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The soil microbiome at the Gi-FACE experiment responds to a moisture gradient but not to CO2 enrichment

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The soil microbiome at the Gi-FACE experiment responds to a moisture gradient but not to CO_2 enrichment

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Abstract

The soil bacterial community at the Giessen free-air CO_2 enrichment (Gi-FACE) experiment was analysed by tag-sequencing of the 16S rRNA gene. No substantial effects of CO_2 levels on bacterial community composition were detected. However, the soil moisture gradient at Gi-FACE had a significant effect on bacterial community composition. Different groups within the Acidobacteria and Verrucomicrobia phyla were affected differently by soil moisture content. These results suggest that modest increases in atmospheric CO_2 may cause only minor changes in soil bacterial community composition and indicate that the functional responses of the soil community to CO_2 enrichment previously reported at Gi-FACE are due to other factors other than changes in bacterial community composition. These results suggest that modest increases in atmospheric CO_2 may cause only minor changes in soil bacterial community composition and indicate that the soil functional responses to CO_2 enrichment previously reported at Gi-FACE are due to factors other than changes in bacterial community composition. The effects of the moisture gradient revealed new information about the relationships between poorly known Acidobacteria and Verrucomicrobia and soil moisture content. This study contrasts with the relatively small number of other temperate grassland FACE microbiome studies in the use of moderate CO_2 enrichment and the resulting minor changes in the soil microbiome. Thus, it will facilitate the development of further climate change mitigation studies. In addition, the moisture gradient found at Gi-FACE contributes new to knowledge in soil microbial ecology, particularly regarding the abundance and moisture relationships of the soil Verrucomicrobia.

Keywords: soil bacteria, microbiome analysis, soil ecology, CO_2 enrichment, soil moisture, 454 sequencing.

1. Introduction

Understanding how ecosystems respond to changes in environmental conditions, particularly those caused by human activity, is essential for predicting impacts of climate change on ecological services. The role of microorganisms in the mitigation or amplification of the effects of climate change caused by rising greenhouse gas levels is of particular concern (Singh *et al.*, 2010, Docherty & Gutknecht, 2012). The effects of rising atmospheric CO_2 levels on soil ecosystems have been investigated in free-air CO_2 enrichment (FACE) experiments at various locations in the world, and although experimental designs vary widely, FACE studies have revealed significant effects of rising CO_2 on soil organisms (Pritchard, 2011). Increased levels of atmospheric CO_2 often lead to 10-25% increases in plant photosynthetic rates (Lee *et al*., 2011) which can increase carbon inputs to soil through litter deposition (Hoosbeek & Scarascia-Mugnozza, 2009), fine root growth (Norby *et al*., 2004) and root exudation (Phillips *et al*., 2009). Increased CO_2 is also associated with increases in soil water availability due to decreased plant stomatal conductance and therefore reduced plant water loss (Nelson *et al*., 2004).

It is generally thought that elevated CO_2 induced changes to soil carbon and moisture levels affect microbial function in soil through either increased microbial growth rates (Dorodnikov *et al*., 2009, Blagodatskaya *et al*., 2010, Pritchard, 2011), or by alteration of microbial composition, for example changing the relative abundance of specific microbial functional groups such as N_2O producers (Regan *et al*., 2011). However, the effects of climate change on soil heterotrophic microbial community composition and function remain unclear (Singh *et al*., 2010). The literature contains examples describing both significant impacts (Feng *et al*., 2010, Dunbar *et al*., 2012, Hayden *et al*., 2012, He *et al*., 2012), and little or no impact (Austin *et al*., 2009, Ge *et al*., 2010, Hagedorn *et al*., 2013) of elevated CO_2 levels on soil microbial community composition.

The Gi-FACE experiment in Giessen, Germany has been running since 1998. It consists of 3 sets of paired rings, and within each pair 1 ring was randomly assigned a moderate CO_2 treatment (20% increase in aboveground CO_2 levels) (Jäger *et al*., 2003).

In addition to the CO_2 enrichment, the Gi-FACE experimental design also includes a moisture gradient, with each replicate FACE ring pair situated at slightly different heights in the water table, whilst soil type, plant cover, land-use and climatic conditions are constant (Jäger *et al*., 2003, Guenet *et al*., 2012). Furthermore, the CO_2 treatments used at Gi-FACE had no effect on soil moisture content (Kammann *et al*., 2005), so the effects of the two variables can be investigated independently. Soil moisture has a central role in biological activity in soil (Sowerby *et al*., 2005, Lennon *et al*., 2012); microorganisms in soil are affected by soil connectivity and resource availability, which are both influenced by moisture content (Treves *et al*., 2003). Higher moisture content soils will often show higher levels of anoxia, forcing a shift in microbial metabolism and hence changes in community composition (Pett-Ridge & Firestone, 2005). In addition, higher water filled pore space under high moisture conditions is associated with lower bacterial diversity, which is likely due to the greater substrate diffusion rates causing higher levels of competition between bacteria, and higher bacterial mobility (Carson *et al*., 2010). Given the importance of moisture in soil microbial ecology, understanding changes in community composition across moisture gradients has the potential to reveal important information about the ecological roles of specific soil microbial taxa. The experimental design of the Gi-FACE experiment provides an ideal setting to investigate such effects of moisture gradients in soil microbial communities as other soil properties are constant across all treatments.

The aim of this study was to examine the relative influence of long-term moderate CO_2 and soil moisture levels on soil bacterial community composition at Gi-FACE using next generation sequencing approaches. The resulting bacterial 16S rRNA gene diversity data set was analysed using both conventional statistical methods and differential abundance analysis using DESeq2 (Anders & Huber, 2010) and SAMSeq (Li & Tibshirani, 2013) to identify links between bacterial groups, and moderate CO_2 and soil moisture.

2. Materials and Methods

2.1 Experimental site

The free-air experiment in Giessen has been described elsewhere in detail (Jäger *et al*., 2003, Guenet *et al*., 2012), briefly it consists of 3 pairs of rings 8 m in diameter; each pair in turn consists of an ambient (currently approx. 400 ppm CO_2) and an moderate CO_2 (20% above ambient, currently approx. 480 ppm) treatment rings, with a total of 6 rings. Samples were taken from moderate CO_2 rings in high (n=3), medium (n=3), and low (n=3) moisture, and an equal number of samples were taken for each moisture level from ambient CO_2 rings (18 samples in total).

