Subsurface microbial habitats in an extreme desert Mars-analogue environment

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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 minerology 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 methylotrophic 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

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

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

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

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

Extreme habitats in Atacama sediment horizons

The on-board Raman spectrometry revealed surface mineral distributions comprising feldspar and quartz. Subsurface sediments, and particularly the playa sites, were elevated in gypsum and anhydrite (Supplementary Material, Table S3, Fig. S5). This was independently corroborated by laboratory Raman measurements (Supplementary Material, Table S3) and geochemical analysis that revealed a positive linear correlation ($r=0.78$, $p<0.001$) across all samples between sulfate-sulfur and calcium, the components of gypsum and anhydrite ($CaSO_4$ +/-$2H_2O$). Anydrite is indicative of drier conditions at depth, and *in situ* soil moisture sensors validated this for horizons in both pavement and playa.

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

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

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

Depth-related trends in DNA recovery and microbial alpha diversity

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

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

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

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

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

moisture levels due to the effects of salt saturation. Conversely the pavement subsurface community was associated largely with pH, and also C and K^+ although the magnitude of variation for these variables was low. This pH-dependent structuring of microbial diversity is consistent with observations for global trends in soil microbial diversity [23]. The BEST multiple rank correlation routine was employed to further examine our data and rank the relative association of abiotic variables with the observed bacterial diversity as follows: extractable cations (Ca^{2+}, Mg^{2+}, Na^+) and phosphorous>sulfate-sulfur> >EC ($_pw$ = 0.595-0.609, p <0.05), thus further validating the ordinations and CCA analysis.

Highly specialised sediment bacterial communities

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

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

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

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

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

There were fewer deep sediment samples from which to make comparisons and this reflected the more challenging subsurface environment, and deeper communities (500+ mm) generally displayed lowest taxonomic diversity. The deepest desert pavement community was similar to those at shallower depths but displayed elevated relative abundance of acidimicrobiales. Conversely the deep playa community shifted to a fundamentally different composition from shallower depths that was dominated almost exclusively by a single facultative methylotrophic and desiccation-tolerant *Methylobacterium radiotolerans* taxon (NCBI GenBank accession number: LT985974) from the phylum Alphaproteobacteria. We speculate the C1 metabolism of this taxon allows it to exploit simple C1 compounds as well as subsurface methane sources[28], a molecule known to be released from subsurface sources on Mars [29]. An overall picture thus emerges of highly-specialised bacterial diversity adapted to and reflecting the challenging subsurface habitat as well as reflecting moisture availability zones determined at least in part by sediment depth and geochemistry.

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

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

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

Implications for detection of biosignatures on Mars

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

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

Methods

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

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

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Author contributions

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 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. conducted field operations and sampling; K.C.L., S.D.J.A., D.C.L-B. and S.B.P. processed sediment samples and conducted geochemical analysis; S.D.J.A. and K.C.L. conducted 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. performed data analysis; K.A.W-R. and S.B.P. wrote the manuscript with inputs from K.C.L., S.D.J.A., D.W. and N.C. (K.A.W-R. and K.C.L. contributed equally).

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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.

