The pH optimum of soil exoenzymes adapt to long term changes in soil pH

Soil extracellular enzymes are locally adapted toward soil pH

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Abstract

Soil extracellular enzymes released by microorganisms break down organic matter and are crucial in regulating C, N and P cycling. Soil pH is known to influence enzyme activity, and is also a strong driver of microbial community composition; but little is known about how alterations in soil pH affect enzymatic activity and how this is mediated by microbial communities. To assess long term enzymatic adaptation to soil pH, we conducted enzyme assays at buffered pH levels (2.5 to 10, 0.5 interval) on two historically managed soils maintained at either pH 5 or 7 from the Rothamsted's Park Grass Long-term experiment). The pH optima for a range of enzymes was found to differ between the two soils, the direction of the shift being toward the source soil pH, indicating the production of pH adapted isoenzymes by the soil microbial community. Soil bacterial and fungal communities determined by amplicon sequencing were found to be clearly distinct between pH 5 and soil pH 7 soils, possibly explaining differences in enzymatic responses. Furthermore, β-glucosidase sequences extracted from metagenomes revealed an increased abundance of Acidobacteria in the pH 5 soils, and increased abundance of Actinobacteria in pH 7 soils; these taxonomic shifts were more pronounced for enzymatic sequences when compared with a number of housekeeping gene sequences. Particularly for the Acidobacteria, this indicates that broad taxonomic groups at phylum level may possess enzymatic adaptations which underpin competitiveness in different pH soils. More generally our findings have implications for modelling the efficiency of different microbial enzymatic processes under changing environmental conditions; and future work is required to identify trade-offs with pH adaptations, which could result in different activity responses to other environmental perturbations.

Keywords: Extracellular enzyme, soil pH, liming, adaptation, Park Grass

1. Introduction

Soil microbes produce exoenzymes to degrade complex plant and soil organic matter (OM) into smaller compounds, which are then assimilated for growth and metabolism (Allison, 2005). These proteins break down large OM compounds through hydrolytic and oxidative processes (Burns et al., 2013; German et al., 2011; Sinsabaugh, 2010) and their activity rates have been hypothesized to be a rate-limiting step in OM decomposition (Bengtson and Bengtsson, 2007). Enzyme activity is predominantly controlled by temperature and pH which affect enzyme kinetics through change in substrate binding and stability. In contrast to intracellular enzymes, the physico-chemical conditions in which exoenzymes operate are poorly controlled by microorganisms and activity rates are thus influenced by local conditions (e.g pH). Thus, to cope with their local environment, microorganisms evolve to produce different types of enzyme (isoenzyme), resulting in equivalent functionality but with altered thermodynamic and kinetic properties. For example, cold adapted enzymes, are believed to exhibit higher conformational flexibility within their active site or protein surface to become more efficient at lower temperatures due to a decrease in the enzyme activation energy (Ea) (Åqvist et al., 2017). However exoenzymes adaptation results in various trade-offs between efficiency and enzyme stability (Åqvist et al., 2017; Zanphorlin et al., 2016); meaning both specific exoenzymes catalyzed processes as well as other non-specific microbial processes may be affected by a changing environment. Though it is known that microbes can tune the properties of EE they produce to adapt to new conditions, little is known about the drivers, mechanisms and timescale of such adaptations in natural habitats such as soil.

To date, in soil systems much research has focused on enzyme adaptation to cold temperatures and extreme environmental conditions (Åqvist et al., 2017) with little reporting of adaption to other edaphic properties. Soil pH is one of the main variables affected by global change through agricultural intensification, climate change and other polluting events such acid rain. In addition, pH is known to be one of the main factors affecting soil microbial diversity and function (Fierer et al, 2017; Griffiths et al., 2011, Malik et al, 2018). How changes in soil pH affect microbial life constraints is poorly understood, but should be addressed to better understand microbial ecophysiology, competition and efficiency in degrading substrates across different soil systems. This is especially true when considering pH constraints on enzyme catalytic efficiency in cycling essential nutrients (C, N, and P) from organic matter compounds, and determining how that may impact soil microbial function and decomposition rates. Moreover, recent C decomposition models now explicitly integrate enzyme kinetics (Allison, 2012; Davidson et al., 2012; Wang et al., 2013) but little empirical data on enzyme kinetic parameters under changing environmental conditions are available. Currently, there is little understanding in the degree to which microbial extracellular enzymes can be or are adapted to their local soil pH, a factor which could help explain different functional responses across different soil systems.

