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Microbial Degradation of Cold Lake Blend and Western Canadian Select Dilbits by Freshwater Enrichments

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Abstract

Treatability experiments were conducted to determine the biodegradation of diluted bitumen (dilbit) at 5 and 25 ºC for 72 and 60 days, respectively. Microbial consortia obtained from the Kalamazoo River Enbridge Energy spill site were enriched on dilbit at both 5 (cryo) and 25 (meso) ºC. On every sampling day, triplicates were sacrificed and residual hydrocarbon concentrations (alkanes and polycyclic aromatic hydrocarbons) were determined by GC-MS/MS. The composition and relative abundance of different bacterial groups were identified by 16S rRNA gene sequencing analysis. While some physicochemical differences were observed between the two dilbits, their biodegradation profiles were similar. The rates and extent of degradation were greater at 25 ºC. Both consortia metabolized 99.9% of alkanes; however, the meso consortium was more effective at removing aromatics than the cryo consortium (97.5 vs 70%). Known hydrocarbon-degrading bacteria were present in both consortia (*Pseudomonas*, *Rhodococcus*, *Hydrogenophaga*, *Parvibaculum*, *Arthrobacter*, *Acidovorax*), although their relative abundances depended on the temperatures at which they were enriched. Regardless of the dilbit type, the microbial community structure significantly changed as a response to the diminishing hydrocarbon load. Our results demonstrate that dilbit can be effectively degraded by autochthonous microbial consortia from sites with recent exposure to dilbit contamination.

Keywords

non-conventional oil, diluted bitumen, dilbit, biodegradation, microbial community structure

Highlights

- Dilbit was effectively degraded by mesophilic and cryophilic bacterial enrichments
- Greater reduction of aromatics was achieved in the mesophilic enrichments
- Different bacterial populations degraded the dilbit based on their abundance levels

1. Introduction

Among the known oil sand deposits distributed worldwide, reservoirs found in Alberta (Canada) are the largest and associated with the highest production. Those deposits are concentrated in the northeastern part and are found primarily in Athabasca, Cold Lake, and Peace River regions [1, 2]. Oil sands are a combination of sand, water, and bitumen, the most viscous, heavily biodegraded form of petroleum crude. For transportation purposes, bitumen is blended with either synthetic crude oil (synbit), or diluents such as natural gas condensate, naphtha, or a mixture of light hydrocarbons (dilbit) [3]. Although dilbit may resemble conventional heavy crude oil, there are significant differences in terms of physical and chemical properties. Dilbit has a greater proportion of resins, asphaltenes, sulfur, and metals in addition to having a higher acid number [4, 5]. The composition of bitumen varies across as well as within reservoirs [6]. The variety of diluents used for blending results in inherently heterogeneous dilbit products.

The commercial levels of oil sand and derived products are escalating due to technological developments in oil recovery and demand for unconventional hydrocarbon sources [7]. According to the Canadian Association of Petroleum Producers [8], production will rise from 2.4 million barrels/day in 2015 to about 3.7 million barrels/day in 2030. Such an increase in production implies a much higher transport demand and as a result a higher risk for the occurrence of accidental spills. In recent years, three major dilbit spills were reported: Kinder Morgan spill in Burnaby, Canada (2007), Kalamazoo River Enbridge spill in Michigan, USA (2010), and ExxonMobil's spill in Mayflower, Arkansas, USA (2013). Along with the preventive measures, preparedness to manage such incidents is equally important. Depending on the degradation rates of the hydrocarbon, bioremediation may be an important remediation practice. For the latter to be a viable in situ remediation option in dilbit spills, knowledge on the fate of

dilbit under different environmental conditions is necessary. When compared to conventional crude oil, dilbit shows several distinct properties and while these differences may impact microbial activity, relatively limited information exists regarding the ease and extent of its biodegradability. More importantly, some of the studies have reached different conclusions as far as the levels and rate of dilbit degradation. For instance, Cobanli et al. [9] and King et al. [10] reported biodegradation of dilbit, which is in contrast with others that suggested limited degradation [3, 11-13]. Although differences in bacterial community structure could in part explain differences in the biodegradation patterns observed thus far, none of these studies have identified the bacterial composition of their microbial enrichments.

To address these issues, the main objectives of this work were to gain insight into the biodegradability of diluted bitumen at different temperatures and to identity bacterial groups potentially linked to dilbit degradation. The two types of dilbit implicated in 2010 Kalamazoo (Michigan, USA) spill were chosen for this study (i.e., Cold Lake Blend, CLB and Western Canadian Select, WCS). These products are commonly transported within the United States and gaining insight into their behavior when exposed to microbial activity would be useful to inform future spill planning [14]. The experiments were carried out in a synthetic freshwater medium with enriched microbial consortia obtained from the dredging operations after the Kalamazoo River Enbridge spill. Since temperature can play a crucial role in biodegradation, two sets of experiments were run at 5 and 25 ºC to study degradation in cryogenic and mesophilic conditions. Microbial composition during the course of the experiment was determined for each microcosm using 16S rRNA gene sequencing analyses.

2 Materials and Methods

2.1 Chemicals and reagents

The U.S. Environmental Protection Agency provided non-weathered CLB and WCS dilbits. These were stored in closed amber glass container with Teflon lid in a well-ventilated area according to MSDS requirements [15]. Mineral salts, dichloromethane (DCM), and hexane were obtained from Fisher Scientific (Pittsburg, PA, USA).

2.2 Media

Bushnell-Haas broth was used as medium for microbial support in the enrichments and treatability tests [16]. This medium is commonly used to enrich hydrocarbon degrading bacteria and has been used in many crude oil degradation studies [17, 18]. It contains excess of nitrogen and phosphate, sufficient to support microbial growth when using hydrocarbons as sole carbon source. It should be noted that nitrogen and phosphate are among the environmental limiting factors in hydrocarbon biodegradation in situ [19]. The broth was prepared by dissolving magnesium sulfate (0.2 g/L), calcium chloride (0.02 g/L), monopotassium phosphate (1.0 g/L), dipotassium phosphate (1.0 g/L), ammonium nitrate (1.0 g/L), and ferric chloride (0.05 g/L) in distilled water. This broth was autoclaved at 120 ºC for 15 min in batches of 1 L.

2.3 Microbial enrichment

The original mixed consortium was obtained from dredged sediment following the Kalamazoo River Enbridge Energy spill (2010). Two separate enrichments were grown on Cold Lake Blend dilbit as the carbon source in Bushnell-Haas broth at 5 (cryo) and 25 ºC (meso). These temperatures are representative of the temperatures of winter and summer of the contaminated site from which the sediments samples were originally collected. Then enrichments were

washed, concentrated tenfold, and stored frozen on 10% glycerol at -80 ºC until ready to use in the degradation experiments.

2.4 Experimental setup and Procedure

The degradation of the two different dilbits (WCS or CLB) was tested at 5 (cryo) and 25 ºC (meso). The experimental design layout for this treatability study is summarized in Table A.1. Each dilbit was individually tested and samples were collected in triplicates for the chemical and bacterial analyses. For the 25 ºC setup, 12 sampling events were conducted at 0, 2, 4, 8, 12, 16, 20, 28, 35, 42, 54, and 60 d, while sampling events at 5 ºC were on days 0, 2, 4, 8, 16, 24, 32, 40, 48, 56, 62, and, 72. To account for abiotic losses, 0.5 mL of sodium azide (500 mg/L stock solution) was added to a separate set of flasks or killed controls (KC), which were run in triplicate for each treatment and sampled at the final sampling day. Aliquots (75 µL) of either WCS or CLB were dispensed into all different shake flasks (including KC flasks) containing 100 mL of sterile broth, and then inoculated with 0.5 mL of the meso or the cryo enrichment for the experimental setup at 25 and 5 °C, respectively. Flasks were mixed (200 rpm) with rotary shakers in temperature-controlled rooms. At a given sampling day, three flasks were sacrificed per dilbit and temperature treatments. Bacterial activity was stopped by adding 0.5 mL of the sterilant to the sample flasks prior to analysis. To separate media and remaining dilbit, liquid-liquid extractions were carried out with DCM. Each replicate was extracted with 60 mL of DCM. Oil extracts were then filtered through anhydrous sodium sulfate to remove any water and concentrated down to 1 mL under nitrogen gas. Solvent exchange was performed by adding 9 mL of hexane to precipitate asphaltenes. These extracts were then analyzed to measure residual alkanes and polycyclic aromatic hydrocarbons (PAHs).

2.5 Hydrocarbon Analysis

2.5.1 Laboratory Methodology

Residual alkane and PAH concentrations were quantified with an Agilent 7890A gas chromatograph and an Agilent 7000 mass selective detector triple quadrupole. This instrument was equipped with a DB-5 capillary column from J&W Scientific (30 m × 0.25 mm I.D. and 0.25 µm film thickness) and operated in splitless mode [20]. The target analytes were normal aliphatics ranging in carbon number from 10 to 35 as well as the branched alkanes pristine (PR) and phytane (PH). Analysis also included 2, 3 and 4-ring PAH compounds and their alkylated homologs [i.e. C_{0-4} naphthalenes (NAP), C_{0-4} phenanthrenes (PHE), C_{0-3} fluorenes (FLU), C_{0-3} dibenzothiophenes (DBT), C_{0-4} napthbenzothiophenes (NBT), C_{0-1} pyrenes (PYR), C_{0-3} chrysenes (CHY)]. Multi reaction monitoring (MRM) mode was used for analyte detection. The reportable detection limit for GC/MS was 0.05 mg/L. Concentrations of the measured alkanes and PAHs were summed up to determine the total alkane and total PAH concentrations, respectively.

2.5.2 Statistical Analyses

The hydrocarbon degradation data was fitted to first order model by non-linear regression. Also, two sample *t*-tests were conducted assuming the following null hypotheses: 1) no differences exist in the biodegradability of two different dilbits and 2) temperature has no influence on the biodegradability of diluted bitumen. Prism 7 (GraphPad Software, CA) was used for all the statistical analyses.

