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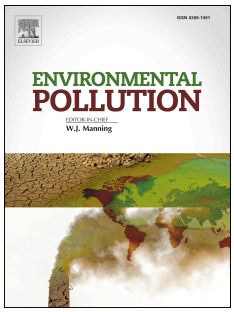

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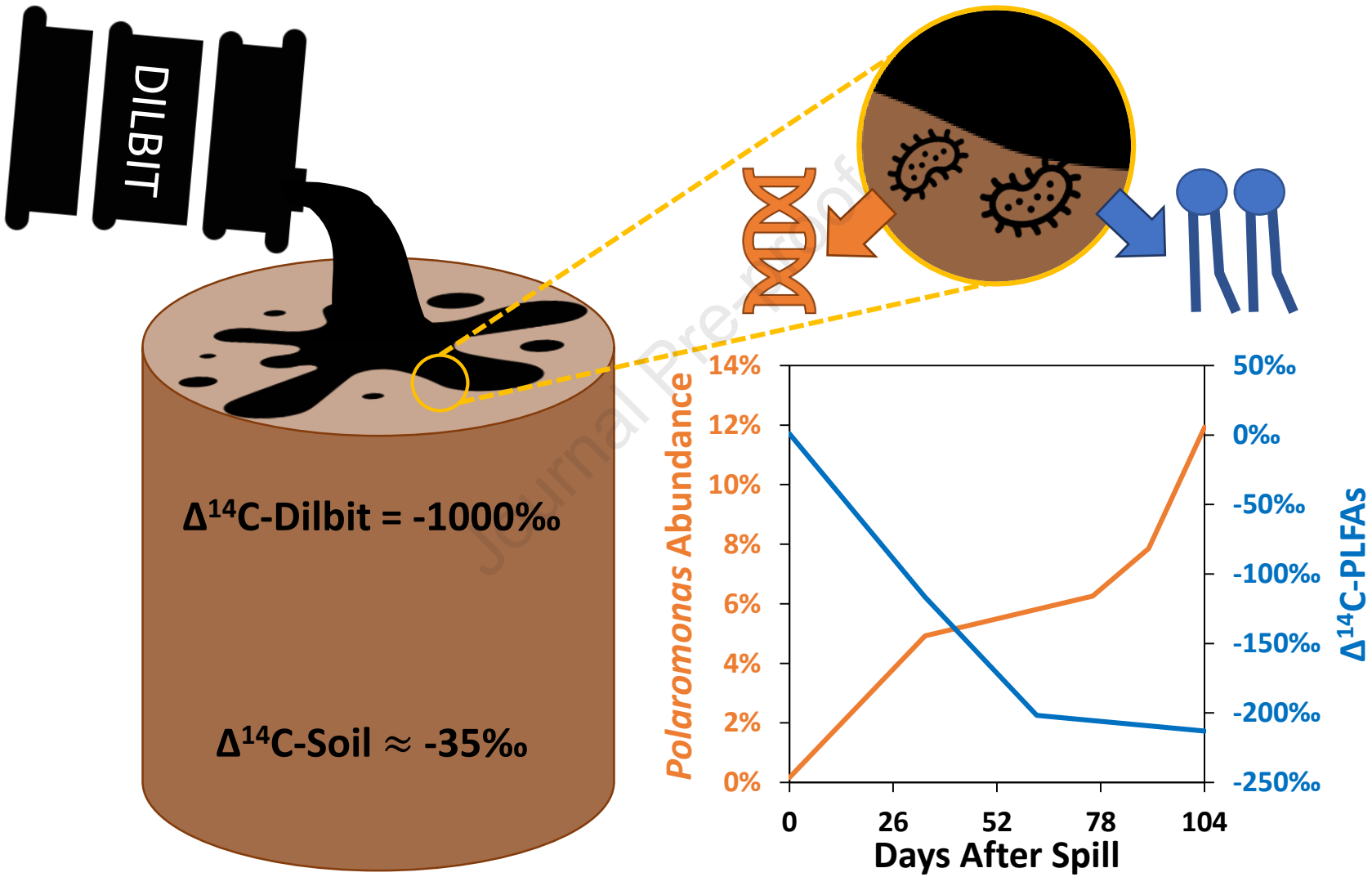

Isotopic and microbial evidence for biodegradation of diluted bitumen in the unsaturated zone

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Abstract

The oil sands region in Western Canada is one of the world's largest proven oil reserves. To facilitate pipeline transport, highly viscous oil sands bitumen is blended with lighter hydrocarbon fractions to produce diluted bitumen (dilbit). Anticipated increases in dilbit production and transport raise the risk of inland spills. To understand the behavior of dilbit in the unsaturated or vadose zone following a surface spill, we ran parallel dilbit and conventional heavy crude exposures, along with an untreated control, using large soil-filled columns over 104 days. Phospholipid fatty acids (PLFAs), biomarkers for the active microbial population, were extracted from column soil cores. Stable carbon isotope contents ($\delta^{13}C$) of individual PLFAs and radiocarbon contents ($\Delta^{14}C$) of bulk PLFAs were characterized over the course of the experiment. The $\Delta^{14}C$-PLFA values in soils impacted by dilbit (-221.1 to -54.7‰) and conventional heavy crude (-259.4 to -97.9‰) indicated similar levels of microbial uptake of fossil carbon. In contrast, $\Delta^{14}C$-PLFA values in the control column (-46.1 to +53.7‰) reflected assimilation of more recently fixed organic carbon. Sequencing of 16S ribosomal RNA genes extracted from soil cores revealed a significant increase in the relative abundance of *Polaromonas*, a known hydrocarbon-degrader, following exposure to both types of oil. This study demonstrates that in the first several months following a surface spill, dilbit has a similar potential for biodegradation by a native shallow subsurface microbial community as conventional heavy crude oil.

1. Introduction

The oil sands deposit in Alberta, Canada, is the world's third largest proven reserve of crude oil (NRCan, 2020). The oil extracted from this region is a heavily degraded, highly viscous form of petroleum known as bitumen. In order to be transported via pipeline, bitumen is diluted

with lighter hydrocarbon fractions such as natural gas condensates and naphtha to yield a less viscous blend commonly referred to as dilbit (Radović et al., 2018). Dilbit is typically manufactured at a ratio of 70-80% bitumen to 20-30% diluent (Crosby et al., 2013; Spalding and Hirsh, 2012). Many of the chemical and physical properties of dilbit differ from those of conventional crude oils, leading to unique behavior in the environment (Dollhopf et al., 2014; Radović et al., 2018). For instance, the generally higher density and viscosity of dilbit can lead to more pronounced sinking in water following a spill (Radović et al., 2018; Utting et al., 2022). As a result, remediation strategies that were largely developed for conventional crude oils may not necessarily be applicable to dilbit spills (Davoodi et al., 2020; Dollhopf et al., 2014). With increases in dilbit production and transport expected in coming years, the risks posed by inland oil spills are of great concern as they have a high potential to occur near populated areas, impact groundwater systems, and affect environments with a much lower capacity to dilute and disperse the oil (Lee et al., 2015).

Biodegradation has been demonstrated to be one of the most cost-effective and least disruptive strategies for containing and removing conventional crude spills from the environment (Hazen et al., 2016; Mahmoudi et al., 2017; Widdel et al., 2010; Yang et al., 2016). The success of this strategy is highly site specific as it depends on both environmental conditions and the abundance and diversity of microbes that possess the enzymes to break down crude oil constituents (Liao et al., 2015; Liu et al., 2017; Yang et al., 2016). Currently, it is unclear to what extent dilbit biodegradation is comparable to that of conventional crude oil. Previous dilbit studies, the vast majority of which have focused on marine and surface freshwater environments, have shown breakdown of the simplest alkane fractions (Schreiber et al., 2019), while evidence for the degradation of larger aromatic fractions of dilbit has been mixed (Deshpande et al., 2018; Schreiber

et al., 2021). In addition, most studies examining the biodegradation of dilbit have only considered short-term (< 30-day) exposures (Davoodi et al., 2020; Deshpande et al., 2017; King et al., 2014; Stoyanovich et al., 2019), which greatly limits our understanding of its long-term susceptibility to microbial breakdown..

The hydrological, geochemical and microbial conditions found in the unsaturated and saturated zones of soils and aquifers compared to marine and surface freshwater environments imply a different behaviour and fate for dilbit following its accidental release in terrestrial ecosystems. However, despite being environments frequently impacted by pipeline ruptures (Owens et al., 1993; Zhao et al., 2020), the behaviour and fate of dilbit in shallow soil and groundwater systems has thus far received little attention. While not dilbit, the large crude oil spill that occurred near the city of Bemidji, MN, USA in August 1979 as a result of a pipeline burst along a seam weld (Essaid et al., 2011) has provided a wealth of information on natural attenuation processes in petroleum-contaminated aquifers. Investigations at this site over the past few decades have shed insight into temporal changes in the geochemical composition (Baedecker et al., 2018; Podgorski et al., 2021), microbial communities involved with *in situ* biodegradation (Beaver et al., 2021; Fahrenfeld et al., 2014), and the lasting toxicological effects of a crude oil plume (McGuire et al., 2018; Zemo et al., 2022). Although previous research carried out at Bemidji and other crude oil-contaminated aquifers provides a valuable reference, the unique chemical and physical properties of dilbit necessitates dilbit-specific controlled spill experiments to better understand its comportment in the subsurface.