In order to evaluate potential exoenzymatic adaptation to local soil pH, we conducted enzymatic assays at a range of buffered pH levels (from 2.5 to 10, 0.5 interval) on soil of the Park Grass long-term experiment (Rothamsted) which had been maintained at either pH 5 or 7 for over 100 years. Hydrolytic exoenzymes studied were selected to correspond to enzymes involved in organic carbon, nitrogen and phosphorus cycling. We hypothesise that enzyme pH optimum will be affected by ancestral soil pH treatment, with soil exoenzymes from soil pH 5 being more adapted

91 towards acidic conditions and exoenzymes from soil pH 7 more adapted towards more neutral or
92 alkaline conditions. To better understand the microbial community relationships underpinning EE
93 pH adaptation, we investigated the change in microbial community composition (bacteria and
94 fungi) with amplicon sequencing, and functional genes using a metagenomics sequencing
95 approach. Specifically, we wished to determine whether change in enzyme activity is associated
96 with change in specific microbial enzyme producers or adaptation of exoenzymes to environmental
97 conditions.
98
99 **2. Method**
100 **2.1 Soil sampling**
101 The Park Grass Long-term experiment (Rothamsted, UK, McDonald 2018), originally started in
102 1856 on permanent pasture to investigate ways of improving hay yields, is managed with a range
103 of fertilisers and pHs with the hay cut twice a year. Soils cores (0-15cm, 4 cm Ø) were sampled
104 on the 27th of November 2015 in subplots 'a' (pH ~ 7) and 'c' (pH ~ 5) of the Nil plot 12, which
105 has never received any fertilisers (Storkey et al., 2016). The soil pH is regularly monitored and
106 controlled by liming, in subplot 'a' to reach pH~7 since 1903 (every 4 yr and then every 3 yr from
107 1976), in subplot 'c' to reach pH~5 since 1965 (every 3 yr). However, because the natural soil pH
108 was 5.4-5.6, the Nil plot received little liming. Five samples were taken in a straight line in each
109 plot.
110
111 **2.2 Basic characterization of bulk soil samples**
112 Gravimetric soil moisture content was determined by drying 15g of soil at 105 °C for 48 h. All
113 other chemical analyses were perform using sieved soil (2mm) and dried (40 °C). Soil pH was

measured in H_2O (1:5 weight:vol) according to the protocol NF ISO 10390 (2005). Soil organic carbon C, total nitrogen (N) and total phosphorus (P) were measured according to CS Technical report No. 3/07 (Emmett et al., 2008). The chemical fingerprint of soil samples was assessed using mid-infrared (MIR) spectroscopy. Dried soil samples were ball-milled and further dried overnight at 40 °C to limit interferences with water, without altering OM chemistry. Milled samples were analyzed using a Nicolet iS10 FT-IR spectrometer (Thermo Fisher Scientific Inc., Madison, WI, USA). Spectral acquisition was performed by diamond attenuated total reflectance (MIR-ATR) spectroscopy over the spectral range 4,000–650 cm^{-1}, with spectral resolution of 8 cm^{-1} and 16 scans per replicate.

2.3 Enzyme assays

Hydrolytic soil extracellular enzyme activities of β-glucosidase (GLU, EC number: 3.2.1.21, substrate: 4-MUB-β-D-glucopyranoside), acetyl esterase (ACE, EC number: 3.1.1.6, substrate: 4-MUB-acetate), phosphatase (PHO, EC number: 3.1.3.1, substrate: 4-MUB-phosphate), and leucine-aminopeptidase (LEU, EC number : 3.4.11.1, substrate: L-Leucine-7-AMC) were measured by fluorogenic methods using methylumbelliferyl (MUB) and 7-amino-4-methylcoumarin (AMC). PHO, GLU, ACE and LEU are involved in phosphorus mineralization, release of glucose from cellulose, deacetylation of plant compound and degradation of protein into amino acids, respectively. Enzyme assays were performed according to Turner et al. (2010) with modifications. A range of buffered pH solutions (from 2.5 to 10, in increments of 0.5) was prepared by adjusting 50mL of modified universal buffer with 1.0M HCl and 1.0M NaOH, at 20°C, then diluting to 100mL with deionized water. For each sample, a soil slurry was prepared by adding 20mL deionized water to 0.5g of soil, then rotary shaking on a magnetic plate for 20min at 28°C.

10mL of this soil solution was diluted in 25mL of deionized water to give a 1:100 soil-to-water ratio. Enzyme reactions were measured in 96-well microplates containing 50 µL of the specific buffer, 50 µL of soil slurry and 100µL of substrate solution (saturated concentration, 200µM). Microplates were then incubated in the dark for 4 hours at 28 °C, with one fluorometric measurement every 30 minutes (BioSpa 8 Automated Incubator) to follow the kinetic of the reaction.

For each sample, three methodological replicates (sample + buffer + substrate) and a quenched standard (sample + buffer + 4-MUB or 7-AMC) were used. Quenching curves were prepared with a serial dilution of 4-MUB solution for different amount of fluorophore in well (3000, 2000, 1000 pmol). For each substrate, a control including the 4-MUB- or 7-AMC-linked substrate and the buffer solution alone were used to check the evolution of fluorescence without enzyme degradation over the duration of assay. The fluorescence intensity was measured using a Cytation 5 spectrophotometer (Biotek) linked to the automated incubator (Biospa 8, Biotek) and set to 330 and 342 nm for excitation and 450 and 440 nm for emission for the 4-MUB and the 7-AMC substrate, respectively. All enzyme activities were calculated in nmol of product per minute per g of dry soil and normalized per the highest enzyme activity value measured at the pH optimum in order to express enzyme activity as relative activity in percentage.