2.6 Molecular Analysis

2.6.1 DNA Extraction

Samples were collected for 16S rRNA gene sequencing analyses on days 0, 2, 16, 35, and 60 for 25 ºC and on days 0, 2, 16, 40, and 72 for 5 ºC. On these sampling days, biomass from each enrichment was obtained by spinning 1.5 mL of media at 10,000 rpm for 10 min. Supernatants were decanted without disrupting cell pellets and the harvested biomass was stored at -80 °C until nucleic acid extractions. DNA was extracted from the frozen biomass with the PowerLyzer™ PowerSoil® DNA kit following the manufacturer's instructions (MoBio Laboratories, Solana Beach, CA).

2.6.2 16S rRNA PCR and Sequencing

The 16S rRNA gene was amplified via the polymerase chain reaction (PCR) for each sample by using DNA extracts and barcoded primers 515F/806R [21] as described by Kapoor et al. [22]. The PCR conditions were as follows: denaturing for 3 min at 95 °C, 35 cycles at 95 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, followed by 10 min of final primer extension at 72 °C. The PCR products were visualized on an agarose gel to confirm product sizes and then pooled in an equimolar ratio to generate the sequencing library. Next generation sequencing was performed at the Cincinnati Children's Hospital DNA Core facility (Ohio, USA) on an Illumina MiSeq sequencer (Illumina, San Diego, CA) using pair-end 250 bp kits. Sequence reads were processed with MOTHUR v1.25.1 [23] as previously described [24]. Sequence reads with overall low-quality scores (Phred ≤ Q30), containing homopolymers larger than eight nucleotides or one ambiguous base were removed from further analysis. Sequences with ≥ 97% similarity were aligned and sorted into operational taxonomic units. Taxonomic classification and identification of nearest neighbors were performed with the Classifier tool in Ribosomal Database Project II (release 10.28), and NCBI BLASTn, respectively [25].

2.6.3 Statistical Analyses

Alpha diversity was determined from the taxa table through observed species and Shannon diversity metrics. One-way ANOVAs were performed for the different enrichments to test the hypothesis that there were no differences in alpha diversity between dilbit types as well as between temperatures. Non-metric multidimensional scaling (NMDS) was used to test the hypothesis that the microbial community structure within a same treatment was similar and significantly different from the other treatment. Taxa tables were used to construct two-dimensional NMDS plots from Bray Curtis dissimilarity. The lowest stress configuration was chosen from 50 iterations of plot construction. Stress values were calculated by the default stress

calculation in the NMDS command in the ecodist package [26]. To test if samples from the same treatment (e.g., CLB, day 2, 5 ºC) were significantly different from each other PERMANOVA [27] analysis was performed on the Bray Curtis dissimilarity matrix with the Adonis function in the vegan package [28] in R. PERMANOVA analysis was performed by using 999 permutations to test if there were any significant differences between the temperature and between the types of dilbit used in this study. Additionally, time-decay analysis of changes in community composition was performed for different temperatures and within temperatures between treatments. This approach is used to further determine if the community composition was changing at different rates between different temperatures. The Bray-Curtis dissimilarity was determined for each sample in a pair-wise manner. These pair-wise dissimilarities were then plotted based on time between each comparison and regression line generated for each case. Differences in the slope of the line are useful in determining if there is a different rate of change in community composition between the selected conditions. ANCOVA analysis was performed to determine if the differences were significant.

3. Results

3.1 Dilbit Characterization

Both WCS and CLB are classified as heavy sour unconventional crude oil. WCS is composed of bitumen from Athabasca basin, whereas CLB has bitumen from Cold Lake region. Bitumen from the Athabasca basin is heavier than the Cold Lake basin because of its higher asphaltene content (~17 vs ~11 %, respectively), and hence it is blended with conventional crude and synthetic crude oils along with the diluent to meet pipeline specification [29]. Whereas bitumen from Cold Lake region is blended with only the diluents.

The physical properties for both CLB and WCS were similar to values previously reported [30-34]. API gravity, density, and sulphur content of two dilbits were similar (within 5 % of each other), but CLB had greater viscosity and total acid number than WCS (Table 1). Relative proportion of total alkanes and total PAHs were similar for both dilbits. Distribution of *n*-alkanes for the two dilbits was similar, with concentrations decreasing with the carbon chain length. Nevertheless, differences in branched alkane composition were observed namely, *n*-C17/PR and *n*-C18/PH ratios for WCS were lower than in CLB (Fig. A.1A). Overall WCS contained 22.5 % more total alkanes as compared to CLB. Also, WCS contained higher content of NAPs (37 vs 26 %) with PHEs (22 vs 28 %) and DBTs (22 vs 30 %) being more abundant in CLB. No differences were noted in the concentrations of other PAH groups (Fig. A.1B). Greater quantities of lower molecular weight hydrocarbons like alkanes and NAPs were observed in WCS which could explain its lower viscosity in contrast to that of CLB [5]. Some minor differences in the amounts of micro carbon residue, sediment, salt, metals, light alkanes, and BTEX (benzene, toluene, ethylbenzene and xylenes) has been reported, which reflects the typical variability in chemical composition of dilbits [35].

3.2 Biodegradation of Alkanes

Panels A and B in Fig. 1 show the time series removal of total alkanes for both dilbits at 5 and 25 ºC, respectively. At 5 ºC, the first-order degradation rate coefficients were calculated to be 0.52 ± 0.09 d^{-1} for CLB and 0.48 ± 0.01 d^{-1} for WCS. No significant difference was observed between the above calculated rate constants for each dilbit (p = 0.232). As expected, rates were higher (2 to 3-fold) at 25 ºC. In this case, the rate coefficients values were 1.13 ± 0.04 and 1.14 ± 0.01 d^{-1} for CLB and WCS, respectively. Again, no significant difference between dilbits was observed at this temperature (p = 0. 7589).

As previously reported, *n*-alkanes were metabolized faster than *iso*-alkanes [36-38]. The meso culture degraded *n*-alkanes to below detection limits within the first two days, with the *iso*-alkanes being removed 8 days later. At 5 ºC the *n*-alkanes disappeared by day 8 while *iso*-alkanes persisted for 40 d (Fig A.2). The *iso*-alkanes degraded 3.5 times faster at 25 ºC than at the lower temperature and the differences in degradation rates as a function of dilbit type were not statistically different at both temperatures ($p = 0.5605$ at 5 ºC and $p = 0.719$ at 25 ºC).

First-order coefficient rates for individual alkanes were not calculated for the 25 ºC treatment as the concentration of the alkane fraction dropped below detection limit within the first 48 hours. Coefficients calculated for individual alkanes at 5 ºC showed a negative correlation with the carbon number suggesting that the degradation rates decreased with the increase in carbon number (Table A.2). Overall, first order rate constants for individual alkanes were marginally higher for CLB.

In terms of degradation extent, the meso culture removed 99.9 % of the total alkanes by day 12, while the cryo culture needed more than 40 d for total alkanes to degrade primarily owing to the persistence of branched alkanes.

3.3 Biodegradation of Aromatics (PAHs)

For total PAHs, the enrichments with both dilbits exhibited comparable degradation rates and *t*-tests confirmed no differences in the biodegradation rates (cryo, Fig. 1C, $p = 0.0944$; meso, Fig. 1D, $p > 0.9999$). As shown in Fig. 1, at 5 ºC, a lag period of 4 d was noted for both dilbits which was not the case at 25 °C. When compared with the microcosm at 5 °C, PAHs were metabolized by the meso culture 2 and 2.5 times faster in the WCS and CLB microcosms, respectively. By the end of the experiment, approximately 97.5 % of total PAHs degraded at 25 ºC, while 30 % remained at 5 ºC.

Figures 2 and 3 show the time series concentrations of different aromatic groups (2-, 3-, and 4-rings and their alkyl derivatives) and per cent removal of individual PAH compounds, respectively. The differences in the rate coefficients of 2- and 3-ring PAHs (i.e., $C_{0\text{-}1}$-NAP, C_0-PHE, C_0-FLU) between the two dilbits at a given temperature were determined to be statistically significant owing to their dissimilar initial concentrations (Table A.3). Conversely, the degradation rates of NBT, PYR, and CHY were similar for both dilbits. In the case of NAPs, complete removal (i.e., below detection limits) was achieved at 25 ºC by 12 d (Fig. 2A). At 5 ºC, $C_{0\text{-}3}$-NAP disappeared within 40 d (Fig. 2B) and the concentration of C_4-NAP was reduced by 80-85 % after 72 d (Fig. 3). The meso culture almost completely degraded (i.e., > 97 %) 3-ring aromatics (PHE, FLU, and DBT) (Fig. 2 D and F), while 35-40 % of these analytes remained at 5 ºC (Fig. 2, C and E) which mainly comprised of $C_{3\text{-}4}$-PHE, C_3-FLU, and, C_3-DBT (Fig. 3). NBT compounds persisted at 5 ºC (Fig. 2G), whereas at 25 ºC, their concentration dropped by 92 % (Fig. 2H). A significant decrease in 4-ring PAH concentration was observed at 25 ºC (Fig. 2J), while at 5 ºC less than 10 % of PYR + CHY was degraded (Fig. 2I).

3.4 Characterization of microbial community

3.4.1 Microbial enrichment

Analysis of 279,807 16S rRNA gene sequences from all the treatments showed that several bacterial groups were present in the meso and cryo enrichments. We used the sequencing data to identify the member of the bacterial community and as a proxy to estimate relative abundance. While difference in rRNA gene copy numbers is different for some of the bacteria identified, we used the data study to overall temporal trends. Although the bacterial composition was similar in both of the enrichments, there were notable differences in the relative abundance of some bacterial groups. On day 0, 10 phyla were detected in both consortia. The dominant phyla were

the Proteobacteria (74 ± 7 and 73 ± 3 % for meso and cryo, respectively), the Actinobaceria (24 ± 7 and 16 ± 4 %), and the Bacteroidetes (3 ± 1 and 10 ± 1%) (Fig. A.4A). The most abundant class of Proteobacteria in the cryo enrichment was the beta-Proteobacteria (41 ± 3%), while in the meso enrichment, gamma-Proteobacteria (41 ± 6%) was the most abundant class (Fig. A.4B). Other classes such as alpha-Proteobacteria, Actinobacteria, and Saprospirae were present in the enrichments at less than 20 % abundance. A total of 190 different genera were identified from the DNA extracts obtained at day 0. *Pseudomonas*, *Rhodococcus*, and *Hydrogenophaga* were the prominent genera present in both enrichments, but their abundances varied (Fig. 4). For example, 21 ± 4 % of the sequences in the meso enrichment were linked to *Pseudomonas* versus 9 ± 1 % in the cryo enrichment. *Rhodocoocus* represented 15 ± 4 and 19 ± 8 % of the sequences in cryo and meso enrichments, respectively. The cryo enrichment was also predominated by *Sulfuritalea* (10 ± 3 %), *Polaromoas* (9 ± 2 %), and *Parvibaculum* (6 ± 1 %), whereas unclassified *Xanthomonadaceae* (5 ± 1 %) was the other dominant genus in the meso enrichment. Genera with abundance less than 1% were grouped together and labeled as other. This group represented more than 150 different genera (e.g., *Acinetobacter*, *Pedobacter*, *Achromobacter*, *Aquabacterium*) out of which around 36 genera had less than 0.1 % in relative abundance (e.g. *Nitrobacter*, *Variovorax*, *Thalassospira*).

