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Effect of soil horizon stratigraphy on the microbial ecology of alpine paleosols

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1 ABSTRACT

There is remarkable potential for research at the interface between the earth sciences and environmental microbiology that may lead to advances in our understanding of the role of bacterial communities in the surface or subsurface environment of our planet. One mainstay of sedimentary classification is the concept of differential soil and/or paleosol horizons being the result of primarily physical and chemical weathering, with relatively little understanding of how microbial communities between these stratified horizons differ, if at all. In this study we evaluate the differences in microbial community taxonomy and biogeochemical functional potential between stratified soil horizons in an alpine paleosol environment using next-generation sequencing (NGS) shotgun sequencing. Paleosols represent a unique environment to study the effect of differences soil horizon environments on the microbial community due to their relative isolation, and the fact that three distinct stratified soil horizons can be identified within the top 30cm of the soil. This enables us to assess variation in microbial community composition that will be relatively distinct from variation

due to distance alone. We test the hypothesis that variation in soil community composition is linked to variation in the physical and chemical parameters that define stratigraphy. Multivariate statistical analysis of sequencing reads from soil horizons across five sampling sites revealed that 1223 microbial genera vary significantly and consistently in abundance across stratified soil horizons at class level. Specifically Ktedonobacter, Bacilli and Betaproteobacteria responded most strongly to soil depth. Alpha diversity showed a positive correlation with soil depth. Beta diversity, however, did not differ significantly between horizons. Genes involved in carbohydrate and nitrogen metabolism were found to be more abundant in Ah horizon samples. Closer inspection of carbohydrate metabolism genes revealed that genes involved in CO_2 fixation, fermentation and saccharide metabolism decreased in abundance with depth while one-carbon metabolism increased down profile.

2 INTRODUCTION

Soil microbiology is a rapidly growing discipline, driven by the advances in high throughput sequencing and bioinformatic techniques. Microbiological studies on alpine soils to date have focused mainly on alpine forest, meadow and grassland soils (Ding et al., 2015; Yashiro et al., 2016; Zhang et al., 2013), comparatively little work has been carried out on cold, high altitude alpine soils and paleosols above the timberline (termed the alpine zone; Mahaney et al., 2016). Studies which have focused on soils/paleosols within the alpine zone, such as glacier forefields, have utilised techniques such as amplicon sequencing of the 16S rRNA gene, restriction fragment length polymorphism (RFLP) and/or GeoChip microarrays (He et al., 2007) to assess microbial populations (Lazzaro et al., 2015; Zhang et al., 2013). These

49 techniques are subject to significant limitations and potential biases, that can now be
50 addressed using shotgun sequencing approaches coupled with bioinformatics.

51 Several studies have shown that the number of high alpine ecosystems may be expanding
52 worldwide, due to an increase in glacier and ice cap thawing rates (King et al., 2011; Byers,
53 2008; Zemp et al., 2006)), although there is a chance that increased temperature may
54 reverse this trend by expanding forest to higher elevations (Körner and Paulsen 2004).
55 Regardless, concerns are rising regarding the potential implications this may have for global
56 atmospheric carbon levels as soils are a major carbon sink: soil organic matter contains
57 approximately 3 times the size of the atmospheric pool of carbon and 4.5 times that of the
58 biotic pool (Lal, 2004). Much more organic carbon exists in soils than in vegetation and the
59 atmosphere combined, and the global carbon budget can be strongly influenced by changes
60 in soil carbon content (Fierer et al, 2009; Serna-Chavez et al, 2013). Thawing permafrost in
61 soils newly exposed to increased temperature may increase the rate at which soil microbes
62 degrade soil organic carbon thus leading to a net increase in CO_2 emissions (Davidson and
63 Janssens, 2006). Additionally, the rate of methanogenesis could potentially increase, due to
64 an increase in available metabolites for methanogens (such as acetate), generated as by-
65 products from organisms carrying out soil organic carbon degradation, which could lead to a
66 net increase in methane emissions. Though these sediments are not completely anaerobic,
67 soils in general become more anoxic with increasing depth (Yu et al., 2006). Additionally
68 even in well oxygenated soils, methanogenesis can still occur due to the presence of anoxic
69 microenvironments within soil aggregates (Fierer 2017). Without a more thorough
70 understanding of the functional potential of the microbial communities present in these
71 soils we cannot make predictions as to the possible effects of an increase in exposed alpine
72 soils.

The alpine paleosols studied in this report have developed from the weathering of parent material deposited during two periods following the last glaciation: The Bølling – Allerød warming of ~15-12.8 ka and the Younger Dryas (YD) from 12.8-~11.5 ka (Mahaney et al., 2017). Moraines (deposits of glacial debris described here) from both pre-YD and YD time are evident at elevations greater than 2400m above sea level in this region of France/Italy. The paleosols in these deposits are classified as Cryochrepts (i.e. cold-climate pedons) with Ah/Bw/Cox profiles over fresh undifferentiated, unweathered substrates. In geological terms the soils are identified as paleosols, and they possess distinct stratigraphy with 'classically' identified Ah/Bw/Cox soil horizons (Mahaney et al., 2013). The soils in this study are generally acidic with pH ranging from 4.0-6.0 (supplementary data file 1).

How microbial communities vary with respect to these soil horizons still remains an underexplored avenue of research in soil-paleosol microbial ecology. Of very few relevant published studies, those which compare soil-paleosol horizons either employed 16S rRNA gene amplicon sequencing (Baldrian et al., 2012; Mahaney et al. 2016) or focused specifically on certain functional enzymes, such as dehalogenation enzymes (Weigold et al., 2016). No studies have focused on changes in overall microbial community structure in relation to paleosol stratigraphy.

The alpine paleosols studied here present a unique opportunity to address this apparent gap in knowledge due to:

i. The fact that they possess well stratified horizons, being distinguishable horizons in physical terms.

ii. The wealth of geochemical data already available for the region (e.g. Mahaney et al., 2016; Mahaney and Keiser, 2013).

iii. Their isolation from the Anthropocene: as paleosols these soils are relatively undisturbed by human activity until now, allowing us to assess the effect of >10,000 years of isolated soil development. Their evolutionary history initiated in the Late Pleistocene coming up to their present state in the Late Holocene entitles them to the 'paleo' designation.

There are a number of soil classification systems in current use (Eswaran, 2003), these varying by country and utility (e.g. assessing the suitability of the soil for agricultural use, or inferring its geological origin). In this study soils have been classified according to the United States Department of Agriculture (USDA) soil taxonomy system (USDA, 1999). In this system, a distinct soil horizon is classified as a layer of minerals and organic material, which differs from the parent material in mineral, physical, chemical, morphological, and biological features.

In this study, using high throughput shotgun sequencing, we first aimed to test the hypothesis that microbial communities vary significantly and consistently between stratified soil horizons in an alpine paleosol environment, both in terms of their taxonomic profile and functional potential. We then test the hypothesis that variations in the soil microbiome can be linked to variations in the physical and chemical properties of the soil horizons. Finally, we investigated which taxa and functional gene categories showed the strongest changes in abundance *between* soil horizons.

3 Methods

Sites were selected on the basis of air photo interpretation (1:20,000 scale) of deposits. Clasts embedded in major landform surfaces and profiles (Fig. 1) were sampled following

118 excavation of sections to depths of ~0.8 m. Profiles extend to depths of ~40-50 cm ± 5 cm.
119 Paleosol descriptions follow standard nomenclature (NSSC, 1995) and Birkeland (1999). The
120 'Cox' horizon designation, originally defined by Birkeland (1999), is applied to strata with
121 detectable levels of secondary Fe hydroxides and oxides, whereas 'Cu' refers to
122 unweathered parent material (Hodgson, 1976). The 'Ah' horizon designation is applied
123 when surface color is stronger than 10YR 3/1, an indication of appreciable organic carbon
124 accumulation (Canada Soil Survey Comm., 1998). Soil colors were assigned using Oyama and
125 Takehara's (1970) soil colour chips. Bulk samples (250-300 g) were collected from paleosol
126 horizons for particle size, clay mineral, geochemical and microbiological analyses.

