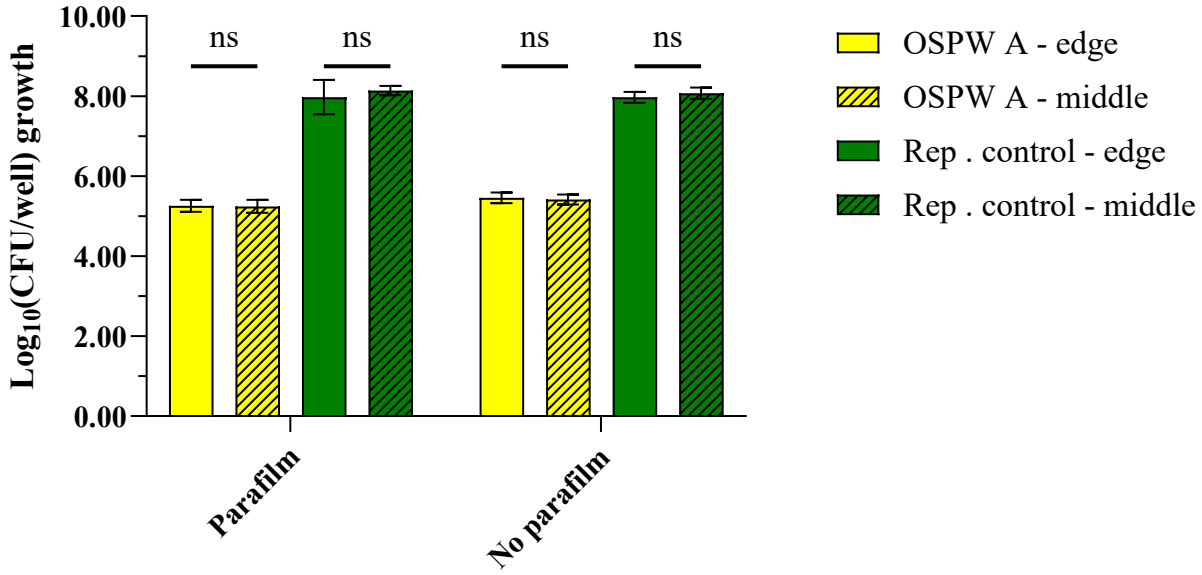

Figure 4.2.8. Effect of evaporation on *Staphylococcus warneri* growth. Bacteria were suspended at an average density of $9.56x10^5$ CFU/mL in TSB. The inoculum was added 1:9 (v:v) to wells containing 100% OSPW A or 100% OSPW replicant. Prior to incubation, one plate was sealed with parafilm. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Bars represent mean Log growth ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a two-way ANOVA was performed with Tukey post test. * represent significance ($p < 0.001$).

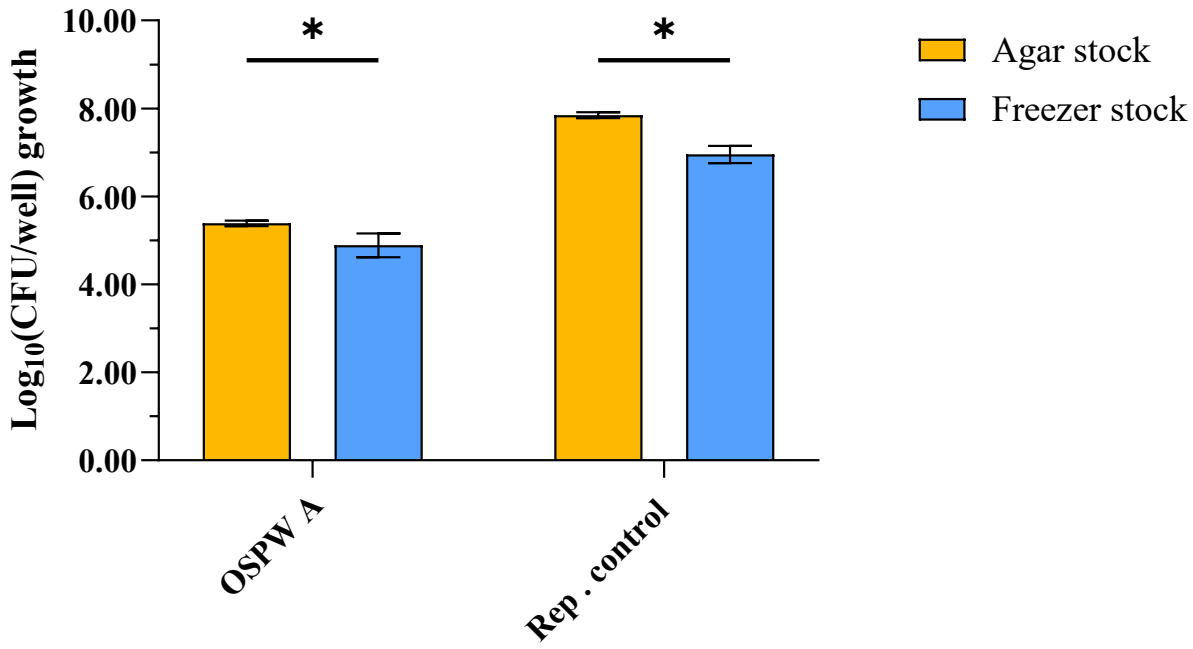

Figure 4.2.9. Comparison of a freezer stock to a live culture for MIC inoculation. Bacteria were suspended at an average density of 1.29×10^6 CFU/mL from a freezer stock inoculum and 1.00×10^6 CFU/mL from a traditional agar inoculum. The inoculum was added 1:9 (v:v) to wells containing 100% OSPW A or 100% OSPW replicant. Challenge plates were incubated for 22.5 hr at 35 °C on a rotary shaker after which the wells were enumerated. Bars represent mean Log growth ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a two-way ANOVA was performed with Tukey post test. * represent significance ($p < 0.001$).

Chapter V

Using a modified microdilution MIC assay to assess the effect of advanced oxidation process treatment on OSPW toxicity

5.1 Introduction

In the previous chapter it was established that a modified microdilution MIC assay using *S. warneri* was sensitive to a whole OSPW with an MIC value between 45 - 22.5%. Additionally, this approach showed high sensitivity to a major component of ecological concern in OSPW with a MIC between 3.8 – 1.9 mg/L for commercial grade NAs. Despite this sensitivity, the assay as performed was robust against variation in salinity, pH, and nutrient conditions that reflect those seen in whole and treated OSPW, as well as regional freshwater.

A broad range of treatment procedures are currently being investigated for their use in NA removal and OSPW remediation. Though many of these approaches have been shown to be successful from a chemical perspective, there remains no accepted standard approach for active or passive remediation of OSPW. There is an urgent requirement for high-throughput, low-technical burden toxicity testing to complement chemical analysis as researchers attempt to optimize treatment parameters for efficiency and efficacy. As such, I intended to use a modified microdilution MIC approach to assess the effectiveness of two unique advanced oxidation treatment approaches on cNAs and three different whole OSPW samples. I also compared the resulting toxicity readouts to chemical analysis data to see if these approaches could detect the removal of organic compounds from OSPW. The growth kinetics in these experiments were also investigated using OD as a proxy for bacterial growth.

Though the condition of initial process water poses several challenges for the organisms relied on for passive remediation, these approaches can be performed at large scales with minimal intervention, making them an appealing option for OSPW treatment (Allen 2008b). Despite this potential, NAs present in OSPW can be highly resilient to biodegradation, resulting in persistent toxicity that greatly extends the time required for passive remediation (Scott et al. 2005). As such, active remediation approaches are still extensively investigated to target recalcitrant compounds, with a potential to serve in concert with passive approaches for full remediation (Scott et al. 2008). One of the most investigated groups of active remediation approaches is oxidation, which uses the production of radicals to degrade organic compounds into less toxic intermediates. While chemical oxidants in isolation have shown to be effective at degrading organic compounds, these techniques are further enhanced in AOPs by encouraging production of radicals by adding UV or electrical current (Deng and Zhao 2015). While these approaches were originally designed for use in wastewater treatment, they have been successfully adapted for use in industrial effluent treatment, reducing organic compounds and resulting toxicity (Deng and Zhao 2015, Scott et al. 2008).

The use of UVA light to induce oxidant production has been used extensively in industrial settings to degrade pharmaceuticals, dyes, and phenols present in effluent (Matafonova and Batoev 2018). These approaches tend to use high-power UV diodes that emit light between the wavelengths of 365 nm – 405 nm. This source of radiation can generate radicals from oxidants such as metal oxides (TiO_2, FeO_2) and hydrogen peroxide which have high absorption at these wavelengths (Matafonova and Batoev 2018). Another strong oxidant that can be used with UV radiation is PMS and PDS, which both generate SO_4^- and HO^- radicals upon activation by an energy source such as UV, thermal, or electrical current. These radicals have already shown a

high capacity to degrade aromatic hydrocarbons and other high molecular weight organic acids, though the efficiency of this process is highly pH dependent (Liang and Su 2009). Though the radicals generated by these approaches have significant toxicity upon production, their high reactivity generally ensures that they do not contribute to additional persistent toxicity. Additionally, the by-products of reactions between radicals and organic compounds are generally less toxic than the original contaminants (Deng and Zhao 2015).

Transition metals can also be used to activate oxidants for removal of contaminants from effluent. The high reactivity of these compounds makes them effective at activating oxidants, but the use of metal oxides and transition metals can result in metal nanoparticle leaching into the substrate, further enhancing the toxicity of effluents (Wang et al. 2019). The use of a biochar support matrix can prevent metal nanoparticle leaching as well as improving dispersion of metal oxides, enhancing electron transfer, and even serving to adsorb degraded by-products for easier removal (Wang et al. 2019; Li et al. 2023). When combined with PMS and PDS oxidants, BC-Fe matrices have been shown to be effective in degrading NAs like those seen in OSPW (Song et al. 2022).

Recently, the use of UVA PDS AOP has been explored for treatment of OSPW and removal of organic contaminants. These approaches have proved to be highly effective at removing organic contaminants from OSPW, resulting in a subsequent decrease in toxicity as assessed by traditional *in vitro* approaches (Sanchez-Montes et al. 2024). Additionally, BC-Fe has been shown to activate PMS and significantly decreases the presence of NAs in whole OSPW with effective adsorption properties for organic contaminants (Vasquez-Aldana 2024). With both AOP approaches discussed, several parameters such as oxidant type/concentration, UV wavelength, biochar composition, and exposure time may be altered to optimize treatment.

Additionally, both approaches have the potential for introduction of toxic by-products as a result of treatment. Treatment of OSPW by AOPs represents a critical point where *in vivo* approaches would not be practical to inform toxicity assessment as several parameters are often modified at once to try and optimize treatment efficacy, with each alteration requiring individual assessment.

In this chapter I used the *S. warneri* modified microdilution MIC protocol established in chapter IV to assess the affects of AOP treatment on OSPW toxicity. Two different AOPs were investigated, a UVA LED stimulated PDS treatment and an BC-Fe stimulated PMS treatment. To support this investigation, I used data from SFS and mass spectroscopy to monitor the removal of organic contaminants from whole OSPW. The specific aims of this chapter were as follows: 1) to treat 3 different whole OSPW samples using AOPs to remove organic contaminants, 2) to validate the efficacy of the treatment using chemical analysis to monitor the removal of organic contaminants, and 3) to monitor toxicity of OSPW samples before and after treatment using a modified microdilution MIC assay to determine treatment efficacy.

5.2 Results

5.2.1 Advanced oxidation process treatment of cNA and OSPW and chemical analysis of organic contaminant removal

The 300 mg/L cNA solution, OSPW B, and OSPW C were treated using a UVA LED activated PDS treatment to remove organic contaminants. The PDS oxidant was added at a concentration of 1.0 mM and was activated using UVA light at a wavelength of 365 nm while being agitated. Samples were exposed to the treatment for a total of 60 min before the oxidant was neutralized using 1.0 mM sodium sulfite. Alternatively, an BC-Fe activated PMS treatment was used to remove organic contaminants from OSPW D. The BC-Fe matrix was added at 1 g/L

before 1.0 mM PMS was added to OSPW D, after which the sample was agitated. Samples were exposed to the treatment for a total of 90 min before the oxidant was neutralized using 1.0 mM sodium sulfite. The presence of organic fluorophore compounds was determined using SFS analysis with excitation and emission wavelength ranges of 200-600$_{nm}$ and 218-618$_{nm}$. Analysis was performed on untreated, 60-min UVA PDS treated, and 90-min BC-Fe PMS treated samples.

For the UT cNA solution, a single peak was observed at ~275 nm with no other major peaks observed (Fig 5.2.1A). Following 60-minute UVA PDS treatment, a peak was still present at 275 nm, but was reduced compared to the UT sample. For OSPW B UT, a large peak that exceeded detection limits was observed at ~275 nm (Fig 5.2.1B). Additionally, a peak was observed at ~310 and ~325 nm, though these were smaller than the peak at ~275 nm. Following 60-minute treatment, the previously mentioned peaks were still present at the same wavelengths, though they had been reduced (Fig 5.2.1B).

A similar trend to that which was seen for OSPW B UT was observed for OSPW C UT. A large peak was present at ~275 nm followed by two smaller peaks at ~310 nm and ~325 nm (Fig 5.2.1C). Following treatment, the peak at ~ 275 nm was reduced greatly compared to the UT sample, and the peaks at ~310 and ~ 325 nm were no longer visible. For the UT OSPW D, a high intensity peak was observed at ~ 275 nm, with smaller peaks at ~310 and ~325 nm (Fig 5.2.1D). Analysis of the treated sample yielded results like what was seen for OSPW C, with a large reduction in the peak at ~275 nm, and no visible peaks at ~310 nm and ~325 nm.

5.2.2 Effects of advanced oxidation process treatment on OSPW and cNA toxicity as assessed by a modified MIC assay

Challenge plates were prepared by filling wells with 33.3 mg/L of cNA solution as well as UT OSPW B, C, and D at concentrations from 100% to 25%. Additional wells were also prepared with 100% AOP treated cNA and OSPW B, C, and D. Control wells were prepared by adding 100% regional freshwater, dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in OSPW replicant, or 100% OSPW replicant. An inoculum with an estimated density of ~$1x10^6$ CFU/mL was generated in TSB and added 1:9 (v:v) to wells in the challenge plates. Challenge plates were incubated for 22.5 hr at 35 °C in a plate reader and had the OD_{600nm} measured in all wells every 30 min. Following incubation all wells were enumerated. Log reduction was calculated by determining the difference in growth between all wells and the OSPW replicant wells. Significance was determined by a *p*-value < 0.001.

The initial stock inoculum was determined to have a concentration of $2.12x10^8$ CFU/mL and was diluted to a final concentration of $1.00x10^6$ CFU/mL. When diluted in the challenge plate, the duplicate growth controls had a density of $2.40x10^4$ CFU/well prior to incubation. There was a significant 4.76 ± 0.42 Log_{10}(CFU/well) reduction in growth for the wells with 22.5 mg/L dichlorophenol compared to the OSPW replicant (Fig 5.2.1.1A-D). No significant log reduction was observed for the regional freshwater control, with a reduction of -0.02 ± 0.22 Log_{10}(CFU/well) compared to the OSPW replicant (Fig 5.2.1.1A-D). The regional freshwater control displayed similar growth kinetics to what is seen for the replicant control based on the observed OD, with both having a distinct log phase and achieving stationary phase with OD_{600nm} values of 0.63 and 0.59, respectively (Fig. 5.2.1.2A-D). Notably, the regional freshwater control entered log growth phase slightly earlier than the replicant control and entered stationary phase around 16 hrs as opposed to the 14 hrs of the control. As measured by OD, growth was completely inhibited by the presence of the dichlorophenol at 22.5 mg/L (Fig 5.2.1.2A-D).

For the UT cNA at 90%, a significant 4.08 ± 0.77 Log_{10}(CFU/well) reduction was observed compared to the OSPW replicant control (Fig 5.2.1.1A). A significant inhibition of growth was observed through all dilutions from 75% – 25%. Following 60-minute AOP treatment, a significant 2.79 ± 0.17 Log_{10}(CFU/well) reduction was observed compared to the OSPW replicant control. Though this value was 1.29 ± 0.26 Log_{10}(CFU/well) lower than the UT sample, it was not significantly different (Fig 5.2.1.1A). For the UT and treated cNA sample a small increase in OD was observed between 30 and 150 min but remained stationary around 0.03 for the remainder of the experiment (Fig. 5.2.1.2A). This suggests a strong inhibitory effect on growth kinetics for these two samples, with no clear log phase observed.

For the OSPW B UT, a MIC value of 45 – 22.5% was determined with a 0.85 ± 0.11 and 0.02 ± 0.32 Log_{10}(CFU/well) log reduction in growth, respectively (Table 6.2). At 90% concentration, OSPW B UT produced a 2.81 ± 0.11 Log_{10}(CFU/well) log reduction in growth compared to the OSPW replicant control (Fig 5.2.1.1B). Following 60-minute AOP treatment, a significant 0.98 ± 0.29 Log_{10}(CFU/well) reduction was observed compared to the OSPW replicant control. This value was 1.83 ± 0.10 Log_{10}(CFU/well) lower than what was observed for the UT OSPW B, a significant decrease following treatment (Fig 5.2.1.1B). The UT OSPW B had an inhibitory effect on the growth kinetics as measured by OD. Over 22.5 hrs the OD slowly increased to a final value of 0.242, with no distinct log or stationary phase (Fig 5.2.1.2B). Following AOP treatment, growth kinetics were improved with a OD_{600nm} of 0.59 in stationary phase. Though this is similar in intensity to what was seen for the negative controls, stationary phase was reached much later at 18 hrs as opposed to 16 and 14 hrs. Additionally, the onset of log phase appeared to be delayed compared to either of the negative control samples (Fig 5.2.1.2B).

OSPW C UT had a MIC value of < 22.5% with a corresponding 2.82 ± 0.19 Log_{10}(CFU/well) log reduction in growth (Table 6.2). At 90% concentration, OSPW C UT produced a 7.98 ± 0.09 Log_{10}(CFU/well) log reduction in growth compared to the OSPW replicant control (Fig 5.2.1.1C). Following 60-minute AOP treatment, a significant 0.27 ± 0.10 Log_{10}(CFU/well) reduction was observed compared to the OSPW replicant control. This value was 7.71 ± 0.04 Log_{10}(CFU/well) lower than what was observed for the UT OSPW C, a significant decrease following treatment (Fig 5.2.1.1C). When comparing the OD values, the UT OSPW C appeared to have a complete inhibitory effect on growth, producing minimal measurable OD like what was seen for the phenol control (5.2.1.2C). Following AOP treatment, there was a slight restoration of growth kinetics, however this sample still had a delayed and more gradual log phase compared to the controls. Additionally, stationary phase was achieved around 17 hrs, later than either control and with a lower final OD of 0.45 (Fig. 5.2.1.2C).

Lastly, OSPW D UT was determined to have a MIC value of 67.5 – 45% with log reduction values of 1.05 ± 0.30 and 0.28 ± 0.43 Log_{10}(CFU/well), respectively (Table 6.2). At 90% concentration, OSPW D UT produced a 2.10 ± 0.45 Log_{10}(CFU/well) log reduction in growth compared to the OSPW replicant control (Fig 5.2.1.1D). Following 90-minute BC-Fe AOP treatment, a 0.29 ± 0.33 Log_{10}(CFU/well) reduction was observed. This significant reduction was 1.82 ± 0.20 Log_{10}(CFU/well) lower than what was observed for the UT OSPW B, and not significantly different than the replicant control (Fig 5.2.1.1D). As measured by OD, the OSPW D UT had strong inhibitory effect on growth kinetics, with a gradual increase being observed between 15 and 22.5 hrs and a final OD value of 0.32 (Fig. 5.2.1.2D). Following 90-min AOP treatment, this inhibitory effect increased, with a very gradual increase in OD being

observed between 10 and 22.5 hrs and a lower final value of 0.08 when compared to the UT OSPW D (Fig. 5.2.1.2D).

5.3 Discussion

In this chapter, AOP treatment was used to remove organic contaminants such as NAs from various OSPW samples as well as to reduce cNA concentrations in lab prepared stocks. This was performed to evaluate if a modified microdilution MIC using *S. warneri* could reliably and sensitively detect changes in cNA and OSPW toxicity in response to changes in water chemistry. OSPW and cNA samples were treated with two different AOP treatments and treatment efficacy was evaluated using SFS analysis. Samples were then assessed using the modified MIC approach to monitor for changes in toxicity that corresponded with changes in organic content in the waters. To follow up on the findings of my previous chapter, an additional readout was implemented by measuring OD throughout the experiment to monitor for the effects of OSPW and cNA exposures on bacterial growth kinetics.

5.3.1 Advanced oxidative process treatment and SFS analysis of cNA and OSPW samples

Based on recently published literature, it was expected that both AOP methodologies would be effective at removing organic contaminants present in OSPW (Sanchez-Montes et al. 2024; Vasquez-Aldana 2024). Traditional spectroscopy approaches rely on measuring the presence of specific fluorescence emission or excitation wavelengths produced by targeted chemical compounds. Though accurate, this approach produces large spectra for target chemicals that experience interference due to overlap in complex mixtures (Samokhalov 2020). Alternatively, SFS scans for changes in both emission and excitation spectra, trading precision quantification of target chemicals for better separation of distinct components of a mixture. The

result is a sensitive, semi-quantitative approach for identifying families of compounds in complex mixtures based on structural motifs (Samokhalov 2020). While effective at measuring NAs as a family, this approach can not directly detect acyclic NAs due to the lack of an aromatic structure. Despite this, these compounds are thought to associate with aromatic organic compounds allowing for a degree of indirect detection through SFS analysis (Kavanagh et al. 2009).

Though the cNA sample had a concentration of 300 mg/L, the intensity of the first peak was lower than what was observed in the UT OSPW B, C, and D, which all contained less than $1/10^{th}$ the total classical NA content (Table 3.2). This is likely due to the cNA solution being composed primarily of alkylated cyclopentane carboxylic acids. These compounds are alicyclic, meaning they possess a ring structure, but may or may not display the physiochemical properties of aromatic compounds depending on conformation and isomerization (Horning 1943). As a result, these compounds are known to have poor fluorescent properties that are dependent on conformation and alkylation, explaining the large discrepancy observed between cNA and OSPW in terms of SFS intensity (Diaz-Garcia and Badia-Laino 2019). This is supported by nuclear magnetic resonance spectroscopy on a compositionally identical cNA solution that revealed only ~7.1% of the NA structures present are aromatic (Rudzinski et al. 2002).

Similar cNA compounds to what was used in this study have been investigated using SFS, though differences in preparation and analysis parameters make comparisons challenging. While intensity varies, the general pattern of a large single peak at ~270 nm is comparable to what is found in literature (Kavanagh et al 2009; Lo et al. 2006). The single peak observed does align with what would be expected of monoaromatic compounds, which are conformationally like the cyclopentanes present in this solution (Martin et al. 2014). Following AOP treatment, the

SFS signature for cNA was reduced, suggesting the degradation of the organic compounds present in solution that are measurable by these approaches.

All three UT OSPW samples showed similar SFS peaks but differed in terms of their overall intensities. These profiles are comparable to what has been observed for OSPW and NA extracts from the Athabasca oil sands, with distinct peaks present at ~270 nm, ~310 nm, and ~325 nm, representing single, double, and triple ringed aromatic compounds (Kavanagh et al. 2009; Frank et al. 2014; Martin et al. 2014). Following AOP treatment, OSPW B had slight reductions in peak intensities, and a maintenance of distinct peaks at 310 nm and 325 nm. This was different than OSPW C and D, which still had a small peak at 270 nm, but had no observable peaks present at 310 nm and 325 nm after AOP treatment. When analyzed using UPLC QTof-MS, AOP treated OSPW has been shown to have reductions in NA content that align with decreases in SFS peak intensities (Sanchez-Montes et al. 2024; Vasquez-Aldana 2024). Given these findings, the data obtained by SFS spectra suggest that the AOP treatments used were effective at removing aromatic organic contaminants from OSPW C and D, but less effective for OSPW B.

There are several considerations that must be evaluated when interpreting SFS data. As previously mentioned, these approaches only account for aromatic organic compounds. Several classical NA compounds are acyclic, and though they may associate with aromatic compounds in a mixture, they cannot be directly detected using these approaches (Jones et al. 2011; Kavanagh et al. 2009). Through use of ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR MS), it was determined that < 50% of organic acid compounds in OSPW are alicyclic or aromatic, resulting in a large fraction of these compounds being unobservable when using SFS (Grewer et al. 2010). In addition to compounds

that cannot be detected, some spectra may overlap between compounds. This prevents distinction between NA species and other aromatic hydrocarbons, preventing interrogation of the contribution of specific compounds to toxicity (Kavanagh et al. 2009). As the concentration of specific compounds cannot be determined, this approach is semiquantitative, though it can still be effective in monitoring changes in contaminant concentrations on an intra-sample basis (Sanchez-Montes et al. 2024).

Overall, SFS analysis serves as a rapid approach to determine the presence of organic contaminants present in samples, with more advanced analytical techniques supporting the relationship between SFS signature intensity and NA concentration under the conditions used in this study (Martin et al. 2014). From the SFS data shown in this study, OSPW C and D, as well as the cNA sample all appeared responsive to the AOP treatment. The reductions in SFS peak intensity for these samples also suggests removal of organic contaminants compared to the untreated state. This trend was not as pronounced in the OSPW B sample, where peak intensities were not significantly reduced, suggesting treatment was less effective at removing organic compounds. This is consistent with unpublished data from our lab that showcases that the same AOP treatment parameters are not equally effective on all OSPW samples.

5.3.2 Effects of advanced oxidative process treatment of OSPW on Staphylococcus warneri growth as determined by enumeration

The effects of growth on *S. warneri* as determined by enumeration show discrepancies between NA content and growth inhibition for the untreated samples. As determined by classical NA concentration, the samples are ordered as cNA, OSPW B, OSPW C and OSPW D (Table 3.2). When comparing the untreated samples in terms of their inhibitory effect on growth, OSPW C was the highest, followed by cNA, OSPW B and OSPW D. These findings are identical in both

the log reduction values produced at the standard 90% v:v exposure concentration, as well as their MIC values in Table 6.2. While it is worth noting that the NA concentration does correlate with observed toxicity for 3 of 4 samples, several factors may contribute to the discrepancy observed between OSPW toxicity and NA concentrations.

The first major consideration is the diversity of NA species and their differential effects on toxicity. Naphthenic acids are a large family of organic acids with high levels of structural diversity. Even when investigating the sub-category of classical NAs, as was done in this study using UPLC QTof-MS, this classification still represents a structurally diverse group of compounds with unique physicochemical properties (Vander Meulen et al. 2021; Headley et al. 2013). This structural diversity presents challenges with chemical analysis, as methods must be precise enough to differentiate structurally similar but distinct compounds (Brown and Ulrich 2015; Vander Meulen et al. 2021; Rudzinski et al. 2002; Huang et al. 2018). Beyond presenting challenges for identification, this structural diversity also makes their toxicity unpredictable. For structurally similar NA compounds, concentration can be a good predictor of toxicity, however; factors such as molecular weight and alkylation have strong influences on toxicity observed in these compounds (Hughes et al. 2017; West et al. 2011; Frank et al. 2009). This variation can greatly alter bioavailability of these compounds, resulting in toxicity endpoints that can differ in orders of magnitude when various species of classical NAs are compared at the same concentration (Jones et al. 2011; Providenti et al. 1993). These complexities are particularly problematic in whole OSPW, where many classes of NAs can be present and are difficult to characterize in the complex mixture (Brown and Ulrich 2015).

