

1 Subsurface microbial habitats in an extreme desert Mars-analogue environment

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22 **Summary**

Sediments in the hyper-arid core of the Atacama Desert represent one of the closest terrestrial analogues to Mars' regolith. Understanding the distribution and drivers of life in the sediment may thus give critical clues on how to search for biosignatures on Mars. Here, we identify the spatial distribution of highly specialised bacterial communities in previously unexplored depth horizons of subsurface sediments and their correlation with moisture and geochemical variables. We deployed an autonomous rover in a mission-relevant Martian drilling scenario with manual sample validation to recover and analyse sediments in two Mars-like terrains, a gravel desert pavement and an evaporite-rich playa. Subsurface communities were distinct from surface-associated communities and were delineated by depth related to sediment moisture. Geochemical analysis indicated soluble salts and mineralogy that influence water bio-availability, particularly in deeper sediments. Colonisation was also patchy and uncolonised sediment was associated with indicators of extreme osmotic challenge. Bacterial diversity reflected strong selection for halotolerant and desiccation tolerant taxa throughout depth horizons. The most diverse communities occurred within the zone of highest moisture, whereas deeper sediments were colonized by methylophilic taxa with limited diversity. The study provides important baseline microbial ecological data on the linkage between biocomplexity, moisture and geochemistry in Mars-like sediments at the limit of habitability and demonstrates feasibility of the rover-mounted drill for future Mars sample recovery.

Introduction

1 The surface of Mars is dry, cold, and exposed to high levels of ionising radiation.
 2 However, data accumulated over the past few decades by orbital and landed missions
 3 have demonstrated that early in its history the planet may have been habitable for
 4 microbial life with abundant sources of energy, carbon, nutrients, and shelter ¹. Mars
 5 supported surface and subsurface water and may still do in some circumstances, as well
 6 as organic molecules required for life ². As a result, Mars 2020 and ExoMars missions will
 7 be searching for biosignatures ^{3,4}, and the investigation of terrestrial analogues can
 8 provide critical insights for the development and testing of exploration strategies.
 9 Among those, the hyper-arid core of the Atacama Desert in Chile is widely regarded as a
 10 tractable Mars analogue in the field of astrobiology. The Atacama is the driest desert
 11 region on Earth ⁵ with extremely low moisture inputs and precipitation events that are
 12 stochastic in nature ⁶. The region has a long history of climatic stability as an extreme
 13 desert ^{7,8} resulting in the build-up of evaporates ^{6,9} and creation of a Mars-like surface.
 14 The desert's hyper-arid core lies at or near the arid limit for soil formation ¹⁰ and thus
 15 surface terrain in this region is regarded as sediment.

16 Animal and plant life are scarce in extreme deserts and instead cyanobacteria-
 17 dominated microbial communities in mineral and rocky refugia including deliquescent
 18 substrates comprise the dominant surface biota and are well-characterised ^{11,12}.
 19 Conversely, evidence for microbial colonisation in hyper-arid Atacama sediment is
 20 scarce, contradictory and almost exclusively limited to surface-associated sediment ^{9,13–}
 21 ¹⁷ (Supplementary Material, Table S1). Cultivation-based approaches are unreliable as
 22 indicators of environmental microbial diversity and have yielded estimates that varied

1 by several orders of magnitude ^{9,14,15,17,18}. Biochemical tests have similarly yielded
 2 inconclusive support for microbial metabolic activity ^{9,14–17}. High throughput sequencing
 3 of environmental DNA/RNA remains the most reliable indicator for microbial diversity in
 4 extreme desert sediments. This approach has provided critical insight on surface-
 5 associated communities in semi-arid, arid and hyper-arid locations ¹⁶. A recent study
 6 also estimated putative microbial metabolic activity in three subsurface samples after
 7 an unusual rain event and this suggested subsurface sediment may also be a habitat for
 8 microbial communities ¹⁷. Major knowledge gaps persist, and importantly from an
 9 astrobiology perspective the question of how microbial occurrence and diversity may
 10 vary with abiotic variables in sediment depth horizons of a Mars analogue and spatially
 11 within terrain, and whether this can be addressed in a simulated robotic sampling
 12 mission scenario.