The vegetation at the Gi-FACE site is described as semi-natural grassland; it harbours 60 vascular plant species and is dominated by *Arrhenatheretum elatioris* and *Filipendula ulmaria*. The rings are situated on a slight moisture gradient such that pair 1 has the lowest moisture content (38.8 ± 10.2) and pair 2 the highest (46.1 ± 13.2) whereas pair 3 is intermediate (40.7% ± 11); the soil moisture values shown in brackets represent volumetric water content averages for 1998 - 2008 determined daily by 4 TDR sensors installed permanently in each ring (Imko, type P2G; inserted vertically a depth of 0 - 15 cm). The experimental site has not been ploughed for more than 100 years. It receives N fertilization (40 kg N ha^{-1} yr^{-1}) once a year since 1995 and is mown twice a year since 1993. The soil at the Gi-FACE site is classified as Fluvic Geysol, its texture is of a sandy clay loam over a clay layer, its pH is 6.2 and its average C and N content is 4.5 and 0.45% as measured in 2001 (Guenet *et. al* 2012, Jäger *et al*. 2003). Jäger *et al*. (2003) provide the averages of soil properties of the site (bulk density, pH, organic C and organic N contents, and C/N ratios) for each CO_2 treatment as well as for each ring pair. Guenet *et al*. (2012) also provides carbon, nitrogen, C/N ratio and phosphorus content averages in addition to several soil enzymatic activities in the Gi-FACE rings. Ambient and moderate CO_2 rings are separated by at least 20 m and each pair is placed at the vertices of an equilateral triangle. Moderate CO_2 treatment is applied all year round during the daytime every day since 1998.

2.2 Soil sampling and DNA extractions

PVC cylinders (20x5 cm) were used to collect bulk soil samples at 3 locations in each ring, and these were stored at -20 °C. The soil samples were collected in September 2010 and stored at -20°C for one year. Once thawed, soils were homogenised and sieved to 2 mm and DNA was extracted using the phenol chloroform method of Griffiths *et al*. (2000). DNA quantity and quality was determined by Nanodrop™ spectrometer (Thermo scientific).

2.3 Tag-sequencing of 16s rRNA genes

Pyrosequencing, including 16S rRNA gene amplification and library preparation was performed at the University of Nebraska-Lincoln Core for Applied Genomics and Ecology using the Roche-454 Titanium platform and following the procedure detailed by Martínez *et al*. (2009). Briefly the V1-V3 regions of the 16S rRNA gene were amplified using the 8F-518R (Lane *et al*., 1985, Muyzer *et al*., 1993) primers containing the Roche-454 Titanium adapter sequences and unique barcodes for each sample. Primer sequences and 454 adaptor details are described in de Menezes *et al*. (2011), whilst PCR reactions contained 2 µM of each primer, 200 µM of each nucleotide, 2 units of Taq polymerase, 2.5 mM of $MgCl_2$, 50 ng of DNA template, buffer and water to 50 µL. The PCR cycling conditions were 1 cycle at 95ºC for 3 min, 25 amplification cycles (95ºC for 1 min, 56ºC for 30 s, 72ºC for 45 s), and a final elongation 72ºC for 7 mins. PCR reactions were quality controlled for saturation by gel electrophoresis and quantified using GENETOOLS software (Syngene, Cambridge, UK); equal quantities of amplicons from each PCR reactions were pooled, gel purified and quantified using picogreen (Invitrogen, Carlsbad) and Qubit fluorimeter (Invitrogen). Sequence files associated with each sample have been submitted to the NCBI Sequence Read Archive (SUB1126458).

2.4 Sequence processing

Sequences were processed using mothur v.1.31.0. with default parameters for 454-Titanium sequence processing (Schloss *et al*., 2009a). Sequence noise was reduced using shhh.flows, chimeric sequences were detected using the Uchime tool built within mothur (Edgar *et al*., 2011) and removed from the dataset. Sequences classified as plastid, mitochondrial, archaeal, eukaryotic or unknown at the kingdom level were also removed from the dataset using the remove.lineage command in mothur. The number of sequences per sample ranged from 4487 to 9892 sequences. Operational taxonomic units (OTUs) were generated by calculating pairwise distances using the dist.seqs and cluster commands in mothur, and sequences were clustered with a distance cutoff of 0.03. An OTU table was generated using the make.shared command in mothur, and finally the sequences were classified in mothur using the SILVA reference files (Schloss, 2009b). The sequence composition of two samples from different rings was found to be substantially different from all samples in the dataset and from their replicates, whilst being similar to each other. These samples (E11, moderate CO_2, low moisture and E31, moderate CO_2, medium moisture) were therefore removed from the dataset as a precaution against the possibility that they may have been affected by technical problems during sequencing.

2.5 Statistical analysis

For alpha-diversity analyses the number of sequences per sample was normalised to 4487 using the subsample command in mothur and the inverse simpson diversity index, as well as the Good's coverage estimator and number of OTUs, was determined. For PERMANOVA and nMDS analysis, from the original OTU table the relative abundance data of OTUs contributing > 0.05% of the sequences in at least one sample was imported into PRIMER-E package for statistical analyis (Clarke & Gorley, 2006), square-root transformed and the Bray-Curtis coeffient of similarity calculated (Kuczynski *et al*., 2010). PERMANOVA was conducted using a fixed-factor design with type II conditional sums of squares and 9999 and unrestricted permutations. Non-metric multidimensional scaling (nMDS) was performed in order to visualise the differences in community composition between treatments.

2.6 Differential abundance analysis

In order to determine which bacterial groups were more abundant at each treatment, we chose the approach outlined in McMurdie and Holmes (2014), which used RNA-Seq statistical methods and raw sequence counts to determine differential abundances in microbiome studies. In particular we chose the microbiome-specific DESeq2 extension available in the phyloseq R package for microbiome analysis (Love *et al*., 2014)**.** DESeq2 was run using the Wald test, with automatic filtering of low abundance OTUs, and an alpha of 0.01. Adjusted p-values were calculated automatically by DESeq2. Differential abundance analysis was carried out at the OTU level as many sequences belonging to abundant taxa at Gi-FACE (such as the Acidobacteria and Spartobacteria) remained poorly classified below the level of phylum and class. In addition we also evaluated differential abundances at family level using SAMseq method of the samr package (Li & Tibshirani, 2013), which is a non-parametric method that uses permutations to assess the false discovery rate (FDR). For SAMseq there were 100 permutations and a q value cut off of 1%.