Another important consideration is that the toxicity of whole OSPW is different than the sum of its toxic components. Environmental factors such as temperature, pH, and salinity can

alter the chemical properties of NAs, and as a result, can induce changes in their observed toxicity (Kavanagh et al. 2012; Celsie et al. 2016). Beyond this, the synergistic and antagonistic interactions between organic and metal pollutants is well established in toxicology and is likely a major factor in the observed toxicity of OSPW (Cedergreen 2014; Wang et al. 2020; Li et al. 2017). Importantly, analysis of the distinct OF and IF of OSPW produce different toxicity endpoints than whole OSPW. This interaction is largely source dependent, as data has demonstrated that the IF has been associated with immunotoxicity, while other research indicates that the OF contributes to cytotoxicity and immunotoxicity (Phillips et al. 2020). Interestingly, fractionation approaches have revealed that the extracted components of OSPW may exhibit toxicity, while the whole OSPW itself does not (Qin et al. 2019). This is contrasted with other literature that shows the opposite phenomenon, where whole OSPW exhibits toxicity, but its fractionated parts do not (Li et al. 2017). Difference in methodology may account for the observed differences in the previously described studies, but these findings do highlight challenges with comparing OSPW samples. Overall, these factors do not significantly impact the value of intra-sample comparison, as is done in monitoring treatment efficacy, but does greatly complicate comparisons between whole OSPW samples.

Based on the SFS data, it was hypothesized that the AOP treatment was effective at removing organic contaminants from OSPW C, D, and the cNA sample, but less effective at removing these compounds from OSPW B. Growth inhibition data largely supports this hypothesis, with detrimental effects on growth being significantly reduced in OSPW C and D following AOP treatment. Though inhibitory effects on growth were reduced following treatment of OSPW B, these effects remained elevated above control levels, suggesting less effective removal of contaminants, further supporting this hypothesis. Despite SFS data suggesting

efficient removal of contaminants from the cNA sample, inhibitory effects on growth were not significantly reduced following AOP treatment.

As previously discussed, it is possible that the production of radicals could contribute to toxicity in the solution, however this seems unlikely given the low inhibition in other treated OSPW samples and the use of 1.0 M Na_2SO_3 to prevent residual oxidation activity (Deng and Zhao 2015). When compounds are degraded using AOPs, transformation products (TPs) can be generated that have unique physiochemical properties compared to the original degradation targets. For example, degradation of pharmaceuticals generally produces TPs that have reduced cytotoxicity and mutagenicity; however, there are scenarios where compounds produce TPs of equal or enhanced toxicity, as was seen with diclofenac and sulfamethoxazole (Fatta-Kassinos et al. 2011; Schmitt-Jansen 2007; Trovo et al. 2009). Similar effects have been observed in PAH degradation, where genotoxicity increased following treatment, though this was largely dependent on starting compound and resulting TP (Chibwe et al. 2015). Investigation of oxidation treatment of classical NAs revealed that treatment reduced the presence of O_2 NAs and decreased the cyclic hydrocarbon content. The TPs of these treatments did show decreased toxicity, though the loss of aromatic structure may reduce their potential for detection through SFS analysis (Meshref et al. 2017). Overall, these findings do align with literature that showcases residual toxicity from OSPW and NA samples despite chemical analysis suggesting effective removal of NA compounds (He et al. 2011; Wang et al. 2013).

5.3.2 Effects of advanced oxidative process treatment of OSPW on Staphylococcus warneri growth as determined by optical density

In addition to enumeration, growth was evaluated using OD to examine detrimental effects on growth kinetics. Bacterial growth kinetics as measured by OD can be divided into 4

major phases. The lag phase represents a period where little to no growth is occurring as the organism adapts to the culturing conditions. As there is limited replication during this time, very gradual or no increase in OD is observed, but the length of this phase is dependent on culture conditions and initial inoculum density (Maier 2004). Once the necessary physiological changes have occurred and sufficient cell density has accumulated, the culture will enter exponential growth. During this phase, the growth rate is directly proportional to the concentration of cells as they double according to the organism's generation time. When investigated using OD or enumeration, this phase will be represented by a linear relationship between OD or CFU as a function of time (Maier 2004).

As nutrients begin to get exhausted and toxic byproducts of metabolism accumulate, the culture will enter stationary phase. During this period, little to no net growth is observed as the culture undergoes several metabolic changes (Maier 2004). Organisms may downregulate metabolism in response to nutrient scarcity or can switch to alternate metabolic pathways (if available), reducing growth during this period. Additionally, cells may begin to die during this period, allowing for nutrients to be scavenged by other members of the culture to continue replication, resulting in population equilibrium (Nystrom 2004).

At this stage of growth, the accumulation of dead cells, changes in cell morphology, and accumulation of metabolic byproducts can have effects on the accuracy of measuring cell density using OD. In several organisms, cell size and shape can change significantly under nutrient starvation, greatly affecting their optical properties (Szermer-Olearnik et al. 2014; Nystrom 2004). Though dead cells and their components do contribute to measured optical density, the optical properties of dead cells are different than those of cells that are living. These properties can depend on the organism and cell death pathway and may or may not result in an observable

death phase where OD decreases (Beal et al. 2020; Biesta-Peters et al. 2010; Maier 2004). In addition to the effects of live and dead cells on photometry, the accumulation of metabolic byproducts, protein aggregates, and even dispersed gasses can affect OD at this stage in culture growth (Szermer-Olearnik et al. 2014; Hernandez and Marin 2002). This phenomenon is further complicated when antimicrobial exposures are assessed using OD, as tested compounds can precipitate or produce byproducts that can change OD by introducing colour or turbidity to the culture (Duedu and French 2017). These factors make OD more suitable for monitoring growth under optimal control conditions rather than monitoring antimicrobial activity in test solutions and environmental samples (Dominguez et al. 2001).

Upon review of my data, a growth curve should have been established using 100% TSB to compare all other solutions to the optimal growth kinetics. Despite this oversight, when compared to available growth curves in literature, the OSPW and regional freshwater control have similar lag and log phase kinetics, though these samples reach stationary phase slightly earlier than the 17 hrs observed in TSB (Baikuni et al. 2023). This finding does highlight potential concerns for the exposure time chosen for these experiments. The 22.5 hr exposure time presents a nearly 5 hr period where organisms are no longer growing. In my data, this is delineated by a clear death phase that is observed as OD decreases from 17.5 hrs – 22.5 hrs. These concerns can be tempered by the enumeration data, as the cell density was similar between the TSB control and both control solutions used in section 4.2.6 and 4.2.7. This growth was lower than what was seen in the overnight culture in section 4.2.9 generated under ATCC recommended conditions, though this is not unexpected given the differences in container volume and culture conditions (Kushkevych 2023).

The exposure time chosen was based on the M07-A10 guidelines, which recommends 20 – 24 hr exposure for CoNS species when performing microdilution MIC assays (CLSI 2018). This was further adjusted by reducing initial inoculum density to account for the lower nutrient conditions that these experiments introduce. The 22.5 hr exposure time also better aligns with other eukaryotic *in vitro* approaches that often use 24 hr exposure time points (Li et al. 2017; Phillips et al. 2020; Paul et al. 2023; Choo-Yin 2021). Overall, the implications of these concerns are that enumeration data may be underestimating control growth at peak density while allowing more time for growth in test wells with inhibitory effects to normalize to control growth levels. This should be addressed by performing enumeration at ~17 hrs where OD estimates peak cell density for the control solutions and comparing it to what is measured at 22.5 hrs.

For the cNA sample, both OD and enumeration showed significant detrimental effects on bacterial growth for both the untreated and treated sample. While enumeration showed a slight reduction in inhibition following treatment of cNA, this was not observed with OD. This growth was likely not detected as all OD values shown are blank adjusted, and both untreated and treated cNA had significant baseline turbidity. For all UT OSPW samples, the OD reflected enumeration data, with OSPW C showing the greatest inhibitory effect on growth, followed by OSPW B and OSPW D. Unlike enumeration, OD was not able to differentiate the inhibitory effects of the untreated cNA and OSPW C, as both samples had cell densities below the detectable limit (as measured for *E. coli*) of ~$5x10^6$ CFU/mL (Sultana and Sanchis 2022). For untreated OSPW B and D, growth was detectable by OD, however, the traditional phases of growth that were previously discussed were not present. Instead, a slow increase in OD was observed over 22.5 hrs, suggesting significant inhibitory effects of these samples on bacterial growth. This is consistent with some of the previously described literature which shows that

many of the compounds present in OSPW have inhibitory effects on microbial growth kinetics in addition to acute cytotoxicity (Sikkema et al. 1995; Sterritt and Lester 1980; Miles et al. 2019). This data supports the previously discussed concerns related to exposure time, as growth in untreated samples continues from 17 – 22.5 hrs while control growth appears to decrease.

Following treatment, OSPW B and C growth was restored to kinetics that resemble what is seen in the control solution, though still appeared to have significant inhibition. The largest difference between the treated OSPW samples and the controls was the significantly reduced growth observed during exponential phase. Enumeration data showed that the treated OSPW B and C had similar cell density as the controls after 22.5 hr exposure, though OD reveals that peak control density may be significantly different than the test samples. As previously mentioned, the control samples reached peak OD intensity at ~16 hrs and began to decline after this point until 22.5 hrs. During this 4.5 hr period, both the untreated and treated samples continue to experience gradual growth, with treated samples eventually normalizing with the control samples. This suggests that these treated samples have residual inhibitory effects on growth. While this was observed in enumeration data for OSPW B, this would not have been identified from the enumeration data for OSPW C. These findings further emphasize the need to perform enumeration at 17 hrs to increases sensitivity and distinguish these inhibitory effects.

The OD data obtained for the treated OSPW D does not align well with the enumeration data. Enumeration showed that the treated sample had significantly better growth than the untreated sample, with a non-significant residual inhibitory effect on growth. The OD data showed inhibition of growth greater than what was observed in the untreated OSPW D and B sample, a result that is seemingly opposite of what was observed for enumeration. This suggests OD is underestimating the cell density present, while the previously described limitations

associated with OD all represent scenarios where these approaches are likely to overestimate cell density (Beal et al. 2020; Biesta-Peters et al. 2010; Maier 2004; Szermer-Olearnik et al. 2014; Hernandez and Marin 2002; Duedu and French 2017). While it is possible for inhibitory effects of tested compounds to change cell shape and decrease cell size (Szermer-Olearnik et al. 2014; Nystrom 2004), these effects were not observed during colony enumeration or light microscopy. These possibilities also seem unlikely to account for the scale of the discrepancies observed, as the calculated CFU from the OD is ~100x lower than what was enumerated.

One important difference between OSPW D compared to the other samples was the method used for treatment. The AOP treatment used iron oxide nanoparticles loaded on a biochar surface to activate PMS, different than the UVA activated PMS treatment used for OSPW B and C. While these compounds were bound to biochar to prevent leaching, it was noted that a small concentration of iron nanoparticles were released into the treated OSPW (Vasquez Aldana 2024). These compounds have been shown to have toxic effects on microorganisms (Ameen et al. 2021), however enumeration data refutes this possibility for these experiments as the cell density was not significantly different than the control solutions. Another important consideration is that nanoparticles have optical properties that have been shown to cause interference in both photometric and colorimetric assays (Ong et al. 2014; Qiu et al. 2017; Kroll et al. 2009). These optical properties may interfere with measurements of OD, though this possibility will be explored in greater detail in chapter VI.

5.4 Future Directions

The data in this chapter suggests that the modified microdilution MIC assay was effective at monitoring treatment efficacy as indicated by SFS analysis. Furthermore, OD revealed inhibitory effects on growth that were not identified by enumeration, though these approaches

appear to be confounded by interference from the BC-Fe treatment. Though seemingly effective for monitoring the presence of NAs in samples before and after treatment, several limitations were discussed that emphasize the need for precise chemical analysis approaches. As NA toxicity is species dependent, these approaches must be able to distinguish between these structurally similar compounds to best interpret some of the differential toxicity observed.

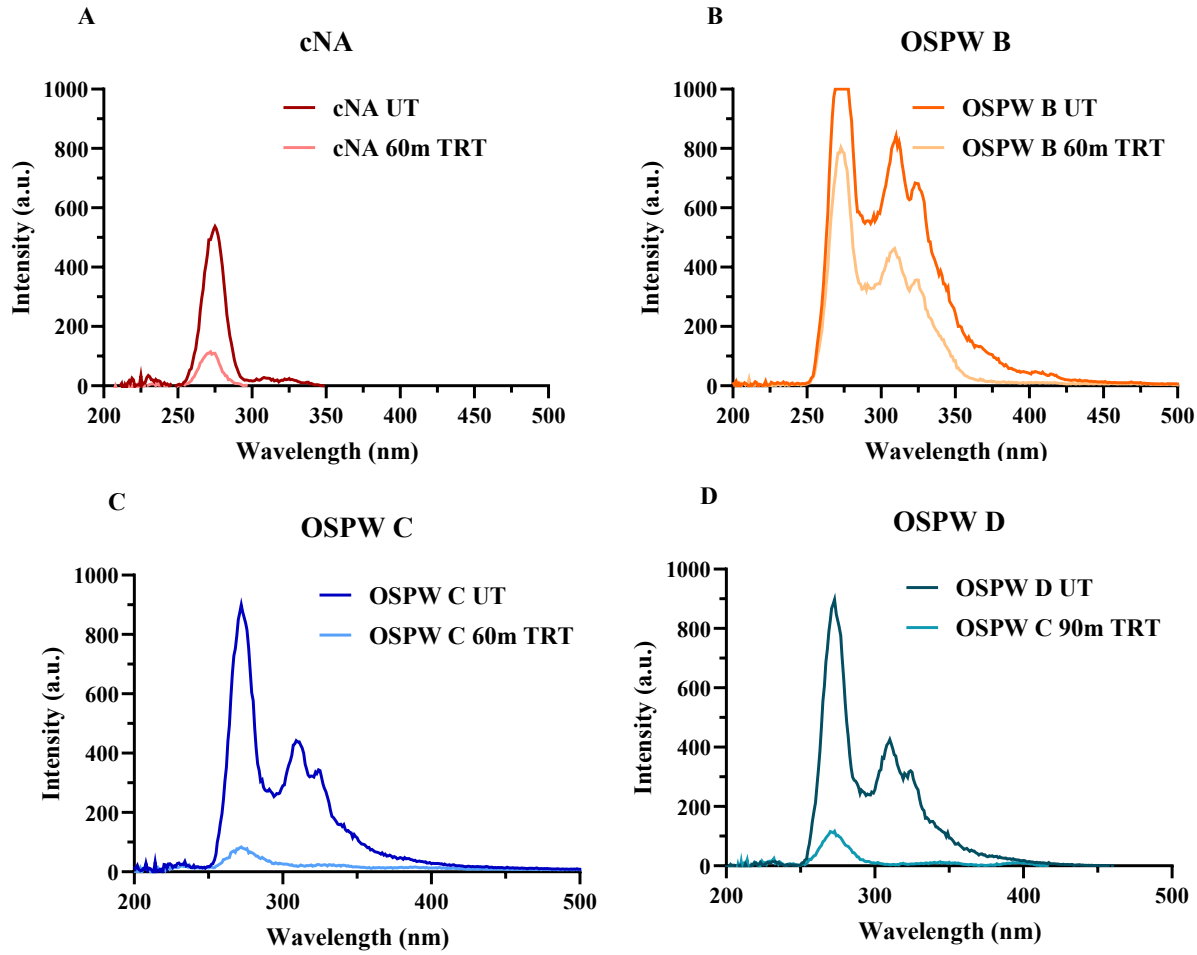

Figure 5.2.1. SFS analysis of fluorophore organic compounds in untreated and treated OSPW and cNA. 50mL of 300 mg/L cNA (A), whole OSPW B (B), or Whole OSPW C (C) was treated by exposure to 1mM PDS oxidant with UV irradiance 0.8mW cm^2 for 60 minutes. 50 mL of OSPW D (D) was treated by exposure to 1mM PMS with BC-Fe stimulant for 90 minutes. Fluorescence of organic compounds was measured at 0 min and following treatment completion at 60 or 90 minutes. Ranges for excitation and emission were 200-600nm and 218-618nm respectively.

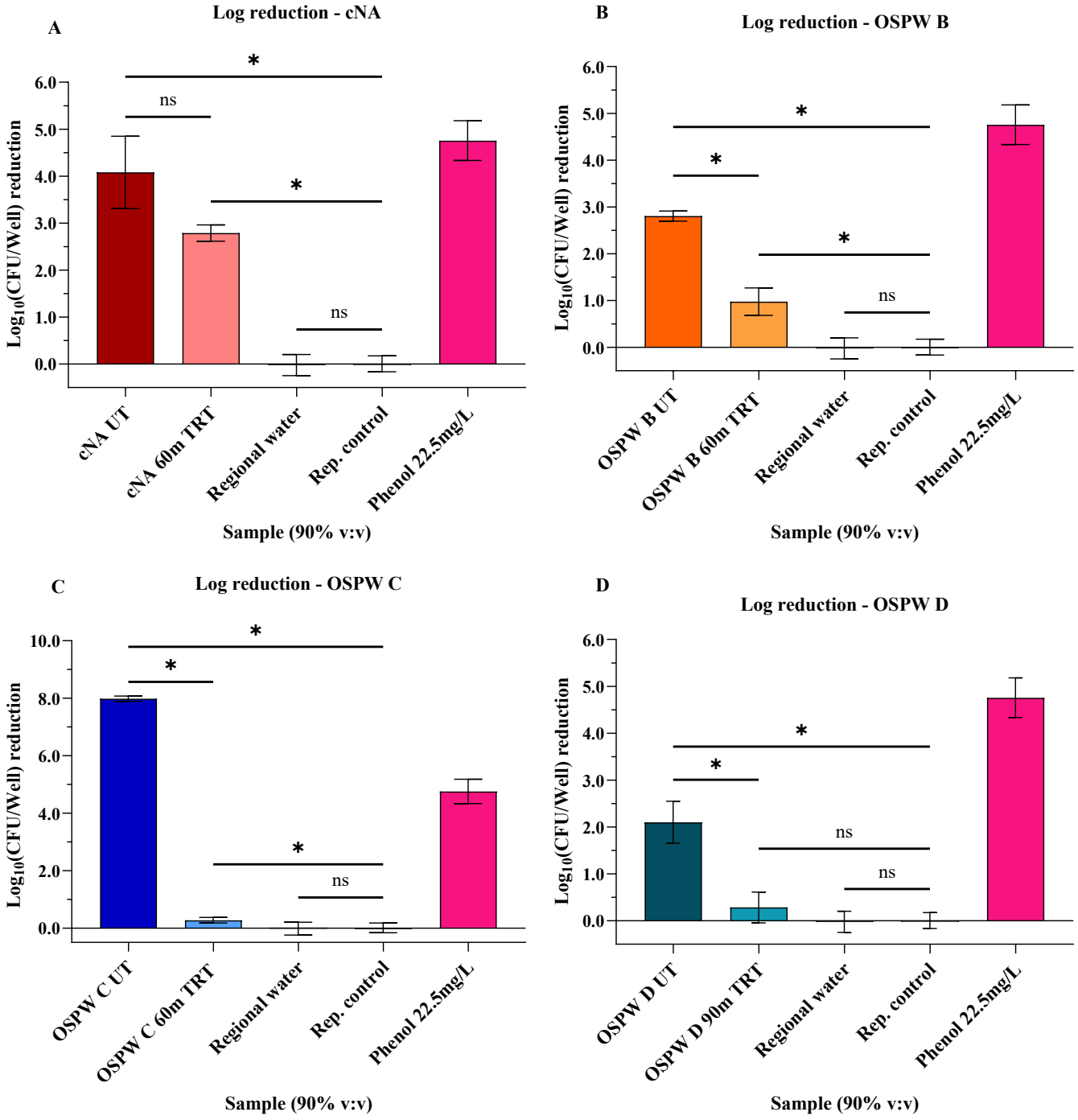
A
Log reduction - cNA
Log10(CFU/Well) reduction
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
ns
*
ns
cNA UT
cNA 60m TRT
Regional water
Rep. control
Phenol 22.5mg/L
Sample (90% v:v)
B
Log reduction - OSPW B
Log10(CFU/Well) reduction
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
*
ns
OSPW B UT
OSPW B 60m TRT
Regional water
Rep. control
Phenol 22.5mg/L
Sample (90% v:v)
C
Log reduction - OSPW C
Log10(CFU/Well) reduction
10.0
8.0
6.0
4.0
2.0
0.0
*
*
*
ns
OSPW C UT
OSPW C 60m TRT
Regional water
Rep. control
Phenol 22.5mg/L
Sample (90% v:v)
D
Log reduction - OSPW D
Log10(CFU/Well) reduction
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
ns
ns
OSPW D UT
OSPW D 90m TRT
Regional water
Rep. control
Phenol 22.5mg/L
Sample (90% v:v)

Figure 5.2.2.1. Effect of treated and untreated cNA and whole OSPW exposure on *Staphylococcus warneri* growth. Bacteria were suspended at an average density of $1.00x10^6$ CFU/mL in TSB. The inoculum was added 1:9 (v:v) to wells containing either untreated or 60-min UVA PDS treated 33.3 mg/L cNA (A), OSPW B (B), OSPW C (C), or untreated and 90-minute BC-Fe PMS treated OSPW D (D). Controls were prepared by suspending the bacterial inoculum 1:9 (v:v) in 100% regional freshwater, 100% OSPW replicant control, dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in OSPW replicant. Challenge plates were incubated for 22.5 hr at 35 °C in a plate reader at 282 rpm orbit. Following incubation each well was serially diluted and spot plated for enumeration. Log reductions were determined by calculating the difference between the test sample and the OSPW replicant control. Bars represent mean Log reduction ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

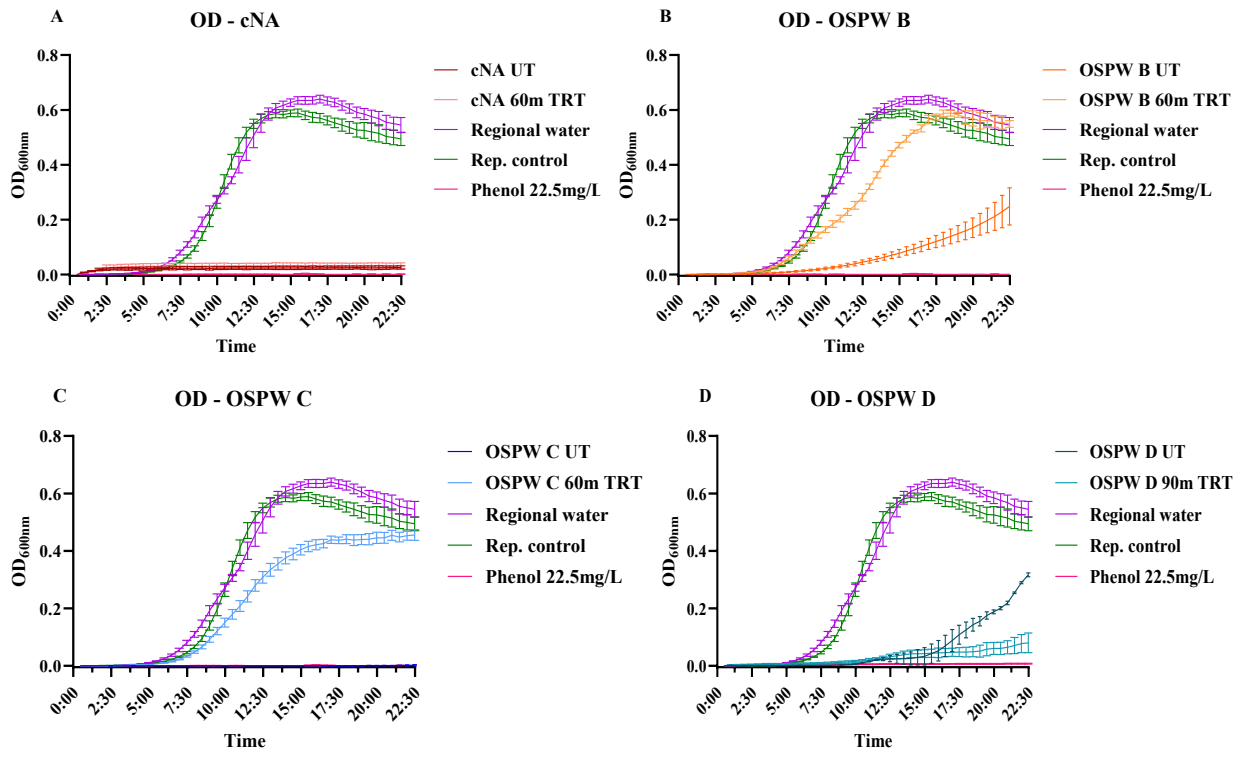

Figure 5.2.2.2. Effect of treated and untreated cNA and whole OSPW exposure on *Staphylococcus warneri* growth kinetics. Bacteria were suspended at an average density of $1.00x10^6$ CFU/mL in TSB. The inoculum was added 1:9 (v:v) to wells containing either untreated or 60-min UVA PDS treated 33.3 mg/L cNA (A), OSPW B (B), OSPW C (C), or untreated and 90-minute BC-Fe PMS treated OSPW D (D). Controls were prepared by suspending the bacterial inoculum 1:9 (v:v) in 100% regional freshwater, 100% OSPW replicant control, dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in OSPW replicant. Challenge plates were incubated for 22.5 hr at 35 °C in a plate reader at 282 rpm orbit. During incubation, OD_{600nm} was read every 30 minutes. Data points represent mean OD_{600nm} ± 95% confidence interval (CI) (n=3). Data points were joined by a connecting curve.

Chapter VI

Using bacterial luminescence to assess the effect of advanced oxidation process treatment on OSPW toxicity

6.1 Introduction

In the previous chapter it was shown that treatment of OSPW and cNA via AOPs produced changes in the profiles of fluorophore organic compounds present in these waters, with treatment generally reducing the signatures detected via SFS. These changes were also sensitively detected via the modified microdilution MIC assay, with treatment reducing the inhibition of bacterial growth. Lastly, investigation of growth kinetics via OD revealed similar results as SFS and enumeration data, with treatment restoring growth kinetics in a sample dependent fashion.

In the field of aquatic toxicity testing, there are several *in vivo* and *in vitro* approaches currently approved for use. In Canada, of the approved approaches only two are *in vitro*, with one being 72-hr algal growth inhibition and the other being 30-min bacterial luminescence inhibition. Due to its low technical burden, inhibition of bacterial luminescence is one of the most employed toxicity approaches to determine NA and OSPW toxicity. Due to this fact, I sought to implement a standardized bacterial luminescence protocol using *A. fischeri* to assess the toxicity of the previously AOP treated samples. I also used bacterial enumeration to determine the relationship between luminescence inhibition and bacterial density under test conditions. Lastly, I compared luminescence inhibition to log reduction for *S. warneri* as well as comparing log reduction values between *S. warneri* and *A fischeri* to evaluate the relationship between the readouts of these two bacterial toxicity approaches.

Made commercially available in 1978, toxicity assessment using luminescent bacteria was popularized by the Microtox assay and has seen widescale implementation. Environment Canada developed its own protocols and guidance for these approaches in 1992, and it remains one of the most common aquatic toxicity tests employed (Viegas et al. 2021; Environment Canada 1992). Thousands of inorganic and organic compounds of environmental concern have been screened with this assay with sensitivity largely being dependent on the compounds tested. Correlation between luminescence inhibition approaches and other toxicity models tends to be strong, though this trend is often exclusive to single toxicant exposures where test compounds are concentrated in the 2% saline control (Kaiser 1998). In general, highly lipophilic compounds and metals tend to produce greater variability and poor correlation for luminescent inhibition approaches, with complex effluents having the poorest sensitivity and correlation (Hao et al. 1995). Despite this variability, the low technical burden and abundance of data using these approaches make them a common first choice for aquatic toxicity screening (Viegas et al. 2021).