2.4 Soil microbial community composition

For sequencing analyses of bacterial and fungal communities, DNA was extracted from 5 replicate soil samples per treatment using 0.25 g of soil and the PowerSoil-htp 96 Well DNA Isolation kit (Qiagen) according to manufacturer's protocols. The dual indexing protocol of Kozich et al (2013), was used for Illumina MiSeq sequencing of the V3-V4 hypervariable regions of the bacterial 16S

rRNA gene using primers 341F (Muyzer et al., 1993) and 806R (Youngseob et al., 2005); and the ITS2 region for fungi using primer ITS7f and ITS4r, (Ihrmark et al., 2012). Amplicon concentrations were normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific) prior to sequencing on the Illumina MiSeq using V3 chemistry. Fungal ITS sequences were analysed using PIPITS (Gweon et al., 2015) with default parameters as outlined in the citation. A similar approach was used for analyses of bacterial sequences, using PEAR (sco.h-its.org/exelixis/web/software/pear) for merging forward and reverse reads, quality filtering using FASTX tools (hannonlab.cshl.edu), chimera removal with VSEARCH_UCHIME_REF and clustering to 97% OTUs with VSEARCH_CLUSTER (github.com/torognes/vsearch). The Illumina MiSeq sequencing generated in average per sample 28205 reads for 16S rRNA gene and 40406 for ITS2 region.

2.5 Metagenome Sequencing

DNA was extracted from 2g of soil from 4 field replicates for the two pH treatments using the PowerMax Soil DNA Isolation kit (Qiagen), and subsequently concentrated and purified using Amicon® Ultra filters. Illumina libraries were constructed using the Illumina TruSeq library preparation kit (insert size < 500- 600 bp) and paired-end sequencing (2 x150 bp) was conducted using the Illumna HiSeq 4000 platform. Prior to annotation, Illumina adapters were removed from raw fastq files using Cutadapt 1.2.1 (Martin, 2011), reads were trimmed using Sickle (Joshi and Fass, 2011) with a minimum window quality score of 20 and short reads were removed (<20bp). Preliminary analysis was conducted using MGRAST to functionally annotate with SEED subsystems and taxonomically annotate with refseq. For more detailed analyses of β-glucosidase sequences, all reads from the 8 samples were co-assembled using MEGAHIT (Li et al., 2015) with

a minimum contig length of 1000. Sequences were translated and open reading frames were predicted using FragGeneScan (Rho et al., 2010). Contigs were assigned CAZY (Carbohydrate-Active enZYmes) subfamilies (Lombard et al., 2014) using a hmmer search (Finn et al., 2011) against dbCan2 profiles with an e-value of 1e-15 (Zhang et al., 2018). Contigs were taxonomically annotated against the NCBI Blast non-redundant protein database using Kaiju, a fast translated method, which identifies protein-level maximum exact matches (MEM's) (Menzel et al., 2016). Regions of contigs annotated as relevant β-glucosidase CAZY domains (GH1, GH2, GH3, GH5, GH9, GH30, GH39, GH116) were extracted. To identify pH associations of these sequences, DNA reads were mapped back to assembled domain protein sequences using BlastX, mappings with an identity percentage of < 97% and/or an e-value of > 0.001 were discarded. Mapping outputs were used to identify the relative abundance of assembled domain sequences across pH5 and pH7 samples, multinomial species classification method (CLAM) (Chazdon et al., 2011) was used to classify pH generalists and specialists and to discount sequences that were too rare for meaningful categorisation.

2.7 Statistical analysis

The effects of assay pH, soil field pH treatment and their interactions were assessed by repeated measures ANOVA. Fixed factors were sampling "assay pH" and "soil field pH", while soil field replicate was added as a random factor. One-way ANOVA was used to test the effects of enzymatic pH reaction on soil enzyme relative at each pH step (from 2.5 to 10). Differences in relative abundances of microbial taxa between soil pH 5 and soil pH 7 was assessed with one-way ANOVA. Assumptions of normality and homoscedasticity of the residuals were verified visually using diagnostic plots and a Shapiro-Wilk test. To identify soil bacterial and fungal community

composition patterns, a principal component analysis (PCA) based on Hellinger-transformed OTU data was performed (Legendre and Gallagher, 2001). Permutational multivariate ANOVA (PERMANOVA) was used to test the effect of soil pH field treatment on soil microbial community composition. All statistical analyses were performed under the R environment software (R Development Core Team 2011), using the R packages vegan (Oksanen et al., 2013) and ade4 (Dray and Dufour, 2007). Fourier-transform infrared spectroscopy (FTIR) spectral data were further processed and analyzed using the hyperSpec package (Beleites and Sergo, 2011),

