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Indigenous microorganisms residing in oil sands tailings biodegrade residual bitumen

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1. Introduction

Due to the rapid development of the oil sands industry in northeastern Alberta, Canada, large quantities of tailings waste and oil sands process-affected water (OSPW) have been produced

over the past five decades (Chalaturnyk et al., 2002; Percy et al., 2012). Tailings waste is often temporarily stored in tailings impoundments called tailings ponds, however the ever-increasing geographical footprints and the urgent demand to reclaim the disturbed landscape has prompted the construction of oil sands end pit lakes (EPLs) (Hrynyshyn, 2012; Aubertin and McKenna, 2016). In EPLs, sand and clay tailings are placed in the bottom of mined-out pits and a water cap (made up of OSPW and fresh water from natural lakes or other healthy water bodies) is placed on top of the tailings (Hrynyshyn, 2012). The water cap is expected to develop into a thriving aquatic ecosystem capable of biodegrading chemicals of potential concern (Hrynyshyn, 2012). Base Mine Lake (BML) is the first commercial-scale demonstration EPL, which was commissioned by Syncrude Canada, Ltd. to support the development of this water capping technique for oil sands reclamation.

Efforts have been made to bioremediate organic compounds in OSPW and oil sands tailings, such as BTEX (benzene, toluene, ethylbenzene and xylene) and naphthenic acids (NAs). The biodegradation of BTEX, n-alkanes (C14-C18) and naphtha (C3-C14) has been confirmed under methanogenic conditions by oil sands tailings microorganisms (Siddique et al., 2011, 2007; Mohamad Shahimin and Siddique, 2017). NAs were known to be the major toxicity contributor in OSPW (Morandi et al., 2015), and its biodegradation tends to be more difficult where the utilization of chemical pre-treatment, such as advanced oxidation, has shown some success (Brown et al., 2013; Brown and Ulrich, 2015; Zhang, 2016; Zhang et al., 2018). Gamma irradiation treatment was also reported to stimulate the hydrocarbon degraders from the tailings microbial community (VanMensel et al., 2017).

A less-studied aspect of EPLs is the bitumen in the tailings, left behind by the successive incomplete extraction processes. Bitumen remaining in the tailings after placement into the pit is carried upwards by biogenic gases (one of the proposed processes), and the viscous

liquid spreads over the water cap (Darling, 2011). This bitumen acts as a hydrocarbon source: as it migrates through the water cap, the hydrocarbons are released and subsequently biodegraded, a process which consumes dissolved oxygen and prevents the establishment of a healthy lake ecosystem. If the bitumen cannot be further degraded or mineralized in situ, the hydrocarbons can contaminate the aqueous environment and the nearby littoral zone.

Hydrocarbon-degrading microbial isolates from sediments of the Athabasca River have been shown to grow on the lighter components of Athabasca bitumen (not on the recalcitrant asphaltene fraction) (Wyndham and Costerton, 1981). Microbial degradation of bitumen (up to 40% removal at 37 °C) was also reported in similarly polluted environments in other regions of the world (Wyndham and Costerton, 1981; Potter and Duval, 2001; Das and Chandran, 2011). Bitumen biodegradation can be enhanced by nutrient addition (nitrogen or phosphorus) and stimulated by addition of more easily biodegradable carbons (Das and Chandran, 2011). The most effective hydrocarbon degradation is usually accomplished under aerobic conditions, while nutrients and temperature are often the most important limiting factors of the process (Das and Chandran, 2011). Because bitumen is complex, and its biodegradation has been demonstrated to occur under various conditions, site-specific factors are important to the feasibility of in situ remediation.

Our previous research showed (Yu et al., 2018), the addition of a proprietary blend of microbes, enzymes and organics to tailings resulted in significant reduction in the petroleum hydrocarbon fractions and tailings pore water toxicity. It was unclear whether these changes were caused by the indigenous microbial community or by the added microbes and organic carrier in the Cypher product. Therefore, the focus of this study was to investigate the ability of the microbial communities in BML to degrade bitumen, and the effectiveness of

biostimulation with acetate. The changes in community composition and community responses were assessed by comparing 16S rRNA gene sequence profiles.

2. Materials and methods

2.1 Materials

All samples were transported to the laboratory in sealed buckets and stored at 4 °C prior to use (bitumen samples were stored for 6 months; water and tailings samples were stored for less than one month). Fluid fine tailings (FFT) were provided by Syncrude Canada Ltd. FFT was sampled at the depth of 12 m below the sediment:water interface at Platform 1 at BML. Extraction technique limitations cause unrecovered bitumen to end up in tailings; this residual bitumen can be observed upon commission of the BML (in this paper, 'bitumen' refers to the residual bitumen in the BML). BML bitumen used in this research was sampled directly from the BML surface. To eliminate moisture content, the bitumen was oven-dried at 105 °C overnight. However, this drying process sacrificed any volatile and semi-volatile hydrocarbon that may have resided within the bitumen. Clays, sands and other small particles were retained, but vegetation and large stones were manually removed. BML cap water used in this research was sampled from the surface of BML at Platform 1.

2.2 Chemical analysis

CO_2 was measured by a gas chromatography thermal conductivity detector. Dissolved organic carbon (DOC) was measured with a Shimadzu Model TOC-L_{CPH}. Acetate was measured by Ion Chromatography. NAs were measured by gas chromatography flame ionization detector (GC-FID) or by reversed-phase chromatography paired with a linear ion trap-Orbitrap mass spectrometer. Detailed procedures and machine conditions for all methods above can be found in the supplementary data: CO_2 measurement (Protocol S1), DOC

91 measurement (Protocol S2), acetate measurement (Protocol S3), and NAs measurements
92 (Protocol S4).