When investigating OSPW and NAs, bacterial luminescence assays have been applied extensively to assess toxicity. Of the literature investigating whole OSPW toxicity, ~25% reported bacterial luminescence IC values as the only toxicity endpoint (Mahaffey and Dube 2006). Similar trends are observed when investigating NA toxicity, as its low technical burden and rapid nature allow for interrogation of the vast structural diversity of NA compounds (Clemente and Fedorak 2005). Bacterial luminescence has revealed highly variable sensitivity to both NAs and OSPW, with many of the results being sample and source dependent. With simpler commercial NA samples, it was observed that toxicity is dose dependent, with treatment approaches that degrade organic compounds resulting in reduced luminescence inhibition (Clemente and Fedorak 2005). Similar trends were not consistently observed in whole OSPW,

where untreated samples can fail to produce inhibitory outcomes, and treatment can increase toxicity (Brown and Ulrich 2015; Mahaffey and Dube 2017; Li et al. 2017). Similar challenges are faced with extracted NAs, where sensitivity was considerably lower for the bacterial luminescence inhibition assays when compared to other toxicity approaches (Bartlett et al. 2017; Bauer et al. 2019).

The challenges faced by bacterial luminescence when interrogating the toxicity of whole OSPW should not be surprising given the previously reported data demonstrating poor performance when investigating both highly lipophilic organic compounds and metals (Hao et al. 1995). Both compounds are present in OSPW at varying concentrations and can contribute to the complex nature of OSPW as an effluent. In addition to these challenges, the emergence of nanoparticles as both a potential remediation approach and an environmental contaminant can pose challenges for toxicity assessment using photometric approaches such as luminescence inhibition (Ong et al. 2014; Monteiro-Riviere 2006).

In this chapter, I implemented a standardized bacterial luminescence inhibition assay using *A. fischeri* to assess the toxicity of both untreated and AOP treated OSPW and cNA. In addition to measuring the luminescence inhibition, bacterial enumeration was also performed to investigate the relationship between luminescence and cell density. Statistical analysis was then done to compare the relationship between luminescence and log reduction for both *S. warneri* and *A. fischeri,* as well as compare the results for enumeration between the two organisms.

6.2 Results

6.2.1 Validation of a standardized bacterial luminescence inhibition protocol using Aliivibrio fischeri

A tube of sterile LBS was inoculated with colonies from a 2° culture of *A. fischeri* and diluted in LBS in ½ step increments. Diluted cultures had OD_{600nm} measured and were subsequently enumerated to determine CFU/mL. The enumerated CFU/mL was graphed as a function of OD to determine the linear relationship between the variables. Simple linear regression analysis was performed to determine the goodness of fit and equation for the line of best fit with 95% confidence intervals. The slope was determined to be significantly non-zero with a p value of < 0.000001. The equation for the line of best fit was calculated to be $y = 2.79x10^8 x - 1.42x10^5$ with an R^2 value of 0.9583 (Fig. 6.2.1.1).

To generate freezer stocks for the luminescence inhibition assay, luminescent colonies from a 2° culture were used to inoculate broth media 5.7 and incubated overnight. Following incubation, the O/N pre-culture was enumerated and was then diluted to achieve an estimated density of $2x10^7$ CFU/mL in broth media 5.7 to create the main culture. The main culture was enumerated prior to incubation and following incubation. After incubation, the main culture was centrifuged twice before being resuspended in protective media 5.9 to achieve an estimated density of $1.5x10^9$ CFU/mL. The inoculum was then enumerated proper to being frozen.

For the O/N preculture, it was determined that the solution had a bacterial density of 2.20 ± 0.10 $x10^9$ CFU/mL. Following dilution and prior to incubation, the main culture had a density of 1.53 ± 0.15 $x10^7$ CFU/mL. After incubation, the main culture had grown to a density of 2.17 ± 0.38 $x10^9$ CFU/mL and was diluted down to a final volume of 1.10 ± 0.27 $x10^9$ prior to being frozen at -80 °C.

To validate the relationship between luminescence and cell density for the freezer stocks as prepared in the protocol, a stock was thawed before diluting in reconstitution solution. The thawed stock was then diluted in ½ step increments in 2% saline (v:v) in an opaque 96-well

plate. This plate was then placed in the plate reader and had the luminescence read as $RLU_{440\text{-}460nm}$ before all wells were enumerated. The enumerated CFU/mL was graphed as a function of RLU to determine the linear relationship between the variables. Simple linear regression analysis was performed to determine the goodness of fit and equation for the line of best fit with 95% confidence intervals. The slope was determined to be significantly non-zero with a p value of < 0.000001. The equation for the line of best fit was calculated to be $y = 62.22\,x - 9.19\text{x}10^5$ with an R^2 value of 0.9418 (Fig. 6.2.1.2).

6.2.2 Effects of advanced oxidation process treatment on OSPW and cNA toxicity as assessed by a standardized bacterial luminescence inhibition assay

Prior to use in this assay, all UT and treated test samples from section 5.2.2 as well as the regional freshwater control had the pH adjusted to ~7 as well as the salinity adjusted to ~2%, resulting in a concentration of 91% (v:v). Following thawing and dilution, the freezer stock inoculum was added to an opaque 96-well plate and had the I_0 luminescence measured. After the I_0 reading, challenge plate wells were filled 9:1 with 33.3 mg/L of cNA solution as well as UT OSPW B, C, and D at concentrations from 90% to 22.5%. Additional wells were also prepared with 90% AOP treated cNA and OSPW B, C, and D. Control wells were prepared by adding 90% regional freshwater, dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in OSPW replicant, or 2% saline. This final dilution resulted in exposure concentrations of 81% (v:v) for all test wells and the regional freshwater. The challenge plate was then incubated for 30 min at 15 °C with luminescence readings being taken at 5, 15, and 30 minutes. Luminescence inhibition was determined by calculating the difference in luminescence between I_0 and I_{30}. Wells were enumerated following the I_{30} reading, and log reduction was calculated by determining the

difference in CFU between all wells and the 2% saline wells. Significance for inhibition and log reduction was determined by a *p*-value < 0.001.

No significant inhibition of luminescence was observed for the regional freshwater control for either of the challenge plates used with values of 0.09 ± 4.45% (Fig. 6.2.2.1A-C) and 1.15 ± 3.86% (Fig 6.2.2.1D). At I_{30}, the dichlorophenol control produced significant inhibition values ranging from 98.20 ± 0.16% to 81.17 ± 4.88% at concentrations of 90.0 mg/L to 11.3 mg/L, respectively, in the first challenge plate (Fig. 6.2.2.1A-C). Like the regional freshwater control, this did not differ between plates, with values in the second challenge plate ranging from 99.16 ±0.50% to 82.06 ± 1.2% % at concentrations of 90.0 mg/L to 11.3 mg/L, respectively (Fig. 6.2.2.1D). At I_5, both challenge plates produced an IC_{50} value of 11.3 mg/L for the dichlorophenol control.

For the enumeration data, the regional freshwater control did not significantly reduce bacterial density compared to the 2% saline control in either plate, with log reduction values of -0.09 ± 0.07 (Fig. 6.2.1.2A-C) and -0.04 ± 0.03 Log_{10}(CFU/well) (Fig. 6.2.1.2D). For the dichlorophenol control, log reduction values ranging from 4.26 ± 1.15 to 0.20 ± 0.17 Log_{10}(CFU/well) and 5.06 ± 0.10 to 0.20 ± 0.20 Log_{10}(CFU/well) were observed in plate 1 and 2, respectively, for concentrations from 90 mg/L to 11.3 mg/L. Despite a similar range of values, the log reduction for plate 1 and plate 2 at 22.5 mg/L dichlorophenol was 1.07 ± 0.24 and 3.23 ± 0.23 Log_{10}(CFU/well), respectively.

The UT cNA sample produced an IC_{50} concentration of 22.9 mg/L and a cutoff for luminescence inhibition between 15.4 mg/L and 7.5 mg/L with inhibition values of 17.91 ± 2.49% and 4.50 ± 2.63%, respectively (Table 6.2). At 81% concentration, the UT cNA had an inhibition value of 98.91 ± 0.36% (Fig. 6.2.1.1A). Following 60-minute AOP treatment, the

inhibition produced by the cNA sample was 53.64 ± 4.42%, a significant 45.27 ± 1.48% decrease in inhibition from the UT cNA sample. For the enumeration data, the UT cNA sample produced a significant 2.54 ± 0.31 Log_{10}(CFU/well) reduction compared to the 2% saline control (Fig. 6.2.1.2A). Following treatment, this log reduction was significantly reduced by 2.48 ± 0.11 Log_{10}(CFU/well) compared to the UT sample. The 0.06 ± 0.06 Log_{10}(CFU/well) reduction observed for the 60-min treated sample was not significant compared to the 2% saline control (Fig. 6.2.1.2).

For OSPW B UT, an IC_{50} concentration could not be generated as the inhibition at the maximum exposure concentration in the assay (81%) only produced an inhibition value of 43.98 ± 2.16% (Fig. 6.2.1.1B). A cut off value for detectable luminescence inhibition was determined to be between 41% and 20% concentration (v:v) with inhibition values of 18.36 ± 2.46% and 4.02 ± 5.30%, respectively (Table 6.2). No significant reduction of luminescence was observed after 60-min AOP treatment of the sample, with a significant 38.81 ± 5.53% inhibition of luminescence being observed compared to the control (Fig. 6.2.1.1B). The UT OSPW B sample produced a log reduction of 0.35 ± 0.14 Log_{10}(CFU/well) compared to the growth control (6.2.1.2B). Following treatment, this was significantly reduced by 0.43 ±0.05 Log_{10}(CFU/well) to a value of -0.07 ± 0.14, which was not significantly different than the 2% control.

Like OSPW B, an IC_{50} concentration could not be generated for OSPW C as the inhibition at 81% concentration only produced an inhibition value of 30.24 ± 2.52% (Fig. 6.2.1.1C). As with OSPW B, the cut off value for detectable luminescence inhibition was determined to be between 41% and 20% concentration (v:v) with inhibition values of 6.24 ± 2.09% and -6.46 ± 2.84%, respectively (Table 6.2). AOP Treatment of OSPW C significantly reduced the inhibition produced by 23.6 ±1.41% to a value of 6.64 ± 3.41%, with the treated

inhibition being determined to be not significant compared to the control. OSPW C UT produced a significant log reduction of 0.73 ± 0.16 Log_{10}(CFU/well) compared to the control (Fig. 6.2.1.2C). Like OSPW B, AOP treatment significantly decreased this reduction by 0.80 ± 0.06 to -0.07 ± 0.09 Log_{10}(CFU/well), a value which as not significant compared to the 2% saline control.

An IC_{50} concentration of 81% (v:v) was determined for OSPW D with an inhibition value of 46.41 ± 7.29% (Fig 6.2.1.1D). The cutoff for inhibition was determined to be at concentrations of 61% and 41% (v:v) with inhibition values of 11.32 ± 1.90% and -2.88 ± 5.25%, respectively (Table 6.2). The 90-min AOP treatment of OSPW D resulted in a significant 99.54 ± 0.14% inhibition of luminescence compared to the 2% control. This value was significantly larger than the inhibition produced by the UT sample by a value of 53.13 ± 2.43% (Fig. 6.2.1.1D). The enumeration data did not follow this trend as the AOP treated sample produced a non-significant 0.03 ± 0.06 Log_{10}(CFU/well) reduction compared to the control (6.2.1.2D). This value was significantly reduced by 1.36 ± 0.12 Log_{10}(CFU/well) compared to the significant 1.39 ± 0.06 Log_{10}(CFU/well) log reduction observed for the UT OSPW D compared to the control.

6.2.3 Comparison of assay readouts between a bacterial luminescence inhibition assay and a microdilution MIC assay for treated and untreated OSPW and cNA

To determine the relationship between the readouts of both the assays used in this thesis the observed log reduction values for each respective organism was plotted as a function of the observed luminescence inhibition for each tested OSPW and cNA sample set. To compare the log reduction readouts produced by both organisms, the log reduction of *A. fischeri* was plotted as a function of the log reduction for *S. warneri* for each sample set. For both investigations simple

linear regression analysis was performed to determine the goodness of fit and equation for the line of best fit with 95% confidence intervals.

When analyzing the observed log reduction as a function of luminescence inhibition for *A. fischeri,* an R^2 value of 0.65, 0.77, 0.77 and 0.16 were obtained for the cNA, OSPW B, C, and D sample sets, respectively (Fig. 6.2.3.1A-D). When analysing the *S. warneri* log reduction values as a function of *A. fischeri* luminescence inhibition, R^2 values of 0.94, 0.89, 0.31, and 0.36 were determined for the cNA, OSPW B, C, and D sample sets, respectively (Fig. 6.2.3.1A-D). When comparing the log reduction values between *A. fischeri* and *S. warneri*, R^2 values of 0.55, 0.82, 0.65, 0.69 were obtained for the cNA, OSPW B, C, and D sample sets, respectively (Fig. 6.2.3.2A-D).

6.3 Discussion

In this chapter, untreated and AOP treated OSPW and cNA samples from chapter V were investigated using a standardized bacterial luminescence inhibition assay. This was done to investigate if correlation was observed between bacterial assay responses to treatment-induced changes in water chemistry. In addition to measuring inhibition of luminescence, the effect of OSPW and cNA exposure was measured using enumeration approaches to investigate the relationship between luminescence inhibition and cell density. Lastly, the enumeration data from both *S. warneri* and *A. fischeri* were compared on a per-sample basis to examine how these two approaches relate in terms of their toxicity endpoints.

6.3.1 Establishment of a standardized bacterial luminescence inhibition assay

As protocols already exist for measuring inhibition of luminescence in response to environmental samples, these approaches were performed as described for toxicity assessment of

OSPW samples. To perform these protocols as written, estimation of cell density was required for culturing and stock preparation. As previously indicated, OD is an accurate tool for estimation of cell density in culturing and control settings, but this approach must be validated to account for organism cell size and arrangement (Szermer-Olearnik et al. 2014). Compared to *E. coli, A. fischeri* are the same bacillus shape, but are slightly smaller in size. These organisms are also known to shrink significantly under adverse culturing conditions, though for the methods used in these experiments this would likely not be a factor due to the optimal growth conditions (Farmer et al. 2015). The online tool initially used for estimation of the test organism cell density proved accurate for measuring *A. fischeri* cell density when compared to the equation calculated based on the linear relationship. Initial investigation of the relationship between cell density and luminescence also revealed a strong linear relationship between these two variables. In the literature, this relationship has been shown to be linear when diluting a luminescent O/N culture at high cell density (Tavares et al. 2010).

6.3.2 Effects of advanced oxidative process treatment of OSPW on Aliivibrio fischeri luminescence

The inhibition of luminescence observed for the untreated cNA and OSPW samples partially aligns with initial classical NA concentrations that were measured. As previously indicated, the classical NA content of the samples tested is ordered as cNA, OSPW B, OSPW C, and OSPW D. The luminescence inhibition data at 81% v:v showed sensitivity in the order of cNA, OSPW D or B, and OSPW C. To better compare these assays in terms of endpoint, the MIC value was determined for the lowest concentration of cNA or OSPW that still produced a significant inhibitory effect on luminescence. The MIC values for *A. fischeri* luminescence inhibition further refines the sensitivity for these samples to be ordered as cNA, OSPW B, D, and

C (Table 6.2). This sensitivity was reflective of what was described for *S. warneri* microdilution MIC values, however bacterial luminescence identified OPSW C as having the smallest inhibitory effect on luminescence while *S. warneri* enumeration data identified this sample as having the greatest inhibitory effect on growth. Only OSPW D and the cNA sample were able to produce valid IC_{50} values, as all other samples produced inhibition lower than 50% at the maximum exposure concentration of 81% v:v.

For the untreated cNA sample, a luminescence MIC value of 15.2 mg/L – 7.6 mg/L was obtained. This value is within the observed range of sensitivity for this assay to cNA compounds with IC_{50} values often between 15 mg/L – 50 mg/L (Swigert et al. 2015; Tollefson et al. 2015). The IC_{25} value was also nearly identical to what has been reported for this same cNA compound in a similar luminescence inhibition assay, with my data providing an IC_{25} value of 14.9 mg/L and literature reporting a value of 16.2 mg/L (Bartlett et al. 2017). While there was no IC_{50} determined for the *S. warneri* microdilution assay, MIC values were lower at 3.8 mg/L – 1.9 mg/L. When evaluating the untreated OSPW samples, luminescence MIC values were nearly identical to what was observed for the *S. warneri* microdilution MIC values. Only the OSPW C showed greater sensitivity in the *S. warneri* microdilution assay with a MIC value of < 22.5% while luminescence MIC values were 41% – 20% (Table 6.2).

When comparing the luminescence inhibition of the treated samples, the data generally aligned with the SFS and *S. warneri* growth inhibition data. As determined by SFS analysis, the cNA sample as well as OSPW C and D had reductions in fluorescent organic compounds following AOP treatment while OSPW B had a maintenance of fluorescence intensity following AOP treatment. Luminescence inhibition data largely supported these trends, as OSPW B treatment did not reduce inhibitory effects while OSPW C saw effective reduction of inhibition

following treatment. While SFS data showed reductions in fluorescent organic compounds for the cNA solution, data in chapter V showed residual inhibitory effects on *S. warneri* growth. This is likely due to SFS analysis underestimating the presence of poorly fluorescent alicyclic compounds present in the cNA sample, or the production of acyclic byproducts that lake fluorescent properties (Rudzinski et al. 2002; Meshref et al. 2017). Similar trends were observed in luminescence data as treatment reduced inhibitory effects, but a significant residual inhibitory effect was still observed in the treated sample.

When investigated using linear regression analysis, the relationship between *S. warneri* growth inhibition and *A. fischeri* luminescence inhibition was largely sample dependent. The cNA and OSPW B samples were strongly correlated with values of 0.94 and 0.89, respectively, while the correlation was poor for OSPW C with a value of 0.31. Despite the weak correlation value, it should be noted that a similar trend was observed in treatment efficacy for OSPW C, with both assays identifying a reduction in inhibitory effects post treatment, however the high sensitivity of *S. warneri* growth to the untreated sample greatly diminishes this relationship. Overall, for the cNA, OSPW B, and OSPW C samples, both approaches appeared effective at detecting detrimental effects of the samples in their untreated state and were responsive to reductions in fluorescent organic compounds in solution.

For OSPW D treatment, SFS data revealed a reduction in fluorescent organic compounds in the sample post treatment, with *S. warneri* enumeration data showing a corresponding decrease in growth inhibition. Luminescence inhibition data did not align with these findings, as the treated sample produced greater luminescence inhibition than any of the untreated OSPW samples, similar to the effect of the untreated cNA and phenol control. This data closely aligns with what was observed for *S. warneri* OD, where this data suggested significant inhibition of

growth due to low measured OD values. As previously mentioned, both the radicals used in AOP treatment and the TPs produced from degradation of target compounds have the potential to introduce toxic effects (Chibwe et al. 2015; Deng and Zhao 2015), though these phenomena would not align with the previous *S. warneri* enumeration data for this sample. To try and better understand the underlying causes of this luminance inhibition, the challenge plates from this assay were enumerated following the 30 min exposure.

6.3.3 Effects of advanced oxidative process treatment of OSPW on Aliivibrio fischeri enumeration

For the *A fischeri* enumeration data, all untreated OSPW as well as the cNA sample had antimicrobial effects after 30 min exposure. Following AOP treatment, these antimicrobial effects were reduced in all samples, including OSPW D. This data suggests that AOP treatment removes or alters compounds that have antimicrobial effects, but conflicts with luminescence inhibition data that showed a large inhibitory response after exposure to the AOP treated OSPW D. This is reflected in the linear regression analysis which showed moderate correlation between *A. fischeri* luminescence and log reduction for all sample sets (0.65 – 0.77) except for OSPW D, which showed poor correlation with a value of 0.15. There are two possibilities for the phenomenon observed in this sample; the first is that contaminants present in the treated sample, either TPs or compounds introduced as part of treatment are interfering with normal metabolism that produces luminescence. However, this data in concert with the observed disparity between OD and enumeration data for *S. warneri* suggests that the inhibitory effects observed from the treated OSPW D may be a result of interference with photometric based approaches.

In principle, the measurement of bacterial growth using optical density involves the emission of light at a wavelength of 600 nm into a solution with bacterial cells suspended. As the

light passes through solution it will be scattered by bacterial cells, resulting in less light reaching the optical detector (Szermer-Olearnik et al. 2014). For luminescence detection, light is produced as part of the luciferase reaction in *A. fischeri* and is measured using an optical detector with filters set to the wavelength of emitted light (Williams et al. 2019). The reliance on photometry in both approaches invites opportunities for interference due to the presence of nanoparticles. One of the properties of nanoparticles that is problematic for photometric and colorimetric assays is their capacity to absorb light in compound-specific wavelengths (Ong et al. 2014). Iron oxide nanoparticles have been shown to have peak absorbance of light at wavelengths between 300 nm – 600 nm, values which encompass both bioluminescence from *A. fischeri* (~440 nm) and the emitted UV light for OD measurement (600 nm), though this absorbance can heavily depend on preparation (Parhizkar and Habibi 2017; Willams et al. 2019; Szermer-Olearnik et al. 2014).

The electrochemical properties of nanoparticles can also result in high light scattering, so much so that dynamic light scattering is one of the approaches for identifying the presence of these compounds in solution. These properties have been observed in iron oxide nanoparticles, which may further complicate assessment of both OD and bioluminescence when these compounds are present in the substrate (Chu et al. 1987; Beal et al. 2020). Adsorption properties of these molecules also can induce aggregation of compounds from tested substrates, interfering with photometric analysis of solutions with high concentrations of suspended solids (Qiu et al. 2017) The potential for these compounds to interfere with a range of *in vitro* toxicity testing has been clearly established, including approaches that rely on bioluminescence (Kroll et al. 2009; Ong et al. 2014; Qiu et al. 2017). It is worth noting that the concentration of iron oxide nanoparticles produced from this treatment were reported as being low (Aldana-Vasquez 2024). Considering that literature did not reveal clear concentration thresholds for interference from the

optical properties of these compounds, it is difficult to determine confidently if these results are a product of photometric interference.

Several concerns have also been identified with the use of bioluminescence for toxicity investigation beyond the potential for photometric interference. Hormesis is a phenomenon in ecotoxicity where low doses of toxicant can induce adaptive responses that appear to be beneficial to organism physiology. These effects are often intermittent, with effects becoming deleterious as time or toxicant dose increases (Hashmi et al. 2014). These effects have already been established for several of the known contaminants in OSPW such as heavy metals and hydrocarbon compounds. *Aliivibrio fischeri* bioluminescence has displayed hormetic effects in response to antimicrobial compounds, with the effects being time dependent (Sun et al. 2018). Given the short endpoint of luminescence inhibition assays (5 – 30 min), these effects can significantly change toxicity interpretations from bioluminescent systems. While it is noted that hormesis has been observed for organism growth, these effects are not as apparent in growth as in bioluminescence, suggesting enhanced stability of growth-based endpoints (Sun et al. 2018; Hashmi et al. 2014).

In addition to hormetic effects, cations such as potassium, sodium, magnesium and calcium can all induce significant luminescence in bioluminescent bacteria (Deryabin and Aleshina 2006). These ions have been shown to alter sensitivity to the reference phenol control in these assays as well as several metals of environmental concern (Berglind et al. 2010) Similar issues were raised when discussing growth-based assays as these ions are of physiological importance in several bacterial systems (Gries et al. 2016). The concentration of these compounds can fluctuate greatly in environmental conditions, though no clear guidelines are present to control for this in bacterial luminescence assays (Ma et al. 2014).

Several analogous compounds have also been shown to competitively inhibit or artificially promote autoinduction of luminescence, posing the potential for compounds to interfere with light production systems in these organisms (Schaefer et al. 1996). These effects can be extended to quorum sensing systems, where naturally occurring compounds can interfere with several of the physiological outputs including luminescence, though it should be noted these compounds often effect organism growth and normal physiology, and as such can be considered toxic in their own regard (McDougald et al. 2007). The effects observed in this dataset may be a product of any combination of these photochemical or physiological factors, complicating interpretation of luminescence inhibition data as it pertains to toxicity.

Despite the large difference in assay exposure times, the best correlation observed across all samples (0.82 – 0.54) was when log reduction values were compared between *A. fischeri and S. warneri*. While the absolute values differed greatly, these datasets largely reflected the predicted toxicity of both the untreated and AOP treated samples as determined by SFS analysis. As previously discussed, culture-based enumeration approaches are resilient against interference seen in photometric approaches (Beal et al. 2020; Szermer-Olearnik et al. 2014). These approaches have also consistently proven to be cost-effective, accurate, and precise for enumeration of culturable bacteria (Davis 2014). However, it must be acknowledged that the use of bioluminescence, which is inherently tied to several physiological processes as well as cell density, does allow for interrogation of effects beyond cytotoxicity and growth inhibition (Hao et al. 1995).

6.4 Future Directions

Overall, the data in this chapter highlights several points of convergence and divergence between the modified microdilution MIC assay and bacterial luminescence inhibition

approaches. In the absence of interference, both assays appear to be effective at detecting toxicity in untreated cNA and OSPW samples, as well as evaluating changes in these waters in response to AOP treatment. Given similarities in technical execution, these assays have the potential to be integrated into a singular approach that can capitalize on the technical and interpretative advantages of both approaches while overcoming several potential limitations. This will be discussed in detail in the next section of this thesis.

Table 6.2. Assay specific endpoint values for treated and untreated cNA and OSPW samples

Assay Endpoint	cNA	OSPW A	OSPW B	OSPW C	OSPW D
Modified microdilution (Growth - MIC)	3.8 – 1.9 mg/L	45 – 22.5%	45 – 22.5%	< 22.5%	67.5 – 45%
Luminescence inhibition (IC_{50})	22.8 mg/L	X	> 81%	> 81%	81%
Luminescence inhibition (Luminescence - MIC)	15.2 – 7.6 mg/L	X	41 – 20%	41 – 20%	61% – 41%

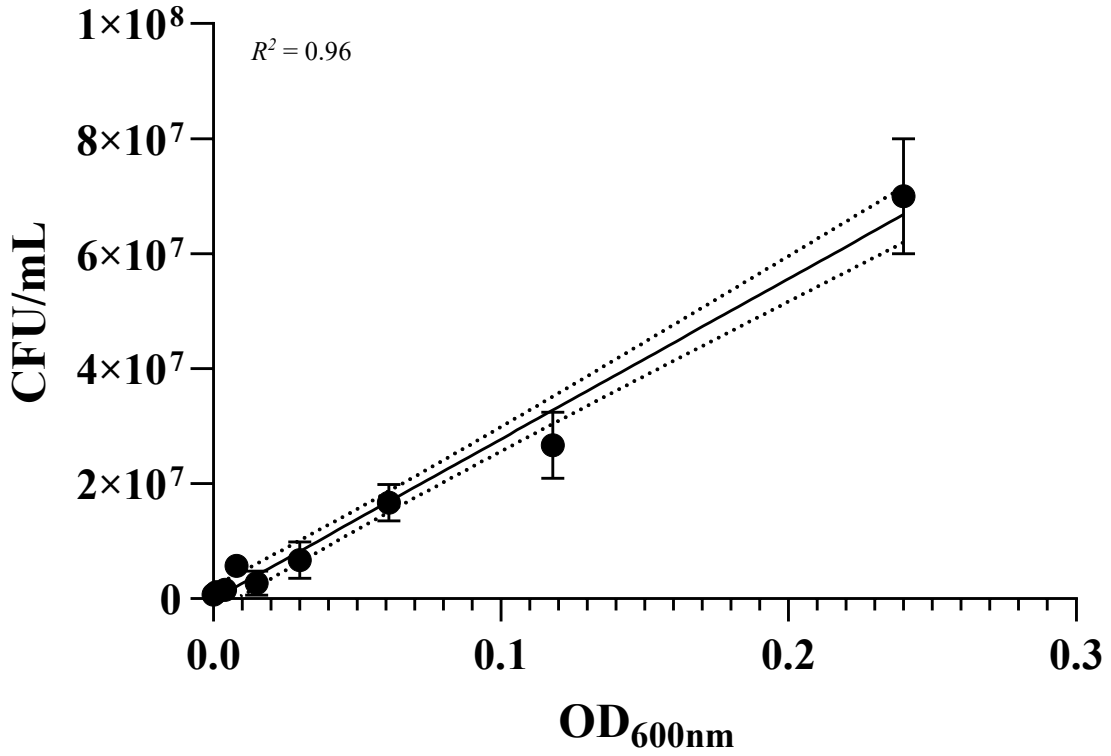

Figure 6.2.1.1. Relationship between CFU/mL and optical density for *Aliivibrio fischeri*. Colonies were suspended from solid agar in LBS and diluted. Following dilution optical density at 600_{nm} was measured. After OD_{600nm} measurement, dilutions were serially diluted and spot plated for enumeration. Simple linear regression analysis was performed. The line represents best-fit with 95% confidence intervals (n=9).

