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Spatial analysis of a hydrocarbon waste-remediating landfarm demonstrates influence of management practices on bacterial and fungal community structure.

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Running title: Microbial community structure in a hydrocarbon-waste landfarm (max 50 char.)

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SUMMARY

Cultivation of dedicated soil plots called "landfarms" is an effective technology for bioremediation of hydrocarbon waste generated by various industrial practices. To understand the influence of soil conditions on landfarm microbial communities, analysis of bacterial and fungal community structure at different sections and depths was performed across a hydrocarbon-waste landfarm in Regina, Saskatchewan, Canada. While a core set of hydrocarbon-associated bacterial and fungal taxa are present throughout the landfarm, unique bacterial and fungal operational taxonomic units (OTUs) are differentially abundant at sections within the landfarm, which correlate with differences in soil physiochemical properties and management practices. Increased frequency of waste application resulted in strong positive correlations between bacterial community assemblages and elevated amounts of oil, grease, and F3 – F4 hydrocarbon fractions. In areas of standing water and lower application of hydrocarbon, microbial community structure correlated with soil pH, trace nutrients, and metals. Overall, diversity and structure of bacterial communities remain relatively stable across the landfarm, while in contrast, fungal community structure appears more responsive to soil oxygen conditions. Results are consistent with the hypothesis that years of bioremediation activity have shaped microbial communities, however several management practices can be undertaken to increase efficiency of remediation, including the removal of standing water and soil tilling across the landfarm.

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INTRODUCTION

Soils naturally contain diverse microorganisms capable of degrading complex hydrocarbons arising from industrial activity. Controlled application of industrial waste to topsoil has provided reliable bioremediation to reduce hydrocarbon waste in various industries (Khan *et al*., 2004). These treatment plots are referred to as "landfarms", and the development of best practices for the maintenance of these systems for *ex-situ* treatment of wastes is attractive as a cost-saving and environmentally-responsible form of waste disposal (Rhykerd *et al*., 1999; Besalatpour *et al*., 2011). Like other bioremediation technologies, treatment efficacy of landfarms depends on multiple physical, chemical, and biological features, including land area, soil aeration, nutrient availability, pollutant mobility and toxicity, and microbial biodiversity (Rhykerd *et al*., 1999; Straube *et al*., 2003; Khan *et al*., 2004; Harmsen *et al*., 2006).

Effective management practices will consider and modulate these soil features to improve the activity of the bacterial and fungal communities, which are the major drivers of the biological degradation of hydrocarbons and other waste compounds (Leahy *et al*., 2003; Strobe *et al*., 2003; Seo *et al*., 2009; Militon *et al*., 2010). However, little is known about the structure and variability among microbial communities in landfarms, thus the contributions of management practices such as tillage (contribution to aeration of soils), waste dosing/application rates, and moisture content towards microbial community activity and stability are poorly understood.

EVRAZ Inc. North America (hereafter EVRAZ), is a leading manufacturer of tubular steel products in Regina, Saskatchewan, Canada. EVRAZ has maintained a landfarm for remediation of hydrocarbon spills since 1994 (**Figure 1**). Routine soil sampling indicates that toxic

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hydrocarbons from applied waste are degraded in the first few centimeters of soil; a success which has been attributed in some part to the landfarm maintenance program that includes nitrogen fertilization, aeration through tilling, regular removal of standing water, occasional application of sanitary sewage treatment plant sludge, and pH monitoring (Schutzman, 2014). The landfarm is divided into three separate management sections, A, B, and C, enabling comparison of differences in waste application and soil characteristics across the landfarm. Such an established landfarm is expected to contain mature microbial communities selected for their ability to metabolize complex hydrocarbons, and which presents an ideal opportunity to characterize the response and stability of the microbial communities to landfarm management activities. Here we profile the bacterial and fungal taxa in the landfarm and analyze community structure and diversity in response to hydrocarbon application, soil depth, and soil saturation.

EXPERIMENTAL PROCEDURES

Site Details

The landfarm located in Regina, Saskatchewan, Canada is divided into three sections (A, B, and C; **Figure 1**) surrounding a waste holding area, where hydrocarbon waste is continuously collected from steel plant activities and stored over the course of fall, winter, and early spring months (**Table 1**). Sections A and C share similar soil characteristics, however Section A has historically received more waste than Section C. Section B has the lowest hydrological grade, with water accumulating in this section after snowmelt or heavy periods of summer precipitation. In 2015, application of accumulated waste to sections A and C began in April and occurred

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periodically until late November. After the majority of standing water was removed, in early summer and fall, soils were tilled, followed by fertilization with urea in November (**Table 1**).