127 DNA was extracted from alpine paleosol samples using a PowerSoil DNA extraction kit (Mo
128 Bio). For each DNA extraction 0.25g of sample was used per extraction, extractions were
129 performed in triplicate and pooled. Extractions were performed according to the
130 manufacturer's protocol with the following modifications: samples were homogenised using
131 a FastPrep 120 cell disrupter system (Thermo-Fisher) at 5.5 $m.s^{-1}$ for 2 minutes, rather than
132 a standard bench top vortex. The eluted and pooled DNA was further purified via two
133 rounds of ethanol precipitation; DNA solution was suspended in 3 volumes of ice cold 100%
134 ethanol, 0.1 volumes 5 M sodium acetate solution (pH 5.2) and 2 µL of linearized
135 polyacrylamide (LPA), the solution was then incubated overnight at -20°C and centrifuged at
136 18000 x g at 4°C for 30 minutes. The supernatant was discarded and the pellet washed in
137 70% ice cold ethanol and again centrifuged at 18000 x g at 4°C for 5 minutes. Finally, the
138 supernatant was discarded and the pellet allowed to air dry for 15 minutes before
139 resuspension in 50 µL molecular grade H_20. Final DNA concentrations were measured using
140 a Quantus Fluorometer (Promega) in conjunction with the Quantiflour DsDNA dye system

141 (Promega). Soil physical and chemical variables were measured as previously described
142 (Mahaney et al., 2016)

143 Sequencing read quality, length and adapter contamination were initially assessed using
144 Fastqc (Andrews, 2010). Adapter trimming was performed using bbduk from the bbtools
145 package (Bushnell, 2015) using the provided library of Illumina adapters. Quality trimming
146 was also performed using bbduk form the bbtools package, reads were trimmed to a
147 minimum quality score of 20 over a sliding window of 10 bases, additionally the flag
148 'modulo = 5' was used to remove trailing single odd numbered bases (i.e. the 301st base in a
149 300bp library, or the 101st base in 100bp library), which are common error bases in Illumina
150 datasets. Read merging was performed using bbmerge from the bbtools package. Human
151 DNA contaminants were removed by using removehuman.sh from the bbtools package.
152 Briefly, raw reads are mapped onto a prebuilt index of the human genome which had been
153 masked to hide 1) any low complexity repeat regions and 2) any regions which showed >
154 85% identity to any sequence in the Silva rRNA database (Quast *et al.*, 2013) over a 70 base-
155 pair window. This method removes potential human DNA contamination while minimising
156 false positive hits to low complexity regions and ribosomal RNA sequences
157

158 Assembly benchmarking was performed using three *de-novo* genome assemblers optimized
159 for metagenomic data, Megahit (Li *et al.*, 2016), SPAdes (Bankevich *et al.*, 2012) (with the
160 flag: – meta), and IDBA-UD (Peng *et al.*, 2012), using kmers ranging from 27-127 in steps of
161 10. Benchmarking was performed against un-normalized raw reads and reads normalized to
162 minimum kmer depth of 3 and maximum kmer depth of 100 for kmers of size 32.
163 Normalization was performed using bbnorm form the bbtools package, with the flags
164 *min=3, max=100, k = 32*. Read recruitment for each assembly was estimated using bbmap

165 with the flags *k=13 vslow=t*. Annotation of quality trimmed reads was achieved using Kaiju
166 (Menzel *et al.*, 2016). A database of all proteins from all bacteria, archaea, single celled
167 eukaryotes and viruses in the NCBI non-redundant protein database
168 (NCBI Resource Coordinators, 2017) was constructed using the makeDB.sh script supplied
169 with Kaiju. Nucleotide reads were translated into amino acid sequence in all 6 reading
170 frames and taxonomically annotated by alignment against this database using Kaiju, with a
171 maximum of 5 mismatches allowed and a minimum bit score of 60. Reads were projected
172 onto all taxonomic ranks from phylum to species and per-sample abundances were
173 compiled into single data tables for downstream statistical analysis using a custom bash
174 script. Functional annotations were assigned by mapping NCBI accession IDs from the Kaiju
175 analysis onto functional classifications from the SEED subsystems protein hierarchy
176 (Overbeek *et al.*, 2005) at levels 1, 2 and 3 using MEGAN 6 (Huson *et al.*, 2016).
177

178 All taxonomic and functional annotations and read counts were concatenated and
179 downstream analysis was performed using R 3.4.1 (R Core Team, 2011). Taxonomic and
180 functional abundances were summed at each taxonomic and functional rank and
181 normalized by dividing counts for each sample by the total number of reads that were
182 annotated for that sample using the *aggregate* function. Prior to statistical analysis,
183 taxonomic and functional abundance values were Hellinger transformed as described in
184 Legendre and Gallagher, (2001) using the function *decostand* from the package vegan
185 (Oksanen *et al.*, 2016). Soil abiotic variables were log transformed and standardized such
186 that each variable had a mean of zero and standard deviation of 1 across all samples.
187

188 Euclidean distance dissimilarity matrices were produced for normalized abundance and
189 abiotic variable tables using the *vegdist* function from vegan. Principal component analysis
190 was performed using the *capscale* function in vegan, samples were plotted in two
191 dimensional space using their first two principal components, plots were produced for
192 taxonomic abundances at the class and genus levels, for functional abundances, and for soil
193 abioitic variables. For taxonomic annotation tables, genus-level richness and Shannon-
194 Weaver diversity indices were computed using the diversity function from the package
195 vegan. Beta diversity for each soil horizon was inferred for all taxonomic and functional
196 ranks as the distance to the group centroid in Euclidean space using the *betadisper* function
197 from the package vegan. Analysis of variance (ANOVA) tests were performed to test for
198 significant differences between group beta dispersions using the function *anova*. Analysis of
199 similarities (ANOSIM) (Clarke, 1993) was performed on all samples grouped by biome type,
200 sample site (for Alpine soils only) and soil horizon using the function *anosim* from the
201 package vegan. Analysis of variance using distance matrices was performed on all samples
202 grouped by, sample site and soil horizon using the *adonis* function from the package vegan
203 with 9999 permutations. Taxa and functions whose abundances differed significantly
204 between horizons depths were identified by applying the Kruskal–Wallis H test (Kruskal and
205 Wallis, 1952) using the function *kruskal.test*, p-values were adjusted for multiple testing
206 using the Benjamin-Hochberg false discovery rate method (Benjamini and Hochberg, 1995)
207 with the function *p.adjust*, only taxa with an adjusted p-value < 0.05 were considered
208 significant.
209

210 **Data availability**

211 Raw Sequence data files are available in the NCBI sequence read archive under Bioproject
212 number PRJNA39461.

213 # 4 RESULTS

214 ## DNA EXTRACTION, SEQUENCING DATA QC AND PROCESSING

215 Vertical sections of weathered sediment (paleosols) in a series of five moraines in the Guil
216 river valley of the French Alps, (G1, G2, G3a, G9, and G11) were sampled at three discrete
217 stratified soil horizons (Ah, Bw and Cox) and were classified as previously described
218 (Mahaney et al., 2016; 2017) (**Figure 1**). The geology of these paleosols is an active area of
219 glacial, cosmic and microbiological research as described in several related geological papers
220 to date (Mahaney et al., 2013, 2016, 2017; Mahaney and Keiser, 2013).

221 Total DNA per gram of soil generally decreased with increasing depth, indicating a lower
222 total biomass at lower depths in the soil profile (supplementary table S3). Average sequence
223 read lengths were as expected (300bp), average sequence quality was found to be > 20 for
224 the full length of each read for all samples. Adapter contamination was also found to be low,
225 on average < 1% per library. Although merging of the paired end reads was attempted, on
226 average < 30% of reads could be merged, indicating that the average insert size of the
227 sequencing libraries exceeded 600bp, therefore downstream processing was performed on
228 interleaved PE reads.

