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Key Points:

- Microbial life in Antarctic surface soils can be severely restricted by constant exposure to cold, dry, salty conditions
- Certain fungi may be better adapted than bacteria or archaea to some of the most challenging soil environments on Earth

Supporting Information:

Supporting Information may be found in the online version of this article.

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Exploring the Boundaries of Microbial Habitability in Soil

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Abstract Microbes are widely assumed to be capable of colonizing even the most challenging terrestrial surface environments on Earth given enough time. We would not expect to find surface soils uninhabited by microbes as soils typically harbor diverse microbial communities and viable microbes have been detected in soils exposed to even the most inhospitable conditions. However, if uninhabited soils do exist, we might expect to find them in Antarctica. We analyzed 204 ice-free soils collected from across a remote valley in the Transantarctic Mountains (84–85°S, 174–177°W) and were able to identify a potential limit of microbial habitability. While most of the soils we tested contained diverse microbial communities, with fungi being particularly ubiquitous, microbes could not be detected in many of the driest, higher elevation soils—results that were confirmed using cultivation-dependent, cultivation-independent, and metabolic assays. While we cannot confirm that this subset of soils is completely sterile and devoid of microbial life, our results suggest that microbial life is severely restricted in the coldest, driest, and saltiest Antarctic soils. Constant exposure to these conditions for thousands of years has limited microbial communities so that their presence and activity is below detectable limits using a variety of standard methods. Such soils are unlikely to be unique to the studied region with this work supporting previous hypotheses that microbial habitability is constrained by near-continuous exposure to cold, dry, and salty conditions, establishing the environmental conditions that limit microbial life in terrestrial surface soils.

Plain Language Summary Microorganisms are the most ubiquitous forms of life on Earth and can be found in even the most challenging environments. As a result, it is often assumed that microbes have come to inhabit every terrestrial surface on Earth. Previous work has hinted that soil environments, without any detectable microorganisms or microbial activity, might exist in Antarctica. To explore this potential limit of habitability, we used a range of approaches, including culturing, DNA sequencing, and metabolic assays to explore patterns of microbial communities in Antarctic surface soils. By testing >200 soils collected across the Shackleton Glacier region, we sought to confirm whether microbial life in Antarctic surface soils is effectively undetectable under certain conditions. While we detected diverse microbial communities in many soils, we could not detect microbes in ~20% of the collected samples. Our results suggest that microbial habitability is limited by the unique combination of cold, dry, salty conditions experienced at inland, higher elevation sites throughout the Transantarctic Mountain. Additionally, the prevalence of fungi at many of the most challenging sites suggests that fungi may be better adapted to some of the most challenging soil environments on Earth than bacteria and archaea.

1. Introduction

Microorganisms are nearly ubiquitous on Earth and can routinely be found in environments that include sediments of the deepest ocean trenches (Pathom-aree et al., 2006), highly acidic geothermal systems

(Hynek et al., 2018), and subsurface aquifers >1 km below the Earth's surface (Sahl et al., 2008). Viable microbes have been detected in even the most inhospitable environments (Merino et al., 2019) and it is widely assumed that all environments on Earth should contain detectable microorganisms (Cockell et al., 2012). This assumption is likely incorrect. Lava flows and lava lakes, e.g., are inhospitable to all microbial life but only remain so for relatively short periods of time until temperatures have cooled sufficiently to permit microbial colonization (Cockell, 2013; Cockell et al., 2012). Few of Earth's stable terrestrial surface environments are expected to experience conditions challenging enough to restrict microbial habitability to the point where organism cannot be detected.

In particular, we would expect that it is very unlikely to find surface soils without detectable microorganisms. Soils typically harbor diverse microbial communities of bacteria, fungi, and archaea (Fierer, 2017). This holds true even for some of the most challenging soil environments found on Earth (Goordial et al., 2016; Schmidt et al., 2018). For example, even soils in the driest regions of the Atacama Desert contain active microbial communities despite these sites receiving ~2 cm of precipitation annually (Schmidt et al., 2018). Soil microbes are impressively adept at tolerating "extreme" conditions, including very high or very low temperatures, high levels of UV radiation, high salt concentrations, very low or high pH levels, and even a near-complete absence of liquid water (Fierer, 2017; Merino et al., 2019; Morozkina et al., 2010; Schmidt et al., 2018). This wide range of microbial tolerances to challenging environmental conditions suggests that, given sufficient time, all surface soils on Earth should harbor active microbial life.

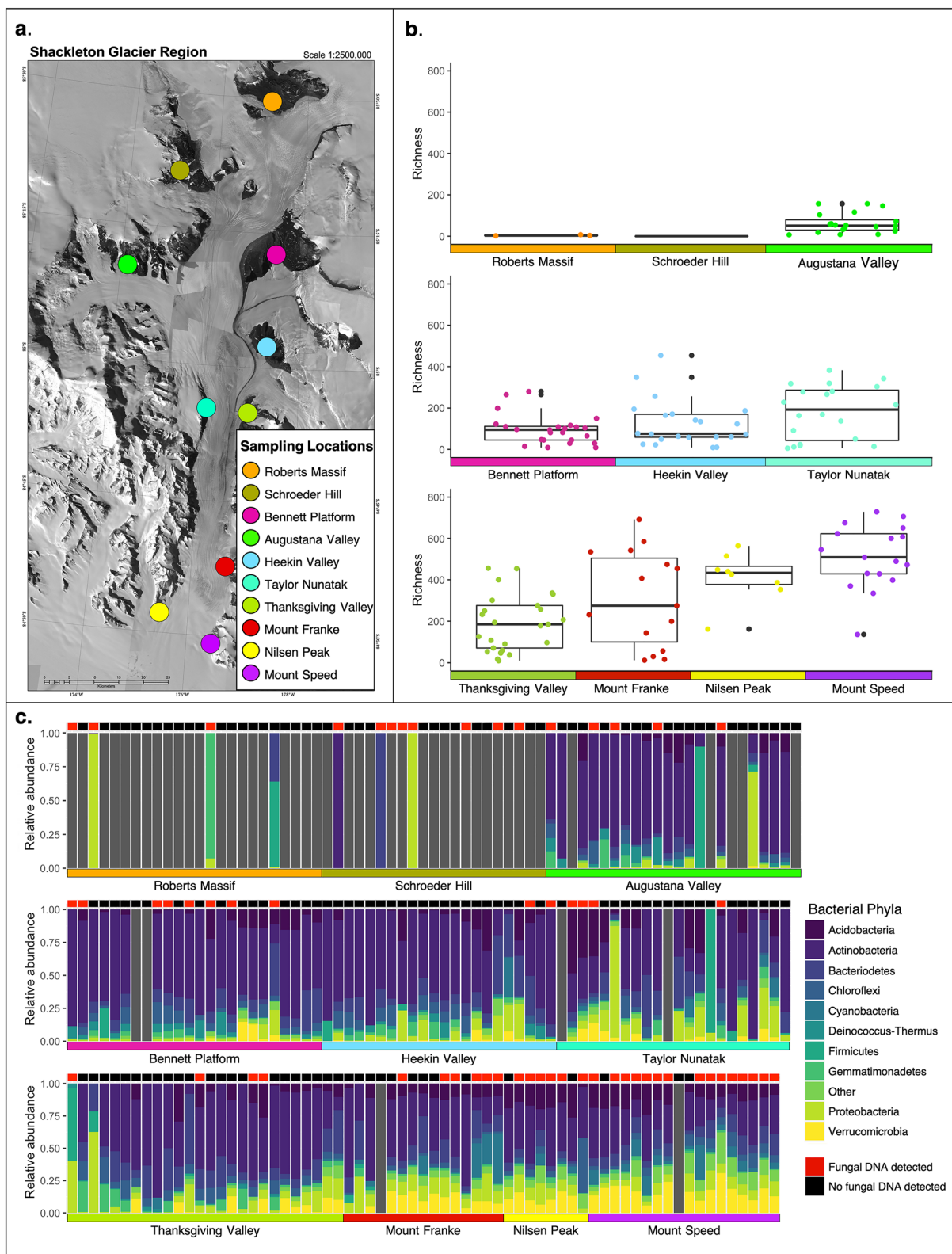
Do surface soils exist where microbial life cannot be detected? If so, Antarctica would be the most likely location to find them. While we know that some Antarctic soils can harbor diverse and metabolically active microbial communities (Cary et al., 2010; Ji et al., 2017), other soils harbor some of the lowest recorded levels of microbial biomass on Earth (Goordial et al., 2016). Many ice-free surface soils in Antarctica are thought to have been exposed for millions of years and have remained largely unchanged over that time (Denton et al., 1989). These soils have generally been unaffected by direct anthropogenic impacts (Bargagli, 2005) and are often very isolated (Archer et al., 2019; Bockheim, 1997). Even if microbes can be dispersed to remote soil patches via aeolian transport (Archer et al., 2019), they often face a unique combination of cold temperatures, extremely low soil water potentials, and high salt concentrations that restrict the activity and survival of all but a few specifically adapted taxa (Goordial et al., 2016; Merino et al., 2019). It seems likely that some isolated Antarctic soils may harbor few, if any, living microorganisms due to these constraints.