Sample collection

Samples were collected with an AMs Frozen Soil Powered Auger Kit at two depths, "surface" (0 – 15 cm) and "deep" (15 – 30 cm), at ten sites in three sections (A, B, and C) of the landfarm. This was done following tilling of the site in June 2015, with "surface" samples (0 – 15 cm) collected again in November (**Table 1**). An additional sample was taken by hand using gloves from the top 5 cm of soil from an area ~ 500 m outside the landfarm for characterization of non-impacted soils (ERW borrow-pit; **Figure 1**). From bagged soil samples for each depth, approximately 50 g was subsampled by hand using gloves and processed for bacterial and fungal community analysis. Soils collected from each of the ten individual sites per landfarm section were sequenced as independent samples and not composited. The remainder of the June samples were composited into landfarm sections (A, B, and C) at each depth and sent to Saskatchewan Research Council Environmental Analytical Laboratories (Saskatoon, SK) for analysis of pH, trace micronutrients (Ca, K, Na), metals (As, Cd, Cu, Co, Fe, Mg, Mn, Mo, Zn), total organic carbon, total nitrogen, phosphorus, oil and grease, and hydrocarbons: benzene, toluene, ethylbenzene, and xylene (BTEX) and Canadian Council of Ministers of the Environment (CCME) hydrocarbon fractions F1 – F4 (**Table S1**; CCME, 2008).

Microbial community profiling

Environmental DNA (eDNA) was isolated from 0.25 g of homogenized soil samples using MoBio Powersoil DNA Isolation (Carlsbad, CA) according to manufacturer's instructions.

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Bacterial and fungal sequencing libraries were prepared according to Kozich *et al.*, (2013), using primers designed for the v4 hypervariable region of the bacterial 16s rRNA gene, and primers for the fungal internal transcribed sequence (ITS-2) region (Schoch *et al.*, 2012; Gweon *et al.*, 2015). Sequencing of a bacterial mock community (BEI Resources; Manassas, VA) facilitated calculation of the sequencing error rate (Kozich *et al.*, 2013). Sequencing was performed on the Illumina MiSeq platform for both bacterial libraries (v2; 250 bp paired-end reads) and fungal libraries (v3; 300 bp paired-end reads).

Bacterial sequencing data was processed using Mothur (v.1.39.5; Kozich *et al.*, 2013) to remove low quality reads and perform operational taxonomic unit (OTU) assignment at the 97% identity level, utilizing the SILVA reference bacterial database (v.123; Quast *et al.*, 2013) for taxonomic identification. Fungal sequencing reads were processed for quality and analyzed using the PIPITS fungal taxonomic assignment software (v.1.4.2; Gweon *et al.*, 2015) with the UNITE fungal ITS reference database (v.7.0; Kõljalg *et al.*, 2013).

Prior to all subsequent analysis, data was rarefied to the level of 5,307 and 11,077 sequences for bacterial and fungal samples, respectively. Inverse Simpson diversity was calculated for all samples, and then analyzed using a generalized linear model (GLM) to determine the effect of section, depth, and month of sampling as factor covariates. An interaction between landfarm section and soil depth was included in the GLM. Sample diversity is a positive real valued response variable with strong mean-variance relationship. To account for this, we assumed the response was conditionally distributed following a Gamma distribution with support on the positive real values. Generalized linear hypothesis tests of differences between selected treatment effects were performed using contrast coding and p-values adjusted for multiple comparisons following via the

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multcomp package (Hothorn et al, 2008) for R (v 3.5.1; R Core Team, 2018) Abundance of bacterial and fungal taxa were visualized via ClustVis (Metsalu and Vilo, 2015).