229 Attempts at assembly of the metagenomic libraries revealed generally poor assemblies
230 (supplementary table S4) and a significant loss of information (i.e. the percentage of raw reads
231 which could be mapped onto the assembly). Since the goal here was to characterize these soil
232 samples in a general sense it was decided that the loss of information during assembly and

233 overall poor assembly statistics were deemed unacceptable. Therefore annotation was
234 performed using only the raw PE read files, since the reads were on average 300bp in length,
235 a reasonable level of taxonomic resolution can still be obtained, and in most cases
236 assignments could be made down to the genus level without much further loss of information
237 **(supplementary figure S1).**

238 On average ~ 80% of all reads could be assigned to at least the Phylum level, the functional
239 gene assignment rates were significantly lower at ranging from 17-26% depending on the
240 SEED subsystem level used. As taxonomic resolution increases so the number of reads which
241 can be confidently assigned to a taxon decreases, with a significant drop-off in the number of
242 reads which can be assigned to the species level, therefor for downstream analysis, the genus
243 level was the minimum taxonomic rank which was analysed..

244 **TAXONOMIC / FUNCTIONAL ANNOTATION**

245 At the class level it is apparent that the community is dominated by four major taxa (**Figure**
246 **2**), the *Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*
247 all of which are common genera of soil microbes which appear to be ubiquitous in soils
248 (Barberán *et al.*, 2014). The Actinobacteria account for roughly 30% of the total annotated
249 reads in all samples. The *Bacilli* and *Acidobacteriia* also appear to be reasonably abundant,
250 as might be expected for paleosols with low pH 4.0-6.0. Certain horizon dependent patterns
251 in the data also emerge even in these boxplots, for example, the *Betaproteobacteria* and
252 *Gammaproteobacteria* appear to be generally more abundant in the Bw and Cox horizon
253 samples while the *Ktedonobacteria* appear to be generally more abundant in the Ah horizon
254 samples.

255 ## ALPHA / BETA DIVERSITY ANALYSIS

256 Alpha diversity was estimated for each sample at each taxonomic level using the Shannon–
257 Weaver diversity index (Shannon and Weaver, 1964), Inverse Simpson diversity index
258 (Simpson, 1949) and genus-level richness (i.e. number of unique genera per sample). Linear
259 regression of alpha diversity measures against sample depth reveals a fairly strong,
260 statistically significant linear relationship between soil depth and alpha diversity (**Figure 3**).
261 Interestingly while richness appears to decrease with depth, the diversity indices appear to
262 increase. Both inverse Simpson and Shannon diversity indices account for taxon abundances,
263 where a lower value indicates more uneven taxon abundances, suggesting that while the
264 number of unique genera decrease while moving down a soil profile, the relative abundances
265 of these genera become more even.

266 In order to test the statistical significance of differences in community structure between
267 horizons, ANOSIM (Figure 3) and Adonis tests (supplementary table S2) were applied. It was
268 necessary to test for homogeneity of group dispersions, i.e. whether or not the multivariate
269 spread of the samples from their group centroids are significantly different (**Figure 3 panel**
270 **B**). Homogeneity of group dispersion tests were performed for samples grouped by both soil
271 horizon and sample site, results were not statistically significant in any case, meaning that
272 downstream ANOSIM tests may be interpreted confidently without any caveats. The results
273 of both ANOSIM and Adonis tests indicate that soil horizon has a much stronger effect on
274 microbial community than sampling site: the ADONIS R statistic is reasonably high for all ranks
275 when grouped by soil horizon and much lower when grouped by sampling site, indicating that
276 the effect is genuine. Statistical significance is strong with all ranks showing three star
277 significance ($P < 0.001$) when grouped by soil horizon, apart from the phylum level which

278 shows two star significance (P < 0.01) (supplementary table S3). When grouped by sampling
279 site, there is a marginally significant effect at the phylum level although this is not seen at
280 lower levels of taxonomic resolution, or at the functional gene level.

281 ## Correlations with Soil Abiotic Variables

282 The effect of soil horizon classification on soil abiotic variables was also assessed (**Figure 4**).
283 Out of the 27 variables which were assessed here [supplementary data file 1], 12 were
284 found to correlate significantly with soil depth and between stratified soil horizons. These
285 12 include 5 of the 6 essential elements for life (C, H, N, P, S), it can also be presumed that
286 available oxygen also decreases with soil depth. Where C,N and P were compered total
287 rather than soluble levels were used, because we wanted to consider insoluble sources of
288 these elements (e.g. lignin). Given this information, it is unsurprising that the total DNA per
289 g of soil decreases with depth and that the microbial communities between soil horizons are
290 significantly different from one another (ANOSIM significance = $3.9e^{-4}$) (**Figure 3**).
291 Conversely, the concentration of numerous elements important for life (Ca, Cu, Na, K and
292 Mn) appears to increase with soil depth – perhaps due to leaching. This is typical during
293 weathering of parent material, when the elements are realised they are leached down
294 profile (Retallack, 2001). The exception is K that follows decreasing trend toward the parent
295 rock. The K is most likely incorporated into secondary clay minerals like Illite, illite-smectite
296 and/or illite-vermiculite (Mahaney et al., 2016).

297 ## Principal Component Analysis

298 Principle component analysis was performed on taxon abundances (at the class and genus
299 levels), functional abundances (at SEED subsystems level 1) and abiotic variables (**Figure 5**),

300 revealing strikingly similar patterns between samples in multivariate space. There is a clear
301 and consistent separation of soil horizons along the first principal component axis in all cases.
302 There is also a slight overlap between Bw and Cox horizons in the case of the Genus level
303 abundance and chemical variables, indicating that the Bw and Cox horizons are less strongly
304 differentiated from each other. This corresponds with the sedimentology - which indicates
305 that the Bw horizon is only weakly differentiated and still developing (hence the w
306 classification), and therefore it is not surprising that these data suggest a closer degree of
307 similarity between population in the Bw and Cox horizons (Mahaney *et al.*, 2013). In all cases,
308 the first two principle component axes cumulatively explain > 70% of the total variation (>
309 80% in the case of the taxonomic variation).

310 In order to identify the taxa and functions which contribute most to the differences between
311 soil horizons the Kruskal-Wallace H test (Kruskal and Wallis, 1952) for differences between
312 groups was applied to taxonomic abundances at the class level, and functional abundances at
313 level 1 Of the SEED subsystems hierarchy. For clarity, plots were only produced for taxa and
314 functions which occurred at an abundance greater than 0.1% in at least one sample.

315 It is clear that the abundances of many classes of bacteria vary along the depth profile (**Figure**
316 **6**). Nine of the top ten most significantly variable taxa appear to increase with soil depth,
317 while the only one which decreases are the *Ktedonobacteria.* Likewise, classes which are
318 known to comprise many obligate and facultative anaerobes (the *Bacilli*, *Clostridia* and
319 *Negativicutes*) increase significantly with depth. The *Betaproteobacteria* are one of the most
320 abundant classes present in the dataset and show a near 3% increase in median abundance
321 between the Ah and Cox horizons. A total of 57 Classes were shown to vary significantly
322 between soil horizons, applying this analysis to genus level abundances revealed that 1223

genera vary significantly between soil horizons. Overall, what has become clear from these data is that a large number taxa vary significantly and consistently between soil horizons over all six sample sites, even when measured at higher taxonomic ranks.