13 To document these questions and support the development and testing of
 14 biosignature exploration strategies, the NASA-funded *Subsurface Life in the Atacama*
 15 *Project* deployed an autonomous rover-mounted robotic drill in Mars-like desert
 16 pavement and playa terrain within the hyper-arid core of the Atacama under
 17 challenging environmental and logistical constraints (Supplementary Material, Fig. S1-
 18 S3). The drill accessed 32 discrete sediment samples to depths of 800mm along a 50km
 19 transect in a realistic simulation of Martian drilling operations and constraints. Over 60
 20 manually excavated sediment samples were recovered in parallel and validated the
 21 automated sampling for abiotic and biotic components of the sediment habitat and
 22 provide important baseline ecological data on the most Mars-like sediments on Earth.

1

2 **Extreme habitats in Atacama sediment horizons**

3 The on-board Raman spectrometry revealed surface mineral distributions comprising
4 feldspar and quartz. Subsurface sediments, and particularly the playa sites, were
5 elevated in gypsum and anhydrite (Supplementary Material, Table S3, Fig. S5). This was
6 independently corroborated by laboratory Raman measurements (Supplementary
7 Material, Table S3) and geochemical analysis that revealed a positive linear correlation
8 ($r=0.78$, $p<0.001$) across all samples between sulfate-sulfur and calcium, the
9 components of gypsum and anhydrite ($\text{CaSO}_4 +/-2\text{H}_2\text{O}$). Anhydrite is indicative of drier
10 conditions at depth, and *in situ* soil moisture sensors validated this for horizons in both
11 pavement and playa.

12 Our chemical analysis focused on geochemical reservoirs readily available and
13 relevant to biological communities and using methodology that was comparable to
14 other microbial ecology studies. A clear depth-dependent pattern in geochemistry was
15 revealed for pavement and playa horizons (Fig. 1, Supplementary Material, Table S2, Fig.
16 S4). Surface sediments were strongly associated with elevated phosphorous
17 concentrations in both playa and pavement units (Fig. 1). Elevated surface P was
18 attributed to presence of surface Fe-oxides and calcite that can adsorb P. These P
19 adsorbing phases were likely present in the subsurface but were diluted by high sulfate
20 concentrations causing lower recorded P concentrations below the surface. All
21 subsurface samples displayed very low to undetectable levels of N and low C throughout
22 horizons. The on-board Raman spectrometer also recorded C as a minor/undetectable

1 phase in all samples. Electrical conductivity (EC) and Na⁺ levels increased with depth in
2 both substrates indicating increased salinity with depth (p = 0.006). Desert pavement
3 subsurface sediments separated mainly due to pH and K⁺, whilst playa subsurface
4 sediments displayed elevated levels of sulfate-sulfur, extractable cations (Ca²⁺, Mg²⁺,
5 Na⁺) and EC indicating increasing osmotic challenge, and most notably in the paleo-playa
6 Site 10 (Fig. 1, Supplementary Material Table S2).

7 Average annual subsurface temperatures ranged from 19.9 – 20.9°C in depth
8 horizons (Supplementary Material, Table S4). At shallow depths (<100mm) temperature
9 varied between 4 – 34°C, whereas in deeper horizons temperature variation was
10 somewhat buffered (Supplementary Material, Fig. S6). Moisture values from depth
11 horizons in representative playa terrain were consistently higher (approximately 4- to 7-
12 fold) at all depths than those in the desert pavement horizons (Supplementary Material,
13 Table S4) and this likely reflects local hydrology given the playa was a relatively low-lying
14 terrain (Supplementary Material, Fig. S1). However, sediment moisture trends in both
15 substrates broadly indicated the existence of depth groupings into distinct moisture
16 zones: (a) a surface zone consisting of the top 200mm, where water availability is
17 typically lowest, except in the short-term following a rain event; (b) a mid-depth zone
18 (300-500mm), where water availability peaks and persists after a rain event; and (c) a
19 deep subsurface zone (≥500-800mm), where water availability is typically lower and,
20 notably for the desert pavement sediments, appeared to be un-impacted by rare large
21 rainfall events (Supplementary Material, Table S4, Fig. S6).