3. Results

3.1 Soil characteristics

The pH values of the two soils were confirmed to be consistent with the treatments applied, with pH measured at 5.5 and 7.5 for the pH 5 and pH 7 plots, respectively (supplementary figure). Liming soil from pH 5 to pH 7 significantly increased by ~20% soil carbon content and soil total nitrogen (Table 1). Soil moisture, total P and C:N were not significantly different between oil pH 5 and soil pH 7 (Table 1). Soil infrared mid-infrared spectroscopy was used to fingerprint soil mineralogy and to assess heterogeneity within and between the two soil pH field treatments. The fingerprints confirm that soil mineralogy is consistent within and between pH field treatments (supplementary figure). The most prominent feature of the FTIR spectra corresponded to peaks indicative of phyllosilicate mineral compound absorption (kaolinite) with peaks at 3696, 3621, 1003, 912, 692 cm^{-1} (Dontsova et al., 2004). The 774 cm^{-1} peak is likely to be an indicator of quartz content and the 1642 cm^{-1} peak corresponds to the H–O–H bending band of water (Stuart, 2004, Dontsova et al., 2004). Small differences in peak amplitude between pH 5 and pH 7 soils are the result of small changes in the relative concentrations of compounds but overall the two soils

presented very similar mineralogy profiles (according to the peak wavelength positions) which indicates a shared ancestral origin.

3.2 Soil microbial community composition

The composition of soil bacterial and fungal community determined by amplicon sequencing (16S rRNA genes and ITS region, respectively) were clearly distinct between soil pH 5 and pH 7 for both communities (Fig.1; PERMANOVA: $R^2 = 0.82$, p-value: <0.001 for fungal community and, $R^2 = 0.51$, p-value: <0.01 for bacterial community). As observed on the PCA (Fig.1) and PERMANOVA results, fungal community structure was more affected than bacterial community by the liming treatment. Stacked bar plots representing the relative proportions of microbial phyla demonstrated relatively greater changes in the fungal compared to the bacterial community from pH 5 to pH 7 (Fig.1). Basidiomycota was significantly more abundant at soil pH 5 (83%, p-value: <0.001, Fig.1) whereas their relative abundance decreased at soil pH 7 (36%) to the advantage of Ascomycota and Zygomycota taxa (30% and 24% at soil pH 7 compared to 4.5% and 4% at soil pH 5, p-value: <0.01, respectively, Fig.1). Concerning the bacterial community, higher relative abundance of the phyla Acidobacteria and Verrucomicrobia was observed at pH 5 versus pH 7 (22% vs 16%, p-value: 0.02; 26% vs 18%, p-value: <0.01, respectively Fig.1). In contrast, higher relative abundance of Proteobacteria and Actinobacteria phylum was observed at pH 7 versus pH 5 (33% vs 27%, p-value: 0.01; 11% vs 7%, p-value: <0.01, respectively Fig.1).

3.3 Extracellular enzyme pH optimum assays

The pH of the enzymatic reaction had a highly significant impact on the catalytic efficiency of all enzymes examined (Fig.2, Table 2). At extremely low pH (2.5), activity was low or could not be

256 detected for leucine aminopeptidase and acetate esterase. For each enzyme, changes in the assay
257 pH strongly impacted the relative enzyme activity with a 15-fold increase between lowest and
258 highest activity at the pH optimum (Fig.2). After reaching the optima, the activity decreased more
259 or less rapidly depending on the assay. Regardless of the initial pH of the soil, pH optima appeared
260 to be specific to the enzyme studied (Fig.2). The pH optimum of leucine aminopeptidase and acetyl
261 esterase enzymes were close to neutrality, with an average pH optimum at 7.2 and 6.7, respectively
262 (Fig.2). The pH optima for β-glucosidase enzyme was acidic with an average of pH 4.3 (Fig.2).
263 Two pH optima were observed for phosphomonoesterase, one acidic (pH 5.7) and the other
264 alkaline (pH 10), although the alkaline optima may not have been fully reached.
265
266 Maintaining field soil at either pH 5 or pH 7 for over 100 years had a strong significant impact on
267 the pH optimum of all enzymes (Table 2). Enzyme pH optima shifted between acidic and alkaline
268 soil whatever the enzyme considered, though this was more pronounced for phosphatase, β-
269 glucosidase and acetate esterase compared to leucine-aminopeptidase. The interaction between
270 enzymatic assay pH and field soil pH was significant for each enzyme assayed, indicating that the
271 magnitude of the difference in enzyme activity between pH 5 and pH 7 soil is dependent upon
272 enzymatic assay reaction pH (Table 2). For each enzyme, optimum activity differed between the
273 two soils by 0.5 pH units. Similar optimal activities were found for acetate esterase and leucine
274 aminopeptidase, while the activity of β-glucosidase and phosphatase was reduced by 4-6% in a pH
275 7 soil. A second optimum at pH 10 was observed for phosphatase and acetyl esterase from pH 7
276 soil, in contrast to little or no activity of these enzymes from pH 5 soil (Fig. 2D). Additionally, the
277 relative activity of enzymes from pH 5 soil was always higher in acidic assay conditions (< pH

5.5), while the relative activity of enzymes from pH 7 soil was always higher in more alkaline conditions (> pH 7).