To explore the limits of microbial habitability in terrestrial soil environments, we sampled Antarctic surface soils from 10 distinct features along the Shackleton Glacier in the Transantarctic Mountains (84–85°S, 174–177°W). We used a suite of microbiological approaches, including cultivation-dependent, cultivation-independent, and metabolic assays, to answer two questions: Are there soils in Antarctica with no detectable signs of microbial life? and, if so, what environmental conditions might be restricting the activity of microorganisms in these inland soils to the point where they cannot be detected using conventional methods?

2. Materials and Methods

2.1. Sample Collection

Soil samples were collected from the Shackleton Glacier region from December 2017 to January 2018. A total of 204 soils were collected from 10 different features running the length of the valley. These features represent a range of elevations (150–2,221 m) across a 120 km north-south distance spanning from the Ross Ice Shelf to the Polar Plateau (Figure 1). Between 14 and 26 soil samples were collected along elevation-al transects located on each of 10 features to maximize variation in soil characteristics and soil exposure times (amount of time at the surface and uncovered by glacial ice) at each feature. Soils (0–5-cm depth) were collected in sterile polyethylene bags using hand trowels that were sterilized with ethanol before each collection. Approximately 2–5 kg of soil was collected from each location and, after thorough homogenization, a 50–100 g subsample was used for downstream microbial analyses (Diaz et al., 2021). GPS coordinates, photographs of the soil surface, elevation, and other metadata were collected at the time of soil sample collection. Frozen immediately after collection and were kept frozen at –20 °C during transport,



with temperature monitored throughout the duration of transport to ensure that the samples never thawed. The soil samples remained frozen until being processed at the University of Colorado in Boulder, Colorado, USA.

2.2. DNA Extractions

DNA was extracted from all of the collected samples in a laminar flow hood. After mixing 1 g of each soil in 1 mL of sterile PCR-grade water, DNA was extracted from a 500 μ L aliquot of the soil slurry using the Qiagen DNeasy[®] Powersoil[®] HTP 96 Kit (Qiagen, Germantown, MD, USA) following the manufacturer's recommendations. A total of six extraction blanks (2 per 96-well plate) were included to test for any possible contamination introduced during DNA extraction.

2.3. Cultivation-Independent Microbial Analyses via Marker Gene Sequencing

The DNA aliquots extracted from each of the 204 soils and the six extraction blanks were PCR-amplified using a primer pair that targets the hypervariable V4 region of the archeal and bacterial 16S rRNA gene (515F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806-R: 5'-GGACTACHVGGGTWTCTAAT-3'). These primers are identical to those used by the Earth Microbiome Project and are routinely used to assess both archeal and bacterial diversity in a wide range of environments (Walters et al., 2016). To assess the fungal communities that may be found in these soils, we also conducted a separate set of PCR amplifications using a primer pair (ITS1-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2-R: 5'-GCTGCGTTCTTCATCGATGC-3') that target the internal transcribed spacer of the fungal ribosomal RNA (rRNA) operon (Bellemain et al., 2010). Three no-template PCR blanks (1 per 96-well plate) were run for each set of amplifications. Both primer sets included the appropriate Illumina adapters and unique 12-bp barcode sequences to permit multiplexed sequencing (Caporaso et al., 2012). PCR was performed using GoTaq[®] Hot Start PCR Master Mix (Promega, Madison, WI, USA) in 25 μ L reaction volumes. The reaction mixture included 10.5 μ L master mix, 12.5 μ L sterile PCR-grade water, 1 μ L primers (0.5 each F and R), and 1 μ L template DNA. Cycling parameters for both primer sets consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C (45 s), annealing at 50 °C (60 s), extension at 70 °C (90 s), and a final extension step at 72 °C for 10 min. Both sets of amplified products were cleaned and normalized to equimolar concentrations using SequalPrep[™] Normalization Plates (Thermo Fisher Scientific, Carlsbad, CA, USA) with the 16S and ITS rRNA gene amplicons sequenced on separate Illumina MiSeq runs (Illumina, San Diego, CA, USA) using the V2 2 $\times$ 150 bp and 2 $\times$ 250 bp paired-end Illumina sequencing kits (for the 16S rRNA gene and ITS amplicons, respectively). All raw sequences can be found in the NCBI Sequence Read Archive, Project Accession No. PRJNA699250.