3.4.2 Treatability results

Throughout the study, the microbial community structure of both enrichments changed considerably; however, there were no observable differences when the two different dilbit types were compared. For example, by day 2, *Pseudomonas* sp. comprised up to ~ 90 % of the total community in meso enrichment, which represented more than a four-fold increase from day 0 measurements. The same group decreased in relative abundance by day 16 (i.e., approximately

60 %). The community profile did not change from day 16 until the end of the experiment (day 60) in either one of the meso treatments. Even though *Pseudomonas* dominated the microbial community in meso consortium throughout the time course, other genera like *Hydrogenophaga*, *Parvibaculum* and unclassified members of *Xanthomonadaceae* and gamma-Proteobacteria showed high relative abundance (Fig. 4A). In contrast, no single genus dominated the cryo enrichment (Fig. 4B) and no substantial change in bacterial dynamics was observed after two days. By day 16, *Pseudomonas*, *Rhodococcus*, *Hydrogenophaga*, *Acidovorax*, and *Arthrobacter* were detected in high relative abundance. Over time, the relative abundance of *Pseudomonas* and *Rhoococcus* decreased whereas it went up for *Hydrogenphaga*, *Acidovorax*, *Arthrobacter*, *Polaromonas*, *Sulfuritalea* and *Verrucomicrobium*.

While there were no differences between the overall community profiles between the two dilbits, differences were noted when temperatures were taken into account. Specifically, no significant differences were detected in observed species and Shannon diversity when treatments with two different dilbits were compared ($p = 0.916$ and $p = 0.901$) (Fig. A.5A and B). Nevertheless, statistical analysis showed significant differences in Shannon diversity between the two temperatures ($p < 0.001$), however, the difference in observed species was not significant ($p = 0.443$) (Fig. A.6A and B). These results indicate that while similar number of taxa was found at 5 and 25 °C, their evenness varied between the two temperature conditions evaluated. Multivariate analysis showed that for a given dilbit treatment similar taxa were present and microbial community shifted with time (Fig. 5). The changes in community structure over time were more significant at 5 °C as the Bray-Curtis dissimilarity between samples within the 5 °C treatments was greater than 25 °C (Fig. 5B). PERMANOVA analysis suggested that there were no significant differences in taxa composition between CLB and WCS ($p = 0.953$) whereas the

taxa composition was statistically different (p = 0.001) between the two temperature conditions. The stress value for these NMDS plots was 0.16 which indicates that the plot provides good representation of the data in reduced dimension. Time-decay analyses showed that there is a significant difference in the rate of change in the community composition for the meso and cryo treatments (Fig. A7). ANCOVA analysis indicated that these differences are significant (p = 3.926×10^{-14}).

4. Discussion

The biodegradation data for two types of dilbit under two temperature conditions were reported in this study. Concentrations for KCs at the end of each experiment were similar to the day 0 sample (alkanes: p = 0.7267, p = 0.5922 and PAHs: p = 0.8192, p = 0.5097 for CLB and WCS, respectively), indicating that abiotic losses did not contribute to dilbit disappearance (Fig. A.3). Although the dilbits herein studied contain bitumen from different origins (Athabasca Basin and Cold Lake Basin), no major differences were observed in their basic properties (e.g., total acid number, total sulphur, API gravity). Also, the extent of biodegradation and the rate of degradation were not statistically different. The biodegradation rates were ranked as follows: linear alkanes > low molecular weight PAHs (C_0-NAP, C_0-PHE) > branched alkanes > other PAHs and their alkylated forms. At 25 °C unsubstituted NAP and PHE degraded within first 4 days of incubation and within 16 days at 5 °C. Degradation of pristane and phytane was slower as compared to these PAHs as they took more than 8 and 40 d to degrade completely at 25 and 5°C respectively (Fig. A.2). This observation did not agree with previous studies in which branched alkanes were more susceptible to microbial uptake compared to these aromatics, although this pattern is not universal [reviewed in 39, 40]. The degradation rates of individual

PAHs were observed to be inversely proportional to number of rings and alkylation degree as previously reported [41, 42].

Other studies suggested that dilbit would withstand microbial degradation given that bitumen has already undergone severe natural weathering [3, 12, 13]. Nevertheless, we observed significant biodegradation of the aliphatic and aromatic fractions of CLB and WCS dilbits. The extent and pattern of dilbit degradation demonstrated by the enrichments used in this study were distinct to previously reported findings. King et al. [10] reported minimal degradation rates for total alkanes (k = 0.0014 d^{-1}) and PAHs (k = 0.0005 d^{-1}) in CLB after 13 days at around 19 °C. King et al. [10] observed approximately 29% decrease in total alkanes and approximately 14% reduction in total PAH concentration whereas in this study, by day 13, total alkanes and PAHs concentrations were reduced by 99.99 and 84% respectively at 25 °C (Fig. 1A and B).The degradation rates obtained in this study at 5 °C (Fig. 1A and C) are greater than the rates reported by King et al. [10] for both alkanes and aromatics (alkanes: 0.52 ± 0.09 vs 0.0014 d^{-1}, PAHs: 0.05 ± 0.02 vs 0.0005 d^{-1}). Cobanli et al. [9] also investigated the biodegradation of naturally and chemically dispersed CLB and found complete degradation of alkanes, while PAH concentration decreased by approximately 20% after 42 d of incubation. Although they observed complete degradation of total alkanes, the degradation rates were much lower compared to values reported in our study at 25 and 5 °C (0.14 d^{-1} vs 0.52 ± 0.09 and 1.13 ± 0.04 d^{-1} at 5 and 25 °C, respectively). These differences can be explained in terms of experimental set-up. We used an artificial freshwater medium and consortia obtained from a dilbit impacted sediment, whereas the other studies used freshly collected surface seawater that was not previously exposed to dilbit. Generally, in clean environments only 0.1% of the microbial community have the ability to uptake hydrocarbons, while in sites with history of oil pollution, oil degraders are often the most

abundant group [19]. Limited availability of hydrocarbon utilizers can explain the lower efficiencies in these studies. For example, many studies have reported the greater degradation potential of microbial communities from hydrocarbon contaminated sites as compared to communities in oil free environment [43-45]. Indeed, chronic exposure to natural oil seeps has been argued as the reason for the fast response to the Gulf of Mexico spill [46]. Thus, while physicochemical factors (e.g., temperature, nutrients, metals) are important in the activity and survival of aquatic microbial groups, previous exposure to hydrocarbon contaminants can impact the extent and rate of hydrocarbon degradation.

When it comes to microbial uptake of heavy oils, Prince et al. [47] studied the biodegradation of heavy fuel oil IF30 that, similar to dilbit, results from a blend of lighter and heavier hydrocarbons and has a density in the same range as CLB and WCS (approximately 930 kg/m^3). The Prince et al. [47] study was carried out with a consortium collected from New Jersey coast. Similar to our observations at 25 °C, the authors reported total degradation of *n*-alkanes within the first week and complete removal of 3-ring PAHs in three weeks. Comparable degradation extents were also achieved for heavier PAHs (CHY ~ 60%). Another biodegradability study conducted with heavy fuel oil IFO-120 and mixed cultures from Gulf of Mexico [48] reported complete removal of alkanes as observed in this study. In our work, the removals of total PAHs at 5 and 25 ºC were 70 and 97.5 %, respectively (Fig. 1C and D), while Zhuang et al. [48] reported approximately 77 and 81% reduction in total PAH concentrations. The differences in removal efficiencies of enrichments at 25 ºC were mainly due to inability of Gulf of Mexico cultures to degrade higher alkylated forms of 3- and 4-ring PAHs. This suggests that dilbit and heavy fuel oils show relatively similar response to the microbial attack, that is, in the succession of degradation of the different hydrocarbon fractions.

Temperature influenced the biodegradation of dilbit. Rapid and almost complete degradation of alkanes and PAHs was observed in the 25 ºC treatments. The cryo culture metabolized most of the alkanes, NAPs and C_{0-2} homologues of 3-ring PAHs, but residual concentrations of C_{3-4}-PHE, C_3-FLU, C_3-DBT and 4-ring compounds were observed. Several studies have reported prolonged biodegradation of crude oils at lower temperatures because of limited microbial activity, slower enzymatic metabolism, and poor solubility of hydrocarbons which results in reduced bioavailability, increased viscosities, and reduced evaporation of toxic volatile petroleum products [49-53], and that temperature can impact microbial community structure [19, 41].

While sediments were collected from the same area (Kalamazoo River), they were enriched separately at two temperatures (5 and 25 ºC) prior to the experimental work. Initially, both meso and cryo enrichments were rich in diverse oil degraders (e.g. *Pseudomonas, Rhodococcus, Parvibaculum, Sphingobium*) and they showed comparable bacterial composition. Previous studies have isolated these bacteria from hydrocarbon polluted sites. For instance, Jurelevicius et al. [54], Patel et al. [55], Kostka et al. [56] and Sutton et al. [57] reported abundance of the members of Proteobacteria and Actinobacteria in petroleum contaminated environments. Golby et al. [58] showed that *Pseudomonas*, *Acidovorax*, and *Hydogenophaga* dominated microbial biofilm communities from the settling basins used to store waste byproducts from oil sand mining. These genera that were also identified in our study. Although similar bacteria were initially present in our two consortia, their abundances were distinct suggesting that they utilized dilbit to different extent. Concomitant with the changes in dilbit concentrations as biodegradation progressed, a pronounced shift in microbial community structure was observed at the genus level, while no significant changes were observed at the

phylum or class levels. The meso consortium was dominated by *Pseudomonas* sp. (> 50%) whereas the microbial community in the cryo enrichment remained diverse. The Shannon index of the cryo consortium (3.8) was higher than the meso (2.8) indicating that the shift in community structure was more pronounced at the lower temperature (Fig. A.6). Interestingly, using samples collected from the Shandong Province (China), Meng et al. [59] reported that lower temperatures decreased bacterial richness in seawater studies. Similar findings were observed by Bargiela et al. [60] in samples collected from sites in the Mediterranean and Red Seas. While several bacterial genera were shared in both seawater and freshwater studies, the different outcome in bacterial richness may be explained by the differences in catabolic diversity of the abundant halotolerant bacteria in marine settings [60].