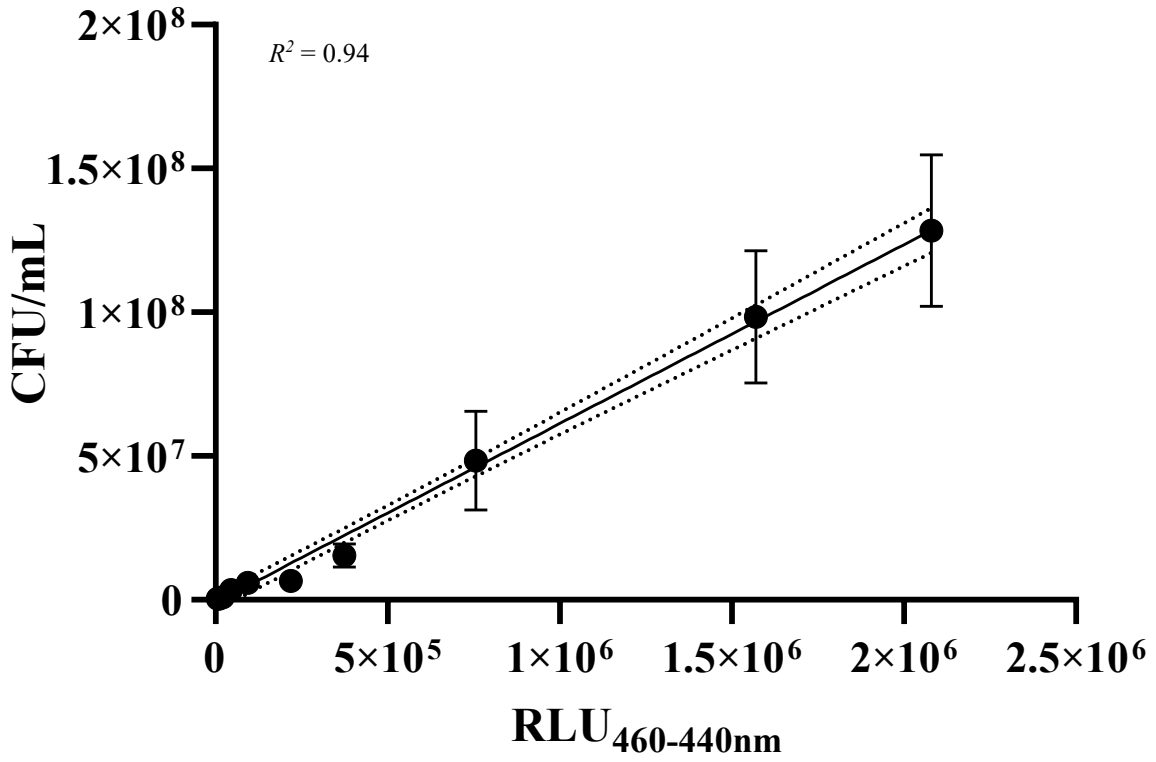

Figure 6.2.1.2. Relationship between CFU/mL and luminescence for *Aliivibrio fischeri* freezer stocks. Freezer stocks of *A fischeri* prepared as per ISO 11348-1 (2007) were thawed and diluted 1:205 (v:v) in reconstitution solution before subsequent ½ step dilution in 2% saline. Following dilution the $RLU_{440\text{-}460nm}$ was measured. After RLU measurement, dilutions were serially diluted and spot plated for enumeration. Simple linear regression analysis was performed. The line represents best-fit with 95% confidence intervals (n=3).

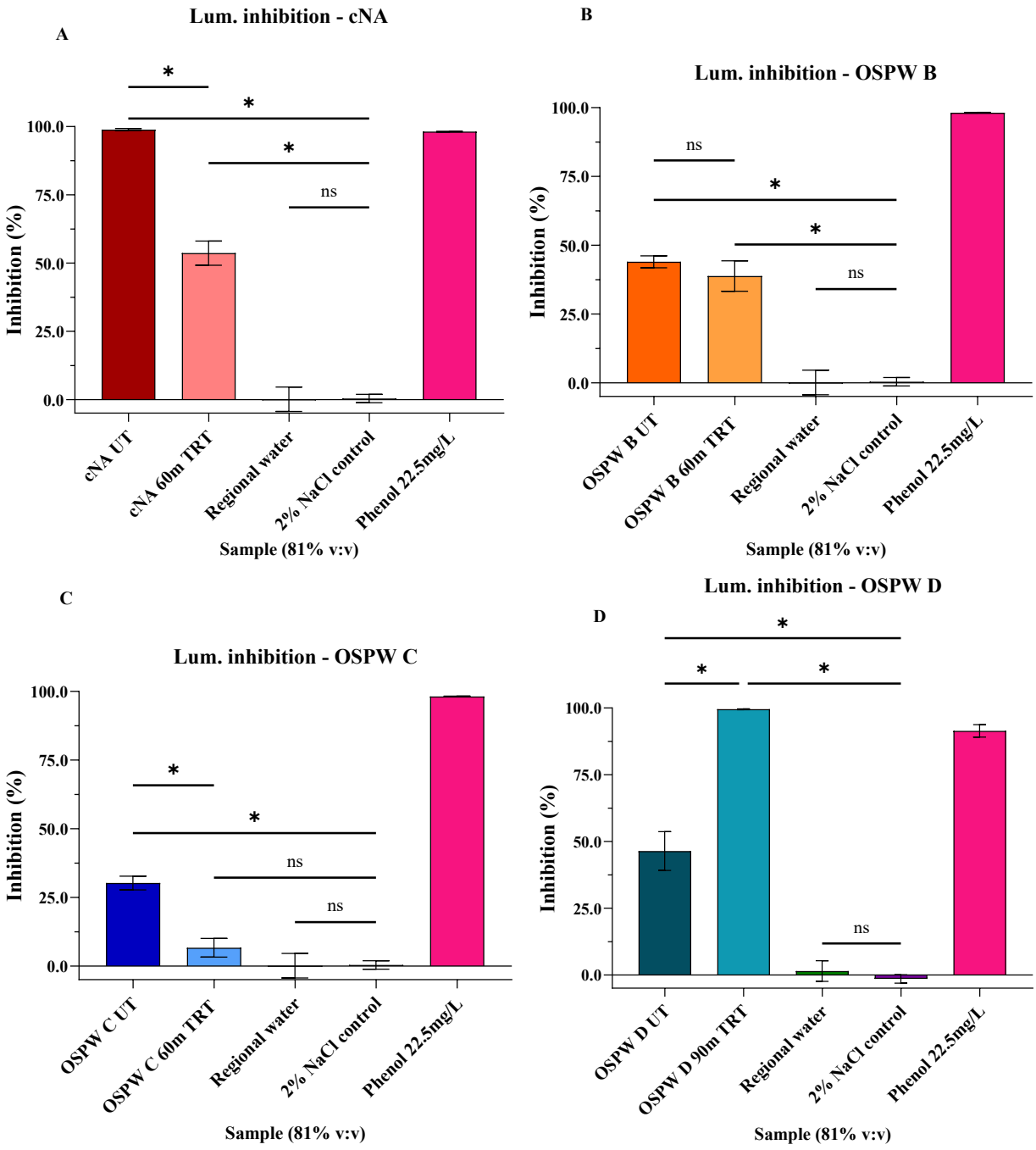
A
Lum. inhibition - cNA
Inhibition (%)
100.0
75.0
50.0
25.0
0.0
*
*
*
ns
cNA UT
cNA 60m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)
B
Lum. inhibition - OSPW B
Inhibition (%)
100.0
75.0
50.0
25.0
0.0
ns
*
*
ns
OSPW B UT
OSPW B 60m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)
C
Lum. inhibition - OSPW C
Inhibition (%)
100.0
75.0
50.0
25.0
0.0
*
*
ns
ns
OSPW C UT
OSPW C 60m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)
D
Lum. inhibition - OSPW D
Inhibition (%)
100.0
75.0
50.0
25.0
0.0
*
*
*
ns
OSPW D UT
OSPW D 90m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)

Figure 6.2.2.1. Effect of treated and untreated cNA and whole OSPW exposure on *Aliivibrio fischeri* luminescence after 30-minute exposure. Freezer stocks of *A. fischeri* prepared as per ISO 11348-1 (2007) were thawed and diluted before being added to a challenge plate. Salinity and pH adjusted test solutions were added 9:1 (v:v) to wells containing either untreated or 60-min UVA PDS treated 33.3 mg/L cNA (A), OSPW B (B), OSPW C (C), or untreated and 90-minute BC-Fe PMS treated OSPW D (D). Controls were prepared by adding 90% regional freshwater, 2% saline control, or dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in 2% saline 9:1 (v:v) in the challenge plate. Challenge plates were incubated for 30 minutes at 15 °C prior to having the $RLU_{440\text{-}460nm}$ read. Percent inhibition was calculated by measuring the difference between the normalized control and test luminescence at 30 minutes. Bars represent mean luminescence inhibition ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

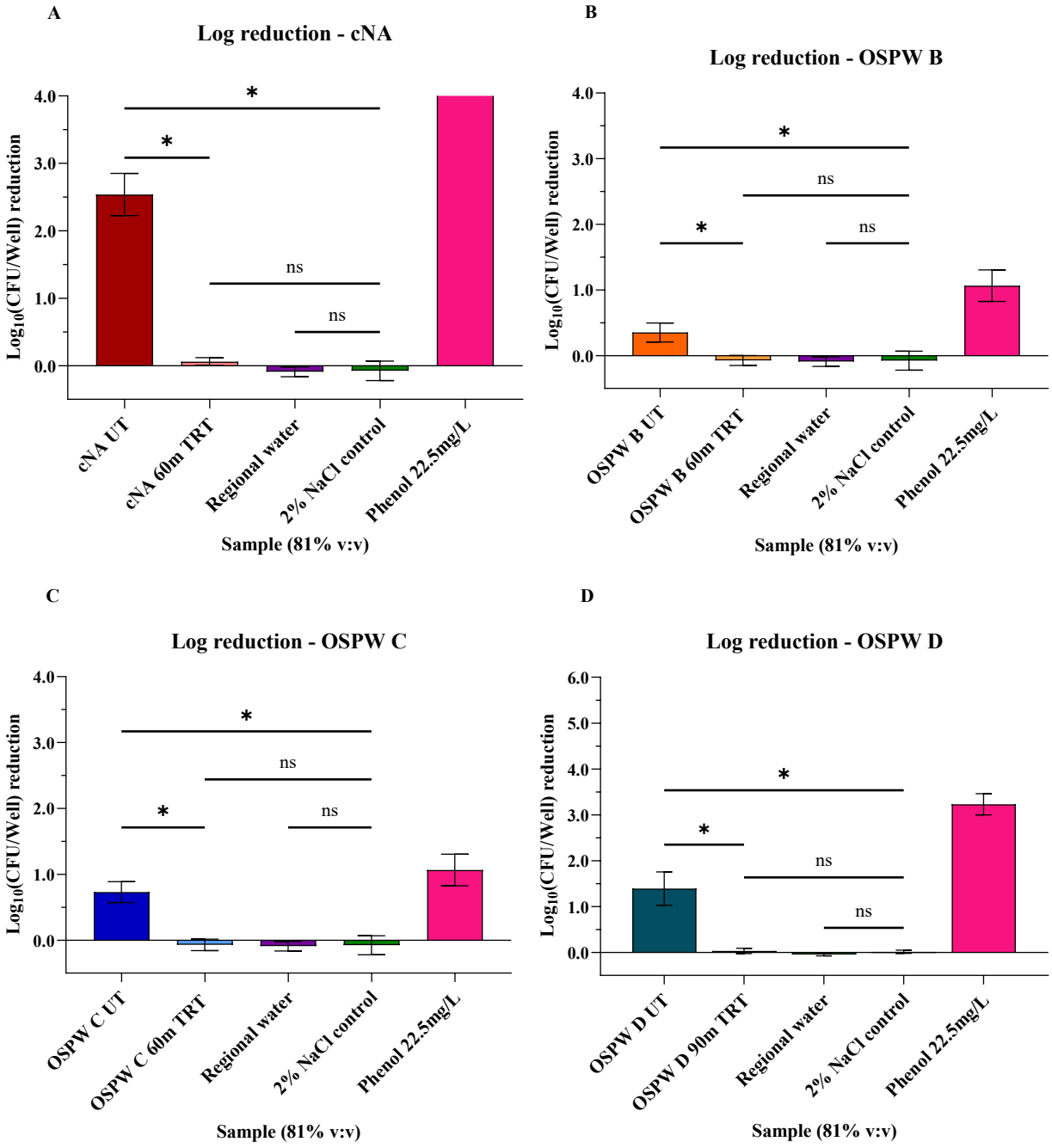
A
Log reduction - cNA
Log10(CFU/Well) reduction
4.0
3.0
2.0
1.0
0.0
*
*
ns
ns
cNA UT
cNA 60m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)
B
Log reduction - OSPW B
Log10(CFU/Well) reduction
4.0
3.0
2.0
1.0
0.0
*
ns
*
ns
OSPW B UT
OSPW B 60m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)
C
Log reduction - OSPW C
Log10(CFU/Well) reduction
4.0
3.0
2.0
1.0
0.0
*
ns
*
ns
OSPW C UT
OSPW C 60m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)
D
Log reduction - OSPW D
Log10(CFU/Well) reduction
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
ns
ns
OSPW D UT
OSPW D 90m TRT
Regional water
2% NaCl control
Phenol 22.5mg/L
Sample (81% v:v)

Figure 6.2.2.2. Effect of treated and untreated cNA and whole OSPW exposure on *Aliivibrio fischeri* luminescence after 30-minute exposure. Freezer stocks of *A. fischeri* prepared as per ISO 11348-1 (2007) were thawed and diluted before being added to a challenge plate. Salinity and pH adjusted test solutions were added 9:1 (v:v) to wells containing either untreated or 60-min UVA PDS treated 33.3 mg/L cNA (A), OSPW B (B), OSPW C (C), or untreated and 90-minute BC-Fe PMS treated OSPW D (D). Controls were prepared by adding 90% regional freshwater, 2% saline control, or dichlorophenol diluted from 100.0 mg/L to 12.5 mg/L in 2% saline 9:1 (v:v) in the challenge plate. Challenge plates were incubated for 30 minutes at 15 °C. Following incubation each well was serially diluted and spot plated for enumeration. Log reductions were determined by calculating the difference between the test sample and the OSPW replicant control. Bars represent mean Log reduction ± SD (n=3). A Shapiro-wilk test for normality was performed, after which a Brown-Forsythe and Welch ANOVA was performed with Dunnett post test. * represent significance ($p < 0.001$).

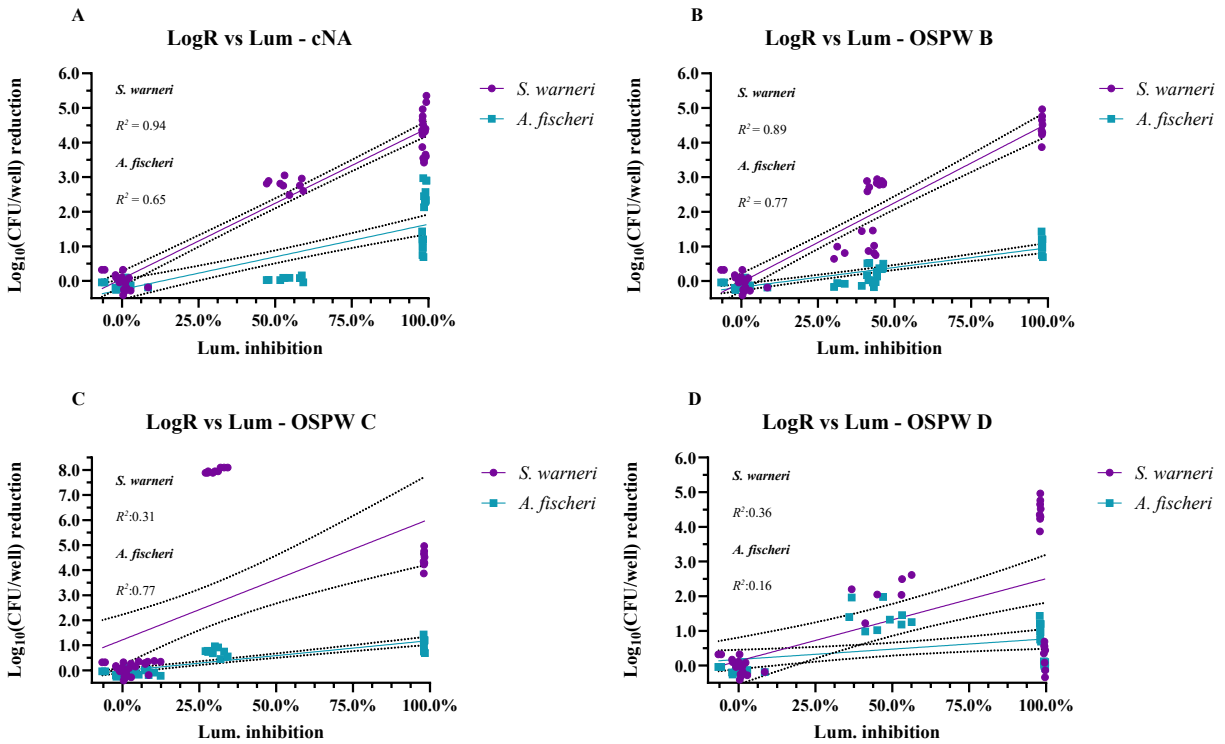

Figure 6.2.3.1. Relationship between luminescence inhibition and log reduction for *Staphylococcus warneri* and *Aliivibrio fischeri*. Values from previously described MIC and luminescence inhibition experiments in section 5.2.2 and 6.2.2 were analyzed with log reduction values of both *S. warneri* and *A. fischeri* as a function of *A. fischeri* luminescence inhibition after exposure to either untreated or 60-min UVA PDS treated 33.3 mg/L cNA (A), OSPW B (B), OSPW C (C), or untreated and 90-minute BC-Fe PMS treated OSPW D (D). Simple linear regression analysis was performed. The line represents best-fit with 95% confidence intervals.

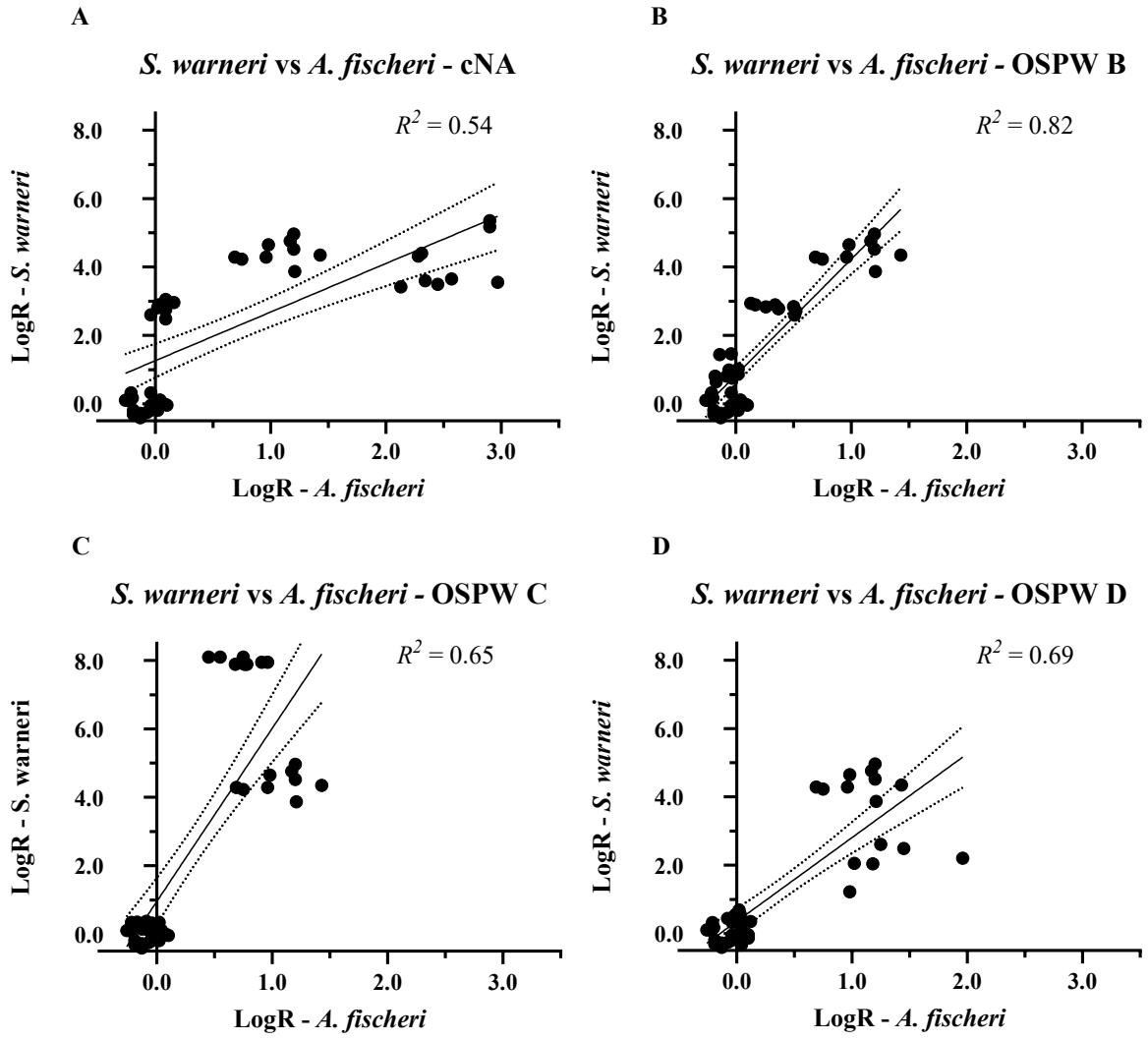

Figure 6.2.3.2. Relationship between *Aliivibrio fischeri* and *Staphylococcus warneri* log reduction. Values from previously described MIC and luminescence inhibition experiments in section 5.2.2 and 6.2.2 were analyzed with log reduction values for *S. warneri* as a function of *A. fischeri* log reduction after exposure to either untreated or 60-min UVA PDS treated 33.3 mg/L cNA (A), OSPW B (B), OSPW C (C), or untreated and 90-minute BC-Fe PMS treated OSPW D (D). Simple linear regression analysis was performed. The line represents best-fit with 95% confidence intervals.

Chapter VII

General Discussion

7.1 Summary of Findings

The Alberta oil sands are the third largest known oil reserve in the world with 20% of the bitumen deposits accessible via surface mining (CAPP 2024b). Industry operators use hot water extraction techniques to separate sand, silt, and clay from the useable bitumen suspended in this mined substrate (Masliyah et al. 2011). During this process, the addition of caustic chemicals as well as the release of sequestered compounds of environmental concern contaminate these waters. As these waters are recycled for multiple extractions, these compounds become concentrated until they can no longer be used for extraction processes (Masliyah et al. 2011; Allen 2008a). These OSPW contain several compounds that have been shown to be detrimental to organism physiology and normal biological function both *in vivo* and *in vitro* (Li et al. 2017; Mahaffey and Dube 2017; Allen 2008a). As several of these recalcitrant compounds such as NAs have no guidelines regarding their presence and release in the environment, these waters have been held under a zero-discharge policy (AER 2023b).

Current environmental guidelines require sustainable remediation of tailings waters within 10 years of site closure (AER 2023b). To facilitate this, significant efforts have been undertaken to investigate new toxicity assessment and treatment approaches for OSPW management. The most commonly implemented *in vitro* assay for measuring OSPW toxicity uses bacterial bioluminescence, though this approach has several limitations that complicate its use in this role (Mahaffey and Dube 2006; Hashmi et al. 2014; Ma et al. 2014; Woutersen et al. 2011). Despite these limitations, bacteria-based toxicity assays are robust, cost-effective, and

high throughput, making them an appealing option for initial toxicity screening (Viegas et al. 2021). In clinical settings, microdilution MIC assays are highly standardized and provide precise, high-throughput, quantitative data for assessment of antimicrobial activity (Balouiri et al. 2016). Given the technical similarities between these clinical approaches and current bacterial ecotoxicity assays, there is high potential for MIC microdilution assays to be adapted for environmental use (Vassallo et al. 2018).

The objective of this thesis was to adapt and implement a growth-based bacterial assay for use in freshwater and OSPW toxicity assessment. Specifically, I sought to 1) establish a modified MIC microdilution assay for use in freshwater and OSPW toxicity settings; 2) perform treatment of OSPW to remove compounds of concern and compare chemical analysis data with toxicity endpoints in a modified MIC assay; and 3) compare chemical analysis data and MIC data to a traditional bacterial luminescence inhibition assay. To achieve these objectives, a modified MIC microdilution assay was adapted from standardized MIC protocols used in a clinical setting. The assay was then validated to be robust under freshwater and OSPW 'like' conditions. Several NA and OSPW samples were then treated, and chemical analysis data was generated via SFS to monitor changes in water chemistry. These untreated and treated waters were then assessed using the modified MIC assay and traditional luminescence inhibition approaches to determine the relative sensitivity in terms of toxicity endpoints and changes in water chemistry.

7.2 Growth-based bacterial toxicity assays

When considering the principles of growth for any bacteria, the chosen parameters for inoculum density, nutrient availability, pH, salinity, temperature, and incubation time all have significant effects on expected growth. Deviation of these conditions from optimal ranges for

organism growth can induce stress that can alter sensitivity to antimicrobial compounds (CLSI 2018). As a result, these same factors have significant impacts on the sensitivity of microorganisms to the toxicity of xenobiotic compounds in the environment (Lenz et al 1986; Ganjian et al. 2012; Levin-Edens et al. 2011; Larsen 1989). As determined from my data, our model organism was shown to experience near optimal growth under freshwater and replicated OSPW conditions. There is still a possibility of the synergistic effects of environmental stressors altering the observed sensitivity to xenobiotic compounds in OSPW (Whiting et al. 1996), though the physiochemical parameters of these waters exist well inside of what was determined to optimal for this organism.