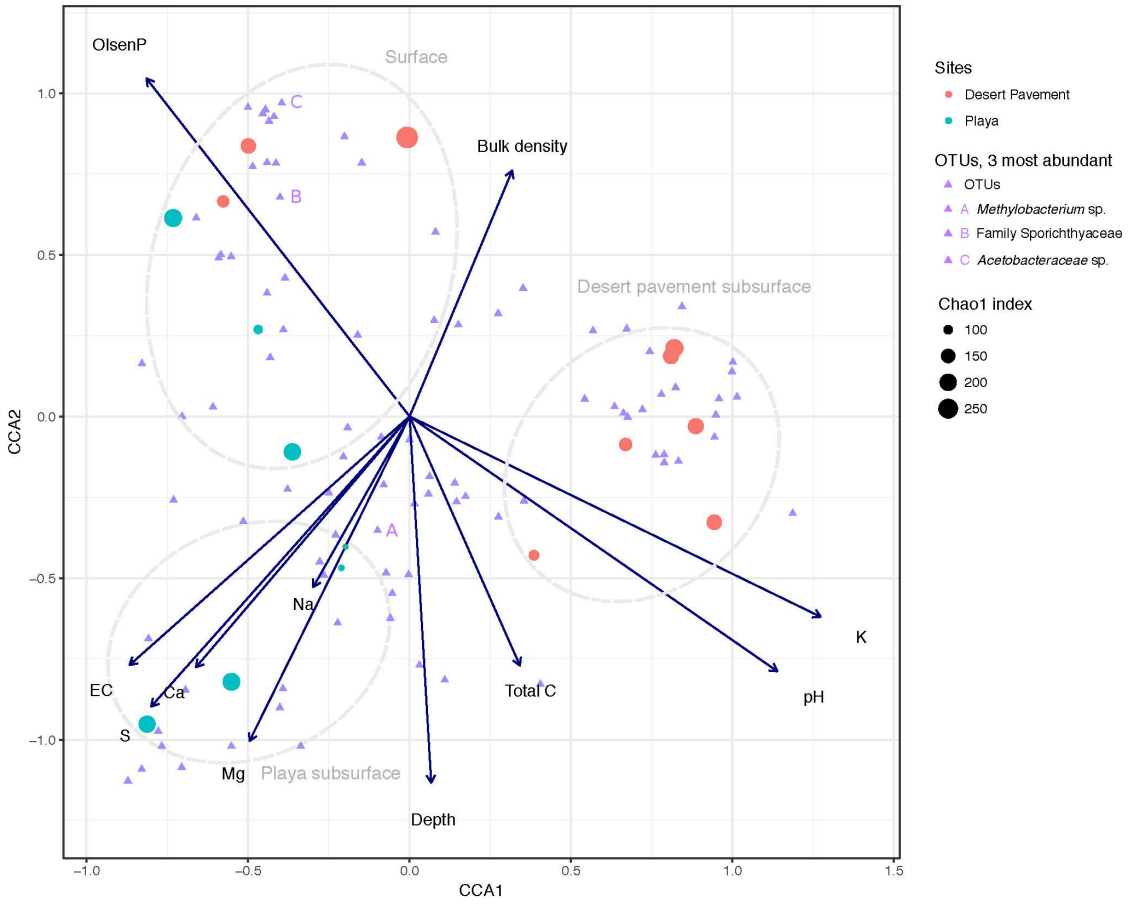

Figure 2. Depth-defined bacterial diversity in Mars analogue sediments of the Atacama Desert. Non-metric multidimensional scaling (NMDS) ordination of Bray Curtis similarities for bacterial diversity versus sediment depth from a) manual recovery, and b) rover recovery. Shaded areas indicate similarity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

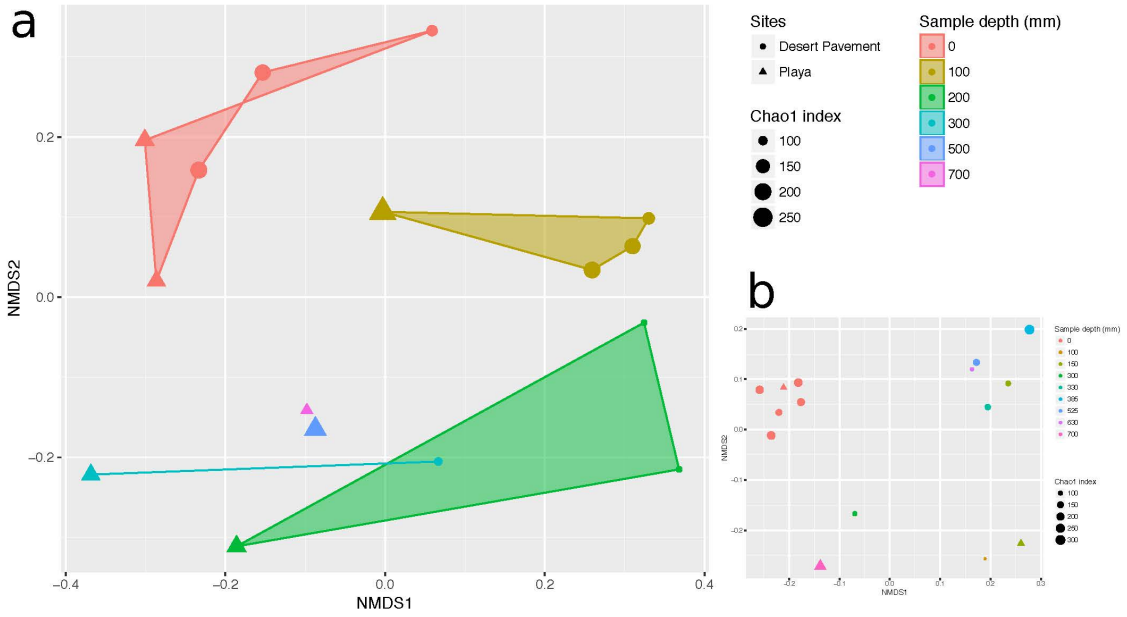

Figure 3. Highly specialised bacterial communities in Mars analogue sediment depth horizons. Distribution of bacterial diversity by taxonomic class with sediment depth for manual (M) and autonomous rover drill (D) recovered samples. Coloured shading indicates relative abundance within each community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