22

1 Depth-related trends in DNA recovery and microbial alpha diversity

2 Surface colonisation was widespread in desert pavement and playa sites but subsurface
3 horizons displayed patchy recovery with low yields of quantifiable DNA in the range
4 0.067-6.5 ng/g sediment, indicating extremely low standing biomass (Supplementary
5 Material, Table S2, S5). Notably, the paleo-playa site yielded no recoverable DNA at any
6 depth after repeated efforts at extraction. Our approach using a DNA recovery method
7 adapted for low-biomass extreme environments highlights for the first time the
8 patchiness of low biomass microbial colonisation in the most extreme subsurface desert
9 sediments where micro-habitat conditions are at or near the limit for life ¹⁹. This may
10 explain, at least in part, why some previous research has concluded this region of the
11 Atacama was lifeless ⁹. Linear Discriminant Analysis was employed to show that
12 variables most strongly associated with potentially lifeless subsurface sediments where
13 environmental DNA was irrecoverable were sulfate-sulfur (substrate) ($p = 0.001$), depth
14 ($p = 0.003$), EC ($p = 0.006$), soluble salts ($p = 0.006$). This strongly suggests that osmotic
15 challenge and limited moisture availability are the major extinction drivers in this Mars
16 analogue sediment. We also demonstrated that substrate-inhibition of DNA recovery
17 was unlikely to have been a significant factor, since bacterial cell suspensions in the
18 range reported for desert soils (Supplementary Material, Table S1) added to these
19 samples were recoverable with 60-80% efficiency compared to a pure laboratory culture
20 and were not significantly inhibited compared to extractions with spiked sediments that
21 did originally yield environmental DNA.

1 Across both terrains any given depth horizon tended to either be colonised
2 almost throughout or display only patchy near-surface colonisation as measured by
3 recoverable DNA. Thus, the ubiquitous surface colonisation was not a predictor for
4 subsurface habitability in these sediments. We postulate that in addition to sediment
5 moisture, extreme geochemistry also influenced habitability in these sediments as
6 evidenced by high soluble salts/salinity indicators and abundance of anhydrite and
7 gypsum mineral signatures (Supplementary Material, Table S2, S3). Temperature
8 variations were not regarded as significant challenges to microbial colonisation
9 compared to other variables (Supplementary Material, Fig. S6). The paleo-playa site 10
10 failed to yield recoverable DNA from any depth, thus highlighting that ancient landforms
11 created under moisture sufficiency, and with elevated anhydrite levels as indicated by
12 the rover's Raman spectrometer (Supplementary Material, Fig. S5) may not support
13 extant genetic biosignatures and do not support recoverable relic DNA. Our overall DNA
14 recovery success was consistent with our expectations for the driest desert location on
15 Earth. It is notable that we have observed Antarctic mineral sediments, another Mars
16 analogue, generally yield higher recovery rates using similar methodologies, e.g. ^{20,21}.
17 However, this is likely due to enhanced standing biomass due to the less extreme nature
18 of the growing season in Antarctic desert where long periods of frozen hibernation are
19 punctuated by periods of moisture sufficiency during the austral summer ²².

