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Tolerance and cytotoxicity of naphthenic acids on microorganisms isolated from oil sands process-affected water

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Abstract

The expansion of oil sands has made remediation of oil sands process-affected water (OSPW) critical. As naphthenic acids (NAs) are the primary contributors to toxicity, remediation is required. Bioremediation by native microorganisms is potentially effective, however, toxicity of NAs towards native microorganisms is poorly understood. The aim of this study was to isolate microorganisms from OSPW, assess tolerance to stressors, including naturally sourced NAs and examine exposure effect of NAs on cell membranes. Microorganisms were isolated from OSPW, including the first reported isolation of a fungus (*Trichoderma harzianum*) and yeast (*Rhodotorula mucilaginosa*). Isolates tolerated alkaline pH, high salinity, and NA concentrations far exceeding those typical of OSPW indicating toxic effects of OSPW are likely the result of interactions between OSPW components. Comparisons of toxicity determined that OSPW exhibited higher cytotoxicity than NAs. The fungal isolate was able to grow using commercial NAs as its sole carbon source, indicating high resistance to NAs' cytotoxic effects. Future studies will focus on the organisms' ability to degrade NAs, and subsequent effects on toxicity. Characterization of OSPW constituents should be investigated with focus on the synergistic toxic effects of dissolved compounds. A better understanding of OSPW toxicity would enable more effective and targeted bioremediation schemes by native microorganisms.

Keywords

bioremediation, toxicity, fungi, yeast, naphthenic acids, oil sands

1.0 Introduction

The oil sands of Northern Alberta, Canada, are home to one of the world's largest proven reserves of crude oil, with an estimated 1.69 billion barrels (269.2m^3) of recoverable bitumen (Allen, 2008; Energy Resources Conservation Board, 2013). A portion of these deposits are shallow and are currently being recovered through surface mining operations. Bitumen is extracted from surface mined oil sand using a modified Clark Hot Water extraction process whereby the bitumen, clay, and sand particles are separated in the presence of heat and chemicals (Dzidic et al., 1988; Schramm et al., 2000; Allen, 2008). This post-extraction water is called oil sands process-affected water (OSPW) and is stored in engineered tailings ponds to limit environmental exposure. With the reuse of OSPW in the extraction process, the tailings ponds become increasingly concentrated with salts, minerals, heavy metals, organics and bitumen (Allen, 2008).

One group of dissolved organics in OSPW are naphthenic acids (NAs) and contribute extensively to toxicity, thus making them the focus of remediation efforts (MacKinnon and Boerger, 1986; Headley and McMartin, 2004; Hughes et al., 2017). NAs are a complex mixture of cyclic and aromatic hydrocarbons possessing a single carboxylated side chain, and have the general formula $C_nH_{2n+Z}O_{2,}$ (where *n* represents the carbon number and *Z* is the related to hydrogen deficiency due to rings and double bonds) (Dzidic et al., 1988; Fan, 1991; St John et al., 1998). Classically defined NAs are often referred to as the O_2^- group as this classical formula is restricted to two oxygen compounds. Commercial NAs are restricted to this category as they mainly conform to

the classical NA formula (but may vary depending on source and method of synthesis). More recent analyses have shown that OSPW not only contains the classically defined NAs, but also a complex mixture of oxidized compounds, containing three or more oxygen atoms and other compounds with atoms such as sulphur and nitrogen (Grewer et al., 2010; Rowland et al., 2011b; Rowland et al., 2011c). This complex mixture of natural NAs in OSPW has different properties and toxicity than classically defined NAs, which is the only group of organics present in commercially available NAs (Scott et al., 2005; Grewer et al., 2010; Quesnel et al., 2011; Hughes et al., 2017). Hughes et al. (2017) determined a link between classically defined NAs fraction within OSPW and the acute toxicity of OSPW sourced organics to rainbow trout (*Oncorhynchus mykiss*). NAs toxicity poses a critical environmental concern to native species within the area; therefore, remediation and monitoring efforts often focus on this organic fraction within OPSW.

Several studies have investigated the process of microbial degradation of NAs in OSPW and have suggested that *in situ* bioremediation of the organic acids is a plausible and cost effective method for the reducing OSPW toxicity (Herman et al., 1994; Scott et al., 2005; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Paulssen and Gieg, 2019). Microorganisms identified in OSPW include *Pseudomonas* sp. (Del Rio et al., 2006; Golby et al., 2012; Demeter et al., 2014) *Thauera* sp., *Hydrogenophaga* sp., *Rhodoferax* sp., *Acidovorax* sp. (Golby et al., 2012), and *Rhodococcus* sp. (Demeter et al., 2014). Unfortunately, the toxicity of NAs may inhibit biodegradation of present organics within OSPW (MacKinnon and Boerger, 1986; Brient et al., 1995; Holowenko et al.,

2002; Clemente et al., 2004; Marentette et al., 2015). Morandi *et al.* (2015) determined that NAs (containing an O_2^- group) contribute most significantly to toxicity, but non-acidic species (containing O^+, O_2^+, SO^+, NO^+) also contribute to toxicity. However, little is known about the mechanism of toxicity exerted by this organic fraction towards microorganisms native to the oil sands tailings ponds at a cellular level (Frank et al., 2008; Rowland et al., 2011c). The chemical structure of NA compounds suggest an amphiphilic character (Holowenko et al., 2002), therefore we examined partitioning into cell membranes to determine if lipid order disruption accounts for toxicity (Goff et al., 2013). Understanding the method of toxicity is critical to understanding how to address remediation of this organic fraction within OSPW. The aim of this study was to isolate aerobic microorganisms from OSPW, assess their tolerance to NAs and other stressors, and to examine effects on cell membranes.