To address this research gap, we integrated molecular and isotopic approaches to investigate the response of indigenous microbial communities to a long-term (104-day) exposure of dilbit using large columns as analogues to the unsaturated or vadose zone of natural groundwater

93 systems. An additional treatment using conventional heavy crude oil was conducted in parallel to
94 compare the biodegradation potential of both oils under identical, controlled conditions. Rather
95 than solely monitoring changes in petroleum hydrocarbon concentrations, an approach which does
96 not unequivocally verify biodegradation, natural abundance stable carbon ($\delta^{13}C$) and radiocarbon
97 ($\Delta^{14}C$) isotope values of microbial lipids were used to provide direct evidence for *in situ* uptake of
98 petroleum carbon. Petroleum products, including dilbit, have no detectable ^{14}C ($\Delta^{14}C$ = -1000 ‰).
99 This contrasts with surface or near-surface soil organic matter, which has a $\Delta^{14}C$ value that more
100 closely reflects the input of recently fixed carbon (~ 0‰) (Ahad et al., 2010; Slater et al., 2005).
101 Thus, the more negative the $\Delta^{14}C$ value of microbial components, the greater the assimilation of
102 petroleum-derived carbon by the microbial community. Temporal changes in microbial
103 community structure and taxonomic composition were tracked using amplicon sequencing of the
104 16S rRNA to identify key dilbit-degrading taxa (Caporaso et al., 2012; Mahmoudi et al., 2013b).
105 To our knowledge, this is the first study to examine the biodegradation of dilbit in the unsaturated
106 zone following a simulated surface spill.
107

108 **2. Materials and Methods**

109 **2.1. Column construction**

110 Approximately 2000 kg of vadose zone soil (~ 0-20 cm depth) was collected in July 2019
111 from the Mount St-Hilaire Nature Reserve, approximately 40 km east of Montréal, Québec,
112 Canada. This site was chosen to provide relatively pristine soil from a region where future dilbit
113 pipeline construction may be considered. The soil was immediately transported to the Laboratories
114 for Scientific and Technological Innovation in Environment (LISTE) facility at INRS in Québec
115 City, Québec, Canada, where it was sieved through a 2 cm mesh and homogenized via mechanical

rotation for 10 min at 25 rpm in 205 L high density polyethylene barrels. The water content following homogenization was 5.38 ± 0.02%. The soil was then evenly distributed between columns that were composed of stainless steel (60 cm width × 100 cm height) lined with polytetrafluoroethylene (PTFE). Within each column, 312.80 ± 0.20 kg of soil (dry mass) was compacted in layers of 2 cm using a jack drill and a stainless steel plate to reproduce field soil density and avoid channeling in the column that would bias water infiltration behavior. Each soil layer was scarified to a depth of 5 mm using a small rake before adding the next one. The average soil density was 1.59 ± 0.02 kg/L and the porosity was 0.4). The total organic carbon (TOC) determined using an elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA) was 1.7 ± 0.6%. Granulometric analysis of the soil indicated > 99% sand-sized particles, 85% of which were larger than 0.5 mm.

Seven 1 cm diameter outflow ports in the bottom of each column directed leachate water into seven 1 L amber glass bottles resting below. Ports were fitted with PTFE pipefittings with fiberglass wicks threaded through them. To limit evaporation, these ports were sealed to the leachate collection bottles with PTFE thread tape. Wicks were in contact with the bottom layer of soil in the column and, relative to the sandy soil, had a net negative capillary pressure which helped to draw the leachate from the column to maintain unsaturated conditions (Everett and McMillion, 1985). A diagram of the column set-up is provided in the Supporting Data (Figure S1).

2.2. Experimental conditions

The columns were designed to be representative of vadose zone systems in the Greater Montréal Area during spring and fall recharge. These seasons were selected as they allow for plenty of groundwater infiltration without interference from the high rates of evapotranspiration

that occur in the summer, or from winter snow and ice cover (Lewis et al., 2009). The columns were maintained in a temperature-controlled room at 10 °C. Artificial rainwater to simulate local precipitation acidity (pH 4.8; Keresztesi et al., 2020; Vet et al., 2014) was prepared using a 3:2 (vol/vol) stock solution of sulfuric:nitric acid and added to each column twice a week for a total of 6.25 L of water per week. Measurements of dissolved oxygen in the column leachate indicated aerobic conditions were maintained in all the columns over the course of the experiment.

Following several column waterings over a period of four days to achieve steady-state flow conditions, conventional heavy crude oil and dilbit were added to the surface of the columns on Day 0. The conventional heavy crude (CC) column received 1.86 kg of conventional heavy crude oil, while the dilbit (DB) column received 1.89 kg of Cold Lake Blend (CLB). These amounts were intended to simulate a medium-scale incident, which ramped up to an area covering 0.25 hectares (2500 m^2) would correspond to an oil spill of approximately 20,000 L. Both samples originated from transmission pipelines: CLB refers to bitumen produced by *in situ* extraction in the Cold Lake region of Alberta, while the conventional heavy crude refers to various Western Canada Sedimentary Basin crude oils with similar physical and chemical properties. The proportions of saturates, aromatics, resins, and asphaltenes in the oils determined gravimetrically following silica gel chromatography were 25.4, 51.9, 9.5, and 13.2% and 30.4, 45.8, 8.5, and 14.2% in the DB and CC samples, respectively. A third column was left unamended to serve as a control. The experiment was carried out for a total of 104 days.

2.3. Soil sample collection

Soil cores were collected from the columns using a stainless-steel soil core sampler (AMS, American Falls, ID, USA) affixed with a slide hammer and a removable 15.2 cm (length) x 3.8 cm

(diameter) aluminum cylinder. The core sampler and removable cylinder were rinsed with acetone and distilled water between columns. The first core series was collected immediately prior to the addition of the crude oil treatments to characterize the initial soil conditions on what is referred to as Day 0. Coring was done on a weekly basis for the first month (Day 0, Day 6, Day 13, and Day 20) and then on a biweekly basis for the remainder of the experiment (Day 34, Day 48, Day 62, Day 76, Day 90, and Day 104) with 10 cores collected in total. A higher frequency of coring was carried out in the first month to coincide with anticipated faster column breakthrough curves for more water-soluble petroleum components such as BTEX (benzene, toluene, ethylbenzene and xylenes). A diagram showing the spatial arrangement of core extractions within the column set-up and further details on coring protocol are provided in the Supporting Data (Figure S2, Text S1).

To minimize the hydraulic disturbance caused by repeated coring, core holes were filled in with a 1:1 mixture of sodium bentonite clay tablets (Volclay® PureGold™ 3/8 inch, CETCO, Bethlehem, PA, USA) and sodium bentonite powder (Envrioplug Grout, Wyo-Ben Inc., Billings, USA) immediately following the collection of each core. This material expanded when mixed with water so that the surrounding soil remained compact while still allowing water to permeate vertically. The downward vertical flow of water (Figure S1), in conjunction with a sampling strategy in which cores were taken at opposite sides of the column from the previous collection (Figure S2), would have limited the potential impact of coring on microbial and geochemical dynamics in non-cored sections of the columns.

2.4. Total petroleum hydrocarbons (TPHs)

Total Petroleum Hydrocarbons (TPHs) from soil cores were extracted and analyzed following protocols similar to those described by (Ahad et al., 2010). TPHs were analyzed using

a gas chromatograph – mass spectrometer (GC-MS; Agilent Technologies Inc., Santa Clara, CA, USA; 7890A GC and 5975C MS detector) equipped with a Zebron (Phenomenex, Inc., Torrance, CA, USA) ZB-5HT column (30 m; 0.25 mm i.d.; 0.25 µm film) at the Delta-Lab of the Geological Survey of Canada (GSC-Québec). The following GC oven temperature program was used: 70 °C (2 min), 8 °C/min to 290 °C (8 min), 10 °C/min to 310 °C (10 min). TPH concentrations were determined by integrating the total area of unresolved complex mixture (UCM) in total ion current (TIC) mode. Further details on TPH analysis are found in the Supporting Data (Text S2).

2.5. Microbial lipid extraction and analysis

Phospholipid fatty acids (PLFAs) are essential membrane lipids of microbial cells and biomarkers for the active microbial population. PLFAs were extracted from sub-samples of homogenized soil cores following a modified Bligh and Dyer method (White et al., 1979) employed by Ahad et al. (2018) and converted to fatty acid methyl esters (FAMEs) by mild alkaline methanolysis adapted from Guckert et al. (1985). Further details on the lipid extraction protocol are found in the Supporting Data (Text S3).

FAMEs were analyzed using the same GC-MS system and column described above for TPH analysis. The following GC oven temperature program was used: 40 °C (1 min), 20 °C/min to 130 °C, 4 °C/min to 160 °C, 8 °C/min to 300 °C (5 min) as per Ahad et al. (2018). Quantification was done in TIC mode using external FAME standards (12:0, 14:0, 16:0, 18:0, and 20:0). Calibration curves and quantitation methods were set up using MSD ChemStation Data Analysis software (Agilent). The distributions of individual PLFAs in each sample are reported here as a mole percentage (mol %) relative abundance. PLFAs were identified by comparing the mass

fragmentation patterns and retention time to a bacterial reference standard (Bacterial Acid Methyl Esters CP Mix, Sigma-Aldrich, Oakville, ON, Canada).

PLFAs were described as Z:nΔx, where Z is the total number of carbon atoms on the fatty acid chain, n is the number of double bonds and Δx indicates the location of the double bond if known. The letters A, B, and C denote different isomers whose double bond position is unknown. Cyclopropyl PLFAs are denoted with the prefix "cyc". Methyl group branching is denoted by the prefixes "i" for the iso-isomer, "a" for the anteiso-isomer, and "br" if the position of the methyl group is unknown (branched isomers are differentiated by letters A, B, and C).

2.6. $\delta^{13}C$ analysis

The stable carbon isotope contents ($\delta^{13}C$) of individual PLFAs were determined using a gas chromatograph – isotope ratio mass spectrometer (GC-IRMS) at the GSC-Québec's Delta-Lab. The system consisted of a TRACE 1310 GC with a HP-5 column (60 m; 0.32 mm i.d.; 0.25 µm film) paired with a Delta V IRMS via a GC IsoLink (Thermo Fisher Scientific, Bremen, Germany). The IsoLink combustion reactor was maintained at 1050 °C. The GC oven temperature program was the same as that used for GC-MS analysis.