16S rRNA gene sequences were processed using the DADA2 pipeline (Callahan et al., 2016). Sequences were quality filtered and clustered into exact sequence variants (ESVs). Taxonomic information was assigned to ESVs using a naive Bayesian classifier method (Wang et al., 2007), which takes the set of ESVs generated and compares them to a training set of reference sequences from the 16S rRNA bacterial and archeal SILVA database (Quast et al., 2012; Yilmaz et al., 2014). A minimum bootstrapping threshold required to return a taxonomic classification of 50% similarity was used for analysis. ESVs associated with chloroplast, mitochondria, eukaryotes, and those unassigned to the phylum level (477 ESVs) were removed prior to downstream analyses. Extraction blanks yielded an average of 420 reads (0–1,831 reads) and these reads came from 14 phylotypes from 11 families *Rubrobacteriaceae*, *Sphingomonadaceae*, *Sulfruspirillaceae*, *Xanthobacteraceae*, *Burkholderiaceae*, *Sporolactobacillus*, *Clostridiaceae*, *Bacillaceae*, *Planococcaceae*, *Deinococcaceae*,

Figure 1. Richness and composition of the soil bacterial communities across the Shackleton Glacier region. (a) A map of the Shackleton Glacier region (Lat: 84–85°S, Long: 174–177°W) with the locations of the 10 features where samples were collected indicated by the colored dots. (b) The richness of the bacterial communities (number of bacterial phylotypes detected per sample) from each of the 10 sampled features. Box and whisker plots indicate the distribution of bacterial richness levels (number of phylotypes per sample) across all samples from each individual feature. (c) Relative abundances of the dominant bacterial phyla from the 204 surface soil samples analyzed. Samples are grouped by feature and organized top to bottom from high elevation to low elevation (higher elevation sites are further south). Samples with no amplifiable bacterial DNA are represented by gray bars. More detailed taxonomic information about the bacterial communities identified in each sample can be found in Data Set S1. The presence/absence of amplifiable fungal DNA is indicated by the red and black boxes over each bar. For more information about the fungal communities identified in each sample see Figure S1, Data Set S2.

Enterobacteriaceae, some of which have been classified as bacterial taxa commonly associated with reagent contamination (Salter et al., 2014). These 14 ESVs found in the extraction blanks represented 100% of the reads from these blanks, while these 14 ESVs only accounted for 0.31% of the reads from all extracted soil samples on average. No-template PCR blanks yielded 311 reads on average (126–586) and ESVs associated with these reads were common in other samples (including taxa within the families: *Blastocatellaceae*, *Soilrubrobacteriaceae*, *Clostridiaceae*, *Paenibacillaceae*, *Chitinophagaceae*, and *Rubrobacteriaceae*) and these taxa are not typical reagent contaminants (Salter et al., 2014), but are instead most likely derived from the soil samples and represent “tag switching” events (Schnell et al., 2015). For this reason, we used 586 reads per sample as a threshold for determining that we could reliably detect prokaryotic DNA using this DNA sequencing approach. All soils with <586 reads were considered to have no detectable PCR-amplifiable prokaryotic 16S rRNA genes. Across the 153 samples that had a sufficient number of bacterial or archaeal 16S rRNA gene reads for downstream analyses, the mean number of reads per sample was 25,912 (629–58,150) while the mean number of reads in the 51 samples that did not meet the threshold determined from the analysis of the “blank” samples was only 125 reads per sample.

Fungal ITS sequences were also processed using the DADA2 pipeline (Callahan et al., 2016). Sequences were quality filtered and clustered into exact sequence variants (ESV). Taxonomic information was assigned to ESVs using the same naive Bayesian classifier method described previously (Wang et al., 2007) but with the UNITE database (Nilsson et al., 2019). A minimum bootstrapping threshold required to return a taxonomic classification was set at 85% similarity (Abarenkov et al., 2010; Nilsson et al., 2019). All six of the extraction blanks and 3 no-template PCR controls had zero reads after processing. We used all ESVs that could be classified to at least a fungal phylum for our analysis. After this filtering, 143 of the 204 samples had no remaining ITS reads. These 143 samples with no identifiable fungal ITS sequences were considered to have no amplifiable fungal DNA. The remaining 61 samples had a mean of 5,886 reads per sample and were considered to have a sufficient number of fungal ITS reads for downstream analyses.

2.4. Quantitative PCR

We measured 16S rRNA gene copy number and ITS gene copy number using the same primers and DNA extracts used for sequencing. Reaction conditions and details followed methods described by Carini et al. (2016). Each of the 204 soil samples, along with corresponding DNA extraction blank, were run for both gene regions and no-template controls were included for each gene region (16 for 16S rRNA, 18 for ITS). Standard curves were calculated using purified genomic DNA from *Escherichia coli* for 16S rRNA copy number and *Aspergillus fumigatus* for ITS copy number. Ct values from the negative controls were used to identify a limit of DNA detection for each gene region. For 16S rRNA, samples with a Ct value greater than 31 were considered below detection limits. For ITS, samples with a Ct value greater than 35 were considered below detection limits. Calculated copy number measurements for each sample are reported as number of *A. fumigatus* or *E. coli* genome equivalents $\times$ g soil⁻¹.

2.5. DNA Extraction and PCR Inhibition Test

From the cultivation independent ITS and 16S rRNA gene sequencing analyses described above, we identified ~20% of samples (40 out of the 204 individual soils) that had no amplifiable DNA from the targeted bacteria, archaea, or fungal marker genes. To confirm that the PCR amplification of microbial DNA from these 40 samples was not simply a product of DNA extraction problems or PCR inhibition, we performed an inhibition test. For this test, we used two 250 mg subsamples of three soils that did not PCR amplify and one soil that successfully amplified. Following the methods described by Warren-Rhodes et al. (2019), one subsample of each of the four soils was extracted after adding a 50 μ L suspension of *E. coli* in trypticase soy broth at a concentration of 10⁷ CFU. The other subsample was extracted with no addition of *E. coli*, with the DNA extractions on all samples conducted as described above. Extracted DNA was amplified using the 16S rRNA gene-targeting primers, reagents, and PCR conditions described above. The concentrations of both the extracted gDNA and amplified product from each soil sample replicate were measured with a Molecular Devices SpectraMax M2 microplate reader (Molecular Devices, LLC, San Jose, CA, USA) using an Invitrogen Quant-iT™ Picogreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Amplified products were also visualized on a 2% agarose gel. No signs of inhibition were detected, i.e., the

DNA from all subsamples amended with *E. coli* cells were successfully PCR-amplified (Figure S4). Thus, we can conclude that our failure to recover 16S and ITS rRNA gene reads from these 44 samples was not likely a result of PCR inhibition, but rather any microbial DNA in these samples was simply not detectable using our cultivation-independent sequencing approach.

2.6. Cultivation-Dependent Analyses

As an added test of the presence of microbial cells, we attempted to cultivate microbes from a subset of 35 soil samples (Supplementary Table S2). These 35 soils were selected to represent the range of environmental gradients encompassed by the larger data set and included samples from all 10 features within the Shackleton Glacier region, including soils with detectable microbial communities and those with microbial communities below detection limits (as determined by the cultivation-independent sequencing). These 35 soil samples were plated on 17 different types of media (Table S1) (Atlas, 1993; Egan et al., 2015; Pulschen et al., 2017; Reasoner & Geldreich, 1985; Singh et al., 2016; Tahon & Willems, 2017) to capture a broad diversity of microbial taxa that could be living in these soils. One gram of each soil sample was homogenized with 1 mL of water and 60 μ L of the resulting soil slurry was pipetted onto each of the 58 cm² plates and spread across the plates using flame sterilized cell spreaders. “Blank” plates inoculated with only 60 μ L sterile water were prepared for each media type and handled in an identical manner to the 595 plates inoculated with the soil slurries. One “positive control” soil sample was collected from a lawn on the University of Colorado Boulder’s main campus and was plated on all media types using the same techniques. The TSA 24 plates, the PH, and PA plates were all left to incubate at 24°C while the rest of the plates were incubated at 4°C. All plates, besides the PA and PH plates, were left to incubate in the dark. Plated samples, and the uninoculated control plates, remained under these conditions for 8 weeks. Photos of the plates were taken each week. Every distinct colony on each of the 629 petri plates was counted weekly from these photos and microbial cell numbers (CFU $\times$ g soil^{−1}) were calculated from these colony counts. The reported total cell numbers are the cumulative number of colonies that grew on each plate over the 8-weeks incubation period. At the end of the 8-weeks incubation, those plates that had no visible colonies (307 of 629 plates) were incubated for an additional month. No colonies grew on any of the blank plates over the 3-month incubation.

