Oil sands process affected water sourced *Trichoderma harzianum* demonstrates capacity for mycoremediation of naphthenic acid fraction compounds

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Abstract

Development of Alberta's oil sands requires large volumes of water, leading to the abundance of oil sands process affected water (OSPW) that must be remediated prior to discharge or reuse. OSPW contains a variety of dissolved organic compounds, however naphthenic acids (NAs) have been found to contribute significantly to the toxicity of OSPW. A fungus, *Trichoderma harzianum*, isolated directly from OSPW, has previously demonstrated a high tolerance and capacity for growth in the presence of commercial NAs. This study conducted microcosm experiments to elucidate and characterize the capacity of *T. harzianum* to degrade labile commercial NAs (Merichem), and OSPW-sourced naphthenic acid fraction compounds (NAFCs). Additionally, two model NA compounds, the simple single ring cyclohexane carboxylic acid (CHCA) and complex diamondoid 1-adamanatane carboxylic acid (ADA), were utilized to determine the influence of NA structure on degradation. *T. harzianum* degraded 14% of CHCA, 13% of ADA, and 23–47% of Merichem NAs. Additionally, Orbitrap mass spectrometry revealed a large change in Z-series within NAFCs. This removal and shift in composition correlated to a 59% and 52% drop in toxicity as per Microtox, for Merichem NAs and NAFCs respectively. This proof of concept experiment confirms that the fungal

species *T. harzianum* can contribute to the biodegradation of complex dissolved organics found in OSPW, including cyclic and diamondoid structures.

Keywords: Mycoremediation; Oil sands; Naphthenic acids; Fungi; Remediation; Bioremediation

1 Introduction

The Albertan oil sands are one of the largest proven reserves of bitumen in the world. The production of this resource requires a substantial amount of water, which leads to an abundance of post extraction water that must be contained and remediated before discharge (Giesy et al., 2010). This post extraction water is termed oil sands process affected water (OSPW) and is stored in engineered tailings ponds. As of 2017, the total volume of accumulated tailings stored in the oil sands tailings ponds was estimated at 1 billion m^3 and occupied an area of approximately 176 km^2 (Foght et al., 2017; Vajihinejad et al., 2017). Storage allows time for clay and sand particles to settle out of the water fraction, and then allows access to OSPW for reuse in the extraction process, thus limiting the use of fresh water (Allen, 2008; Giesy et al., 2010). With the continual reuse of OSPW in bitumen extraction, the water becomes concentrated with compounds including salts, minerals, heavy metals, residual bitumen, and organic compounds such as naphthenic acids (NAs) (Allen, 2008). NAs are the principal group of compounds within OSPW that contribute extensively to its toxicity (MacKinnon and Boerger, 1986; Holowenko et al., 2002; Clemente et al., 2004; Kavanagh et al., 2013; Marentette et al., 2015).

NAs are defined as a complex mixture of cyclic and aromatic alkanes, with a carboxylic acid moiety (Dzidic et al., 1988; St John et al., 1998). NAs are naturally present in bitumen and become solubilized and concentrated in OSPW during the extraction process (Frank et al., 2008; Yang et al., 2019). Classical NAs are described as O_2^- species and denoted by the general formula $C_nH_{2n+z}O_2$, where n represents the carbon number and Z is the number of hydrogen atoms in the structure which are replaced (substituted) by the presence of a ring and/or double bond (Dzidic et al., 1988; Fan, 1991; St John et al., 1998). More recently, the definition of OSPW-sourced organics has been expanded to not only include classical NAs (O_2^- species), but also a complex mixture of oxidized organics that contain three or more oxygen-atoms, sulphur and nitrogen heteroatoms and is termed naphthenic acid fraction compounds (NAFCs) (Grewer et al., 2010; Rowland et al., 2011a, 2011b). NAFCs are more chemically diverse than classical NAs, such as those found in commercial mixtures like Merichem NAs, which consist solely of O_2^- species. As a result, NAFCs have different properties and toxicity profiles than the classical NAs (Scott et al., 2005a; Grewer et al., 2010; Quesnel et al., 2011a; Hughes et al., 2017). Regardless of the source, the majority of studies attribute toxicity directly to classical NAs (O_2^- species) (Morandi et al., 2017).

Hughes et al. (2017) compared the toxicity of different fractions of NAFCs to rainbow trout (*Oncorhynchus mykiss*), and found that the O_2^- species were primarily responsible for toxicity. Although the majority of classical NAs present were compounds with a carbon number ≤16, the toxicity mainly corresponded to the larger (i.e. higher molecular weight), more complex compounds with ≥17 carbons, indicating a higher priority for remediation (Hughes et al., 2017). Morandi et al. (2017) found that while other non-acidic species (O^+, O_2^+, SO^+, NO^+) contributed to the toxicity for *Escherichia coli*, it was much less compared to that of the O_2^- chemical class. Clearly, the unique components in NAFC plays a role in toxicity, and therefore remediation methods must aim to address the complete mixture of organic compounds within OSPW.

Treatment technologies for NAFCs have traditionally utilized chemicals, through the process of advanced oxidation such as ozonation (Wang et al., 2013), UV photocatalysis (Leshuk et al., 2016), and potassium ferrate

(VI) (Wang et al., 2016), or physical processes through the use of adsorbents such as activated carbon (Niasar et al., 2019). While these treatments are quick and reliable, they may be cost prohibitive for large scale treatment of OSPW. Alternatively, with the abundance of organic compounds found in tailings ponds, microbial communities are very active and diverse, indicating that *in situ* bioremediation is a plausible method for treating toxicity within OSPW (Herman et al., 1994; Scott et al., 2005a; Del Rio et al., 2006; Hadwin et al., 2006; Biryukova et al., 2007; Whitby, 2010; Kannel and Gan, 2012).