An external standard mixture containing 5-α-androstane obtained from the Biogeochemical Laboratories at Indiana University and five in-house FAME isotopic standards (12:0, 14:0, 16:0, 18:0, and 20:0) was injected into the GC-IRMS after every six sample injections to assess accuracy. Samples were run in duplicate, and peaks were manually integrated using Isodat software (Thermo Fisher Scientific) to obtain $\delta^{13}C$ ratios for specific PLFAs. The precision (1σ) for replicate standard and sample injections was ± 0.5‰. All $\delta^{13}C_{PLFA}$ values were corrected

for the isotopically characterized methyl group added to each FAME during mild alkaline methanolysis by the following equation:

$$\delta^{13}C_{PLFA} = [\delta^{13}C_{measured} - (f_{MeOH} \times \delta^{13}C_{MeOH})]/1\text{-}f_{MeOH} \quad (1)$$

where f_{MeOH} is the fraction of C atoms derived from methanol (MeOH). The $\delta^{13}C_{MeOH}$ value was -51.3‰.

The $\delta^{13}C$ values of total organic carbon (TOC) in decarbonated soil samples were measured using an elemental analyser (Costech Analytical Technologies Inc.) interfaced with a Delta V (Thermo Fisher Scientific) IRMS system. Further details on sample preparation are provided in the Supporting Information (Text S4).

2.7. $\Delta^{14}C$ analysis

The masses of individual PLFAs were too low for compound-specific radiocarbon analysis; consequently, ^{14}C contents were determined in the bulk PLFA fractions (Ahad et al., 2010; Mahmoudi et al., 2013a). In some cases, samples from multiple depths were combined to meet mass requirements for bulk ^{14}C analyses. Samples for radiocarbon analysis were selected at four different time series covering the full length of the experiment.

FAMEs dissolved in dichloromethane (DCM) were transferred by syringe into a 40 µL rigid silver capsule (IVA-Analysentechnik e.K.), dried in an oven at 50 °C for 30 min, and sealed with pliers following a protocol similar to that previously used for preparation of organic extracts for ^{14}C analysis (Ahad et al., 2020; Ahad et al., 2021). Samples were placed in quartz tubes with CuO oxidizer, sealed under vacuum, and combusted to CO_2 at 900 °C. CO_2 was then reduced to

graphite and measured for its $^{14}C/^{12}C$ ratio using a 500 kV compact accelerator mass spectrometer (AMS) unit (National Electrostatics Corporation, Middleton, WI, USA) at the W.M. Keck Carbon Cycle AMS facility at the University of California Irvine. Ratios were then corrected with the ratio of Oxalic Acid I (SRM 4990) and normalized for ^{13}C fractionation to be presented in the standard $\Delta^{14}C$ notation (Stuiver and Polach, 1977).

The radiocarbon contents of total organic carbon (TOC) in freeze-dried soil samples (also reported in $\Delta^{14}C$ notation) were determined using the same AMS system described above. TOC samples were decarbonated with 1N HCl at 70 °C, washed with ultrapure MilliQ water and dried prior to combustion to CO_2.

To assess the accuracy and precision of $\Delta^{14}C$ analyses, both modern (butter-derived FAMEs) and fossil (Ordovician shale aromatic hydrocarbons) standards of similar masses to those of samples were prepared in the same manner as described above. Based on replicate analyses of these standards, the error incorporating both accuracy and precision for $\Delta^{14}C$ analyses was < 20‰. All $\Delta^{14}C_{PLFA}$ values were corrected for the isotopically characterized methyl group added to each FAME during mild alkaline methanolysis by the following equation:

$$\Delta^{14}C_{MeOH} = [\Delta^{14}C_{measured} - (f_{MeOH} \times \Delta^{14}C_{MeOH})]/1\text{-}f_{MeOH} \quad (2)$$

where f is the fraction of C atoms derived from MeOH on each FAME. The $\Delta^{14}C_{MeOH}$ value was -998.2‰. Contributions of potential carbon sources to ^{14}C content of bulk PLFA fractions were estimated using a two end-member mass balance:

$$\Delta^{14}C_{PLFA} = (1 - f_{soil}) \times \Delta^{14}C_{petroleum} + f_{soil} \times \Delta^{14}C_{soil} \quad (3)$$

where $\Delta^{14}C_{PLFA}$ corresponds to the isotopic value of the PLFAs, f_{soil} is the fraction of carbon contributed by the background soil TOC, 1- f_{soil} is the fraction of carbon contributed by petroleum, $\Delta^{14}C_{petroleum}$ is the isotopic value of DB or CC (both assumed to be -1000‰) and $\Delta^{14}C_{soil}$ is the isotopic value of the background soil TOC.

2.8. Genomic DNA extraction and sequencing of 16S rRNA genes

Genomic DNA was extracted from 45 soil cores, in replicate, using the DNeasy PowerSoil DNA extraction kit from Qiagen (Hilden, Germany) following the manufacturer's protocol. The V4 hypervariable region of the 16S rRNA gene in both bacteria and archaea (primer pair 548F and 806R) (Kozich et al., 2013) was amplified and sequenced in duplicate for each soil core. Amplicons were sequenced on an Illumina MiSeq (San Diego, CA, USA) in the Ronholm Lab (McGill University). Sequence data was analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) v2021.2 (Bolyen et al., 2019). Files generated in the QIIME2 pipeline were then exported for use in downstream statistical analyses. Details of sequence data processing using QIIME2 can be found in the Supporting Information (Text S5). Sequencing data were submitted to the National Center for Biotechnology Information (NCBI) under BioProject PRJNA922993.

2.9. Statistical analyses of sequencing data

All statistical analyses and visualizations were done in R, version 4.0.2. Amplicon sequence variant (ASV) counts were rarefied (subsampled without replacement) to 4162, which equated to the sample with the lowest number of reads, using the phyloseq R package (McMurdie and Holmes, 2013). Beta diversity metrics were calculated using Weighted UniFrac distance with

phyloseq. Results of the distance matrix were visualized by Principal Coordinate Analysis (PCoA) using the phyloseq and ggplot2 R packages (Wickham, 2011). Differences in microbial community composition between metadata groups (treatment type/sample depth/sample day) were assessed using permutational multivariate analysis of variance (PERMANOVA). The homogeneity of within-group dispersions was assessed by permutation multivariate analysis of dispersion (PERMDISP). Both PERMANOVA and PERMDISP were implemented using the vegan R package (Oksanen et al., 2013).

Normality and homogeneity of variance of the relative abundance of individual taxonomic groups were evaluated with the Shapiro-Wilk test ($P < 0.05$) and the Levene's test ($P < 0.05$), respectively. For all datasets, the assumptions of normality and equal variance required for analysis of variance (ANOVA) tests were not met. As an alternative to ANOVA, the non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparison test with Holm adjustment was done to reveal if significant differences existed between metadata groups (treatment type/sample depth/sample day) in these datasets. These calculations were done using the rstatix package (Kassambara, 2021).

3. Results

3.1. Concentrations of TPHs

Concentrations of TPHs extracted from soil cores ranged from 19 to 568 mg/kg in DB, 19.5 to 605 mg/kg in CC, and 24 to 27.6 mg/kg in the control (Table S2). TPH concentrations were highest in the top depths of DB and CC cores (404 to 605 mg/kg) and were comparable to the lower range of those reported in soils contaminated by crude oil near pipelines (Iturbe et al., 2007; Pernar et al., 2006). TPH concentrations at middle and bottom depths remained at similar levels to

the control over the course of the experiment apart from the bottom depth of DB, which spiked to 91 mg/kg on Day 34. The background TPH concentration in the control (24.0 to 27.6 mg/kg) is attributed to the presence of non-petroleum hydrocarbons (e.g., alkanes from plant leaf waxes) found in the soil.

3.2. Microbial PLFAs

The total concentrations of PLFAs extracted from individual samples varied widely over the course of the exposure but did not show any trends across metadata groups (treatment type, sample depth, sample day). Total PLFA concentrations ranged from 8.18 to 37.57 ng/g soil, with much of this variability likely attributable to differences in grain size from sample to sample (Figure S3). Overall, PLFA distributions (as mol %) for the most abundant compounds were similar across all samples (Figure 1). The most abundant PLFAs across all DB, CC, and control samples were 16:0 (17.0 to 23.0%) and 18:1B (10.4 to 16.2%). In all columns, 16:0, a general bacterial biomarker, and 18:1B, a biomarker for gram-negative bacteria (Wilkinson and Ratledge, 1988), showed a slight increase in abundance over the exposure period. The gram-positive bacterial biomarkers, i-15:0 and a-15:0 (O'Leary et al., 1988; Vestal and White, 1989) both decreased for DB and CC columns. In contrast, cyc17:0, a biomarker for gram-negative bacteria and petroleum-degrading bacteria (Ahad et al., 2018; Cowie et al., 2010; Greenwood et al., 2009), only increased in CC and DB columns (Figure 1).

Principle Component Analysis (PCA) was performed on mol % data for the top 12 PLFAs from each depth separately (Text S6). PCA biplots showed a progressive change in PLFA distributions over time in oil-impacted samples and the control (Figure S4). Greater mol % of cy17:0, 16:0, 16:1Δ9, and 18:1B were associated with CC and DB samples in the later stages of

the exposure period (after day 48). All other PLFAs were associated with control and early-stage (before day 48) DB and CC samples. The PCA biplots indicate changes in microbial PLFA distributions in response to oil exposure but no distinction between DB- and CC-impacted communities.

3.3. $\delta^{13}C$ values of PLFAs and TOC

The $\delta^{13}C$ values of individual PLFAs ranged from -30.5 to -24.0‰ (Figure 2). In general, the $\delta^{13}C$ values of individual PLFAs showed no clear trends across depths or time. The $\delta^{13}C$-PLFA values have thus been grouped to highlight potential differences between treatments (Figure 2). The most enriched PLFA was a-15:0, with an average $\delta^{13}C$ value from all three columns (DB, CC, and control) of -25.2 ± 0.5‰ over the entire length of the experiment. The most depleted PLFA was 19:1A, with an average $\delta^{13}C$ value of -29.0 ± 0.7‰.