93 Petroleum hydrocarbons (PHC) are grouped into these fractions by using a Canada-Wide
94 Standard: F1 (C6 - C10), F2 (C10 - C16), F3 (C16 - C34), F4 (C34 - C50), and F4G-SG (>
95 C50) (CWS, 2003). F4 and F4G-SG fractions are classified into bitumen content in the Dean
96 Stark extraction (industry accepted method) (Dean, E. W., 1920). F1 fractions were measured
97 prior to submission to Maxxam Analytics and were non-detectable in all samples. All
98 samples were mixed with organic solvent (toluene) and sonicated for greater homogeneity
99 prior to submission to Maxxam. Maxxam then further homogenized the samples. One
100 duplicate was submitted for analysis due to the sample size limitations.

101 2.3 Microbial analysis

102 2.3.1 Toxicity bioassay

103 The toxicity of aqueous samples was analyzed using the Microtox® bioassay. The 81.9%
104 Basic Test protocol was followed (Microtox® 500 Analyzer, Azur Environmental) with an
105 incubation time of 5 min (Anderson et al., 2011). Light emission was measured with
106 MicrotoxOmni software to determine inhibitory concentration 20% (IC_{20}) or inhibitory
107 concentration 50% (IC_{50}) value. Toxicity units, derived from IC_{50} ($TU = 100 \div IC_{50}$), was
108 used to visualize high-level toxicity trends.

109 2.3.2 DNA extraction

110 DNA was isolated from the tailings phase using the FastDNA™ SPIN Kit for Soil (MP
111 Biomedicals). Up to 500 mg of tailings were used per extraction, following the DNA
112 isolation protocol suggested by the manufacturer.

113 2.3.3 Bacterial population by qPCR assay

114 The Bacterial population was determined by the qPCR amplification of the RNA polymerase
115 beta subunit (*rpoB*) gene, utilizing *rpoB* 1698f (5'-AACATCGGTTTGCTCAAC-3') and
116 *rpoB* 2041r (5'-CGTTGCATGTTGGTACCCAT-3') primers (Nava et al., 2011; Brown et al.,
117 2013). The qPCR assay was performed using a Bio-Rad CFX96 optical reaction module
118 conversion of the C1000 Touch thermal cycler. All samples and standards were completed in
119 triplicate, and the amplification data was analyzed using Bio-Rad CFX Manager™ 3.0
120 software. The reaction followed the protocol suggested by the manufacturer (detailed
121 description can be found in Protocol S5). The qPCR assay was performed on DNA samples
122 extracted from the tailings/solid phase at the start and end of the experiment. Each biological
123 duplicate was measured three times (n = 6) for the end of the experiment, and the original
124 tailings samples were measured six times (n = 6) to determine the initial bacterial population
125 density for all groups.

126 2.3.4 Microbial communities

127 DNA samples were used for PCR amplification of the V4 hypervariable regions of bacterial
128 16S rRNA genes, using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R
129 (5'-GGACTACVSGGGTATCTAAT-3') (Caporaso et al., 2011). The PCR conditions were
130 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min of extension;
131 72 °C for 6 min. The PCR products were purified and combined in equimolar ratios with the
132 quantitative DNA binding method to create a DNA pool that was further used for sequencing
133 from the adapter. The 16S rRNA gene fragments were sequenced using the Illumina MiSeq
134 platform. The sequences were deposited in Sequence Read Archive (SRA) within the study
135 with accession number SRP131750. Operational taxonomic units (OTUs) were constructed
136 using an identity threshold of 97% and assigned to taxa using the UPARSE pipeline (Edgar,

2013). The community matrix was normalized with the DESeq package (Love et al., 2014). The bacterial communities were ordinated with non-metric multidimensional scaling based on the Bray-Curtis distance matrix using phyloseq (McMurdie and Holmes, 2013).

2.4 Biodegradation experiments

Aerobic conditions were used for the biodegradation experiments since the BML water cap is aerobic during the summer and fall season. A second benefit is because oxidative biodegradation is also considered more effective and occurs at higher rates than anaerobic biodegradation (Ait-Langomazino et al., 1991; Wolf and Bachofen, 1991). Experiments were set up in 500 mL Fisherbrand™ reusable glass media bottles with customized septum caps for sample withdrawal. All bottles were placed on a horizontal shaker at 150 rpm at room temperature (20 °C). Aerobic conditions were maintained by a sequential renewal of air (0.22 μm filtered-sterile, every 10 d for the active groups and every month for control groups). Bottles, caps, and glass beads were sterilized by autoclave (121 °C, 100 kPa). Filtered BML water (500 mL), and glass beads (40 g) were used for all groups. When indicated, the following was also added: sodium acetate (250 mg measured as carbon), 10 g bitumen, and 40 g FFT. FFT was used as the indigenous inoculation used in this study. The four test groups were BML-B, BML-BT, BML-BTC and BML-T (BML: 0.22 μm-filtered BML water, B: bitumen addition, T: tailings addition and C: sodium acetate addition). Two replicates were set up for each group.

BML-B shows the effect of bitumen on water quality and how the bitumen composition changes over time with exposure to the BML water. BML-T contains tailings and BML water, to reflect the conditions of an oil sands end pit lake near the mud line level (presumably with little bitumen). BML-BT represents an area of a bitumen-impacted oil sands end pit lake

where bitumen accumulates over time. BML-BTC represents the situation described in BML-BT with acetate biostimulation.

3. Results and discussion

3.1 CO_2 and DOC

Microbial degradation of an undefined and complex substrate (i.e. BML bitumen) can be quantified by monitoring CO_2 production (Wolf and Bachofen, 1991). Biodegradation experiments were set up in sealed bottles with customized caps allowing for sample withdrawal. The pressure in the headspace was also measured to convert the CO_2 concentration within the sealed bottle to the concentration under the ambient atmosphere pressure. Air renewal introduced low amounts of CO_2 into the system but did not significantly contribute to the CO_2 concentration within the bottle. Headspace CO_2 concentration and DOC are shown in Fig. 1.