Similarly, many functional gene categories vary in abundance with respect to soil horizon, although the effect is much less pronounced than for taxon abundances, with only 6 major categories showing significant variation (**Figure 7**). Nitrogen metabolism and carbohydrate metabolism are the two which can be most obviously linked to ecosystem level processes. Both decrease with increasing depth, corresponding to the decrease in soil nitrogen and organic carbon with depth recorded by Mahaney, et al. (2016). The low measured N (supplementary data file 1) concentrations in these soils would suggest that the environment is extremely nitrogen limited. Therefore we would expect that Ammonia oxidation may be an extremely important component of the nitrogen cycle in these soils. Genes relating to central metabolism (i.e. metabolic pathways essential for organism survival) and secondary metabolism (i.e. pathways which are non-essential) show a near inverse relationship, with secondary metabolism decreasing with depth while central metabolism genes increase with depth. This may be explained by the fact that in lower soil horizons, where nutrients are less abundant (Fierer, 2017), functions which are non-essential for survival are less vital and thus are selected against over time - core metabolism genes become more important.

Additionally, in the Ah horizon where C N and P are more abundant we might expect anti-microbial secondary metabolites biosynthesis genes to be more prevalent due to the higher abundance of microbes competing for these nutrients. In lower soil horizons with less microbial activity, competition for nutrients is likely to be lower leading to a drop in the

346 abundance of anti-microbial biosynthesis genes, although this cannot be confirmed from the
347 results of this study. This does however suggest the possibility that nutrient-rich surface soil
348 horizons may be ideal locations to screen for the presence of novel antimicrobial compounds
349 and future work should endeavour to test this hypothesis. Fatty acid and isoprenoid synthesis
350 genes are more abundant in the Ah horizon, this may be due to the fact these genes are
351 heavily involved in cell membrane maintenance and manufacture (Kaneda, 1991), and may
352 indicate that the Ah horizon hosts the most actively growing microbial cells. Finally,
353 membrane transport related genes appear to be most abundant in the Cox horizons. The
354 reason for this trend is not immediately obvious, though one could speculate that it may be
355 connected to an increase in chemo-lithotrophic lifestyles in the lower mineral soil horizons –
356 where alternative electron donors (such as sulfide, ferric Iron or ammonia) must be
357 transported across the cell membrane before they can be used by the cell for ATP synthesis
358 (Peck 2003).

359 Carbohydrate metabolism is one of the largest SEED subsystem categories and contains many
360 subcategories of genes which are linked to the carbon cycle, therefore carbohydrate
361 metabolism genes were analyzed in more detail (**Figure 8**). After multiple test corrections
362 were applied 5 categories within carbohydrate metabolism show significant variation. Poly,
363 mono, di and oligo saccharide metabolism genes all decrease moving down the soil profile, as
364 do carbon dioxide fixation genes. This is hardly surprising due to the fact that organic carbon,
365 and carbon dioxide are generally present at higher concentrations in upper soil horizons.
366 However, one-carbon metabolism genes appear to increase with soil depth, as might be
367 expected given the importance of the methane cycle in low organic carbon environments
368 (Serrano-Silva *et al.*, 2014). A picture of the carbon cycle in these soils then begins to emerge
369 whereby organic carbon is primary energy and provides carbon sources for many microbes in

upper soil horizons as might be expected, while organisms in lower soil horizons gain energy and carbon by utilizing reduced one-carbon compounds (e.g. methane) as energy sources which are liberated from the anaerobic turnover of waste products from the upper horizons.

5 Discussion

It is clear that the abundances of many bacterial vary consistently between stratified soil horizons (Figure 6). We found that the *Ktedonobacter* showed the largest differential abundance between stratified soil horizon and was generally most abundant in the Ah horizons, members of this class are thought to be aerobic filamentous, spore-forming, gram-positive, heterotrophic bacteria (Yabe et al. 2017). Conversely, many classes of obligate and facultative anaerobes decreased in abundance with soil depth (eg. the *Bacilli*, *Clastridiales*) (Figure 3), suggesting a strong influence of oxygen availability on the community composition. Interestingly, the most abundant classes of microbes across all samples (eg: *Acinobacteria*, *Proteobacteria*) (Figure 2) did not show a statistically significant variation in abundance between soil horizons (Figure 6), perhaps suggesting that the more successful microbes in these environments are ones with mixotrophic lifestyles who can adapt to variable conditions between soil horizons.

When comparing functional profiles between soil horizons, differences in certain biogeochemical cycling genes become clear. It is evident that many gene abundances correlate either positively or negatively with depth, which is understandable given that most key nutrients will vary down profile. Nitrogen metabolism appears to decrease with increasing depth, and likewise, carbohydrate metabolism genes appear to be significantly

392 more abundant in the Ah horizon, corresponding to the zone of highest organic carbon
393 turnover (evident from CNP measurements, Mahaney et al., 2016). Taking a more detailed
394 look at carbohydrate metabolism (**Figure 8**) revealed that there are significant variations
395 even within this category. Carbon dioxide (autotrophy) and central carbohydrate
396 metabolism genes appear to be relatively abundant in the Ah horizon, while genes for other
397 one-carbon metabolism becomes more abundant in the Cox horizon. This is consistent with
398 increasing abundance of methane (associated with both methylotrophy and
399 methanogenesis) turnover processes in deeper soils (Dunfield, 2007). This is likely due to the
400 fact that, as complex plant polymers are broken down by microbes in the upper soil
401 horizons, reduced carbon compounds are produced as waste products and filter down to
402 the lower horizons where they are then utilized by methylotrophic microbes.
403
404 It should be noted that there is significant co-linearity between the many abiotic and biotic
405 variables measured and compared here. As an example, looking specifically at almost all other
406 abiotic and biotic parameters correlate with soil depth (in cm) to some degree (Figure 5).
407 Therefore it was not possible to investigate and compare correlations between individual
408 taxa/functions and abiotic variables as disentangling this co-linearity to reveal the true source
409 of variation is not possible without carefully designed laboratory experiments to control for
410 the effect of specific co-linear variables identified here. Indeed, any attempt to do so may be
411 statistically dubious and may therefore be considered as an example of "p-hacking" (Head *et*
412 *al.*, 2015). However, this analysis makes it abundantly clear that soil horizon matters: in terms
413 of chemistry, taxonomy and functionality of the community.
414

415 There are several important caveats to the results presented here that must also be
416 considered. Firstly, metagenomes are not expected to be static over time, so caution must
417 be taken when making broad statements about a microbial community. In extreme cases,
418 we might expect drastic changes in microbial community structure to (Mahaney et al.,
419 2017). Otherwise, previous studies have established that temporal variation within soil
420 microbiomes is generally much lower than spatial variation (Lauber *et al.*, 2013), so the
421 patterns observed here are likely to persist across seasons, at least in the short term.
422 Secondly, for any enzyme-catalysed processes to occur, transcription and translation of
423 protein coding genes are essential requirements. Therefore, a positive relationship between
424 the abundance of gene or transcripts and corresponding process rate is not always
425 necessarily true, though it is often presumed. Rocca *et al.*, (2015) indicated that functional
426 gene abundances are only weakly correlated to process rates, but are consistently
427 correlated across multiple environments. Finally, the relatively low depth of sequencing per
428 sample means that complete genomes cannot be resolved, (a common limitation for diverse
429 soil metagenomes (Nesme et al. 2016) and annotations are based on ~300bp fragments.
430 Much more robust results could be obtained with more complete genomes and functional
431 genes, allowing investigation of functional genes in their genomic context, and analysis of
432 complete metabolic pathways within certain species. The relatively short read length used
433 here may be useful for taxonomic classification but full length genes are preferable for
434 functional annotation. Despite this limitation, there is a growing body of evidence to suggest
435 that shallower sequencing across many samples with suitable replication levels is sufficient
436 to answer key questions about microbial ecology (Knight *et al.*, 2012).
437