The data in this thesis emphasizes some of the major advantages that are inherent in using growth-based toxicity assessment. One such advantage is that the longer exposure time used to assess organism growth allows for greater potential sensitivity to compounds of concern. While it is possible that these longer exposures can allow for organisms to adapt to the presence of toxicants and environmental stress, this adaptation process often has severe implication for bacterial growth and morphology, allowing for detection of these effects in growth-based assays (Marles-Wright et al. 2007; Aertsen et al. 2004). While hydrocarbons have shown acute toxicity to bacteria, the systems used by bacteria to survive these stresses such as energy-dependent efflux use valuable ATP that cannot be used for growth and reproduction (Heipieper et al. 2010; Isken et al 1999). This has been demonstrated in organisms such as *Pseudomonas putida,* where exposure to volatile hydrocarbon compounds resulted in greatly reduced growth but stable cell energy charge and ATP production (Neumann et al. 2006). The relationships between toxicant stress, energy usage, and bacterial growth are combined in models based on Dynamic Energy Budget (DEB), which can connect molecular processes to whole organism physiology. These

DEB models consistently predict the cumulative effects of toxicants on multiple physiological systems as converging on inhibitory effects on the onset, rate, and total growth of the organism (Birnir et al. 2018; Klanjscek et al. 2012). This was observed in our data, where growth inhibition data showed greater MIC values when compared to those generated using luminescence inhibition for untreated OSPW and cNA (Table 6.2).

Beyond the advantage of increased sensitivity, the use of longer exposure times in growth-based assessment also has advantages. The inhibition of bacterial luminescence as assessed in standardized assays for 30 minutes at sub-optimal growth temperatures reflects acute detrimental effects of toxicants on crucial bacterial systems such as membrane integrity or electron transport chain activity. While the short exposure period offers low technical burden, it does not reveal the cumulative, multi-generational effects of chronic toxicant exposure on physiological systems (Gellert 1999; Tjes 2008). This has been demonstrated in *A. fischeri* bioluminescence specifically, where extended exposure times revealed sensitivities an order of magnitude greater than what was observed for the acute 30-minute test (Backhaus et al. 1997). Not only are these longer exposure times more consistent with the 24 – 72-hr exposures used in other *in vitro* and *in vivo* aquatic toxicity tests (Government of Canada 2024), but they are also more ecologically relevant as they better model the chronic exposure that would occur for an organism exposed to OSPW and whole effluent (Gellert 2000).

Due to growth inhibition being the convergent result of deleterious effects on several physiological processes, these approaches lack the resolution to investigate the mechanism of toxicity (Houck and Kavlock 2008; Wiegand et al. 2008). This makes growth-based assessment better suited for toxicity screening purposes, with other assays and approaches serving to investigate toxicity mechanisms. The potential value of the methods described in this thesis are

supported by current trends in WET approved methodologies. Currently, approved methods for growth-inhibition of algae are the only other available *in vitro* approach alongside bacterial luminescence. These approaches are acknowledged as having similar advantages to what has been described previously for growth-based toxicity testing, though are limited by their technical burden. High culturing costs, 72-hr exposure times, and variation in stock culture growth and sensitivity all limit the practicality and reproducibility of these approaches (Nyholm and Kallqvist 1989; Eisentraegar et al. 2003, Environment Canada 2007). The methods presented in this thesis leverage the strength of bacterial culturing and growth to provide an endpoint of similar biological relevance as what is used in algal tests, but with decreased cost, shorter exposure times, and high reproducibility. However, the use of *S. warneri* does limit the ecological relevance of these approaches compared to freshwater algae that naturally inhabit ecosystems affected by OSPW (Mahdavi et al. 2015).

The efforts made in this thesis are supported by a small body of literature that has attempted to adapt growth-based MIC assays for environmental use. These investigations are primarily focused on nanoparticle and metal toxicity, citing several of the limitations of bacterial luminescence assays identified in this thesis. These approaches do deviate significantly from traditional MIC guidelines, using different organisms, short exposure times, and relying on enumeration approaches such as measured biomass or OD (Vassallo et al. 2018; Qiu et al. 2017). The closely related and equally robust minimum biofilm inhibitory concentration (MBIC) assay has been used extensively for investigating metal toxicity and has been specifically used to evaluate metal tolerance of organisms isolated from OSPW (Frankel et al. 2016). While this literature helps to highlight the potential for adapting growth-based toxicity assessment, these

approaches remain methodologically distinct from what is described in this thesis, where considerable effort was made to adhere to the protocols described in M07-A10 (CLSI 2018).

7.3 Culture-dependent enumeration approaches

Even when growth is used as a toxicity endpoint, the data present in this thesis also emphasizes the challenges associated with indirect assessment of growth and metabolic activity through photometric approaches. Based on the data obtained for OD and luminescence, it is likely that compounds present in the treated OSPW D sample had optical properties that interfered with measurement of light emission and absorption. This was consistent with established literature that has shown that both approaches may be subject to optical interference by nanoparticles due to their optical properties (Qiu et al. 2017; Kroll et al. 2009; Parhizkar and Habibi 2017; Willams et al. 2019; Szermer-Olearnik et al. 2014). These concerns are further compounded by literature which demonstrates the accuracy of OD is affected by cell debris, morphology changes, and byproducts from tested compounds and metabolic substrates (Szermer-Olearnik et al. 2014; Hernandez and Marin 2002; Beal et al 2020). These concerns were also observed in my data, where factors such as initial turbidity, values outside of detectable limits, and underestimation of cell density limited the reliability of this approach.

In some settings determining the presence and number of bacteria is not possible using culture-dependent approaches, especially when organisms require complex culture conditions or are present in mixed populations. In these settings, culture-independent approaches such as flow cytometry, staining, and RT-qPCR serve as some of the only precise methods for identifying bacterial populations. Though effective, these approaches require specialized equipment and training, are costly, and lack the throughput required for large scale antimicrobial testing (Lee et al. 2021: Thomas et al. 2015; Prakash et al. 2013). In settings where enumeration is possible to

perform using culture-dependent techniques, plate-based enumeration remains the most cost-effective, precise, and efficient approach for bacterial enumeration with detection limits that exceed what is established for molecular techniques (Davis 2014; Beal et al. 2020; Szermer-Olearnik et al. 2014). No fact better emphasizes this than the persistence of disk diffusion, agar dilution, and broth microdilution supported by plate-based enumeration as some of the only standardized and accepted methodologies for antimicrobial assessment in clinical settings (Balouiri et al. 2016; CLSI 2018).

Using the protocol described in this thesis, 32 samples prepared in triplicate can be serially diluted, spot platted, and counted in under 20 minutes, not including incubation time. This is far more efficient than what is described for many molecular techniques, though admittedly these approaches do not need to wait for bacterial growth for enumeration (Rompre et al. 2001). While molecular techniques can certainly serve critical roles in investigating the toxicity of OSPW, the methods used for enumeration in this thesis provide precise, interference-free determination of inhibitory effects on organism growth.

One of the major limitations of growth-based enumeration and colony counting is the presence of VBNC cells. Limited literature exists that specifically discusses the induction of VBNC in *S. warneri*, but it has been shown to enter this state under similar conditions as other *Staphylococcus* spp., requiring a combination of salinity, temperature, nutrient, and antibiotic stress to induce this state (Franca et al. 2021). Most of the literature cites low temperature as a requirement for induction of VBNC in *Staphylococcus*, while these experiments were performed at the optimal 35 °C for this organism, though it is possible that conditions created in these exposures could apply similar physiological pressures and induce this state (Li et al. 2020; Yan et al. 2021). Ultimately, the implications of cells in a VBNC state on environmental toxicity

assessment are not as severe as in clinical settings. The risk to human health is greatly increased by VBNC bacteria, as these cells may evade detection in food processing and healthcare settings, as well as possessing increased survivability against antimicrobial compounds. The methods used for enumeration in this thesis would not have the ability to distinguish dead cells from those in a VBNC, though this is irrelevant for toxicity screening, as both outcomes are a result of the detrimental effects of the samples on organism physiology (Ramamurthy et al. 2014).

7.4 Limitations of luminescence based bacterial assays

The data presented in this thesis also emphasize several limitations with current bacterial bioluminescence inhibition assays. The use of a marine organism such as *A. fischeri* requires significant modification of samples that have similar physiochemistry to what is observed in freshwater systems. In addition to diluting the samples, these changes in pH and salinity can change the nature of compounds present in solution, altering their toxicity (Environment Canada 1992). The comparison between luminescence inhibition and enumeration data revealed that luminescence is not a reliable predictor of cell density. Even in samples where photometric interference was not likely, significant luminescence inhibition was observed without any impact on cell density. This can be a result of detrimental effects on metabolic and biochemical pathways associated with luminescence, and while this certainly can be considered toxicity this distinction between metabolic interference and cell death is seldom identified, complicating toxicity interpretations (Weitz et al. 2002; Monteiro-Riviere 2006). These concerns are compounded by the results for OSPW D which appear to show interference due to the optical properties of nanoparticles. Here, the absorption, adsorption and light scattering properties of these compounds can greatly decrease luminescence while having limited effect on organism health (Ong et al. 2014; Qiu et al. 2017; Kroll et al. 2009).

In addition to the complications observed directly in the data of this thesis, several outstanding limitations have been identified for luminescence inhibition assays. While large volumes of data exist showing strong predictive outcomes between luminescence and other *in vitro* and *in vivo* approaches, these correlations are much weaker for several known toxicants present in OSPW as well as complex mixtures such as industrial effluent (Hao et al. 1995). This has been associated with samples that have high chemical oxygen demand (COD), suggesting the potential for organic compounds to interfere with bioavailability of toxicants (Bennett and Cubbage 1992). This has been demonstrated for OSPW specifically, where sensitivity of luminescence-based approaches was much lower than what was seen in other *in vivo* and *in vitro* approaches (Bartlett et al. 2017; Frank et al. 2008; Bauer et al. 2019). The limitations associated with nanoparticles and substrates such as OSPW must be carefully considered when interpreting toxicity data from these approaches. Overall, these limitations do not make luminescence-based toxicity assessment entirely obsolete but do greatly limit their significance when used for toxicity testing in isolation (Mahaffey and Dube 2017).

7.5 Future Directions

Several future directions are clearly apparent from the information presented in this thesis. As previously discussed in chapter IV and V, several small modifications of the microdilution MIC assay should be made to improve sensitivity and validity of its readouts. The first is that the OSPW replicant media should be supplemented with additional potassium. Given the role of this ion in several physiological systems (Gries et al. 2016), it should be added to the control in concentrations 10 mg/L, similar to what is seen in freshwater and OSPW conditions (Table 3.2). The OD data revealed that under these test conditions, *S. warneri* reached stationary phase at ~16 hrs, resulting in a nearly 6 hr period where the control cell density appeared to

decrease as the tested samples continued to grow. Intervention for enumeration at this 16 hr timepoint may allow for increased sensitivity and better resolution between samples compared to the controls.

The chemical analysis provided by SFS served an initial rapid, semi-quantitative tool for monitoring the presence of aromatic organic compounds. While convenient, these approaches lack the precision found in more high-resolution techniques such as mass spectroscopy. It has been consistently shown that the use of various forms of mass spectroscopy are effective in differentiating organic compounds present in OSPW, including NAs (Headly et al. 2002; Vander Meulen et al. 2021; Grewer et al. 2010; Huang et al. 2018). Structurally different NAs can have dramatically different contributions to toxicity, making delineation of NA speciation crucial for interpreting toxicity data (Jones et al. 2011; Lo et al. 2006). Additionally, structure of NA species has been shown to have significant impacts on removal efficacy using passive and active treatment techniques (Han et al. 2008; Meshref et al. 2017; Islam et al. 2014). Techniques such as Fourier transform infrared spectroscopy (FTIR), gas chromatography (GC), and high-performance liquid chromatography (HPLC), and negative ion electrospray orbitrap mass spectroscopy all provide the resolution required to further investigate structural influences on OSPW toxicity, making these important analytical outputs for investigating OSPW toxicity and treatment (Brown and Ulrich 2015).

The majority of the discussion in this thesis focuses on the contribution of the organic contaminants to OSPW toxicity, but it must be acknowledged that inorganic compounds such as trace metals are present in OSPW at concentrations that are considered a health risk to aquatic organisms (Allen 2008a; CCME 1999). Trace metals have significant detrimental effects on microorganism physiology and can contribute to the toxicity observed in OSPW when

investigated using bacterial assays (Igiri et al. 2018; Frankel et al. 2016). However, with no knowledge of the concentrations of trace metals present in these waters, it is difficult to predict what their contribution to toxicity may be. Inductively coupled plasma-optical emission spectroscopy (ICP-OES) and atomic absorption spectroscopy (AAS) are two currently used approaches for detecting trace metals in water, though these are cited as being slow and expensive. Recently, laser-induced breakdown spectroscopy (LIBS) has been proposed as a method to measure trace elements in the environment with a lower cost, less sample preparation, and similar sensitivity (Parmar et al. 2023; Khan et al. 2022). These approaches can provide crucial insight into the presence of trace metals in OSPW, combing with chemical analysis on NA composition to help discern the individual contributions of these compounds to toxicity.

As previously indicated, growth-based assays are a powerful tool in toxicity testing as they can recognize the sum of detrimental effects on multiple physiological systems. However, using growth as an endpoint makes it challenging to delineate the mechanism of toxicity for compounds. One potential approach for determining mechanism of toxicity is using genetic biosensors. These approaches involve selecting genes associated with physiological stress (efflux, DNA repair, membrane repair, etc.) and attaching genetic reporters to monitor transcription of these genes. These genetic reporters can produce fluorescence upon excitation, or may simply produce bioluminescence, allowing for relatively easy measurement of stress responses in bacteria, though they may be susceptible to photometric interference (Vollmer et al. 2004; Ong et al. 2014). Beyond creation of biosensors, approaches such as RNA-seq may also be used to directly investigate genes that are upregulated in response to compounds of concern. This has already been performed for cNA and organic extracts from OSPW using *Pseudomonas* sp., where exposure to NAs and extracted organics induced upregulation of genes associated with

efflux and membrane repair and maintenance (Bookout et al. 2024). These approaches allow for the determination of sub-inhibitory stress on organisms and may further elucidate mechanisms of toxicity by understanding physiological systems that these compounds effect.

From an ecotoxicity perspective, the significance of *in vitro* methods is often built upon their ability to serve as predictive models for toxicity outcomes in *in vivo* toxicity (Frank et al. 2009). This is one of the major cited strengths in bacterial luminescence-based toxicity tests, where large volumes of data have been published comparing this model to whole organism studies for various toxicants (Hao et al. 1995). As such, I propose the inclusion of additional toxicants such as trace metals, as well as the incorporation of *in vivo* models to test alongside the modified microdilution MIC approach described in this thesis. Preferentially, the use of organisms and methods approved for WET testing would substantially improve our understanding of how this model may fit into a battery of toxicity tests for OSPW.

While much of this thesis is critical of bacterial luminescence inhibition assays, these assays still serve a valuable role in ecotoxicity assessment. Though endpoints and test organisms may differ, there are a number of technical similarities between luminescence inhibition assays and the microdilution MIC assay. As such, several investigations have already been done to combine the advantages of these approaches. Using *A. fischeri,* growth-based assessments have been performed with 6-hr and 19-hr exposures. These assessments used OD to monitor growth and revealed similar sensitivity to what is observed for luminescence, though salinity and media appeared to have large effects on assay sensitivity (Gellert 2000; Gellert et al. 1999). Rather than change the toxicity endpoints, assessments have also been performed by extending the exposure length for luminescence inhibition. This data revealed increased sensitivity to toxicants tested, though the lack of supporting cell viability and culturing data makes it hard to determine if these

methods allowed for optimal growth of the organism in the absence of toxicants (Backhaus et al. 1997). These efforts deserve renewed investigation to potentially introduce a more ecologically and technically relevant exposure time to an assay that already is widely accepted for toxicity assessment.

Give the poor suitability of *A. fischeri* for toxicity assessment under freshwater conditions, an alternative approach could be the introduction of bioluminescence into a more robust organism such as *S. warneri*. These approaches are frequently used in the creation of biosensors, with recombinant strains of *E. coli* with the *lux* operon already existing (Deryabin and Aleshina 2008). Similar efforts have been made in *Staphylococcus* spp., though it has been noted that light production is generally low in *Staphylococcus* spp. and Gram-positive bacteria due to differences in fatty acid metabolism in these organisms (Mesak et al. 2009; Beard et al. 2002). Nonetheless, these approaches should be investigated to provide lower technical burden and better integration with currently approved toxicity tests when photometric interference is not suspected.

7.6 Final Conclusions

In this thesis I adapted and optimized a bacterial assay that used culture-dependent enumeration techniques to monitor the inhibitory effects of OSPW and cNA on organism growth. These approaches were heavily adapted from CLSI guidelines that clearly define test parameters for assessing antimicrobial susceptibility in clinical settings via microdilution MIC assays. Using the organism *S. warneri*, the methods were determined to be robust in salinity and pH ranges observed in freshwater and OSPW, while also being stable under low nutrient conditions required for high exposure concentrations. To further establish the validity of the assay, a control was generated that had similar physiochemical parameters to what is observed in OSPW but

lacked several toxicants of concern. This control had improved growth over the saline previously used and had growth comparable to what is seen in 100% OSM. This assay showed sensitivity to whole OSPW comparable to what is seen in the literature for other *in vivo* and *in vitro* approaches, though the variation in OSPW physiochemistry and source made comparison challenging. When a more standardized cNA sample was used for toxicity assessment, sensitivity greater than what is seen in several other model systems was observed.

To better understand the ability of this assay to track organic contaminants in OSPW, a cNA sample and several OSPWs were treated using AOPs. As monitored by SFS analysis, AOP treatment reduced the presence of organic compounds in these samples, but efficacy appeared largely sample dependent. When investigated using a modified microdilution MIC assay, enumeration data reflected SFS profiles for treatment efficacy. The use of OD allowed for determination of additional inhibitory effects of the treated and untreated samples that were not immediately apparent from enumeration data. The treated OSPW D sample presented OD data that did not align with enumeration data, suggesting possible photometric interference.

These samples were then analyzed using traditional bacterial luminescence inhibition approaches to compare the adapted methods to standardized bacterial models for toxicity assessment. Luminescence inhibition data was strongly correlated with *S. warneri* enumeration data for the cNA sample and OSPW B, though it predicted lower toxicity from untreated OSPW C. Overall, similar trends were observed between treated and untreated samples when compared to chemical analysis data, though treated OSPW D produced similar inhibitory data as was seen in OD assessment. Enumeration data for *A. fischeri* revealed no loss in cell density for treated OSPW D, suggesting that photometric interference was influencing the high inhibition measured by OD and luminescence readouts. While there were stronger examples of sample specific

correlation between luminescence and enumeration, the comparison of enumeration data between both organisms showed the best overall correlation.

The work described in this thesis displays a robust, high-throughput, photometry independent approach for assessing OSPW and cNA toxicity. These approaches had equal if not better sensitivity than bacterial luminescence inhibition assays and were stable under freshwater and simulated OSPW conditions than can disrupt organisms used in luminescence inhibition approaches. With several promising future directions, these approaches may serve to inform revision of currently used bacterial assays or provide an alternative.

Literature Cited

[AER]. Alberta Energy Regulator. 2023a. ST39 2023: Alberta mineable oil sands plant statistics monthly supplement. Alberta Energy Regulator. https://static.aer.ca/prd/documents/sts/ST39-2023.pdf

[AER]. Alberta Energy Regulator. 2023b. state of fluid tailings management for mineable oil sands, 2022. Alberta Energy Regulator. https://static.aer.ca/prd/documents/reports/State-Fluid-Tailings-Management-Mineable-OilSands.pdf

[AER]. Alberta Energy Regulator. 2023c. Water use performance. Alberta Energy Regulator. https://www.aer.ca/protecting-what-matters/holding-industry-accountable/industry-performance/water-use-performance.

[ATCC] American Type Culture Collection. 2022. *Enterococcus faecalis* (Andrew and Horder) Schleifer and Kilpper-Balz 19433™. American Type Culture Collection. https://www.atcc.org/products/19433

[BD] Becton Dickinson. 2022. Tryptic soy broth (TSB) prepared bottled media, ready-to-use. Becton Dickinson GmbH. https://static.bd.com/documents/eifu/ZMG_L012112_EN_B_02.pdf

[CAPP] Canadian Association of Petroleum Producers. 2024a. Oil and natural gas in Canada. [accessed 2024 May 26]. https://www.capp.ca/en/oil-natural-gas-you/oil-natural-gas-canada/.

[CAPP] Canadian Association of Petroleum Producers. 2024b. oil Sands. [accessed 2024 May 26]. https://www.capp.ca/en/oil-natural-gas-you/oil-natural-gas-canada/oil-sands/.

[CCME] Canadian Council of Ministers of the Environment. 1999. water quality guidelines for the protection of aquatic life. Government of Canada [accessed 2024 May 28]. https://ccme.ca/en/summary-table.

[CLSI] Clinical Laboratory Standards Institute. 2018. M07-A10, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 11th edition. Wayne (PA). 112 p.

[EBPI]. Environmental Bio-detection Products Inc 2024a. SOS-ChromoTest Kit. https://biotoxicity.com/index.php/ebpi-toxicity-tests/sos-genotoxicity-tests/sos-chromotest-kit

[EBPI]. Environmental Bio-detection Products Inc. 2024b. Toxi-ChromoTest kit. https://www.biotoxicity.com/biotoxicity/index.php/ebpi-toxicity-tests/acute-toxicity/toxi-chromotest-kit

[ECCC] Environment and Climate Change Canada. 2023. Federal environmental quality guidelines: benzene, toluene, ethylbenzene, xylene (BTEX). Government of Canada. https://www.canada.ca/en/environment-climate-change/services/evaluating-existing-substances/cepa-feqg-benzene-toluene-ethylbenzene-xylene.html

[ECCC] Environment and Climate Change Canada. 2024a. Mainstem water quality - oil sands region. Government of Canada. https://data-donnees.az.ec.gc.ca/data/substances/monitor/surface-water-quality-oil-sands-region/mainstem-water-quality-oil-sands-region.

[ECCC] Environment and Climate Change Canada. 2024b. Biological test method: toxicity tests using aquatic life stages of frogs (*Lithobates pipiens*). Government of Canada.

https://www.canada.ca/en/environment-climate-change/services/wildlife-research-landscape-science/biological-test-method-publications/toxicity-tests-aquatic-life-stages-frogs.html

[ECHA] European Chemicals Agency. 2017. Chapter R.7a: Endpoint specific guidance - guidance on information requirements and chemical safety assessment chapter: Endpoint specific guidance. http://echa.europa.eu/guidance-documents/guidance-on-reach.

[EPA] Environmental Protection Agency. 2024. ECOTOX Knowledgebase. [accessed 2024 May 29]. United States of America. https://cfpub.epa.gov/ecotox/search.cfm.

[ISO] International Organization for Standardization. 2007. ISO 11348-1 Water quality — determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test) — Part 1: Method using freshly prepared bacteria, second edition. (SW). 30 p.

[NRC] National Research Council. 2011. Committee for the update of the guide for the care and use of laboratory animals. 8th edition. Institute for Laboratory Animal Research. Washington (DC): National Academies Press. https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf

Abdalrhman AS, Gamal El-Din M. 2020. Degradation of organics in real oil sands process water by electro-oxidation using graphite and dimensionally stable anodes. Chem Eng J. 389:124406. https://doi.org/10.1016/j.cej.2020.124406

Achten C, Andersson JT. 2015. Overview of polycyclic aromatic compounds (PAC). Polycycl Aromat Comp. 35(2–4):177–186. https://doi.org/10.1080/10406638.2014.994071

Adriana Marcela Vasquez Aldana. ZVI-loaded biochar composite for effective peroxymonosulfate activation: application in the removal of naphthenic acids from real oil sands process water [dissertation]. University of Alberta. https://era.library.ualberta.ca/items/810b668f-8dd6-41cf-8db1-bc2871075700

Aertsen A, Michiels CW. 2004. Stress and How Bacteria Cope with Death and Survival. Crit Rev Microbiol. 30(4):263–273. https://doi.org/10.1080/10408410490884757.