Studies of microbial communities that exhibit tolerance to and/or degradation of NAFCs within OSPW have traditionally focused on bacteria and algae (Herman et al., 1994; Del Rio et al., 2006; Quesnel et al., 2011b; Demeter et al., 2014; Mahdavi et al., 2015; Paulssen and Gieg, 2019). However, numerous studies in different fields have indicated that fungal species are often also present and possess the capacity for degradation of complex organics such as PAHs (Zafra et al., 2014; Lee et al., 2015; Andreolli et al., 2016; Marchand et al., 2017), but this has not yet been demonstrated for NAs. A comprehensive sampling study by Richardson et al. (2019) revealed substantial genetic diversity of the microbial community present in OSPW. Analysis of 18S rRNA revealed that, although limited in classification below the phylum level, two of the most abundant operational taxonomic units (OTU's) of the entire dataset were fungi (Richardson et al., 2019). This major presence of fungal activity within the water fraction of the tailings pond indicates that fungi possess the capacity to resist the harsh OSPW environment and as such there is an opportunity to understand their potential for mycoremediation. Repas et al. (2017) isolated *T. harzianum* from plant roots growing in coarse tailings, and found it had the capacity to remediate complex organic compounds, indicating the potential for novel fungal remediation of NAFC within OSPW.

Previous work from our research group found 6 microbial isolates within OSPW with the capacity for growth at high salinity, pH and NA concentration, including the fungal isolate *Trichoderma harzianum,* which was the first report of a fungi being isolated from OSPW (Miles et al., 2019). *T. harzianum* demonstrated the ability to withstand high salinity (≥60 g/L), pH range (2–9) and NA concentration (inhibitory concentration of 2400 mg/L of OSPW NAs (Miles et al., 2019). In addition, this isolate was also able to grow on a pure drop of Merichem NAs as a sole source of carbon on an agar plate (Miles et al., 2019). These results indicated that *T. harzianum* could be a prime candidate for mycoremediation of the complex and toxic organic fraction found in OSPW. The aim of this study was to: 1) determine if the OSPW sourced isolate *T. harzianum* possess the capacity to degrade complex OSPW sourced NAFCs; 2) use Merichem NAs to determine the specific impact of classical NAs (O_2^- species) on degradation; and, 3) use model NA compounds to determine how the complexity of chemical structure effected degradation.

2 Materials and methods

2.1 Fungal isolate

The microbial isolate used in this study was obtained through selective enrichment of OSPW for NA degrading microorganisms. Details of this enrichment, confirmation of isolate purity and subsequent tolerance testing of *T. harzianum* are described previously by Miles et al. (2019) (GenBank database, 2015/03/25, accession #KR011318). Biomass for microcosms was obtained by cultivating *T. harzianum* on Lysogeny broth (LB) nutrient agar plates. Frozen stock (0.100 mL) of *T. harzianum* was inoculated into 250 mL of LB liquid media containing 200 mg/L Merichem NAs and incubated for 48 h at room temperature (20 ± 2 °C). Approximately 100 μL of LB media was then transferred to each LB nutrient agar plate and incubated for 48 h at room

temperature. Dry cell mass was carefully scraped off plates to ensure no agar was selected and weighed before inoculating each individual microcosm.

2.2 NA sources

NAFCs were extracted as described previously (Grewer et al., 2010). Concentration of the final extract was analyzed using Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) (see Section 2.4). A 100 mg/mL stock was prepared by volume in 1 N NaOH and stored at 4 °C until use.

Merichem NAs (gifted from Merichem Chemicals and Refinery Services LLC) were chosen as a positive control as previous research has demonstrated this mixture is comparable to the O_2^- species found in OSPW, and is readily biodegradable (Clemente et al., 2004; Scott et al., 2005a; Toor et al., 2013b).

Model NAs cyclohexane carboxylic acid ($C_7H_{12}O_2$) (CHCA) (99%; Sigma-Aldrich; St. Louis, MO, USA) was chosen as a single ring compound analogous to simple structures naturally found in OSPW, while 1-adamantane carboxylic acid ($C_{11}H_{16}O_2$) (ADA) (99%; Sigma-Aldrich; St. Louis, MO, USA), was chosen as a diamondoid (three-ring) model compound to represent the more complex ring structures found in OSPW (Rowland et al., 2011a).

2.3 Microcosm set up

1L Fisherbrand™ reusable glass media bottles with cap (modified with a 20 mm blue butyl septa for periodic gas and liquid sampling) were filled aseptically with 500 mL of modified Bushnell Haas media (compound list can be found in the Supplemental Materials). All bottles were incubated at room temperature (20 ± 2 °C) with light restriction on the benchtop. Unless otherwise stated, all materials were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Bottles were fed 60 mg/L of NAFCs, 80 mg/L of commercial NAs (Merichem), or 40 mg/L of either ADA or CHCA. All microcosms were inoculated with 35 mg (dry cell weight) of *Trichoderma harzianum*. These biotic bottles are herein referred to as "Live" bottles (denoted with an L) and were conducted with four or five replicates. Abiotic controls were set-up (with four replicates) identically to each live test bottle condition with the addition of 0.04% sodium azide to kill *T. harzianum* and are referred to as "Killed" bottles (denoted with a K). Dissolved oxygen was monitored periodically to ensure aerobic conditions were maintained, and it was found that the 50/50 liquid/headspace set up provided sufficient oxygen throughout the experiment (data not shown). Liquid samples (5mL–25mL) were taken periodically, filtered with 0.22 μm nylon filters and stored at 4 °C until analysis.

2.4 Merichem NAs analysis

Merichem was extracted under methods previously described (Grewer et al., 2010) using 5 mL of sample, derivatized by 50 μL of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC-FID) (Agilent J&W 122–5512 column: 15 m × 250 μm x 0.25 μm). The temperature gradient was as follows: 50 °C for 2 min, then increasing at a rate of 30 °C/min to 280 °C which was maintained for 8 min. Helium was used as carrier gas with the following flow program: 0.15 mL/min for 2 min, then decreased to 0.063 mL/min which was maintained until the end of the separation. Total run time was 17.7 min. The detector was maintained at 300 °C. Column flow plus makeup gas (helium) was set to 32.1 mL/min, air flow 450 mL/min and H_2 flow 40 mL/min. A typical liquid injection volume was 1 μL. Merichem NAs were used as standards for quantification, and fluorene-9-carboxylic acid (97% FCA) was used as an internal standard to determine extraction efficiency.