438 Fundamentally we know that soil stratigraphy is a function of soil chemistry (USDA 1999).
439 Our full metagenome data, for the first time, show that soil stratigraphy is strongly
440 correlated with variation in microbial community composition – as shown in figures 3 and 5.
441 Furthermore we find that the variation seen between stratified soil horizons is greater than
442 the variation seen between soil sampling sites when taken as a whole (Figure 3).
443
444 Ultimately, the physical and chemical properties of a soil are the true drivers of microbial
445 diversity and soil horizons are a useful method for categorizing soils with similar
446 physicochemical characteristics. While the gene analysis presented here has provided an
447 overview of microbial community structure and functionality in alpine soils, it has also
448 highlighted a need for more specific methods in order to make definitive statements about
449 biogeochemical processes occurring between soil horizons. The use of more specific and
450 informative functional gene ontologies or the use of meta-transcriptomic information will
451 certainly add a further level of understanding to this field. One correlation that we did not
452 investigate in this study, is the relationship between oxygen concentration and/or redox
453 potential with metagenome composition down profile. In general we would expect these to
454 decrease with depth. Clearly there is abundant microbial activity in the sediments, and
455 oxygen – as the ultimate acceptor – would certainly become limiting to microbial
456 metabolism within a cm or two of the Ah horizon (Birkeland, 1999).
457

458 **CONCLUSION**

459 In conclusion, our study begins to demonstrate a clear relationship between the physico-
460 chemical parameters that are typically used to define soil pedons, and parallel the structure
461 of complex microbial communities. The ability to do so has only become available to us in

recent years due to the advent of shotgun sequencing and metagenomic analysis. The limitations of culture-dependant microbial analysis, have previously not allowed any such connection to be established. However, in this study some dependency is evident: the reduction between organic carbon availability and the related genetic profile of functional genes involved in C1 turnover is especially clear and is quite rational. The results here may lead us to consider that the formation of paleosol horizons is inextricably linked to the biological, not just physical and chemical transformations that occur. Critically, we emphasise here a key finding in this study – that variation in microbial gene populations between palesol samples sites, sometimes hundreds of metres apart, is significantly less than that seen between soil pedons, just centimetres apart at specific sample sites. This remarkable metagenome diversity may be exploited for gene mining applications.

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FIGURE LEGENDS

Figure 1: Overview of Sampling sites and soil stratigraphy. Panel A: Satellite map of sampling area. Panel B: Stratigraphy of soil profiles for each sample (from Mahaney *et al*., 2016). Sampling sites are shown at the top of each profiles, depth in centimeters is displayed on the left and soil horizon classification is displayed on the right of each profile.

Figure 2: Boxplot showing the relative abundances of the 20 most abundant Taxa across all samples at the Class level. Jittered points overlaid on boxplots represent individual sample and are coloured by sample soil horizon classification. The bottom and top of the boxes represent the first and third quartiles, with the central band representing the median, whiskers represent 1.5 times the interquartile range according to Tukey's schematic boxplot method (Tukey, 1977)

Figure 3: Diversity analysis of alpine soils. **Panel A:** Alpha diversity plots for taxonomic abundance tables at each taxonomic level. Y axis represents inverse Simpson diversity index, Shannon-Weaver diversity index, and Genus level richness (i.e. the number of unique genera detected per sample) respectively. X axis represents depth in cm of soil sample. Blue lines represent linear regressions, shaded area represents the 95% confidence interval for the regression analysis. R^2 values and P-values for linear regressions along with significance codes are displayed for each plot. **Panel B:** homogeneity of group dispersions for samples grouped by soil horizon, Y axis represents distance to the group centroid in 2d Euclidean space, standard error of the mean across all samples, analysis of variance (ANOVA) test P

687 value for differences between soil horizons value is displayed on the plot. Panel C: Boxplot
688 of analysis of similarities (ANOSIM) results, Y axis represents ranked order of dissimilarities
689 ANOSIM R statistic and significance level are shown on the plot, significance of the R statistic
690 was assessed by permutation for 9999 replicates. The bottom and top of the boxes
691 represent the first and third quartiles, with the central band representing the median,
692 whiskers represent 1.5 time the interquartile range according to Tukeys schematic boxplot
693 method (Tukey, 1977).

694 **Figure 4:** Analysis of effect of paleosol depth and paleosol horizon on paleosol abiotic
695 variables. **Panel A:** Spearmans rank correlation matrix for pairwise correlations between soil
696 geochemical variables. Points are coloured and scaled according to the value of the
697 correlation coefficient, only correlations with a P value < 0.05 are displayed. Variables which
698 correlate significantly with soil depth are highlighted in red. **PanelB:** Boxplots of
699 geochemical variables which vary significantly between stratified soil horizons. The bottom
700 and top of the boxes represent the first and third quartiles, with the central band
701 representing the median, whiskers represent 1.5 time the interquartile range according to
702 Tukeys schematic boxplot method (Tukey, 1977).

703 **Figure 5:** PCoA plots of abundance tables at each taxonomic and functional rank. Constrained analysis
704 of principal components was performed on dissimilarity matrices using the function capscale from the
705 package Vegan (Oksanen et al., 2016) in R version 3.4.1 (R Core Team, 2011). In each case the x and y
706 axis represent the first and second principal component axis respectively. Points are coloured by soil
707 horizon and the convex hulls are drawn and highlighted for each horizon grouping.

708 **Figure 6:** Boxplots showing relative abundance of microbial classes which differed significantly
709 between soil horizons. Relative abundance is expressed as a percentage of the total annotated reads.
710 The bottom and top of the boxes represent the first and third quartiles, with the central band

711 representing the median, whiskers represent 1.5 time the interquartile range according to Tukey's
712 boxplot method (Tukey, 1977). All data points are also plotted as points.

713 **Figure 7:** Boxplots of relative abundance of SEED level 1 functional categories. Relative abundance is
714 expressed as a percentage of the total annotated reads. The bottom and top of the boxes represent
715 the first and third quartiles, with the central band representing the median, whiskers represent 1.5
716 times the interquartile range according to Tukey's boxplot method (Tukey, 1977). All data points are
717 also plotted as jittered points.

718 **Figure 8:** Abundance of SEED level 2 categories related to carbon metabolism. Relative abundance is
719 expressed as a percentage of the total annotated reads. The bottom and top of the boxes represent
720 the first and third quartiles, with the central band representing the median, whiskers represent 1.5
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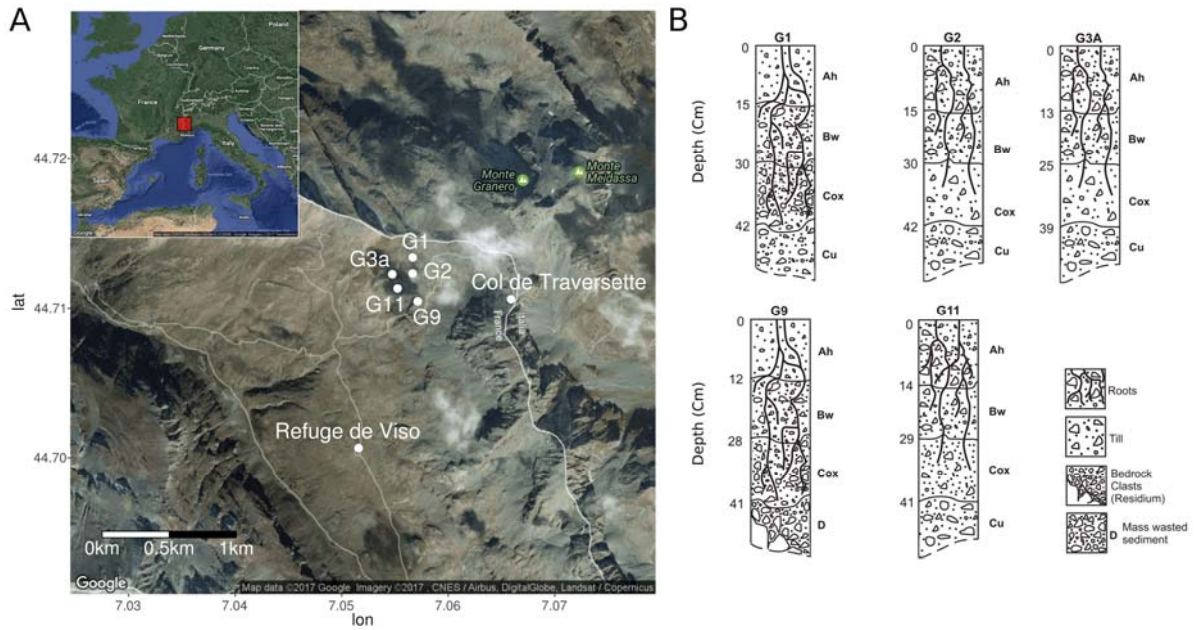
723