Redundancy analysis for soil chemistry correlation

To assess relationships among geochemical parameters and community structure, RDA was performed on select parameters of the geochemical dataset (**Table S1**), using the vegan package (v.2.41; Oksanen *et al* 2016) for R. Bacterial and fungal OTU data were screened to remove taxa present in fewer than five samples in the data set and subsequently Hellinger transformed (Legendre and Gallagher, 2001). The month of sampling was used as a partial covariate to remove the effect of sample collection date from the data prior to analysis

The direction and magnitude of greatest change in RDA ordination space for each geochemical variable was calculated using a least squares regression. Each geochemical variable, in turn, was used as the response variable and the coordinates of the samples on RDA1 and RDA2 were used as covariates. The two resulting regression coefficients provide vector coordinates in the RDA space, which represent the direction of maximal change. The R^2 statistic provides indication of the strength of the correlation between the configuration of samples in ordination space and each geochemical variable (assuming a linear relationship). Correlations were assessed statistically using a randomization test with 999 permutations.

RESULTS

Landfarm microbial diversity

Sequencing of 16S rDNA and ITS-2 taxonomic markers was conducted for all soil samples. Goode's coverage estimates, which are the proportion of DNA sequences belonging to OTUs that

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have been observed more than once, averaged 0.956 for bacterial and 0.990 for fungal communities, indicating sufficient DNA sequencing coverage to profile microbial communities in all samples. Up to 1200 bacterial and 400 fungal OTUs were identified in each sample, with a total of twenty-three bacterial and 7 fungal phyla identified in the landfarm.

Bacterial diversity is significantly higher in surface soil within landfarm sections A (effect estimate -1.557; $p < 0.00001$) and B (effect estimate -1.2044; $p = 0.0254$) (**Table 2**), which receives more frequent application (**Figure 2**). Alternatively, it is expected that the deep section B soils are the most anaerobic within the landfarm, given the soil-saturating presence of standing water, which corresponds to the statistically modelled difference between the surface and deep samples of section B (**Figure 2; Table 2)**. Interestingly, there was no significant difference in fungal diversity between surface or deep soils within any of the three landfarm sections (**Table 2**). However, an effect of standing water on section B fungal communities was observed when we examined differences in diversity between sections by combining surface and deep samples from each section. By this measure, bacterial diversity was not significantly different across the landfarm, but fungal diversity was significantly lower in section B compared to sections A (effect estimate 0.62161; $p = 0.00146$) and C (effect estimate -0.92973; $p < 0.00001$) (**Table 2; Figure 2b**).

Landfarm microbial taxonomy

Differential abundance analysis of bacterial and fungal OTUs across landfarm sections demonstrate that landfarm sites are distinct from the control ERW site (**Figure 1**), which is presumed to represent the natural and ancestral state of the landfarm soils (**Figure 3a,b)**. When considering the 50 most abundant bacterial OTUs, landfarm sections do not form natural clusters (**Figure 3a**), revealing that despite the similarities in overall diversity described above, there is

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heterogeneity among the most abundant taxa between sections and at both depths within sections. Section B samples contained the most distinct OTU abundance profiles, as section B surface and deep OTUs did not cluster with each other, nor closely to the other sections and control soils. Multiple classes of bacteria that metabolize hydrocarbons in the absence of oxygen were found only in the water-saturated soils of section B. For instance, Anaerolineaceae, which include anaerobic bacteria associated with *n*-alkane degradation (Liang *et al*., 2016), was abundant and common, particularly in deep soils (**Figure 3a**). Although it did not rank in the few most abundant illustrated in **Figure 3a**, Hydrogenophilales (*Thiobacillus*), a microaerophilic hydrogen-oxidizing bacterial group (Orlygsson and Kristjansson, 2014), that was also particularly abundant in section B (**Table S2**).

Across the landfarm, common soil residents Actinomycetales were the most abundant order of bacteria (**Table S2**) and soils were highly enriched in taxonomic groups identified in other hydrocarbon-contaminated soils and water (Peng *et al*., 2015), consistent with the hypothesis that EVRAZ landfarm soils communities have been refined by years of bioremediation activity. These include (but are not limited to) bacterial taxonomic groups of Pseudomonadales (Leahy *et al.*, 2003; Seo *et al.*, 2009), Acidobacter (Seo *et al*., 2009; Peng *et al*., 2015), Gammaproteobacteria and Actinobacteria (Militon *et al*., 2010; Peng *et al*., 2015), Chloroflexi, Planctomycetes, and Bacteroidetes (Peng *et al*., 2015) (**Table S2**). Specific genera previously demonstrated to be associated with hydrocarbon-impacted sites and high molecular weight polyaromatic hydrocarbons, such as bacterial OTUs identified as Anaerolineaceae, Caldilineaceae (Zhang *et al.*, 2017), and *Lutibacter* (McFarlin *et al*., 2015) are shown to be elevated in abundance in samples from shallow and deep soils of A and B relative to section C (**Figure 3a**). Additionally, hydrocarbon-associated OTUs *Terrimonas* (Adrion *et al*., 2016) and *Agromyces* (Zhang *et al*.,

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2010) were found abundant in section A soil; while *Thiobacillus* (Militon *et al*., 2010), and *Petrimonas* (Grabowski *et al*., 2005), were found in higher abundances in section B soil.