2.7. ¹³C Glucose Metabolism Assay

To further verify whether there were, indeed, some soil samples with no detectable microbes and to complement the cultivation-independent and dependent approaches described above, we conducted a ¹³C-glucose metabolic assay with the subset of 35 soil samples described above. We used ¹³C-glucose as a substrate because glucose should be readily catabolized by most heterotrophic microbes. Two 1 g replicate subsamples of each of the 35 soils were placed in sterile glass tubes. Three soil samples (including the one used in the culturing experiment) collected from the University of Colorado Boulder’s main campus were prepared in the same way as the Shackleton Glacier samples to serve as “positive” controls. One of the replicate subsamples from each of the 38 soil samples, plus 6 soil-free blank tubes, was autoclaved on a gravity cycle with a 60-min sterilization and a 30-min drying cycle so we could assess metabolic activity in paired autoclaved versus unautoclaved subsamples of each soil. A 250 μ L solution ¹³C-glucose (99 atom% U-¹³C, Cambridge Isotope Laboratories, Tewksbury, MA, USA) dissolved in H₂O was pipetted directly into each soil, an addition of $\sim$ 250 μ g glucose C per gram soil. This amount of glucose added to the soils is comparable to the glucose amendments used in other comparable soil metabolic assays (Bastida et al., 2013; Derrien et al., 2014). A 2 mL cryotube containing 1 mL of 1N sodium hydroxide solution was placed in each tube before all tubes were sealed with an airtight cap. We prepared a total of 76 soil incubations (35 Antarctic soils + 3 Colorado soils with and without soil autoclaving) and six blanks (three tubes with NaOH traps without soil or glucose, and three tubes without NaOH traps without soil but with glucose). All tubes were incubated for 1 month at 4°C after which an aliquot of the NaOH trap from each tube was transferred to preevacuated glass vials filled with helium gas. The NaOH traps were acidified with 1 mL of concentrated phosphoric acid to release CO₂ into the evacuated headspace. The quantity and isotopic composition of the released ¹³CO₂ were determined using a ThermoFisher Scientific Gasbench II (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Delta V Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Data from the mass spectrometer was processed using the R package “isoproccCUBES.” Sample readings were

corrected using the IAEA reference standard NBS18 (Crowley, 2010). The delta ^{13}C values were converted to fraction of ^{13}C per mil, expressed as ‰, using the international standards of V-PDB (Vienna Pee Dee Belemnite, Coplen et al., 2006). Positive glucose mineralization was detected in all measurements, including in control blanks who had an average reading of $1.095 \text{ ‰ } ^{13}\text{C} \times \text{g soil}^{-1}$. Samples were identified as harboring active microbes based on comparisons between the replicate unautoclaved and autoclaved samples and positive controls. Significance was calculated using Kruskal-Wallis tests as implemented in R with differences between groups determined by a Nemenyi test calculated using the `kruskalmc` function in the R package “pgirmess.”

2.8. ATP Assay

To further confirm the results of the ^{13}C glucose metabolism assay, we conducted an ATP assay with the same subset of 35 soils to test for any bacterial activity. One-gram subsamples of each of the soils were placed in sterile 2 mL microcentrifuge tubes. Tubes were homogenized into a soil slurry with 1 mL of PBS. Three 100 μL aliquots of each homogenized soil slurry (35 Antarctic soils + 3 Colorado soils) were pipetted into black, 96-well Costar Assay plates (Corning Inc., Corning, NY, USA) and ATP production was analyzed using the Promega BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. This assay has been used in other studies to detect low levels of bacterial activity on simulated Martian environments (Schuergel et al., 2008). Luminescence readings were performed using a Biotek Synergy™ HT Plate Reader (Biotek Instruments, Inc., Winooski, VT, USA). Six reagent “blanks” (three wells containing just the sterile PCR-grade water used to make the soil slurries, three wells with water and glucose), and six blank wells were measured using the same methods. After this “baseline” reading, a 250 μL filter-sterilized solution of glucose dissolved in H_2O was added to each of the soil slurries, an addition of $\sim 250 \mu\text{g}$ glucose C per gram soil. Soils were mixed and were then left to incubate at 4°C . After a 24-h incubation, the soil slurries were measured again following the previously described methods, with the same reagent blanks and blank wells.

ATP concentrations were calculated from luminescence readings based on a standard curve ($R^2 = 0.99$) generated from a 10-fold serial dilution (10^{-10} – 10^{-15} mols) of a Tris-buffered ATP standard (Thermo Fisher Scientific, Hampton, NH, USA). Based on this standard curve, we determine that we were effectively able to detect ATP concentrations $>10^{-15}$ mol ATP $\times$ g soil $^{-1}$.

2.9. Soil Geochemical Analyses

To determine soil water content, 50 g of soil was put into aluminum weigh dishes and dried at 105°C for 24 h. % H_2O was determined by the difference between the dried and initial weights of the soils. Since studies have shown that organic matter can and does degrade at this temperature, the water content analysis was done on a separate aliquot of samples.

Soils were leached at a 1:5, soil to DI water ratio and filtered through 0.45 μm nucleopore filters. Anions were analyzed using a Dionex ICS-2100 ion chromatograph and an AS-DV automated sampler. Cations were analyzed by optical emission spectrum on a PerkinElmer Optima 8300 inductively coupled plasma mass spectrometer (ICP-OES) (Diaz et al., 2021). Relative soil exposure ages were estimated using perchlorate concentrations in 1:5 soil to water leaches and annual fluxes from the McMurdo Dry Valleys, Antarctica. Fluxes are estimated to range from 1 to 4 $\mu\text{g m}^{-2} \text{ yr}^{-1}$ and average 2 $\mu\text{g m}^{-2} \text{ yr}^{-1}$. Perchlorate salts are highly soluble in water and the primary source is wet and dry atmospheric deposition (Jackson et al., 2015, 2016). Therefore, surface soil concentrations can be used to calculate a rough estimate of the amount of time which has passed since the soil was last inundated or wetted.