Agilent Genomics. *E. coli* cell culture concentration from OD600 calculator. [accessed 2024]. https://www.agilent.com/store/biocalculators/calcODBacterial.jsp?_requestid=869835

Ahad JME, Macdonald RW, Parrott JL, Yang Z, Zhang Y, Siddique T, Kuznetsova A, Rauert C, Galarneau E, Studabaker WB, et al. 2020. Polycyclic aromatic compounds (PACs) in the Canadian environment: A review of sampling techniques, strategies and instrumentation. Environ Pollut. 266. https://doi.org/10.1016/j.envpol.2020.114988

Ajaero C, Peru KM, Simair M, Friesen V, O'Sullivan G, Hughes SA, McMartin DW, Headley J V. 2018. Fate and behavior of oil sands naphthenic acids in a pilot-scale treatment wetland as characterized by negative-ion electrospray ionization Orbitrap mass spectrometry. Sci Total Environ. 631–632:829–839. https://doi.org/10.1016/j.scitotenv.2018.03.079

Albakistani EA, Nwosu FC, Furgason C, Haupt ES, Smirnova A V, Verbeke TJ, Lee E-S, Kim J-J, Chan A, Ruhl IA, et al. 2022. seasonal dynamics of met hanotrophic bacteria in a boreal oil sands end pit lake. Appl Environ Microb. 88(3). https://doi.org/10.1128/aem.01455-21

Allam NE, Romaniuk N, Tate M, Meshref MNA, Dhar BR, Ulrich AC. 2021. Impact of lime treatment on tailings dewatering and cap water quality under an oil sands end pit lake scenario. Sci Total Environ. 781. https://doi.org/10.1016/j.scitotenv.2021.146699

Allen EW. 2008a. Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. J Environ Eng Sci. 7(2):123-138. https://doi.org/10.1139/S07-038

Allen EW. 2008b. Process water treatment in Canada's oil sands industry: II. A review of emerging technologies. J Environ Eng Sci. 7(5):499-524. https://doi.org/10.1139/S08-020

Ameen F, Alsamhary K, Alabdullatif JA, ALNadhari S. 2021. A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi. Ecotoxicol Environ Saf. 213. https://doi.org/10.1016/j.ecoenv.2021.112027

Anderson J, Wiseman SB, Moustafa A, Gamal El-Din M, Liber K, Giesy JP. 2012. Effects of exposure to oil sands process-affected water from experimental reclamation ponds on Chironomus dilutus. Water Res. 46(6):1662-1672. https://doi.org/10.1016/j.watres.2011.12.007

Andreozzi R, Caprio V, Insola A, Marotta R. 1999. Advanced oxidation processes (AOP) for water purification and recovery. Catal Today. 53(1):51-59. https://doi.org/10.1016/S0920-5861(99)00102-9

Archibald K. 2018. Animal research is an ethical issue for humans as well as for animals. Journal of Animal Ethics. 8(1):1-11. https://doi.org/10.5406/janimalethics.8.1.0001

Backhaus T, Froehner K, Altenburger R, Grimme LH. 1997.Toxicity testing with *Vibrio fischeri*: a comparison between the long term (24 h) and the short term (30 min) bioassay. Chemosphere. 35(12):2925-2938. https://doi.org/10.1016/S0045-6535(97)00340-8

Baikuni A, Hawari FL, Sutriyo, Ramadon D, Malik A. 2023. Untargeted LC-QTOF-MS/MS based metabolomic profile approach of bacterial ferment lysates and skin commensal bacterial cocktail ferment lysates. Hayati. 30(3):576–587. https://doi.org/10.4308/hjb.30.3.576-587

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 6(2):71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Barrow K, Escher BI, Hicks KA, König M, Schlichting R, Arlos MJ. 2023. Water quality monitoring with in vitro bioassays to compare untreated oil sands process-affected water with unimpacted rivers. Environ Sci-Wat Res. 9(8):2008–2020. https://doi.org/10.1039/d2ew00988a

Bartlett AJ, Frank RA, Gillis PL, Parrott JL, Marentette JR, Brown LR, Hooey T, Vanderveen R, McInnis R, Brunswick P, et al. 2017. Toxicity of naphthenic acids to invertebrates: Extracts from oil sands process-affected water versus commercial mixtures. Environ Pollut. 227:271–279. https://doi.org/10.1016/j.envpol.2017.04.056

Bauer AE, Frank RA, Headley J V., Milestone CB, Batchelor S, Peru KM, Rudy MD, Barrett SE, Vanderveen R, Dixon DG, et al. 2019b. A preparative method for the isolation and fractionation of dissolved organic acids from bitumen-influenced waters. Sci Total Enviro. 671:587–597. https://doi.org/10.1016/j.scitotenv.2019.03.244

Bauer AE, Hewitt LM, Parrott JL, Bartlett AJ, Gillis PL, Deeth LE, Rudy MD, Vanderveen R, Brown L, Campbell SD, et al. 2019a. The toxicity of organic fractions from aged oil sands process-affected water to aquatic species. Sci Total Environ. 669:702–710. https://doi.org/10.1016/j.scitotenv.2019.03.107

Bauer AE, Hewitt LM, Roy JW, Parrott JL, Bartlett AJ, Gillis PL, Norwood WP, Rudy MD, Campbell SD, Rodrigues MR, et al. 2022. The acute toxicity of bitumen-influenced groundwaters from the oil sands region to aquatic organisms. Sci Total Environ. 848:157676. https://doi.org/10.1016/j.scitotenv.2022.157676

Beal J, Farny NG, Haddock-Angelli T, Selvarajah V, Baldwin GS, Buckley-Taylor R, Gershater M, Kiga D, Marken J, Sanchania V, et al. 2020. Robust estimation of bacterial cell count from optical density. Commun Biol. 3(1). https://doi.org/10.1038/s42003-020-01127-5

Beard SJ, Salisbury V, Lewis RJ, Sharpe JA, MacGowan AP. 2002. Expression of lux genes in a clinical isolate of *Streptococcus pneumoniae*: Using bioluminescence to monitor gemifloxacin activity. Antimicrob Agents Chemother. 46(2):538–542. https://doi.org/10.1128/AAC.46.2.538-542.2002

Beddow J, Johnson RJ, Lawson T, Breckels MN, Webster RJ, Smith BE, Rowland SJ, Whitby C. 2016. The effect of oil sands process-affected water and model naphthenic acids on photosynthesis and growth in *Emiliania huxleyi* and *Chlorella vulgaris*. Chemosphere. 145:416–423. https://doi.org/10.1016/j.chemosphere.2015.11.046

Bennet J, Cubbage J. 1992. Review and evaluation of Microtox® Test for Freshwater Sediments. Washington State Department of Ecology. https://apps.ecology.wa.gov/publications/documents/92e04.pdf

Bennett EO. 1959. Factors Affecting the Antimicrobial Activity of Phenols. In: Umbreit WW, editor. Advances in Applied Microbiology. Vol. 1. University of Houston Academic Press. p 123–140. https://doi.org/10.1016/S0065-2164(08)70477-5

Berglind R, Leffler P, Sjöström M. 2010. interactions between pH, potassium, calcium, bromide, and phenol and their effects on the bioluminescence of *Vibrio fischeri*. J Toxicol Env Heal S. 73(16):1102–1112. https://doi.org/10.1080/15287394.2010.482918.

Biesta-Peters EG, Reij MW, Joosten H, Gorris LGM, Zwietering MH. 2010. Comparison of two optical-density-based methods and a plate count method for estimation of growth parameters of *Bacillus cereus*. Appl Environ Microb. 76(5):1399–1405. https://doi.org/10.1128/AEM.02336-09

BioRender. BioRender – Figure generation; [Accessed 2024 June 30th]. https://app.biorender.com/

Birnir B, Carpio A, Cebrián E, Vidal P. 2018. Dynamic energy budget approach to evaluate antibiotic effects on biofilms. Commun Nonlinear Sci. 54:70–83. https://doi.org/10.1016/j.cnsns.2017.05.016

Bitton G, Dutton R, Koopman B. 1988. Cell permeability to toxicants: An important parameter in toxicity tests using bacteria. Crit Rev Env Contr. 18(3):177–188. https://doi.org/10.1080/10643388809388347

Bitton G, Koopman B. 1992. Bacterial and enzymatic bioassays for toxicity testing in the environment. Ware GW, editor. Rev Environ Contam Toxicol. 125:1–22. https://doi.org/10.1007/978-1-4612-2890-5

Blum DJW, Speece RE. 1991. A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. Res J Water Pollut C. 63(3):198-207. https://www.jstor.org/stable/25043983

Blumer M. 1976. Polycyclic Aromatic Compounds in Nature. Sci Am. 234(3):34–45. https://www.jstor.org/stable/24950303

Bookout T, Shideler S, Cooper E, Goff K, Headley J V, Lewenza S. 2024. Construction of whole cell bacterial biosensors as an alternative environmental monitoring technology to detect naphthenic acids in oil sands process-affected water. Department of Microbiology, Immunology and Infectious Diseases, University of Calgary. Preprint. https://doi.org/10.1101/2024.04.05.588297.

Borghesan F, Bernardi D, Plebani M. 2007. *In vivo* and *in vitro* allergy diagnostics: It's time to reappraise the costs. Clin Chem Lab Med. Vol. 45. p. (3):391–395. https://doi.org/10.1515/CCLM.2007.077

Bright DA, Meier M. 2007. CAPP freshwater salinity working group and the salt technical advisory sub-committee of the British Columbia upstream petroleum committee: A review of the toxicological literature for salt-2002 to 2007. UMA Engineering Ltd. https://www2.gov.bc.ca/assets/gov/environment/air-land-water/site-remediation/docs/bulletins/review_salt_toxicity07.pdf

Brodl E, Winkler A, Macheroux P. 2018. Molecular mechanisms of bacterial bioluminescence. Comput Struct Biotechnol J. 16:551–564. https://doi.org/10.1016/j.csbj.2018.11.003

Brown LD, Ulrich AC. 2015. Oil sands naphthenic acids: A review of properties, measurement, and treatment. Chemosphere. 127:276–290. https://doi.org/10.1016/j.chemosphere.2015.02.003

Cancelli AM, Gobas FAPC. 2022. Treatment of naphthenic acids in oil sands process-affected waters with a surface flow treatment wetland: mass removal, half-life, and toxicity-reduction. Environ Res. 213. https://doi.org/10.1016/j.envres.2022.113755

Casey JR, Grinstein S, Orlowski J. 2010. Sensors and regulators of intracellular pH. Nat Rev Mol Cell Biol. 11(1):50–61. https://doi.org/10.1038/nrm2820

Cedergreen N. 2014. Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. PLoS One. 9(5):e96580. https://doi.org/10.1371/journal.pone.0096580

Celsie A, Parnis JM, Mackay D. 2016. Impact of temperature, pH, and salinity changes on the physico-chemical properties of model naphthenic acids. Chemosphere. 146:40–50. https://doi.org/10.1016/j.chemosphere.2015.11.122

Chakraborty R, Wu CH, Hazen TC. 2012. Systems biology approach to bioremediation. Curr Opin Biotech. 23(3):483–490. https://doi.org/10.1016/j.copbio.2012.01.015.

Chegounian P, Zerriffi H, Yadav VG. 2020. Engineering microbes for remediation of oil sands tailings. Trends Biotechnol. 38(11):1192–1196. https://doi.org/10.1016/j.tibtech.2020.04.007

Chibwe L, Geier MC, Nakamura J, Tanguay RL, Aitken MD, Simonich SLM. 2015. Aerobic bioremediation of PAH contaminated soil results in increased genotoxicity and

developmental toxicity. Environ Sci Technol. 49(23):13889–13898. https://doi.org/10.1021/acs.est.5b00499.

Choo-Yin YY. Monitoring macrophage immune gene expression profiles as an early indicator system for examining the bioactivity of oil sands process affected waters [dissertation]. University of Alberta. https://era.library.ualberta.ca/items/b4f2503c-f757-4ff1-a309-b1c7d39f6440

Chu B, Xu R, Dinapoli A. 1987. Light Scattering Studies of a Colloidal Suspension of Iron Oxide Particles. J Colloid Interf Sci. 116(1):182-195. https://doi.org/10.1016/0021-9797(87)90109-3

Clemente JS, Fedorak PM. 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere. 60(5):585–600. https://doi.org/10.1016/j.chemosphere.2005.02.065

Dalgaard P, Ross Laura Kamperman T, Neumeyer K, McMeekin TA. 1994. Estimation of bacterial growth rates from turbidimetric and viable count data. Int J Food Microb. 23(3-4):391-404. https://doi.org/10.1016/0168-1605(94)90165-1

Davis C. 2014. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. J Microbiol Methods. 103:9–17. https://doi.org/10.1016/j.mimet.2014.04.012.

De P, Kar S, Ambure P, Roy K. 2022. Prediction reliability of QSAR models: an overview of various validation tools. Arch Toxicol. 96(5):1279–1295. https://doi.org/10.1007/s00204-022-03252-y.

Debenest T, Turcotte P, Gagné F, Gagnon C, Blaise C. 2012. Ecotoxicological impacts of effluents generated by oil sands bitumen extraction and oil sands lixiviation on Pseudokirchneriella subcapitata. Aquatic Toxicology. 112–113:83–91. https://doi.org/10.1016/j.aquatox.2012.01.021.

Dejonghe W, Goris J, Elfantroussi S, Hofte M, Hofte H, De Vos P, Verstraete W, Top EM. 2000. Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. Appl Env Microb. 66(8):3297-3304. https://doi.org/10.1128/AEM.66.8.3297-3304.2000.

Demeter MA, Lemire JA, Yue G, Ceri H, Turner RJ. 2015. Culturing oil sands microbes as mixed species communities enhances ex situ model naphthenic acid degradation. Front Microbiol. 6:936. https://doi.org/10.3389/fmicb.2015.00936.

Deng Y, Zhao R. 2015. Advanced oxidation processes (AOPs) in wastewater treatment. Curr Pollut Rep. 1(3):167–176. https://doi.org/10.1007/s40726-015-0015-z.

Deryabin DG, Aleshina ES. 2008. Effect of salts on luminescence of natural and recombinant luminescent bacterial biosensors. Appl Biochem Microb. 44(3):292–296. https://doi.org/10.1134/S0003683808030113.

Dhakar K, Pandey A. 2016. Wide pH range tolerance in extremophiles: towards understanding an important phenomenon for future biotechnology. Appl Microb Biotechnol. 100(6):2499–2510. https://doi.org/10.1007/s00253-016-7285-2.

Di Toro DM, Mcgrath JA, Hansen DJ. 2000. Technical basis for narcotic chemicals and polycyclic aromatic hydrocarbon criteria. I. Water and tissue. Environ Toxicol Chem. 19(8):1951–1970. https://doi.org/10.1002/etc.5620190803.

Díaz-García ME, Badía-Laíño R. 2019. Fluorescence: Fluorescence Derivatization. In: Worsfold P, Poole C, Townshend A, Miró M, editors. Encyclopedia of Analytical Science. 3rd edition. Oxford: Academic Press. p. 256–265. https://doi.org/10.1016/B978-0-12-409547-2.11180-1

Dominquez CM, de la Rosa M, Borobio VM. 2001. Application of a spectrophotometric method for the determination of post-antibiotic effect and comparison with viable counts in agar. J Antimicrob Chem. 47(4):391-398. https://doi.org/10.1093/jac/47.4.391

Duedu KO, French CE. 2017. Data for discriminating dead/live bacteria in homogenous cell suspensions and the effect of insoluble substrates on turbidimetric measurements. Data Brief. 12:169–174. https://doi.org/10.1016/j.dib.2017.04.003.

Eisentraeger A, Dott W, Klein J, Hahn S. 2003. Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth-inhibition assays. Ecotoxicol Environ Saf. 54(3):346-354. https://doi.org/10.1016/S0147-6513(02)00099-4

Environment Canada. 1992. Biological test method - toxicity test using luminescent bacteria (*Photobacterium phosphoreum*). Government of Canada. https://publications.gc.ca/site/eng/9.579452/publication.html

Environment Canada. 2007. biological test method - growth inhibition test using a freshwater alga. Government of Canada. https://www.canada.ca/en/environment-climate-change/services/wildlife-research-landscape-science/biological-test-method-publications/growth-inhibition-test-freshwater-alga.html

Farmer III JJ, Michael Janda J, Brenner FW, Cameron DN, Birkhead KM. 2015. Vibrio. In: Bergey's Manual of Systematics of Archaea and Bacteria. p. 1–79. https://doi.org/10.1002/9781118960608.gbm01078.

Fatta-Kassinos D, Vasquez MI, Kümmerer K. 2011. Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes - Degradation, elucidation of byproducts and assessment of their biological potency. Chemosphere. 85(5):693–709. https://doi.org/10.1016/j.chemosphere.2011.06.082.

Ferenci T. 2001. Hungry bacteria - Definition and properties of a nutritional state. Environ Microbiol. 3(10):605–611. https://doi.org/10.1046/j.1462-2920.2001.00238.x.

Fernandes A, Makoś P, Boczkaj G. 2018. Treatment of bitumen post oxidative effluents by sulfate radicals based advanced oxidation processes (S-AOPs) under alkaline pH conditions. J Clean Prod. 195:374–384. https://doi.org/10.1016/j.jclepro.2018.05.207.

Foght JM, Gieg LM, Siddique T. 2017. The microbiology of oil sands tailings: Past, present, future. FEMS Microbiol Ecol. 93(5). https://doi.org/10.1093/femsec/fix034.

Fort DJ, Stover EL. 1995. Impact of toxicities and potential interactions of flocculants and coagulant aids on whole effluent toxicity testing. Water Environment Research. 67(6):921–925. https://doi.org/10.2175/106143095X133149

Foster T. 1996. Staphylococcus. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. https://www.ncbi.nlm.nih.gov/books/NBK8448/

França A, Gaio V, Lopes N, Melo LDR. 2021. Virulence factors in coagulase-negative staphylococci. Pathogens. 10(2):1–46. https://doi.org/10.3390/pathogens10020170.

Frank RA, Kavanagh R, Kent Burnison B, Arsenault G, Headley J V., Peru KM, Van Der Kraak G, Solomon KR. 2008. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere. 72(9):1309–1314. https://doi.org/10.1016/j.chemosphere.2008.04.078.

Frank RA, Roy JW, Bickerton G, Rowland SJ, Headley J V., Scarlett AG, West CE, Peru KM, Parrott JL, Conly FM, et al. 2014. Profiling oil sands mixtures from industrial developments and natural groundwaters for source identification. Environ Sci Technol. 48(5):2660–2670. https://doi.org/10.1021/es500131k.

Frank RA, Sanderson H, Kavanagh R, Burnison BK, Headley J V, Solomon KR. 2009. Use of a (quantitative) structure–activity relationship [(Q)Sar] model to predict the toxicity of naphthenic acids. J Toxicol Environ Health A. 73(4):319–329. https://doi.org/10.1080/15287390903421235.

Frankel ML, Demeter MA, Lemire JA, Turner RJ. 2016. Evaluating the metal tolerance capacity of microbial communities isolated from Alberta oil sands process water. PLoS One. 11(2). https://doi.org/10.1371/journal.pone.0148682.

Fu F, Cheng VWT, Wu Y, Tang Y, Weiner JH, Li L. 2013. Comparative proteomic and metabolomic analysis of *Staphylococcus warneri* SG1 cultured in the presence and absence of butanol. J Proteome Res. 12(10):4478–4489. https://doi.org/10.1021/pr400533m.

Fu L, Li C, Lillico DME, Phillips NAI, Gamal El-Din M, Belosevic M, Stafford JL. 2017. Comparison of the acute immunotoxicity of nonfractionated and fractionated oil sands process-affected water using mammalian macrophages. Environ Sci Technol. 51(15):8624–8634. https://doi.org/10.1021/acs.est.7b02120.

Gagné F, André C, Turcotte P, Gagnon C, Sherry J, Talbot A. 2013. A comparative toxicogenomic investigation of oil sand water and processed water in rainbow trout hepatocytes. Arch Environ Contam Toxicol. 65(2):309–323. https://doi.org/10.1007/s00244-013-9888-2.

Ganiyu Soliu O, Arslan M, Gamal El-Din M. 2022a. Combined solar activated sulfate radical-based advanced oxidation processes (SR-AOPs) and biofiltration for the remediation of dissolved organics in oil sands produced water. Chemical Engineering Journal. 433(1). https://doi.org/10.1016/j.cej.2022.134579.

Ganiyu Soliu O, Sable S, Gamal El-Din M. 2022b. Advanced oxidation processes for the degradation of dissolved organics in produced water: A review of process performance, degradation kinetics and pathway. Chemical Engineering Journal. 429(1). https://doi.org/10.1016/j.cej.2021.132492.

Ganjian H, Nikokar I, Tieshayar A, Mostafaei A, Amirmozafari N, Kiani S. 2012. Effects of salt stress on the antimicrobial drug resistance and protein profile of *Staphylococcus aureus*. Jundishapur J Microbiol. 5(1):328–331.

Garcia-Garcia E, Ge JQ, Oladiran A, Montgomery B, El-Din MG, Perez-Estrada LC, Stafford JL, Martin JW, Belosevic M. 2011b. Ozone treatment ameliorates oil sands process water

toxicity to the mammalian immune system. Water Res. 45(18):5849–5857. https://doi.org/10.1016/j.watres.2011.08.032.

Garcia-Garcia E, Pun J, Hodgkinson J, Perez-Estrada LA, El-Din MG, Smith DW, Martin JW, Belosevic M. 2012. Commercial naphthenic acids and the organic fraction of oil sands process water induce different effects on pro-inflammatory gene expression and macrophage phagocytosis in mice. Journal of Applied Toxicology. 32(12):968–979. https://doi.org/10.1002/jat.1687.

Garcia-Garcia E, Pun J, Perez-Estrada LA, Din MG-E, Smith DW, Martin JW, Belosevic M. 2011a. Commercial naphthenic acids and the organic fraction of oil sands process water downregulate pro-inflammatory gene expression and macrophage antimicrobial responses. Toxicol Lett. 203(1):62–73. https://doi.org/10.1016/j.toxlet.2011.03.005.

Garde S, Chodisetti PK, Reddy M. 2021. Peptidoglycan: structure, synthesis, and regulation. EcoSal Plus. 9(2). https://doi.org/10.1128/ecosalplus.esp-0010-2020.

Gault IGM, Sun C, Martin JW. 2023. Persistent cytotoxicity and endocrine activity in the first oil sands end-pit lake. ACS ES and T Water. 3(2):366–376. https://doi.org/10.1021/acsestwater.2c00430.

Geier MC, Chlebowski AC, Truong L, Massey Simonich SL, Anderson KA, Tanguay RL. 2018. Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons. Arch Toxicol. 92(2):571–586. https://doi.org/10.1007/s00204-017-2068-9.

Gélinas M, Museau L, Milot A, Beauregard PB. 2021. The *de novo* purine biosynthesis pathway is the only commonly regulated cellular pathway during biofilm formation in TSB-based

medium in *Staphylococcus aureus* and *Enterococcus faecalis.* Microbiol Spec. 9(3):e00804-1. https://doi.org/10.1128/Spectrum.00804-21

Gellert G, Stommel A, Trujillano AB. 1999. Development of an optimal bacterial medium based on the growth inhibition assay with *Vibrio fischeri*. Chemosphere. 39(3):467-476. https://doi.org/10.1016/s0045-6535(99)00002-8

Gellert G. 2000. Sensitivity and significance of luminescent bacteria in chronic toxicity testing based on growth and bioluminescence. Ecotoxicol Environ Saf. 45(1):87-97. https://doi.org/10.1006/eesa.1999.1849

Gentes ML, McNabb A, Waldner C, Smits JEG. 2007a. Increased thyroid hormone levels in tree swallows (*Tachycineta bicolor*) on reclaimed wetlands of the Athabasca oil sands. Arch Environ Contam Toxicol. 53(2):287–292. https://doi.org/10.1007/s00244-006-0070-y.

Gentes M-L, Waldner C, Papp Z, Smits JEG. 2006. Effects of oil sands tailings compounds and harsh weather on mortality rates, growth and detoxification efforts in nestling tree swallows (*Tachycineta bicolor*). Environmental Pollution. 142(1):24–33. https://doi.org/10.1016/j.envpol.2005.09.013

Gentes M-L, Waldner C, Papp Z, Smits JEG. 2007b. Effects of exposure to naphthenic acids in tree swallows (*Tachycineta bicolor*) on the Athabasca oil sands, Alberta, Canada. J Toxicol Environ Health A. 70(14):1182–1190. https://doi.org/10.1080/15287390701252709.

Golby S, Ceri H, Gieg LM, Chatterjee I, Marques LLR, Turner RJ. 2012. Evaluation of microbial biofilm communities from an Alberta oil sands tailings pond. FEMS Microbiol Ecol. 79(1):240–250. https://doi.org/10.1111/j.1574-6941.2011.01212.x.

Government of Alberta. 2023a. Historical royalty revenue. [accessed 2024 May 25]. https://open.alberta.ca/opendata/historical-royalty-revenue#detailed.

Government of Alberta. 2023b. Oil sands facts and statistics. [accessed 2024 May 25]. https://www.alberta.ca/about-oil-sands.

Government of Alberta. 2024. Surface water quality - discrete data. [accessed 2024 May 24]. http://osmdatacatalog.alberta.ca/dataset/surface-water-quality-discrete.

Government of Canada. 2020. Crude oil. [accessed 2024 May 24]. https://natural-resources.canada.ca/our-natural-resources/energy-sources-distribution/fossil-fuels/crude-oil/what-are-oil-sands/18089.

Government of Canada. 2024. Biological test methods publications. [accessed 2024 May 25]. https://www.canada.ca/en/environment-climate-change/services/wildlife-research-landscape-science/biological-test-method-publications.html

Grewer DM, Young RF, Whittal RM, Fedorak PM. 2010. Naphthenic acids and other acid-extractables in water samples from Alberta: What is being measured? Sci Total Environ. 408(23):5997–6010. https://doi.org/10.1016/j.scitotenv.2010.08.013.

Gries CM, Sadykov MR, Bulock LL, Chaudhari SS, Thomas VC, Bose JL, Bayles KW. 2016. Potassium uptake modulates *Staphylococcus aureus* metabolism. mSphere. 1(3). https://doi.org/10.1128/msphere.00125-16.

Haaber J, Cohn MT, Petersen A, Ingmer H. 2016. Simple method for correct enumeration of *Staphylococcus aureus*. J Microbiol Methods. 125:58–63. https://doi.org/10.1016/j.mimet.2016.04.004.

Hach. 2024. ToxTrack Toxicity Reagent. Hach Company/Hach Lange GmbH. https://www.hach.com/p-toxtrak-toxicity-reagent-set-25-49-tests/2597200?srsltid=AfmBOop7-PgHhXZ_OMH3Z1l3n6X-P9wUwUalb1pL2QjhHGwTC-7omqs8

Hagen MO, Katzenback BA, Islam MDS, Gamal El-Din M, Belosevic M. 2014. The analysis of goldfish (*Carassius auratus* L.) innate immune responses after acute and subchronic exposures to oil sands process-affected water. Toxicol Sci. 138(1):59–68. https://doi.org/10.1093/toxsci/kft272.

Han X, Scott AC, Fedorak PM, Bataineh M, Martin JW. 2008. Influence of molecular structure on the biodegradability of naphthenic acids. Environ Sci Technol. 42(4):1290–1295. https://doi.org/10.1021/es702220c.

Hao OJ, Lin Cheng-Fang, Jeng Fu-Tien, Shih Chien-Jen. 1995. A review of Microtox test and its applications. Toxicol Environ Chem. 52(1–4):57–76. https://doi.org/10.1080/02772249509358249.

Harder W, Dijkhuizen L. 1983. Physiological responses to nutrient limitation. Annu Rev Microbiol. 37:1-23. https://doi.org/10.1146/annurev.mi.37.100183.000245.

Harrop AJ, Lilly MD. 1989. Biotransformation in organic solvents: a difference between Gram-positive and Gram-negative bacteria. Biotechnol Lett. 11:807-810. https://doi.org/10.1007/BF01026102

Hasegawa H, Naganuma K, Nakagawa Y, Matsuyama T. 2003. Membrane filter (pore size, 0.22-0.45 μm; thickness, 150 μm) passing-through activity of *Pseudomonas aeruginosa* and

other bacterial species with indigenous infiltration ability. FEMS Microbiol Lett. 223(1):41–46. https://doi.org/10.1016/S0378-1097(03)00327-6.

Hashmi MZ, Naveedullah, Shen H, Zhu S, Yu C, Shen C. 2014. Growth, bioluminescence and shoal behavior hormetic responses to inorganic and/or organic chemicals: A review. Environ Int. 64:28–39. doi:10.1016/j.envint.2013.11.018.

Hassell KL, Kefford BJ, Nugegoda D. 2006. Sub-lethal and chronic salinity tolerances of three freshwater insects: *Cloeon* sp. and *Centroptilum* sp. (Ephemeroptera: Baetidae) and *Chironomus* sp. (Diptera: Chironomidae). J Exp Biol. 209(20):4024–4032. https://doi.org/10.1242/jeb.02457.

Hatfield Consultants. 2019. Ecotoxicity assessment of treated oil sands process-affected water (OSPW): 2019 toxicity and mesocosms studies. Government of Alberta. https://open.alberta.ca/publications/9781460144503.

Hauser B, Schrader G, Bahadir M. 1997. Comparison of acute toxicity and genotoxic concentrations of single compounds and waste elutriates using the Microtox/Mutatox test system. Ecotoxicol Environ Saf. 38(3):227–231. https://doi.org/10.1006/eesa.1997.1594.

He Y, Patterson S, Wang N, Hecker M, Martin JW, El-Din MG, Giesy JP, Wiseman SB. 2012. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (*Pimephales promelas*). Water Res. 46(19):6359–6368. https://doi.org/10.1016/j.watres.2012.09.004.

He Y, Wiseman SB, Hecker M, Zhang X, Wang N, Perez LA, Jones PD, Gamal El-Din M, Martin JW, Giesy JP. 2011. Effect of ozonation on the estrogenicity and androgenicity of

oil sands process-affected water. Environ Sci Technol. 45(15):6268–6274. https://doi.org/10.1021/es2008215.

He Y, Wiseman SB, Zhang X, Hecker M, Jones PD, El-Din MG, Martin JW, Giesy JP. 2010. Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line. Chemosphere. 80(5):578–584. https://doi.org/10.1016/j.chemosphere.2010.04.018.

Headley J V, Peru KM, Mcmartin DW, Winkler M. 2002. Determination of dissolved naphthenic acids in natural waters by using negative-ion electrospray mass spectrometry. J AOAC Int. 85(1):182-187. https://doi.org/10.1093/jaoac/85.1.182

Headley J V., Goudey S, Birkholz D, Linton LR, Dickson LC. 2001. Toxicity screening of benzene, toluene, ethylbenzene and xylene (BTEX) hydrocarbons in groundwater at sour-gas plants. Canadian Water Resources Journal. 26(3):345–358. https://doi.org/10.4296/cwrj2603345.