The data indicate that CO_2 production rates are 5.3–58 times greater in the other three groups relative to the control (Fig. 1). BML-B and BML-T groups both had linear CO_2 production with rates of 0.17 mg L^{-1} d^{-1} (R^2 = 0.97) and 0.91 mg L^{-1} d^{-1} (R^2 = 0.97), respectively. BML-BTC and BML-BT had linear CO_2 production during the first 10 d with rates of 9.97 mg L^{-1} d^{-1} (R^2 = 0.99) and 6.19 mg L^{-1} d^{-1} (R^2 = 0.99), respectively. BML-BTC and BML-BT rates decreased rapidly and concentrations plateaued at 40 d with final concentrations of approximately 130 mg L^{-1} and 90 mg L^{-1}, respectively.

Studies have shown that co-oxidation can occur during petroleum hydrocarbon degradation (Herbes and Schwall, 1978; Atlas, 1981; Varjani, 2017). Large amounts of partially oxidized metabolites from petroleum hydrocarbon biodegradation accumulate instead of full mineralization of the parent compounds to CO_2 (Herbes and Schwall, 1978; Atlas, 1981;

183 Varjani, 2017). As a result, CO_2 production would grossly underestimate the degradation
184 rates of the parent compounds (Herbes and Schwall, 1978).

185 DOC decrease was only observed in the BML-BTC group (from approximately 500 mg L^{-1} to
186 approximately 380 mg L^{-1}). However, this decrease was not due to the presence of acetate
187 (DOC in the form of acetate: from about 290 mg L^{-1} to about 320 mg L^{-1}). Acetate might be
188 utilized by indigenous microbes and also produced as a by-product during incubation.
189 Therefore, the removal of DOC was more likely associated with the indigenous carbon
190 source originating from BML water or tailings pore water via co-oxidation processes
191 stimulated by the acetate addition.

192 There were three possible sources of organic carbon in this experiment: DOC in BML water,
193 DOC or particulate carbon in the tailings, and dissolved or particulate carbon in the added
194 bitumen. DOC measurements only represent the soluble organic carbon originating from
195 BML and tailings pore water. Previous research indicates that 80% of DOC in OSPW are
196 NAs (Nelson et al., 1993; Allen, 2008). NAs contribute to the acute toxicity of OSPW, and
197 remain recalcitrant to microbial degradation (Morandi et al., 2015). Therefore, removal of
198 DOC including NAs is one target for remediation in BML. Slightly soluble or insoluble
199 organics likely originate from the FFT and the added bitumen: organics leaching from
200 bitumen usually have more complex chemical structures and lower solubility (Hayes et al.,
201 1972; Ait-Langomazino et al., 1991; Das and Chandran, 2011). Therefore, microbial
202 degradation of these hydrocarbons often requires an increase in their bioavailability, for
203 example via the secretion of polysaccharides by microbes that enhance adhesion and
204 emulsify hydrocarbons (Wyndham and Costerton, 1981; Neu Thomas R., 1996). Although
205 microbial colonization of bitumen surfaces has been demonstrated, microbial degradation of

bitumen has not yet been shown experimentally (Wyndham and Costerton, 1981). However, microbial activity was observed, and was further investigated in section 3.2.

3.2 Non-aqueous phase organics

Insoluble or slightly soluble hydrocarbons might also be microbially degraded or altered during incubation. Therefore, tailings samples were analysed for petroleum hydrocarbon composition. F2, F3, F4, and F4G-SG data are shown in Fig. 2.

The BML-T group had the lowest concentrations of all hydrocarbon fractions. Bitumen was added to the other three groups. Heavier hydrocarbons were present in the bitumen than in the tailings. Bitumen addition greatly influenced the petroleum hydrocarbon distribution: BML-B, BML-BT and BML-BTC had a similar distribution of these four classifications: F2: 1%, F3: 21–22%, F4: 10% and F4G-SG: 67–68%, while BML-T had a unique distribution of: F2: 5%, F3: 28%, F4: 12% and F4G-SG: 55%.

Concentrations of all hydrocarbon fractions on day 100 followed a similar trend: BML-B > BML-BT, BML-BTC > BML-T. The decrease in hydrocarbon concentration in the BML-B group represents any baseline abiotic (desorption) and biotic (microorganisms could be attached to the bitumen surface) processes occurring in this fraction. The BML-T group displayed a > 90% removal of all hydrocarbon fractions. This is likely due to microbial degradation, since FFT is a known source of microorganisms. Greater reduction in hydrocarbon fractions was seen in the BML-BTC group (reductions: F2: 64%, F3: 58%, F4: 58% and F4G-SG: 68%) when compared to the BML-BT group (reductions: F2: 23%, F3: 26%, F4: 24% and F4G-SG: 35%). Due to the intrinsic complexity of the analysis, PHC change was more statistically significant for BML-BTC group, not in BML-B and BML-BT groups. The PHC results indicates that the addition of acetate may have triggered co-metabolic processes and that more hydrocarbons were catabolized in the presence of acetate.

Removal of dissolved organics was not improved in the presence of acetate. Therefore, although acetate may have stimulated the degradation of heavier non-aqueous organic compounds from residual bitumen, it did not substantially affect the removal of dissolved organics as shown by DOC in Section 3.1. Previous studies have shown that acetate addition to oil sands tailings resulted in reduced anaerobic degradation of lower end PHCs (Stasik et al., 2015). The delay in biodegradation may be linked to pH reduction as a result of acetate accumulation and competition for limited nutrients and electron acceptors (Stasik et al., 2015). However, this inhibition of acetate was not seen in this research, potentially because of the different redox level or because of different metabolic pathways of the various PHCs.

3.3 AEOs and O_2^- compounds

Acid Extractable Organics (AEOs) were measured by GC-FID, which comprises a broad class of organic compounds (e.g., O_2^- compounds, nitrogen-containing species (NO_n and N_2O_n), and sulfur-containing species (O_nS and O_nS_2)), and AEOs include NAs as defined by O_2^- compounds, which were more specifically measured with an Orbitrap mass spectrometer as described in Section 2.2.4 (Headley et al., 2011). Start and end data were shown in Fig. 3. NAs solubility is influenced by pH (Headley et al., 2002), so pH was also tracked. On day 0, pH was about 8.3 for all groups. After 100 d, pH was 8.15 ± 0.22, 7.53 ± 0.25, 7.70 ± 0.01, and 8.04 ± 0.03 for BML-B, BML-BT, BML-BTC, and BML-T respectively. This pH change is not significant enough to greatly influence NA solubility (Headley et al., 2002). Therefore, the main influence on NAs concentration changes should be physiochemical and biological processes.

Unextracted bitumen has long been suspected a source of petroleum acids including NAs (Quagraine et al., 2005a). BML-B group demonstrated that bitumen was a source of AEOs

but not of O_2^- compounds. 20-40% removal of O_2^- compounds was observed in groups containing tailings (BML-BT, BML-BTC, BML-T), demonstrating the ability of indigenous microbes to remove both NAs and bitumen-sourced organic acids.

3.4 Toxicity

Liquid phase toxicity was measured on day 0, 48, and 100, as shown in Fig. 4. Day 0 samples were taken within 3 h of setting up the bottles. On day 0, differences could be observed: BML-B had the highest toxicity tested by Microtox®, indicating that bitumen may significantly contribute to toxicity. In the BML-T group, aqueous toxicity was reduced over 100 d from 1.0 TU to about 0.2 TU, which indicates the aqueous phase could be detoxified by exposure to the native microbial activities in cap water. In other groups, bitumen was likely the primary cause of the increased toxicity over time.

In BML-B and BML-BT groups, toxicity increased 3.5 times and 25 times, respectively. The higher final toxicity in the sample containing the tailings may have been caused by toxic metabolic intermediates produced by the microbial degradation of organics in the tailings. In the BML-BTC group, a different trend was observed: after 48 d, toxicity increased 8.3 times (10 TU, similar to BML-B and BML-BT) but did not significantly increase further after 100 d (7.9 TU ± 4.2 TU). Different microbial degradation pathways may have resulted from the addition of acetate, which resulted in different metabolic intermediates, indicating that the addition of acetate could help detoxify bitumen-polluted aqueous environments. In previous research (Yu et al., 2018), the addition of a proprietary blend of microbes and organics allowed the detoxification of bitumen-containing cultures, suggesting that the addition of readily-degradable organic compounds could help detoxify bitumen-polluted aqueous environments in the presence of proper microbial communities.

3.5 qPCR

277 DNA extraction from the BML-B group was not successful, suggesting low bacterial
278 populations. DNA from other groups was extracted and *rpoB* gene copy numbers were
279 measured by qPCR (Fig. 5). BML-BT had a 70% reduction in the bacterial density, while this
280 group had the highest CO_2 production. No DNA samples were tested between day 0 and day
281 100, so it is unknown how the bacterial population changed over time. As shown in Fig. 4,
282 the toxicity increased significantly over time, which might have caused the decrease in
283 bacterial density (Fig. 5). Bacterial growth showed a reduction in the bacterial density in the
284 presence of the bitumen. BML-BTC had a 3.8 times bacterial density increase, which was
285 stimulated by the addition of acetate. Although complete mineralization of hydrocarbons
286 (observed as CO_2 generation) was less effective in the BML-BTC group, more effective
287 removal of heavier hydrocarbons (Fig. 2) and more rapid population growth of bacteria was
288 observed. BML-T group's bacterial density remained relatively constant (1.3 times denser).
289 BML-T group had the lowest available hydrocarbons, but also the lowest toxicity levels due
290 to the lack of bitumen.

291 3.6 Microbial community analysis

292 Oxidative culture conditions were used in this study. Therefore, archaeal species, which have
293 been reported to be mostly methanogens in oil sands tailings (Penner and Foght, 2010;
294 Siddique et al., 2012), were rarely detected in this set of experiments (e.g., relative abundance
295 about 0.1% in BML-T group). This discussion focuses on bacterial communities.

296 Microbial community composition profiles, shown in relative abundance (%), of BML-T,
297 BML-BT, BML-BTC groups and the original tailings microbial community (labeled as "Day
298 0 Tailings") are shown in Fig. 6. The microbial community composition profile in the Day 0
299 Tailings sample represents the indigenous tailings microbial community, used as a reference
300 for the other three groups. The change in the microbial community composition profile

301 during the experiment in groups BML-T, BML-BT and BML-BTC compared to Day 0
302 Tailings represents the response of the indigenous microbial community under the conditions
303 described in Section 2.4. Microbial communities in these three groups have changed
304 significantly from the original tailings microbial community (Day 0 Tailings).

305 Not surprisingly, the relatively more abundant species found in this study are well-known
306 oil/hydrocarbon degraders which have been reported in a variety of oil-contaminated
307 environments (Sánchez et al., 2006; Yakimov et al., 2007; Bartram et al., 2011; Gray et al.,
308 2011; Kostka et al., 2011; Siddique et al., 2012; Yergeau et al., 2012). These bacteria have
309 also been reported to exist in oil sand tailings ponds and in the Athabasca River and its
310 tributaries (Penner and Foght, 2010; Ramos-Padrón et al., 2011; Siddique et al., 2012;
311 Yergeau et al., 2012; Chávez, 2014; Foght et al., 2017). Many of these species are facultative
312 anaerobes. However, nitrate and sulphate levels were constant for these two electron
313 acceptors, and methane in the headspace was below detection limit (< 1mg L^{-1}) during the
314 incubation period.