For comparison, we repeated DESeq2 analysis comparing OTU abundances between ambient and moderate CO_2 without adjusting p-values for multiple comparisons (alpha = 0.05), as the p-value correction may have led to a lack of detection of real changes in OTU abundance across treatments. A Welch two-sample t-test was then conducted in R to determine the statistical significant of any OTUs that were found to be enriched at moderate or ambient CO_2 when not using adjusted p-values (alpha = 0.05) (supplementary Table S1). We also repeated DESeq2 analysis without adjusting p-values for data aggregated at genus, family, order and class levels, however only the genus and class level results are shown (Table S1).

3. Results:

3.1 Pyrosequencing

A total of 144,767 sequences were obtained; after quality screening, noise reduction, removal of chimeric and plastid sequences and singletons there was a total of 98,559 sequences. Table 1 shows the number of OTUs clustered at 0.03 similarity level obtained in each sample after normalisation using the subsample procedure in mothur (Schloss *et al*., 2009a), as well as sample coverage and the inverse-Simpson diversity index. There were no significant differences in the number of OTUs or richness between ambient or moderate CO_2 samples, or between the different levels of moisture.

3.2 Bacterial community composition

Fig. 1 shows the overall phylum-level bacterial community composition at each moisture and CO_2 level. Bacterial assemblages were typically dominated by the Verrucomicrobia (ca. 35-56% of the community 16S rRNA genes), Proteobacteria (18-24%), Acidobacteria (7-10%) and Actinobacteria (5-12%). No clear trend could be discerned between ambient and moderate CO_2 levels. When bacterial assemblages were compared at different moisture levels, the Verrucomicrobia were relatively less abundant in high moisture rings whereas the relative abundance of the Actinobacteria was higher in medium moisture particularly in the ambient rings. The relative abundance of the Planctomycetes increased from low to high moisture especially in the ambient CO_2 rings (Fig. 1).

3.3 Effect of CO_2 enrichment on soil bacterial communities

Non-metric multidimensional scaling (nMDS) was used to visualise the effects of moderate CO_2 and moisture on the bacterial community composition (Fig. 2). Although no separation of samples according to CO_2 treatment was observed, the samples clearly clustered based on moisture content (Fig. 2). PERMANOVA failed to show any statistically significant effect of the CO_2 treatment, and there was no interaction between the effects of CO_2 and moisture (Table 2). The lack of statistical significance in PERMANOVA test for differences in community composition between ambient and moderate CO_2 treatments was observed at all taxa levels analysed (OTU, genus, family, class and phylum levels) (Table 2). Differential abundance analysis of OTUs using the DESeq2 extension in phyloseq showed that there were no differentially abundant OTUs between ambient and moderate CO_2 treatments when analysing each moisture level separately, or in combination (using an alpha = 0.01 and adjusted p-values). Likewise,

differential abundance analysis using SAMSeq failed to reveal any enriched bacterial family at either ambient or moderate CO_2 levels.

Determining the presence of differentially abundant OTUs with DESeq2 and non-adjusted p-values followed by t-test showed the presence of two OTUs that were more abundant in moderate CO_2 (from the classes Spartobacteria and Deltaproteobacteria), and five that were more abundant in ambient rings (one unclassified bacteria and three Planctomycetacia OTUs) (Table S1). When testing for differentially abundant groups with sequences aggregated at each taxa level, the *Gemmatimonas* and *Hyphomicrobium* genera were found to be significantly more abundant in ambient rings (Table S1).

3.4 Effect of soil moisture on soil bacterial communities

Soil moisture content had a much greater effect on bacterial community composition, and PERMANOVA showed significant differences between moisture levels (Table 2). Pairwise PERMANOVA also indicated that moisture had a significant effect on the microbial community composition when comparing all moisture levels to each other (P-value < 0.01). The PERMANOVA results were consistent across all taxa levels analysed except that at phylum level the differences in community composition between low and high and low and medium moisture rings were not significant.

The abundance of the 30 most abundant bacterial classes at each moisture level is shown in Fig. 3 and supplementary Fig. S1. Overall, the Deltaproteobacteria, Acidobacteria group 5, 11, 17, the Anaerolineae (phylum Chloroflexi), Betaproteobacteria, Gammaproteobacteria and Nitrospira were relatively more abundant in soils with high moisture content. The Spartobacteria (Phylum Verrucomicrobia) were relatively more abundant in soil from the low and medium moisture rings. The Acidobacteria group 1, 2 and 3 as well as the Gemmatimonadetes and the Sphingobacteria were more abundant in low moisture. The Actinobacteria was the only major group that was distinctly more abundant in medium moisture rings.

Analysis of differential abundance with DESeq2 revealed that only one OTU was significantly enriched in high and medium moisture content soil, whereas 14 OTUs were significantly enriched at low moisture and 7 at medium moisture (Table 3). Of the Acidobacteria, groups 1, 2 and 3 tended to favour

low moisture, group 6 favoured either medium or high moisture whereas different OTUs of group 5 favoured low or high moisture. Of the Proteobacteria, most OTUs affected by moisture were from the Alphaproteobacteria, including members of the Rhizobiales and Rhodospirillales. OTUs from Deltaproteobacteria (Desulfuromonadales and Myxococcales), Betaprotebacteria (Burkholderiales) and Gammaproteobacteria favoured high moisture, as was the case with one Nitrospira and two Chloroflexi (Anaerollinae) OTUs. Three actinobacterial OTUs (Kribella, Leifsonia and unclassified OTUs) were enriched at medium moisture levels and one firmicute OTU (Bacillales) favoured low moisture.

Differential abundance analysis of individual families using the SAMseq method generally supported the results obtained with DESeq2 (Table 4). In low moisture content soil, SAMseq showed enrichment of Acidobacteria groups 1, 2 and 3, Bradyrhizobiaceae, Acetobacteraceae, and unclassified Bacillales. At medium moisture, several actinobacterial families, Acidobacteria group 6 and Rhodospirillales were enriched, whereas in high moisture the Nitrospiraceae, the Anaerolineaceae and Caldilineaceae from the phylum Chloroflexi and the Geobacteraceae (order Desulfuromonadales, Deltaproteobacteria) were more abundant.