Headley J V., Peru KM, Mohamed MH, Frank RA, Martin JW, Hazewinkel RRO, Humphries D, Gurprasad NP, Hewitt LM, Muir DCG, et al. 2013. Chemical fingerprinting of naphthenic acids and oil sands process waters-A review of analytical methods for environmental samples. J Environ Sci Heal A. 48(10):1145–1163. https://doi.org/10.1080/10934529.2013.776332.

Hebben T, Alberta. Alberta Environment. 2009. Analysis of water quality conditions and trends for the long-term river network: Athabasca River, 1960-2007. Water Policy Branch, Alberta Environment. https://open.alberta.ca/dataset/9780778576891

Heipieper HJ, Cornelissen S, Pepi M. 2010. Surface properties and cellular energetics of bacteria in response to the presence of hydrocarbons. In: Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg. p. 1615–1624.

Hernández A, Marín M. 2002. New turbidimetric method for estimating bacterial growth in heterogeneous media. Process Biochem. 37(10):1125–1128. https://doi.org/10.1016/S0032-9592(01)00328-4.

Holden AA, Haque SE, Mayer KU, Ulrich AC. 2013. Biogeochemical processes controlling the mobility of major ions and trace metals in aquitard sediments beneath an oil sand tailing pond: Laboratory studies and reactive transport modeling. J Contam Hydrol. 151:55–67. https://doi.org/10.1016/j.jconhyd.2013.04.006.

Holowenko FM, MacKinnon MD, Fedorak PM. 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Res. 36(11):2843–2855. https://doi.org/10.1016/S0043-1354(01)00492-4.

Honda M, Suzuki N. 2020. Toxicities of polycyclic aromatic hydrocarbons for aquatic animals. Int J Environ Res Public Health. 17(4):1363. https://doi.org/10.3390/ijerph17041363.

Horning EC. 1943. Alicyclic-aromatic isomerization. Chem Rev. 33(2):89-135. https://doi.org/10.1021/cr60105a002

Houck KA, Kavlock RJ. 2008. Understanding mechanisms of toxicity: Insights from drug discovery research. Toxicol Appl Pharmacol. 227(2):163–178. https://doi.org/10.1016/j.taap.2007.10.022.

Huang R, Chen Y, Meshref MNA, Chelme-Ayala P, Dong S, Ibrahim MD, Wang C, Klamerth N, Hughes SA, Headley J V, et al. 2018. Characterization and determination of naphthenic acids species in oil sands process-affected water and groundwater from oil sands development area of Alberta, Canada. Water Res. 128:129–137. https://doi.org/10.1016/j.watres.2017.10.003.

Hughes SA, Mahaffey A, Shore B, Baker J, Kilgour B, Brown C, Peru KM, Headley J V, Bailey HC. 2017. Using ultrahigh-resolution mass spectrometry and toxicity identification techniques to characterize the toxicity of oil sands process-affected water: The case for classical naphthenic acids. Environ Toxicol Chem. 36(11):3148–3157. https://doi.org/10.1002/etc.3892

Humphrey TJ, Cruickshank JG. 1985. Antibiotic and deoxycholate resistance in *Campylobacter jejuni* following freezing or heating. J Appl Bacteriol. 59:65-71. https://doi.org/10.1111/j.1365-2672.1985.tb01777.x

Hussain NAS, Stafford JL. 2023. Abiotic and biotic constituents of oil sands process-affected waters. J Environ Sci. 127:169–186. https://doi.org/10.1016/j.jes.2022.06.012.

Igiri BE, Okoduwa SIR, Idoko GO, Akabuogu EP, Adeyi AO, Ejiogu IK. 2018. Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: a review. J Toxicol. 2018. https://doi.org/10.1155/2018/2568038.

Impey RE, Hawkins DA, Sutton JM, Soares da Costa TP. 2020. Overcoming intrinsic and acquired resistance mechanisms associated with the cell wall of Gram-negative bacteria. Antibiotics. 9(9):1–19. https://doi.org/10.3390/antibiotics9090623.

Incardona JP. 2017. Molecular mechanisms of crude oil developmental toxicity in fish. Arch Environ Contam Toxicol. 73(1):19–32. https://doi.org/10.1007/s00244-017-0381-1.

Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. J Ferment Bioeng. 73(3):194-196. https://doi.org/10.1016/0922-338X(91)90109-T

Ishii S. 2020. Quantification of antibiotic resistance genes for environmental monitoring: Current methods and future directions. Curr Opin Environ Sci Health. 16:47–53. https://doi.org/10.1016/j.coesh.2020.02.004.

Isken S, Derks A, Wolffs PFG, De Bont JAM. 1999. Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. Appl Environ Microbiol. 65(6): 2631-2635. https://doi.org/10.1128/aem.65.6.2631-2635.1999

Islam MdS, Moreira J, Chelme-Ayala P, Gamal El-Din M. 2014. Prediction of naphthenic acid species degradation by kinetic and surrogate models during the ozonation of oil sands process-affected water. Sci Total Enviro. 493:282–290. https://doi.org/10.1016/j.scitotenv.2014.05.138.

Iyer V, Raut J, Dasgupta A. 2021. Impact of pH on growth of *Staphylococcus epidermidis* and *Staphylococcus aureus in vitro*. J Med Microbiol. 70(9). https://doi.org/10.1099/jmm.0.001421.

James R, Dindal A, Willenberg Z, Riggs K. 2003. Environmental technology verification report: Strategic Diagnostics inc. Microtox® Rapid Toxicity Testing System. Environmental Protection Agency, United States of America. https://nepis.epa.gov/Exe/ZyPDF.cgi/P100W0H2.PDF?Dockey=P100W0H2.PDF

Jensen-Fontaine M, Norwood WP, Brown M, Dixon DG, Le XC. 2014. Uptake and speciation of vanadium in the benthic invertebrate *Hyalella azteca*. Environ Sci Technol. 48(1):731–738. https://doi.org/10.1021/es403252k.

Jones D, Scarlett AG, West CE, Rowland SJ. 2011. Toxicity of individual naphthenic acids to *Vibrio fischeri*. Environ Sci Technol. 45(22):9776–9782. https://doi.org/10.1021/es201948j.

Jurtshuk P. 1996. Bacterial metabolism. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. https://www.ncbi.nlm.nih.gov/books/NBK7919/

Kagan J, Kagan ED, Kagan IA, Kagan PA, Quigley S. 1985. The phototoxicity of non-carcinogenic polycyclic aromatic hydrocarbons in aquatic organisms. Chemosphere. 14(11):1829–1834. https://doi.org/10.1016/0045-6535(85)90125-0.

Kaiser KLE. Correlations of *Vibrio fischeri* bacteria test data with bioassay data for other organisms. Environ Health Perspect. 206(2):583-591. https://doi.org/10.1289/ehp.98106583

Kanuparthy A, Challa T, Meegada S, Siddamreddy S, Muppidi V. 2020. *Staphylococcus warneri*: skin commensal and a rare cause of urinary tract infection. Cureus. 12(5):e8337 https://doi.org/10.7759/cureus.8337.

Kavanagh RJ, Burnison BK, Frank RA, Solomon KR, Van Der Kraak G. 2009. Detecting oil sands process-affected waters in the Alberta oil sands region using synchronous fluorescence spectroscopy. Chemosphere. 76(1):120–126. https://doi.org/10.1016/j.chemosphere.2009.02.007.

Kavanagh RJ, Frank RA, Burnison BK, Young RF, Fedorak PM, Solomon KR, Van Der Kraak G. 2012. Fathead minnow (*Pimephales promelas*) reproduction is impaired when exposed to a naphthenic acid extract. Aquat Toxicol. 116–117:34–42. https://doi.org/10.1016/j.aquatox.2012.03.002.

Kelly EN, Short JW, Schindler DW, Hodson P V, Ma M, Kwan AK, Fortin BL. 2009. Oil sands development contributes polycyclic aromatic compounds to the Athabasca River and its tributaries. P Natl A Sci USA. 106(52):22346–22351. https://doi.org/10.1073/pnas.0912050106

Khan ZH, Ullah MH, Rahman B, Talukder AI, Wahadoszamen M, Abedin KM, Haider AFMY. 2022. Laser-Induced Breakdown Spectroscopy (LIBS) for trace element detection: A review. J Spectrosc. 2022. https://doi.org/10.1155/2022/3887038.

Kinley CM, McQueen AD, Rodgers JH. 2016. Comparative responses of freshwater organisms to exposures of a commercial naphthenic acid. Chemosphere. 153:170–178. https://doi.org/10.1016/j.chemosphere.2016.03.002.

Kjelleberg S, Albertson N, Flardh K, Holmquist L, Jouper-Jaan A, Marouga R, Ostling J, Svenblad B, Weichart D. 1993. How do non-differentiating bacteria adapt to starvation? A Van Leeuw. 63(3-4):333-341. https://doi.org/10.1007/BF00871228

Klanjscek T, Nisbet RM, Priester JH, Holden PA. 2012. Modeling physiological processes that relate toxicant exposure and bacterial population dynamics. PLoS One. 7(2):e26955. https://doi.org/10.1371/journal.pone.0026955.

Kohanski MA, Dwyer DJ, Collins JJ. 2010. How antibiotics kill bacteria: From targets to networks. Nat Rev Microbiol. 8(6):423–435. https://doi.org/10.1038/nrmicro2333.

Kroll A, Pillukat MH, Hahn D, Schnekenburger J. 2009. Current *in vitro* methods in nanoparticle risk assessment: Limitations and challenges. Eur J Pharm Biopharm. 72(2):370–377. https://doi.org/10.1016/j.ejpb.2008.08.009.

Krulwich TA, Sachs G, Padan E. 2011. Molecular aspects of bacterial pH sensing and homeostasis. Nat Rev Microbiol. 9(5):330–343. https://doi.org/10.1038/nrmicro2549.

Kushkevych I. 2023. Chapter 3 - Bacterial nutrition and growth. In: Kushkevych I, editor. Bacterial Physiology and Biochemistry. Academic Press. p. 91–150. https://www.sciencedirect.com/science/article/pii/B9780443187384500036.

Laceby P, Emmerton C. 2023. 2021 Status of surface water quality, Lower Athabasca region, Alberta. Government of Alberta. https://open.alberta.ca/publications/status-of-surface-water-quality-lower-athabasca-region-alberta

Lari E, Pyle GG. 2017. Rainbow trout (*Oncorhynchus mykiss*) detection, avoidance, and chemosensory effects of oil sands process-affected water. Environmental Pollution. 225:40–46. https://doi.org/10.1016/j.envpol.2017.03.041.

Lari E, Steinkey D, Razmara P, Mohaddes E, Pyle GG. 2019. Oil sands process-affected water impairs the olfactory system of rainbow trout (*Oncorhynchus mykiss*). Ecotoxicol Environ Saf. 170:62–67. https://doi.org/10.1016/j.ecoenv.2018.11.105.

Lari E, Wiseman S, Mohaddes E, Morandi G, Alharbi H, Pyle GG. 2016. Determining the effect of oil sands process-affected water on grazing behaviour of Daphnia magna, long-term consequences, and mechanism. Chemosphere. 146:362–370. https://doi.org/10.1016/j.chemosphere.2015.12.037.

Larsen J. 1989. The influence of growth phase and culture conditions of *Tetrahymena* on effects of cadmium. Toxicology. 58(2):211–223. https://doi.org/10.1016/0300-483X(89)90010-3.

Lau CY, Zahidi AAA, Liew OW, Ng TW. 2015. A direct heating model to overcome the edge effect in microplates. J Pharm Biomed Anal. 102:199–202. https://doi.org/10.1016/j.jpba.2014.09.021.

Le Loir Y, Baron F, Gautier M. 2003. *Staphylococcus aureus* and food poisoning. Genet Mol Res. 2(1):63-67. https://www.funpecrp.com.br/gmr/year2003/vol1-2/pdf/sim0009.pdf

Leblanc GA. 1980. Acute toxicity of priority pollutants to water flea (*Daphnia magna*). Bull Environ Contam Toxicol. 24(5):684-691. https://doi.org/10.1007/BF01608174

Leclair LA, Pohler L, Wiseman SB, He Y, Arens CJ, Giesy JP, Scully S, Wagner BD, Van Den Heuvel MR, Hogan NS. 2015. In vitro assessment of endocrine disrupting potential of naphthenic acid fractions derived from oil sands-influenced water. Environ Sci Technol. 49(9):5743–5752. https://doi.org/10.1021/acs.est.5b00077.

Lee J, Kim HS, Jo HY, Kwon MJ. 2021. Revisiting soil bacterial counting methods: Optimal soil storage and pretreatment methods and comparison of culturedependent and -independent methods. PLoS One. 16(2). https://doi.org/10.1371/journal.pone.0246142.

Lee S-H, Lee S-S, Kim C-W. 2002. Changes in the cell size of *Brevundimonas diminuta* using different growth agitation rates. PDA J Pharm Sci Technol. 56(2):99. http://journal.pda.org/content/56/2/99.abstract.

Lenz P, Süssmuth R, Seibel E. 1986. Development of sensitive bacterial tests, exemplified by two mycotoxins. Toxicology. 40(2):199–205. https://doi.org/10.1016/0300-483X(86)90079-X.

Leshuk T, Krishnakumar H, Livera D de O, Gu F. 2018. Floating photocatalysts for passive solar degradation of naphthenic acids in oil sands process-affected water. Water. 10(2). https://doi.org/10.3390/w10020202.

Levin-Edens E, Bonilla N, Meschke JS, Roberts MC. 2011. Survival of environmental and clinical strains of methicillin-resistant *Staphylococcus aureus* (MRSA) in marine and fresh waters. Water Res. 45(17):5681–5686. https://doi.org/10.1016/j.watres.2011.08.037.

Li C, Fu L, Lillico DME, Belosevic M, Stafford JL, Gamal El-Din M. 2019. Exposure to organic fraction extracted from oil sands process-affected water has negligible impact on pregnancy and lactation of mice. Environ Sci Technol. 53(12):7083–7094. https://doi.org/10.1021/acs.est.9b01965.

Li C, Fu L, Stafford J, Belosevic M, Gamal El-Din M. 2017. The toxicity of oil sands process-affected water (OSPW): A critical review. Sci Total Environ. 601–602:1785–1802. https://doi.org/10.1016/j.scitotenv.2017.06.024.

Li Q, Mahendra S, Lyon DY, Brunet L, Liga M V., Li D, Alvarez PJJ. 2008. Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications. Water Res. 42(18):4591–4602.https://doi.org/10.1016/j.watres.2008.08.015.

Li X, Zhou Q, Luo Y, Yang G, Zhou T. 2013. Joint action and lethal levels of toluene, ethylbenzene, and xylene on midge (*Chironomus plumosus*) larvae. Environ Sci Pollut R. 20(2):957–966. https://doi.org/10.1007/s11356-012-1264-7.

Li Y, Huang TY, Mao Y, Chen Y, Shi F, Peng R, Chen J, Yuan L, Bai C, Chen L, et al. 2020. Study on the viable but non-culturable (VBNC) state formation of *Staphylococcus aureus* and its control in food system. Front Microbiol. 11. https://doi.org/10.3389/fmicb.2020.599739.

Li Y, Wu Z, Zhao C, Zhang Y, Peng D, Gong Z. 2023. Facile fabrication of zero-valent-iron biochar from red mud for bisulfite activation in wastewater treatment: Performance and mechanism. Environ Technol Innov. 30. https://doi.org/10.1016/j.eti.2023.103110.

Liang C, Su HW. 2009. Identification of sulfate and hydroxyl radicals in thermally activated persulfate. Ind Eng Chem Res. 48(11):5558–5562. https://doi.org/10.1021/ie9002848.

Lillico DME, Hussain NAS, Choo-Yin YY, Qin R, How ZT, El-Din MG, Stafford JL. 2023. Using immune cell-based bioactivity assays to compare the inflammatory activities of oil sands process-affected waters from a pilot scale demonstration pit lake. J Environ Sci. 128:55–70. https://doi.org/10.1016/j.jes.2022.07.018.

Liu C, Zhao X, Xie H, Zhang X, Li K, Ma C, Fu Q. 2020. Whole genome sequence and comparative genome analyses of multi-resistant Staphylococcus warneri GD01 isolated from a diseased pig in China. PLoS One. 15(5). https://doi.org/10.1371/journal.pone.0233363.

Liu H, Yu T, Liu Y. 2015. Sulfate reducing bacteria and their activities in oil sands process-affected water biofilm. Sci Total Environ. 536:116–122. https://doi.org/10.1016/j.scitotenv.2015.06.135.

Lo CC, Brownlee BG, Bunce NJ. 2006. Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. Water Res. 40(4):655–664. https://doi.org/10.1016/j.watres.2005.12.008.

Lordelo R, Branco R, Gama F, Morais P V. 2024. Assessment of antimicrobial resistance, biofilm formation, and surface modification potential in hospital strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Heliyon. 10(9). https://doi.org/10.1016/j.heliyon.2024.e30464.

Lzroaie MM. 2010. Multiple responses of Gram-positive and Gram-negative bacteria to mixture of hydrocarbons. Brazilian J Microbiol. 41(3):649–667. https://doi.org/10.1590/S1517-83822010000300016

Ma XY, Wang XC, Ngo HH, Guo W, Wu MN, Wang N. 2014. Bioassay based luminescent bacteria: Interferences, improvements, and applications. Sci Total Environ. 468–469:1–11. https://doi.org/10.1016/j.scitotenv.2013.08.028.

Mack D, Becker P, Chatterjee I, Dobinsky S, Knobloch JKM, Peters G, Rohde H, Herrmann M. 2004. Mechanisms of biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*: Functional molecules, regulatory circuits, and adaptive responses. Int J Med Microbiol. 294(2–3):203–212. https://doi.org/10.1016/j.ijmm.2004.06.015.

MacKinnon MD, Boerger H. 1986. Description of two treatment methods for detoxifying oil sands tailings pond water. Water Qual Res J Can. 21(4):496–512. https://doi.org/10.2166/wqrj.1986.043.

Mackinnon MD. A public service of a study of the chemical and physical properties of Syncrude's tailings pond, Mildred Lake, 1980. Syncrude Environmental Research Monograph 1981-1. p. 81

Madill REA, Brownlee BG, Josephy PD, Bunce NJ. 1999. Comparison of the Ames *Salmonella* assay and Mutatox genotoxicity assay for assessing the mutagenicity of polycyclic aromatic compounds in porewater from Anthabasca oil sands mature fine tailings. Environ Sci Technol. 33(15):2510–2516. https://doi.org/10.1021/es981343o.

Mahdavi H, Prasad V, Liu Y, Ulrich AC. 2015. In situ biodegradation of naphthenic acids in oil sands tailings pond water using indigenous algae–bacteria consortium. Bioresour Technol. 187:97–105. https://doi.org/10.1016/j.biortech.2015.03.091.

Maier RM. 2000.Chapter 3 – Bacterial growth. In:Maier RM, Pepper Il, Gerba CP, editor. Environmental Microbiology 2nd edition. Academic Press. p. 37–54. https://doi.org/10.1016/B978-0-12-370519-8.00003-1

Marentette JR, Frank RA, Bartlett AJ, Gillis PL, Hewitt LM, Peru KM, Headley J V., Brunswick P, Shang D, Parrott JL. 2015. Toxicity of naphthenic acid fraction components extracted from fresh and aged oil sands process-affected waters, and commercial naphthenic acid mixtures, to fathead minnow (Pimephales promelas) embryos. Aquatic Toxicology. 164:108–117. https://doi.org/10.1016/j.aquatox.2015.04.024.

Marentette JR, Sarty K, Cowie AM, Frank RA, Hewitt LM, Parrott JL, Martyniuk CJ. 2017. Molecular responses of Walleye (*Sander vitreus*) embryos to naphthenic acid fraction components extracted from fresh oil sands process-affected water. Aquatic Toxicology. 182:11–19. https://doi.org/10.1016/j.aquatox.2016.11.003.

Margesin R, Schinner F. 2001. Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles. 5(2):73–83. https://doi.org/10.1007/s007920100184.

Marles-Wright J, Lewis RJ. 2007. Stress responses of bacteria. Curr Opin Struct Biol. 17(6):755–760. https://doi.org/10.1016/j.sbi.2007.08.004.

Martin N, Burkus Z, Mceachern P, Yu T. 2014. Naphthenic acids quantification in organic solvents using fluorescence spectroscopy. J Environ Sci Heal A. 49(3):294–306. https://doi.org/10.1080/10934529.2014.846631.

Masliyah JH, Czarnecki J, Xu Z. 2011. Handbook on theory and practice on bitumen recovery from Athabasca oil sands volume I: Theoretical basis. Kingsley Knowledge Publishing. https://era.library.ualberta.ca/items/9bbcd0ba-9a7f-4622-a361-073435439350

Matafonova G, Batoev V. 2018. Recent advances in application of UV light-emitting diodes for degrading organic pollutants in water through advanced oxidation processes: A review. Water Res. 132:177–189. https://doi.org/10.1016/j.watres.2017.12.079.

Mathivanan K, Chandirika JU, Vinothkanna A, Yin H, Liu X, Meng D. 2021. Bacterial adaptive strategies to cope with metal toxicity in the contaminated environment – A review. Ecotoxicol Environ Saf. 226. https://doi.org/10.1016/j.ecoenv.2021.112863.

McDougald D, Rice SA, Kjelleberg S. 2007. Bacterial quorum sensing and interference by naturally occurring biomimics. Anal Bioanal Chem. 387(2):445–453. https://doi.org/10.1007/s00216-006-0761-2.

Menzie CA, Potocki BB, Santodonato J. 1992. Exposure to carcinogenic PAHs in the environment. Environ Sci Technol. 26(7):1278–1284. https://doi.org/10.1021/es00031a002

Mesak LR, Yim G, Davies J. 2009. Improved lux reporters for use in *Staphylococcus aureus*. Plasmid. 61(3):182–187. https://doi.org/10.1016/j.plasmid.2009.01.003.

Meshref MNA, Chelme-Ayala P, Gamal El-Din M. 2017. Fate and abundance of classical and heteroatomic naphthenic acid species after advanced oxidation processes: Insights and indicators of transformation and degradation. Water Res. 125:62–71. https://doi.org/10.1016/j.watres.2017.08.007.

Microbics. 1995. Microtox ® acute toxicity basic test procedures. Microbics Corporation. https://www.modernwater.com/assets/Technical%20Support/Toxicity/Manuals/ACUTE%20User's%20Manual.pdf

Miles SM, Hofstetter S, Edwards T, Dlusskaya E, Cologgi DL, Gänzle M, Ulrich AC. 2019. Tolerance and cytotoxicity of naphthenic acids on microorganisms isolated from oil sands process-affected water. Sci Total Environ. 695. https://doi.org/10.1016/j.scitotenv.2019.133749.

Miller SI. 2016. Antibiotic resistance and regulation of the Gram-negative bacterial outer membrane barrier by host innate immune molecules. mBio. 7(5). https://doi.org/10.1128/mBio.01541-16.

MilliporeSigma. 2020. Effect of membrane filter pore size on microbial recovery and colony morphology. Merck kGaA. https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/marketing/global/documents

/510/554/membrane-filter-pore-size-tn6309en-ms.pdf?srsltid=AfmBOoobOAO03RcnSwILL-p2LmsdfxIbao0RgvJ4DuKwRlCyq9wJh1cN

Miyamoto CM, Lin YH, Meighen EA. 2000. Control of bioluminescence in *Vibrio fischeri* by the LuxO signal response regulator. Mol Microbiol. 36(3):594–607. https://doi.org/10.1046/j.1365-2958.2000.01875.x.

Miyashiro T, Ruby EG. 2012. Shedding light on bioluminescence regulation in *Vibrio fischeri*. Mol Microbiol. 84(5):795–806. https://doi.org/10.1111/j.1365-2958.2012.08065.x.

Modrzyński JJ, Christensen JH, Brandt KK. 2019. Evaluation of dimethyl sulfoxide (DMSO) as a co-solvent for toxicity testing of hydrophobic organic compounds. Ecotoxicology. 28(9):1136–1141. https://doi.org/10.1007/s10646-019-02107-0.

Mohamad Shahimin MF, Foght JM, Siddique T. 2021. Methanogenic biodegradation of iso-alkanes by indigenous microbes from two different oil sands tailings ponds. Microorganisms. 9(8). https://doi.org/10.3390/microorganisms9081569.

Monteiro-Riviere NA, Inman AO. 2006. Challenges for assessing carbon nanomaterial toxicity to the skin. Carbon N Y. 44(6):1070–1078. https://doi.org/10.1016/j.carbon.2005.11.004.

Nyholm N, Källqvist T. 1989. Methods for growth inhibition toxicity tests with freshwater algae. Environ Toxicol Chem. 8(8):689–703. https://doi.org/10.1002/etc.5620080807.

Nyström T. 2004. Stationary-phase physiology. Annu Rev Microbiol. 58:161–181. https://doi.org/10.1146/annurev.micro.58.030603.123818.

Ong KJ, MacCormack TJ, Clark RJ, Ede JD, Ortega VA, Felix LC, Dang MKM, Ma G, Fenniri H, Veinot JGC, et al. 2014. Widespread nanoparticle-assay interference: Implications for nanotoxicity testing. PLoS One. 9(3). https://doi.org/10.1371/journal.pone.0090650.

Padilla-Martínez F, Carrizosa-Villegas LA, Rangel-Serrano Á, Paramo-Pérez I, Mondragón-Jaimes V, Anaya-Velázquez F, Padilla-Vaca F, Franco B. 2015. Cell damage detection using Escherichia coli reporter plasmids: fluorescent and colorimetric assays. Arch Microbiol. 197(6):815–821. https://doi.org/10.1007/s00203-015-1119-y.

Parhizkar J, Habibi MH. 2017. Synthesis, characterization and photocatalytic properties of Iron oxide nanoparticles synthesized by sol-gel autocombustion with ultrasonic irradiation. Nanochem Res. 2(2):166–171. https://doi.org/10.22036/ncr.2017.02.002.

Parmar D, Srivastava R, Baruah PK. 2023. Laser induced breakdown spectroscopy: A robust technique for the detection of trace metals in water. Mater Today Proc. 77:234–239. https://doi.org/10.1016/j.matpr.2022.11.267.

Pasquaroli S, Zandri G, Vignaroli C, Vuotto C, Donelli G, Biavasco F. 2013. Antibiotic pressure can induce the viable but non-culturable state in Staphylococcus aureus growing in biofilms. Journal of Antimicrobial Chemotherapy. 68(8):1812–1817. https://doi.org/10.1093/jac/dkt086.

Patel AB, Shaikh S, Jain KR, Desai C, Madamwar D. 2020. Polycyclic aromatic hydrocarbons: Sources, toxicity, and remediation approaches. Front Microbiol. 11. https://doi.org/10.3389/fmicb.2020.562813.

Paul S, Hussain NAS, Lillico DME, Suara MA, Ganiyu SO, Gamal El-Din M, Stafford JL. 2023. Examining the immunotoxicity of oil sands process affected waters using a human macrophage cell line. Toxicology. 500. https://doi.org/10.1016/j.tox.2023.153680.

Peterson BW, Sharma PK, van der Mei HC, Busscher HJ. 2012. Bacterial cell surface damage due to centrifugal compaction. Appl Environ Microbiol. 78(1):120–125. https://doi.org/10.1128/AEM.06780-11.