2.10. Statistical Analyses

We performed a random forest analysis to determine if any of the measured environmental and geochemical variables could be used to predict whether a soil would have no detectable microorganisms or detectable microorganisms. The factors used in our models were chosen from a total of 37 different measurements. Highly correlated variables, edaphic factors, and variables that were not measured on at least 80% of the

204 samples were not included in the analysis. The final variables used for analysis included elevation, the concentration of certain water-soluble salts (NO_3^- , Cl^- , ClO_3^- , ClO_4^-) leached at a 1:5 soil: water ratio, and a sum of total salt, total anions, and total cations calculated from these leached results. Cation and anion concentrations were log transformed before analysis. To determine what environmental variables might best predict which soils would have detectable microorganisms, we used the R package “rfPermute” and performed a random forest analysis with 100 trees and three variables tried at each split to identify the most important predictors. As we were unable to perform all of our habitability tests on our full data set of 204 samples, the random forest model was based on whether we could detect microbial DNA in each sample using the cultivation-independent marker gene sequencing approach (i.e., whether we could detect bacterial, archeal, or fungal sequences).

3. Results

3.1. Microbial Communities of the Shackleton Glacier Region

The 204 individual soils collected for this study represent a broad range of ice-free sites found across the Shackleton Glacier region. Sites ranged in elevation from ~150 to 2,221 m above sea level (m.a.s.l) and most soils are likely to have been exposed for prolonged periods of time, with the approximate time since last wetting (estimated from ClO_4^- concentrations, Jackson et al., 2015, 2016) ranging from <10 years to >2 million years (mean: ~20,000 years). Not surprisingly given the absence of plants in this region, the measured soil organic carbon concentrations were low, from ~3 to 60 $\text{mg} \times \text{g soil}^{-1}$ (mean = 13 $\text{mg} \times \text{g soil}^{-1}$), and most of the collected soils have high soluble salt concentrations (Diaz et al., 2021). As expected, the soils contained almost no water at the time of collection (0.001–0.11 $\text{g H}_2\text{O} \times \text{g dry soil}^{-1}$, mean of 0.02 $\text{g H}_2\text{O} \times \text{g dry soil}^{-1}$). In general, soils located further inland at higher elevations were drier, saltier, and contained less organic carbon (Diaz et al., 2021). These soil characteristics are not unique to the Shackleton Glacier region as ice-free soils found in other regions of Antarctica have comparable edaphic characteristics (Bockheim, 1997; Magalhães et al., 2012).

Of the 204 soil samples analyzed, 80% of the samples yielded sufficient amounts of PCR-amplifiable DNA to characterize the bacterial and/or fungal taxa found in these soils using cultivation-independent marker gene sequencing. As expected, the number of taxa identified per soil sample (microbial richness) was low with a mean of 205 bacterial phylotypes (2–729 exact sequence variants, ESVs) and only 5.5 fungal phylotypes (1–45 ESVs) detected per sample. In general, the soils from higher elevation features farther away from the Ross Ice Shelf had less diverse fungal and bacterial communities (Figures 1 and S1, Data Sets S1 and S2).

The bacterial phyla with the highest relative abundances across all soils were: Bacteroidetes, Proteobacteria, Actinobacteria, and Acidobacteria, with the dominant taxa members of the families *Solirubrobacteraceae*, *Blastocatellaceae*, *Chitinophagaceae*, and *Rubrobacteriaceae* (Figure 1c, Data Set S1). The composition of the bacterial communities in these soils is consistent with results obtained using similar cultivation-independent analyses of other Antarctic soils, including those found in the McMurdo Dry Valley region (Aislabie et al., 2013; Cary et al., 2010; Lambrechts et al., 2019). The fungal communities in these soils were dominated by members of the Ascomycota and Basidiomycota phyla, including members of the families *Herpotrichiellaceae*, *Trapeliaceae*, *Verrucariaceae*, *Filobasidiaceae*, *Mortierellaceae*, *Stereocaulaceae* (Figure S1, Data Set S2). While there have been relatively few comparable studies of fungi in Antarctic soils, the dominant taxa identified are generally similar to those found in other Antarctic soils (Baeza et al., 2017; Rosa et al., 2019). Archeal sequences associated with the phyla Thaumarchaeota, family Nitrososphaeraceae, were detected in 60 samples, but these archeal sequences made up a maximum of 1.5% of all the 16S rRNA gene reads per sample (mean = 0.08% of reads per sample, Data Set S1).

We found that ~20% of the soils (40 out of 204 soils) had no amplifiable microbial DNA as determined by our cultivation-independent sequencing approach (see methods for more details). This is an unusual result for a culture-independent analysis of soil as the microbial communities found in a wide range of soils across the globe have been successfully characterized using similar techniques (Delgado-Baquerizo et al., 2018), and we have shown that the soils themselves do not appear to be inhibiting DNA extraction or PCR amplification (Figure S4). We recognize that these results do not confirm that these soils are completely sterile

and we acknowledge that we could have further optimized our methods to try to detect microbial DNA (e.g., extracting DNA from larger sample volumes, testing a range of different extraction kits and PCR protocols). Instead, we note that while all of these soils have very low biomass, 20% of the soils failed to yield detectable amounts of amplifiable bacterial, archaeal, or fungal DNA using techniques that are routinely used to successfully characterize microbial communities in soils from different regions of Antarctica (Goordial et al., 2016; Lambrechts et al., 2019; Smith et al., 2006).