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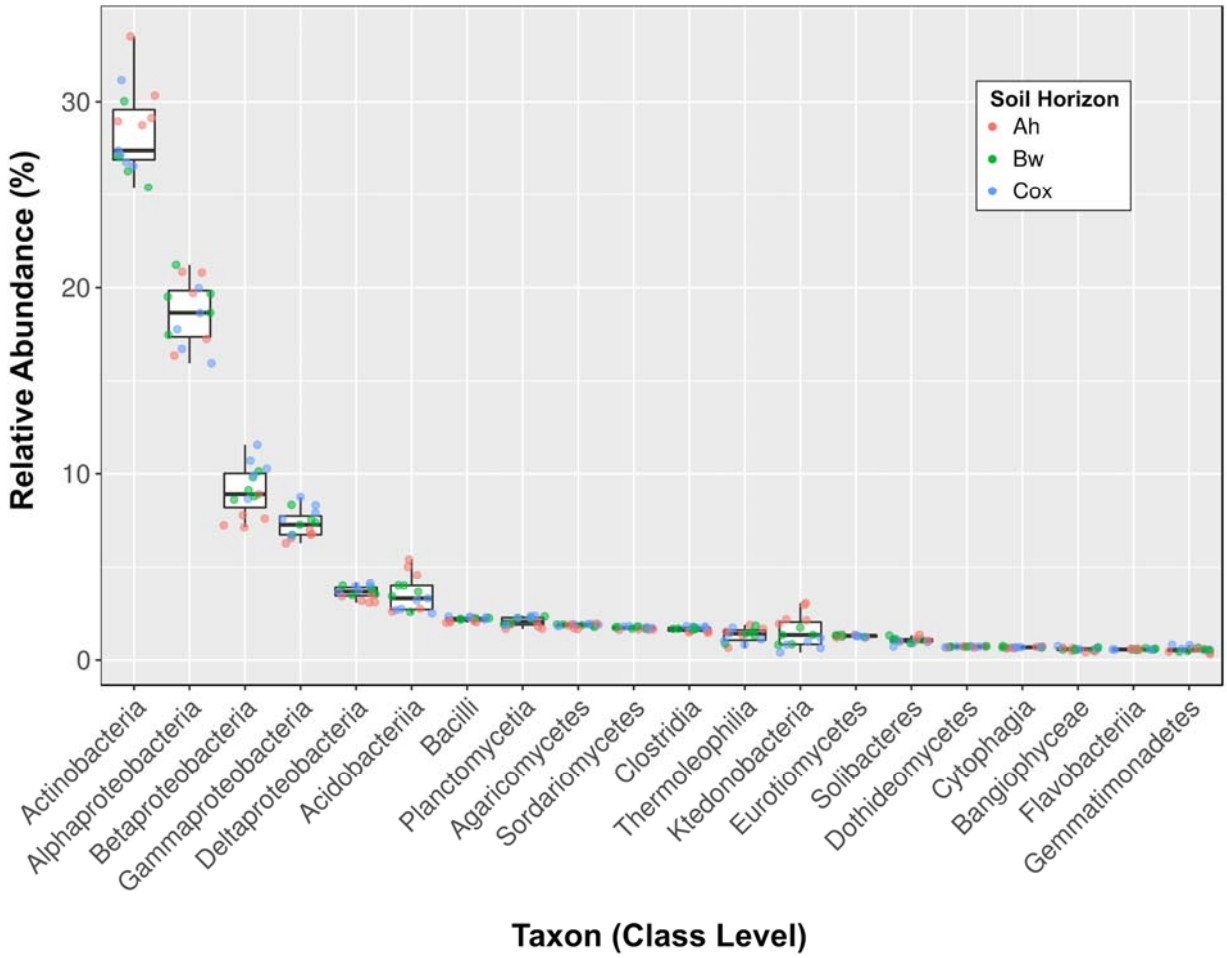

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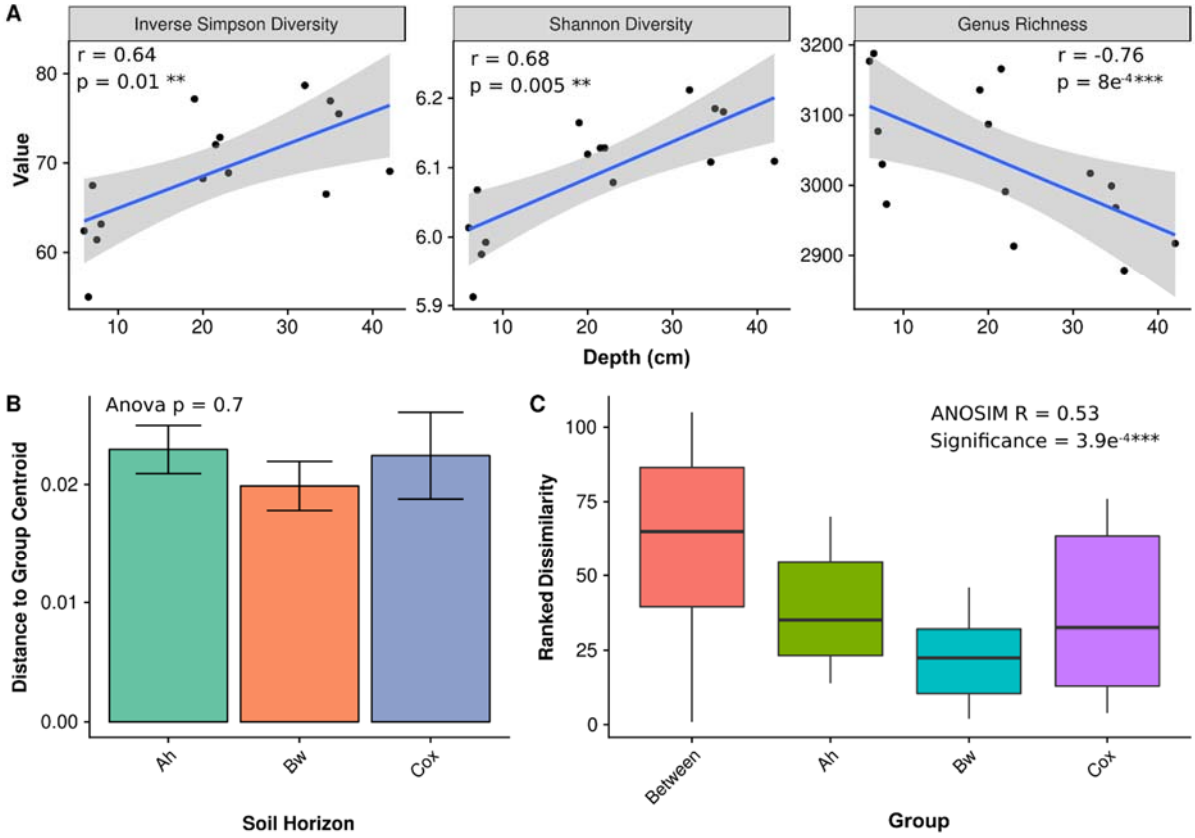