20 Clear patterns were discernible from manually excavated sediment pits. Bacterial
21 diversity decreased significantly with depth across both terrains (Chao1 richness: $r =$
22 0.508, $p = 0.044$; Shannon's H: $r = 0.731$, $p = 0.001$), thus indicating depth as a major

1 driver of bacterial diversity (Supplementary Material, Fig. S8, Table S6). Bacterial
 2 communities formed six diversity clusters that associated with clearly defined depth
 3 ranges and zones of variability in sediment moisture and geochemistry (Fig. 2a).
 4 Subsurface communities were more distinct between habitat types but also more
 5 heterogenous overall than surface-associated communities, due largely to the lower
 6 bacterial diversity within each depth-defined sediment micro-habitat. Community
 7 evenness displayed little clear pattern with depth for desert pavement but for playa
 8 deep samples displayed very low evenness reflecting their highly specialised diversity
 9 (Supplementary Material, Table S6). In all cases the rover-acquired samples showed a
 10 generally similar pattern in depth profile and diversity clusters although these were less
 11 clearly defined and we attributed this to mixing of sediment during recovery using the
 12 bite-drilling approach (Fig. 2b; Supplementary Material, Fig. S8).

13 To further unravel the influence of sediment environment on bacterial diversity,
 14 we performed canonical correspondence analysis (CCA) to establish the association of
 15 distinct geochemistry for desert pavement and playa samples on the assembly of
 16 bacterial communities (Fig. 1). Surface communities were strongly influenced by P levels
 17 that were elevated compared to the subsurface (Fig. 1). Bacterial diversity in subsurface
 18 sediment habitats correlated with two groups of geochemical variables: The playa
 19 subsurface community was strongly associated with EC and extractable cations (Ca^{2+} ,
 20 Mg^{2+} , Na^+) and the rover-mounted Raman data indicated elevated anhydrite and
 21 gypsum (Supplementary Material, Table S3, Fig. S5). This strongly suggests that bio-
 22 availability of water may be limited in this subsurface habitat despite relatively high

1 moisture levels due to the effects of salt saturation. Conversely the pavement
 2 subsurface community was associated largely with pH, and also C and K⁺ although the
 3 magnitude of variation for these variables was low. This pH-dependent structuring of
 4 microbial diversity is consistent with observations for global trends in soil microbial
 5 diversity²³. The BEST multiple rank correlation routine was employed to further
 6 examine our data and rank the relative association of abiotic variables with the
 7 observed bacterial diversity as follows: extractable cations (Ca²⁺, Mg²⁺, Na⁺) and
 8 phosphorous>sulfate-sulfur> >EC ($\rho w = 0.595-0.609$, $p < 0.05$), thus further validating the
 9 ordinations and CCA analysis.

10

11 Highly specialised sediment bacterial communities

12 The taxa identified in sediment indicated highly specialised and relatively low diversity
 13 bacterial communities in both desert pavement and playa, and this low diversity reflects
 14 global trends in soil microbial diversity where deserts are considered to be relatively
 15 depauperate^{24,25}. Overall the drill samples yielded weaker depth resolution than
 16 manual sampling but still corroborated observations from the manually collected
 17 samples (Fig. 3, Supplementary Material Fig. S9). Bacterial taxonomic diversity varied
 18 significantly with subsurface habitat as compared to a generally consistent surface
 19 community (Fig. 3)¹⁶. Overall, communities were dominated largely by only three phyla:
 20 Chloroflexi, Actinobacteria and Alphaproteobacteria.

21 All surface communities were dominated by the AKIW781 lineage of the
 22 photoheterotrophic Chloroflexi, a known desert soil crust taxon (NCBI GenBank

1 accession number: JQ402146), and overall community structure contributed additional
 2 taxonomic resolution to previous observations of surface sediment communities ^{16,17}.
 3 AKIW781 has also been recorded in desert varnish on rock surfaces ²⁶ as well as a
 4 keystone taxon of hypolithic communities ²⁷ in the Atacama. This indicates a
 5 cosmopolitan distribution and broad habitat preference among surface niches in this
 6 extreme desert. Conversely, the Chloroflexi were minor components of subsurface
 7 communities, decreasing in relative abundance with depth, and mainly comprised an
 8 uncharacterised candidate class Ellin6529, likely adapted to non-phototrophic
 9 metabolism in the subsurface microhabitat.