There were statistically significant differences in $\delta^{13}C$ values for cyc17:0 ($P < 0.05$) and the combined PLFAs 16:1Δ9 and 16:1B ($P < 0.05$) between the control and both oil-impacted columns. The $\delta^{13}C$ values of cyc17:0 were significantly more depleted in both CC and DB compared to the control ($P < 0.05$). The combined 16:1Δ9 and 16:1B group was significantly more depleted in ^{13}C in CC compared to the control ($P < 0.05$).

The $\delta^{13}C$ values of the control soil TOC on Day 0 were -26.8, -27.6 and -27.4‰ in the top, middle and bottom core sections, respectively, and remained unchanged over the course of the experiment (-27.2 ± 0.1‰ on Day 104). On Day 6, the $\delta^{13}C$-TOC values in the in the top, middle and bottom core sections of the DB and CC columns were -29.0, -27.5 and -27.7‰ and -29.2, -27.9 and -27.2‰, respectively. The more depleted values in the top sections reflected a greater contribution from either dilbit (-29.9‰) or conventional heavy crude (-29.3‰). This trend

remained consistent over the course of the experiment, as evident from a similar suite of values measured in top, middle and bottom core sections on Day 104 (DB: -29.3, -27.3 and -26.8‰; CC: -29.1, -27.4 and -27.4‰).

3.4. $\Delta^{14}C$ values of bulk PLFAs

Bulk PLFA ^{14}C contents for DB and CC columns were assessed from individual top, middle and bottom depths on Days 34 and 62 and from combined depths on Day 104 (Table S3, Figure 3). Bulk PLFA ^{14}C contents in the control were determined from individual top, middle and bottom depths on Days 34 and 62 and from combined depths on Days 0 and 104 (Table S3, Figure 3). The $\Delta^{14}C$ values of bulk PLFAs in the control ranged from -46.1 to +53.7‰ and for most samples were more positive than the $\Delta^{14}C$ value of the bulk soil TOC (-34.7‰). The $\Delta^{14}C$ values of bulk PLFAs ranged from -221.1 to -54.7‰ in the DB column and from -259.4 to -97.9‰ in the CC column. In contrast to the control, there was a discernible decrease in $\Delta^{14}C$ values for both CC and DB columns over the exposure period (Figure 3).

3.5. Microbial community composition

Sequencing of 16S amplicons yielded a total of 6,382,607 reads. Following quality control, the total read count was reduced to 3,812,693 with an average of 39,715 reads per sample. The total number of amplicon sequence variants (ASVs) present in the filtered dataset was 26,641. The overwhelming majority of ASVs (17,736) were assigned to *Bacteria* and only 69 were assigned to *Archaea*. The dominant bacterial phylum across all samples was *Proteobacteria*, making up 32.7 to 60.1% of ASVs across all samples (Figure S5). Other abundant phyla (> 1% average abundance) included *Actinobacteriota* (8.5 to 21.4%), *Acidobacteriota* (6.8 to 17.0%), *Chloroflexi* (2.6 to

9.7%), *Bacteroidota* (1.7 to 19.8%), *Myxococcota* (0.8 to 7.8%), *Verrucomicrobiota* (1.2 to 6.9%), *Planctomycetota* (0.8 to 5.9%), *Gemmatimonadota* (1.3 to 3.7%), and *Firmicutes* (0.4 to 6.0%). *Gammaproteobacteria* was the dominant class (Figure S6) across all samples. There were significant differences in the relative abundance of *Gammaproteobacteria* between control and oil-impacted columns ($P < 0.05$). During the exposure period, the average relative abundance of *Gammaproteobacteria* increased by 11.7 and 16.1% in the DB and CC columns, respectively. In contrast, the average relative abundance of *Gammaproteobacteria* remained at 19.4% in the control column. Within *Gammaproteobacteria*, the predominant order was *Burkholderiales* (Figure S7) whose relative abundance significantly increased by 11.5% and 15.3% in both the DB and CC columns ($P < 0.05$). At the genus level, dramatic increases in relative abundances of *Polaromonas* from within the order *Burkholderiales* were observed over time (Figure 4). At day 0, *Polaromonas* accounted for < 1% of sequences but by day 104 it comprised 8.9 to 18.2% of all sequences in the DB samples (across all depths) and 8.0 to 17.2% in the CC samples (across all depths) while accounting for < 1% of sequences in the control column.

Several other groups became more abundant in the oil-impacted columns compared to the control column over time (Figure 4). There were significantly higher proportions of *Phenylobacterium (Alphaproteobacteria)*, *Mycobacterium* (*Actinobacteria*), and an unknown genus from the family *Comamonadaceae* (the same family as *Polaromonas*) in both the DB and CC samples relative to the control ($P < 0.05$).

Changes in microbial community structure over time were visualized for each sampling depth (top, middle and bottom) using PCoA of weighted unifrac distances based on the relative abundance of ASVs (Figure S8). For all three depths, the DB and CC samples were significantly different from the control samples ($P < 0.05$) but not from each other. This implies that although

413 microbial communities were strongly affected by the presence of oil, the type of oil (DB vs. CC)
414 did not lead to specific changes in community composition. Likewise, PERMANOVA tests found
415 no significant differences between the microbial communities exposed to DB and CC but did
416 clearly differentiate them from Day 0 and control samples ($P < 0.05$).
417
418 **4. Discussion**
419 Although subtle (Figure 1), the distributions of PLFAs in DB, CC, and control columns
420 changed over the course of the experiment. The increases in the mol % of cyc17:0, 16:0, 18:1B
421 and 16:1Δ9 and decreases in a-15:0, and i-15:0 were associated with microbial communities
422 exposed to CC and DB. Cyc17:0, 18:1B, and 16:1Δ9 are biomarkers for gram-negative bacteria
423 (Wilkinson and Ratledge, 1988). Previous studies have reported similar increases in the abundance
424 of gram-negative PLFA biomarkers following exposure to petroleum (Bastida et al., 2016; Green
425 and Scow, 2000; Li et al., 2018; Margesin et al., 2007). In addition to being a biomarker for gram-
426 negative bacteria, cyc17:0 has been associated with petroleum-degrading microbes in previous
427 studies (Ahad et al., 2018; Cowie et al., 2010; Greenwood et al., 2009). Correspondingly, this
428 increase in gram-negative was confirmed by the amplicon sequencing data which revealed that the
429 gram-negative bacterial phylum, *Proteobacteria*, dominated the microbial community and their
430 relative abundances increased over the exposure period in DB and CC columns.
431 *Proteobacteria* are known to include a multitude of hydrocarbon-degrading bacteria that
432 contribute to crude oil degradation (Ahad et al., 2018; Bastida et al., 2016; Deshpande et al., 2018;
433 Hazen et al., 2016; Mahmoudi et al., 2013b). The dominant DB and CC-degrader identified in our
434 study was *Polaromonas*, a genus within the order *Burkholderiales*. In addition to *Polaromonas*,
435 several other genera significantly increased in abundance during the DB and CC exposures. These

included *Phenylobacterium*, *Mycobacterium*, and an unknown genus from the family *Comamonadaceae* – all of which are known oil-degrading bacteria (Atlas et al., 2015; Kweon et al., 2011; Rodgers-Vieira et al., 2015; Yang et al., 2016). Previous genomic analyses have shown that numerous bacteria within the taxa *Burkholderiales*, including *Polaromonas*, have the metabolic potential to degrade a broad spectrum of aromatics as they possesses genes used in many peripheral and central ring-cleavage pathways (Hanson et al., 2012; Jeon et al., 2003; Pérez-Pantoja et al., 2012). Moreover, *Polaromonas* spp. were identified as potential dilbit-degraders in a recent study monitoring biodegradation of two types of dilbit (CLB and Western Canadian Select) by bacterial enrichments in freshwater microcosms (Deshpande et al., 2018). In their 72-day experiment using an enriched microbial consortium obtained from sediment contaminated by the 2010 Kalamazoo River dilbit spill, Deshpande et al. (2018) noted that relative abundance of *Polaromonas* and other bacterial genera increased as concentrations of larger aromatic and branched alkane fractions decreased. *Polaromonas* reached its highest abundance on day 40 for CLB and day 72 for Western Canadian Select dilbit. In our study, *Polaromonas* reached its highest relative abundance more than three months following the spill, on Day 104. This slow but consistent increase suggests *Polaromonas* was potentially metabolizing the larger, more complex hydrocarbon fractions that did not undergo immediate weathering.

Hydrocarbon metabolism by bacteria under aerobic conditions results in δ^{13}C-PLFA values that closely mirror that of the carbon source (typically PLFAs are depleted by < 3‰) (Ahad and Pakdel, 2013; Cowie et al., 2010; Hayes, 2001). The observation that all δ^{13}C PLFA values were within 3‰ of the potential carbon sources thus reflects the oxic conditions in soil columns and aerobic biodegradation of DB and CC (Figure 2). The depleted δ^{13}C values for cyc17:0 and the combined 16:1Δ9 and 16:1B in DB and CC relative to the control pointed to uptake of ^{13}C-depleted

459 oil (DB: -29.9‰; CC: -29.3‰) relative to more ^{13}C-enriched soil TOC (-27.3‰). Both PLFAs are
460 biomarkers for gram-negative bacteria and were previously observed to be associated with oil-
461 degrading microbial communities (Ahad et al., 2018; Greenwood et al., 2009). Interestingly,
462 *Polaromonas* strains have been shown to predominantly feature 16:1Δ9 and cyc17:0 in their PLFA
463 profiles (Kämpfer et al., 2006; Sizova and Panikov, 2007). The more positive δ^{13}C values for i-
464 15:0 and a-15:0 show the carbon source to be background soil TOC rather than the ^{13}C-depleted
465 oil. The growth of the subset of soil microbes preferring soil organic matter was not promoted by
466 the presence of DB or CC, and as such the relative abundances of i-15:0 and a-15:0 decreased as
467 PLFAs associated with oil degradation (e.g., cyc17:0) increased (Figure 1). There were no
468 discernable trends in δ^{13}C values over time or between depths, likely due to the small differences
469 between the δ^{13}C of TOC and the two oils (2.0 and 2.6‰ for DB and CC, respectively), relative to
470 the margin of error in δ^{13}C measurements (± 0.5‰).