Pseudomonas was observed to be one of the major bacterial groups which can be linked to the bio-transformation of dilbit at both temperatures tested. The capability of *Pseudomonas* to metabolize hydrocarbons was recognized more than 75 years ago [61]. Studies have reported degradation of alkanes and various PAHs by *Pseudomonas* spp. at both cold (4-5 °C) and warm (20-25 °C) temperatures [62-65]. In our study,, *Pseudomonas* increased considerably in the 25 ºC enrichment by day 2 (from 21 ± 4 to 91 ± 1%) and remained the most abundant genus thereafter suggesting that they may have played an important role in dilbit degradation (Fig. 4A). At 5 °C, we saw an increase in the abundance of *Pseudomonas* until day 16 where degradation of linear alkanes and naphthalenes was noted, but this genus decreased gradually which suggests it might not contribute to the degradation of the higher aromatics and branched alkanes. Depletion of these fractions started around day 16 and was concomitant with an increase in the abundance of other potential aromatic hydrocarbon utilizers such as *Rhodococcus*, *Arthrobater*, *Acidovorax*, *Hydrogenophaga*, and *Polaromonas* genera (Fig 5B)[63, 66-69].

Our study demonstrated that dilbit can be degraded by bacteria from freshwater sediments. As the original enrichment was generated from a dilbit spill, our data suggest that dilbit degradation may be faster in freshwater environments that have previously been exposed to crude oil hydrocarbons, as the environment may be selecting for bacterial communities capable of a rapid response [70]. These findings are also relevant to those interested in exploring the potential of autochthonous microbial consortia in bioaugmentation studies [71]. Sequencing analyses identified several bacteria that have previously been identified as hydrocarbon degraders, some of which have been detected in both freshwater and marine systems (e.g., *Hydrogenophaga*, *Alcalinovorax*, *Novosphingobium*, *Pseudomonas*, *Dokdonella*, *Parvibaculum*, *Microbacterium*). The cosmopolitan nature of these species and their known degradation capabilities could be used as part of the remediation toolbox as far as predicting the biodegradation potential of a given site and their use as sentinel groups when evaluating remediation practices [72]. Knowing the identity of these bacteria (i.e., via sequencing analyses) is of great value in the development of novel molecular methods (e.g., qPCR assays) that could be used to detect and quantify bacterial populations implicated in dilbit degradation.

Although many well-known hydrocarbons degraders were present in both consortia, meso enrichment metabolized dilbits more effectively, further highlighting the importance of temperature as previously suggested [73]. Our data also showed shifts in the abundant bacterial taxa over time, suggesting that successions in population abundance may be linked to the sequence of specific alkanes and aromatic hydrocarbons degraded during the incubation period. While additional studies are needed to confirm the latter, several studies have shown the presence of multiple species of hydrocarbon degraders in oil polluted ecosystems, and that this functional redundancy is needed for efficient hydrocarbon removal [74, 75]. As biodegradation

of any crude oil depends on various biotic and abiotic factors, in depth research is required to gain more knowledge regarding microbial degradation of diluted bitumen under various conditions (e.g., different levels of nitrogen and phosphorus), and how these conditions impact the microbial ecology of hydrocarbon degrading bacteria in natural settings. Future studies should also consider studying other classes of dilbit to determine how different variations in their chemical composition may impact their degradation rates.

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Table 1. Physical and chemical properties of two diluted bitumen

Properties		Cold Lake Blend (CLB)	Western Canadian Select (WCS)
API Gravity at 15 ºC	º API	21.7	22.1
Absolute Density at 15 ºC	kg/m^3	922.8	920.7
Total Acid Number (TAN)	mg KOH/g	1.03	0.86
Total Sulphur,	mass %	3.68	3.40
Kinematic Viscosity at 15 ºC	cSt	230.7	199.7
Total Alkanes	µg/g of dilbit	3100	4000
Total PAHs	µg/g of dilbit	3540	3845

***Note**: Dilbit characterization was done by Maxxam Analytical International Corp. (Petroleum Technology Center, Edmonton, Canada)

Figure Legends

Figure 1. Biodegradation of total alkanes (A, B) and total PAHs (C, D) at 5 ºC (A, C) 25 ºC (B, D)

Figure 2. Biodegradation of naphthalene (NAP) homologues (A, B), phenantheren (PHE) + fluorene (FLU) homologues (C, D), dibenzothiophenes (DBT) homologues (E, F), napthbenzothiophene (NBT) homologues (E, F), and pyrene (PYR) + chrysene (CHY) homologues (G, H) at 5 ºC (left panels) and 25 ºC (right panels)

Figure 3. Percent removal of individual PAH compound

Figure 4. Microbial community composition at genus level

Figure 5. NMDS Plots - Comparison of molecular Community Structure between (A) dilbit type and (B) temperature

Figure 1. Biodegradation of total alkanes (A, B) and total PAHs (C, D) at 5 ºC (A, C) 25 ºC (B, D)

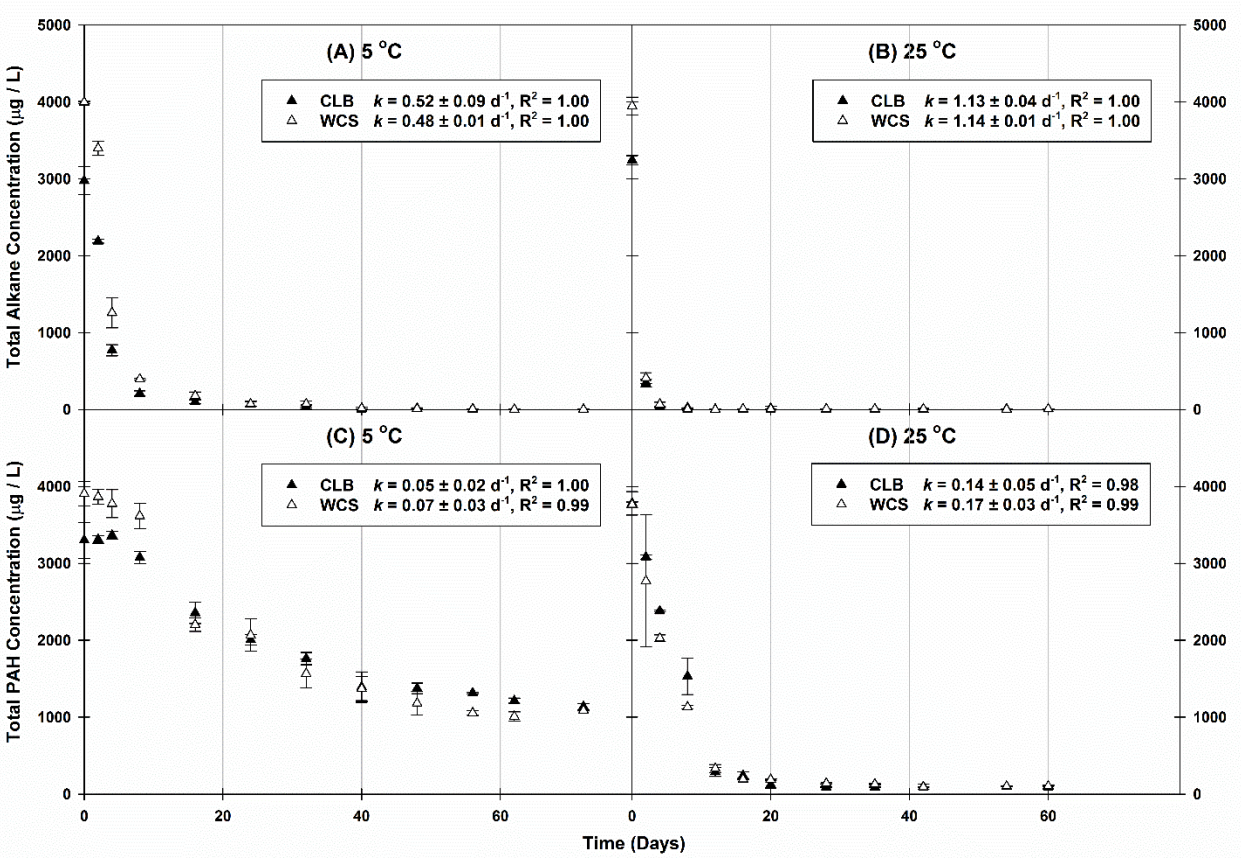


Figure 2. Biodegradation of naphthalene (NAP) homologues (A, B), phenantheren (PHE) + fluorene (FLU) homologues (C, D), dibenzothiophenes (DBT) homologues (E, F), napthbenzothiophene (NBT) homologues (E, F), and pyrene (PYR) + chrysene (CHY) homologues (G, H) at 5 ºC (left panels) and 25 ºC (right panels)