2.0 Materials and methods

2.1 OSPW characterization

A sample (20 L; pH 8.4) of OSPW from the Athabasca oil sands region of northern Alberta, Canada, was collected in 2009 and stored in 4L amber glass bottles at 4°C until used. OSPW was analyzed for dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) concentrations using a Shimadzu TOC-5000A (Shimadzu Corporation, Kyoto, Japan) as described previously (Clescerie et al., 1999). Phenol was measured by Exova, (Edmonton, Alberta, Canada) following the APHA 5530D method (American Public Health Association 2005) for phenols. Polyaromatic hydrocarbons (PAH) were

measured by Maxxam Analytical Laboratories, (Edmonton, Alberta, Canada), following the EPA 3510C/8270D method (United States Environmental Protection Agency 1996; United States Environmental Protection Agency 2007) for PAHs. Analysis of dissolved trace metals was completed by inductively coupled plasma-mass spectrometry (ICP-MS) (ELAN 9000; PerkinElmer/SCIEX, Waltham, MA) as described previously (Abolfazlzadehdoshanbehbazari, 2013).

2.2 Extraction of NAs from OSPW

Naturally occurring NA fractions from OSPW were extracted as described previously (Grewer et al., 2010). Additional OSPW sources were used in order to obtain sufficient amounts of organic extract for initial selective enrichment of microorganisms. The final extract was dried, weighed, and stored at 4˚C until needed.

2.3 Selective enrichment of NA-degrading microorganisms

Selective enrichment was conducted using progressively increasing concentrations of natural NA compounds. Prior to selective enrichment, OSPW (500 mL) was incubated aerobically at ~21˚C for six weeks to stimulate the microbial community after long-term storage. The natural NA fraction was dissolved in dichloromethane (DCM), added to flasks, and DCM was allowed to evaporate; 125 mL of Bushnell-Haas (BH) media(Bushnell and Haas, 1941) and 14 mL OSPW were added to arrive at a final concentration of 50 mg L^{-1} of NAs. Samples were incubated at room temperature (~21°C), shaking at 200 rpm, for two weeks. Due to a shortage of natural NAs, after the

initial enrichment, Merichem commercial NAs (Merichem Chemicals and Refinery Services LLC, Houston, TX) were utilized. The initial enrichment culture was sequentially transferred (10% v/v) in sequence into Bushnell-Haas media (125 mL) containing increasing concentrations of commercial NAs (100 mg L^{-1}, 150 mg L^{-1}, and 300 mg L^{-1}). Each transfer was allowed to incubate at room temperature (~21°C), shaking at 200 rpm, for two weeks until microbial isolation.

2.4 Isolation of Microorganisms

Selective enrichment cultures were plated onto general and selective media to isolate microorganisms. Two high-protein media were chosen in order to facilitate growth of a broad range of fast-growing microorganisms, similar to the approach used previously (Biryukova et al., 2007): Brain Heart Infusion (Difco, Becton, Dickinson and Company, Sparks, USA) and Nutrient Broth (Difco, Becton, Dickinson and Company, Sparks, USA). Gen-1 media was used as the general growth media. Gen-1 media contained (per L): 10 g malt extract, 5 g tryptone, 1 g dextrose, 1 g yeast extract, and 5 g NaCl. Selective growth media used were Sabouraud Dextrose (Difco, Becton, Dickinson and Company, Sparks, USA), Cooke Rose Bengal (Difco, Becton, Dickinson and Company, Sparks, USA) supplemented with Antimicrobic A (Difco, Becton, Dickinson and Company, Sparks, USA; 35 µg mL^{-1} chlortetracycline), and Brain Heart Infusion and Nutrient Broth supplemented with cyclohexamide (Sigma-Aldrich, St Louis, MO; 100 mg L^{-1}) or chloramphenicol (Fisher Scientific, Toronto, Canada; 300 mg L^{-1}).