3.4 Soil metagenomics

The amplicon sequencing results revealed large shifts in broad taxa between the two soils.,To determine whether similar shifts were also observed in associated enzymatic gene production shotgun metagenomes generated from the same soils was utilized. We focussed our analyses on bacterial β-glucosidases, since the bacteria dominate soil metagenomics gene libraries (Malik et, 2017) and the β-glucosidases are genetically well characterized enzymes, known to be important for soil carbon transformations. Analyses of the functional and taxonomic annotations of β-glucosidase related genes using MGRAST revealed they were relatively more abundant insequences from the Acidobacteria in the pH 5 compared to pH 7 soils (15.9% vs 1.9%, p-value: 7.4×10^{-5}; Fig.3 A), while this was reversed in sequences from Actinobacteria in pH 7 compared to pH 5 soils (34.6% vs 43.4%, p-value: 6×10^{-3}; Fig.3 A). When normalized by housekeeping genes abundances, Acidobacteria β-glucosidase gene abundance were significantly enriched in pH 5 soil compared with pH 7 soil (Fig. 3 B) being on average twice as abundant.

It is clear that Acidobacterial β-glucosidases are a unique feature of the more acid soils and therefore can be highly implicated as responsible for the pH related differences in enzyme activity. However, this does not rule out that other phyla may have distinct pH-selected sub clades which could also be responsible. To address this, we sought to classify individual taxa according to pH association, by assembling contigs based on the pooled metagenomics sequence reads (all samples from pH 5 and pH 7 soils); extracting β-glucosidase sequences using a hmmer search against dbCan2 profiles; and then mapping back individual reads to these sequences. Sequences were then

classified as pH specialists, generalists or too rare to categorise using multinomial species classification method (CLAM). The majority of Acidobacteria sequences were classed as pH 5 specialists, this suggests that not only isthere a higher relative abundance of Acidobacteria β-glucosidase sequences at pH 5 but that the majority of these sequences appear to be unique to pH 5 soils (Fig. 4). Sequences annotated as other dominant phyla such as Actinobacteria and Proteobacteria appeared to have a higher proportion of pH 5 specialist and generalist sequences (supplementary table), whilst Verrucomicrobia included a clear sub-clade of pH 7 specialist sequences (Fig. 4).

4. Discussion

The activity of enzymes involved in C, N and P cycles were all found to be strongly dependent on the pH of the assay. Beta-glucosidase had an acidic pH optimum (pH=4.3), which is generally observed for glycosidase enzymes (Niemi and Vepsäläinen, 2005; Sinsabaugh et al., 2008; Turner, 2010), whereas leucine aminopeptidase had an alkaline pH optimum (7.2) as is commonly reported for proteases (Niemi and Vepsäläinen, 2005; Sinsabaugh et al., 2008). Acetyl esterase pH optima were at pH 7 for both soils studied, also in line with previous findings(Degrassi et al., 1999 and Humberstone and Briggs, 2000 respectively). However, source soil pH had a significant and strong impact on soil exoenzyme pH optimum response curves. For each enzyme studied, extracellular enzymes originally from pH 5 soil were more adapted towards acidic pH conditions, whereas pH 7 soil possessed enzymes adapted towards more alkaline conditions (Fig.2). Interestingly, the enzymatic pH optima observed in this study did not correspond exactly to the local soil pH, presumably due to constraints within the active sites that enable physicochemical function to be maintained. It is possible that the responses observed are due to

the presence of isoenzymes, which have different kinetic properties adapted toward the local soil pH. Alkaline and acid phosphatases are the most studied example of soil isoenzymes (Nannipieri and al, 2011), and our phosphatase pH response curves illustrate this with a marked bimodal distribution, and extremely low activity for the pH 7 soil compared to the pH 5 soil, at acidic assay pH. Acetyl esterase also exhibited a bimodal response but only in the pH 7 soil, which also exhibited a second pH optimum developing at pH 10.

Previous studies have observed different pH optima for the same enzyme across different soil types (Niemi and Vepsäläinen, 2005; Turner, 2010), though the specific causes were not empirically assed. Mechanisms proposed include either abiotic stabilization by soil chemical properties which alter the conformation of the enzyme and thus kinetics; or differences in the microbes that produce the enzymes. Our experiment, conducted on the same soil type, provides strong evidence for microbial control, mediated through altered soil pH. Shifts in enzyme pH optima due to enzyme sorption to different clay types (Leprince and Quiquampoix, 1996; Ramirez-Martinez and McLaren, 1966; Skujins and al., 1974) was discounted as IR based soil chemistry fingerprints (incorporating information on clay content) were very similar between the pH 5 and pH 7 soils (Supplementary Fig.2). Moreover, the dilution factor used to perform enzyme assays 1:100 soil-to-water ratio helped to reduce potential effect of small increases in soil organic matter content and total nitrogen observed between the pH 5 and pH 7 soils. Further strong evidence for biotic mechanisms is provided by the consistent non-random shift in optima towards the source soil pH and the presence of bi-modal pH optimum curve indicating clearly the presence of isoenzyme.