4. Discussion

4.1 Effects of CO_2 enrichment on the Gi-FACE soil bacterial community

Moderate CO_2 had only subtle effects on the bacterial community structure at Gi-FACE, which was only evident when performing differential abundance analysis without adjusted p-values, followed by t-test. The potential enrichment of one Spartobacteria OTU in moderate CO_2 rings is in agreement with the study of Lipson *et al*. (2005) and Austin *et al*. (2009). The limited data available regarding Verrucomicrobia ecosystem function suggests that these bacteria may play a role in organic matter metabolism (Ranjan *et al*., 2015, Janssen *et al*., 2002). It is possible therefore that their greater abundance in moderate CO_2 rings is connected with increased plant biomass yield in these rings (Kammann *et al*., 2008), which may have led to greater plant matter inputs in these soils. However, no changes soil carbon

was detected in the moderate CO_2 rings in previous studies at Gi-FACE (Angel *et al*., 2012). In the ambient rings, the enrichment of one OTU classified to the genus *Gemmata* (Planctomycetes) is in agreement with the study of Lesaulnier *et al*. (2008), and He *et al.* (2012) also found a general decrease of Planctomycetes OTUs in elevated CO_2, as observed here. Planctomycete diversity is affected by soil nitrogen (Buckley *et al*. 2006), and the changes in soil nitrogen dynamics observed at Gi-FACE (Müller *et al*., 2009) may be connected to their lower abundance in moderate CO_2 rings. The decrease in abundance of *Hyphomicrobium* in the moderate CO_2 rings is surprising, as these bacteria are methylotrophs, which are known to colonise the plant rhizosphere (Turner *et al*. 2013) in order to utilise C1 compounds such as methanol which are produced by plants (Galbally & Kristine, 2002).

The overall lack of substantial changes in the soil bacterial community is somewhat surprising as moderate CO_2 induced changes in plant biomass yields (Kammann *et al*., 2008) and increased abundance of grasses compared to forbs (Grüters *et al*., 2006). In addition, moderate CO_2 led to two-fold increase in N_2O emissions (Kammann, *et al*., 2015), changed soil nitrogen dynamics (Müller *et al*., 2009), decreased methane uptake (Kolb *et al*,., 2005), and increased soil acid phosphatase activity (Guenet *et al*., 2012). However, the lack of changes in total soil carbon and nitrogen contents in the moderate CO_2 rings at Gi-FACE (Angel *et al*., 2012) likely contributed to the lack of a more substantial change in soil bacterial communities as observed here. In addition, the effects of moderate CO_2 on soil function are more likely to be related to factors other than a change in the bacterial community composition. For example, it is possible that changes in bacterial metabolism took place with no significant change in the bacterial community composition itself, or that the observed changes in soil function were due to the effect of moderate CO_2 on soil fungi and archaea, which were not the target of this study. Indeed, soil fungi and archaea diversity are known to respond to elevated CO_2 (Hayden *et al*. 2012, Weber *et al*. 2013, Lesaulnier 2008, Drigo *et al*. 2009), and soil fungi in particular have been linked to changes in soil enzymatic activity (Lipson *et al*., 2005) and the incorporation of increased plant-derived carbon linked to elevated CO_2 (Hagedorn *et. al*., 2013).

296 The responses of soil microbial communities to elevated CO_2 can vary significantly depending on
297 experimental conditions, analysis techniques used, which component of the community is analysed, or
298 whether bulk soil or rhizosphere are investigated (Weber *et al*., 2013). Studies using next-generation
299 sequencing, PhyloChip or GeoChip have gathered more robust evidence of changes in below-ground
300 microbial communities with elevated CO_2 (He *et al*., 2010, Deng *et al*., 2012, Dunbar *et al*., 2012,
301 Hayden *et al*., 2012, He *et al*., 2012). Importantly, the studies above used higher CO_2 enrichment levels
302 than used in this study (>534 ppm compared to the 480 ppm at Gi-FACE). The CO_2 enrichment values
303 used at Gi-FACE represent a similar level of atmospheric CO_2 projected for the year 2050 under different
304 scenarios (Meinshausen *et al.*, 2011), and therefore tests a more immediate impact of CO_2 increase on the
305 soil microbiome compared to most other FACE studies.

306 Alternatively, the increased heterogeneity of the microbial community caused by the moisture
307 gradient at Gi-FACE may have obscured effects of CO_2 enrichment in the overall experiment. Likewise,
308 the effect of moderate CO_2 may vary seasonally, as warming and elevated CO_2 are known to have
309 synergistic effects on soil microbial communities (Hayden *et al*., 2012). Future increased sampling effort
310 within each moisture level and across a seasonal cycle may allow the detection of CO_2 induced changes in
311 soil microbial community composition currently undetected.
312

313 **4.2 Moisture effects on the microbial community**

314 Multivariate statistical analysis showed a stronger effect of moisture compared to CO_2 level on the soil
315 bacterial community. Analysis of the overall bacterial relative abundances at class level suggests that at
316 high moisture there was a decrease in oxygen levels, as several groups enriched at high moisture belonged
317 to groups often associated with wastewater sediments, such as Nitrospira, the Desulfuromonadales
318 (Deltaproteobacteria), and the Anaerolineae and Caldilineae (Phylum Chloroflexi) (Kuever *et al*., 2005,
319 Yamada & Sekiguchi, 2009, Luecker *et al*., 2010).

The Acidobacteria at Gi-FACE appears to be more sensitive to moisture than other bacterial groups, as this phylum had the highest number of OTUs enriched at any moisture level despite having a considerably lower total number of OTUs than the Proteobacteria and the Verrucomicrobia. Furthermore, differential abundance analysis revealed that Acidobacteria groups 1, 2 and 3 favoured low moisture levels, whereas the other Acidobacteria classes present at Gi-FACE favoured either medium or high moisture levels. The data from Gi-FACE therefore provides evidence of ecological distinctness for individual acidobacterial classes, which contributes to our knowledge of these poorly known, albeit abundant soil bacteria (Quaiser *et al.*, 2003, Janssen, 2006, Jones *et al.*, 2009, Castro *et al.*, 2010, George *et al.*, 2011, Griffiths *et al.*, 2011).

The class Spartobacteria (phylum Verrucomicrobia) included some of the most abundant bacteria in these soils, however no consistent relationship was observed between Spartobacteria OTUs with moisture, with different OTUs belonging to this class favouring high, medium and low moisture levels. The lack of a consistent pattern of enrichment for the Spartobacteria OTUs at specific moisture levels suggests a wide moisture niche-space for these organisms. Indeed, whilst a recent study suggested that members of this phylum favour higher moisture soils (Maestre *et al.*, 2015), another study noted that their abundance increased following droughts and heat-waves (Acosta-Martinez *et al.*, 2014), and Buckley *et al*. (2001) suggest that they are negatively associated with moisture. As with the Acidobacteria, the ecology of soil Verrucomicrobia is poorly understood, however evidence for their abundance in soil is increasing, in particular in grassland biomes (Buckley & Schmidt, 2001, Bergmann *et al.*, 2011, Fierer *et al.*, 2013, Carbonetto *et al*., 2014, Navarrete *et al*., 2015). This study provides further support for the Verrucomicrobia dominance of soil microbial communities, and suggests that members of this phylum, and more specifically the class Spartobacteria, show variability in their response to moisture, even within a single habitat, suggesting that they may have a diverse functional role in soil.