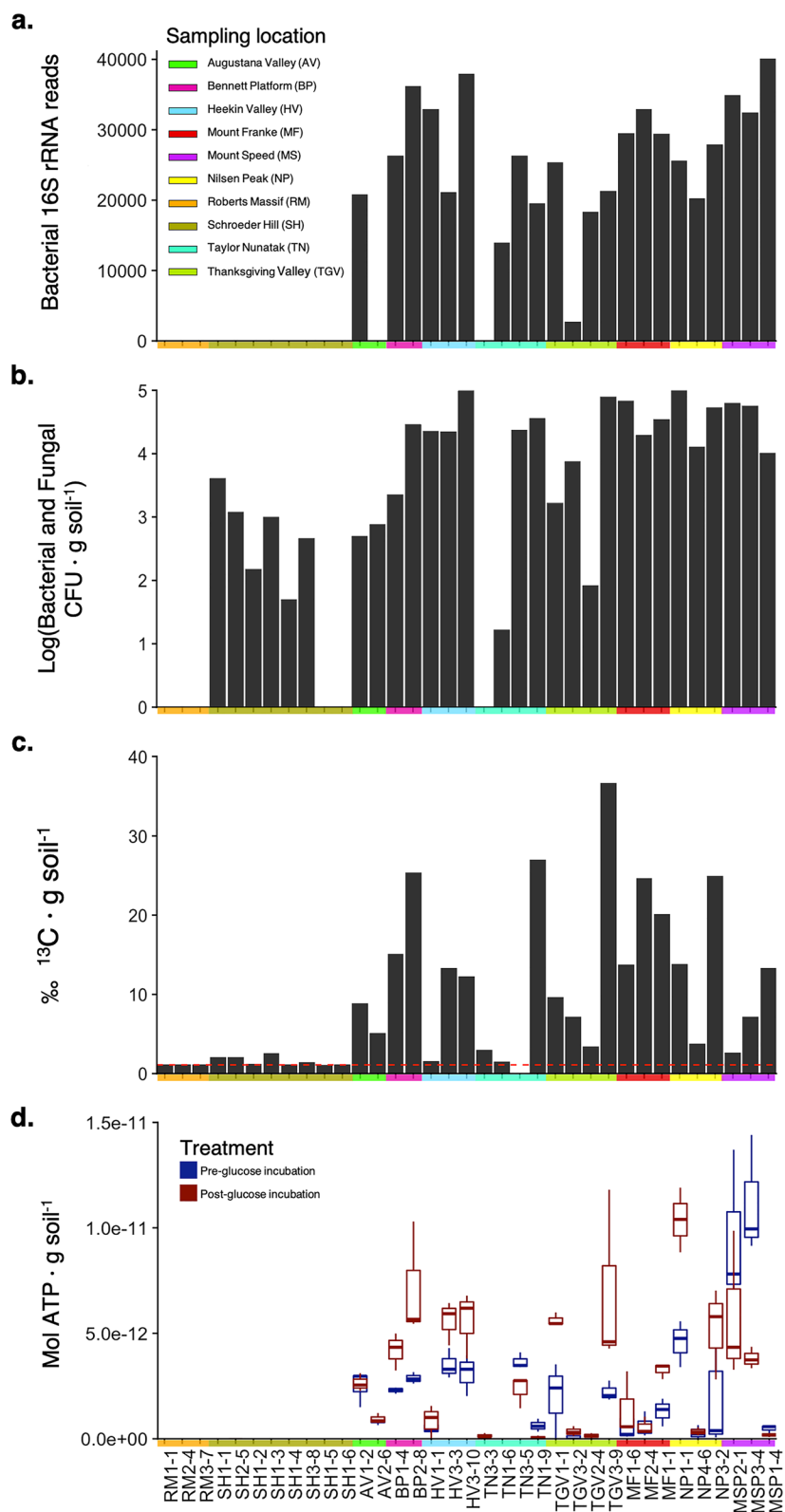
To complement the amplicon sequencing effort, we also conducted quantitative PCR (qPCR) assays to measure concentrations of bacterial and fungal DNA in each of the 204 soil samples. The qPCR results (Data Set S4) confirm the sequencing-based results presented in Figure 1c in that those samples found to have no detectable microbial DNA via sequencing were nearly always the same samples found to have no detectable microbial DNA via qPCR (Data Set S4). Specifically, bacterial DNA concentrations varied from 10^{-1} to 10^4 genome equivalents $\times$ g soil $^{-1}$ across the 204 samples, with 40 of the 50 samples found to have no detectable bacterial DNA via amplicon sequencing also having no detectable bacterial DNA as determined from the qPCR assays. In general, there were more samples that had qPCR DNA concentrations below detectable limits, but had detectable DNA as determined by amplicon sequencing, than vice versa. For fungi, measured fungal DNA concentrations varied from 10^{-1} to 10^6 genome $\times$ g soil $^{-1}$, but 96 of the 143 samples found to have no detectable fungal DNA via amplicon sequencing also had fungal DNA concentrations near or below the level of qPCR detection.

3.2. Habitability Tests

To test whether we can detect microbial life and activity in soils where microbial DNA was near or below detectable levels (using cultivation-independent sequencing), we analyzed a subset of 35 samples with culture-based methods, metabolic assays to detect ^{13}C -glucose mineralization, and ATP quantification (in soils amended with and without glucose). This subset of 35 samples included both soils with and without microbial DNA (as determined by the cultivation-independent sequencing and qPCR, Figures 1, 2, and S3, Data Set S4) with these soils collected from transects on each of the 10 features and spanning a range of edaphic properties (Table S2).

The 35 soil samples were plated on 17 different types of solid culture media. All media targeted aerobic microorganisms (both heterotrophs and autotrophs), as obligate anaerobes are unlikely to be present in these extremely dry surface soils. This cultivation effort supports the results from the cultivation-independent analyses (Figure 2b). Of the 35 samples tested; four samples did not grow any colonies on any of the different media types after three months. In general, those samples which did not yield any detectable microbial DNA also failed to yield any colonies, even when using such a broad range of aerobic media and relatively long incubation times. We acknowledge that our cultivation-based survey will not have captured all taxa potentially living in these samples as alternative cultivation strategies could always be employed, but the extensive cultivation-based analyses broadly agree with our cultivation-independent analyses in demonstrating that microbes are undetectable in an appreciable subset of the soil samples.

Of those soils (29 out of 35) which yielded visible bacterial or fungal colonies after 8 weeks, estimated cell numbers were highly variable, ranging from <20 to $>130,000$ CFU $\times$ g soil $^{-1}$ (Figure 2c, Data Set S3). Even the soil with the highest cultivable cell numbers had values that were 50 times smaller than the “positive control” soil collected from a lawn in Colorado (7.4×10^6 CFU $\times$ g soil $^{-1}$). These results are not surprising as we would expect ice-free Antarctic soils to have relatively low cultivable cell numbers (Lambrechts et al., 2019). However, it is worth noting that, while bacterial isolates dominated the culture collection, we were also able to culture and isolate viable fungi from 14 of these soils including some soils from which no bacteria could be cultured (Figure 2b). These results extend the cultivation-independent fungal analyses and the qPCR results highlighted above in demonstrating that even viable fungi are often present and that bacteria are not the sole members of these Antarctic soil microbial communities. This prevalence of fungi in soils from higher elevation sites is consistent with the culture-independent results (Figures 1b and S2, Data Set S4) and suggests that certain fungi may in fact be better adapted than bacteria or archaea to some of the most challenging soil environments on Earth.



To further assess microbial activity, we performed an assay to detect microbial activity in these soils based on the catabolic mineralization of ^{13}C -labeled glucose added to soils (Figure 2c, Data Set S3). We found that the 32 of the 35 samples tested had ^{13}C -CO₂ values that were significantly higher than their autoclaved replicates and the corresponding control blanks. Three of the 35 samples had nonautoclaved ^{13}C -CO₂ values that were indistinguishable from their paired autoclaved replicates or from the soil-free blanks. This suggests that we were unable to detect glucose mineralization resulting from microbial activity in these three soil samples (Table S3). This same subset of soils also had no culturable bacteria or fungi (Figure 2b), confirming that microbial cells were not detectable using the methods employed.

As a final test for microbial activity, we measured soil ATP concentrations both with and without glucose amendments (Figure 2d, Data Set S3). In theory, ATP assays should be able to detect very low levels of microbial activity that might not be detectable using other methods (Cowan et al., 2002; Schuerger et al., 2008). We found that 21 of 35 of the soils had measurable ATP concentrations with the addition of the PBS buffer only, while 23 of 35 had measurable ATP activity after amendment with glucose and incubated for 24 h. ATP production increased after glucose amendment in nearly all of the samples that had measurable ATP in the corresponding unamended soil. All reagent blanks and blank wells were below ATP detection limits of 10^{-15} mol ATP $\times$ g soil⁻¹. ATP concentrations in 12 of the 35 samples were below detection both with and without glucose amendment. These results suggest that there was no measurable microbial ATP in these soils, further evidence for a lack of actual or potential microbial activity in a subset of the samples collected.

Together these five distinct methods (cultivation-independent marker gene sequencing, qPCR, the extensive cultivation effort, the whole-soil ^{13}C -glucose metabolic assay, and the ATP assays, Figure 2) yielded similar results. We were unable to detect microorganisms or microbial activity in a subset of the samples (3 of 35) regardless of the methods employed: no colonies grew on any aerobic media type, no microbial metabolic activity was detected, and we could not detect any measurable ATP production. Although microscopy-based cell counting can be used to quantify microbial cell numbers in higher biomass soils, we did not use such an approach here given the problems associated with distinguishing between cells and soil particles in these soil types (Klauth et al., 2004; Li et al., 2004).