Our data suggest that differences in microbial communities underpin the observed functional responses. Bacterial and fungal communities were found to be clearly distinct between

the two pH soils investigated, as anticipated from previous work in the Park Grass long-term experiment (Zhalnian et al., 2015; Liang et al., 2015). Such differences in microbial community composition are likely to be responsible for the production of different versions of the same enzyme (Fig.2). For example, the Acidobacteria phylum has been reported to possess more diverse and abundant genes encoding for carbohydrate-decomposing enzymes than Proteobacteria (Lladó et al., 2019; Lladó et al., 2016) which could be responsible for shift observed in bulk soil beta-glucosidase pH optimum between pH 5 and pH 7 soils. The metagenomics results clearly showed that different proportions of bacterial phyla produced β-glucosidases across the two soils. Notably, the Acidobacteria contributed more to the β-glucosidase gene pool in the acid soil, and this contribution was more marked than would be expected from examining abundances based on housekeeping genes alone. Furthermore, sub clades of acidobacterial glucosidase were unique in being exclusively found in acid soils, with other broad taxa possessing both generalist enzymes, and a mix of pH specialized genes for either acid or neutral pH. This indicates that acidophilic acidobacterial lineages may possess enzymatic adaptations which underpin their demonstrated competitiveness in acidic soils (Griffiths et al, 2011), and confirms recent genomic studies which have identified enzyme production for carbohydrate degradation as a key feature of these organisms (Eichorst et al, 2018).

As soil microorganisms can depend upon proximate decomposition agents for acquiring resource and energy, the efficiency of (costly) extracellular enzymes may represents a fundamental competitive trait (Wallenstein et al. (2011). Here we provide evidence that beta-glucosidase enzymes from pH 5 soil are half as efficient as those from pH 7 soil relative to their total activity (Fig.3, 4% vs 8% of total activity at a pH 7 assay, soil pH5 vs soil pH7 respectively) at neutral assay pH; and these functional changes are accompanied by large changes in the relative

abundance of enzyme producing bacteria. We note however that our data does not empirically prove that the taxa detected through metagenomics are directly responsible for altered efficiency. Further support could be achieved through new computational approaches predicting pH optima based on amino acid sequence composition (Yan and Wu, 2012; Lin et al, 2013), or in vitro enzyme testing on novel isolates or expressed metagenomic sequences. We also cannot discount evolutionary processes acting within populations contribute to the observed soil pH optima, e.g. through mutations affecting enzyme active sites (Ohara et al., 2014). Whilst a number of evolutionary adaptations to pH have been documented for bacterial strains (Harden et al, 2015), we found only one study addressing experimentally evolved enzymatic adaptions, which was refuting (Gale & Epps, 1945). Comparatively more is known about the adaptation of microbial enzymes to temperature (Åqvist et al, 2017), and local temperature adaptation has been, in comparison, extensively studied in bulk soil enzyme assays across thermal gradients (Blagodatskaya et al, 2016; Alvarez et al, 2018; Nottingham et al 2016 ; Allison et al, 2018a). However, few studies have examined adaptive capacities of individual populations (Allison et al, 2018b). Clearly more detailed testing of community selection versus evolutionary processes in governing enzymatic adaptation to environmental factors is required, but our data strongly implicate a role for the Acidobacteria in carbohydrate-degrading processes in acidic soils. Linking (meta)genetic information to explicit enzymatic functional potential is an exciting new area, where advances could allow prediction of soil function from microbial biodiversity data. More specifically, we feel it is of utmost importance to determine whether the enzymatic adaptations to soil pH observed here, give rise to other functional outcomes or trade-offs, such as alteration of temperature optima. Such knowledge will allow better prediction of decomposition processes in response to changing climate, across global pH-defined soil systems.

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TABLES

Table 1. Effect of soil field pH treatment (soil pH 5 vs soil pH 7) on soil properties. Values represent the mean (n=5) with the associated standard error (SE). Bold letters indicate significant differences ($p<0.05$).

	units	Low pH (5)	High pH (7)
pH (H_2O)	-	**5.5 ± 0 a**	**7.3 ± 0.1 b**
Soil moisture	%	30.2 ± 1.1	31.5 ± 1.2
Carbon content	%	**3 ± 0.1 b**	**3.9 ± 0.3 a**
CN ratio	-	10.7 ± 0.1	11 ± 0.1
Total Nitrogen	%	**2.8 ± 0.1 b**	**3.5 ± 0.2 a**
Total phosphorus	mg/Kg	54 ± 12.9	59.3 ± 2.5