It is possible that the effects of moisture on the microbial communities described in this study were due to other soil variables that co-correlated with moisture, or to the location of the three rings within the experimental site. Soil pH and organic carbon and nitrogen content also varied somewhat

between the different moisture levels, and pH in particular may have contributed to the differences observed between ring pairs, as the low moisture rings had a relatively lower pH compared to high and medium moisture rings (approximately 5.4-6.0 in low moisture vs. 5.8-6.2 in medium and high moisture rings) (Jäger *et al*., 2003). However, changes in microbial community structure seen here, such as increases sediment-associated bacteria in high moisture rings, are consistent with moisture effects, and previous studies have demonstrated the effect of moisture on soil PLFA profiles as well as soil enzyme activities at Gi-FACE (Guenet *et al*., 2012). Given the importance of moisture to soil microbes (Pett-Ridge & Firestone, 2005, Carson, *et al*., 2010, de Menezes 2015), it would be unexpected if the changes shown here were not at least partly related to the well-established differences in soil moisture between the three ring pairs at Gi-FACE.

5. Conclusions

In conclusion, this study has shown that the effects of moderate CO_2 on soil bacterial community composition can be subtle, however we have gathered evidence that shows that soil bacterial community composition is relatively resilient to moderate increases in atmospheric CO_2 levels similar to those predicted to occur by 2050. Clearer evidence for the effect of moderate CO_2 on soil bacterial communities may be obtained by increased sampling effort at each moisture level and across seasons in Gi-FACE. Furthermore this study provides new insight into the relationships of poorly known but abundant and globally important soil bacteria, the Acidobacteria and the Verrucomicrobia with soil moisture content. The latter phylum in particular was found to be very abundant in the soils at Gi-FACE. Although we only have information about their responses to moisture and CO_2 levels, these results are in broad agreement with other recent studies on the ecology of the Verrucomicrobia and highlight the global importance of this phylum in temperate grassland ecosystems, as well as the need for targeted studies designed to elucidate their role in soil ecosystem function.

6. Acknowledgements

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7. Conflicts of interest

The authors of this manuscript would like to declare that there are no conflicts of interest in the production and submission of this work.

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588 **Tables**

589 Table 1. Number of sequences, Good's coverage, number of OTUs and inverse Simpson diversity index
590 for all samples. OTUs were clustered at 0.03 dissimilarity. For each sample, label letters A or E represent
591 ambient or moderate samples, followed by the ring pair number (1-3) and the replicate number (1-3).
592 Prior to the calculation of alpha diversity the dataset was subsampled to the lowest number of sequences
593 (4487).

Ring	CO_2	Moisture	No. of sequences	Coverage	No. of OTUs	Inv. Simpson
A11	Ambient	Low	6194	0.92	785	6.62
A12	Ambient	Low	5593	0.85	1263	27.05
A13	Ambient	Low	7359	0.89	1197	10.22
A31	Ambient	Medium	7878	0.89	1342	12.08
A32	Ambient	Medium	5801	0.87	1206	18.19
A33	Ambient	Medium	4925	0.92	743	10.97
A21	Ambient	High	8773	0.93	1009	8.71
A22	Ambient	High	7887	0.92	1030	7.96
A23	Ambient	High	7036	0.86	1449	33.81
E12	Moderate	Low	9892	0.93	1221	10.93
E13	Moderate	Low	4487	0.90	756	6.59
E32	Moderate	Medium	4853	0.85	1155	34.20
E33	Moderate	Medium	6639	0.90	1021	8.35
E21	Moderate	High	5774	0.87	1134	16.26
E22	Moderate	High	8132	0.90	1428	20.04
E23	Moderate	High	5326	0.89	936	11.03

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Table 2. PERMANOVA analysis of the effects of CO_2 and moisture levels on soil bacterial community composition. Values shown are P values.

Treatment		Taxa Level				
		OTU	Genus	Family	Class	Phylum
CO_2	Ambient vs. Moderate	0.384	0.166	0.156	0.105	0.132
Moisture	All groups	< 0.001	< 0.001	< 0.001	< 0.001	0.012
	Low-High	0.001	0.001	0.002	0.002	0.095
	Low-Medium	0.004	0.005	0.004	0.004	0.266
	High-Medium	0.002	< 0.001	< 0.001	< 0.001	0.004
CO_2 vs. moisture interaction		0.346	0.770	0.752	0.790	0.689

Table 3. Number of differentially abundant OTUs at each moisture level. Differential abundance was analysed by comparing every moisture level with each other using DESeq2 (alpha = 0.01). In brackets are the combined abundances (%) of the OTUs that were significant for a particular genus/treatment.

Moisture level comparison	Class	Genus	No. of differentially abundant OTUs	
			High moisture	Medium moisture
High vs. Medium	Actinobacteria	*Kribbella*	0	1 (1.19)
	Anaerolineae	Anaerolineaceae unclassified	1 (0.39)	0
			Low moisture	High moisture
High vs. Low	Acidobacteria Gp1	Unclassified	4 (0.74)	0
	Acidobacteria Gp2	Unclassified	3 (0.32)	0
	Acidobacteria Gp3	Unclassified	1 (0.19)	0
	Acidobacteria Gp5	Unclassified	1 (0.27)	1 (0.43)
	Acidobacteria Gp6	Unclassified	0	4 (0.67)
	Bacteria unclassified	Bacteria unclassified	0	1 (0.35)
	Anaerolineae	Unclassified	0	1 (0.26)
	Nitrospira	*Nitrospira*	0	1 (0.69)
	Alphaproteobacteria	Rhodospirillales unclassified	1 (0.14)	0
	Alphaproteobacteria	Rhizobiales unclassified	1 (0.13)	0
	Alphaproteobacteria	Bradyrhizobiaceae unclassified	1 (1.89)	0
	Alphaproteobacteria	*Dongia*	0	1 (1.10)
	Alphaproteobacteria	Hyphomicrobiaceae unclassified	0	1 (0.10)
	Betaproteobacteria	Burkholderiales unclassified	0	1 (0.09)
	Deltaproteobacteria	Myxococcales unclassified	0	1 (0.16)
	Deltaproteobacteria	Desulfuromonadales unclassified	0	2 (0.18)
	Gammaproteobacteria	Gammaproteobacteria unclassified	0	1 (0.20)
	Spartobacteria	Unclassified	5 (7.82)	2 (4.39)
	Verrucomicrobia subdivision 3	Unclassified	0	1 (0.13)
			Low moisture	Medium moisture
Low vs. Medium	Acidobacteria Gp1	Unclassified	5 (1.06)	0
	Acidobacteria Gp2	Unclassified	1 (0.12)	0
	Acidobacteria Gp5	Unclassified	1 (0.27)	0
	Acidobacteria Gp6	Unclassified	0	1 (0.14)
	Actinobacteria	Unclassified	0	1 (2.28)
	Actinobacteria	*Kribbella*	0	1 (1.19)
	Actinobacteria	*Leifsonia*	0	1 (0.17)
	Bacteria unclassified	Bacteria unclassified	2 (0.27)	1 (0.35)
	Bacilli	Bacillales unclassified	1 (0.49)	0
	Alphaproteobacteria	*Dongia*	0	1 (1.14)
	Spartobacteria	Unclassified	4 (5.06)	1 (0.54)