Aliquots (100 µL) of selective enrichment culture were plated, in triplicate, onto general and selective media. Plates were incubated at room temperature (~21°C) and visually inspected for growth every 24 h for a total of 144 h. Every 24 h, individual colonies were aseptically picked and triple streaked, in duplicate, onto plates of corresponding media and incubated at room temperature (~21°C) for a total of 144 h. This process was repeated six times for each individual colony on triple-streaked plates to ensure isolate purity. Where fungal growth was observed, mycelium was picked from outer margins of growth. Final colonies were picked, wet mount slides prepared (Fisher Scientific, Toronto, Canada), and inspected for purity using light microscopy (Zeiss AXIOVision Imager, Toronto, Ontario, Canada). Pure cultures were inoculated into corresponding broth media, incubated at room temperature (~21°C) for 24-72 h, transferred to 60% glycerol, and frozen at -80°C until needed.

2.5 Isolation of DNA, PCR, and identification

To obtain samples for DNA extraction, isolates were inoculated into a corresponding broth media from -80°C frozen stocks and incubated at room temperature (~21°C) for 48 h. Total genomic DNA from bacterial and yeast samples was extracted using Qiagen DNeasy Blood and Tissue Kit (QIAGEN Sciences, Maryland, USA) and from fungal samples using Qiagen DNeasy Plant Kit (QIAGEN Sciences, Maryland, USA) following manufacturer protocols. Fungal samples were placed in 2mL screw cap tubes and lysed by grinding with lysis buffer and 2 tungsten carbide beads to release DNA from cells; no liquid nitrogen was used. PCR amplification and DNA sequencing

were done at the Molecular Biology Facility (MBSU) at the Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada.

The 16S rDNA gene sequence was amplified from bacterial samples using primers 27F/1492R (Lane, 1991). The ITS region was amplified from fungal samples using primers ITS1/ITS4 (Sandhu et al., 1995). PCR reactions contained (final volume of 15 µl) 2.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 0.1 µmol L^{-1} primers, 1X PCR buffer (Fisher Scientific, Ottawa, Canada) and 1.25 U Taq polymerase (Fisher Scientific, Ottawa, Canada), and cycling was performed in an Eppendorf Mastercycler (Eppendorf, Ontario, Canada) as follows: 94°C for 2 min, 30 cycles of 94°C for 20 s, 51°C for 30 s, and 72°C for 30 s, and 10 min final extension. Whole PCR products were visually confirmed via agarose gel electrophoresis (1% agarose gel), and subsequently purified with ExoSAP (Affymetrix, California, USA) prior to sequencing. Amplicons were sequenced using original amplification primers, BigDye V3.1 chemistry and resolved on an ABI 3730 DNA Analyzer (Life Technologies, Ontario, Canada). Basecalling was completed using ABI DNA Sequencing Analysis Software. Sequenced PCR products were edited using Chromalign software, aligned using ClustalOMEGA (http://www.ebi.ac.uk/Tools/msa/clustalo/), and submitted for BLAST analysis (23/05/14) (http://blast.ncbi.nlm.nih.gov/) and Ribosomal Database Project (23/05/14) (http://rdp.cme.msu.edu/) for identification. Species identity was only listed where a match of 99% or greater was obtained; identification was otherwise listed at genus level (Janda and Abbott, 2007). Sequences were submitted to the GenBank database (2015/03/25; Accession numbers Table 1)

2.6 Tolerance of microbial isolates to pH, salinity, and NAs

Serial dilution assays to investigate the tolerance of microbial isolates to stressors commonly encountered in OSPW were performed as described previously (Gänzle et al., 2000). All assays were done in glass scintillation vials using Gen-1 media. Microbial isolates were exposed to a range of pH, salinity, and NA concentrations. Glass scintillation vials containing Gen-1 broth (5 mL / vial) were adjusted to pH 2–11 using NaOH (1 mol L^{-1}) or HCl (1 mol L^{-1}), or a NaCl content of 1–10% (w/v). For pH and saline trials, cultures (48 h; Gen-1 broth) of isolates were inoculated (1% v/v) in triplicate. To test tolerance to NAs, a known weight of NA fraction was dissolved in a known volume of 1 mol L^{-1} NaOH and stored at 4°C until needed. The pH in blank samples confirms that the addition of NaOH stock during the assay did not shift pH out of growth range (data not shown). Samples were incubated at room temperature (~21°C) and visually scored for growth at 24, 48, and 72 h, as described previously (Gänzle et al., 2000). To further confirm these findings, dilution assays were also scored visually via light microscopy. At each time point, five 10 µL aliquots were withdrawn from each sample vial. Aliquots were placed on glass slides, covered with cover slips and examined for the presence of cells using a light microscope (Zeiss AXIOVision Imager, Toronto, Ontario, Canada). It should be noted that the *T. harzianum* was macroscopically visible, making microscopic observation unnecessary. Samples with positive growth of *T. harzianum* quickly grew to fill the vial.