471 Sorption onto organic matter and heterogeneous distributions between samples make
472 determining mass losses of TPHs in soils and sediments difficult to quantify (Ahad et al., 2010;
473 Slater et al., 2005). In the shallow subsurface, dispersion and advection play important roles in
474 controlling contaminant concentrations over time (Kalbe et al., 2008). As shown on Table S2, there
475 were no consistent changes in TPH concentrations in the top, middle and bottom core sections
476 over the course of this experiment. In contrast, the significant differences in Δ^{14}C-PLFA values
477 between DB/CC and control columns on Days 34, 62, and 104 (Figure 3) provided unequivocal
478 evidence for microbial uptake of petroleum-derived carbon throughout the exposure period.
479 Although TPH concentrations showed no clear temporal trends, in general the Δ^{14}C values of bulk
480 PLFAs reflected the level of petroleum contamination in the soil. For example, higher

concentrations of TPHs in the top depths of DB and CC cores corresponded to lower Δ^{14}C-PLFA values that indicated greater levels of microbial uptake of oil (Tables S2 and S3).

Using the two end-member mass balance (Eq. 3), we calculated the relative contribution of carbon sources to the ^{14}C contents of the PLFAs in the DB and CC samples (Table S3). Between 2 and 19% and 7 and 23% of microbial PLFA carbon was derived from petroleum in columns amended with DB and CC, respectively. Thus, while there was certainly uptake of DB and CC, the background soil organic matter remained the dominant carbon source for microbial communities. The slightly more positive Δ^{14}C-PLFA values in the control (+10.7 ± 38.6‰) compared to bulk soil TOC (-34.7‰) indicated that microbes were preferentially degrading the more modern (i.e., more recently fixed from atmospheric CO_2) and ostensibly labile constituents within the background soil TOC, a finding observed in previous studies (Cowie et al., 2010; Kramer and Gleixner, 2008).

The preferential uptake of more modern background soil TOC at petroleum-contaminated sites has been observed in other studies that determined Δ^{14}C values in bulk PLFAs. In an investigation into carbon sources utilized by the active microbial communities in shallow groundwater systems underlying three petroleum service stations, Ahad et al. (2010) found that higher petroleum concentrations and lower soil TOC levels corresponded to greater utilization of fossil carbon (up to 43%). The preferential microbial utilization of relatively more modern carbon sources was also observed in soils surrounding a former industrial site, where maximum contributions of fossil PAH-derived carbon in microbial PLFAs were found to range from 12 to 71% (Mahmoudi et al., 2013a). In Athabasca oil sands region tailings ponds – systems dominated by fossil carbon sources – Ahad and Pakdel (2013) reported Δ^{14}C values of up to around -600‰ for non-specific PLFAs (e.g., 16:0). Dissolved and particulate organic matter from the Athabasca

River, the main source of freshwater used by bitumen mining companies, was considered the main source for the relatively modern carbon preferred by tailings sediment microbes.

The $\Delta^{14}C$ values of bulk PLFAs in the control at middle, bottom and combined depths ranged from +0.3 to +53.7‰. However, the $\Delta^{14}C$ values in the top depths of the control were significantly more negative with Day 34 and Day 62 values at -28.5 and -46.1‰, respectively (Figure 3, Table S3). This discrepancy can be attributed to the release of volatile organic compounds from neighbouring DB and CC columns contaminating the top depth of the control, leading to potential uptake of ^{14}C-depleted hydrocarbons by control column microbes. This theory is supported by the trends in amplicon sequencing results. During the exposure period, microbial community composition in the top depth of the control was significantly different than in middle and bottom depths ($P < 0.05$). In addition, the microbial community in the control on Day 0 was significantly different from all other days of the exposure ($P < 0.05$).

5. Conclusions

The production and transportation of dilbit is expected to increase in coming decades (CAPP, 2019), leading to a greater risk for accidental release into the environment. Understanding the behaviour of dilbit in the shallow subsurface following a spill is a key component of responsible resource development. Over a 104-day exposure period using large-scale columns, we observed direct evidence of continuous microbial uptake of dilbit in simulated vadose zone systems. Similarities in the microbial response to DB and CC spills demonstrated that, under aerobic conditions, the biodegradation potential of these two oils in the shallow subsurface is equal.

The experiments reported here were carried out using a sandy soil with a TOC content of 1.7 ± 0.6%. Variations in these and other soil parameters (e.g., microbial community composition,

moisture level, etc.) may have led to different levels of natural attenuation in both DB and CC columns. A goal of future work should thus be to examine the shallow subsurface behaviour of DB under a variety of different experimental conditions. Data generated by this study and additional controlled spill experiments can be used to inform the development of effective DB spill response strategies, specifically the potential for biodegradation by natural soil microbial communities in the unsaturated zone to act as a remediation strategy.

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Appendix A. Supplementary data

The following is the Supplementary data to this article:

References

Ahad, J.M.E., Burns, L., Mancini, S., Slater, G.F., 2010. Assessing Microbial Uptake of Petroleum Hydrocarbons in Groundwater Systems Using Natural Abundance Radiocarbon. Environmental Science & Technology 44, 5092-5097.

Ahad, J.M.E., Pakdel, H., 2013. Direct evaluation of in situ biodegradation in Athabasca oil sands tailings ponds using natural abundance radiocarbon. Environmental Science & Technology 47, 10214−10222.

Ahad, J.M.E., Pakdel, H., Gammon, P.R., Mayer, B., Savard, M.M., Peru, K.M., Headley, J.V., 2020. Distinguishing Natural from Anthropogenic Sources of Acid Extractable Organics in Groundwater near Oil Sands Tailings Ponds. Environmental Science & Technology 54, 2790-2799.

Ahad, J.M.E., Pakdel, H., Gammon, P.R., Siddique, T., Kuznetsova, A., Savard, M.M., 2018. Evaluating in situ biodegradation of 13C-labelled naphthenic acids in groundwater near oil sands tailings ponds. Science of the Total Environment 643, 392-399.

Ahad, J.M.E., Pakdel, H., Labarre, T., Cooke, C.A., Gammon, P.R., Savard, M.M., 2021. Isotopic Analyses Fingerprint Sources of Polycyclic Aromatic Compound-Bearing Dust in Athabasca Oil Sands Region Snowpack. Environmental Science & Technology 55, 5887-5897.

Atlas, R.M., Stoeckel, D.M., Faith, S.A., Minard-Smith, A., Thorn, J.R., Benotti, M.J., 2015. Oil biodegradation and oil-degrading microbial populations in marsh sediments impacted by oil from the Deepwater Horizon well blowout. Environmental Science & Technology 49, 8356-8366.

Baedecker, M.J., Eganhouse, R.P., Qi, H., Cozzarelli, I.M., Trost, J.J., Bekins, B.A., 2018. Weathering of oil in a surficial aquifer. Groundwater 56, 797-809.

Bastida, F., Jehmlich, N., Lima, K., Morris, B., Richnow, H., Hernández, T., Von Bergen, M., García, C., 2016. The ecological and physiological responses of the microbial community from a semiarid soil to hydrocarbon contamination and its bioremediation using compost amendment. Journal of Proteomics 135, 162-169.

Beaver, C.L., Atekwana, E.A., Bekins, B.A., Ntarlagiannis, D., Slater, L.D., Rossbach, S., 2021. Methanogens and their syntrophic partners dominate zones of enhanced magnetic susceptibility at a petroleum contaminated site. Frontiers in Earth Science 9, 598172.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology 37, 852-857.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal 6, 1621-1624.

586 CAPP, 2019. 2019 Crude Oil Forecast, Markets and Transportation. Canadian Association of
587 Petroleum Producers, p. 26.

588 Cowie, B.R., Greenberg, B.M., Slater, G.F., 2010. Determination of Microbial Carbon Sources
589 and Cycling during Remediation of Petroleum Hydrocarbon Impacted Soil Using Natural
590 Abundance ^{14}C Analysis of PLFA. Environmental Science & Technology 44, 2322-2327.

591 Crosby, S., Fay, R., Groark, C., Kanī, '., Smith, J.R., Sullivan, T., Pavia, R., Shigenaka, G.,
592 2013. Transporting Alberta oil sands products : defining the issues and assessing the risks.
593 NOAA technical memorandum NOS-OR&R ; 44.

594 Davoodi, S.M., Miri, S., Taheran, M., Brar, S.K., Galvez-Cloutier, R., Martel, R., 2020.
595 Bioremediation of Unconventional Oil Contaminated Ecosystems under Natural and Assisted
596 Conditions: A Review. Environmental Science & Technology 54, 2054-2067.

597 Deshpande, R.S., Sundaravadivelu, D., Campo, P., SantoDomingo, J.W., Conmy, R.N., 2017.
598 Comparative study on rate of biodegradation of diluted bitumen and conventional oil in fresh
599 water, International Oil Spill Conference Proceedings. International Oil Spill Conference, pp.
600 2256-2267.

601 Deshpande, R.S., Sundaravadivelu, D., Techtmann, S., Conmy, R.N., Santo Domingo, J.W.,
602 Campo, P., 2018. Microbial degradation of Cold Lake Blend and Western Canadian select dilbits
603 by freshwater enrichments. Journal of Hazardous Materials 352, 111-120.