10 Low and mid-depth subsurface horizons for both desert pavement and playa
 11 were dominated by the low G-C gram positive taxonomic class Actinobacteria (Fig. 3,
 12 Supplementary Material, Fig. S9). They are typically thick-walled bacteria commonly
 13 encountered in soil. Communities encountered were specific to each depth horizon and
 14 shifts in diversity clearly reflected the moisture zones identified for both pavement and
 15 playa horizons. At shallow depths (<200mm) halotolerant, alkalotolerant, spore-forming
 16 desiccation-tolerant actinobacterial groups were abundant and included the orders
 17 acidimicrobiales, gaiellales, nitriliruptorales and solirubrobactales in desert pavement
 18 and acidimicrobiales and nitriliruptorales in playa. Mid-depth sediments (300-<500 mm)
 19 corresponding to the zone of greatest moisture availability in both substrates supported
 20 highly variable bacterial diversity between samples and also the most diverse
 21 communities (Supplementary Material, Table S6). Of note was the elevated relative
 22 abundance of bacillales at a single pavement site and this may reflect the relatively high

1 altitude of this site and limited moisture input to the subsurface. The bacillales-
 2 dominated community was more similar to those encountered at greater depths at
 3 other locations. The bacillales are characterised by their ability to form highly resistant
 4 endospores and so may indicate an increasingly challenging micro-habitat in deeper
 5 subsurface sediments.

6 There were fewer deep sediment samples from which to make comparisons and
 7 this reflected the more challenging subsurface environment, and deeper communities
 8 (500+ mm) generally displayed lowest taxonomic diversity. The deepest desert
 9 pavement community was similar to those at shallower depths but displayed elevated
 10 relative abundance of acidimicrobiales. Conversely the deep playa community shifted to
 11 a fundamentally different composition from shallower depths that was dominated
 12 almost exclusively by a single facultative methylotrophic and desiccation-tolerant
 13 *Methylobacterium radiotolerans* taxon (NCBI GenBank accession number: LT985974)
 14 from the phylum Alphaproteobacteria. We speculate the C1 metabolism of this taxon
 15 allows it to exploit simple C1 compounds as well as subsurface methane sources²⁸, a
 16 molecule known to be released from subsurface sources on Mars ²⁹. An overall picture
 17 thus emerges of highly-specialised bacterial diversity adapted to and reflecting the
 18 challenging subsurface habitat as well as reflecting moisture availability zones
 19 determined at least in part by sediment depth and geochemistry.

20 Other bacteria typically encountered in desert surface communities and
 21 generally regarded as tolerant to extreme conditions were not major components of
 22 subsurface communities. A complete absence of cyanobacterial taxa was consistent

1 with their adaptation to surface mineral refugia rather than subsurface sediment
 2 habitats that preclude photoautotrophy ¹², and the highly desiccation-tolerant
 3 *Deinococcus*-*Thermus* group were represented by only a single lineage of Trueperaceae
 4 candidate genus B-42 recovered in just a few subsurface samples with low abundance
 5 (0.6 – 4.8%), compared to a relatively diverse assemblage of cultivable *Deinococci*
 6 recovered from surface sediments in other less arid desert locations ³⁰. This further
 7 supports our evidence for highly specialised subsurface communities selected for by the
 8 distinct geochemistry and microclimate in the subsurface Mars analogue sediments of
 9 the Atacama. They may thus be broadly indicative of the type of microbial consortia that
 10 could exploit Martian subsurface habitats.

11 In the deepest and least-diverse playa communities biocomplexity was reduced
 12 almost to a single taxon, reflecting extreme selective pressure and also highlighting a
 13 possible lack of resilience to environmental change given that recruitment to deep
 14 subsurface sediments may be limited. The minimum biocomplexity may comprise
 15 multiple ecotypes of a single taxon, each adapted to exploit a given suite of
 16 microclimate and geochemical conditions ³¹. They likely exhibit a strong preference for
 17 C1 and/or autotrophic taxa that are somewhat de-linked from their immediate
 18 surroundings in terms of carbon sequestration and reflect the extreme oligotrophic
 19 nature of these microhabitats. Given the highly specialised diversity recovered, the
 20 association of putative physiology with environmental variables, and lack of DNA
 21 recovery in paleo-playa samples; we conclude that our estimates are likely to represent
 22 resident microbial communities rather than relic DNA^{17,32}.