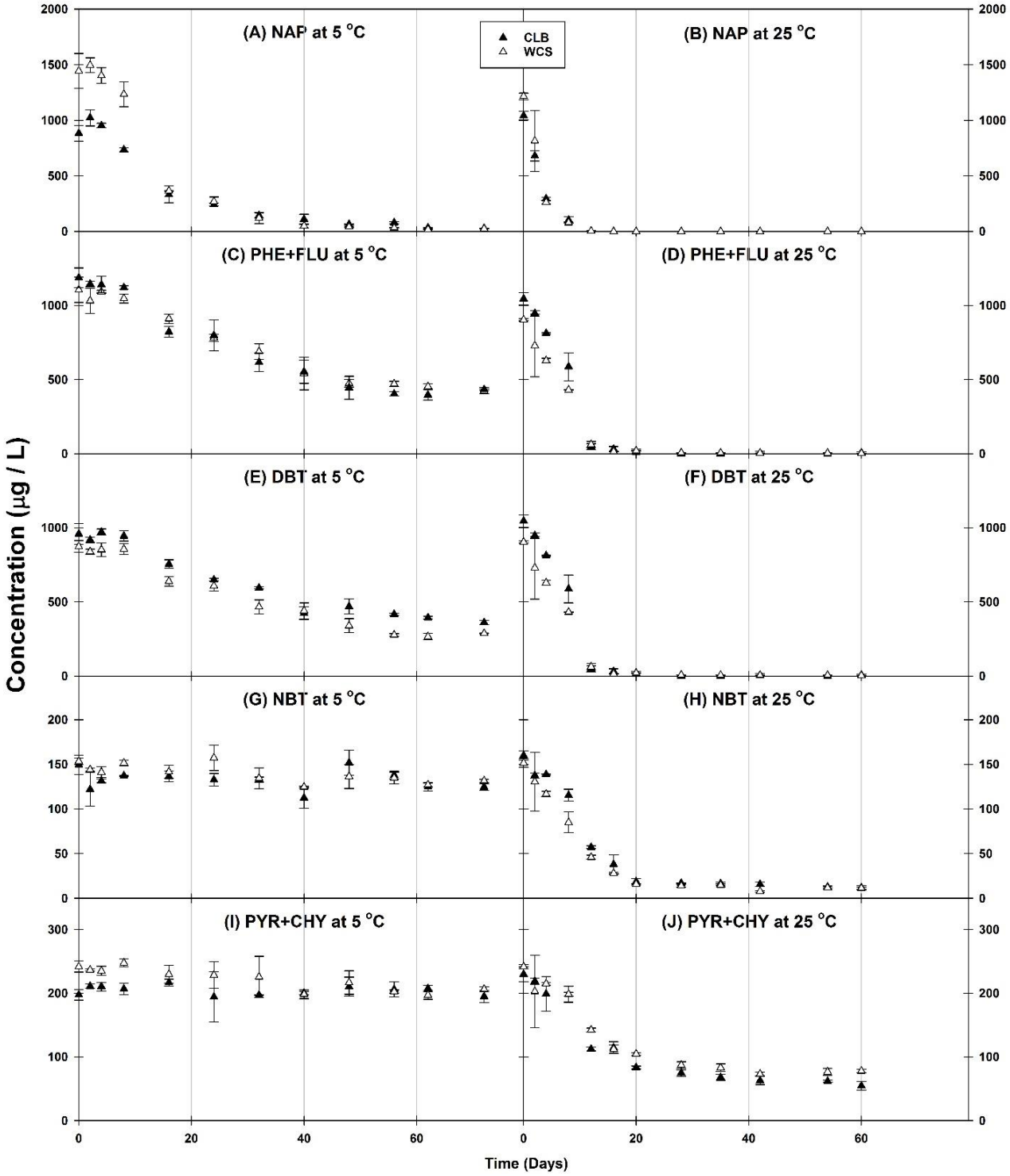


Figure 3. Percent removal of individual PAH compound

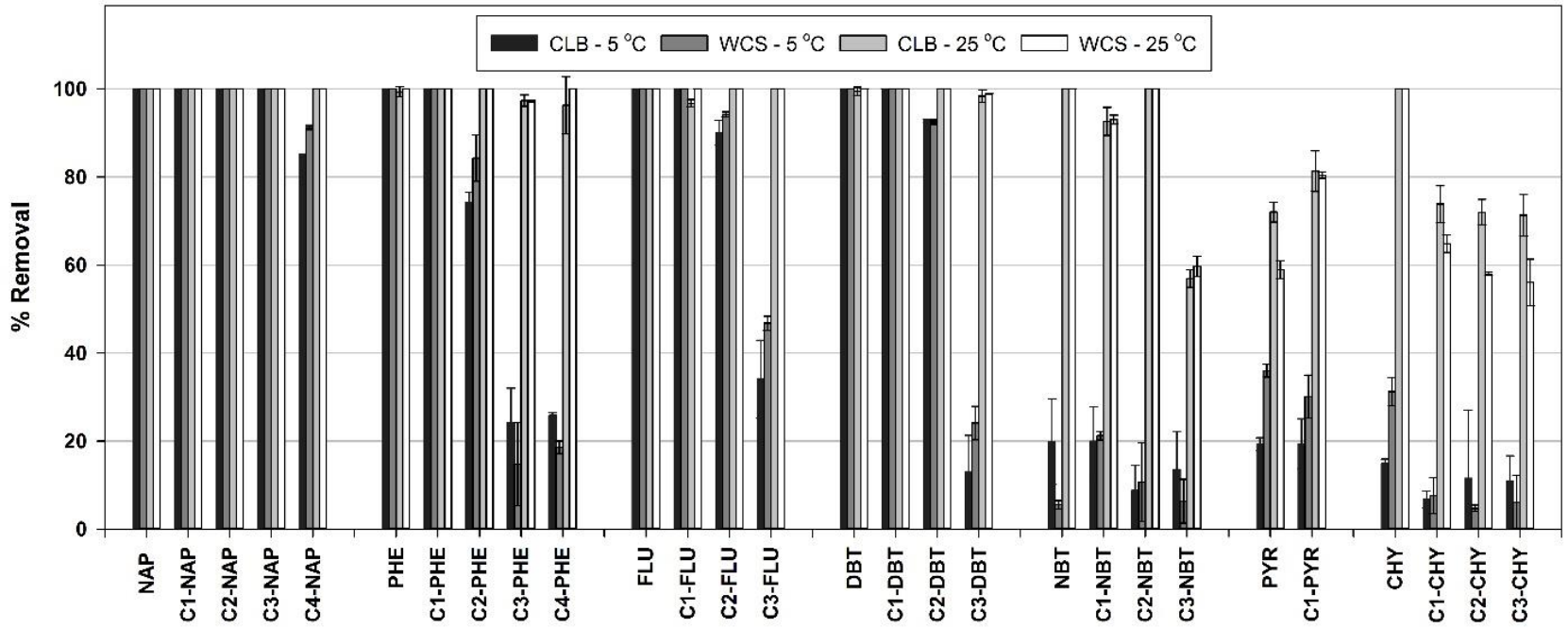


Figure 4. Microbial community composition at genus level

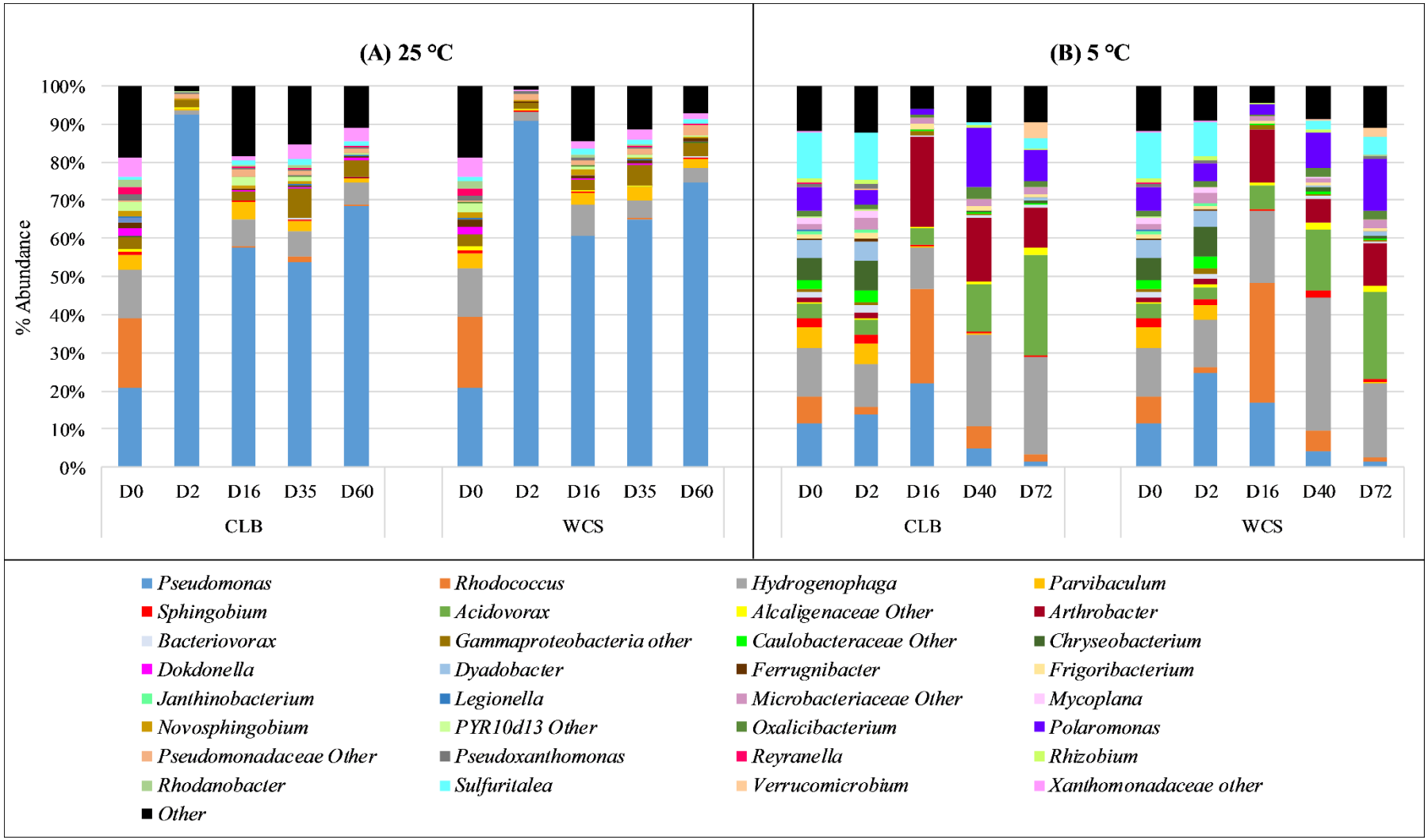
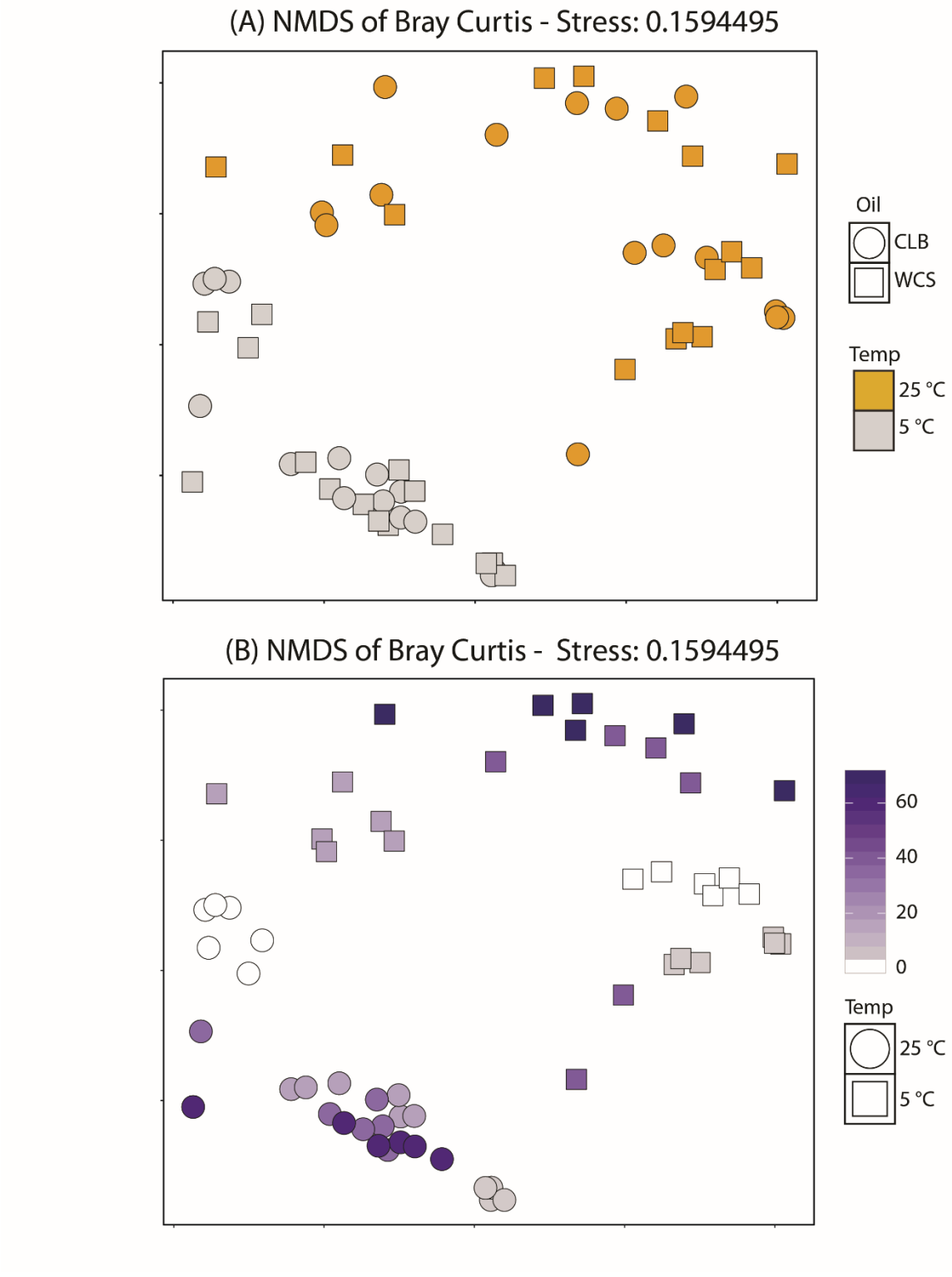