604 Dollhopf, R.H., Fitzpatrick, F.A., Kimble, J.W., Capone, D.M., Graan, T.P., Zelt, R.B., Johnson,
605 R., 2014. Response to heavy, non-floating oil spilled in a Great Lakes river environment: a
606 multiple-lines-of-evidence approach for submerged oil assessment and recovery, International
607 Oil Spill Conference Proceedings. American Petroleum Institute, pp. 434-448.

608 Essaid, H.I., Bekins, B.A., Herkelrath, W.N., Delin, G.N., 2011. Crude Oil at the Bemidji Site:
609 25 Years of Monitoring, Modeling, and Understanding. Groundwater 49, 706-726.

610 Everett, L.G., McMillion, L.G., 1985. Operational ranges for suction lysimeters. Groundwater
611 Monitoring & Remediation 5, 51-60.

612 Fahrenfeld, N., Cozzarelli, I.M., Bailey, Z., Pruden, A., 2014. Insights into biodegradation
613 through depth-resolved microbial community functional and structural profiling of a crude-oil
614 contaminant plume. Microbial Ecology 68, 453-462.

615 Green, C.T., Scow, K.M., 2000. Analysis of phospholipid fatty acids (PLFA) to characterize
616 microbial communities in aquifers. Hydrogeology Journal 8, 126-141.

617 Greenwood, P.F., Wibrow, S., George, S.J., Tibbett, M., 2009. Hydrocarbon biodegradation and
618 soil microbial community response to repeated oil exposure. Organic Geochemistry 40, 293-300.

619 Guckert, J.B., Antworth, C.P., Nichols, P.D., White, D.C., 1985. Phospholipid, ester-linked fatty
620 acid profiles as reproducible assay for change in prokaryotic community structure of estuarine
621 sediments. FEMS Microbiology Ecology 31, 147-158.

Hanson, B.T., Yagi, J.M., Jeon, C.O., Madsen, E.M., 2012. Role of nitrogen fixation in the autecology of Polaromonas naphthalenivorans in contaminated sediments. Environmental Microbiology 14, 1544-1557.

Hayes, J.M., 2001. Fractionation of carbon and hydrogen isotopes in biosynthetic processes, Stable Isotope Geochemistry. Mineralogical Soc America, Washington, pp. 225-277.

Hazen, T.C., Prince, R.C., Mahmoudi, N., 2016. Marine Oil Biodegradation. Environmental Science & Technology 50, 2121-2129.

Iturbe, R., Flores, C., Castro, A., Torres, L.G., 2007. Sub-soil contamination due to oil spills in six oil-pipeline pumping stations in northern Mexico. Chemosphere 68, 893-906.

Jeon, C., Park, W., Padmanabhan, P., DeRito, C., Snape, J., Madsen, E., 2003. Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. Proceedings of the National Academy of Sciences 100, 13591-13596.

Kalbe, U., Berger, W., Eckardt, J., Simon, F.-G., 2008. Evaluation of leaching and extraction procedures for soil and waste. Waste management 28, 1027-1038.

Kämpfer, P., Busse, H.-J., Falsen, E., 2006. Polaromonas aquatica sp. nov., isolated from tap water. International journal of systematic and evolutionary microbiology 56, 605-608.

Kassambara, A., 2021. Pipe-friendly framework for basic statistical tests, R Package Rstatix, Version 0.7.0.

Keresztesi, Á., Nita, I.-A., Boga, R., Birsan, M.-V., Bodor, Z., Szép, R., 2020. Spatial and long-term analysis of rainwater chemistry over the conterminous United States. Environmental Research 188, 109872.

King, T.L., Robinson, B., Boufadel, M., Lee, K., 2014. Flume tank studies to elucidate the fate and behavior of diluted bitumen spilled at sea. Marine Pollution Bulletin 83, 32-37.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and Environmental Microbiology 79, 5112-5120.

Kramer, C., Gleixner, G., 2008. Soil organic matter in soil depth profiles: distinct carbon preferences of microbial groups during carbon transformation. Soil Biology and Biochemistry 40, 425-433.

Kweon, O., Kim, S.-J., Holland, R.D., Chen, H., Kim, D.-W., Gao, Y., Yu, L.-R., Baek, S., Baek, D.-H., Ahn, H., 2011. Polycyclic aromatic hydrocarbon metabolic network in Mycobacterium vanbaalenii PYR-1. Journal of Bacteriology 193, 4326-4337.

Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., Venosa, A., 2015. Expert Panel Report on the Behaviour and Environmental Impacts of Crude Oil Released into Aqueous Environments. Royal Society of Canada, Ottawa, ON, Canada, ISBN: 978-1-928140-02-3.

Lewis, J., Martel, R., Trépanier, L., Ampleman, G., Thiboutot, S., 2009. Quantifying the transport of energetic materials in unsaturated sediments from cracked unexploded ordnance. Journal of Environmental Quality 38, 2229-2236.

Li, X., Fan, F., Zhang, B., Zhang, K., Chen, B., 2018. Biosurfactant enhanced soil bioremediation of petroleum hydrocarbons: design of experiments (DOE) based system optimization and phospholipid fatty acid (PLFA) based microbial community analysis. International Biodeterioration & Biodegradation 132, 216-225.

Liao, J., Wang, J., Huang, Y., 2015. Bacterial community features are shaped by geographic location, physicochemical properties, and oil contamination of soil in main oil fields of China. Microbial Ecology 70, 380-389.

Liu, Q., Tang, J., Gao, K., Gurav, R., Giesy, J.P., 2017. Aerobic degradation of crude oil by microorganisms in soils from four geographic regions of China. Scientific Reports 7, 1-12.

Mahmoudi, N., Beaupré, S.R., Steen, A.D., Pearson, A., 2017. Sequential bioavailability of sedimentary organic matter to heterotrophic bacteria. Environmental Microbiology 19, 2629-2644.

Mahmoudi, N., Fulthorpe, R.R., Burns, L., Mancini, S., Slater, G.F., 2013a. Assessing microbial carbon sources and potential PAH degradation using natural abundance 14C analysis. Environmental Pollution 175, 125-130.

Mahmoudi, N., Porter, T.M., Zimmerman, A.R., Fulthorpe, R.R., Kasozi, G.N., Silliman, B.R., Slater, G.F., 2013b. Rapid degradation of deepwater horizon spilled oil by indigenous microbial communities in louisiana saltmarsh sediments. Environmental Science and Technology 47, 13303-13312.

Margesin, R., Hammerle, M., Tscherko, D., 2007. Microbial activity and community composition during bioremediation of diesel-oil-contaminated soil: Effects of hydrocarbon concentration, fertilizers, and incubation time. Microbial Ecology 53, 259-269.

McGuire, J.T., Cozzarelli, I.M., Bekins, B.A., Link, H., Martinović-Weigelt, D., 2018. Toxicity assessment of groundwater contaminated by petroleum hydrocarbons at a well-characterized, aged, crude oil release site. Environmental Science & Technology 52, 12172-12178.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8, e61217.

NRCan, 2020. Natural Resources Canada. Crude oil facts. https://www.nrcan.gc.ca/our-natural-resources/energy-sources-distribution/fossil-fuels/crude-oil/what-are-oil-sands/18089.

O'Leary, W., Wilkinson, S., Ratledge, C., 1988. Microbial lipids.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. Package 'vegan'. Community ecology package, version 2, 1-295.

Owens, E., Taylor, E., Marty, R., Little, D., 1993. An inland oil spill response manual to minimize adverse environmental impacts, International Oil Spill Conference. American Petroleum Institute, pp. 105-109.

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D.H., González, B., 2012. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environmental Microbiology 14, 1091-1117.

Pernar, N., Baksic, D., Antonic, O., Grubesic, M., Tikvic, I., Trupcevic, M., 2006. Oil residuals in lowland forest soil after pollution with crude oil. Water, Air, and Soil Pollution 177, 267-284.

Podgorski, D.C., Zito, P., Kellerman, A.M., Bekins, B.A., Cozzarelli, I.M., Smith, D.F., Cao, X., Schmidt-Rohr, K., Wagner, S., Stubbins, A., 2021. Hydrocarbons to carboxyl-rich alicyclic molecules: A continuum model to describe biodegradation of petroleum-derived dissolved organic matter in contaminated groundwater plumes. Journal of Hazardous Materials 402, 123998.

Radović, J.R., Oldenburg, T.B., Larter, S.R., 2018. Environmental Assessment of Spills Related to Oil Exploitation in Canada's Oil Sands Region, Oil Spill Environmental Forensics Case Studies. Elsevier, pp. 401-417.

Rodgers-Vieira, E.A., Zhang, Z., Adrion, A.C., Gold, A., Aitken, M.D., 2015. Identification of anthraquinone-degrading bacteria in soil contaminated with polycyclic aromatic hydrocarbons. Applied and Environmental Microbiology 81, 3775-3781.

Schreiber, L., Fortin, N., Tremblay, J., Wasserscheid, J., Elias, M., Mason, J., Sanschagrin, S., Cobanli, S., King, T., Lee, K., 2019. Potential for microbially mediated natural attenuation of diluted bitumen on the coast of British Columbia (Canada). Applied and Environmental Microbiology 85, e00086-00019.

Schreiber, L., Fortin, N., Tremblay, J., Wasserscheid, J., Sanschagrin, S., Mason, J., Wright, C.A., Spear, D., Johannessen, S.C., Robinson, B., 2021. In situ microcosms deployed at the coast of British Columbia (Canada) to study dilbit weathering and associated microbial communities under marine conditions. FEMS Microbiology Ecology 97, fiab082.

Sizova, M., Panikov, N., 2007. Polaromonas hydrogenivorans sp. nov., a psychrotolerant hydrogen-oxidizing bacterium from Alaskan soil. International journal of systematic and evolutionary microbiology 57, 616-619.