2.5 O_2^- Orbitrap- MS analysis

Instrumental analysis. Reversed-phase High Performance Liquid Chromatography (HPLC) was paired with a hybrid linear ion trap-Orbitrap mass spectrometer (Orbitrap Elite, Thermo Fisher Scientific, San Jose) and operated using a modified version of the method of (Pereira et al., 2013). HPLC separation was performed on a Hypersil Gold C18 Selectivity Column (Thermo Scientific, Edmonton, AB, 50 × 2.1 mm, particle size 1.9 μm), using an Accela HPLC system (Thermo Scientific, San Jose, CA). The column was maintained at 40 °C and a flow rate of 0.5 mL/min was used with 20 μL injection volumes. Initial mobile phase composition was 95% A (0.1% acetic acid in water) and 5% B (100% methanol) for 30 s followed by linear gradient ramp to 90% B over 10 min, and a final ramp to 99% B over 5 min. Percent composition of B was then decreased to 5% over 2 min 30 s and held for 2 min for column re-equilibration. Although electrospray ionization (ESI) is commonly used, matrix effects were reduced using atmospheric pressure chemical ionization (Headley et al., 2011; Peru et al., 2019). All analyses were performed in negative (−) ionization mode. Nominal resolution was set to 240 000 at m/z 400 and mass spectral data was acquired in full scan mode between 100 and 500 m/z. Capillary temperature was 300 °C, needle voltage was set to 2.25 kV, and sheath, auxiliary and capillary gas flow was set to 20, 15, and 3 (arbitrary units), respectively.

Qualitative and quantitative analysis. Xcalibur ™ software was used for data acquisition. Mass spectral peak intensities were used to quantify relative concentrations by taking total spectra over the window during which most species eluted (7–13 min). Empirical formulas were assigned using the exact mass of all detected ions using the following restrictions: minimum (C-5, H-10), maximum (N-1, S-1, O-6) and the maximum error tolerance for formula prediction was 5 ppm. By this method, NAs are those species with two oxygens detected in negative ion mode (i.e. O_2^- species). Data analyses were based exclusively on mass spectra rather than chromatographic peaks, so chromatograms were only examined for the purposes of review separation of isomers for a given species.

2.6 Model NAs analysis

Concentrations of ADA and CHCA were analyzed by HPLC. The HPLC was an Agilent 1200 Infinity Series HPLC with an autosampler, thermostated column compartment and UV–visible diode array detector. The HPLC analytical column ThermoScientific Acclaim Organic LC (DX062902902) (Thermo Scientific, Waltham, Massachusetts, USA) was kept at 30 °C. Mobile phase was a programmed mix of 40:60 acetonitrile:2 mM phosphate solution, pH adjusted to 2.5 (KH_2PO_4, Fisher Scientific, Fair Lawn, NJ, USA) with a flow rate of 1 mL/min. Total run time was 7.002min for CHCA, and 18.002min for ADA with injections of 25 μL and 100 μL respectively. Mobile phase and samples were filtered at 0.22 μm nylon filters and sonicated for 30min to remove all particles and air. Stock solutions of respective model NAs (1000 mg/L in water) were used for standards and development of calibration curves. The detector was set to detect wavelengths between 200 nm and 260 nm, and 210 nm was most accurate for the model compounds. Software for operation was ChemStation for LC. Integration was done using baseline hold.

2.7 Kinetics analysis

The reaction rate constants for Merichem and model NA live bottles were determined by comparing the coefficient of determinations for zero order, first order and second order plots. Biodegradation of organics followed first order kinetics, as described by Equation (1).

$$ln\left(\frac{C_t}{C_o}\right) = -Kt$$

Equation (1)

Where: t refers to the time of expeirmental measurement (days); C_o and C_t are the concentration (mg/L) of organics at time zero and a given day t; and K is the first order reaction rate constant (day^{-1}). Values of K were obtained from the slope of plots of the natural log of the concentration of organics versus time.

2.8 COD

Chemical oxygen demand (COD) was measured with HACH COD High Range (HR; limits of 20–1500 mg/L) digestion solution vials (product 2125925, Fisher Scientific, Fair Lawn, NJ, USA) following method 8000, using a HACH digestor reactor and UV–Vis Hach DR/4000 spectrophotometer (Hach, Loveland, CO, USA).

2.9 Fungal growth analysis

Headspace $CO_{2(g)}$ was measured to track CO_2 production as a metabolic byproduct of biodegradation with an Agilent 7890A gas chromatograph equipped with a thermal conductivity detector (GC-TCD) (Agilent HP-PLOT/Q column: 30 m × 320 μm x 0.2 μm). The oven temperature gradient was as follows: 50 °C for 2 min, then increased at a rate of 30 °C/min to 150 °C which was maintained for 2 min. Helium was used as carrier gas with the following flow program: 8.83 mL/min for 2 min, decreasing to 5.67 mL/min until the end of the separation. Total run time was 7.33 min. The detector was maintained at 200 °C, and the injection port at 300 °C. The makeup gas (helium) was set to 5 mL/min. The injector split ratio was set to 5:1 (no gas saver), with a column flow of 8.89 mL/min, split vent flow of 44.4 mL/min, and a septum purge flow of 58.3 mL/min under a pressure of 30 psi. A typical injection volume was 100 μL. Gas standards were created using various concentrations of CO_2 and N_2 gas mixes.

2.10 Microtox™ assay

Microtox™ is routinely used to assess the toxicity of OSPW and NAFCs (Herman et al., 1994; Lo et al., 2006; Frank et al., 2008; Toor et al., 2013a). Toxicity of samples at the beginning and end of the experiment was determined using the Microtox™ toxicity assay (Osprey Scientific, Edmonton, Alberta, Canada). Day 0 samples were taken within 2 h of set up, filtered at .22 μm to sterilize and stored at 4 °C until analysis. The 81.9% Basic Test 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. Both 5 and 15 min acute toxicity was measured, however there was no significant difference in toxicity measured between tests and therefore only the 5 min data is described. The IC_{50} values were recorded representing percentage of the sample resulting in a 50% decrease in bioluminescence of the target microorganism *Vibrio fischeri*. Toxicity units (TU) were then derived from IC_{50} ($TU = 100 \div IC_{50}$) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