1

2 **Implications for detection of biosignatures on Mars**

3 The autonomous rover drilling platform yielded subsurface sediment samples that
 4 allowed for the first time a combined investigation of sediment abiotic properties and
 5 microbial diversity at an unprecedented level of detail. The parallel manual sampling
 6 and analysis demonstrated feasibility and fidelity of the autonomous rover approach.
 7 Highly specialised but low-diversity subsurface bacterial communities were encountered
 8 patchily and this was strongly associated with abiotic variables which suggests that
 9 “follow the water” is only part of the biosignature exploration solution in the search for
 10 potential habitable refuges on Mars. Consideration of subsurface micro-habitat
 11 variability in geochemistry, originating with and adapted to possible water availability
 12 zones may also be key. Whilst the geochemistry of our analogue sites was similar to that
 13 of a habitable Martian regolith, moisture in the Atacama is surface-sourced by fog
 14 and/or rain events ⁶, whereas on Mars subsurface sources may provide an upward
 15 migration of moisture similar to that observed in Antarctic mineral sediment overlaying
 16 permafrost ^{33,34}. Thus, extrapolating habitable subsurface locations on Mars would need
 17 to consider this along with the incident radiation regime and other Martian
 18 environmental variables. Based on the exploratory design consideration for this NASA-
 19 funded research, drill depth was constrained at 800mm as a proof of concept study.
 20 However, current plans are for both NASA and ESA to target depths of up to 2m on Mars
 21 in order to target potential subsurface habitats that account for Martian environmental
 22 variables.

1 The relevance of ecology and microbial habitats to past and possible extant life
2 on Mars are finally coming to the fore in the robotic search for biosignatures on Mars
3 ^{1,35}. As our study suggests, detecting such life or its residual biosignatures may prove
4 highly challenging, given that in the most extreme deserts on Earth these communities
5 are extremely patchy in distribution and occur with exceedingly low biomass. The drill
6 apparatus employed in this study has demonstrated that subsurface sediment
7 biosignatures can be autonomously recovered, although precise depth delineation
8 requires refinement with the bite-drilling approach used in this study. Whilst genetic
9 biosignatures such as DNA used in our study may not ultimately be the primary method
10 employed to search for traces of life on Mars, they are the most reliable and widely
11 used method currently available for microbial diversity estimation ^{24,25}. This approach
12 provided essential first proof of concept that an incontrovertible biological signature
13 within the likely range for geochemical variables in a habitable subsurface environment
14 can be recovered from a Mars-like sediment using an autonomous rover.

15

16 **Methods**

17 A detailed description of all field and experimental approaches is presented in the
18 Supplementary Material. Briefly, the automated rover completed a 50km traverse and
19 used a robotic drill to sample subsurface sediments in depth horizons to 800mm. On-
20 board mineralogical characterisation was achieved using Raman spectroscopy and
21 samples were recovered for further laboratory geochemical analysis and estimation of
22 bacterial diversity from sequencing of environmental DNA. Parallel manually excavated

1 samples were recovered for validation of rover-acquired sampling, and the first detailed
 2 characterisation of subsurface bacterial habitats in the Mars analogue sediment.
 3 Automated microclimate recording was achieved using dataloggers deployed *in situ* in
 4 the depth horizons.

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2 **Acknowledgements**

3 The work was completed with funding from NASA Astrobiology Science and Technology
4 for Exploring Planets (ASTEP) Program Grant NNX11AJ87G and a Yale-NUS College Start-
5 Up Grant. The authors are grateful to Brad Sutter (NASA) for advice on interpretation of
6 geochemical analysis, and Craig Cary (University of Waikato) for assistance with
7 biosecurity compliance and sample custody.