Table 4. Differential abundance of bacterial families determined using the SAMseq method of the samr package. q value < 1%. Only families containing at least 50 sequences across all samples were considered.

	Class	Order	Family
Low moisture	Acidobacteria Gp1	Acid. Gp1 incertae sedis	Acid. Gp1 incertae sedis
	Acidobacteria Gp2	Acid. Gp2 incertae sedis	Acid. Gp2 incertae sedis
	Acidobacteria Gp3	Acid. Gp3 incertae sedis	Acid. Gp3 incertae sedis
	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae
	Sphingobacteria	Sphingobacteriales	Chitinophagaceae
	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae
	Bacilli	Bacillales	unclassified Bacillales
Medium moisture	Actinobacteria	Propionibacteriales	Nocardioidaceae
	Actinobacteria	Actinomycetales	unclassified Actinomycetales
	Actinobacteria	Actinomycetales	Microbacteriaceae
	Actinobacteria	unclassified Actinobacteria	unclassified Actinobacteria
	Actinobacteria	Actinomycetales	Micromonosporaceae
	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae
	Acidobacteria Gp16	Acidobacteria Gp16	Rhodospirillaceae
	Acidobacteria Gp16	Acidobacteria Gp16	Acid. Gp16 incertae sedis
	Acidobacteria Gp6	Acidobacteria Gp6	Acid. Gp6 incertae sedis
High moisture	Acidobacteria Gp17	Acidobacteria Gp17	Acid.Gp17 incertae sedis
	Nitrospira	Nitrospirales	Nitrospiraceae
	Anaerolineae	Anaerolineales	Anaerolineaceae
	Deltaproteobacteria	Desulforomonadales	Geobacteraceae
	Caldilineae	Caldilineales	Caldilineaceae

Figure Legends

Fig. 1. Relative abundance of bacteria phyla. The 5000 most abundant OTUs, which corresponded to 97% of the total sequences in the dataset, were used. Bad quality, plastid and chimeric sequences were removed.

Fig. 2. Non-metric multidimensional scale plots showing the similarity between soil samples. The A and E above each sample represent ambient and moderate CO_2 levels respectively; numbers identify ring pairs, whereas symbols represent moisture level. Only OTUs contributing > 0.05% of the community were included.

Fig. 3. Box plot showing the relative abundance of the 10 most abundant classes in the overall dataset at low, medium and high moisture rings. Upper and lower box limits represent the first and third quartiles, whilst the upper and lower lines represent the maximum and minimum abundances, and filled circles represent outliers.