3 Results and discussion

3.1 NAFCs and merichem NAs

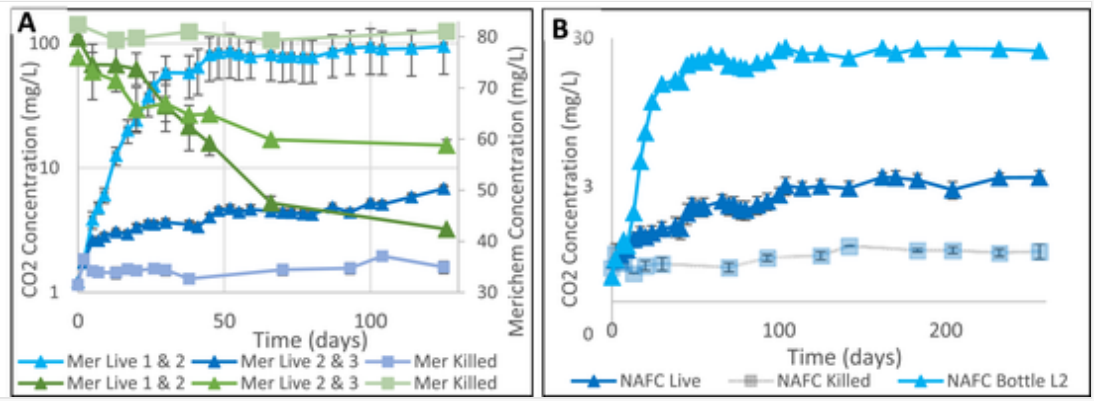
NAFCs are a mixture of organic acids with structures of varying carbon numbers and degrees of unsaturation in the form of double bonds, aliphatic and aromatic rings. Due to their complexity, characterization of NAFCs

requires methods with high resolving power (Rowland et al., 2011a; Headley et al., 2013; Peru et al., 2019). The use of commercial NA mixtures, such as Merichem NAs, allows for a more focused comparison of changes within the mixture, as they are comprised of solely of O_2^- species (Scott et al., 2005b). Focusing on O_2^- species, or classical NAs, allows focused efforts on remediation of this fraction which is known to be the major contributor to toxicity in OSPW (Marentette et al., 2015; Hughes et al., 2017; Morandi et al., 2017).

In addition to difficulties characterizing NAFC degradation, fungal growth is challenging to track and measure. However, consistent monitoring of CO_2 production in the headspace can be used to estimate mineralization of organics and serve as a proxy indicator of *T. harzianum* growth. Fig. 1 shows CO_2 production linked to substrate degradation of Merichem NAs (1A), and CO_2 production for NAFCs (1B). GC-FID was used to monitor Merichem NAs concentration; however, this method is limited that it lumps all carboxylic acids within in a sample together. Consequently, specific changes in the profile of organics cannot be assessed and thus quantification of complex NAFCs is inaccurate. Therefore, for NAFCs, only CO_2 production was regularly monitored, and Orbitrap-MS was used to determine organic profile shifts. NAFCs-Live bottles L1, L3, and L4 performed similarly and averaged 3 mg/L of CO_2 production in 250 days, indicating that a portion of the NAFCs were completely mineralized. NAFCs-Live bottle L2 produced 30 mg/L of CO_2 in 250 days, a considerable difference to the other replicate treatments, likely due to variability in the inoculated mass or other inherent biological ambiguity.

alt-text: Fig. 1

Fig. 1



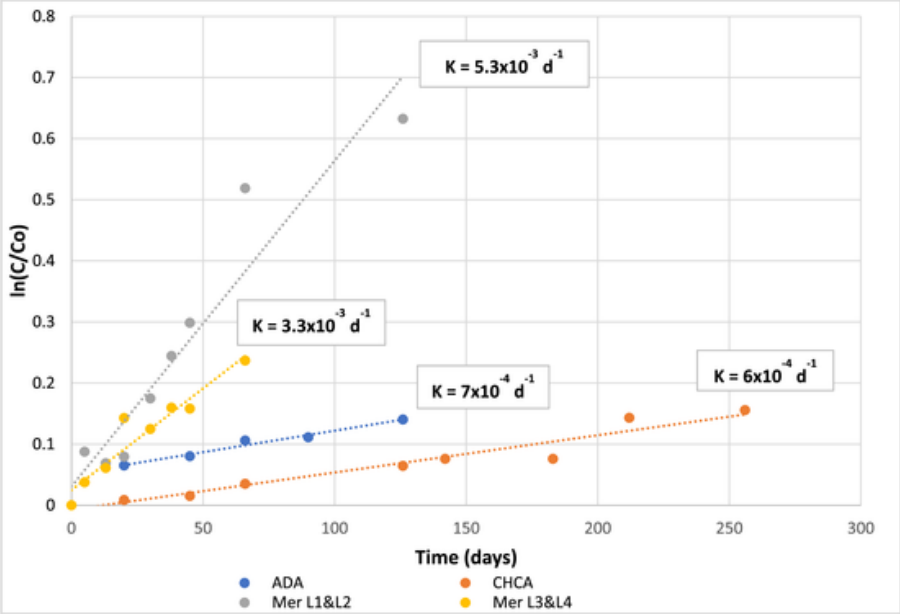
Degradation of Merichem NAs and NAFCs with subsequent CO_2 production by *T. harzianum*. (A) Merichem NAs degradation (green; as per GC-FID) with CO_2 headspace production (blue) *by T. harzianu*m. (B) NAFCs CO_2 headspace production by *T. harzianum.* Results are presented as average ± standard error of replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Microcosms with Merichem NAs showed variable growth of *T. harzianum* over time, with pairs of replicate treatments growing differently from each other (Fig. 1A). Therefore, Merichem-Live bottles that performed most similarly have been combined for discussion (Mer-L1&L2 and Mer-L3&L4). Largely, all four live treatment replicates produced CO_2 and demonstrated removal of Merichem NAs over time, indicating mineralization of a portion of Merichem NAs. Conversely, all replicates of the killed controls remained consistent throughout the time series, indicating the production of CO_2 and removal of Merichem NAs was due to microbial activity, not abiotic processes. Live bottles Mer-L1&L2 produced 93 ± 38 mg/L of CO_2 in 183 days and removed 47 ± 1% of Merichem NAs (as per GC-FID) in 126 days, while Mer-L3&L4 produced 6 ± 0.5 mg/L of CO_2 in 183 days and removed 23 ± 1% of Merichem NAs (as per GC-FID) in 126 days. Merichem NA degradation was found

to follow first order kinetics, with reaction rate constants (K) found from the slope of the graph shown in Fig. 2. Mer-L1&L2 was found to have a faster reaction rate constant of 5.3×10^{-3} d^{-1} ($r^2 = 0.92$) compared to that of Mer-L3&L4. Albeit slower, the first order reaction rate constant of $3.3.0 \times 10^{-3}$ d^{-1} ($r^2 = 0.91$) for Mer-L3&L4 indicates that microorganisms in these replicates were also actively degrading the Merichem NAs present.