We recognize that our tests do not prove the absence of living microbes and that there are always more strategies that could be employed. However, our use of multiple distinct methods suggests that any microbial life that may exist in these soils is below the limit of detection using methods that are routinely used in other low biomass microbial systems (Caporaso et al., 2012; Delgado-Baquerizo et al., 2018; Dineen et al., 2010; Hynek et al., 2018; Schmidt et al., 2018; Thompson et al., 2017; Vimercati et al., 2019). These soils may contain low levels of microbial biomass that we were unable to detect with these analytical approaches, which is why we do not conclude that the soils are sterile. Rather we emphasize that our results show that microbial life is restricted in certain soils to the point where we cannot detect microbes using the methods employed.

3.3. Why Might Microbial Life be Restricted in Certain Soils?

We next sought to identify patterns in microbial habitability and determine what soil or site characteristics differentiated those soils that had no detectable microorganisms from those in the same region that had detectable microorganisms. As previously mentioned, we were unable to perform all of our habitability

Figure 2. The four tests used to detect microorganisms and/or microbial activity in soils. (a) The number of bacterial 16S rRNA reads that were classified to at least the phylum level of resolution for the subset of samples ($n = 35$) used to verify the results of the culture-independent sequencing. Samples with a read number less than the threshold determined to identify and reliably detect prokaryotic DNA (see Section 2) have been omitted. For all panels, samples are arranged on the x-axis by elevation (higher elevation features on the left). (b) The log transformed total colony counts (Log(CFU $\times$ g soil⁻¹)) of microorganisms (both bacteria and fungi) grown from each of the 35 samples. The reported total cell numbers are the cumulative number of colonies that grew on each plate over the 3-month incubation period. Samples with no bar had no growth, i.e., no colonies detected. (c) The ^{13}C ‰ released as CO₂ from the glucose-amended soils as calculated from the unautoclaved replicates of each of the 35 samples. The dashed red line indicates the ^{13}C ‰ measured in the blank samples. Three samples (RM3-7, SH1-5, SH1-6) had ^{13}C ‰ from their unautoclaved replicates that were not significantly different from the values calculated from either their autoclaved replicated or the blank tubes and were below detection limits. Sample TN3-5, indicated by a star, was not successfully measured. (d) ATP concentrations measured from the soils. Blue boxes were baseline readings performed on the soils before any glucose was added and before any incubation. Red boxes are from paired soil subsamples amended with glucose and incubated for 24 h. Samples that are blank indicate that the ATP readings were below detection limits.

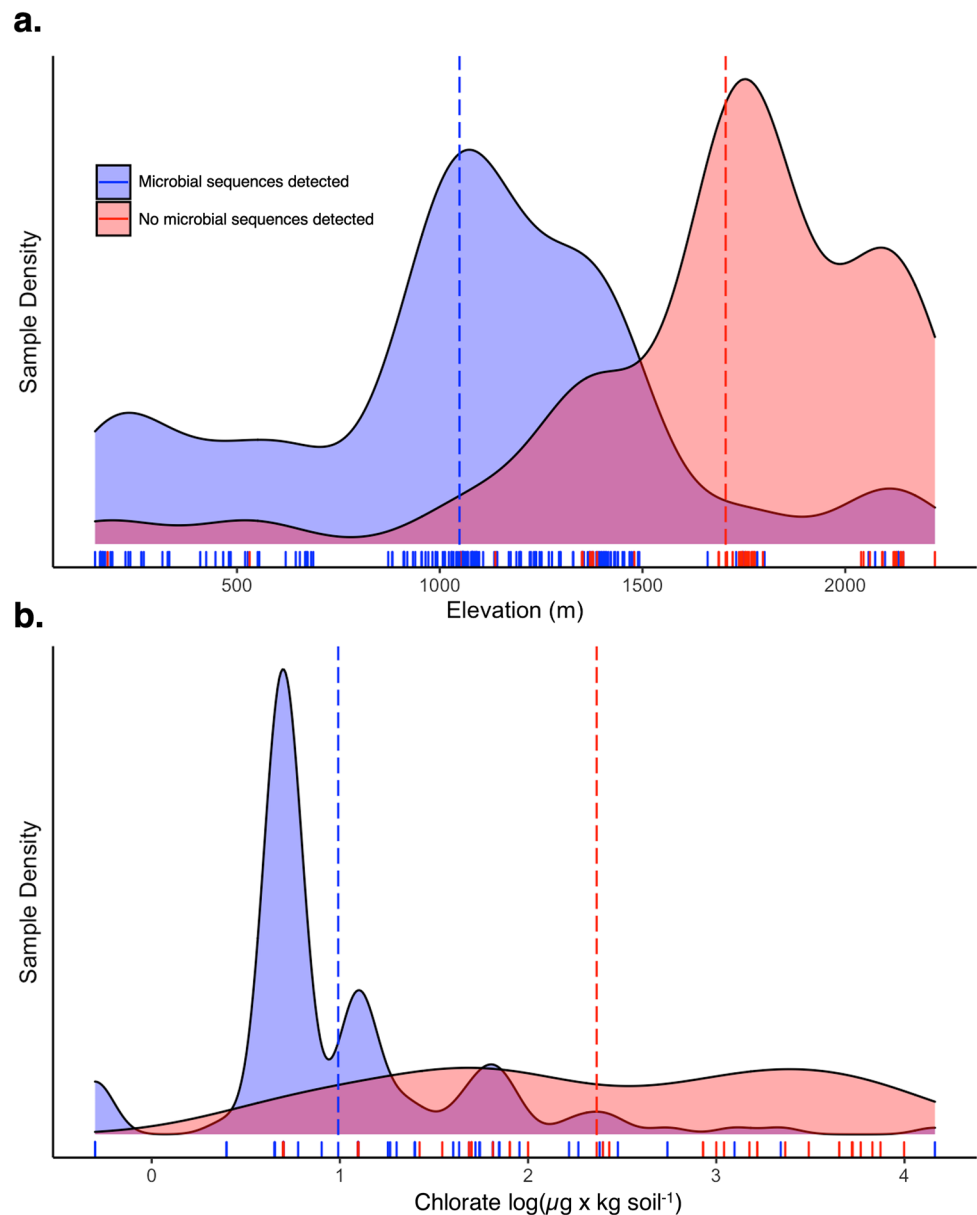


Figure 3. The best predictors of the habitability of a soil are elevation and chlorate concentration based on the random forest model. The distribution of samples with or without microbial DNA detected across the Shackleton Glacier region in relationship to elevation (panel a) and soil chlorate concentrations (panel b). Y values show frequency density of each group with $n = 204$ for elevation and $n = 169$ for chlorate. The area under each curve is equal to 1. Concentrations of chlorate ions ($\mu\text{g} \times \text{kg soil}^{-1}$) have been log transformed. Dashed lines indicate the mean value for each group. To see the distribution of all other variables tested in the random forest model and the model results, see Figure S5 and Table S4.