When fungal OTU abundance is considered, surface and deep samples cluster together within each section, while ERW forms the most divergent cluster (**Figure 3b**). Ascomycota were the most abundant order of fungi (**Table S3**), and fungal hydrocarbon-related taxonomic orders including Pezizomycetes (Bell *et al*., 2014), Pleosporales (Bourdel *et al*., 2016; Kolařík *et al*., 2016; Mohammadian *et al*., 2017), Hypocreales (Prenafeta-Boldu *et al*., 2005; Mohammadian *et al*., 2017), Capnodiales (Mohammadian *et al*., 2017), Chaetothyriales (Badali *et al*., 2008), and Tremellales (de Garcia *et al*., 2012) (**Table S3**). Specific OTUs belonging to *Sordariomycetes* previously demonstrated to be associated with hydrocarbon-impacted sites and high molecular weight polyaromatic hydrocarbons (Marchand *et al*., 2017), are increased in abundance in deep A and B section soils relative to section C (**Figure 3b**).

Influence of soil parameters on community structure

BTEX, F1 (number of carbons ranging from: C6–C10), and F2 (C10–C16) fractions were below detection limits across all sites (**Table S1**). More-recalcitrant hydrocarbon fractions (longer carbon chain, polyaromatics) of F3 (C16–C34) and F4 (C34–C50) were in greater abundance within deep soils relative to surface soils, though F3 fractions were more abundant than F4 fractions across all samples (**Table S1**).

RDA of fungal OTUs showed a tighter clustering of individual samples within sections, and greater spatial distance between sections compared to bacterial OTUs (**Figure 4a,b),** in agreement with the clustering observed in **Figure 3a,b.** For both bacterial and fungal OTUs in their respective ordination spaces, F3 and F4 hydrocarbon fraction vectors are strongly with the

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centroid of all surface samples, with section A samples showing strong positive correlation (**Figure 4a**). Similarly, Oil and Grease is correlated with surface and section A samples, and negatively correlated with the centroid of section B fungal samples. Parameters of Oil and Grease and F3 and F4 are negatively correlated with Metals, Trace Nutrients, and pH for both bacterial and fungal communities, though Trace Nutrients are well correlated with the centroid of section B bacterial samples (**Figure 4a,b**). Interestingly, moisture is negatively correlated with bacterial B samples, and correlated in the same direction as CCME-classified hydrocarbon fractions, Oil and Grease, and Total Nitrogen.

DISCUSSION

High-resolution sequencing of microbial taxonomic markers has facilitated spatial analysis of bacterial and fungal communities in an active hydrocarbon-remediating landfarm. Results suggests that the sustained application of hydrocarbons and management practices at the EVRAZ landfarm have selected specialized microbial communities that are highly distinct from microbial communities in a nearby soil borrow-pit (ERW) used as a control (**Figures 1 and 2**). Because of the regular application of industrial hydrocarbon waste to the farm soils along with the removal of plant life, we anticipated that the landfarm would be characterized by simple and highly refined microbial communities dominated by specialist species, but surprisingly the landfarm communities are composed of a greater diversity of OTUs than the neighbouring ERW pit soil (**Figures 2 and 3**). The microbial communities of the landfarm are comprised of taxonomic groups that have been previously associated in hydrocarbon-impacted and hydrocarbon-remediating soils. This pattern was consistent in samples collected at the beginning (June) and end (November) of the landfarm operational season, with no statistical influence of month on microbial community

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structure detected by the statistical GLM (**Table 2**). This stability confirms the hypothesis that seasonal changes and operational activities such as tillage, fertilization, and hydrocarbon application are not disruptive over time, but rather work to actively maintain the communities that have established under these management conditions.