alt-text: Fig. 2

Fig. 2



First order reaction kinetics for Merichem NAs (Mer-Live replicates) and Model NAs (CHCA and ADA). The natural log of NA concentration at each time point relative to initial concentration was plotted over time and the slope of the line of best fit was established to give reaction rate constants.

Although there was variability within live treatments, overall, *T. harzianum* grew and degraded Merichem NAs as a sole source of carbon, which a fungus has never demonstrated before. *T. harzianum* exhibited a period of faster growth and degradation within the first 105 and 50 days for both NAFCs and Merichem NAs respectively. Considering the structural diversity of NAFCs, it is likely that the fungi could only degrade a portion, after which their growth slowed and plateaued, and/or a intermediate product built up which inhibited further growth (Sanchez and Demain, 2008).

A common method for determining the quantity of oxidizable organics within a water sample is to measure the chemical oxygen demand. One limitation to using COD is that it only measures organics that are oxidizable by the reagent (dichromate ions). While dichromate ions are considered the best available oxidizing agent for measuring COD, there are still exceptions of organics that cannot be oxidized (Sawyer et al., 2003). Fig. 3A shows NAFCs with 3 replicates (NAFC-L1-L3) producing 36±4 mg/L over 256 days, a 17% increase in COD. However, one replicate (NAFC -L4) was removed from this analysis as COD increased by 435 mg/L, a 67% increase in oxidizable organics in 256 days. Killed controls remained constant throughout the experiment. It should be noted the initial measurements of COD in killed controls were higher than live treatments, as the *T. harzianum* cells lysed at death and released measurable organics. Measurements of COD for Merichem NAs shows the continuing trend of two pairs of replicates with significant differences between each other (Mer-L1&L2 vs Mer-L3&L4). Over the course of 256 days, there was an increase of 249±6 mg/L and 120 ± 10 mg/L of measurable COD, a 172% and 81% increase for Mer-L1&L2 and Mer-L3&L4 respectively.

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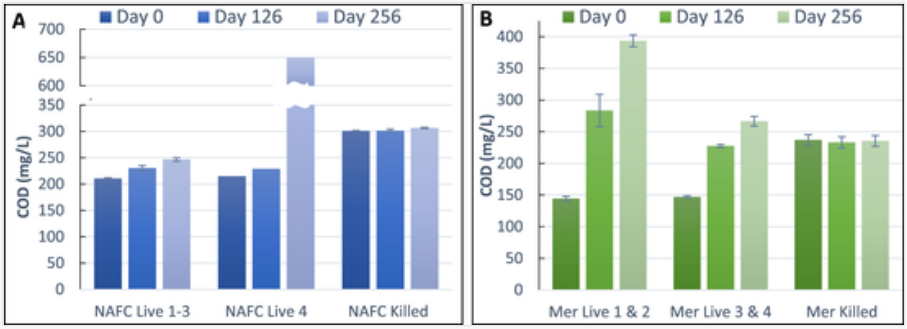
Fig. 3



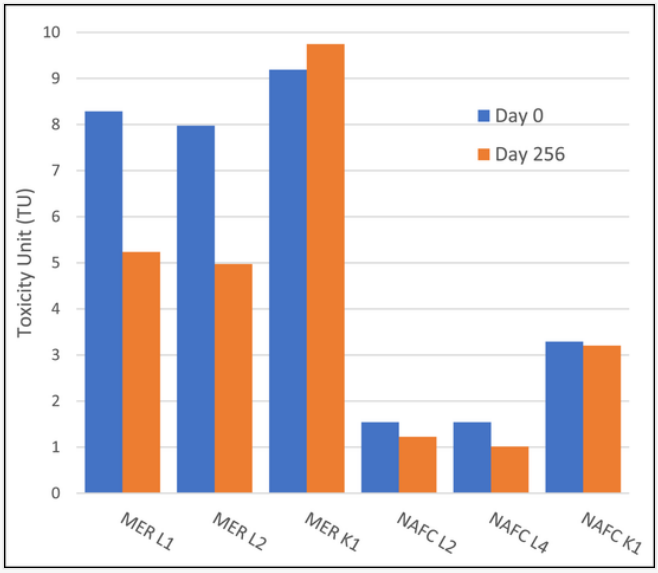
COD analysis of *T. harzianum* live treatments and killed controls for NAFC and Merichem NAs. (A) NAFCs COD change over time, bars represent averaged data ±standard error of replicates. A single bottle NAFC-Live 4 is shown outside the averaged replicates as a notable outlier on day 256. (B) Merichem NAs (Mer-replicates) COD change over time, bars represent averaged data ±standard error of replicates.

As this is the first study to demonstrate NAs degradation by a fungus, the mechanism or pathway *T. harzianum* used to degrade and consume the NAs is not yet fully understood, and the uncommon increase in COD should be explored in more depth in future studies. The initial decrease in Merichem NA concentration and subsequent plateau combined with the increase in COD indicates that a metabolite or intermediate is being produced and is accumulating within the microcosm. Therefore, it is possible that *T. harzianum* is utilizing a substrate that was not measurable by COD and producing intermediates that are measurable, and/or a metabolite such as an exopolysaccharide (EPS) is being produced (Mahapatra and Banerjee, 2013). Further characterization of organics measured by COD is needed to determine what exactly is being generated. Despite the large difference of COD production between the Mer-L1&L2 and Mer-L3&L4 replicates, the fraction of COD increase to Merichem NA removal is approximately the same, 3.7 and 3.5 respectively. This suggests that the two parameters are correlated and as *T. harzianum* degrades Merichem NAs, it is consistently producing the same fraction of metabolite, measured as COD.