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9 **Author contributions**

10 K.A.W-R., N.C. and S.B.P. conceived the study; D.W., K.Z., S.V., M.W. and G.F. built and
11 operated the rover; K.A.W-R.; C.D., J.M., G.C., C.T., K.T., T.H., C.G.T., A.W. and J.W.
12 conducted field operations and sampling; K.C.L., S.D.J.A., D.C.L-B. and S.B.P. processed
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14 molecular biology experiments; K.A.W-R, K.C.L., S.D.J.A., D.C.L-B, L.N-B. and S.B.P.
15 performed data analysis; K.A.W-R. and S.B.P. wrote the manuscript with inputs from
16 K.C.L., S.D.J.A., D.W. and N.C. (K.A.W-R. and K.C.L. contributed equally).

17

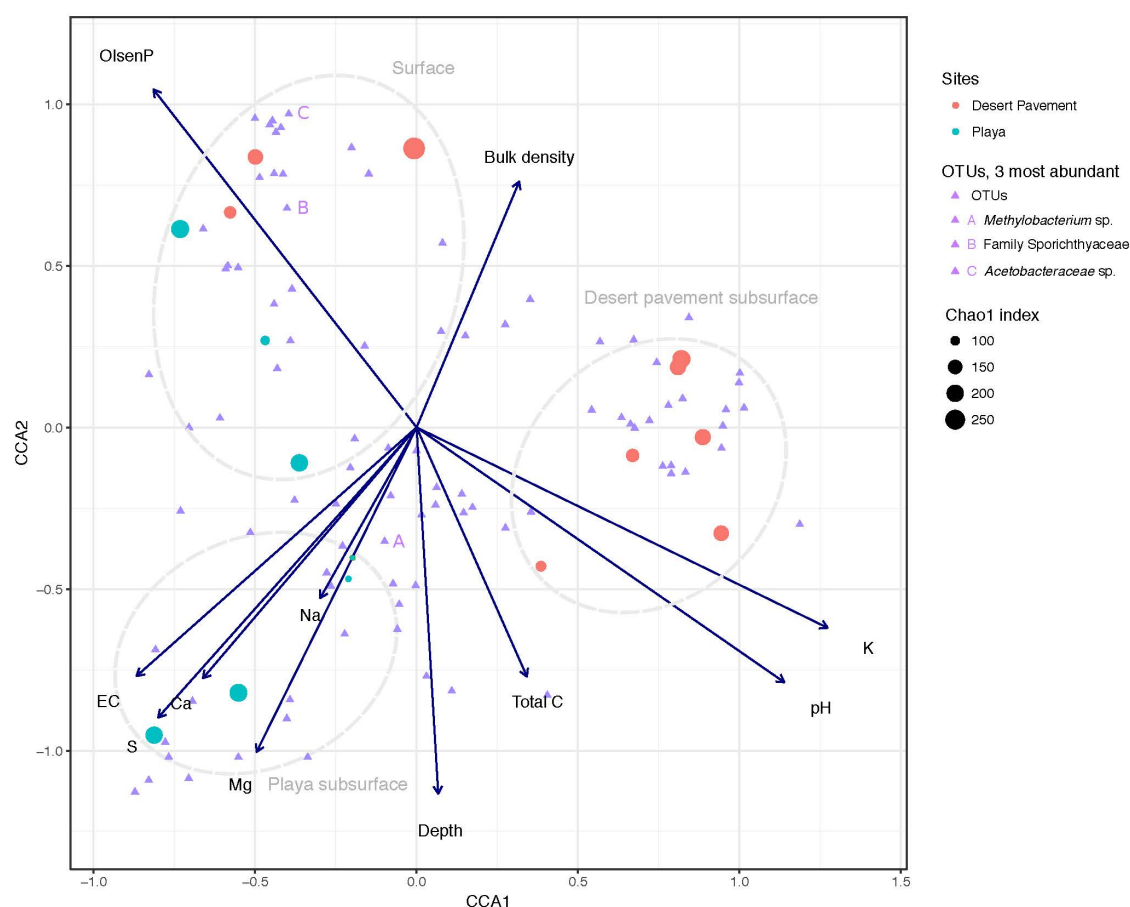
18 **Materials & Correspondence**

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Figure 1. Correlation of Mars analogue sediment geochemistry with bacterial diversity.