tests on our full data set of 204 samples. Thus, our random forest model was based on the results from the cultivation-independent marker gene sequencing, with those samples that yielded no archaeal, bacterial, or fungal reads considered to represent soils with the potential to have no detectable microbial DNA. We found that elevation and soil chlorate concentrations were the best predictors of whether microbes could be detected in a soil (Figures 3 and S5, Table S4). Soils from lower elevations were more likely to have detectable microorganisms (37% of soils from sites $>1,190$ m.a.s.l. had detectable microbial DNA while only 3% of soils from sites $<1,190$ m.a.s.l. had detectable DNA) and soils with lower chlorate concentrations were more likely to have detectable microbial DNA (soils with no detectable DNA or detectable DNA had average chlorate

concentrations of $1,675 \pm 2,595$ and $161 \pm 1,273 \mu\text{g} \times \text{kg}^{-1}$, respectively). Soils with no detectable microbial DNA also had higher median total salt and perchlorate concentrations (Figure S5), but these differences were not significant (Table S4).

Elevation and chlorate concentrations may be the best predictors of whether a soil contains detectable microorganisms, but it is unlikely that these factors, singly or in combination, are solely responsible for restricting microbial life in these soils. We know, e.g., that there are soils at much higher elevations that have active microbial communities (Schmidt et al., 2018; Vimercati et al., 2019) and microorganisms have been found in soils with higher concentrations of deposited chlorate in the Atacama Desert and in the McMurdo Dry Valleys (Coates & Achenbach, 2004; Rao et al., 2010). A more parsimonious explanation is that these variables are indicative of a suite of environmental properties that together restrict microbial life. Elevation likely correlates with a number of variables that increase in magnitude further inland and higher in elevation in the Shackleton Glacier region that may reduce microbial habitability: higher UV radiation, lower temperatures, lower water availability, and increased soluble salt concentrations (Diaz et al., 2021). The accumulation of high concentrations of chlorate salts, on the other hand, may influence habitability due to its toxic properties but this seems unlikely due to the fact that we observed soils with detectable and undetectable microbial communities in close proximity across this region (Figure 1). Instead, high concentrations of chlorate, a highly soluble molecule, may indicate that these soils seldom, if ever, have had sufficient amounts of liquid water to sustain active microbial communities (Rao et al., 2010).

There is not likely a single environmental factor that restricts microbial life in Antarctic soils. Instead, it is likely that the constant exposure to deposited salts, low temperatures, and extremely low water potentials over thousands of years have created surface environmental conditions that repress the effective colonization, activity, and establishment of those microbes that may reach these sites via aeolian transport (Archer et al., 2019). The impact of these constraints likely changes constantly, due to transient fluctuations in temperature, water availability, and salt concentrations. Clearly microbial activity in challenging soil environments is not ubiquitously distributed and local, landscape-level differences in environmental conditions can impose limits on microbial habitability in certain Antarctic soils.

4. Discussion

Our finding that some ice-free soils in the Shackleton Glacier region of Antarctica contain not detectable microbial life using standard methods is significant. These soils were neither recently formed, nor did they come from completely isolated areas. Instead, these soils are likely very old, and some were found on features in reasonably close proximity (within 50 m) to soils that contained detectable microbial life. This distinction is noteworthy as young soils may not have had a sufficient amount of time to accumulate microbial biomass. Instead it appears that a unique set of environmental conditions, those associated with higher elevation sites further inland and a near complete lack of liquid water over time, create conditions unfavorable to microorganisms. Soils typically contain large numbers of active microorganisms (often $>1,000 \text{ kg}$ of microbial biomass carbon per hectare, Fierer, 2017) and even dry polar desert soils from the McMurdo Dry Valleys tend to have detectable, diverse, and active microbial communities (Cary et al., 2010; Goordial et al., 2016). To find soils with such restricted microbial habitability is unexpected.

Ice-free soils are not unique to the Shackleton Glacier region. More than $45,000 \text{ km}^2$ of ice-free terrestrial surfaces can be found in Antarctica $>5 \text{ km}$ from the coast (Brooks et al., 2019). The environmental and geochemical characteristics acting on these inland, high elevation, ice-free soils are similar to those we observed in the Shackleton Glacier region (Bockheim, 1997; Magalhães et al., 2012) which suggests that the patterns in habitability we observed may be common elsewhere in Antarctica. This possibility is supported by the research of Goordial et al. (2016), whose work on soils in the University Valley also suggests that the unique combination of conditions found in Antarctica can severely limit microbial activity and survival. A similar constraint on habitability may also exist at higher elevation sites in the Altiplano of South America (Schmidt et al., 2018), but the absence of detectable microbial life in these soils remains hypothetical. Regardless, soils with such restricted microbial biomass and activity are likely very rare outside of Antarctica, as other ultra-arid regions, like the Atacama and Namib Deserts, harbor detectable microbial communities (Bull & Asenjo, 2013; Schmidt et al., 2018).

We hypothesize that the microbial life in even the most challenging soils may not remain below detectable limits for long. Antarctic terrestrial systems have changed very little since the Last Glacial Maximum due to relatively stable climatic conditions (Thompson & Solomon, 2002; Turner et al., 2014). Increases in temperature, precipitation, and moisture availability are predicted to accelerate at high elevations in coming years due to ongoing climate change (Turner et al., 2014). These anticipated environmental changes would likely expand the range of habitable soil environments in Antarctica (Le Roux & McGeoch, 2008) and decrease the likelihood of finding soils with no detectable microbial life in the future.

5. Conclusions

The existence of Antarctic soils lacking detectable microbial life is significant to the study of terrestrial biology and astrobiology. We are not suggesting that we have found “lifeless” or “sterile” soils, nor have we identified the low temperature threshold for life (many locations on Earth maintain lower temperatures than the Shackleton Glacier region and contain active microorganisms, Merino et al., 2019). However, our inability to detect microbes in certain soils suggests that microbial activity and survival in Antarctic may be restricted by exposure to extremely cold, dry, and salty conditions. This hypothesis is supported by previous work performed elsewhere in Antarctica (Goordial et al., 2016) and is a different type of “limit to life” than what might be found in a hot, acidic environment (Belilla et al., 2019; Merino et al., 2019). Acknowledging that certain Antarctic soils may be more or less habitable will allow us to better understand the adaptations that allow organisms to survive and remain active in these unique, challenging environments and predict what other soils on Earth may be similarly restrictive to life. Finally, understanding patterns of terrestrial habitability on our planet will set the groundwork to better predict where microbes might, or might not, be found on other planets.

Data Availability Statement

All data used in this study are publicly available in the main text or the supplementary material with the raw sequence data from the cultivation-independent marker gene sequencing publicly available in the NCBI Sequence Read Archive; Project Accession No. PRJNA699250. The supplemental data tables are publicly available on Figshare (<https://doi.org/10.6084/m9.figshare.14597535.v1>, <https://doi.org/10.6084/m9.figshare.14597571.v1>, <https://doi.org/10.6084/m9.figshare.14597523.v2>, <https://doi.org/10.6084/m9.figshare.14597526.v1>).

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