Vaiopoulou et al. (2015) demonstrated that with chemical oxidation or biodegradation using a mixed microbial community there was no residual metabolite retention over time or correlated COD increase (Vaiopoulou et al., 2015). However, these treatment options involve chemical agents which oxidize metabolites produced during the treatment, or a mixed microbial community where syntrophic relationships exist between microorganisms that allow for any produced metabolites to be consumed. Mycoremediation of Merichem NAs showed a reduction of approximately 59% in toxicity, with very little variation between replicates (Fig. 4). However, the live *T. harzianum* treatments of NAFCs showed variability between replicates ranging from 26% to 52% reduction of total toxicity in NAFC-L2 and NAFC-L4 respectively. As discussed previously, NAFC-L4 showed a greater increase in COD production compared the other treatment replicates. This trend of greater toxicity reduction paired with the increased COD production also suggests that the more toxic and complex NAFCs were degraded into simpler, less toxic metabolites. Initial toxicity measurements indicate commericial Merichem NAs are almost 5 times more toxic than NAFCs. This is reasonable as Merichem NAs are composed almost exclusively of O_2^- species, which has been determined to be the main fraction of organics within OSPW that contribute to toxicity (Morandi et al., 2017).

alt-text: Fig. 4

Fig. 4



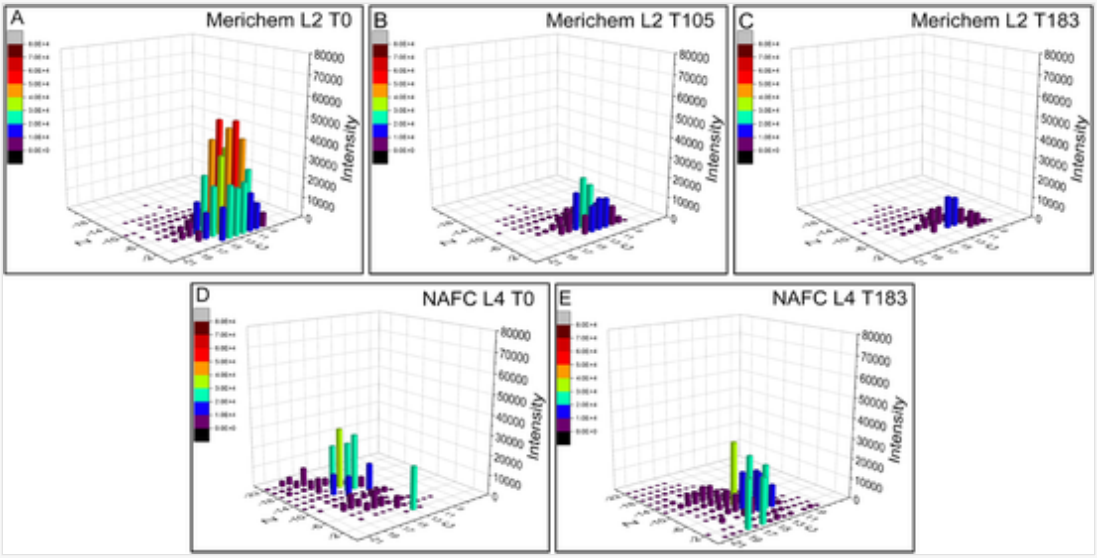
5-minute acute toxicity Microtox™ analysis of NAFCs and Merichem NAs (Mer) at Day 0 and Day 256. Data represents beginning and end of *T. harzianum* degradation treatment. Toxicity units were derived from IC_{50} (TU = 100÷IC_{50}) to visualize high level toxicity trends. Phenol toxicity was measured as a positive control prior to measurements (data not shown).

Characterization of NAFCs has been evolving for the last 20 years, and the advent of powerful mass spectrometers, like the Orbitrap, has enabled better separation of these organics. The Orbitrap-MS has high resolution (high resolving power) meaning it can differentiate between species beyond the 3rd decimal place (i.e. 240 000 at m/z 400). In this way, subtle changes in the profile of organic species can be detected and more accurately monitored over time (Ross et al., 2012; Headley et al., 2013; Pereira et al., 2013). Select samples of live treatments which demonstrated the largest change (Mer-L2 and NAFC-L4) and controls (data not shown) were sent for Orbitrap-MS analysis. As toxicity has been attributed to classical NAs (O_2^- species), this was the focus of Orbitrap analyses.

The output of Orbitrap analysis is summarized in three-dimensional profile plots displaying carbon number (C), hydrogen-deficiency (Z) and intensity (related to concentration). The number and distribution of Merichem NAs show major changes between days 0, 105 and 183 of the experiment (Fig. 5). Merichem NAs decreased by 88% in 183 days; however, 80% of removal was completed before 105 days of treatment with *T. harzianum* (Fig. 5A and B). GC-FID data indicated that most of the degradation occurred relatively quickly (by day 66) and then tapered off (Fig. 1). By Day 105 there was a drastic 100%, 68% and 51% decrease in the Z = 0, Z = −2 and Z = −4 series, respectively. These results suggest *T. harzianum* preferentially degraded organics with simple structures (i.e. those with fewer than 4 bonds and/or rings). By Day 183, 83% and 67% organics in the Z = 0, Z = −2 and Z = −4 series were removed (Fig. 5C). As toxicity has been attributed to classical NAs containing ≥17 carbons, remediation of these heavier organics is critical. Over 183 days there was a 77% reduction in classical NAs containing 16 or less carbons; however, more importantly there was an 85% reduction in organics containing 17 or more carbons (Fig. 5C). This reduction of larger compounds containing ≥17 carbons could be the primary contributor to the 59% reduction of toxicity as per Microtox (Morandi et al., 2016; Hughes et al., 2017). Overall, *T. harzianum* was able to degrade a wide range of Merichem NAs that have varying carbon number and complexity.

alt-text: Fig. 5

Fig. 5



Three dimensional plots showing changes in O_2^- distribution of Merichem NAs and NAFCs in *T. harzianum* treated samples. Plots denote ion intensity vs carbon number (C) and Z-series as measured per Orbitrap-MS across time. (A), (B) and (C) are commercial Merichem NAs through Day 0, 105 and 183 respectively. (D), and (E) are NAFCs through Day 0 and 183 respectively. Data represents time series of a single live treatment of *T. harzianum*, control data not shown.

Extrapolation of the Merichem NAs degradation results to the natural environment is limited as it is known that commercial NAs are more easily biodegradable than those in OSPW (Scott et al., 2005a). To establish a more comprehensive picture of mycoremediation, samples from microcosms containing NAFCs and *T. harzianum* were also analyzed by Orbitrap-MS. Fig. 5D shows that the initial profile of O_2^- species in NAFCs is different than Merichem NAs (Fig. 5A), with more NAs with $Z \leq -4$ and carbons above 17. Furthermore, the initial intensities appear to be considerably different between Merichem NAs and NAFCs on Day 0. This is possibly due to the fact Orbitrap-MS data shown is entirely O_2^- species, where as OSPW sourced NAFCs contain more organic compounds than just O_2^- species. Although microcosms were given the same concentration of organics (Merichem or NAFCs), a much smaller portion of organics present in NAFCs were strictly O_2^- species (Morandi et al., 2015). Overall, NAFCs showed a 30% net increase in O_2^- intensities, the opposite to what was observed for Merichem NAs. On Day 0, NAFCs contained no species in the $Z = 0$ series, however after 183 days there was a large intensity increase in this family of organics ($Z = 0$). The production of simpler, less complex organics was not observed in the Merichem microcosms. As only the O_2^- species were being analyzed by Orbitrap and NAFCs contain numerous other organic species, it is possible that the non- O_2^- species were partially degraded and produced detectable O_2^- species as intermediates. In addition, as discussed above, the COD increases in live treatments also suggest that a metabolite such as EPS or an intermediate potentially could be produced. EPS produced by *T. harzianum* could be detected by both Orbitrap-MS and COD measurements. However, no production of species in the $Z = 0$ series was noted in Merichem NA treatments, which also had a large increase in COD, indicating it is likely an intermediate product and not EPS metabolites. Regardless, since *T. harzianum* has demonstrated the ability to degrade $Z = 0$ species in the Merichem NA microcosms, given enough time removal of these species would likely occur. Several mixed community degradation studies have also demonstrated that species in the $Z = 0$ series are readily biodegraded by bacteria(Han et al., 2008; Toor et al., 2013b; Mahdavi et al., 2015). Therefore, the addition of a secondary isolate could use these and other intermediates as a primary carbon source.

Despite the net increase in O_2^- intensities in NAFC microcosms, there was a 13% decrease in species containing ≥17 carbons (apart from C = 18, which saw a 294% increase in 183 days). This decrease may have

contributed to the 52% decrease in toxicity of this sample (as per Microtox). However, the removal of the more complex O_2^- species would not be the only factor for toxicity reduction as it is likely chemical classes other than O_2^- were degraded (Fig. 4). A shift in species not quantified by Orbitrap-MS analysis here may also have contributed to toxicity reduction (Morandi et al., 2017). Review of all organics by Orbitrap-MS could identify species being degraded in other chemical classes which may have contributed in the decrease in toxicity.

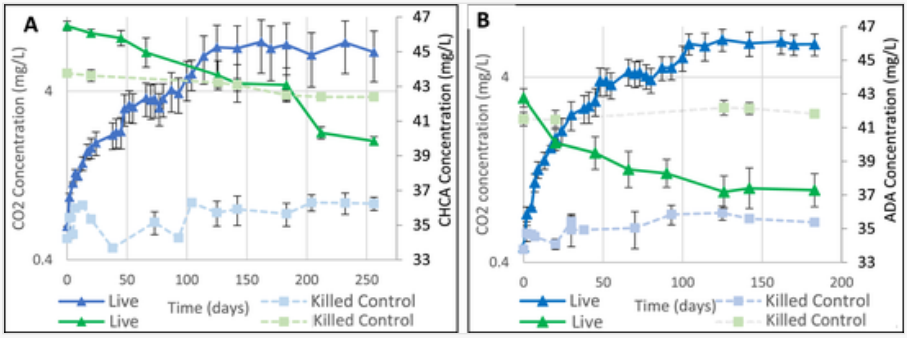
3.2 Model NA compounds

Two model NA compounds were selected to showcase the range of classical NA structures found in OSPW, using an analog of the simple single ring (CHCA) compared to a significantly more complex diamondoid ring structure (ADA) (Rowland et al., 2011a). CHCA is commonly used as an organic substrate that is a simpler carbon source and has demonstrated to be readily degradable by microorganisms native to OSPW (Herman et al., 1994; Del Rio et al., 2006; Demeter et al., 2014; Yu et al., 2019). Conversely, ADA has frequently demonstrated to be recalcitrant to biodegradation (Demeter et al., 2015; Frankel et al., 2016). With the surge of interest in utilizing bioremediation for reduction of OSPW toxicity, greater emphasis needs to be placed on finding microorganisms capable of degrading the more complex organics in OSPW, such as ADA.

For model NA experiments, CO_2 production was used as an approximation of both microbial growth and mineralization of model NA compounds and was linked to substrate degradation measured by HPLC (Fig. 6). Killed controls remained consistent throughout the time series, indicating the production of CO_2 and removal of substrate was due to microbial activity, not abiotic processes for both model NAs.

alt-text: Fig. 6

Fig. 6



Degradation of Model NAs and subsequent CO_2 production by *T. harzianum*. (A) cyclohexane carboxylic acid (CHCA) degradation (green) with CO_2 headspace production (blue) *by T. harzianu*m. (B) 1-adamantane (ADA) degradation (green) with CO_2 headspace production (blue) *by T. harzianu*m. Results are presented as average ± standard error of replicates (5 live treatments, 4 killed controls). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Over 256 days, 14 ± 0.5% of CHCA was removed and 6.8±2 mg/L of CO_2 produced in the headspace of the microcosm, indicating a portion of the CHCA was completely mineralized by *T. harzianum* (Fig. 6A). Similar to the Merichem NA and NAFCs results, degradation of CHCA exhibited more rapid initial degradation in 100 days, followed by a plateau at which point no further significant degradation occurred, potentially due to the accumulation of intermediates. The first order reaction rate constant for CHCA was found to be 6×10^{-4} d^{-1} (r^2 = 0.94) (Fig. 2). Reaction rates for CHCA degradation by specialized microbial communities consisting primarily of bacteria are often as high as 0.41 d^{-1} (Kannel and Gan, 2012), which is considerably higher than the observed rate for this experiment. Overall, these results indicate that *T. harzianum* cannot efficiently degrade

CHCA in contrast to most CHCA degradation studies that utilize bacteria and suggest CHCA is readily biodegradable.