Phillips NAI, Lillico DME, Qin R, McAllister M, El-Din MG, Belosevic M, Stafford JL. 2020. Inorganic fraction of oil sands process-affected water induces mammalian macrophage stress gene expression and acutely modulates immune cell functional markers at both the gene and protein levels. Toxicology in Vitro. 66. https://doi.org/10.1016/j.tiv.2020.104875.

Plesiat P, Nikaido H. 1992. Outer membranes of Gram-negative bacteria are permeable to steroid probes. Mol Microbiol. 6(10):1323-1333. https://doi.org/10.1111/j.1365-2958.1992.tb00853.x

Pouliot R, Rochefort L, Graf MD. 2012. Impacts of oil sands process water on fen plants: Implications for plant selection in required reclamation projects. Environmental Pollution. 167:132–137. https://doi.org/10.1016/j.envpol.2012.03.050.

Prakash O, Shouche Y, Jangid K, Kostka JE. 2013. Microbial cultivation and the role of microbial resource centers in the omics era. Appl Microbiol Biotechnol. 97(1):51–62. https://doi.org/10.1007/s00253-012-4533-y.

Providenti MA, Lee H, Trevors JT. 1993. Selected factors limiting the microbial degradation of recalcitrant compounds. J Ind Microbiol. 12(6):379-395. https://doi.org/10.1007/BF01569669

Puttaswamy N, Liber K. 2012. Influence of inorganic anions on metals release from oil sands coke and on toxicity of nickel and vanadium to *Ceriodaphnia dubia*. Chemosphere. 86(5):521–529. https://doi.org/10.1016/j.chemosphere.2011.10.018.

Qin R, How ZT, Gamal El-Din M. 2019b-. Photodegradation of naphthenic acids induced by natural photosensitizer in oil sands process water. Water Res. 164. https://doi.org/10.1016/j.watres.2019.114913.

Qin R, Lillico D, How ZT, Huang R, Belosevic M, Stafford J, Gamal El-Din M. 2019a. Separation of oil sands process water organics and inorganics and examination of their acute toxicity using standard in-vitro bioassays. Science of the Total Environment. 695. https://doi.org/10.1016/j.scitotenv.2019.07.338.

Qiu TA, Nguyen THT, Hudson-Smith N V., Clement PL, Forester DC, Frew H, Hang MN, Murphy CJ, Hamers RJ, Feng ZV, et al. 2017. Growth-based bacterial viability assay for interference-free and high-throughput toxicity screening of nanomaterials. Anal Chem. 89(3):2057–2064. https://doi.org/10.1021/acs.analchem.6b04652.

Quagraine, E. K., Peterson, H. G., & Headley, J. V. (2005). *In situ* bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region—demonstrated field studies and plausible options: A review. J Environ Sci Heal A. 40(3):685–722. https://doi.org/10.1081/ESE-200046649

Quesnel DM, Oldenburg TBP, Larter SR, Gieg LM, Chua G. 2015. Biostimulation of oil sands process-affected water with phosphate yields removal of sulfur-containing organics and detoxification. Environ Sci Technol. 49(21):13012–13020. https://doi.org/10.1021/acs.est.5b01391.

Quinlan PJ, Tam KC. 2015. Water treatment technologies for the remediation of naphthenic acids in oil sands process-affected water. Chemical Engineering Journal. 279:696–714. https://doi.org/10.1016/j.cej.2015.05.062.

Radlinski L, Conlon BP. 2018. Antibiotic efficacy in the complex infection environment. Curr Opin Microbiol. 42:19–24. https://doi.org/10.1016/j.mib.2017.09.007.

Rainbow PS. 2007. Trace metal bioaccumulation: Models, metabolic availability and toxicity. Environ Int. 33(4):576–582. https://doi.org/10.1016/j.envint.2006.05.007.

Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S. 2014. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Front Public Health. 2(JUL). https://doi.org/10.3389/fpubh.2014.00103.

Ramos JL, Duque E, Gallegos M-T, Godoy P, Ramos-González MI, Rojas A, Terán W, Segura A. 2002. Mechanisms of solvent tolerance in Gram-negative bacteria. Annu Rev Microbiol. 56:743–768. https://doi.org/10.1146/annurev.micro.56.012302.161038.

Ray B, Speck ML, John Ordal Z. Freeze-injury in bacteria. Crc Cr Rev Cl Lab Sc. 4(2):161-213. https://doi.org/10.3109/10408367309151556

Redman AD, Parkerton TF, Butler JD, Letinski DJ, Frank RA, Hewitt LM, Bartlett AJ, Gillis PL, Marentette JR, Parrott JL, et al. 2018. Application of the target lipid model and passive

samplers to characterize the toxicity of bioavailable organics in oil sands process-affected water. Environ Sci Technol. 52(14):8039–8049. https://doi.org/10.1021/acs.est.8b00614.

Reygaert W. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microb. 4(3):482–501 https://doi.org/10.3934/microbiol.2018.3.482.

Robinson CE, Elvidge CK, Frank RA, Headley J V, Hewitt LM, Little AG, Robinson SA, Trudeau VL, Vander Meulen IJ, Orihel DM. 2023. Naphthenic acid fraction compounds reduce the reproductive success of wood frogs (*Rana sylvatica*) by affecting offspring viability. Environmental Pollution. 316:120455. https://doi.org/10.1016/j.envpol.2022.120455.

Rogers V V, Wickstrom M, Liber K, Mackinnon MD. 2002. Acute and subchronic mammalian toxicity of naphthenic acids from oil sands tailings. Toxicol Sci. 66(2):347-355. https://doi.org/10.1093/toxsci/66.2.347

Rompré A, Servais P, Baudart J, De-Roubin M-R, Laurent P. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods. 49(1):31-54. https://doi.org/10.1016/s0167-7012(01)00351-7.

Ross MS, Pereira A dos S, Fennell J, Davies M, Johnson J, Sliva L, Martin JW. 2012. Quantitative and qualitative analysis of naphthenic acids in natural waters surrounding the Canadian oil sands industry. Environ Sci Technol. 46(23):12796–12805. https://doi.org/10.1021/es303432u.

Roy JW, Bickerton G, Frank RA, Grapentine L, Hewitt LM. 2016. Assessing Risks of Shallow Riparian Groundwater Quality Near an Oil Sands Tailings Pond. Groundwater. 54(4):545–558. https://doi.org/10.1111/gwat.12392.

Rudzinski WE, Oehlers L, Zhang Y, Najera B. 2002. Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil. Energy and Fuels. 16(5):1178–1185. https://doi.org/10.1021/ef020013t.

Russell WMS, Burch RL, Hume CW. 1992. The Principles of Humane Experimental Technique. Boulder (CO): Universities Federation for Animal Welfare.

Rusydi AF. 2018. Correlation between conductivity and total dissolved solid in various type of water: A review. In: IOP Conference Series: Earth Environ Sci. 118. Institute of Physics Publishing. https://doi.org/10.1088/1755-1315/118/1/012019

Saborimanesh N. 2021. Toward sustainable remediation of oil sands fine tailings-A review. J Environ Manage. 288. https://doi.org/10.1016/j.jenvman.2021.112418.

Salton MRJ, Kim KS. 1996. Structure. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston. https://www.ncbi.nlm.nih.gov/books/NBK8477/

Sánchez-Montes I, Mokarizadeh H, Paul S, Moghrabi K, Hussain N, Chelme-Ayala P, Stafford JL, Lanza MRV, Gamal El-Din M. 2024. UVA LED-assisted breakdown of persulfate oxidants for the treatment of real oil sands process water: Removal of naphthenic acids and evaluation of residual toxicity. Chemical Engineering Journal. 481. https://doi.org/10.1016/j.cej.2024.148631.

Sansom B, Vo NTK, Kavanagh R, Hanner R, MacKinnon M, Dixon DG, Lee LEJ. 2013. Rapid assessment of the toxicity of oil sands process-affected waters using fish cell lines. In Vitro Cell Dev Biol Anim. 49(1):52–65. https://doi.org/10.1007/s11626-012-9570-4.

Scarlett AG, West CE, Jones D, Galloway TS, Rowland SJ. 2012. Predicted toxicity of naphthenic acids present in oil sands process-affected waters to a range of environmental and human endpoints. Sci Total Environ. 425:119–127. https://doi.org/10.1016/j.scitotenv.2012.02.064.

Schaefer AL, Hanzelka BL, Eberhard A, Greenberg EP. 1996. Quorum sensing in *Vibrio fischeri*: Probing autoinducer-LuxR interactions with autoinducer analogs. J Bacteriol. 178(10): 2897-2901. https://doi.org/10.1128/jb.178.10.2897-2901

Scheutz F, Strockbine NA. 2015. Escherichia. In: Bergey's manual of systematics of Archaea and Bacteria. p. 1–49. https://doi.org/10.1002/9781118960608.gbm01147.

Schlegel M, Mcintosh J, Petsch S, Orem W, Jones E, Martini A. 2013. Extent and limits of biodegradation by in situ methanogenic consortia in shale and formation fluids. Appl Geochem. 28:172–184. https://doi.org/10.1016/j.apgeochem.2012.10.008.

Schleifer K-H, Bell JA. 2015. Staphylococcus. In: Bergey's manual of systematics of Archaea and Bacteria. p. 1–43. https://doi.org/10.1002/9781118960608.gbm00569.

Schmitt-Jansen M, Bartels P, Adler N, Altenburger R. 2007. Phytotoxicity assessment of diclofenac and its phototransformation products. Anal Bioanal Chem. 387(4):1389–1396. https://doi.org/10.1007/s00216-006-0825-3

Schuytema GS, Nebeker A V, Stutzman TW. 1996. Salinity Tolerance of *Daphnia magna* and potential use for estuarine sediment toxicity tests. Environ Contam. 33:194–198. https://doi.org/10.1007/s002449900242

Scott A. 2007. Biodegradation, ozonation, and characterization of naphthenic acids [dissertation]. University of Alberta. https://era.library.ualberta.ca/items/13c01a29-9936-497d-a6f8-5c7610948378

Scott AC, MacKinnon MD, Fedorak PM. 2005. Naphthenic acids in athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environ Sci Technol. 39(21):8388–8394. https://doi.org/10.1021/es051003k.

Scott AC, Zubot W, Davis CW, Brogly J. 2020. Bioaccumulation potential of naphthenic acids and other ionizable dissolved organics in oil sands process water (OSPW) – A review. Science of the Total Environment. 712. https://doi.org/10.1016/j.scitotenv.2019.134558.

Scott AC, Zubot W, MacKinnon MD, Smith DW, Fedorak PM. 2008. Ozonation of oil sands process water removes naphthenic acids and toxicity. Chemosphere. 71(1):156–160. https://doi.org/10.1016/j.chemosphere.2007.10.051.

Semrau JD. 2011. Bioremediation via methanotrophy: Overview of recent findings and suggestions for future research. Front Microbiol. 2. https://doi.org/10.3389/fmicb.2011.00209.

Seneviratne CJ, Yip JWY, Chang JWW, Zhang CF, Samaranayake LP. 2013. Effect of culture media and nutrients on biofilm growth kinetics of laboratory and clinical strains of *Enterococcus faecalis*. Arch Oral Biol. 58(10):1327–1334. https://doi.org/10.1016/j.archoralbio.2013.06.017.

Shimizu K. 2013. Regulation systems of bacteria such as *Escherichia coli* in response to nutrient limitation and environmental stresses. Metabolites. 4(1):1–35. https://doi.org/10.3390/metabo4010001.

Shinn-Thomas JH, Scanga SE, Spica PS, Nariya HK, Klempic E, Brockett MR. 2019. Wrapping culture plates with Parafilm M® increases *Caenorhabditis elegans* growth. BMC Res Notes. 12(1). https://doi.org/10.1186/s13104-019-4854-3.

Siddique T, Penner T, Klassen J, Nesbø C, Foght JM. 2012. Microbial Communities Involved in Methane Production from Hydrocarbons in Oil Sands Tailings. Environ Sci Technol. 46(17):9802–9810. doi:10.1021/es302202c. https://doi.org/10.1021/es302202c.

Sikkema J, De Bont JAM, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev. 59(2):201-222. https://doi.org/10.1128/mr.59.2.201-222.1995

Silva V, Caniça M, Capelo JL, Igrejas G, Poeta P. 2020. Diversity and genetic lineages of environmental staphylococci: a surface water overview. FEMS Microbiol Ecol. 96(12). https://doi.org/doi:10.1093/femsec/fiaa191

Sinskey TJ, Silverman GJ. 1970. characterization of injury incurred by *Escherichia coli* upon freeze-drying. J Bacteriol. 101(2):429-437. https://doi.org/10.1128/jb.101.2.429-437.1970

Slonczewski JL, Fujisawa M, Dopson M, Krulwich TA. 2009. Cytoplasmic pH measurement and homeostasis in bacteria and archaea. In: Poole RK, editor. Advances in Microbial Physiology. Academic Press. p. 1–317. https://doi.org/10.1016/S0065-2911(09)05501-5.

Song J, How ZT, Huang Z, Gamal El-Din M. 2022. Biochar/iron oxide composite as an efficient peroxymonosulfate catalyst for the degradation of model naphthenic acids compounds. Chemical Eng J. 429. https://doi.org/10.1016/j.cej.2021.132220.

Song J, Huang Z, Gamal El-Din M. 2021. Adsorption of metals in oil sands process water by a biochar/iron oxide composite: Influence of the composite structure and surface functional groups. Chem Eng J. 421:129937. https://doi.org/10.1016/j.cej.2021.129937.

Song J, Messele SA, Meng L, Huang Z, Gamal El-Din M. 2021. Adsorption of metals from oil sands process water (OSPW) under natural pH by sludge-based biochar/chitosan composite. Water Res. 194. https://doi.org/10.1016/j.watres.2021.116930.

Stasik S, Wick LY, Wendt-Potthoff K. 2015. Anaerobic BTEX degradation in oil sands tailings ponds: Impact of labile organic carbon and sulfate-reducing bacteria. Chemosphere. 138:133–139. https://doi.org/10.1016/j.chemosphere.2015.05.068.

Sterritt RM, Lester JN. 1980. Interactions of heavy metals with bacteria. Sci Total Environ. 14(1):5-17. https://doi.org/10.1016/0048-9697(80)90122-9

Suara MA, Ganiyu SO, Paul S, Stafford JL, Gamal El-Din M. 2022. Solar-activated zinc oxide photocatalytic treatment of real oil sands process water: Effect of treatment parameters on naphthenic acids, polyaromatic hydrocarbons and acute toxicity removal. Sci Total Environ. 819. https://doi.org/10.1016/j.scitotenv.2022.153029

Suedel BC, Rodgers JH. 1996. Toxicity of Fluoranthene to *Daphnia magna*, *Hyalella azteca*, *Chironomus tentans*, and *Stylaria lacustris* in water-only and whole sediment exposures. Bull Environ Contam Toxicol. 57(1):132-138. https://doi.org/10.1007/s001289900166

Sultana L, Sanchis AG. 2022. Establishing the lower bacterial concentration threshold for different optical counting techniques. J Microbiol Methods. 203. https://doi.org/10.1016/j.mimet.2022.106620.

Sun C, Shotyk W, Cuss CW, Donner MW, Fennell J, Javed M, Noernberg T, Poesch M, Pelletier R, Sinnatamby N, et al. 2017. Characterization of naphthenic acids and other dissolved organics in natural water from the Athabasca oil sands region, Canada. Environ Sci Technol. 51(17):9524–9532. https://doi.org/doi:10.1021/acs.est.7b02082

Sun H, Calabrese EJ, Zheng M, Wang D, Pan Y, Lin Z, Liu Y. 2018. A swinging seesaw as a novel model mechanism for time-dependent hormesis under dose-dependent stimulatory and inhibitory effects: A case study on the toxicity of antibacterial chemicals to *Aliivibrio fischeri*. Chemosphere. 205:15–23. https://doi.org/10.1016/j.chemosphere.2018.04.043.

Suncor Energy Inc. 2024. Tailings management. https://www.suncor.com/en-ca/sustainability-new/environment/tailings-management

Sunuwar S, Manzanares CE. 2021. Excitation, emission, and synchronous fluorescence for astrochemical applications: Experiments and computer simulations of synchronous spectra of polycyclic aromatic hydrocarbons and their mixtures. Icarus. 370:114689. https://doi.org/10.1016/j.icarus.2021.114689.

Swigert JP, Lee C, Wong DCL, White R, Scarlett AG, West CE, Rowland SJ. 2015. Aquatic hazard assessment of a commercial sample of naphthenic acids. Chemosphere. 124(1):1–9. https://doi.org/10.1016/j.chemosphere.2014.10.052.

Szermer-Olearnik B, Sochocka M, Zwolińska K, Ciekot J, Czarny A, Szydzik J, Kowalski K, Boratyński J, Janusz B. 2014. Comparison of microbiological and physicochemical methods for enumeration of microorganisms. Advances in Hygiene and Experimental Medicine. 68:1392-1396. https://doi.org/10.5604/17322693.1130086

Tavares A, Carvalho CMB, Faustino MA, Neves MGPMS, Tomé JPC, Tomé AC, Cavaleiro JAS, Cunha Â, Gomes NCM, Alves E, et al. 2010. Antimicrobial photodynamic therapy: Study of bacterial recovery viability and potential development of resistance after treatment. Mar Drugs. 8(1):91–105. https://doi.org/10.3390/md8010091.

Tejs S. 2008. The Ames test: a methodological short review. Environ Biotechnol. 4(1):7-14. https://www.researchgate.net/publication/267961221_The_Ames_test_a_methodological_short_review

Thomas P, Sekhar AC, Upreti R, Mujawar MM, Pasha SS. 2015. Optimization of single plate-serial dilution spotting (SP-SDS) with sample anchoring as an assured method for bacterial and yeast CFU enumeration and single colony isolation from diverse samples. Biotechnology Reports. 8:45–55. https://doi.org/10.1016/j.btre.2015.08.003

Tollefsen KE, Petersen K, Rowland SJ. 2012. Toxicity of synthetic naphthenic acids and mixtures of these to fish liver cells. Environ Sci Technol. 46(9):5143–5150. doi:10.1021/es204124w. https://doi.org/10.1021/es204124w.

Trovó AG, Nogueira RFP, Agüera A, Fernandez-Alba AR, Sirtori C, Malato S. 2009. Degradation of sulfamethoxazole in water by solar photo-Fenton. Chemical and toxicological evaluation. Water Res. 43(16):3922–3931. https://doi.org/10.1016/j.watres.2009.04.006.

Vaish M, Jereen A, Ali A, Krulwich TA. 2019. The alkaliphilic side of *Staphylococcus aureus*. Pharmacological Sciences, Icahn School of Medicine at Mount Sinai. Preprint. https://doi.org/10.1101/735191.

Vander Meulen IJ, Schock DM, Parrott JL, Mundy LJ, Pauli BD, Peru KM, McMartin DW, Headley J V. 2021. Characterization of naphthenic acid fraction compounds in water from Athabasca oil sands wetlands by Orbitrap high-resolution mass spectrometry. Science of the Total Environment. 780. https://doi.org/10.1016/j.scitotenv.2021.146342.

Vassallo J, Besinis A, Boden R, Handy RD. 2018. The minimum inhibitory concentration (MIC) assay with Escherichia coli: An early tier in the environmental hazard assessment of nanomaterials? Ecotoxicol Environ Saf. 162:633–646. https://doi.org/10.1016/j.ecoenv.2018.06.085.

Viegas CA. 2021. Microbial bioassays in environmental toxicity testing. In: Advances in Applied Microbiology. Vol. 115. Academic Press Inc. p. 115–158.

Vollmer AC, Van Dyk TK. 2004. Stress responsive bacteria: biosensors as environmental monitors. Adv Microb Physiol. 49:131–174. https://doi.org/10.1016/S0065-2911(04)49003-1

Wallace SJ, de Solla SR, Head JA, Hodson P V., Parrott JL, Thomas PJ, Berthiaume A, Langlois VS. 2020. Polycyclic aromatic compounds (PACs) in the Canadian environment: Exposure and effects on wildlife. Environ Pollut. 265(B). https://doi.org/10.1016/j.envpol.2020.114863.

Wang C, Alpatova A, McPhedran KN, Gamal El-Din M. 2015. Coagulation/flocculation process with polyaluminum chloride for the remediation of oil sands process-affected water: Performance and mechanism study. J Environ Manage. 160:254–262. https://doi.org/10.1016/j.jenvman.2015.06.025.

Wang C, Klamerth N, Messele SA, Singh A, Belosevic M, Gamal El-Din M. 2016. Comparison of UV/hydrogen peroxide, potassium ferrate(VI), and ozone in oxidizing the organic fraction of oil sands process-affected water (OSPW). Water Res. 100:476–485. https://doi.org/10.1016/j.watres.2016.05.037.

Wang N, Chelme-Ayala P, Perez-Estrada L, Garcia-Garcia E, Pun J, Martin JW, Belosevic M, Gamal El-Din M. 2013. Impact of ozonation on naphthenic acids speciation and toxicity of oil sands process-affected water to vibrio fischeri and mammalian immune system. Environ Sci Technol. 47(12):6518–6526. https://doi.org/10.1021/es4008195.

Wang S, Zhao M, Zhou M, Li YC, Wang J, Gao B, Sato S, Feng K, Yin W, Igalavithana AD, et al. 2019. Biochar-supported nZVI (nZVI/BC) for contaminant removal from soil and water: A critical review. J Hazard Mater. 373:820–834. https://doi.org/10.1016/j.jhazmat.2019.03.080.

Wang Y, Puthussery J V., Yu H, Verma V. 2020. Synergistic and antagonistic interactions among organic and metallic components of the ambient particulate matter (PM) for the cytotoxicity measured by Chinese hamster ovary cells. Sci Total Environ. 736. https://doi.org/10.1016/j.scitotenv.2020.139511.

Wani PA, Ebudola AN, Olusebi YK, Rafi N, Fawzhia O SJ, Oluwaseun OI. 2023. Hydrocarbon utilizing and metal tolerant bacteria simultaneously degrade hydrocarbons and detoxify metals in petroleum contaminated soil. Geomicrobiol J. 40(4):372–381. https://doi.org/10.1080/01490451.2023.2176573.

Weelink SAB, van Eekert MHA, Stams AJM. 2010. Degradation of BTEX by anaerobic bacteria: Physiology and application. Rev Environ Sci Biotechnol. 9(4):359–385. https://doi.org/10.1007/s11157-010-9219-2.

Weitz HJ, Campbell CD, Killham K. 2002. Development of a novel, bioluminescence-based, fungal bioassay for toxicity testing. Environ Microbiol. 4(7):422-429. https://doi.org/10.1046/j.1462-2920.2002.00315.x

West CE, Jones D, Scarlett AG, Rowland SJ. 2011. Compositional heterogeneity may limit the usefulness of some commercial naphthenic acids for toxicity assays. Sci Total Environ. 409(19):4125–4131. https://doi.org/10.1016/j.scitotenv.2011.05.061.

Whiting RC, Sackitey S, Calderone S, Merely K, Phillipsb JG. 1996. Model for the survival of *Staphylococcus aureus* in nongrowth environments. Int J Food Eng. 31(1-3):231-243. https://doi.org/10.1016/0168-1605(96)01002-1

Wiegand I, Hilpert K, Hancock REW. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 3(2):163–175. https://doi.org/10.1038/nprot.2007.521.

Williams CF, Geroni GM, Lloyd D, Choi H, Clark N, Pirog A, Lees J, Porch A. 2019. Bioluminescence of Vibrio fischeri: bacteria respond quickly and sensitively to pulsed microwave electric (but not magnetic) fields. J Biomed Opt. 24(05):1. https://doi.org/10.1117/1.jbo.24.5.051412.

Wiseman SB, Anderson JC, Liber K, Giesy JP. 2013. Endocrine disruption and oxidative stress in larvae of *Chironomus dilutus* following short-term exposure to fresh or aged oil sands

process-affected water. Aquat Toxicol. 142–143:414–421. https://doi.org/10.1016/j.aquatox.2013.09.003.

Wood JM, Bremer E, Csonka LN, Kraemer R, Poolman B, Van Der Heide T, Smith LT. 2001. Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comp Biochem Physiol A Mol Integr Physiol. 130(3):437-460. https://doi.org/10.1016/s1095-6433(01)00442-1

Woodworth APJ, Frank RA, McConkey BJ, Müller KM. 2012. Toxic effects of oil sand naphthenic acids on the biomass accumulation of 21 potential phytoplankton remediation candidates. Ecotoxicol Environ Saf. 86:156–161. https://doi.org/10.1016/j.ecoenv.2012.09.007.

Woutersen M, Belkin S, Brouwer B, Van Wezel AP, Heringa MB. 2011. Are luminescent bacteria suitable for online detection and monitoring of toxic compounds in drinking water and its sources? Anal Bioanal Chem. 400(4):915–929. https://doi.org/10.1007/s00216-010-4372-6.

Xu J, Zheng L, Yan Z, Huang Y, Feng C, Li L, Ling J. 2020. Effective extrapolation models for ecotoxicity of benzene, toluene, ethylbenzene, and xylene (BTEX). Chemosphere. 240. https://doi.org/10.1016/j.chemosphere.2019.124906.

Xue J, Huang C, Zhang Y, Liu Y, Gamal El-Din M. 2018. Bioreactors for oil sands process-affected water (OSPW) treatment: A critical review. Science of the Total Environment. 627:916–933. https://doi.org/10.1016/j.scitotenv.2018.01.292.

Yan H, Li M, Meng L, Zhao F. 2021. Formation of viable but nonculturable state of *Staphylococcus aureus* under frozen condition and its characteristics. Int J Food Microbiol. 357. https://doi.org/10.1016/j.ijfoodmicro.2021.109381.

Yergeau E, Lawrence JR, Sanschagrin S, Waiser MJ, Korber DR, Greer CW. 2012. Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities. Appl Environ Microbiol. 78(21):7626–7637. https://doi.org/10.1128/AEM.02036-12.

You Z, Xu H, Zhang S, Kim H, Chiang PC, Yun W, Zhang L, He M. 2018. Comparison of petroleum hydrocarbons degradation by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Appl Sci. 8(12). https://doi.org/10.3390/app8122551.

Young RF, Orr EA, Goss GG, Fedorak PM. 2007. Detection of naphthenic acids in fish exposed to commercial naphthenic acids and oil sands process-affected water. Chemosphere. 68(3):518–527. https://doi.org/10.1016/j.chemosphere.2006.12.063.

Yue Z, Economy J. 2005. Nanoparticle and nanoporous carbon adsorbents for removal of trace organic contaminants from water. J Nano Res. 7:477-487. https://doi.org/10.1007/s11051-005-4719-7

Zhang L, Zhang Y, Gamal El-Din M. 2018. Degradation of recalcitrant naphthenic acids from raw and ozonated oil sands process-affected waters by a semi-passive biofiltration process. Water Res. 133:310–318. https://doi.org/10.1016/j.watres.2018.01.001.

Zhang Y, McPhedran KN, Gamal El-Din M. 2015. Pseudomonads biodegradation of aromatic compounds in oil sands process-affected water. Sci Total Environ. 521–522:59–67. https://doi.org/10.1016/j.scitotenv.2015.03.068.

Zisanur Rahman ASM, Liu C, Sturm H, Hogan AM, Davis R, Hu P, Cardona ST. 2022. A machine learning model trained on a high-throughput antibacterial screen increases the hit rate of drug discovery. PLoS Comput Biol. 18(10). https://doi.org/10.1371/journal.pcbi.1010613.

Zubot W, MacKinnon MD, Chelme-Ayala P, Smith DW, Gamal El-Din M. 2012. Petroleum coke adsorption as a water management option for oil sands process-affected water. Sci Total Environ. 427–428:364–372. https://doi.org/10.1016/j.scitotenv.2012.04.024.