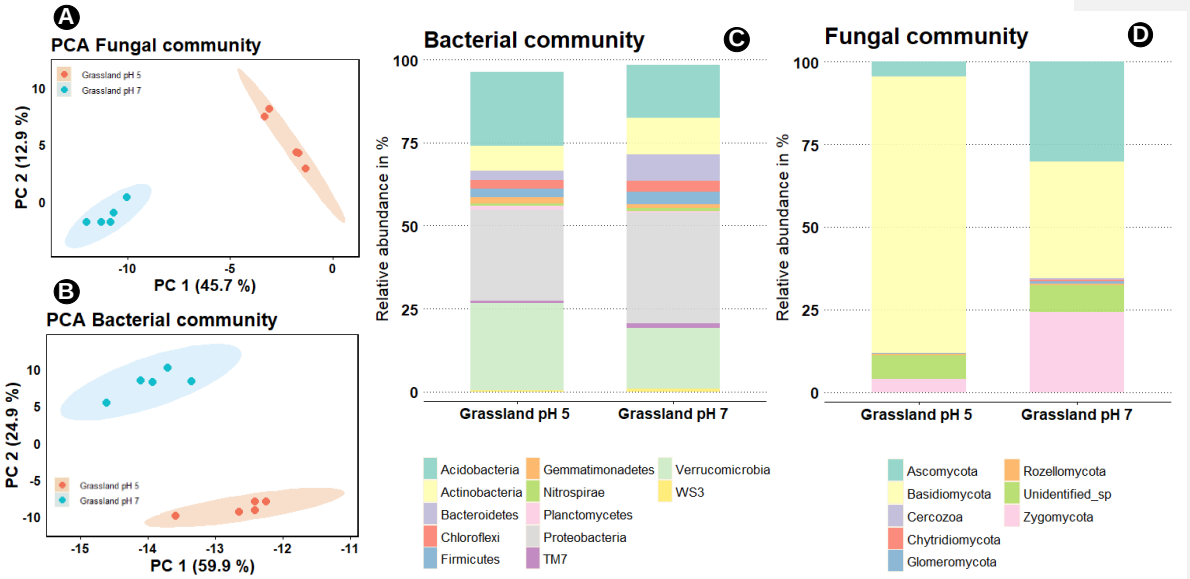

Table 2. Effects of pH, soil treatment and interactions of both factors on relative enzyme activity at different assay pH (mixed model, overall repeated measures ANOVA tests).

	Assay pH		Field soil pH		Assay pH x field soil pH	
	F-value	P-value	F-value	P-value	F-value	P-value
Leucine amino-peptidase	190.1	**<0.001**	6.9	**0.03**	3.42	**<0.001**
Phosphatase	89.1	**<0.001**	51.4	**<0.001**	44.2	**<0.001**
ß-glucosidase	88.4	**<0.001**	23.4	**<0.01**	33.7	**<0.001**
Acetate esterase	397.2	**<0.001**	30.9	**<0.001**	38.4	**<0.001**

FIGURES:

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585 **Fig 1. Principal component analysis (PCA) ordination of soil bacterial (A) and fungal (B)**

586 **communities. Stacked bar plots show the relative proportion of the main abundant phyla**

587 **(>0.5 %) for C) bacterial and D) fungal community.**

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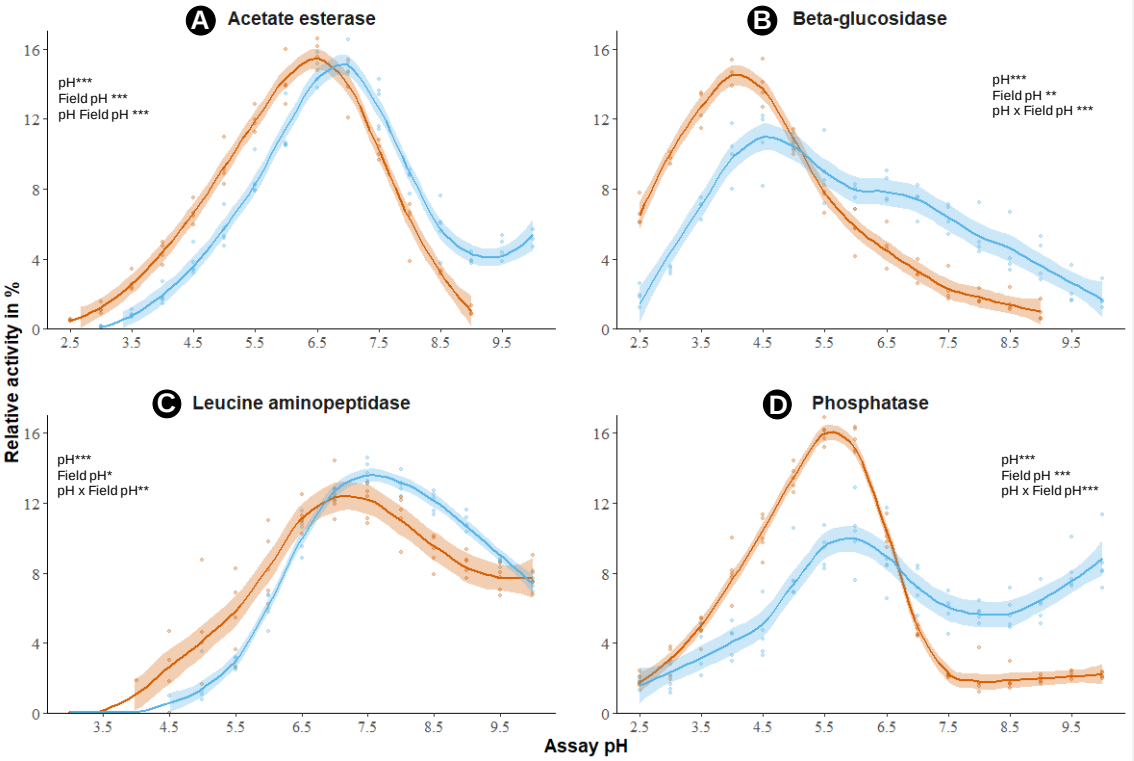