The present study provides strong data that supports the expectation that landfarm maintenance practices of both tillage and frequency of waste application influence the structure and profile of resident communities. The increased loading of hydrocarbon waste and fertilization at the surface of section A is what likely selects for specific hydrocarbon-related OTUs of *Agromyces*, *Micrococcacea*, and *Terrimonas* in A relative to B and C (**Figure 3a,b**). Despite tillage to levels of 10 cm across the entire landfarm, the influence of oxygen conditions is most evident on fungal soil communities in deep B anaerobic soils (30 cm), which has noted enrichment of several OTUs, not identified to genus level. The significant influence of water level on soil physiochemical properties and microbial community structure of non-hydrocarbon impacted forest and freshwater wetland soils have been previously demonstrated (Brockett *et al.*, 2012; Ma *et al.*, 2018). Thus, the combined presence of standing water and lack of tillage to this depth contribute to landfarm community structure, in addition to the selective presence of hydrocarbon waste.

Interestingly, across the three landfarm sections, bacterial communities remain relatively more diverse across landfarm sites and depths compared to fungal communities (**Figure 2**; **Table S2**). Fungal genera are naturally highly heterogeneous and metabolically specialized, which could explain the observation that the presence and abundance of specific genera and OTUs are more greatly influenced by differences between site conditions (**Figures 3 and 4**). RDA was used to assess the true influence of these parameters on bacterial and fungal community structures, and we found that the

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strongest correlation occurs with CCME-defined F3 and F4 fractionations to all surface samples, and notably all section A samples (**Figure 4)**. As F3 and F4 molecules are typically more difficult to degrade, even with increased aeration (i.e., at the soil surface) (Seo *et al*., 2009), it stands to reason that the frequent application of hydrocarbon at section A contributes to increased concentration of these recalcitrant products. Thus, while complete remediation of these levels of F3 and F4 by section A microbial communities may require an increased treatment period, the differentially abundant bacterial and fungal OTUs of section A are uniquely adapted to higher concentration of recalcitrant hydrocarbons. Interestingly, increased moisture content corresponds with section A surface samples, and not with section B, indicating some relationship between hydrocarbon waste and resultant soil moisture. This finding provides context and support for previous analysis indicating that remediation activity is optimal with increased soil moisture, in addition to aeration (Bahmani *et al*., 2018).

These results suggest that effective management practices would include removal of standing water from section B and spread section A soils to seed sites B and C, to maintain and potentially increase the efficiency of bioremediation. Furthermore, the more anaerobic deep soil B, which also contains specific hydrocarbon-associated OTUs, if spread throughout section B, and potentially to other sections, may contribute to increased remediation activity. Ultimately, it is evident that management practices and geochemical parameters contribute to the diversity and stability of microbial community structure, and that current conditions of the operational landfarm maintained by EVRAZ supports bacterial and fungal communities capable of contributing to effective remediation of hydrocarbon waste.

SUPPLEMENTAL MATERIAL

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Table S1. Soil analysis results for samples collected in June.

Table S2. Rarefied OTU abundance table of bacterial sequences.

Table S3. Rarefied OTU abundance table of fungal sequences.

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Table 1. Timeline of events at landfarm between April and November 2015.

Date	Event	Details	Location[1]
6 – 15 Apr	Application of waste	Hydrocarbon impacted clay	Section A
20 Apr	Application of waste	Hydrocarbon impacted clay	Section C
5 Jun	Tilling	Landfarm soil turned using a smooth blade grader at a depth of 10 cm	Entire landfarm
9 Jun	Application of waste	Hydrocarbon impacted clay	Section A
10 Jun	Samples collected for biological and chemical analysis	Surface 0 – 15 cm; Deep 15 – 30 cm	26 stations
23 Oct	Samples collected for biological analysis	Sampling of ERW Control site; 0 – 5 cm	Control
2 Nov	Application of waste	Diesel, gas, oil, and hydraulic fluid impacted absorbent pads, oil and diesel impacted sand, slag, soil, contaminated ice	Sections A and C
2 Nov	Fertilization	~2500 kg of 43% NO_3 (containing urea) was used to apply 94.87 to A, 35.11 to B, and 59.52 kg to C	Entire landfarm
20 Nov	Samples collected for biological analysis	0 – 15 cm	24 stations

[1] Refer to Figure 1.

Table 2. Simultaneous tests of general linear hypotheses for differences between and effect of landfarm section and depth[1].