Figure 5. NMDS Plots - Comparison of molecular Community Structure between (A) dilbit type and (B) temperature



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Supplementary Material

Microbial degradation of Cold Lake Blend and Western Canadian Select Dilbits Using Freshwater Enrichments

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Table A.1. Summary of experimental layout

Test	Temperature	Treatment	Sampling Events	Sample Replicates	Total Experimental unit (EU)
1	5°C	Dilbit	12	3	36
2	5°C	Kill Control	1	3	3
				Subtotal EU's	39
3	25°C	Dilbit	12	3	36
4	25°C	Kill Control	1	3	3
				Subtotal EU's	39
			Total EU's for one type of dilbit		78

Table A.2. Calculated first order rate constants (k) for individual *n*-alkanes at 5 ºC

Individual *n*-alkanes	CLB		WCS	
	k **(day^{-1})**	**Std. Error (day^{-1})**	***k*** **(day^{-1})**	**Std. Error (day^{-1})**
***n*c10**	0.42	0.04	0.30	0.06
***n*c11**	0.40	0.06	0.30	0.06
***n*c12**	0.37	0.05	0.28	0.06
***n*c13**	0.35	0.06	0.30	0.07
***n*c14**	0.23	0.05	0.24	0.06
***n*c15**	0.28	0.06	0.27	0.06
***n*c16**	0.27	0.06	0.28	0.06
***n*c17**	0.25	0.05	0.27	0.04
***n*c18**	0.28	0.05	0.29	0.05
***n*c19**	0.26	0.05	0.29	0.06
***n*c20**	0.28	0.05	0.28	0.05
***n*c21**	0.30	0.04	0.27	0.05
***n*c22**	0.30	0.04	0.26	0.05
***n*c23**	0.30	0.04	0.26	0.05
***n*c24**	0.29	0.03	0.26	0.05
***n*c25**	0.30	0.04	0.25	0.05
***n*c26**	0.31	0.03	0.23	0.06
***n*c27**	0.28	0.03	0.24	0.05
***n*c28**	0.30	0.03	0.24	0.05
***n*c29**	0.36	0.03	0.26	0.05
***n*c30**	0.28	0.06	0.25	0.05
***n*c31**	0.26	0.05	0.23	0.05
***n*c32**	0.33	0.05	0.22	0.04
***n*c33**	0.20	0.06	0.21	0.04
***n*c34**	0.25	0.05	0.23	0.05
***n*c35**	0.19	0.05	0.21	0.04

Note: First order rate constants were not calculated at 25 ºC due to rapid degradation of *n*-alkanes

Table A.3. Calculated first order rate constants (k) for individual PAHs at 5 and 25 ºC

Individual PAH Compound	CLB at 5ºC		WCS at 5ºC		CLB at 25ºC		WCS at 25ºC	
	k **(day^{-1})**	**Std. Error (day^{-1})**	***k*** **(day^{-1})**	**Std. Error (day^{-1})**	***k*** **(day^{-1})**	**Std. Error (day^{-1})**	***k*** **(day^{-1})**	**Std. Error (day^{-1})**
NAP	0.88	0.00	0.73	0.07	2.16	0.00	1.56	0.00
C1-NAP	0.49	0.01	0.61	0.01	2.14	0.00	1.99	0.00
C2-NAP	0.44	0.00	0.63	0.18	0.61	0.05	0.61	0.00
C3-NAP	0.12	0.00	0.18	0.01	0.41	0.00	0.59	0.00
C4-NAP	0.03	0.01	0.05	0.02	0.13	0.03	0.16	0.03
PHE	0.32	0.00	0.43	0.00	0.74	0.00	0.70	0.00
C1-PHE	0.10	0.01	0.18	0.03	0.42	0.02	0.38	0.00
C2-PHE	0.03	0.01	0.04	0.01	0.13	0.03	0.21	0.01
C3-PHE	*NA**	*NA*	*NA*	*NA*	0.11	0.03	0.21	0.01
C4-PHE	*NA*	*NA*	*NA*	*NA*	0.13	0.03	0.14	0.02
FLN	0.41	0.00	0.32	0.02	0.89	0.00	0.76	0.00
C1-FLN	0.06	0.01	0.11	0.00	0.22	0.01	0.43	0.00
C2-FLN	0.05	0.01	0.03	0.01	0.19	0.02	0.29	0.01
C3-FLN	*NA*	*NA*	*NA*	*NA*	0.11	0.04	0.13	0.03
DBT	0.28	0.05	0.14	0.00	0.49	0.00	0.82	0.00
C1-DBT	0.08	0.01	0.12	0.02	0.31	0.01	0.32	0.00
C2-DBT	0.03	0.00	0.04	0.01	0.13	0.03	0.11	0.01
C3-DBT	*NA*	*NA*	*NA*	*NA*	0.09	0.03	0.16	0.01
NBT	*NA*	*NA*	*NA*	*NA*	0.10	0.02	0.17	0.03
C1-NBT	*NA*	*NA*	*NA*	*NA*	0.08	0.02	0.11	0.02
C2-NBT	*NA*	*NA*	*NA*	*NA*	0.09	0.01	0.08	0.01
C3-NBT	*NA*	*NA*	*NA*	*NA*	0.00	0.02	0.00	0.02
PYR	*NA*	*NA*	*NA*	*NA*	0.10	0.02	0.06	0.02
C1-PYR	*NA*	*NA*	*NA*	*NA*	0.11	0.02	0.09	0.01
CHY	*NA*	*NA*	*NA*	*NA*	0.11	0.03	0.11	0.03
C1-CHY	*NA*	*NA*	*NA*	*NA*	0.08	0.03	0.06	0.02
C2-CHY	*NA*	*NA*	*NA*	*NA*	0.04	0.01	0.06	0.01
C3-CHY	*NA*	*NA*	*NA*	*NA*	0.04	0.02	0.06	0.02

Note: *NA* denotes that the compound concentration did not decrease significantly due to which first order rate constants were not calculated

Table A.4 ANCOVA analysis of time decay rates comparing temperatures and oil types within a temperature.

Comparison	F value	Pr(>F)
Meso versus Cryo	57.671	3.93E-14
CLB versus WCS (Cryo)	0.17	0.6802
CLB versus WCS (Meso)	0.0451	0.8318

Figure A.1. Hydrocarbon Composition for CLB and WCS

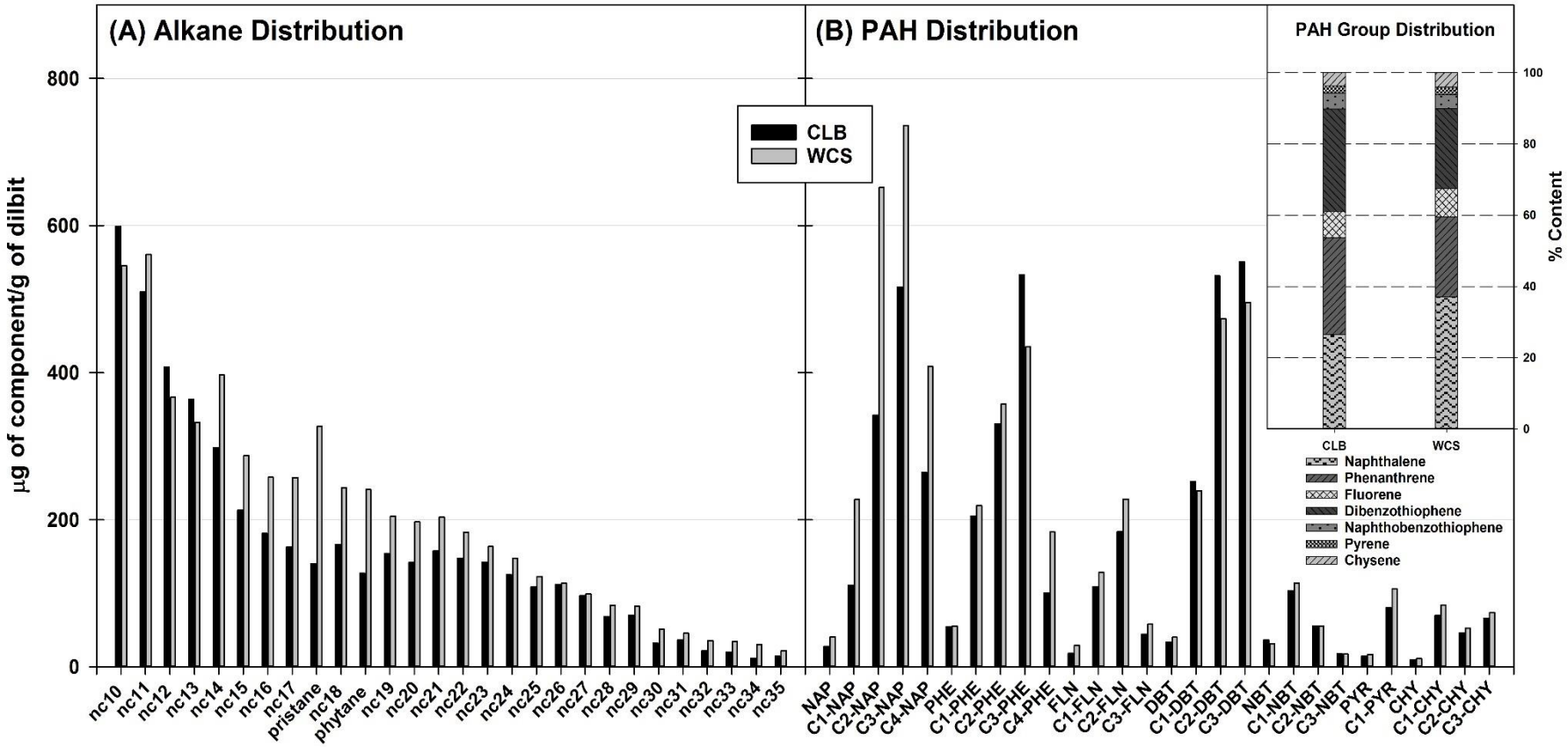


Figure A.2. Biodegradation Branched alkanes pristane (PR) + phytane (PH) at 5 ºC (A) 25 ºC (B)

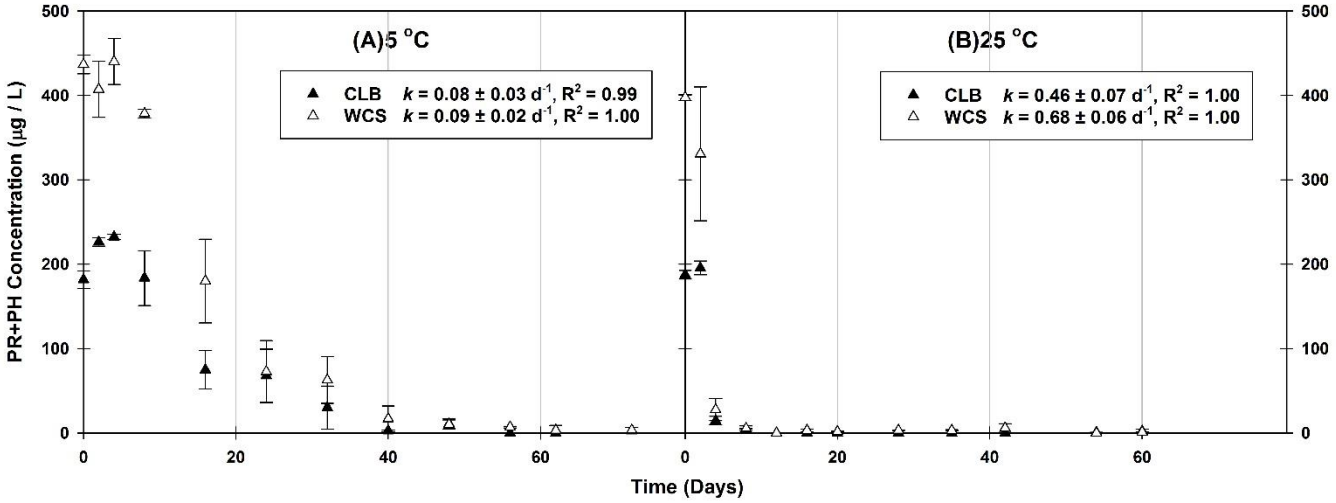


Figure A.3. Comparison between day 0 and KC concentrations of total alkanes (A), total PAHs (B)

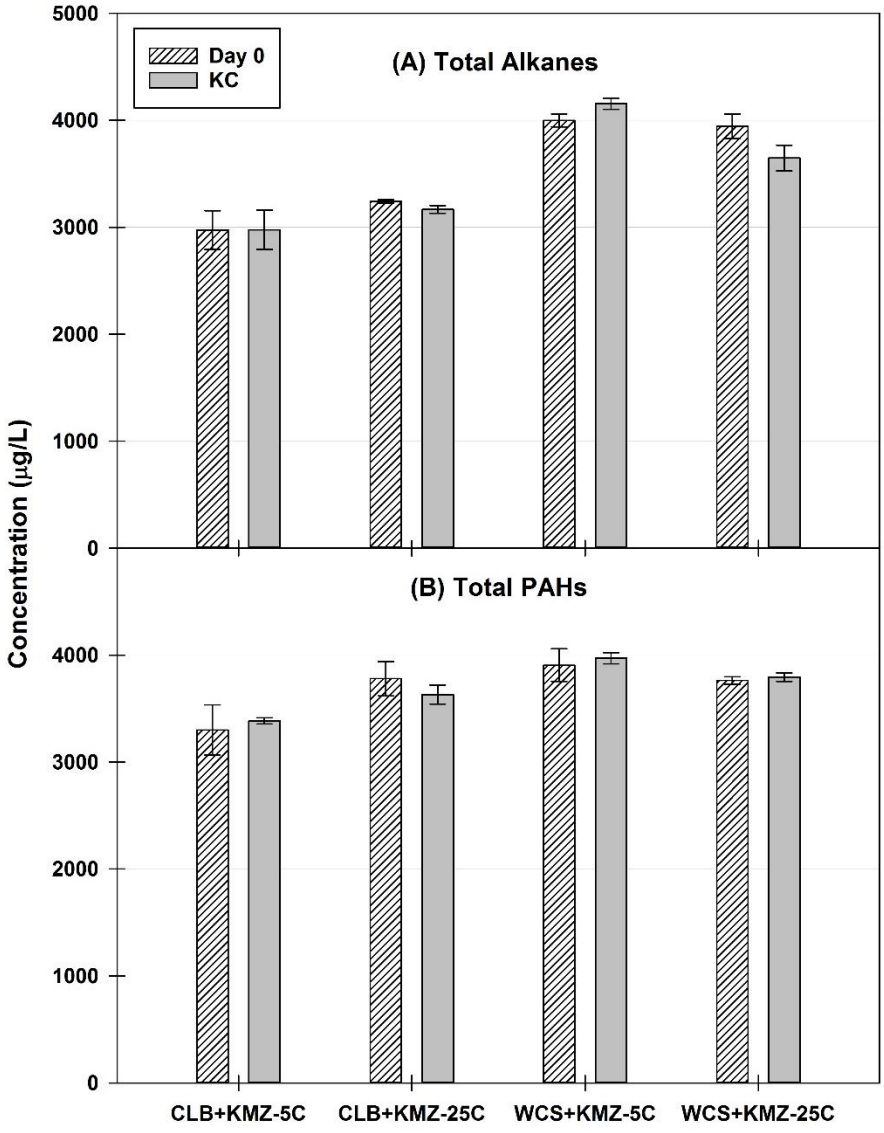
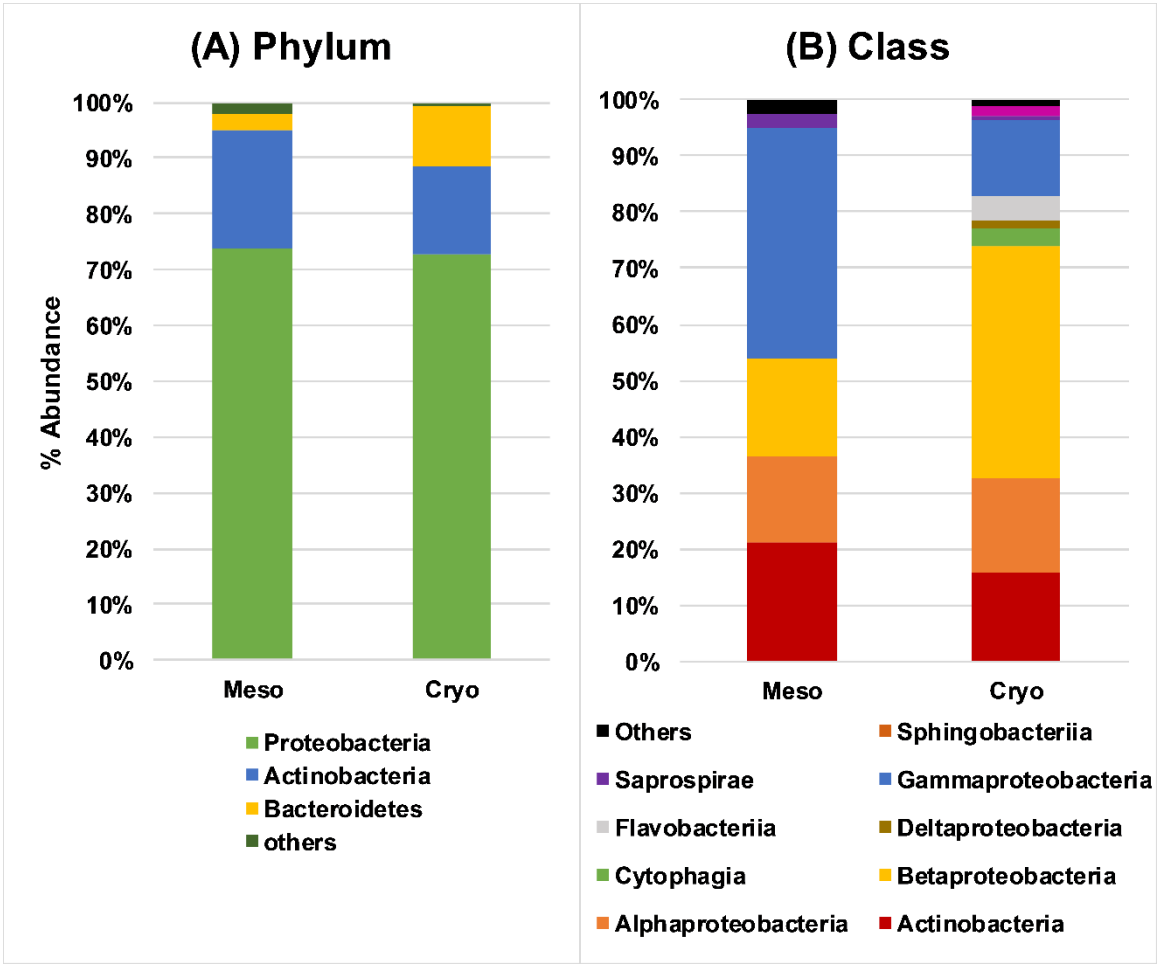


Figure A.4. Initial (day 0) microbial community composition of meso and cryo enrichments at phylum (A) and class (B) level



Note: All groups with less than 1% abundance are grouped together and labeled as other

Figure A.5. Comparison of alpha diversity between dilbit types – observed species (A) and Shannon index (B)

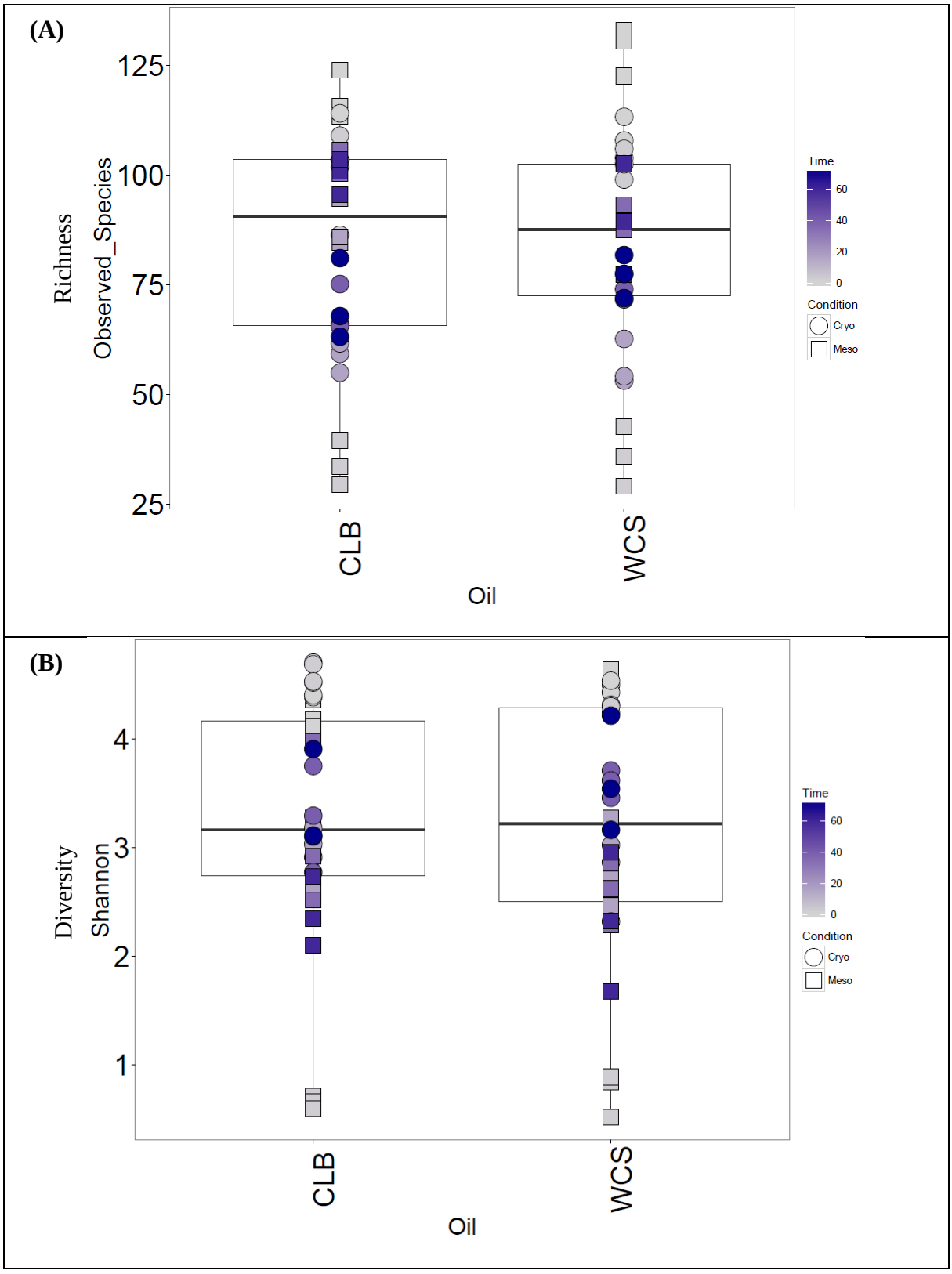


Figure A.6. Comparison of alpha diversity between temperature – observed species (A) and Shannon index (B)

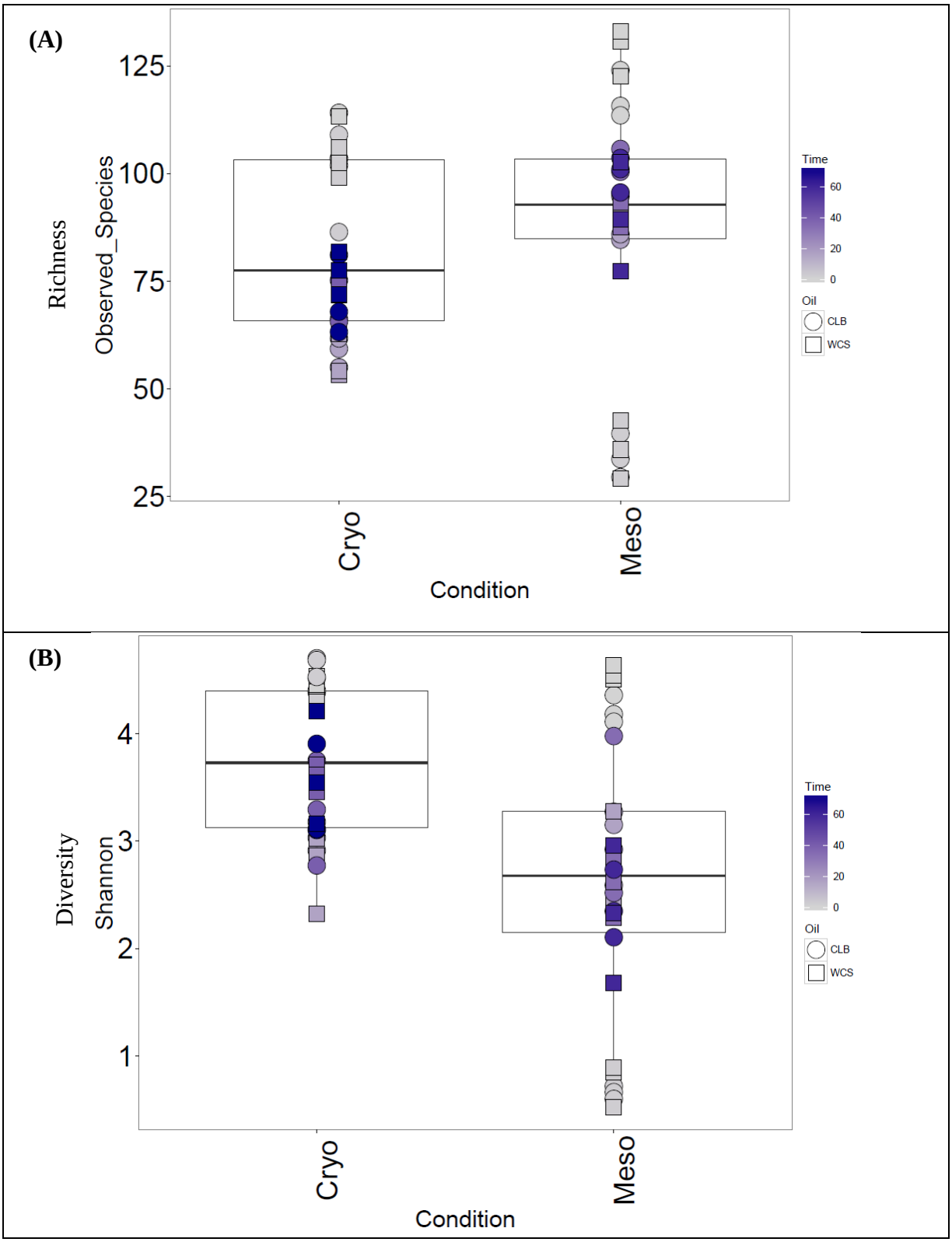


Figure A.7. Time Decay Analysis