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Anaerobic biodegradation of surrogate naphthenic acids

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ABSTRACT

Surface bitumen extraction from the Alberta's oil sands region generates large settling basins known as tailings ponds. The oil sands process-affected water (OSPW) stored in these ponds contain solid and residual bitumen-associated compounds including naphthenic acids (NAs) that can potentially be biodedgraded by indigenous tailings microorganisms. While the biodegradation of some NAs is known to occur under aerobic conditions, little is understood about anaerobic NA biodegradation even though tailings ponds are mainly anoxic. Here, we investigated the potential for anaerobic NA biodegradation by indigenous tailings microorganisms. Enrichment cultures were established from anoxic tailings that were amended with 5 single-ringed surrogate NAs or acid-extractable organics (AEO) from OSPW and incubated under nitrate-, sulfate-, iron-reducing, and methanogenic conditions. Surrogate NA depletion was observed under all anaerobic conditions tested to varying extents, correlating to losses in the respective electron acceptor (sulfate or nitrate) or the production of predicted products (Fe(II) or methane). Tailings-containing cultures incubated under the different electron-accepting conditions resulted in the enrichment and putative identification of microbial community members that may function in metabolizing surrogate NAs under the various anoxic conditions. In addition, more complex NAs (in the form of AEO) was observed to drive sulfate and iron reduction relative to controls. Overall, this study has shown that simple surrogate NAs can be biodegraded under a variety of anoxic conditions, a key first step in understanding the potential anaerobic metabolism of NAs in oil sands tailings ponds and other industrial wastewaters.

1. Introduction

The Athabasca region of northeastern Alberta is home to the world's second largest heavy oil deposits in the form of bitumen (containing > 100 billion barrels of recoverable bitumen; Whitby, 2010; Brown and Ulrich, 2015). Surface bitumen extraction occurs via an alkaline hot water extraction process wherein natural surfactants associated with the bitumen, known as naphthenates, help to separate bitumen from the sands, silts, and clays (Schramm and Smith, 1985). These naphthenates, along with the remaining water, solids, residual bitumen, and associated hydrocarbon diluent are released into holding areas referred to as tailings ponds that are currently estimated to contain ~77 km^2 liquid wastes and whose total land areas occupy >170 km^2 in the Athabasca region (http://www.oilsands.alberta.ca/tailings.html). The tailings ponds operate under a zero-discharge policy and are managed to promote solids densification and water recycling for further bitumen extraction (Ramos-Padrón et al., 2011). The introduction of Directive 074 by the Alberta Energy Regulator in 2009 placed a focus on the importance of tailings pond management and reclamation (AER, 2009) and environmentally-responsible water management is a major focus of oil sands operators (https://www.cosia.ca/). Naphthenates, or naphthenic acids (NAs), have known toxicity to aquatic life such as minnows and phytoplankton (e.g., Lai et al., 1996; Leung et al., 2003) thus their removal is important to any tailings reclamation strategies.

Naphthenic acids have been classically defined as acyclic, alkyl-substituted, and cycloaliphatic carboxylic acids adhering to the formula $C_nH_{2n+Z}O_2$, where n is the carbon number and Z is hydrogen deficiency, indicative of the cyclization present in the structures (Clemente and Fedorak, 2005; Whitby, 2010). These compounds, naturally present in crude oils and other fossil energy resources (e.g., coal deposits, Clemente and Fedorak, 2005) and in a variety of industrial wastewaters (Misiti et al., 2013a), are now known to be even more complex. Naphthenic acids can also contain more than 2 oxygen atoms and/or other heteroatoms such as

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nitrogen and sulfur (Clemente and Fedorak, 2005; Grewer et al., 2010) and can be aromatic in nature (Whitby, 2010; West et al., 2014). Due to the structural complexity of NAs, the exact structures of many naturally occurring NAs are poorly defined and have posed analytical difficulties (Headley et al., 2015). However, recent high resolution analytical approaches have identified various di-, tri-, tetra-, and pentacyclic diamondoid NAs (Rowland et al., 2011a, 2011b; Wilde et al., 2015), along with sulfur-containing NAs (West et al., 2014) in OSPW.

Several studies have shown that NAs can be biodegraded at least to some extent by aerobic microorganisms (e.g., as reviewed in Whitby, 2010; Brown and Ulrich, 2015). Simple, single-ringed NAs (referred to as surrogate NAs in this study) can be readily biodegraded by aerobic microorganisms retrieved from tailings ponds (e.g., Herman et al., 1993, 1994; Demeter et al., 2014; Yue et al., 2015) and such compounds have been used to elucidate the pathways of NA biodegradation (Blakley and Papish, 1982; Rontani and Bonin, 1992; Taylor and Trudgill, 1978; Quesnel et al., 2011). In general, most simple surrogate NAs are metabolized by β-oxidation (Whitby, 2010). However, increasing levels of structural complexity such as increased alkyl side chain branching and cyclization (Han et al., 2008; Johnson et al., 2012; Misiti et al., 2014) along with stereochemical differences amongst isomers (Smith et al., 2008) can vastly reduce NA biodegradability. In examining the aerobic biodegradability of complex NA mixtures, Scott et al. (2005) found that commercially-available mixtures of NAs were readily biodegraded by aerobic tailings pond-derived microorganisms; however, the organisms were only partially able to consume the NAs naturally present in oil sands process-affected water (OSPW). Del Rio et al. (2006) showed that two *Pseudomonas* isolates incubated in conjunction could fully biodegrade OSPW NAs.