	Null Hypotheses[2]	Bacteria			Fungi		
		Effect Estimate	Z value	Adjusted p-value	Effect Estimate	Z value	Adjusted p-value
Differences in depth averaged over sections	Surface – Deep = 0	0.5325	2.104	0.121	0.08738	0.567	0.92978
Effect of depth within land section	$A_{Deep} - A_{Surface} = 0$	-1.5557	-4.240	< 0.0001***	-0.04721	-0.221	0.9947
	$B_{Deep} - B_{Surface} = 0$	-1.2044	-2.630	0.0254*	-0.57704	-2.162	0.0892
	$C_{Deep} - C_{Surface} = 0$	0.8326	1.718	0.2361	0.32737	1.215	0.5332
Differences between sections averaged over depths	A – B = 0	0.3031	1.071	0.665	0.62161	3.558	0.00146 **
	A – C = 0	-0.1165	-0.389	0.976	-0.30811	-1.715	0.26786
	B – C = 0	-0.4196	-1.302	0.510	-0.92973	-4.783	< 0.0001***

[1]Effect of month (June and November) insignificant, and small replicate number of control samples excluded from analysis.
[2] Null hypotheses tested is no difference between groups (= 0). Null hypotheses rejected at level of $p < 0.05$.

Figure 1. Sample locations within the EVRAZ landfarm.
Soil samples were collected from ten sites within A, B, and C sections of the landfarm, and from an excavation site (ERW; Control) 500m southeast of the landfarm. Map data attributed to Google Earth and DigitalGlobe ©2016.

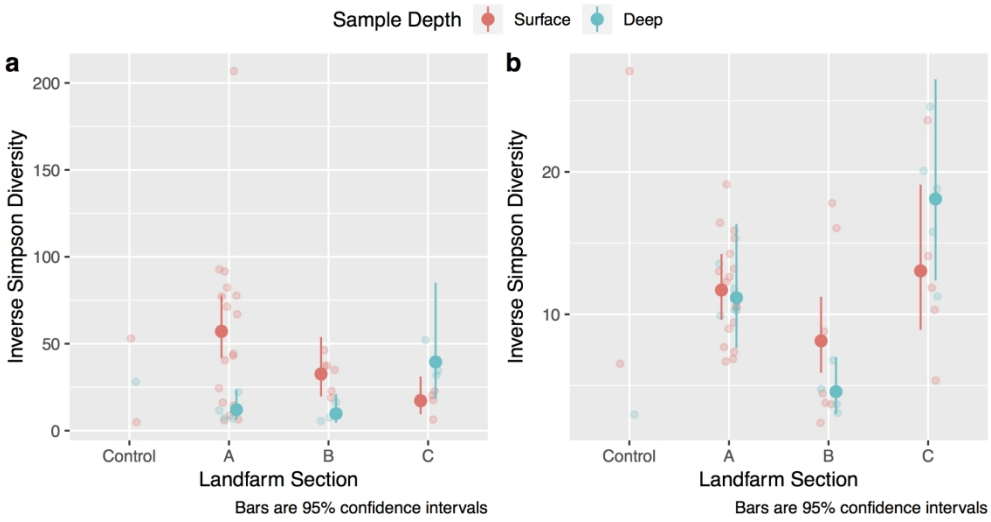

Figure 2. Bacterial (a) and fungal (b) OTU diversity of EVRAZ landfarm soil measured by Inverse Simpson's diversity index.
Inverse Simpson's diversity indices for all samples (June and November) are plotted; points are estimated effects from the GLM. Bars indicate 95% confidence intervals. ERW control, n = 3; Surface samples, n = 8; Deep samples, n = 4.