While CHCA is a simple single ring, ADA is a triple ring diamondoid structure, more representative of the complex ring structures found in OSPW. Over 183 days, 13 ± 2% of ADA was removed and 6.1 ± 0.5 mg/L of CO_2 was produced in the headspace of the microcosm (Fig. 6B), indicating complete mineralization of a portion of the ADA by *T. harzianum* (Demeter et al., 2015). ADA followed the trend of a more rapid degradation phase followed by a plateau; however, the most substantial degradation occurred within the first 50 days, compared to the first 100 days for CHCA. Similarly, ADA exhibited a higher first order reaction rate constant of 7×10^{-4} d^{-1} ($r^2 = 0.97$) compared to 6×10^{-4} d^{-1} for CHCA (Fig. 2). This is notable as CHCA is has been shown to be more readily biodegradable than ADA.

Recently, Paulssen et al. (2019) evaluated the ability of a native photosynthetic microbial community found in OSPW to degrade ADA. They found microbial communities that were dominated by algae of the order *Chlorella/Chlorellales* and genus *Actuodesmus (Scenedesmus)* (~90% relative abundance) removed approximately 80% of ADA in 90 days, demonstrating that biodegradation of diamondoid NAFCs is possible by photosynthetic communities native to OSPW (Paulssen and Gieg, 2019). A study by Folwell et al. (2019) also found that communities sourced from OSPW could degrade ADA and 3-ethyl adamantane carboxylic acid. While Paulssen et al. (2019) and Folwell et al. (2019) evaluated the capacity for communities of microorganisms to degrade diamondoid structure carboxylic acids, our study demonstrates that a single microorganism can degrade ADA, although much less efficiently. The advantage of microbial community dynamics is in the synergistic relationships amongst microorganisms whereas an isolate must either be provided with or create all co-factors needed for efficient degradation of substrates (Demeter et al., 2015). *T. harzianum* exhibited greater Merichem NA removal (23–47% removal) at much faster rates ($K = 2.0 \times 10^{-3}$ - 5.3×10^{-3} d^{-1}) compared to both CHCA (14% removal; $K = 6 \times 10^{-4}$ d^{-1}) and ADA (13% removal; $K = 7 \times 10^{-4}$ d^{-1}). It is possible that mixtures of compounds are better removed by single microorganisms as they can preferentially degrade the compounds that are most suited to them.

While *T. harzianum* initially used the supplied organics as a carbon source, a metabolite or intermediate may have built up within the bottles that inhibited further degradation. Folwell et al. (2019) identified metabolites produced by ADA degradation, however within their microbial community this inhibitory metabolite could be consumed by another species and not accumulate. This synergistic metabolic process within a microbial community would allow *T. harzianum* to continue to degrade the provided organics. Regardless, the fact that a single microorganism, *T. harzianum,* consumed even a portion of ADA is novel and makes it a desirable option to add to other specialized microbial consortia for degrading various NAFCs. In particular, its slight preference for ADA over CHCA is notable and implies this fungus prefers different, and sometimes more complex, compounds than bacteria.

4 Conclusion

This study determined that *T. harzianum* isolated from OSPW could utilize CHCA, ADA, Merichem NAs and NAFCs as a sole source of carbon, as seen by the 14%, 13% and 23–47% reduction of CHCA, ADA and Merichem NAs respectively. Additionally, Orbitrap-MS revealed shifts in the chemical profiles of Merichem NAs and NAFCs that led to a 59% and 52% decrease in toxicity over the course of the experiment, respectively. In addition, results suggested a build up of nontoxic metabolites or intermediates during the degradation process.

A significant outcome of this study was that a single microorganism, the fungus *T. harzianum,* degraded a portion of ADA, which was previously thought to be recalcitrant, at a rate faster than for the more labile CHCA.

Overall, this indicates that *T. harzianum* may be an ideal addition to a microbial community, with synergistic relationships where metabolites are consumed, leading to more comprehensive NAFC removal. However, treatment technologies and applications of *T. harzianum* requires further research, such as elucidating the degradation pathways for model compounds CHCA and ADA, to improve understanding of how fungi can support the remediation of OSPW.

With the continuing development of the oil sands region, an effective remediation strategy will be important for stored OSPW. Biological treatment of this water fraction has become an attractive alternative to costly chemical options. This proof of concept experiment showed that a fungal species associated with oil sands tailings ponds can degrade complex organics found within OSPW including multi-ring diamondoid structures, which has never been demonstrated before.

CRediT authorship contribution statement

Sarah M. Miles: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Evelyn Asiedu:** Methodology, Formal analysis, Writing - review & editing. **Amy-lynne Balaberda:** Formal analysis, Writing - review & editing. **Ania C. Ulrich:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127281.

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Highlights

- A fungus isolated from OSPW, *Trichoderma harzianum*, previously grew with pure NAs as the sole source of carbon.
- ~~A m~~Microcosm study u~~stiliz~~ing *T. harzianum* characterized the capacity to degrade NAFCs and Merichem NAs, as well as two model NAs.
- Merichem NAs showed 23–47% removal and 59% drop in toxicity in 183 days.
- Cyclohexane carboxylic acid and 1-adamantane carboxylic acid showed 14% and 13% removal in 256 and 183 days respectively.
- NAFCs demonstrated a shift in O_2^- profile, with a large increase in Z = 0 species.

Appendix A Supplementary data

The following is the Supplementary data to this article:

Multimedia Component 1

Multimedia component 1

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Paulssen, J.M., Gieg, L.M., 2019. Biodegradation of 1-adamantanecarboxylic acid by algal-bacterial microbial communities derived from oil sands tailings ponds. Algal Research 41, 101528.

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