Oil sands tailings ponds are typically oxic at the surface, but are anoxic ~1 m below the surface and deeper (Ramos-Padrón et al., 2011). Anoxic tailings harbor genera known to have nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic activities (Foght et al., 1985; Penner and Foght, 2010; Ramos-Padrón et al., 2011) that can vary as a function of depth depending upon the availability of electron acceptors (Ramos-Padrón et al., 2011). Studies have shown that hydrocarbons in naphtha, a diluent added during bitumen extraction that is frequently present in tailings ponds, can drive methanogenesis in tailings ponds (Siddique et al., 2006, 2007, 2011). Given that NAs are typically present in tailings surface and interstitial waters at concentrations ranging from 40 to 70 mg/L (Allen, 2008), these acids can also potentially be used as carbon sources by anaerobic tailings pond microbial communities. Holowenko et al. (2001) showed that the addition of AEO-NA or surrogate NAs to microcosms containing anoxic tailings material did not result in increased methanogenesis relative to controls although the communities tolerated high levels of NAs. However, sewage sludge-inoculated incubations showed increased methanogenesis when surrogate NAs were added (Holowenko et al., 2001). Misiti et al. (2013b) did not observe NA biotansformation under nitrate-reducing and methanogenic conditions when studying the inhibitory effects of surrogate NAs on wastewater treatment plant microbial communities. However, Gunawan et al. (2014) showed that a model surrogate NA could be readily metabolized under nitrate-reducing conditions in bioreactors. Aside from these reports, little is known about the potential for anaerobic NA biodegradation.

In this study, we aimed to determine if oil sands tailings pond-associated microbial communities can biodegrade NAs (mainly surrogate NAs) under nitrate-reducing, iron-reducing, sulfate-reducing, and methanogenic conditions and to identify microorganisms potentially involved in this metabolism. In addition, a major goal of the work was to establish anaerobic NA-degrading enrichments for further study of anaerobic NA metabolic mechanisms and for the biodegradation of more complex NA compounds and mixtures.

2. Methods and materials

2.1. Samples

The samples used for all experiments were collected from the anoxic zone of an active oil sands tailings pond treated with gypsum ($CaSO_4 \bullet 2H_2O$) for solids densification (Ramos-Padrón et al., 2011). The samples were stored at room temperature in an anaerobic glove bag (90% N_2/10% CO_2) until NA-amended enrichments were prepared. Sulfate-reducing and methanogenic activity, sulfate and sulfide concentrations, and microbial community composition of the samples were previously described (Ramos-Padrón et al., 2011). Samples collected from 3 to 4.5 m depths were mixed and used for incubations under sulfate-reducing conditions, while samples collected from the 13.7 m depth were used for incubations established under nitrate-reducing, iron-reducing, and methanogenic conditions. Samples used to construct incubations were chosen based on available volume of material. Microbial community analysis showed that diverse anaerobes representing all electron-accepting conditions being tested were present in all samples used in this study (Ramos-Padrón et al., 2011).

2.2. Naphthenic acid substrates

Several NA stock solutions were prepared to test for anaerobic NA biodegradation. The five surrogate NAs used for this study were cyclohexanecarboxylic acid (CHCA), cyclohexaneacetic acid (CHAA), cyclohexanepropionic acid (CHPA), cyclohexanebutyric acid (CHBA), and cyclohexanepentanoic acid (CHPeA). Surrogate NA stock solutions were prepared either as individual compounds (final concentration of 6 g/L for each NA), or as a mixture of the 5 compounds (1.2 g/L of each CHCA, CHAA, CHPA, CHBA, CHPeA). The surrogate NAs were dissolved in sterile 0.1 N NaOH that was flushed aseptically with N_2 to create anoxic solutions.

To test NAs naturally present in tailings ponds, a stock solution of acid extractable organics or 'AEO' (previously referred to as 'oils sands tailings water acid-extractable organics' or OSTWAEO in Grewer et al., 2010) was prepared by extracting 1 L of acidified (pH < 2) OSPW with 3 volumes of dichloromethane (DCM) in a manner similar to that previously described (Clemente et al., 2004; Grewer et al., 2010). Combined extracts were concentrated by rotary evaporation and transferred to a pre-weighed glass vial. The DCM was allowed to evaporate completely and the vial was weighed to determine the weight of the extracted residue. The residue was then reconstituted in 10 mL sterile 0.1 N NaOH and made anoxic by flushing with N_2. The final AEO stock solution concentration prepared was 6.5 g/L.

2.3. Establishment of anaerobic NA-amended enrichments

Incubations under different anaerobic electron-accepting conditions were established at different times. Sulfate-reducing enrichments were established first, since sulfate was initially identified as a key electron acceptor in the sampled tailings due to the gypsum addition for solids densification (Ramos-Padrón et al., 2011). In an anaerobic glove bag, 10 mL tailings were aseptically added to 20 mL of sterile, anoxic minimal salts medium (McInerney et al., 1979) in glass serum bottles that were closed with butyl rubber stoppers and crimp-sealed. The medium was reduced with 20 mL/L of a 2.5% cysteine-sulfide solution (Bryant and Robinson, 1961) prior to the addition of tailings. Sulfate (480 mg/L) and