Figure 1

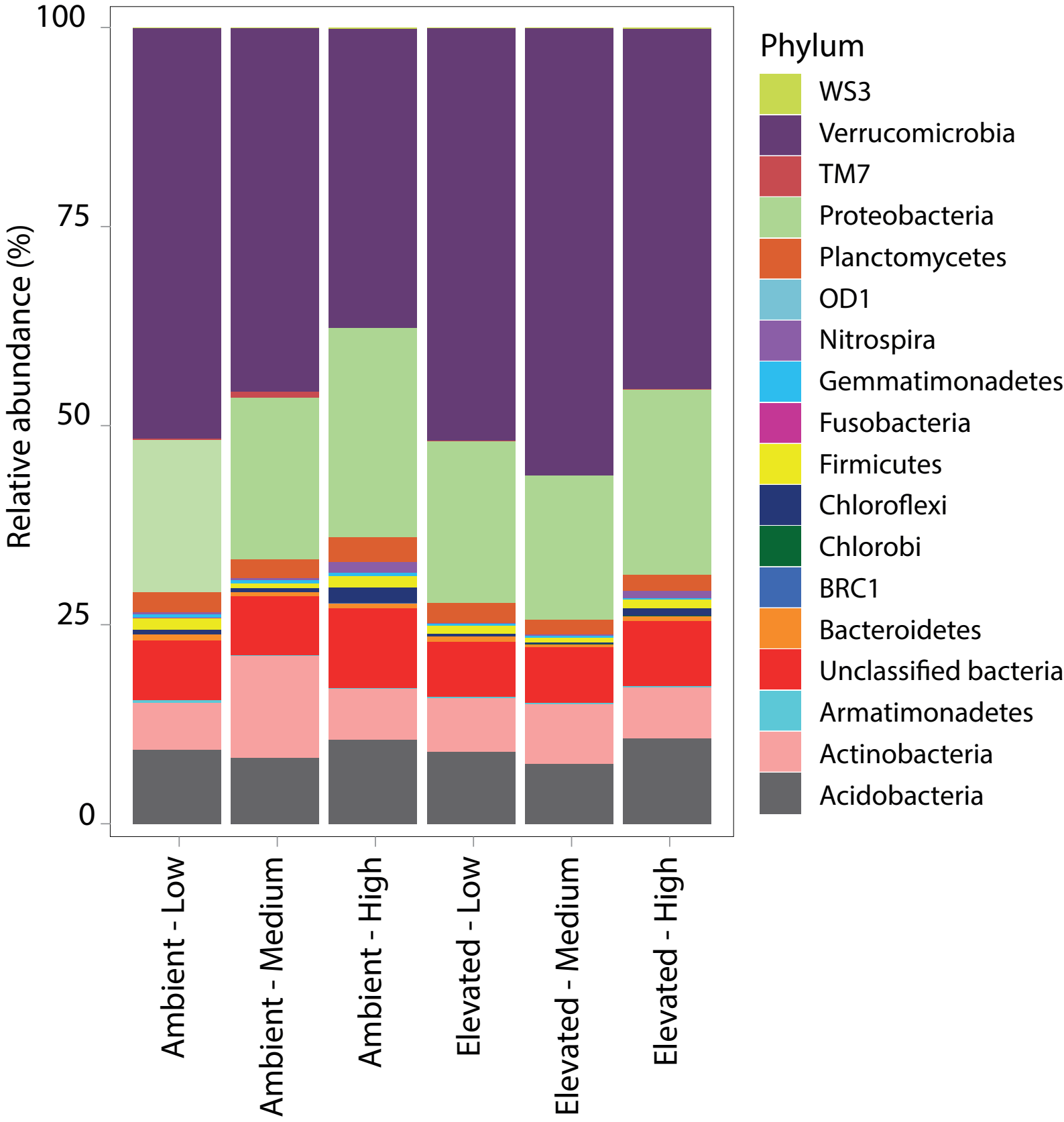


Figure 2

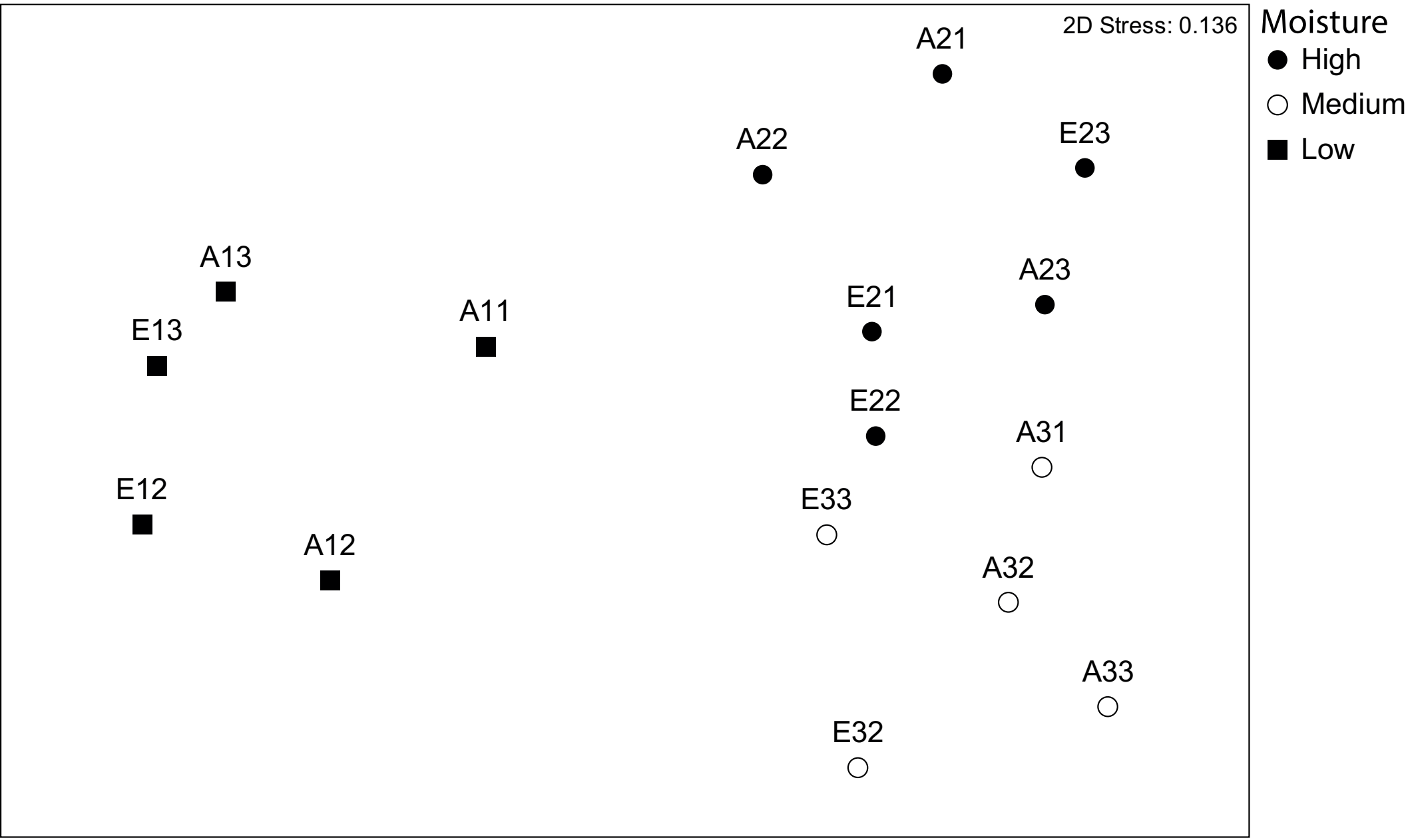


Figure 3

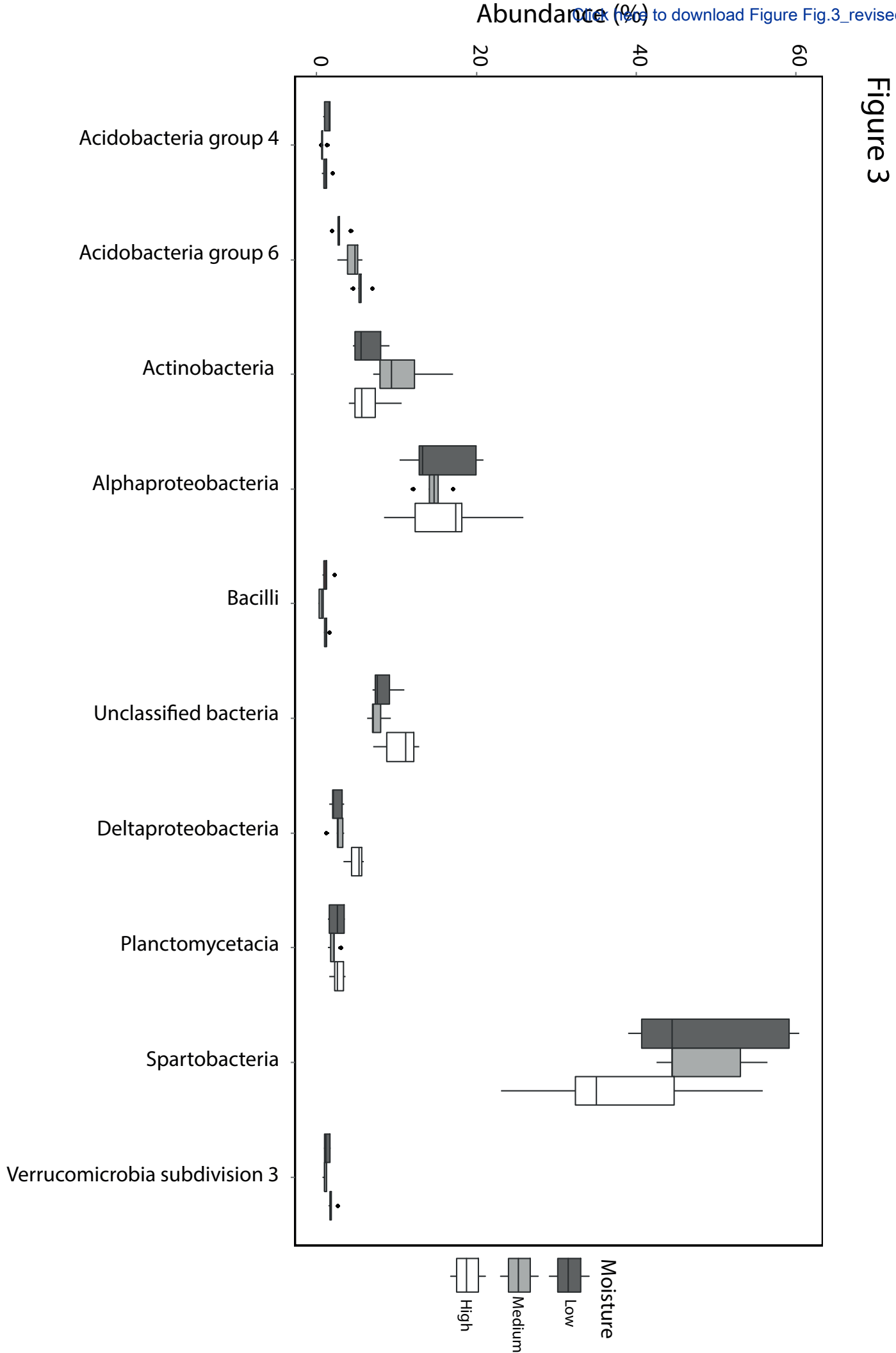
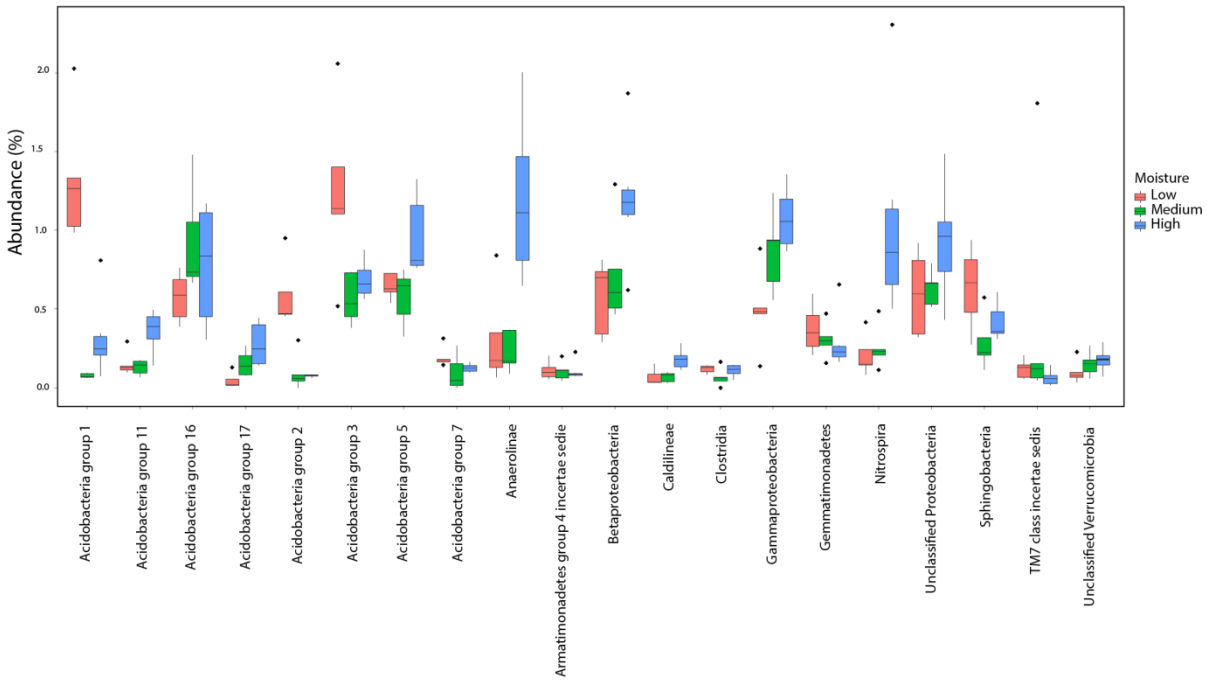


Figure 3

Supplementary Material

Supplementary Table S1. Number of differentially abundant OTUs or bacterial genera in ambient and moderate CO_2. Differential abundance was analysed using DESeq2 without correction for multiple testing (alpha = 0.05), followed by a Welch's t-test (alpha = 0.05). The combined relative abundances (%) of the OTUs or genera that were significant for a particular treatment are shown in brackets.

OTU	Class	Genus	OTU/genus relative abundance (%)	
			Ambient CO_2	Moderate CO_2
OTU0034	Unclassified	Unclassified	0.19	0.06
OTU0076	Spartobacteria	Spartobacteria_genera_incertae_sedis	0.07	0.24
OTU0090	Planctomycetacia	Planctomycetaceae_unclassified	0.17	0.07
OTU0423	Planctomycetacia	Planctomycetaceae_unclassified	0.03	0.00
OTU0723	Planctomycetacia	Gemmata	0.02	0.00
OTU0869	Deltaproteobacteria	Myxococcales_unclassified	0.00	0.01
Genus	Class			
Gemmatimonas	Gemmatimonadetes	-	0.40	0.22
Hyphomicrobium	Alphaproteobacteria	-	0.06	0.02



Supplementary Figure S1: box plot showing the abundances of the 10-30 most abundant classes in the overall dataset at low, medium and high moisture rings. The abundance data was scaled to the lowest number of sequences in an individual sample (4198) prior to the calculation of the relative abundances. Boxplots were generated using the ggplot2 (Wickham, 2009) package in R. Upper and lower box limits represent the first and third quartiles, the upper and lower lines represent the maximum and minimum abundances, and filled circles represent outliers.