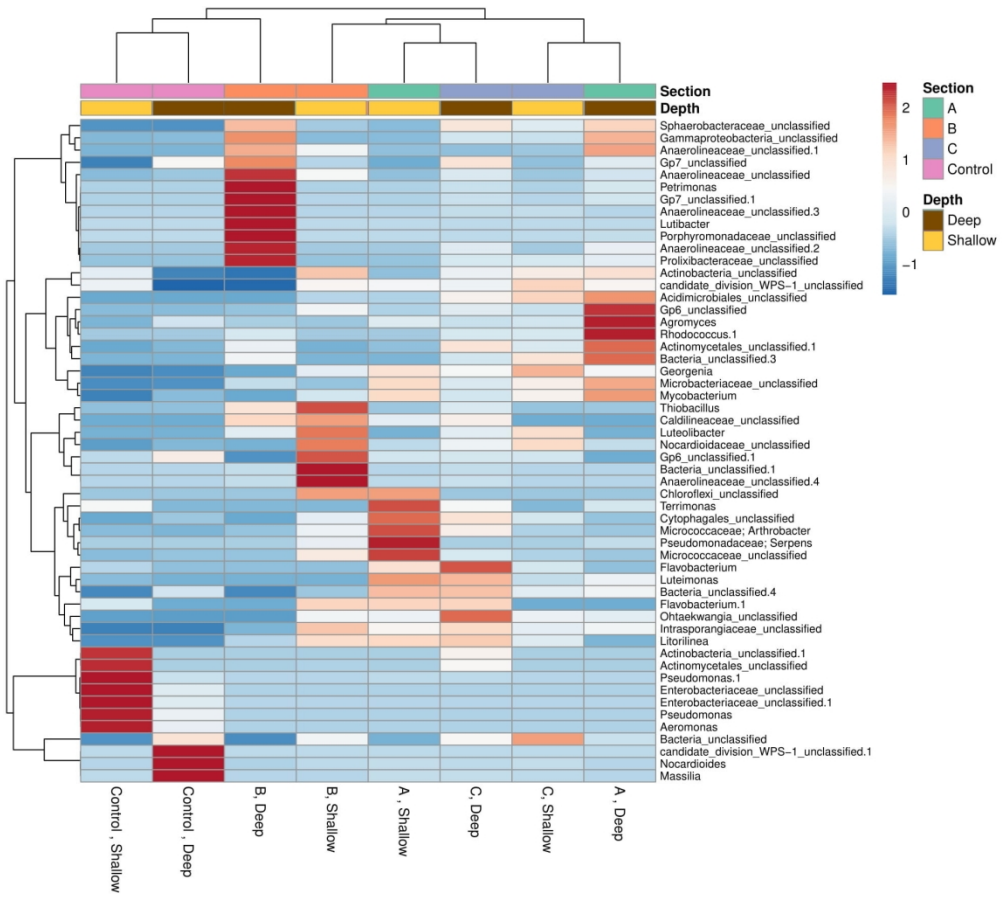

Figure 3(a). Clustering of top 50 most abundant bacterial OTUs.
Columns are labelled according to each section and depth, and the median calculated for each OTU in these sample types. OTU abundance values are centered across rows. Heatmap legend indicates result of unit variance scaling, where the standard deviation from a mean (0) is calculated across all columns and can be compared for each OTU between sample type. Both rows and columns are clustered using correlation distance and average linkage. For each OTU, the most refined taxonomic annotation is provided, with p_; c_; o_; f_; g_ indicating phylum, class, order, family, and genus, respectively.

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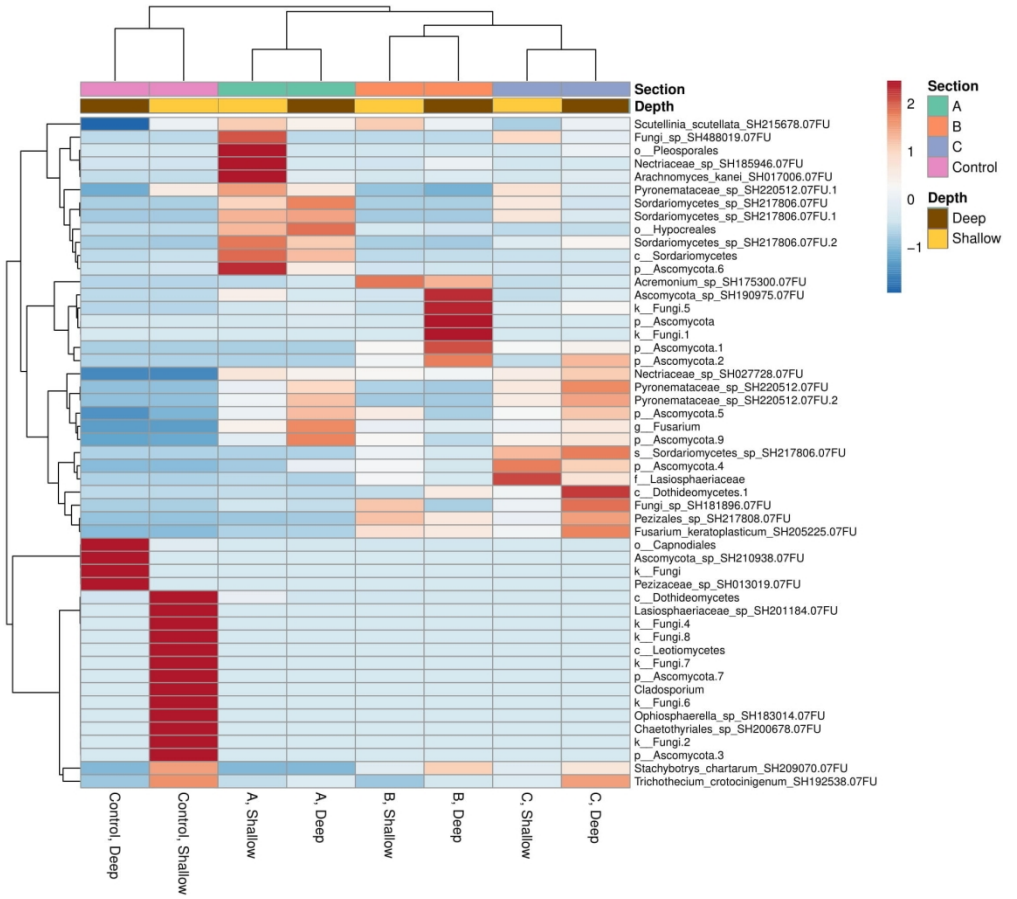