2.7 Laurdan fluorescence during exposure to inhibitory concentrations of NAs

The effect of NAs on cell membranes was investigated by labeling microbial isolates with the fluorescent probe Laurdan and exposing them to inhibitory concentrations of the organic fraction. Inhibitory concentrations of natural NA fractions used were determined from serial dilution assays as described above. Isolates were grown in Gen-1 broth, and labeled with Laurdan as described in Molina-Höppner *et al.* (2013). Staining of fungi with Laurdan was done as described previously using 10 mg of mycelia per sample. Suspensions (100 µL) of labeled cells were aliquoted into 96-well microtitre plates in triplicate. Generalized polarization (GP) values of solutions were calculated from emission measurements at 440 nm and 490 nm following excitation at 360 nm using a spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada); GP = $(I_{440}-I_{490})/(I_{440}+I_{490})$, where I is the intensity reading at given wavelength (Bagatolli et al., 2003). The inherent fluorescence of NAs did not interfere with measurements as the excitation wavelength used was below that reported for NAs (Lu et al., 2013).

2.8 Microtox™ assay

Microtox™ is routinely used to assess the toxicity of OSPW and NA compounds (Herman et al., 1994; Lo et al., 2006; Frank et al., 2008; Toor et al., 2013a). Toxicity of OSPW and the natural NA fraction (obtained from multiple OSPW sources, extracted as described above, and resuspended in 1M NaOH to a final concentration of 131 mg L^{-1}) was determined using the Microtox™ toxicity assay (Osprey Scientific, Edmonton, Alberta, Canada). The basic protocol for 5 min and 15 min acute exposure toxicity assay

were performed on a Microbics model M500 analyzer (AZUR Environmental Corporation, Fairfax, California, USA) according to the manufacturer's recommendations. The IC_{50} values reported represent the natural NA fraction or OSPW concentration resulting in a 50% decrease in bioluminescence of the target microorganism *Vibrio fischeri*. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

2.9 Plating of fungal isolate *T. harzianum* on BH agar and commercial NAs

The ability of fungal isolate *T. harzianum* to grow using commercially available Merichem NAs was assessed. This organism was selected because it is the first fungal isolate obtained from OSPW. *T. harzianum* was inoculated, in triplicate, from -80°C frozen stocks into Gen-1 broth (5 mL) and incubated at room temperature (~21°C) for 48 h. Aliquots of the culture (100 µL) were spread onto BH agar plates and three drops of pure Merichem NAs were placed on agar surface using a sterile glass pipette. Plates were incubated at room temperature (~21°C) for 60 d. Plates were photographed using a Binocular Model 570 Microscope (American Optical Corporation, Buffalo, NY, USA) 24 h and 60 d post-inoculation.

3.0 Results & Discussion

3.1 Characterization of OSPW

DOC and DIC concentrations in the original OSPW were 56.0 and 161.5 mg L^{-1}, respectively. Concentrations of phenols and PAHs were below detection limits of 0.002

mg L^{-1} and 0.2 µg L^{-1}, respectively. Dissolved trace metal analysis did not detect Pb or Cd, but Ni (10.8 µg L^{-1}), Mo (296.2 µg L^{-1}), Al (145.1 µg L^{-1}), As (38.2 µg L^{-1}), and Se (124.6 µg L^{-1}) were detected at levels exceeding guidelines for surface water quality (Canadian Council of Ministers of the Environment, 2006) The total concentration of natural NAs (using FT-ICR-MS) in the OSPW was 1.92 mg L^{-1}.

A sample of OSPW and corresponding natural NA fraction were subjected to a Microtox™ toxicity assay. The acute 5min and 15min toxicity of the concentrated (131 mg L^{-1}, as measured gravimetrically) NAs fraction were recorded as 64.7% (5min IC_{50}) and 48.5% (15min IC_{50}). These values correspond to an NAs content of 131 mg L^{-1} x 0.647 = 84.7 mg L^{-1} (5min IC_{50}) and 131 mg L^{-1} x 0.485 = 63.5 mg L^{-1} (15min IC_{50}) (Table 2). The IC_{50} 5min and 15min acute exposure toxicity of the OSPW, from which the natural NAs tested were extracted, was reported as 95.30%, and 54.1%. Assuming that all the measured toxicity in the OSPW sample is attributed to the NA fraction, these percentage values of OSPW responsible for the IC_{50} values correspond to NA concentrations of 1.92 mg L^{-1} x 0.953 = 1.83 mg L^{-1} (5min toxicity), and 1.92 mg L^{-1} x 0.541 = 1.04 mg L^{-1} (15min toxicity) (Table 2).

3.2 Isolation and identification of microorganisms from OSPW

A sample of OSPW was subjected to selective enrichment using natural NA fractions (derived from multiple OSPW sources) and commercial NAs to isolate aerobic microorganisms with the capacity for growth on or in the presence of NAs. Bacteria, yeast, and fungi grew on plates inoculated with the OSPW enrichment, and pure

cultures were observed by microscopic analyses of individual colonies. Isolates were genetically identified by sequencing PCR products obtained using the primer pairs 27F/1492R (for putative bacteria) and ITS1 / ITS4 (for putative fungi and yeast). All four putative bacterial isolates that yielded positive PCR reactions and identifiable sequence identities are listed in Table 1. One yeast isolate was identified (*Rhodotorula mucilaginosa*) as well as one fungal isolate (*T. harzianum*) (Table 1).

All microorganisms identified, apart from the yeast and fungus, are consistent with those typically found in contaminated environments and wastewater treatment facilities. For example, *Pseudomonas putida*, *Pseudomonas fluorescens*, and other indigenous microflora have been isolated from OSPW using commercial NAs as the sole carbon source, and have demonstrated the ability to remove these NAs from solution (Clemente et al., 2004; Scott et al., 2005; Del Rio et al., 2006; Johnson et al., 2013). The species isolated in our study are consistent with findings by Golby et al., which found that biofilms of mixed microbial communities formed in oil sands tailings (Golby et al., 2012). A rich variety of microorganisms have been identified in fluid fine tailings including *Pseudomonas* sp., *Acinetobacter* sp., and *Brevundimonas* sp. in the aerobic portion of an oil sands tailings sample (Golby et al., 2012). Demeter et al. (2014) isolated a *Rhodococcus* sp. and *Pseudomonas* sp. from OSPW, with the *Pseudomonas* sp. able to degrade a cyclohexane carboxylic acid model NA compound (Demeter et al., 2014).

3.2.1 Growth of *T. harzianum* on BH agar and Merichem NAs

Although bacterial isolates and mixed community biofilms have demonstrated the ability to degrade commercial NAs, little information exists on the relative capabilities of fungal isolates. After a 24h incubation on BH media with Merichem NAs, no growth of *T. harzianum* was observed, but after a 60d incubation period growth was observed in all drops of pure NAs (Figure 1). Growth after 60d shows not only can *T. harzianum* isolated in this study use commercially sourced NAs as a sole source of carbon, it can withstand the immense acute toxicity of a pure drop of NAs. This indicates a strong potential for use of *T. harzianum* as a mycoremediation treatment.

Although this is the first demonstration of an OSPW sourced fungi, several studies have indicated similar resilience and capacity of NA degradation with fungi in soils at much lower NA concentrations. For example, enrichments of rhizosphere microbial populations (both bacteria and fungi) obtained from soils on undisturbed, surface-exposed oil sands deposits have been shown to exhibit enhanced degradation of Merichem NAs (Biryukova et al., 2007). *Trichoderma sp.* is of particular interest due to its known resilience to toxic compounds and ability to degrade organics such as PAHs and diesel in contaminated soils (Cerniglia, 1997; van Elsas et al., 2000; Hadibarata et al., 2007; Fijalkowski et al., 2008). In a recent study, Repas *et al.* (2017) isolated *T. harzianum* from the roots of a dandelion successfully growing on coarse tailings, and the fungus greatly increased germination speed, percent germination, and biomass accumulation of three study plants grown in coarse tailings. Repas *et al.* (2017) also successfully tested growth of *T. harzianum* on autoclaved coarse tailings, bitumen, and other petrochemicals as a sole carbon source. The demonstrated ability to degrade

other complex organic compounds indicates the potential of *T. harzianum* to remediate NAs within OSPW.

The yeast isolated, *R. mucilaginosa*, is a species commonly found in the environment whether it be soil, lakes or oceans. Several studies have found *Rhodotorula* at sites with hostile environments, such aquatic, high metal, hypersaline and high temperature environments (Kutty and Philip, 2008; Irazusta and de Figueroa, 2014). Presence of *R. mucilaginosa* and its successful isolation using enrichments of NAs in OSPW are an exciting finding, indicating their potential for remediation efforts. Chandran and Das (2012) isolated *R. mucilaginosa* from soil contaminated with petroleum hydrocarbons. The isolate was able to degrade 93% of diesel in Bushnell Haas media containing 2% diesel in 10 days, demonstrating a capacity for this yeast to survive the toxic conditions of the hydrocarbons and also degrade and use diesel as carbon source (Chandran and Das, 2012). *R. mucilaginosa* has shown potential remediation capacity, encouraging the potential for OSPW sourced NAs remediation.

To our knowledge, the *R. mucilaginosa* isolate in our study represents the first incidence of a yeast successfully isolated from OSPW, and the *T. harzianum* isolate the first successful isolation of a fungi from OSPW. Here, *T. harzianum* grew on a pure drop of commercial NAs, not only as a sole source of carbon, but tolerating the acute toxicity of the pure NAs extract. These results indicate that *T. harzianum* could be used to remediate similar organic compounds within oil sands tailings and resist the toxicity of the target organics. However, further characterization is required to fully understand and characterize the potential application of *T. harzianum* for remediation technologies.