Slater, G.F., White, H.K., Eglinton, T.I., Reddy, C.M., 2005. Determination of microbial carbon sources in petroleum contaminated sediments using molecular ^{14}C analysis. Environmental Science & Technology 39, 2552-2558.

727 Spalding, R.F., Hirsh, A.J., 2012. Risk-Managed Approach for Routing Petroleum Pipelines:
728 Keystone XL Pipeline, Nebraska. Environmental Science & Technology 46, 12754-12758.

729 Stoyanovich, S.S., Yang, Z., Hanson, M., Hollebone, B.P., Orihel, D.M., Palace, V., Rodriguez-
730 Gil, J.L., Faragher, R., Mirnaghi, F.S., Shah, K., 2019. Simulating a spill of diluted bitumen:
731 environmental weathering and submergence in a model freshwater system. Environmental
732 Toxicology and Chemistry 38, 2621-2628.

733 Stuiver, M., Polach, H.A., 1977. Discussion: Reporting of ^{14}C data. Radiocarbon 19, 355-363.

734 Utting, N., Namsechi, B., McMullen, C., Brydie, J., Ahad, J.M.E., 2022. Comparing simulated
735 shallow subsurface spills of diluted bitumen and conventional crude oil. Journal of Contaminant
736 Hydrology 251, 104099.

737 Vestal, J.R., White, D.C., 1989. Lipid analysis in microbial ecology. BioScience 39, 535-541.

738 Vet, R., Artz, R.S., Carou, S., Shaw, M., Ro, C.-U., Aas, W., Baker, A., Bowersox, V.C.,
739 Dentener, F., Galy-Lacaux, C., 2014. A global assessment of precipitation chemistry and
740 deposition of sulfur, nitrogen, sea salt, base cations, organic acids, acidity and pH, and
741 phosphorus. Atmospheric Environment 93, 3-100.

742 White, D.C., Davis, W.M., Nickels, J.S., King, J.D., Bobbie, R.J., 1979. Determination of the
743 sedimentary microbial biomass by extractible lipid phosphate. Oecologia 40, 51-62.

744 Wickham, H., 2011. ggplot2. WIREs Computational Statistics, 3 (2), 180–185.

745 Widdel, F., Knittel, K., Galushko, A., 2010. Anaerobic hydrocarbon-degrading microorganisms:
746 an overview. Handbook of hydrocarbon and lipid microbiology, 1998-2022.

747 Wilkinson, S., Ratledge, C., 1988. Microbial lipids. Academic Press.

748 Yang, S., Wen, X., Shi, Y., Liebner, S., Jin, H., Perfumo, A., 2016. Hydrocarbon degraders
749 establish at the costs of microbial richness, abundance and keystone taxa after crude oil
750 contamination in permafrost environments. Scientific Reports 6, 1-13.

751 Zemo, D.A., Patterson, T.J., Kristofco, L., Mohler, R.E., O'Reilly, K.T., Ahn, S., Devine, C.E.,
752 Magaw, R.I., Sihota, N., 2022. Complex mixture toxicology: Evaluation of toxicity to freshwater
753 aquatic receptors from biodegradation metabolites in groundwater at a crude oil release site,
754 recent analogous results from other authors, and implications for risk management. Aquatic
755 Toxicology 250, 106247.