Figure 3(b). Clustering of top 50 most abundant fungal OTUs.Columns are labelled according to each section and depth, and the median calculated for each OTU in these sample types. OTU abundance values are centered across rows. Heatmap legend indicates result of unit variance scaling, where the standard deviation from a mean (0) is calculated across all columns and can be compared for each OTU between sample type. Both rows and columns are clustered using correlation distance and average linkage. For each OTU, the most refined taxonomic annotation is provided, with p_; c_; o_; f_; g_ indicating phylum, class, order, family, and genus, respectively.

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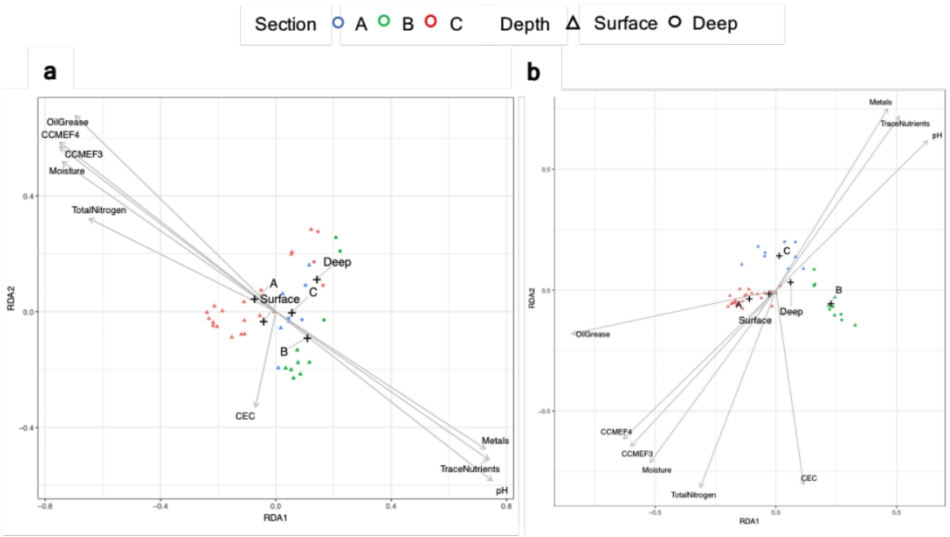

Figure 4. Redundancy analysis of bacterial (a) and fungal (b) OTU abundance and correlation with geochemical parameters.
The origin (0.0, 0.0) can be considered as relative abundance, with symbols further away from the origin having greater relative abundance. Cross marks represent the fitted centroid ("mean effect") of the two factors (section and depth) on variation in OTUs. "Metals" = sum of As, Cd, Co, Mn, Mo; "Trace Nutrients" = sum of Ca, K, Na. Arrow length is scaled according to R2 value, thus the length of each vector represents the strength of correlation or magnitude of change in the direction of each arrow.

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