Canonical Correspondence Analysis (CCA) triplot with symmetrical scaling indicating differences in sediment geochemistry within sediment pits, and influence of these abiotic variables on bacterial communities and individual taxa. The three most abundant taxa are labelled (A, B, C). The circle size of each sample indicates species richness index (Chao 1) of the respective community.



1 **Figure 2. Depth-defined bacterial diversity in Mars analogue sediments of the**
2 **Atacama Desert.** Non-metric multidimensional scaling (NMDS) ordination of Bray Curtis
3 similarities for bacterial diversity versus sediment depth from a) manual recovery, and
4 b) rover recovery. Shaded areas indicate similarity clusters for sediment communities at
5 the same depth. The size of each symbol (circle or triangle) indicates species richness
6 index (Chao 1) of the respective community. Mid-range values were used for drill
7 samples where depth ranges instead of individual depths were generated.

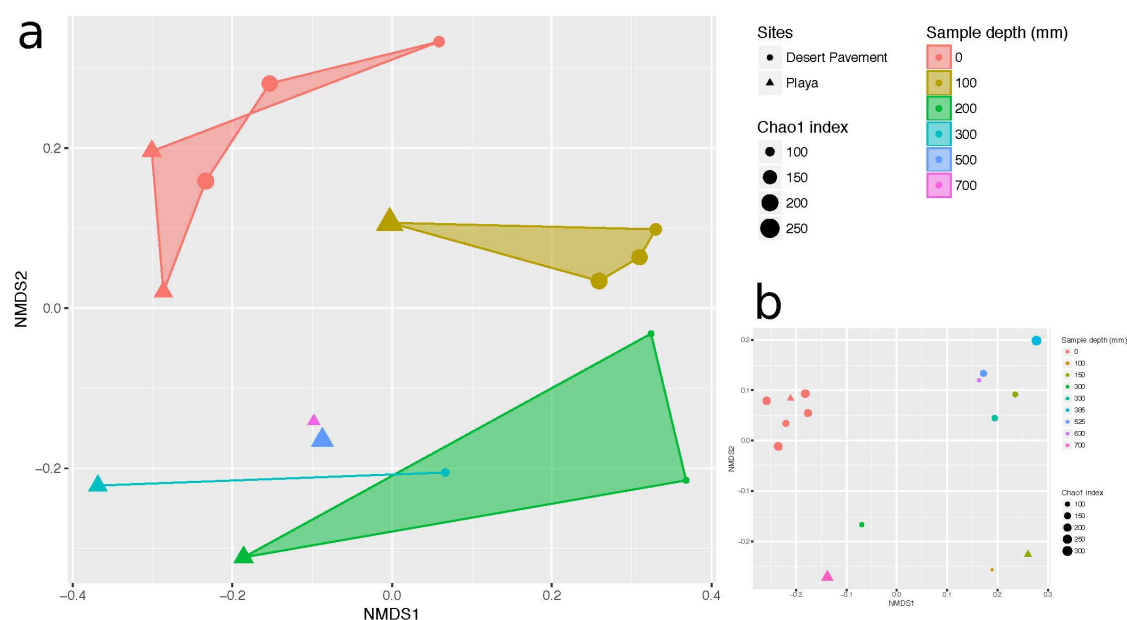


Figure 3. Highly specialised bacterial communities in Mars analogue sediment depth horizons. Distribution of bacterial diversity by taxonomic class with sediment depth for manual (M) and autonomous rover drill (D) recovered samples. Coloured shading indicates relative abundance within each community for a given bacterial class. Grey shading indicates no recoverable bacteria.