756 Zhao, J., Verma, M., Verter, V., 2020. Pipeline transportation of crude oil in Canada:
757 Environmental risk assessment using modified diffusion models. Human and Ecological Risk
758 Assessment: An International Journal 27, 1206-1226.
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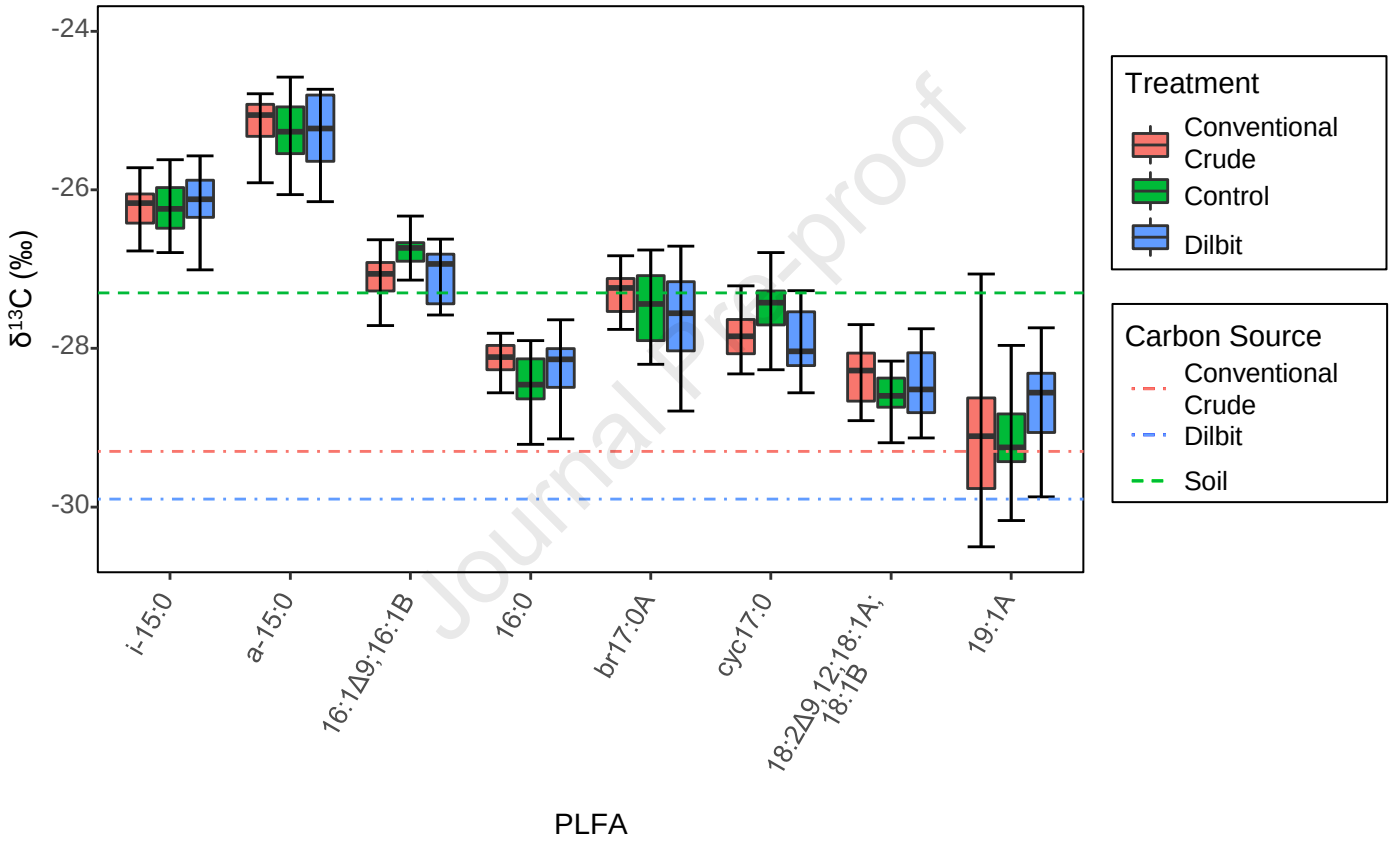
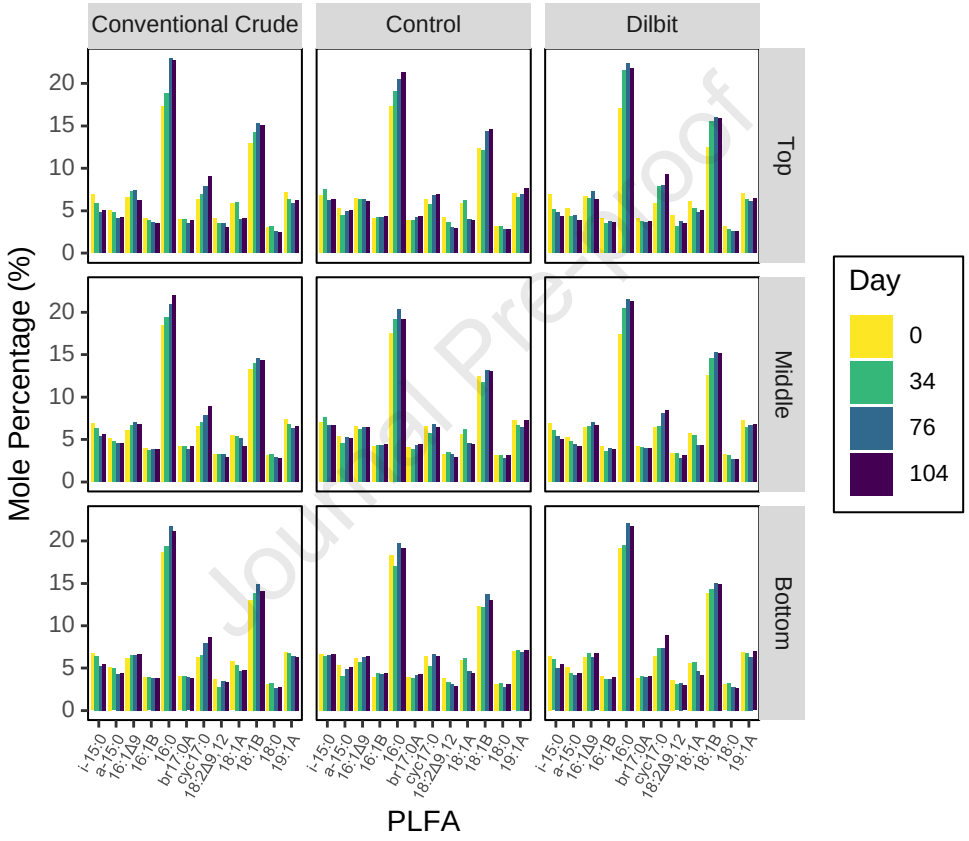
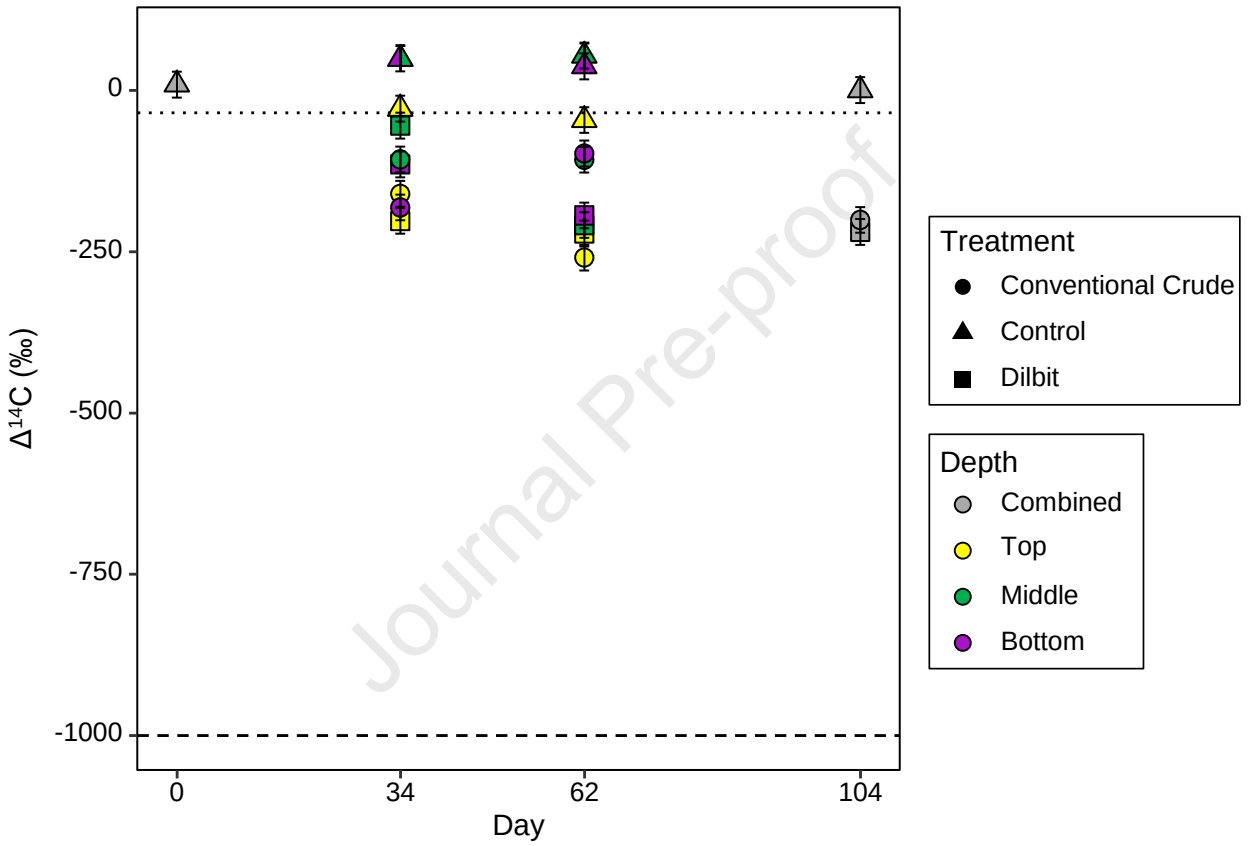
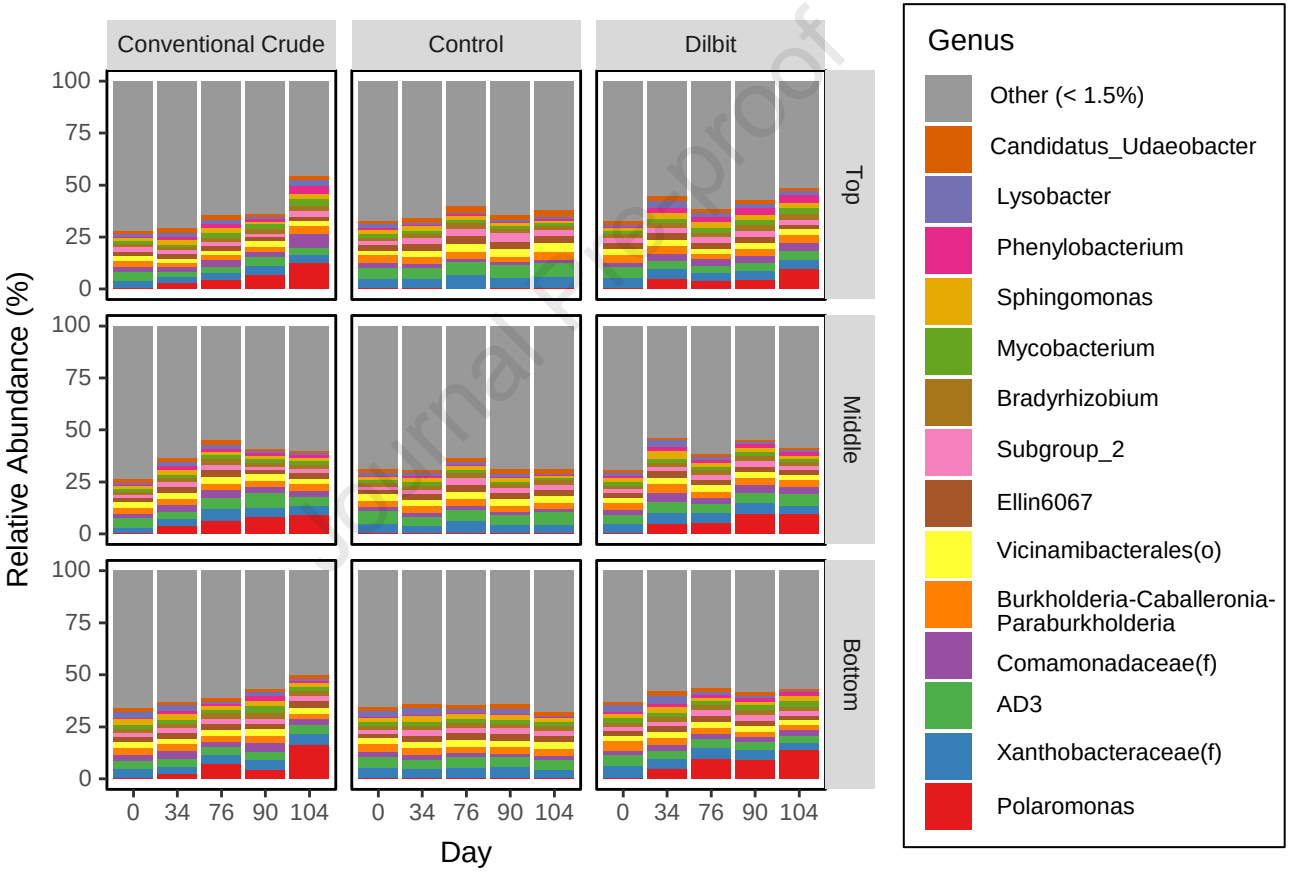
763 **Figure Headings**
764
765 **Figure 1.** Relative abundance distributions (mole percentage) of 12 of the most abundant PLFAs
766 in samples collected from the top, middle and bottom sampling depths during the 104-day exposure
767 period to dilbit and conventional crude.
768
769 **Figure 2.** The average stable carbon isotope ($\delta^{13}C$) values for the most abundant PLFAs over the
770 course of the entire 104-day experiment. Error bars represent standard deviations between samples.
771 Outlier points are not shown but were included in calculations. The horizontal lines denote the
772 $\delta^{13}C$ values of the potential carbon sources: conventional crude (red; -29.3‰), dilbit (blue; -
773 29.9‰) and soil TOC (green; -27.3‰).
774
775 **Figure 3.** Radiocarbon ($\Delta^{14}C$) values for PLFAs at days 0, 34, 62, and 104. Top, middle, and
776 bottom sampling depths were combined for Days 0 (control) and 104 (control, dilbit and
777 conventional heavy crude). Top, middle, and bottom sampling depths were analysed individually
778 for Days 34 and 64 samples. The dotted line represents the $\Delta^{14}C$ value of the soil TOC (-35‰)
779 while the dashed line represents the $\Delta^{14}C$ value of dilbit and conventional heavy crude (-1000‰).
780 Error bars represent ± 20‰ accuracy and precision of $\Delta^{14}C$ measurements.
781
782 **Figure 4.** Microbial community composition at the genus level. Relative abundances are expressed
783 as a percent (%). Facets group column treatments (top x-axis), and depths (y-axis). Genera with
784 an average relative abundance less than 1.5% are collectively labelled as 'Other'. Significant
785 increases in the abundance of *Polaromonas* were observed over time for both the dilbit and
786 conventional crude columns.









Highlights

- Controlled vadose zone spill experiments carried out using large soil-filled columns.
- Dilbit (DB) & conventional heavy crude (CC) showed similar biodegradation over 104 d.
- Up to ~ 20% of carbon in microbial phospholipid fatty acids derived from CC or DB.
- Abundances of *Polaromonas*, a known hydrocarbon-degrader, increased over time.
- Natural attenuation potential for DB similar to CC following a vadose zone spill.

Author statement

Leah M. Mindorff: Writing – original draft, Formal analysis, Investigation, Methodology; **Nagissa Mahmoudi**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing, Resources; **Scott L. J. Hepditch**: Formal analysis, Investigation, Methodology, Writing – review & editing; **Valerie S. Langlois**: Conceptualization, Writing – review & editing; **Samrat Alam**: Formal analysis, Writing – review & editing; **Richard Martel**: Conceptualization, Resources, Writing – review & editing; **Jason M. E. Ahad**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing, Project administration, Resources.

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: