Local fungi, willow and municipal compost effectively remediate petroleum-contaminated soil in the Canadian North

Kawina Robichaud[a], Catherine Girard[d], Dimitri Dagher[b], Katherine Stewart[c], Michel Labrecque[b], Mohamed Hijri[b], Marc Amyot[a*]

[a] *Center for Northern Studies, Département de sciences biologiques, Université de Montréal, Pavillon Marie-Victorin, 90 Vincent d'Indy, Montréal QC, H2V 2S9*

[b] *Institut de Recherche en Biologie Végétale, Université de Montréal, 4101 Rue Sherbrooke Est Montréal, QC H1X 2B2*

[c] *Department of Soil Science, University of Saskatchewan, 51 Campus Dr. Saskatoon, SK, S7N 5A8 and Yukon Research Center, Yukon College, 500 College Dr, Whitehorse, Yukon, Y1A 5K4*

d Sentinelle Nord, Center for Northern Studies, Département de biochimie, de microbiologie et de bio-informatique, Université Laval, 1030 avenue de la Médecine, Québec QC, G1V 0A6

* Corresponding author: Marc Amyot; phone: 514-343-7496; e-mail: m.amyot@umontreal.ca

KEY WORDS - Northern bioremediation; soil microbiome; mycoremediation; phytoremediation; petroleum hydrocarbons

ABSTRACT

Low energy-input alternatives based on locally available products are needed for treating petroleum-hydrocarbon spills in northern regions. We tested the efficacy of three local biological components (municipal compost, white-rot fungus: *Pleurotus ostreatus* and willow: *Salix planifolia*) to remediate diesel-contaminated soils in a subarctic climate (Whitehorse, YT, Canada), and compared their efficacy to natural attenuation and chemical fertilizers (industry standard). After the first growing season, biologically amended treatments (BAT) that contained >2 biological components, had decreased 69-73 % of the diesel's F2 fraction (C10-C16), which is more than natural attenuation or fertilizer (48 and 51 %). By the third growing season, the BAT dropped below the Canadian Council of Ministers of the Environment's (CCME) Agricultural & Residential/Parkland guideline (<150 mg kg^{-1}) and 86% of willows had survived and developed extensive roots. MiSeq amplicon sequencing of fungal (ITS) and bacterial (16S) rRNA genes showed the BAT's microbial communities were significantly more abundant and diverse. We found 132 bacterial and 35 fungal genera unique to the BAT. Readily-available local biological components such as municipal compost, fungi and willows may provide an effective alternative to applications of imported chemical fertilizers for the bioremediation and revegetation of diesel-contaminated soil in northern environments.

INTRODUCTION

Petroleum hydrocarbons (PHC) have become one of the most common and widely distributed contaminants worldwide due to their extensive use, distribution, and associated spills (Aislabie et al. 2008; Abioye 2011). In northern regions, human activities rely heavily on petroleum fuels, and spills have occurred at many sites (Mohn et al. 2001; Aislabie et al. 2006). Attenuation of PHC occurs naturally through volatilization and degradation by endemic microorganisms, particularly bacteria and fungi. Nonetheless, this is a slow process; especially in the North (above 50° latitude), where cold temperatures are present for the majority of the year (a frost-free period of 50 to 90 days $year^{-1}$) (Britannica 2018). These low temperatures increase PHC viscosity, which limits their bioavailability to degrading microbes and slows down enzymatic reactions. Other factors limiting microbial activity in these environments include poor moisture and nutrient availability (Walworth & Ferguson 2008; Walworth et al. 2007; Aislabie et al. 2008). Conventional northern soil remediation methods in North America include the addition of nutrients in the form of synthetic fertilizers (often with repetitive tilling), which stimulate PHC degradation by native microorganisms. In northern soils, fertilizer amendments have shown varying results from very effective to detrimental (Walworth et al. 2007; Juwarkar et al. 2010; Paudyn et al. 2008; Lladó et al. 2012); results are often site-specific and linked to initial PHC concentration (Walworth & Ferguson 2008). This could be due to varying requirements between microbial taxa since many cold-climate microbes are adapted to oligotrophic soil conditions (Aislabie & Foght 2008). The most important drivers for PHC-degradation seem to be microbial competition, initial community structure, and soil properties (Bento et al. 2005), which can all be manipulated through site-specific soil amendments. In 2013, the Northern Contaminated Sites Program reported that there

were 166 contaminated sites in northern Canada (Yukon, NWT, and Nunavut), worth $2.3Billion in liability (AANDC). While there is a clear need for remediation and revegetation of contaminated soils in northern regions, spill sites are often remote and traditional remediation can be prohibitively costly (Mohn et al. 2001). Additionally, traditional strategies may be questionable in terms of broader environmental impacts such as: (1) tilling-induced volatilization which can simply displace the problem from soil to air; (2) greenhouse gas emissions related to soil displacement and the transport of chemical fertilizers; and (3) impact of fertilizer mining of finite resources or energy-intensive synthesis (Rafiqul et al. 2005; Sanscartier et al. 2010). The need for combining complementary approaches in order to effectively decontaminate soils in the North has been recognized for some time (Robertson et al. 2007). Low energy-input, *in situ* materials adapted to northern climates and based on locally available products may provide affordable and effective alternative remediation strategies.

Soil treatment approaches using multiple biological components have been shown to be effective for contaminant-remediation in other studies (Germaine et al. 2015; Asemoloye et al. 2017; Palmroth et al. 2006). Based on the literature, we identified (1) local municipal compost, (2) fungi and (3) willows as potential biological agents of interest that might complement each other when used for northern remediation. (1) Compost provides microbial communities and slow-release nutrients that can accelerate contaminant degradation, and it acts as a physical support for other bioremediation elements such as willows and fungi (Guidi et al. 2012; Aislabie et al. 2008; BATTELLE Environmental Restoration Department 1996; Zubillaga et al. 2012). Additionally, municipal compost represents a key opportunity for cities to increase waste diversion from

88 burdened landfills (Giroux 2014). (2) Mycoremediation is the use of fungi for decontamination.
89 White-rot fungi, like *Pleurotus ostreatus* are saprotrophs that specialize in the degradation of the
90 complex plant polymer lignin, through the exudation of aggressive and non-specific lignolytic
91 enzymes such as laccases and peroxidases (Stamets 2005). These enzymes have been shown to
92 target organic contaminants like aromatic and aliphatic PHC (Pointing 2001; Colombo et al. 1997).
93 Although *P. ostreatus* is a wood saprotroph, its mycelium is adapted for some soil colonization
94 (Baldrian 2008; Kendrick 2000). (3) Phytoremediation is the use of plants to assist in contaminant-
95 remediation. Willows are fast-growing hardy plants that are extensively used for revegetation and
96 in a wide range of bioremediation projects (Slater et al. 2011; Pugh et al. 2002; Cunha et al. 2012).
97 *Salix planifolia* was chosen because it was identified as a PHC-resistant species (Kershaw &
98 Kershaw 1986; McKendrick 1987; McKendrick 2002) and it was the most vigorous of four local
99 species we examined in growth trials (data not shown). It has been used in revegetation projects
100 (Collet 2004), but to our knowledge represents a new species for the direct use of cuttings in a
101 PHC bioremediation project.
102
103 The goal of this project was to test the efficacy of these three biological components (municipal
104 compost, fungi and willow) individually and together, in a subarctic climate, and to compare their
105 efficacy to a fertilizer treatment as well as natural attenuation. Since the components were
106 chosen to each fill a different role in the plant-soil system, it was hypothesized that their
107 respective actions and contributions would strengthen the system as a whole, making it more
108 resilient over time, and more effective for decreasing PHC contamination. To obtain a profile of
109 the soil microbial community, we used MiSeq amplicon sequencing of fungal and bacterial

markers (ITS and 16S respectively). Our aim was to identify the influence the different biological components had on the microbial communities, and if certain changes in community composition and diversity could be linked to greater PHC reduction. Finally, we compared degradation between treatments and related the contamination levels to regulatory guidelines from Canada (Canadian Council of Ministers of the Environment (CCME 2008)). The approach examined here could be advantageous because it is less energy-intensive than the traditional tilling and fertilizing methods and could be implemented at remote sites, eventually as a low-maintenance *in-situ* technology.

MATERIALS AND METHODS

Site description

The experimental trial occurred at a Land Treatment Unit (LTU) owned by Arctic Backhoe Services Ltd. (60° 40' 15.29'' N 135° 6' 8.93'' W) located near the City of Whitehorse, Yukon, Canada, from June 2013 to July 2015. Starting total PHC concentration in the soil was 2200 mg kg^{-1}. The initial soil used for the trial was received in August 2012 from the Klondike area (YT), where an accidental diesel spill had just occurred. The CCME (2008) defines their environmental guideline fractions as: F2 (C10-C16), F3 (C16-C34), and F4 (C34-C50). Since F3 and F4 fractions were present in low concentrations and were well below the guidelines for Commercial and Industrial sites (C&I), our target for remediation was the F2 fraction, which was above CCME C&I guidelines at the beginning of the trial (Table 1).

Initial soil pH was 7.72 ± 0.08 (tabletop Oakton PCD650, equipped with an Oakton epoxy pH probe. H_2O:soil ratio of 1:2 (Kalra & Maynard 1991)). The soil contained 1.5 % organic matter (loss on incineration) and its textural description was sandy muddy gravel, unsorted with a median size of 0.1075 mm and a density of 1.39 g cm^{-3} (ASTM Standard test method (Anon 2008), analysed with GRADISTAT (Blott & Pye 2001)). Initial moisture was 7.5 ± 0.6 % by mass (determined gravimetrically).

Experimental design

Treatment descriptions and amendments

Treatments included: **C**-Compost (15 % mature municipal compost (v/v)); **CW**-Compost and Willow (15 % compost (v/v) and six dormant *S. planifolia* cuttings); **CF**-Compost and Fungi (15 % compost and 15 % fungal treatment (composed of 5 % pure sawdust spawn of *P. ostreatus,* 7.1 % sundried *Populus spp.* woodchips, and 2.9 % naturalized *P. ostreatus* mycelium (v/v)); **CWF**-Compost, Willow and Fungi (15 % compost, 15 % fungal treatment and six *S. planifolia* cuttings); **FERT**-Fertilizer (C:N:P ratio 100:9:1)) and **CTRL**-Control (no amendment added). When the four biologically amended treatments (C, CW, CF, CWF) are mentioned together, they are hereinafter referred to as **BAT**, and the FERT and CTRL as **NoC** (no compost). Due to logistical constraints, it was not possible to conduct all 9 possible combinations of willow, compost and fungi. The choice was made to add compost to the fungal and willow treatments to act as a colonization support for the willow and fungi in this nutrient-poor soil. Furthermore, we hypothesized that the effects of these biological components would be additive in PHC-reducing efficiencies.

153 Since heterogeneous distribution of PHC in contaminated soils if often observed (Aislabie et al.
154 2008; Rike et al. 2008), our six treatments were replicated seven times each. They were then
155 placed in seven randomly distributed blocks (n=42) (see Figure. S1A for ground layout). A total of
156 42 Rubbermaid polypropylene bins (189 L each) were perforated with 0.64 cm holes at regular
157 intervals to promote drainage and aeration (Fig. S1B). Each bin received 150 L of soil.
158
159 Municipal compost produced by Boreal Compost Enterprises Ltd. was used. It was analyzed by
160 A&L Canada Laboratories Inc. and met the CCME (CCME 2008) and the Bureau de Normalisation
161 du Québec (BNQ) (2005) standards for maximum allowable microbiological levels and trace metal
162 content in Category A compost. It was fine textured (99.9 % < 0.64 cm (1/4 in.)), with a pH of 8.8,
163 initial moisture of 7.3 %, C:N ratio of 12:1 and qualified as mature compost (CO2 respiration <0.01
164 mg CO2-carbon g^{-1} organic matter day^{-1}) (see SI for A&L report).
165
166 *Salix planifolia* cuttings were harvested near Whitehorse (YT) in their dormant state in March
167 2013 and stored in thick black plastic bags for 3 months at -18 °C. Willow branches (1.3 to 2.5 cm
168 wide) were thawed and cut into 30 cm-long segments. They were then soaked in 20 cm of water
169 for 24 h prior to planting 15 cm deep in the experimental bins (soaking time based on lab trials
170 and practicality). Essentially no other plants colonized the soil surface. No additional
171 management (such as weeding) was required.
172
173 For the fungal amendment, woodchips were soaked for 24 h in tap water and then inoculated
174 with pure sawdust spawn of *P. ostreatus* (spawn origin: Champignons Advitam inc. Qc, Canada).

This was incubated for 28 days (18-22 °C) to allow the mycelium to colonize the woodchips (hereinafter referred to as "naturalized mycelium"). The objective of naturalization is to acclimatize the mycelium to a non-sterile environment and hence stimulate its immune system (Stamets 2005). A second woodchip mix was incorporated directly in the treatment piles to serve as a primary carbon source for the fungi. (See SI for full details on fungi source and woodchip origin and handling.)

Nitrogen and phosphorus are the two most commonly added nutrients in fertilizers. Reported ratios vary from C:N 200:1 to 9:1 and C:P 500:1 to 12:1 (Walworth & Ferguson 2008). In this experiment, urea (46-0-0) and Teeble Super phosphate (0-45-0) were added to a ratio of 100:9:1.

Soil preparation

The diesel-contaminated soil was thoroughly mixed in an attempt to reduce the heterogeneous distribution of PHC and then split in six piles. Each pile received the amendments for one treatment and was then turned over nine times. The amended soils were randomly loaded in wheelbarrows by hand shovels and placed in their respective treatment bins.

Four temperature sensors (Thermacron ibuttons) were placed at a depth of 25 cm in the center of CTRL and CWF treatment bins to monitor their core temperature over 12 months, from June 2013 to June 2014 (Figure SI.B).

During the willow and fungi establishment phase, irrigation was conducted twice with watering cans, to simulate a remote-site condition where irrigation frequency may be limited. The water was from the City of Whitehorse's municipal supply. In 2013, 2.4 L were applied to each of the 42 bins on June 28, while 8 L were applied on July 15. Moisture readings were taken gravimetrically (oven-dry, 105 °C) on PHC sampling days.

Sampling

Soil samples for PHC concentrations were collected eight times over three years, when the ground was not frozen. Two soil-column samples were taken from a depth of 5 to 25 cm and homogenized into one composite sample for each treatment bin. The homogenized samples were placed in a 125 mL glass jar with a Teflon-lined lid and kept at -20 °C until analysis (n = 7 per treatment, per sampling time). Sub-samples of that frozen soil were taken from the beginning and end of the growing seasons of the first two years to conduct the DNA extraction (n = 4 per treatment, per sampling time). Willow cuttings were monitored for leaf emergence at the beginning of the growing seasons and for survival at the end of each season. The dead cuttings were replaced once in the spring after the first growing season. In July of the last year of treatment, the willow cuttings were pulled from the ground and the shoots growing from the initial cuttings were cut and weighed separately from the cuttings to measure the biomass produced without the initial introduced biomass (dry weight).

Hydrocarbon quantification

The CCME fractions F2, F3, and F4 (C10-C16, C16-C34 and C34-C50, respectively) (2008) were extracted by rotary evaporator methods and then analyzed by GC-FID (Gas Chromatography – Flame Ionization Detector, Agilent 6890 GC systems). (See Supplementary Methods in SI). Only alkanes are reported since analysis revealed that aromatic hydrocarbons were absent from the site (data not shown). Samples were preserved at -20 °C until time of analysis.

Amplicon sequencing of fungal ITS and bacterial 16S DNA

DNA extraction and sequencing

Fungal and bacterial DNA was extracted from soil samples using the NucleoSpin® Soil Kit from Macherey-Nagel. Primers ITS1F CS1 (CTTGGTCATTTAGAGGAAGTAA) and 58A2R CS2 (CTGCGTTCTTCATCGAT) were used for fungi, while primers 341F CS1 (CCTACGGGNGGCWGCAG) and 805R CS2 (GACTACHVGGGTATCTAATCC) were used for the V3-V4 region of the 16S rRNA gene in bacteria. Extracted DNA was sent for paired-end next generation sequencing by Illumina MiSeq PE250 (Genome Quebec, See Supplementary Methods in SI.)

Sequence processing and analyses

Primers were trimmed and only sequences with a Q score greater than Q33 (16S) and Q30 (ITS) were retained. Chimeric sequences were identified and removed (vsearch{} (Rognes et al. 2016)). Open-reference OTU (Operational Taxonomic Unit) picking was performed and OTU tables were rarefied to 20,000 and 7,700 reads/sample for 16S and ITS, respectively (QIIME (Caporaso et al. 2010)) (see Supplementary Methods in SI). Raw 16S and ITS sequences have been deposited in GenBank-NCBI under study ID 384633

239 (http://www.ncbi.nlm.nih.gov/bioproject/384633).

240

241 **Statistical analysis**

242 A linear mixed-effects repeated measures model fitted by Restricted Maximum Likelihood (REML)
243 with blocking was performed with nlme{} (Pinheiro et al. 2017) in R (R Core Team et al. 2016) to
244 compare : decreases in PHC concentrations (LOG of [F2]) ; temperature; and moisture levels
245 between treatments. Treatment was considered a fixed factor and blocking was a random one.
246 Measures were repeated eight times over three years. Post-hoc multiple comparison tests were
247 performed with the HSD Tukey method (lsmeans{} (Lenth 2016) and mltcompview{} (Hothorn et
248 al. 2008)).

249

250 Fungal and bacterial alpha diversity as well as differences between degradation rates (k) were
251 also tested with a linear mixed effects model followed by the HSD Tukey method. Principal
252 coordinates analyses (PCoA) followed by permutational multivariate analysis of variance
253 (PERMANOVA in vegan{}) (Oksanen et al. 2016) were conducted to compare fungal and bacterial
254 beta diversity metrics (the compare_categories.py script in QIIME was also used for 16S).
255 Assumptions of normality and homoscedasticity were met for all tests. Figures were built with
256 ggplot2{} (Wickham 2009) and phyloseq{} (McMurdie & Holmes 2013) in R (R Core Team et al.
257 2016).

258

259 ## RESULTS AND DISCUSSION

260 **Diesel removal**

Temporal trends

This study shows that local biological components (compost, plants and fungi) led to higher rates of hydrocarbon removal than inorganic fertilizer or natural attenuation for the field remediation of diesel-contaminated soil in a subarctic climate. The Biologically amended treatments (BAT) brought the contamination level below 260 mg kg^{-1} (the CCME's Commercial and Industrial (C&I) guideline,) in 14 months and below 150 mg kg^{-1} (CCME's Agricultural & Residential/Parkland limit (A&R/P)) within three growing seasons. We chose to compare our results to the national guideline values for our study sites. Note that comparisons with guidelines from other jurisdictions is difficult, because of the different purposes underlying these guidelines (see Cavanagh & Halloran (2002) for a discussion on guidelines). For instance, the purpose of a guideline may be to reach a remediation goal or assess the urgency of remediation. We present F2 results on a yearly basis, to highlight PHC removal in the remediation system (Fig. 1).

After the first growing season, treatments containing two or more biological components had lost significantly more F2 (71.4 ± 2.6 % (CWF), 72.9 ± 2.8 % (CF) and 67.8 ± 2.0 % (CW)) ($p < 0.05$), than the NoC treatments (51.1 ± 9.1 % (FERT), and 48.2 ± 2.1 % (CTRL)) (Table 2). These results pointed to the additive efficacy of multiple biological components used concomitantly. Biodegradation followed pseudo first-order kinetics (linear slopes were obtained with: $Ln[F2]t / Ln[F2]t_0 = -kt$). The BAT treatments generally had steeper slopes (faster removal rates) than the NoC. The fastest removal rate in year one was observed for the CWF treatment (0.78 ± 0.08 day^{-1}), which was significantly faster than the NoC treatments ($p < 0.05$) (Table 2). Our results indicate that within one growing season in a subarctic setting, our approach, incorporating multiple biological

components, performed better than a chemical fertilizer or natural attenuation. One year and two months following treatment, the remediation target was reached for all the BAT treatments; the CCME's C&I guideline (260 mg kg^{-1}) (Fig. 1, Table 1). The BAT led to significantly more F2 reduction (C: 87.5 ± 2.2 %, CW: 84.4 ± 4.6 %, CF: 83.2 ± 3.6 % and CWF: 84.0 ± 2.5 %) than both the CTRL (71.0 ± 5.6 %) and the FERT (72.4 ± 18.4 %) treatments. A final sampling was performed a year later to see the long-term effectiveness of the remediation methods. At this point, the CTRL, representing natural attenuation, showed the lowest overall F2 removal (87.5 ± 1.1 %). The BAT's F2 removal was statistically equivalent, and the C treatment led to significantly more than the two NoC ($p < 0.05$) for a total of 95.0 ± 0.4 % removal, the highest overall (Table 2). By the end of the experiment, the BAT had fallen below the CCME's A&RP limits (150 mg kg^{-1}), while the NoC were below C&I limits (Fig. 1).

Early in the experiment, the highest removal rates and percentages where observed for the CWF treatment. Bell et al. (2013) determined that the natural evolution of a contaminated soil's microbial community structure does not always naturally foster an environment conducive to organisms which possess the strongest biodegradation efficacies. This type of selection may have occurred in the CWF soil, as it did not keep the lead for the following years. Additionally, it is possible that during the last 12 months of the study a nutrient deficiency occurred in the CWF, leading to increased competition between the organisms and a decrease in their ability to degrade PHCs.

304 Multiple factors can impact the rate and total PHC-removal achieved in a given system, and field
305 studies regularly yield different results than laboratory studies (Aislabie & Foght 2008; Rike et al.
306 2008; Paudyn et al. 2008; Facundo et al. 2001). While most studies have focussed on approaches
307 using one or two techniques, we tested the efficacy of phytoremediation, mycoremediation and
308 compost-aided bioremediation, simultaneously. In a field experiment conducted over 94 days in
309 Ontario, Canada, Gomez and Sartaj (2013) found that the addition of compost and a microbial
310 consortium stimulated a removal of 82 % (of the initial 940 mg kg^{-1} of heating fuel) and their
311 compost-only treatment yielded 52 % (Gomez & Sartaj 2013). In the same time span our compost-
312 only treatment had removed 69 % and our CWF treatment had removed 73 % (Table 2). In Finland,
313 a local species of pine was used to treat soil contaminated with 5000 mg kg^{-1} diesel. In 330 days,
314 this phytoremediation resulted in the removal of at least 88% of original fuel (Palmroth et al.
315 2002). Over a longer period of time, the course of two years, research conducted in the Canadian
316 Arctic (Resolution Island, Nunavut) by a landfarming approach (with tilling every 4 days), attained
317 removal rates >90 % (Paudyn et al. 2008). Our passive compost-only treatment led to a 95 %
318 decrease of the initial 1715 mg kg^{-1} of F2 from diesel, in two years and 10 days. The age of
319 contaminants can also have an impact on the remediation as weathered contaminants tend to be
320 more recalcitrant to bioremediation. As contaminants become less available to degradation they
321 can also be less available to local flora and fauna, hence possibly reducing risks (Brassington et al.
322 2007). A large advantage of our method resides in the fact that no amendment addition, tilling
323 or handling is required after initial set-up and additionally re-vegetation takes place
324 concomitantly with decontamination.
325

326 *Effect of compost vs. fertilizer*

327 FERT led to a smaller F2 percent decrease than the four BAT ($p < 0.05$). The 234 mg kg^{-1} of nitrogen
328 added to our soil is well below the reported inhibitory levels of 1200 mg N kg^{-1} in sub-polar soils
329 (Walworth et al. 2007) and within the commonly used C:N range of 100:9 (Snape et al. 2008a;
330 Karppinen et al. 2017b). The addition of fertilizer has been reported to yield mixed degradation
331 results, from very good to inhibitory, especially in cold climates where soil microorganisms are
332 usually adapted to low nutrient levels (Lladó et al. 2012; Paudyn et al. 2008; Walworth et al. 2007).
333 Compost on the other hand, can not only provide a moisture-retaining matrix in the soil, but also
334 nutrients and microbial communities that can accelerate contaminant degradation as well as
335 serve as a physical support for other bioremediation elements such as willows and fungi (Guidi et
336 al. 2012; Aislabie et al. 2008; BATTELLE Environmental Restoration Department 1996; Zubillaga et
337 al. 2012). Vouillamoz et al., (2001) also found that the addition of compost reduced the toxicity
338 of diesel to plants and increased its degradation. Similarly, Chirakkara and Reddy (2015)
339 determined that biomass amendments like compost improved plant growth in PHC-contaminated
340 soil. Although we did not have willows growing in a control soil, we hypothesize their good
341 survival rates could be linked in part to compost addition, in accordance with the previous
342 author's findings. Overall, compost appears to play a key role in our approach. Although not all
343 northern towns and cities have composting facilities, in Canada there is a growing trend to
344 increase diversion of waste from landfill through composting (a 125% increase between 2000 and
345 2010) (Giroux 2014). Diversion of 66% of residential waste can usually be achieved, while reducing
346 the production of greenhouse gases, such as methane (produced by the anaerobic degradation

of compostable materials within a landfill), which is very relevant within the current global climate change context (Lou & Nair 2009; Giroux 2014).

In remote northern locations the long-term efficacy of in-situ treatments, as well as impact on the ecological integrity of the surrounding environment, absolutely need to be considered. Leewis et al. (2013) observed that initial plant colonization of a contaminated site had long-term influences on the site's future plant-community composition, and most importantly that fertilizer applications favoured non-native plant establishment, even 15 years after treatment (Leewis et al. 2013). The use of locally available organic materials, such as compost rather than inorganic fertilizers, may be used to effectively improve PHC degradation and promote recovery of native vegetation. Further, fertilizer transport has been shown to be one of the main sources of pollution (Sanscartier et al. 2010). By using local materials, which reduced transport, we also aimed to minimize this project's carbon footprint.

Effect of environmental drivers in a cold climate

Moisture and temperatures above freezing are important environmental drivers of microbial activity in the soil. Microorganisms require water for their metabolic functions; a system with adequate moisture will encourage their growth. In our 42 bins, mean soil moisture varied between 8.5 and 14.6 % in the sampling months (Table 2). The BAT treatments generally had higher moisture levels than the NoC ($p < 0.05$). This is most likely explained by the presence of additional organic matter in the soil and possible water retention by fungal mycelium.

No temperature differences were denoted between the two treatments monitored (CTRL, CWF) (p > 0.05). The bins were aboveground, and comparisons of our recorded temperatures with those of a nearby weather station indicate that bin core temperatures followed air temperatures (Fig. S2). The center of the bins was buffered against the most extreme temperatures but were still subjected to a wide range of temperatures (-27 to 28 °C). The key temperature-related consideration in the North is the limited season when the ground, and its water content, are not frozen. Studies in northern locations, including near our research site in Whitehorse, have shown that the addition of certain amendments such as biochar could extend PHC-remediation into the winter months by maintaining pockets of liquid water in the soil where microbes remain active, even when the temperature is below zero (Karppinen, et al. 2017a; Karppinen et al. 2017b). It should be noted that it is challenging to assess the role of soil temperature alone in the study of PHC-degradation since temperature will influence the properties of both soil and petroleum hydrocarbons (Aislabie & Foght 2008; Rike et al. 2008).

Progression of biological components

Salix planifolia

Of the 84 *S. planifoli*a cuttings planted, 100 % showed bud and leaf emergence, and 79 % survived the first winter. Dead cuttings were replaced in the spring of the second year (May 2014) since the priority was to measure their influence on remediation, not their survival rates. A total of 96 % were alive before going into the second winter and 86 % survived into the third growing season. The total aboveground dry biomass per cutting (all new branches and leaves but excluding the initial cutting) was an average of 4.60 ± 0.56 g and 4.18 ± 0.44 g, for CW and CWF respectively.

391 No differences between treatments were observed ($p > 0.05$). Although underground biomass
392 was not quantified, the root systems that developed were extensive and traveled throughout the
393 bins.
394
395 The presence of petroleum in soil can slow or hinder plant growth or germination (Gillian & Harry
396 2002; van Gestel et al. 2001). Despite the presence of diesel and large temperature fluctuations
397 observed in our bins, good post-winter survival rates were observed for *S. planifolia* (79 (CWF)
398 and 86% (CW)). These survival rates in diesel-contaminated soil are within the range that Houle
399 and Babeux (1994) observed in non-contaminated soil (77 % and 96 %) at two different sites in
400 subarctic Canada. Different authors have observed this species' ability to survive or colonize
401 petroleum-contaminated soils. Kershaw and Kershaw (1986) found this species to be one of the
402 dominant colonizers on a 35-year-old crude oil spill in the Northwest Territories of Canada and
403 McKendrick (1987) found it was one of the few shrub species that survived a diesel spill in the
404 Alaskan Arctic. Although it has not yet been investigated, we hypothesize that *S. planifolia's*
405 success for diesel-remediation may result from its ability to structure the microbial community in
406 its rhizosphere, as it was recently described by Leewis et al. (2016) for *Salix alaxensis* in diesel-
407 contaminated soil. We found 9 fungal and 1 bacterial genera unique to the treatments that
408 contained willow cuttings, but we did not specifically analyze the rhizosphere soil. Our results
409 confirm this species' resilience to petroleum-contaminated soil; its resistance to relatively dry soil
410 conditions; and most importantly, the possibility for the direct use of its cuttings in diesel-
411 contaminated soil in the North.
412

413 *Pleurotus ostreatus*

414 Large fruiting bodies appeared in multiple locations on the surface, as well as from aeration holes
415 on all CF and CWF bins at the end of the first summer (see abstract image), indicating mycelial
416 growth was distributed throughout the bins. Sporophores also appeared in 2014, but they were
417 much smaller and scattered. This second fruiting confirmed the survival of the fungi, but their
418 trifled state could indicate a reduced access to nutrients. Our results show that *P. ostreatus* can
419 grow in a northern bioremediation system. To our knowledge, white-rot fungi has rarely been
420 used for the bioremediation of diesel in subarctic climates (Winquist et al. 2014; Camenzuli &
421 Freidman 2015), and our work confirms its potential.
422
423 Low-nutrient soils that support smaller densities of microbes may favour the initial establishment
424 of white rot fungi, which can be more successful at colonizing sterile soils because of reduced
425 competition (Baldrian 2008). Other field-scale studies at lower latitudes have found this species
426 to be an effective degrader of PHC both in *in vitro* and *in situ* settings. Thomas et al. (1998) found
427 that the addition of *P. ostreatus* to large outdoor biopiles noticeably altered the appearance of
428 the soil in comparison to the control and to bacterial bioaugmentation treatments over four
429 months. The scent of oil disappeared, multiple large fungi fruiting bodies regularly flushed, and
430 by the 10th week volunteer vegetation had begun colonizing the pile, while the other two
431 treatments remained black with a smell of PHC (Thomas et al. 1998; Stamets 2005). *Pleurotus*
432 *ostreatus* is specialized in the primary degradation of dead wood (Kendrick 2000) and a willow
433 cutting end can offer an entry way for the fungi. The replacement rate of willows in the treatment
434 with fungi (28.5 %) was double than without fungi (14.2 %). Planting seedlings or using rooted

435 cuttings could possibly diminish this additional loss in the presence of a white rot fungus.
436 *Pleurotus ostreatus* proved to be an adequate species in this climate, but because of the
437 naturalization process used, we found it required more handling than municipal compost.
438 Although we ultimately deemed the addition unnecessary in our particular case, *P. ostreatus* may
439 still be an appropriate and profitable option for other diesel spills, especially where toxic and
440 recalcitrant organic polycyclic aromatic hydrocarbons (PAH) molecules are present (we initially
441 expected them in our soil).
442
443 *Soil fungal and bacterial communities*
444 The different biological components added to the soil had clear and lasting impacts on the soil
445 fungal and bacterial community composition and diversity. It comes to no surprise that the
446 initial addition of amendments would have some effect, since the BAT supply their own new
447 microbial communities. Nonetheless, since most bio-augmentation experiments yield little
448 success in community manipulation over time (Thompson et al. 2005), we were surprised to see
449 the fungal and bacterial communities remain very different in the BAT versus the NoC, even by
450 the end of the second growing seasons (Fig. 2).
451
452 In total, 390 fungal genera were identified of which 35 are exclusive to the BAT treatments. The
453 most abundant phyla across all treatments were *Ascomycota* and *Basidiomycota* followed by
454 *Zygomycota*, *Chytridiomycota* and *Rozellomycota*, which make up a small portion of the
455 remaining diversity (Fig. S3A). Compost does not appear to be a driver of fungal richness within
456 bins (alpha diversity) in our experimental trial ($p > 0.05$), but the presence of *P. ostreatus*

457 reduced fungal richness (Fig. S4A, “Observed”). The FERT has the largest range of observed
458 OTUs (≈175 to ≈450); this may be linked to the high heterogeneity of PHC removal performance
459 discussed above. Compost shaped the fungal community’s structure and composition with clear
460 clusters separating NoC (cluster CA.1; Fig. 2A) and BAT treatments (Cluster CA.2) (beta diversity
461 calculated with Jensen-Shanon Divergence metric, Fig. 2A. PERMANOVA, $p < 0.05$). Interestingly
462 this strong difference was maintained over two years and the community did not appear to
463 move back to its original composition. On the other hand, there is a clear change over time in
464 the treatments amended with *P. ostreatus* (cluster CA.3); they display a steady decrease in total
465 fungal genera present (269, 244, 219, and 204 for the four sampling times; a 25% decrease). The
466 ITS sequence of the genus *Pleurotus* is clearly detectable at the start of the experiment in the CF
467 and WF treatments (6.8 ± 3.2 % of the total abundance), but it drops to nearly zero (0.03 ± 0.04
468 %) by the end of the second growing season. This indicates that the addition of *P. ostreatus* can
469 have a lasting effect on a soil’s fungal community structure and composition (Fig. 2A, cluster
470 CA.3), by decreasing diversity, even after it is no longer present in the system.
471
472 We found there were 132 bacterial genera unique to the BAT treatments (absent from NoC).
473 These may not be bacteria directly responsible for petroleum degradation, but may promote
474 degradation pathways, and further work should be conducted to examine the presence and role
475 of these bacteria. The presence of compost had a clear impact on the number of observed
476 bacterial OTUs (Kruskal-Wallis test, Bonferroni-corrected, $p < 0.05$) (alpha diversity, Fig. S4.B). The
477 FERT reduced the minimum richness below the CTRL; again this is possibly linked to soil pockets
478 with higher fertilizer composition, which could have reduced richness. The BAT had markedly

more observed OTUs than the NoC (≈1660 to 2200 compared to ≈800 to 1500, respectively) (p < 0.05). We used the Bray-Curtis dissimilarity index to measure similarity across groups. The PCoA on bacterial beta diversity (based on Bray-curtis) indicates that compost is a strong driver of community structure and composition as clear differences are visible between the BAT and the NoC (PERMANOVA, p < 0.05) (Fig. 2B). At the start, the CTRL and FERT clustered together, and then gradually moved in opposite direction through time, indicating that the fertilizer is likely driving bacterial community structure and composition (Fig 2B, clusters CB.2 and CB.3). Although there is a slight divergence of the treatments with *P. ostreatus*, the BAT treatments mainly cluster together (Fig 2B, cluster CB.1). Six commonly recognized cold climate PHC-degrading bacterial genera were identified in all treatments: *Acinetobacter*, *Arthrobacter*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas* and *Variovorax*.

Our results indicate that the addition of compost had an immediate and lasting influence (over two years) on fungal and bacterial community composition and structure. *Pleurotus ostreatus* also had a dramatic impact on the fungal community's structure. Overall, the biologically amended treatments (BAT) led to higher F2 removal than fertilizer or natural attenuation. Higher soil microbial diversity does not always lead to greater PHC degradation (Bell et al. 2013). But in this case the BAT, which led to the greatest F2 percent-decrease, also had significantly more diverse and abundant fungal and bacterial communities. In our results, *S. planifolia* was not as important as compost for stimulating PHC-removal, but it proved to be an adequate species for the re-vegetation of diesel-contaminated soils whilst bioremediation occurred.

This study shows that a diesel-contaminated subarctic soil can be successfully remediated utilizing compost alone or with plants and fungi simultaneously. Despite promising research results with local organic amendments in northern regions, the fertilization and tilling method is still the most widely used for soil remediation in North America. This research paper proposes another angle on existing bioremediation tools by using multiple local biological amendments concomitantly, to best serve a specific site's requirements. As global resources continue to decrease and carbon emissions linked to global warming are taken more into account, research and remediation projects working to reduce their environmental footprints will become increasingly necessary (Sanscartier et al. 2010; Sauvé et al. 2015; Sauvé et al. 2016). Circular economy practices (Sauvé et al. 2016) and ecosystemic principle (Secretariat De La Convention Sur La Diversite Biologique 2004) can serve as guiding tools to increase sustainability in research practices; they helped guide our approach using local materials, rather than synthetic fertilizers. Finally, a variety of different local resources surrounds each northern contaminated site, and the use of site-specific amendments is recommended.

515 **Table 1.** Initial PHC concentrations measured (mg kg^{-1} dry soil) across hydrocarbon fractions in
516 different treatments (n=7). The contamination is a 12-month old diesel fuel in soil. In the listed
517 CCME fractions for Commercial & Industrial limits, the C indicates the number of carbons in the
518 molecules. Letters denote the ranks of the statistical differences between treatments,
519 determined by a Tukey HSD test ($p < 0.05$), following a linear mixed-effects model. SD stands for
520 standard error.

Fraction	**F2** (C10-C16)		**F3** (C16-C34)	**F4** (C34-C50)
CCME limit	*260 mg kg^{-1}*		*1700 mg kg^{-1}*	*3300 mg kg^{-1}*
Detection limit	*30 mg kg^{-1}*		*50 mg kg^{-1}*	*50 mg kg^{-1}*
Treatment	**Mean ± SD**	**Rank**	**Mean ± SD**	**Mean ± SD**
CTRL	1697 ± 178	ab	482 ± 36	43 ± 17
C	1715 ± 298	ab	496 ± 65	66 ± 9
CW	1475 ± 227	ab	476 ± 71	58 ± 23
CF	1222 ± 126	a	447 ± 34	86 ± 27
CWF	1394 ± 261	ab	480 ± 72	73 ± 12
FERT	2057 ± 378	b	547 ± 68	42 ± 16

C = Compost; F = Fungi; W = Willow; CTRL = Control; FERT = Fertilizer.

521

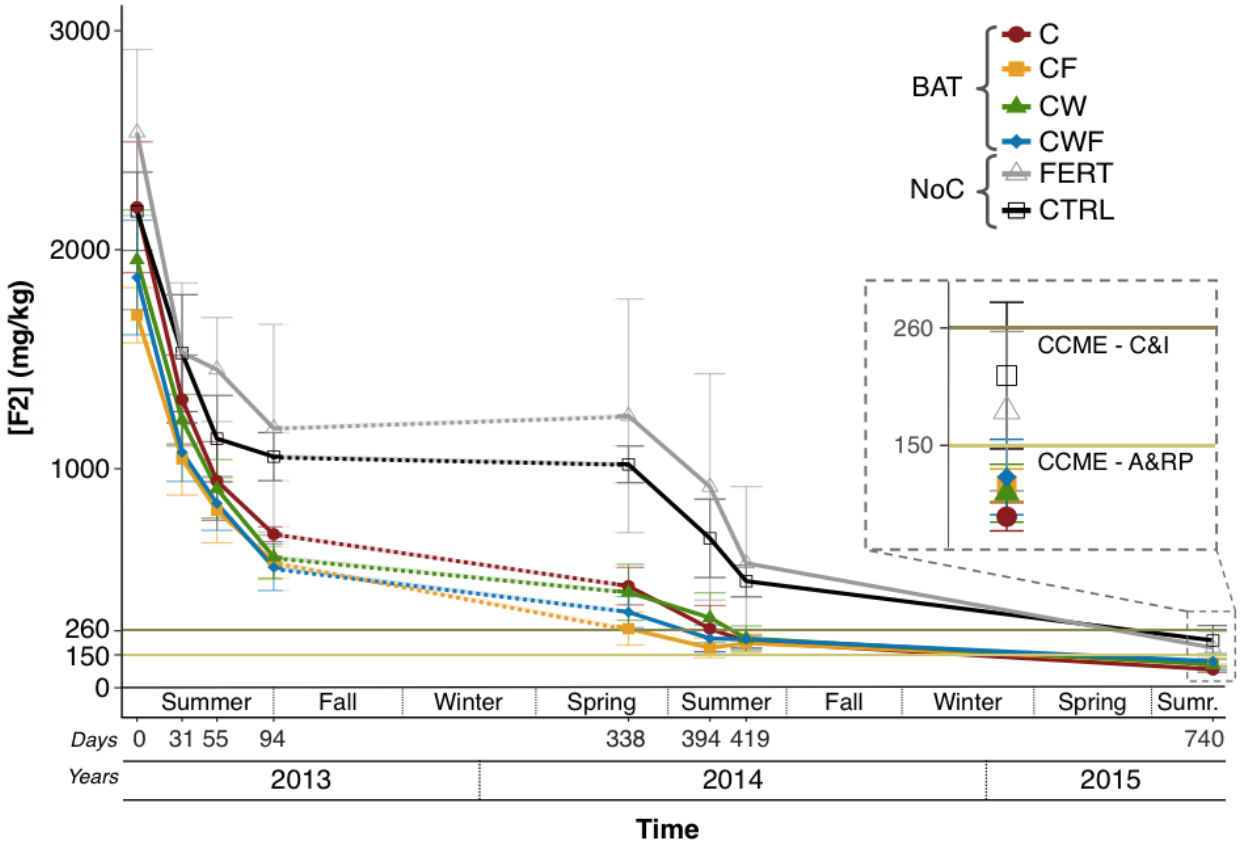
Table 2. Cumulative percent decreases (% dcr.) in the concentrations of the F2 fraction in relation to the first sampling event (June 2013) are presented for the beginning and end of each year. Removal rates (over the first 94 days are presented with the constant k, which represents mean percent removal in mg kg^{-1} per day. Letters denote the ranks (r) of the statistical differences between treatments' F2 concentrations, determined by a Tukey HSD test ($p < 0.05$), following a linear mixed-effects model with repeated measures. Soil moisture (%) means represent the averages measured per treatment between June 27, 2013 and July 4, 2015 (n=56 per treatment, measure on the same days as PCH-sampling occurred), as well as the minimum and maximum moisture concentrations measured.

Date	2013					2014				2015		2013-2015		
	June	Sept.		Removal rates		May		Aug.		Sept.		Soil Moist. (%)		
Treatment	% dcr.	% dcr.	r	k (day^{-1})	r	% dcr.	r	% dcr.	r	% dcr.	r	mean	min	max
CTRL	0	48 ± 5	a	0.51 ± 0.06	c	40 ± 4	c	71 ± 6	b	88 ± 3	c	9	3	16
C	0	69 ± 6	b	0.73 ± 0.06	ab	72 ± 7	b	88 ± 2	a	95 ± 1	a	12	6	20
CW	0	71 ± 7	b	0.76 ± 0.07	a	70 ± 10	b	84 ± 5	a	93 ± 2	a	11	4	27
CF	0	68 ± 5	b	0.72 ± 0.06	ab	78 ± 5	a	83 ± 4	a	91 ± 1	ab	15	7	21
CWF	0	73 ± 7	b	0.78 ± 0.08	a	75 ± 5	ab	84 ± 3	a	91 ± 3	ab	13	5	35
FERT	0	51± 24	a	0.54 ± 0.26	bc	40 ± 25	c	71 ± 6	b	91 ± 4	bc	9	5	17

C= Compost; F=Fungi; W=Willow; CTRL=Control; FERT=Fertilizer.

532 **TOC/ABSTRACT ART**

533



534

535 **Figure 1.** Diesel fraction F2 (C10-C16) removed over 740 days for the six treatments at the ABH

536 site. Error bars represent standard deviation (SD, n=7). The dashed lines during the first winter

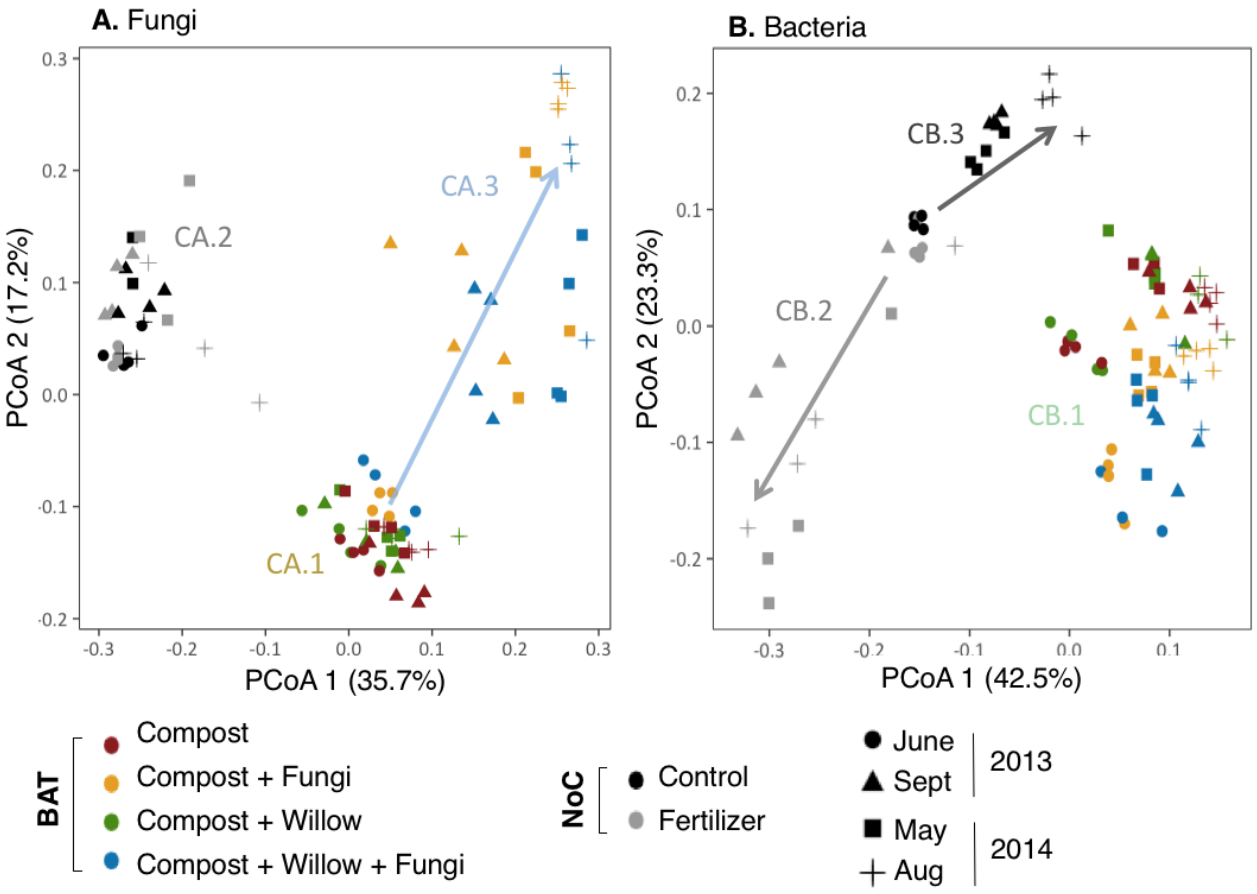
537 indicate a change in PHC extraction method (Text S2). For graphical representation only, year one

538 values were increased 30 % to palliate lower recovery rates of that year. The lines at 260 and 150

539 mg kg^{-1} indicate the CCME's Commercial & Industrial (C&I), and Agricultural &

540 Residential/Parkland (A&RP) limits, respectively.

541



542

543 **Figure 2. A.** A principal coordinates analysis (PCoA) was performed on fungal OTU datasets using

544 Jensen-Shannon divergence. **B.** Another PCoA was performed on bacterial OTU datasets using

545 Bray-Curtis dissimilarity. Arrows indicate temporal shifts.

546

547

548

ASSOCIATED CONTENT

Declaration of interests

This project was funded through NSERC's College and Community Innovation (CCI) grant to S. Mooney (Cold Climate Innovation, YRC); an NSERC CREATE Mine of knowledge scholarship to KR; NSERC Discovery grants to MA and MH; NSERC northern supplement and Canada Research Chair grants to MA; the Northern Scientific Training Program to KR, and in-kind contributions from Boreal Compost Enterprises Ltd. and Arctic Backhoe Services Ltd, who also provided land space.

Acknowledgements

Special thanks go out to Robert McPhee and Kira Beukeboom the 2013 and 2014 summer students, as well as to Isobel Ness, David Silas and Corey Théoret, for their positive presence, clever contributions and hard work. Many thanks go out the Amyot laboratory for their kind and continued support, and logistical help. Thank you to the Y2C2 crews, the Yukon Research Center members and Stephen Mooney, and to the countless other people that have generously contributed their time, knowledge and resources to this project. Thank you to Stéphane Daigle and Raphaël Lavoie for their meticulous assistance through the statistical analyses. Finally, thank you to Garret Gillespie for logistical support and contributions that initially inspired this project. We appreciate that working directly with over a dozen local students, scientists and residents, enabled this project to be more holistic and to work with an ecosystem-based approach within circular economy principles (Sauvé et al. 2016).

SUPPORTING INFORMATION

Figure S1. A. Field layout. **B.** Experimental bin scheme.
Figure S2. Temperature in bins.
Figure S3. Relative abundance of major phyla. **A** Fungal. **B.** Bacterial.
Figure S4. Alpha diversity **A.** Fungal. **B.** Bacterial.
SUPPLEMENTARY METHODS.

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