3.3 Tolerance of microbial isolates to pH and salinity

Microbial isolates were exposed to various levels of pH, salinity, and NA compounds in order to determine relative tolerance levels (Table 3). Isolates exhibited varying pH tolerance. Both *R. mucilaginosa* and *T. harzianum,* were able to grow within a wide range of pH conditions (3–10, and 2–9, respectively). Salinity tolerance varied, with the highest tolerance displayed by *Rhodococcus* sp. and *Pseudomonas* sp., which were inhibited only when NaCl content reached 80 g L^{-1}. The isolates identified in this study had the ability to grow in common conditions found of OSPW, pH 8–8.4 (Allen, 2008) and salinity (>2 g L^{-1}) (Leung et al., 2001). All microbial isolates were capable of growth at alkaline conditions (pH >8), with the exception of the isolates *B. diminuta* and *S. granuli*. All isolates exhibited the ability to grow in salt concentrations higher than marine environments (>3.5% w/v). Tolerance to acidic growth conditions among the isolates was variable. Overall, these results confirm the isolates identified can adapt to the conditions encountered in a tailings pond, and their growth may be limited only by the bioavailability and relative toxicity of carbon sources.

3.4 Tolerance of microbial isolates to NAs and toxicity

Tolerance to natural NA fractions varied among isolates, with *B. diminuta* exhibiting the highest tolerance (9,600 mg L^{-1}) (Table 3). To our knowledge, the microbes isolated in this study demonstrate the highest observed tolerance to NAs (600 - 9600 mg L^{-1}). Tolerance to varying NA concentrations does not appear to correspond to

pH or salinity tolerance. While not a direct comparison, the exceptionally high tolerance of the isolates to NAs is further highlighted by the findings of the Microtox™ assay. The acute toxicity of natural NAs measured by Microtox™ at 84.7 mg L^{-1}(5min) and 63.5 mg L^{-1}(15min), are much lower than the inhibitory concentrations to growth of native OSPW microbial isolates (≥ 600 mg L^{-1}). It is expected that OSPW isolates accustomed to growth in OSPW should exhibit a higher tolerance to the organic fraction than microorganisms not chronically exposed to these compounds, such as *Vibrio fischeri* used in the Microtox™ assay. This assay is meant to assess the overall toxicity of a given compound as measured by the inhibition of growth of an established bacterial culture (measured indirectly as the decreased luminescence of *V. fischeri* upon exposure to toxic compound). It is also a short-term acute toxicity assay, lasting a maximum of 15 minutes. The serial dilution assay conducted to determine tolerance to NAs (Table 3), on the other hand, assesses the ability of a small inoculum of tolerant species to establish growth over a 24–72 h period. However, taking both the NA tolerance test and Microtox™ assay together, these results provide strong evidence that (1) NA fractions are toxic at the levels observed in active tailings ponds, and (2) microorganisms native to tailings ponds are able to survive and grow in the presence of relatively high levels of these compounds.

Differences in acute exposure toxicity 5min and 15min IC_{50} values between raw OSPW and NA fractions is also noteworthy. The natural NA fraction concentration tested (131 mg L^{-1}) is more than ten times higher than that of the raw OSPW, and yet OSPW exhibits higher toxicity (Table 2). The observation that indigenous microorganisms

tolerate high levels of NAs in this study, and the discrepancies between the toxicity of NAs and OSPW following biodegradation in previous studies, has led to the postulation that OSPW toxicity involves interactions between additional components of OSPW, not simply the NA organics fraction (Energy Resources Conservation Board, 2009; Toor et al., 2013a; Toor et al., 2013b; Gauthier et al., 2014). Here, salinity and dissolved metals could be a potential contributing factor as the levels of Ni, Mo, Al, As, and Se in the OSPW tested in this study exceeded guidelines for surface water quality (Canadian Council of Ministers of the Environment, 2006). A possible combined toxic effect in OSPW have been observed in studies with the algal species *Chlamydomonas reinhardtii* , suggesting that toxicity is related to the surfactant activity of the NA fraction in addition to environmental factors in OSPW (Goff et al., 2013). The same study also observed the interaction of NA molecules with the cell membrane contributes to toxicity. Thus, further investigation of the role of the cell membrane overall toxicity of OSPW and NAs is required.

3.5 Generalized polarization (GP) values of isolates exposed to natural NA compounds

Microbial isolates were dyed with Laurdan and exposed to inhibitory concentrations of the naturally sourced NA fraction to assess effects on cell membranes. All microbial isolates dyed with Laurdan in the absence of added NAs exhibited cell membrane lipid order consistent with a fluid (undamaged) cellular membrane; exposure to inhibitory NA concentrations had variable effects (Table 4).

The lipid bilayer order of cellular membranes is indicative of membrane fluidity and is therefore related to the proper function of cell membranes. Compounds that share similar structural attributes to NAs have been shown to act on cell membranes, and often exert a change to lipid order. For example, reutericyclin is an antimicrobial proton-ionophore that possesses a tetramic acid head group and acts as an antimicrobial agent by dissipating proton gradients across cell membranes (Gänzle et al., 2000). In order to do so, reutericyclin incorporates into cell membranes and decreases lipid order (Hofstetter et al., 2013). Although not an antimicrobial compound, cholesterol is another compound that exhibits structural similarity to NAs and preferentially partitions into lipid membranes (Yu et al., 1996; Rowland et al., 2011c). Cholesterol is crucial to modulating cell membrane fluidity in living cells, and typically increases lipid order (Harris et al., 2002). The fluorescent probe Laurdan has historically been used to measure lipid bilayer order and its corresponding fluidity (Ulmer et al., 2002). Laurdan is also structurally similar to NAs, being a naphthalene-based amphiphile (Weber and Farris, 1979), and is therefore a useful surrogate to explore the ability of NAs to affect the lipid bilayer of cell membranes.

All microbial isolates dyed with Laurdan in the absence of NAs exhibited lipid order and GP values consistent with that of fluid cellular membranes (Sanchez et al., 2007) and/or values typical of those previously observed experimentally (Parasassi et al., 1998). Exposure to inhibitory levels of NAs did not have a statistically significant effect on lipid order for most isolates, with the exception of *S. granuli*, where a large degree of lipid order was introduced. This increase was consistent with a transition from

a fluid state to that of a gel-phase lipid bilayer (Parasassi et al., 1998). Although the NA fraction appears to interact with lipid membranes to some extent, there is no consistent relationship between lipid order of the isolates and corresponding inhibitory concentrations of NAs and appears to be species-specific. The NA fraction does not appear to compromise lipid order, and corresponding membrane fluidity, in a way that solely accounts for a generic mechanism of toxicity. Our understanding of the toxicity of the NAs fraction may be more appropriately reframed as a low-level toxic effect exacerbated by the effects of other compounds and ions present in OSPW. The toxicity of OSPW, including resident NAs, may be the result of an as yet uncharacterized interaction between these organic compounds and other stressors (e.g. heavy metals, salinity, pH, etc.) present in the environment (Macfie et al., 1994; Macfie and Welbourn, 2000; Headley and McMartin, 2004).

4.0 Conclusions

With continued development of the Alberta oilsands, a feasible and efficient method to remediate OSPW is greatly needed. NAs are the common target for remediation strategies as they have been shown to be a major contributor to OSPW toxicity. Bioremediation using native microbial cultures has been shown to be possible, however little is known about the mechanism of NA toxicity towards native oil sands tailings microorganisms at a cellular level (Frank et al., 2008; Rowland et al., 2011a). In this study, aerobic microorganisms were isolated from oil sands OSPW in order to examine their tolerance to pH, salinity, and the NA fraction of OSPW, to understand the

potential mechanism of cytotoxicity. Fluorescence measurements were used to assess the effects of inhibitory NA concentrations on the cell membranes of microbial isolates to determine if cytotoxicity was caused by lipid disruption. Toxicity of natural NAs and OSPW measured by Microtox™ were compared to determine if factors other than organics contributed to toxicity. Overall, the natural NA fraction investigated in this study was found to have a variable effect on membrane order and fluidity of resident microflora. The effects of this fraction on lipid bilayer order appear to be due to a combination of species-specific membrane profiles and the prevailing chemical composition of the fraction itself. The exact mechanism by which NA fractions cause cellular toxicity remains unclear.

Despite toxicity, microorganisms exist within OSPW, and can thrive in the presence of organic compounds. *T. harzianum,* the first fungus isolated from OSPW, and *R. mucilaginosa*, the first yeast isolates from OSPW, may be a promising microbial tool for the biodegradation of toxic NA compounds. Our results indicate that although the NA fraction appears to interact with lipid membranes to some extent, there is no consistent relationship between lipid order of the isolates and corresponding inhibitory concentrations of NAs and appears to be species-specific. The NA fraction does not appear to compromise lipid order, and corresponding membrane fluidity, in a way that solely accounts for a generic mechanism of toxicity. Other components within OSPW likely contribute to an overall toxic effect greater than that of the NA fraction alone. Future studies should characterize OSPW and examine toxic effects of mixtures of constituents to identify the cause of OSPW toxicity.

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Conflict of interest

None declared.

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Table 1: Identification of microorganisms isolated from OSPW.

Primers	Identification (NCBI BLAST)[a]	Homology (%)[a]	Identification (RDP)[b]	Confidence Interval (%)[b]	GenBank Accession Number[c]
27F / 1492R	*Rhodococcus* sp.	99	*Rhodococcus* sp.	95	KR011313
	Brevundimonas diminuta	99	*Brevundimonas* sp.	95	KR011314
	Pseudomonas sp.	99	*Pseudomonas* sp.	95	KR011315
	Shinella granuli	99	*Shinella* sp.	95	KR011316
ITS 1 / ITS 4	*Rhodotorula mucilaginosa*	99	n/a	n/a	KR011317
	Trichoderma harzianum	99	n/a	n/a	KR011318

[a] Based on NCBI BLAST database analyses (26/08/16). Species identity omitted where a match less than 99% was obtained, or where two different species were listed (Janda and Abbott 2007).

[b] Based on Ribosomal Database Project (26/08/16). Only the genus level or higher was confirmed using this database.

[c] Sequences submitted to GenBank database and assigned accession number (25/03/15).

Table 2: Microtox™ toxicity assay of OSPW and NA fraction.

	5min Toxicity		15min Toxicity	
	IC_{50}	Corresponding Concentration (mg L^{-1})	IC_{50}	Corresponding Concentration (mg L^{-1})
OSPW [a]	95.3	1.83	54.1	1.04
NA Solution [b]	64.7	84.7	48.5	63.5

[a] Concentration of NAs in OSPW = 1.92 mg L^{-1} (determined by FT-ICR MS)

[b] Concentration of NAs stock solution obtained from the same OSPW source = 131 mg L^{-1} (determined gravimetrically).

Table 3: Summary of tolerance of isolates to pH, salinity, and NA fraction. [a]

Isolate	pH Growth Range	Inhibitory salinity ($g\ L^{-1}$)	Inhibitory NA Concentration ($mg\ L^{-1}$)
Rhodococcus sp.	5-10	≥80	1200
B. diminuta	6-10	≥20	9600
Pseudomonas sp.	5-9	≥80	4800
S. granuli	5-10	≥20	4800
R. mucilaginosa	3-10	≥60	2400
T. harzianum	2-9	≥60	2400

[a] All isolates tested in Gen-1 media (n=3); no standard deviations calculated as serial dilution assay scores growth / no growth at given concentrations.

Table 4: Generalized polarization (GP) values of Laurdan fluorescence observed in cells exposed to inhibitory concentrations of NA fraction.

Isolate	GP Values		Change in Membrane Order
	Control	Inhibitory NA Concentration [a]	
Rhodococcus sp.	0.22 ± 0.095	0.30 ± 0.030	Insignificant
B. diminuta	-0.13 ± 0.0069	-0.11 ± 0.078	Insignificant
Pseudomonas sp.	0.038 ± 0.11	-0.00094 ± 0.079	Insignificant
S. granuli	-0.13 ± 0.0036	0.75 ± 0.0033	Increase [b]
R. mucilaginosa	0.017 ± 0.037	-0.14 ± 0.013	Decrease [b]
T. harzianum	0.38 ± 0.039	0.18 ± 0.045	Insignificant

[a] Inhibitory concentrations determined by serial dilution assay (Table 2)

[b] Statistically significant (P value < 0.05) as per paired t-test (n=3).

Highlights:

- Isolates tolerated alkaline pH, high salinity and NA concentrations far exceeding typical of OSPW
- *Trichoderma harzianum* can grow with pure NAs as sole source of carbon
- OSPW toxicity showed higher cytotoxicity than just NAs alone
- Possible synergistic effect of other components of OSPW with NAs attributed to toxicity
- Membrane disruption and fluidity not the cause of toxic effects of NAs

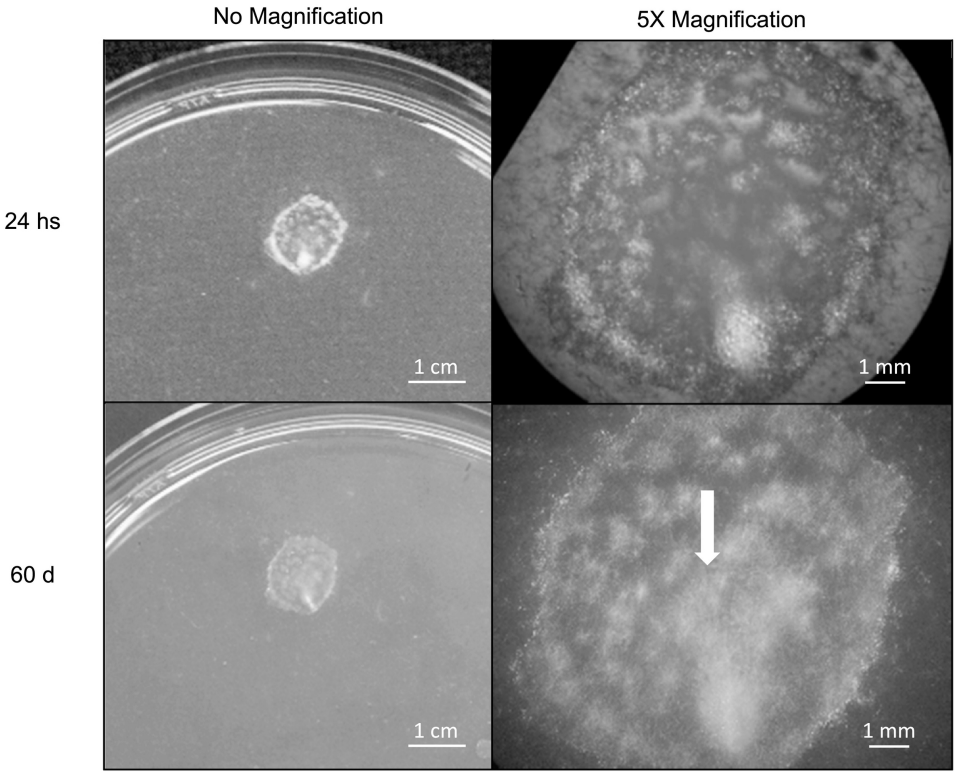

Figure 1