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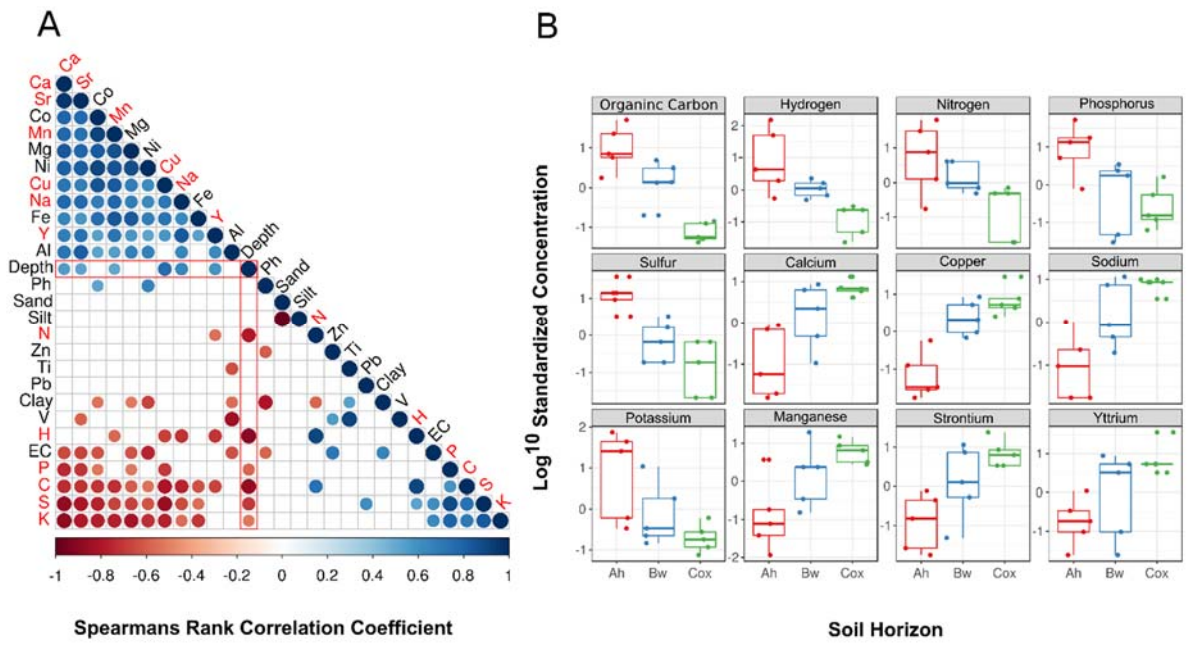

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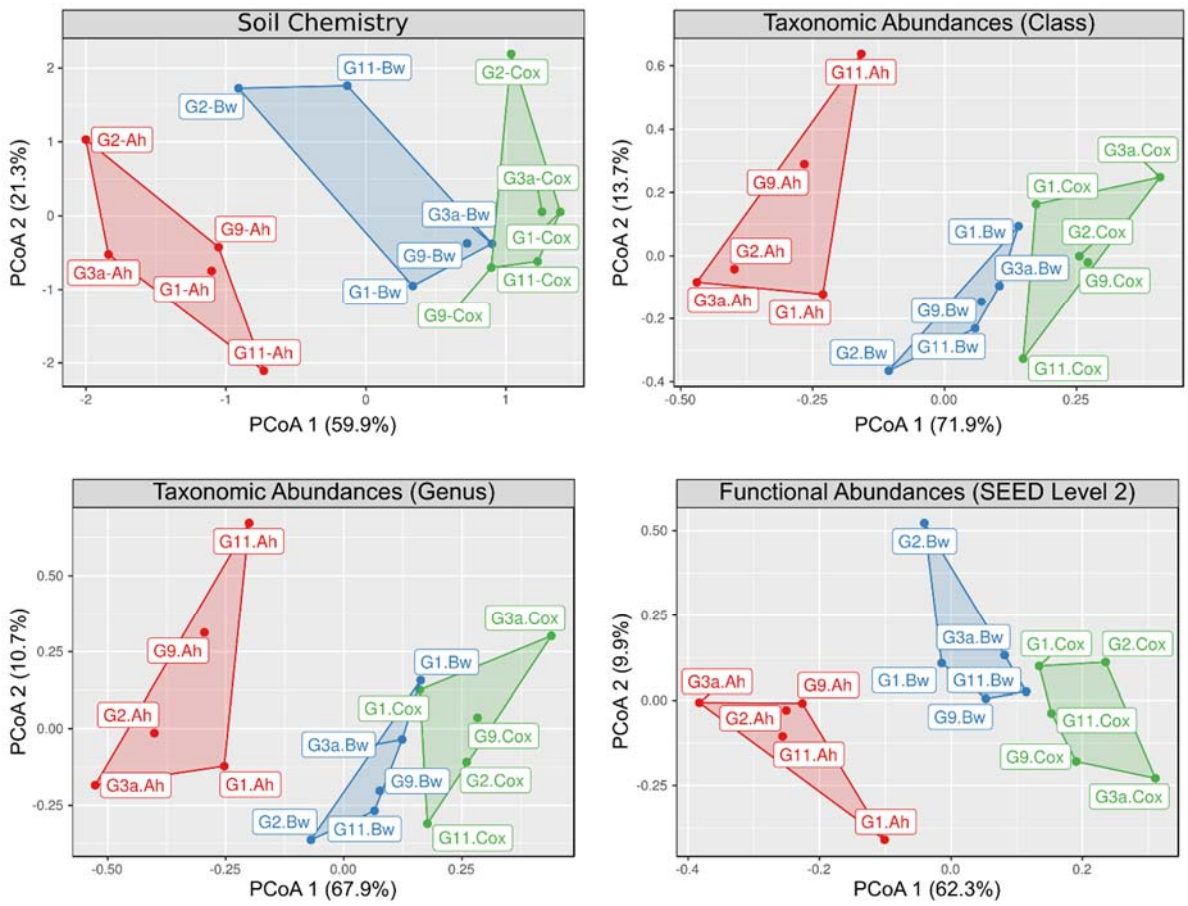