315 *Marinobacter* was the most abundant genus found in the Time 0 Tailings (> 29%).
316 *Marinobacter* has been found in many studies to be an effective oil degrader, and is
317 recognized to play a role in the degradation of hydrocarbons from oil polluted marine waters
318 (Sánchez et al., 2006; Yakimov et al., 2007; Gray et al., 2011; Kostka et al., 2011). However,
319 the abundance of *Marinobacter* decreased in all three groups after 100 d, especially in the
320 cultures with bitumen.

321 In the BML-T group, the most abundant genera were *Acidovorax* (> 15%), *Pseudomonas* (>
322 12%), *Marinobacter* (> 8%) and *Parvibaculum* (> 6%). The BML-T group showed a similar
323 trend to those seen in previous investigations of West In-Pit (WIP) tailings and Mildred Lake
324 Settling Basin (MLSB) tailings (Penner and Foght, 2010). WIP was a previous tailings

impoundment at the Mildred Lake Mine site, and was later commissioned as BML. It contains FFT mainly transferred from MLSB and water transferred from Beaver Creek Reservoir (Dompierre and Barbour, 2016). *Acidovorax* spp. and *Pseudomonas* spp. are frequently detected in hydrocarbon-contaminated environments (Eriksson et al., 2003; Penner and Foght, 2010). *Acidovorax* is a denitrifier and facultative lithoautotroph, which can use molecular hydrogen, and has been found in anaerobic sites contaminated with toluene (Aburto and Peimbert, 2011). This genus has also been found in mineral oil hydrocarbon-contaminated soil (Popp et al., 2006). *Pseudomonas* spp. are found ubiquitously in natural soil environments as well as hydrocarbon-contaminated sites, and certain species are capable of degrading model and commercial NAs (Lai et al., 1996; Kato et al., 2001; Quagraine et al., 2005; Del Rio et al., 2006; Popp et al., 2006; Whitby, 2010). *Pseudomonas* is also involved in biofilm formation, which provides advantages for growth in extreme environments (Golby et al., 2012).

In the BML-BT group, *Rhodoferax* (> 28%), *Acidovorax* (> 23%), *Pseudoxanthomonas* (> 18%), and *Pseudomonas* (> 7%) were detected at the highest abundance. Iron-reducing *Rhodoferax* spp. have been identified as effective hydrocarbon degraders and also are abundant in tailings pond or enriched oil sands tailings cultures (Penner and Foght, 2010; Aburto and Peimbert, 2011; Golby et al., 2012; Yergeau et al., 2012). *Pseudoxanthomonas* spp. have been found in oil contaminated sites, and identified as benzene, toluene, ethylbenzene, and o-, m-, and p-xylene (BTEX) degraders. Members of this genus can also produce biosurfactants and degrade crude oil (Sánchez et al., 2006; Kim et al., 2008; Nayak et al., 2009; Mortazavi et al., 2013; Nopcharoenkul et al., 2013). There are no publications regarding *Pseudoxanthomonas* spp. in the context of oil sands tailings. The presence of this genus might be correlated with the high dose of the bitumen added in this group.

In the BML-BTC group, *Pseudomonas* (> 31%), *Acidovorax* (> 17%), *Petrimonas* (> 8%), and *Rhodoferax* (> 7%) were detected at the highest abundance. Acetate addition likely stimulated *Pseudomonas* spp., which dominated this group, and the growth of Pseudomonas may have contributed to the significant bacterial growth (qPCR results shown in Fig. 5) and the highest rate of removal of PHC in the BML-BTC group (shown in Fig. 2). *Petrimonas* has not been reported in environmental samples, however, this genus has been reported in previous bioreactor studies (Sun et al., 2015; Li et al., 2016). Intermittent anoxic conditions might have occurred in this group because of the rapid bacterial growth and effective removal of hydrocarbons.

A recent study using metatranscriptomics correlated highly expressed genes with energy metabolism and hydrocarbon degradation from samples collected along the Athabasca River freshwater tributaries, and indicated that the expression of *alkB* (alkane monooxygenase) could potentially serve as a bioindicator gene for active hydrocarbon degradation potential (Reid et al., 2018). The *alkB* is responsible for aerobic hydrocarbon degradation in the oil-polluted sites and abundantly distributed among bacteria belonging to Alpha-, Beta- and Gammaproteobacteria (Nie et al., 2014). Alpha- (>7% for BML-T), Beta- (>28% for BML-BTC, >54% for BML-BT, >26% for BML-T) and Gammaproteobacteria (>31% for BML-BTC, >27% for BML-BT, >39% for BML-T) were also the three most abundant classes found in this study.

4. Conclusions

Bitumen in the BML would significantly contribute to the PHC level, especially in the presence of tailings. Bitumen in this study increased the aquatic toxicity (measured by Microtox®) by four times when mixed with the BML water, and by 20 times when mixed with the BML water and tailings. Through the on-site monitoring program carried by

Syncrude, the acute toxicity of BML has been decreasing every year indicating that in situ remediation occurring (Syncrude Canada Ltd., 2017). Acetate addition mitigated this toxicity and effectively removed the PHC compounds. The quantitative increases in bacterial populations and the increase of the relative abundances of known oil-degrading bacteria indicated a strong selective response of indigenous microbial communities in the presence of the bitumen obtained from BML. *Rhodoferax, Acidovorax, Pseudomonas* and *Pseudoxanthomonas* were genera that were best able to tolerate bitumen-derived toxicity. *Rhodoferax, Acidovorax* and *Pseudomonas* spp. showed more potential for biostimulation treatment with acetate to remove PHC/bitumen. *Pseudomonas* spp. were the most significantly stimulated species by acetate and might serve as the biggest contributor to bitumen removal and toxicity mitigation.

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594

Fig. 1 Microbial degradation of bitumen measured by CO_2 production in the headspace and DOC concentration in the aqueous phase over a period of 100 d. For BML-BTC group, acetate carbon was also included. Results are presented as an average ± one standard deviation (n = 2). Black circles represent CO_2, and star symbols represent DOC.

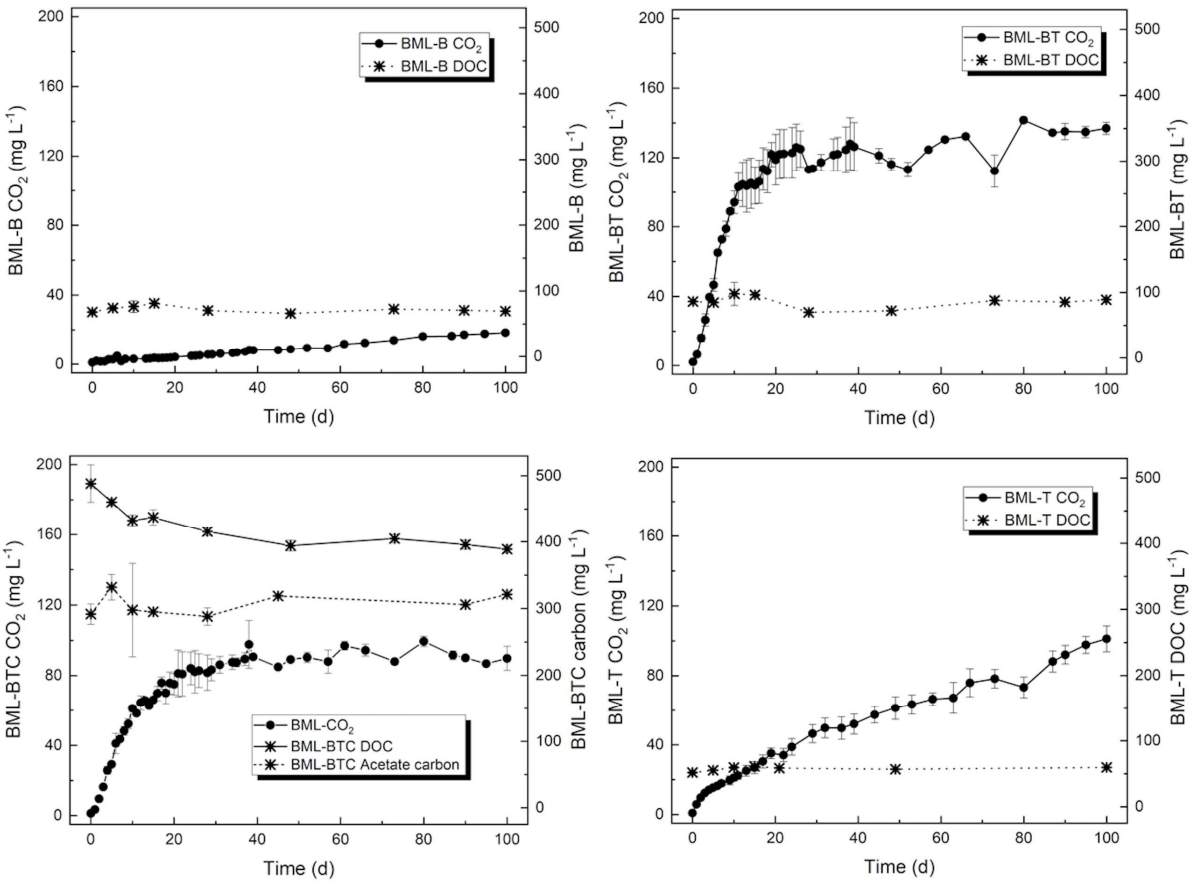
Fig. 2 Petroleum hydrocarbon contents (F2, F3, F4 and F4G-SG) in all groups on day 0 and day 100. Different y axis scales were used. The white columns represent day 0 data, and shadow columns represent day 100 data. Results were based on one duplicate and the error bars represented the measurement uncertainty.

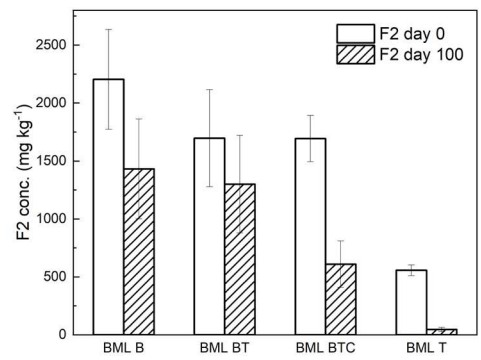
Fig. 3 NAs in the liquid phase measured as AEOs (left) and O_2^- compounds (right) on day 0, and after 100 d in all groups respectively. Results are presented as an average ± one standard deviation (n = 4 for all AEOs, n = 2 for BML-BT and BML-BTC O_2^- compounds, n = 1 for Day 0, BML-BTC and BML-T O_2^- compounds due to the limited volume).

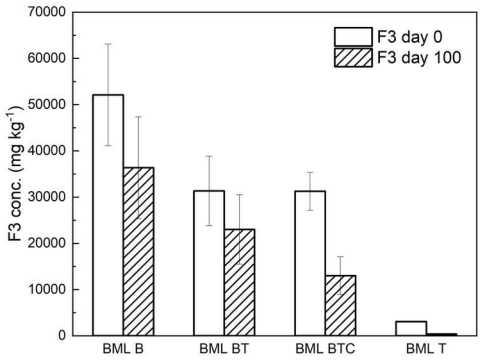
Fig. 4 Aqueous toxicity over a period of 100 d (day 0, day 48 and day 100). Results are presented as an average ± one standard deviation (n = 2). The open bars, shadow bars, and black bars represent day 0, day 48, and day 100, respectively.

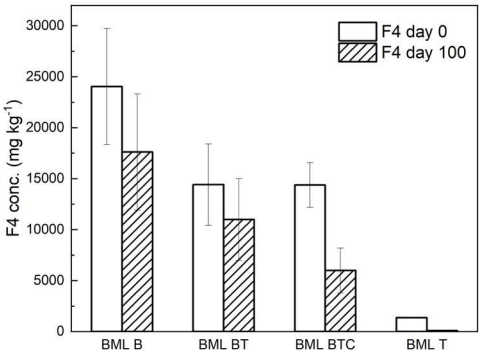
Fig. 5 qPCR results targeting at *rpoB* gene at time 0, and after 100 d in other three groups respectively. Results are presented as an average ± one standard deviation (n = 6).

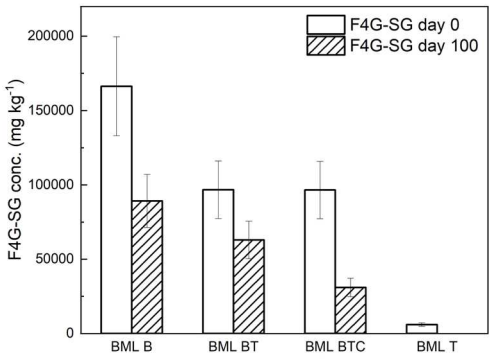
Fig. 6 Microbial community profiles of the original tailings microbial community (Day 0 Tailings), and BML-BT, BML-BTC and BML-T microbial communities after 100 d incubation. Phylum, class and genus information is shown in bold black, black and grey text, respectively. The size of the bubble represents the relative abundance (%). The microbial community richness (n = observed operational taxonomic units (OTUs)) is shown.

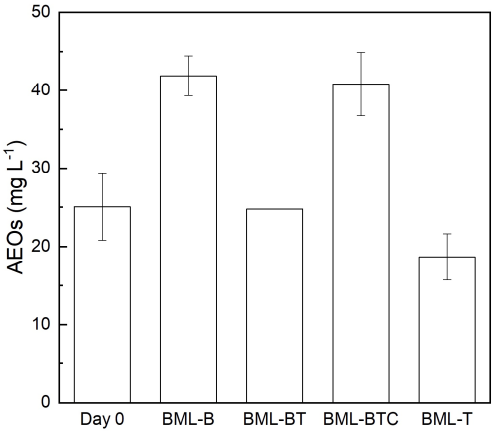


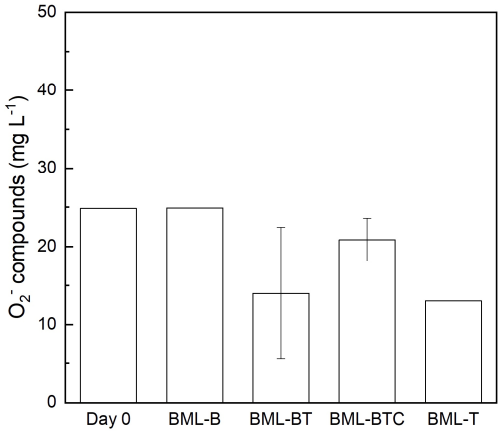


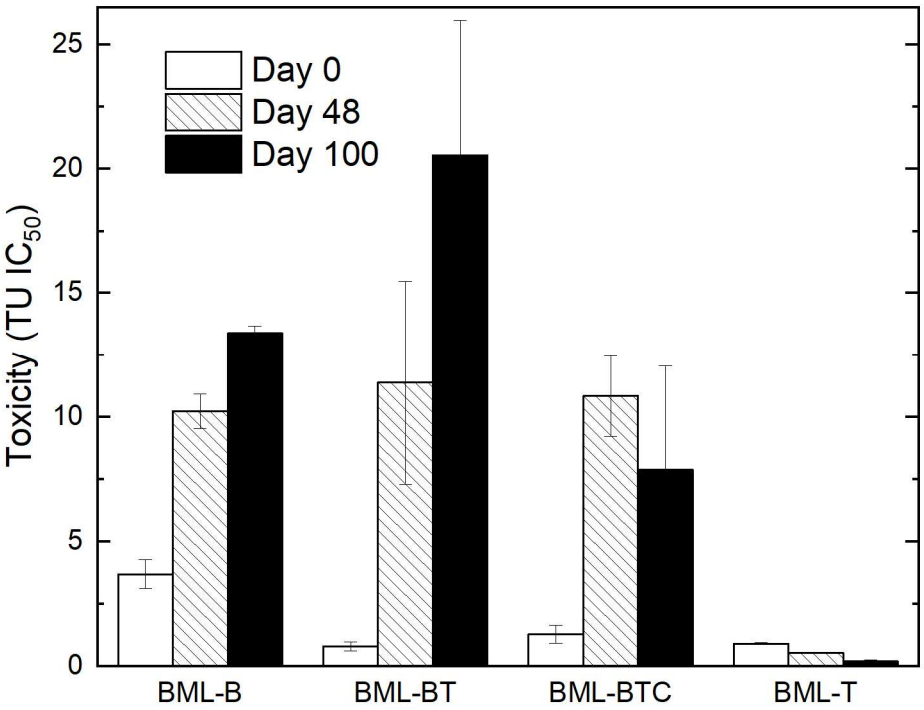


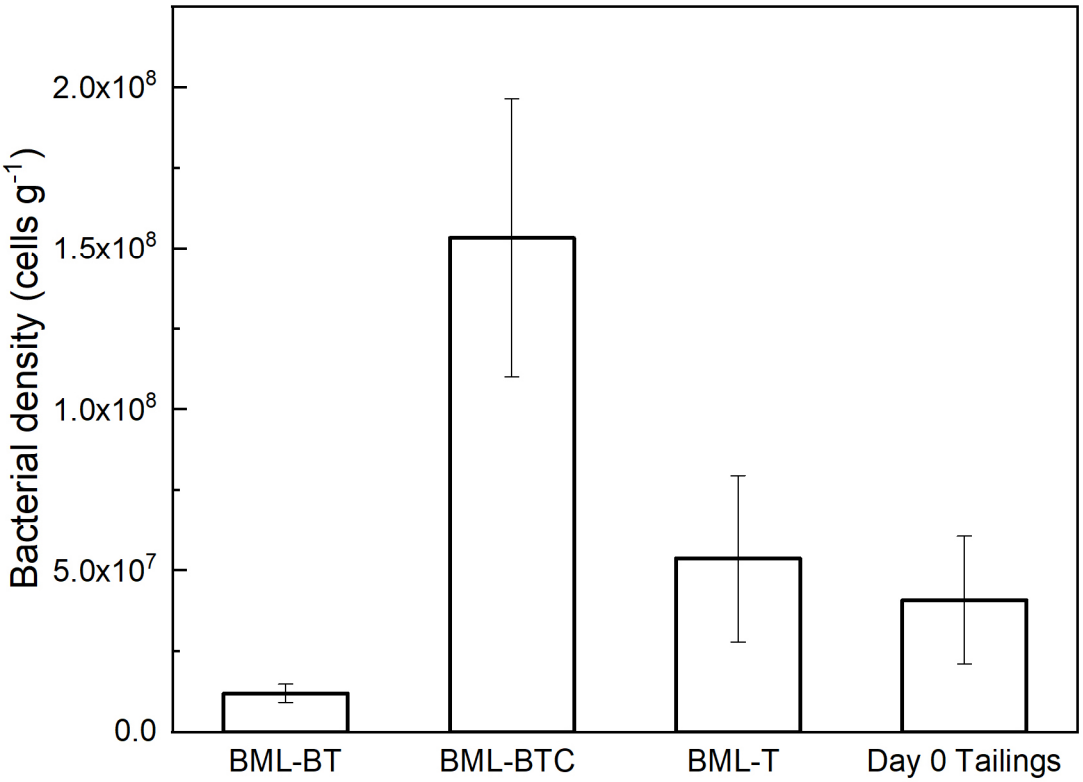


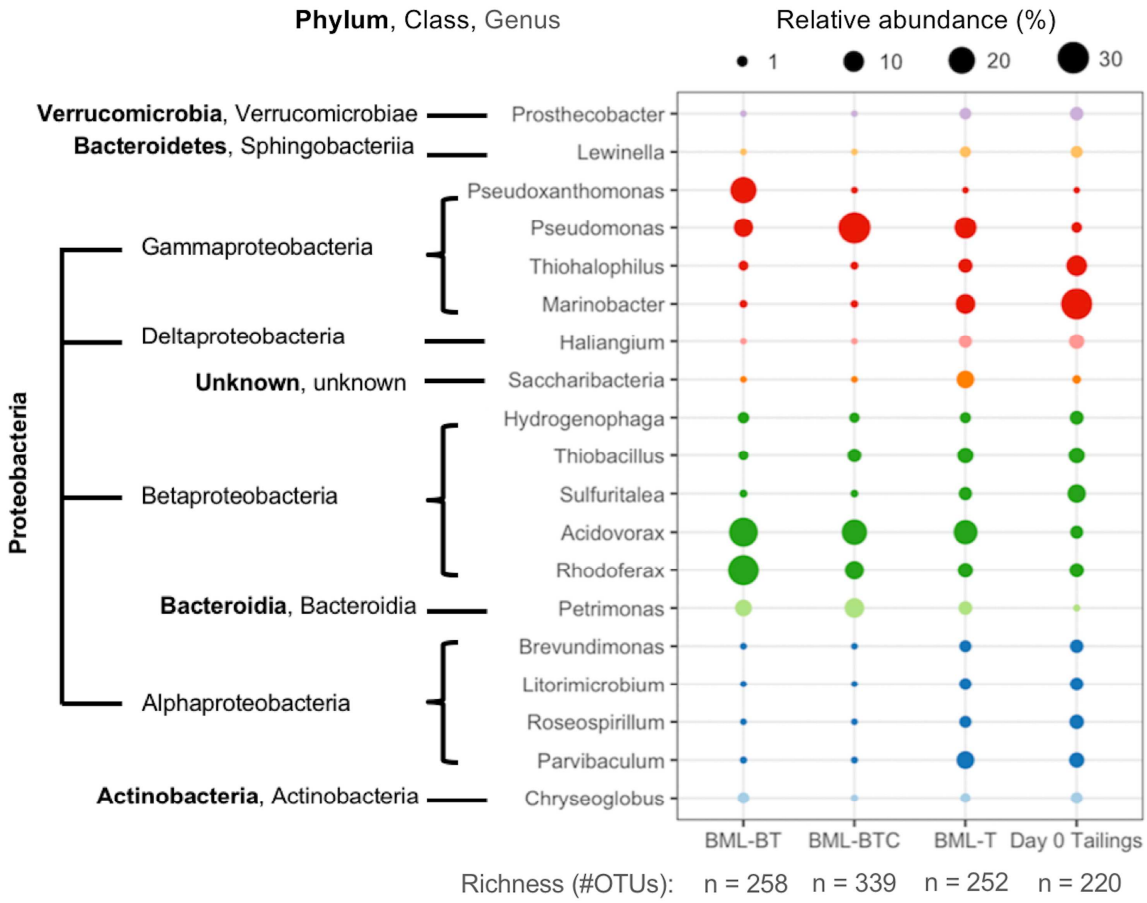












1 **Highlights**

2 Indigenous microorganisms removed PHCs (>58%) from bitumen.

3 Bitumen addition increased tailings toxicity by 25 times.

4 Acetate stimulated microbial growth and bitumen degradation.

5 *Pseudomonas*, *Acidovorax*, and *Rhodoferax* were potential bitumen degraders.

6