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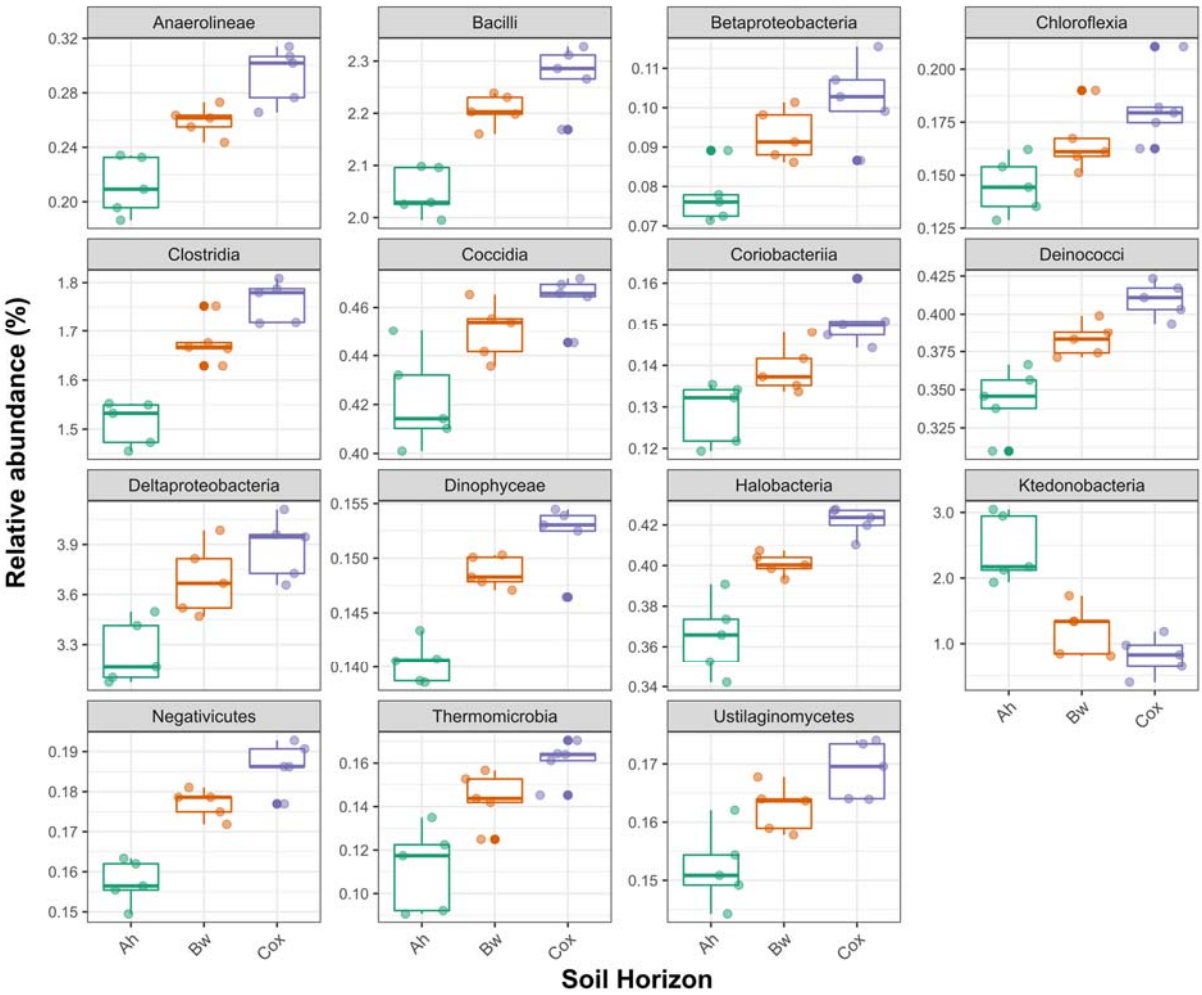

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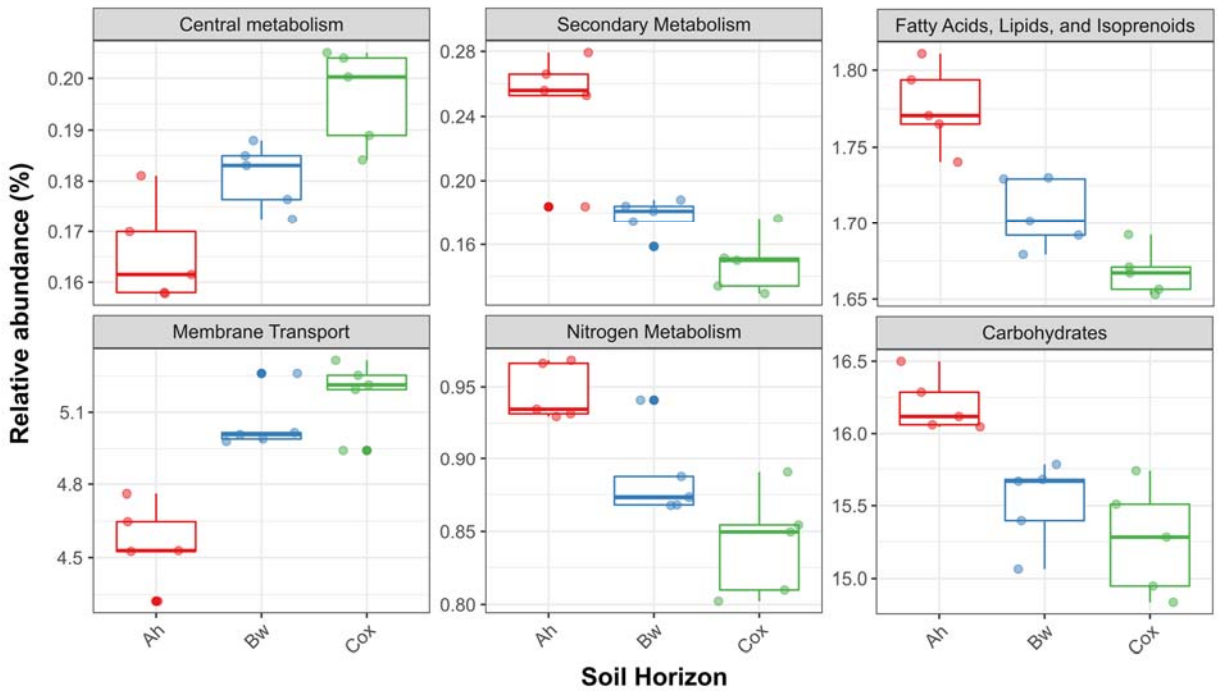

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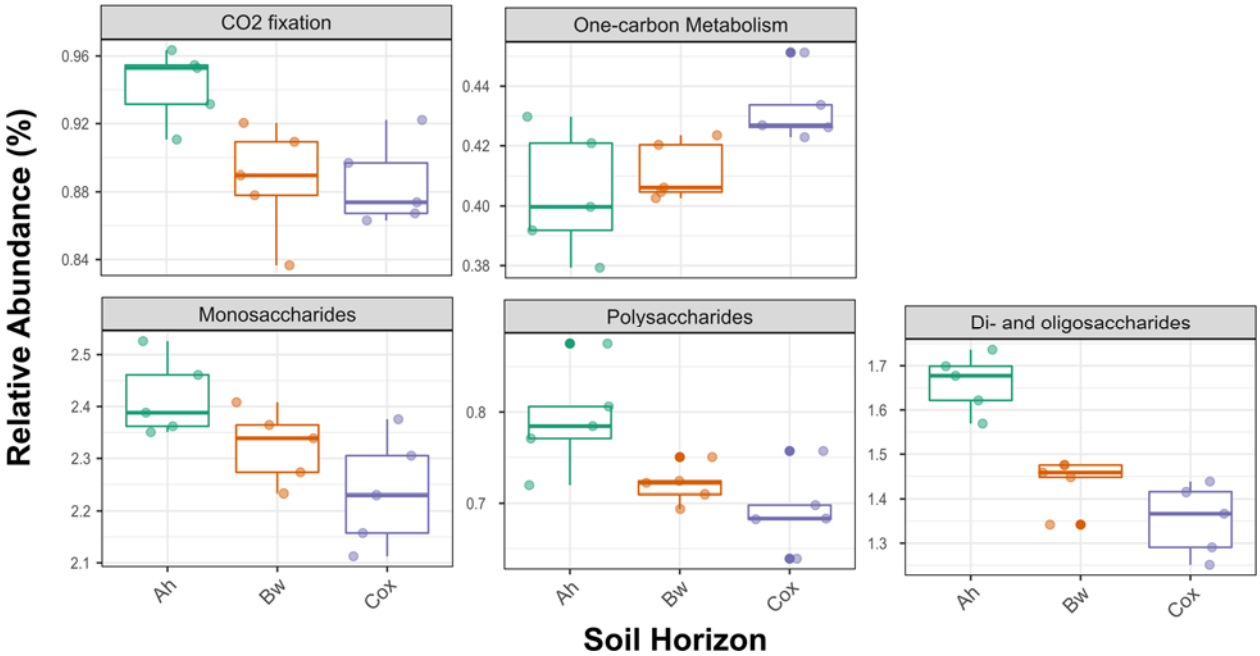

Figure 8: Abundance of SEED level 2 categories related to carbon metabolism. Relative abundance is expressed as a percentage of the total annotated reads. The bottom and top of the boxes represent the first and third quartiles, with the central band representing the median, whiskers represent 1.5 time the interquartile range according to Tukey's boxplot method (Tukey, 1977). All data points are also plotted as points.

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