596 **Fig 2. Enzyme pH optima of acetylesterase (A), beta-glucosidase (B), leucine aminopeptidase**
597 **(C), phosphomonoesterase (D).** Activity is expressed as a percentage of the total activity
598 measured across the entire pH range (from pH 2.5 to pH 10). The orange and blue lines correspond
599 to pH 5 and soil pH 7 soils respectively. Shaded area represents 95% confidence intervals around
600 the trend line using a t-based approximation (LOESS smoothing). Stars indicate result of the
601 mixed model used to evaluate the effects of assay pH, soil field pH treatment and their interactions
602 were assessed by repeated measures ANOVA. Fixed factors were sampling "assay pH" and "soil

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603 field pH", while soil field replicate was added as a random factor. Significance codes for
604 ANOVA's are (***) p<0.001; (**) p<0.01; (*) p<0.05.
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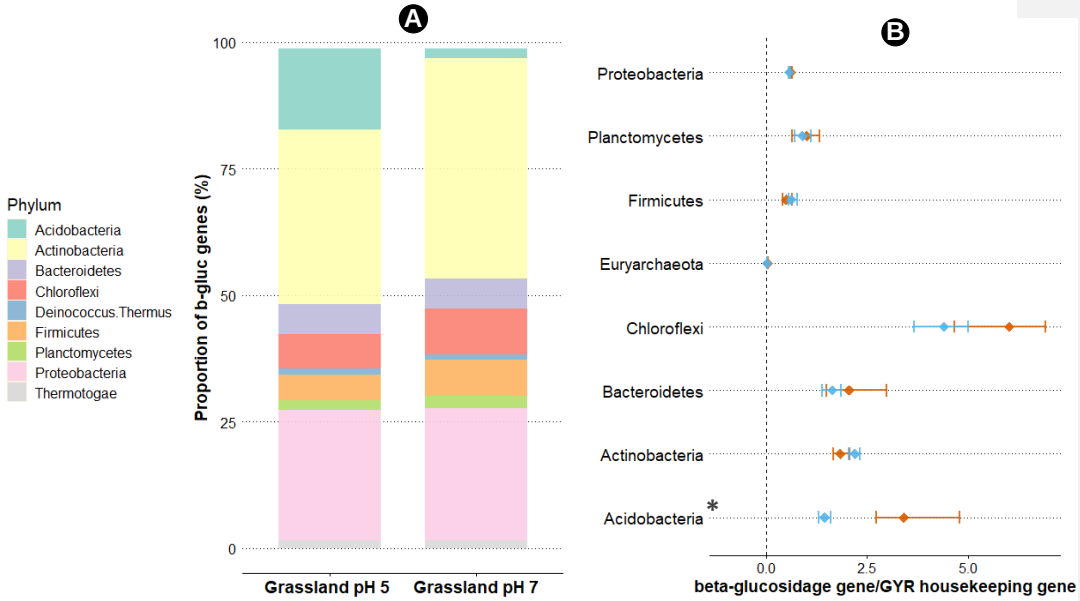
608 **Fig 3. Abundances of beta-glucosidase genes from different microbial taxa, from MG-RAST**



609 **annotated metagenomes (SEED Subsystems).** A: Stacked plot representing the total proportion
610 of beta-glucosidase genes from dominant bacterial phyla. B: The proportional change of beta-
611 glucosidase gene abundance compared to the abundance of the DNA gyrase subunit B gene.
612 Orange and blue colors correspond to pH 5 and pH 7 soil respectively. The x-axis shows the
613 relative fold change on log2 scale. Error bars indicate +/- standard deviation and the means are

indicated by filled diamond shape. Asterisks indicate significance difference between pH 5 and pH 7 soil (ANOVA $p<0.05$).

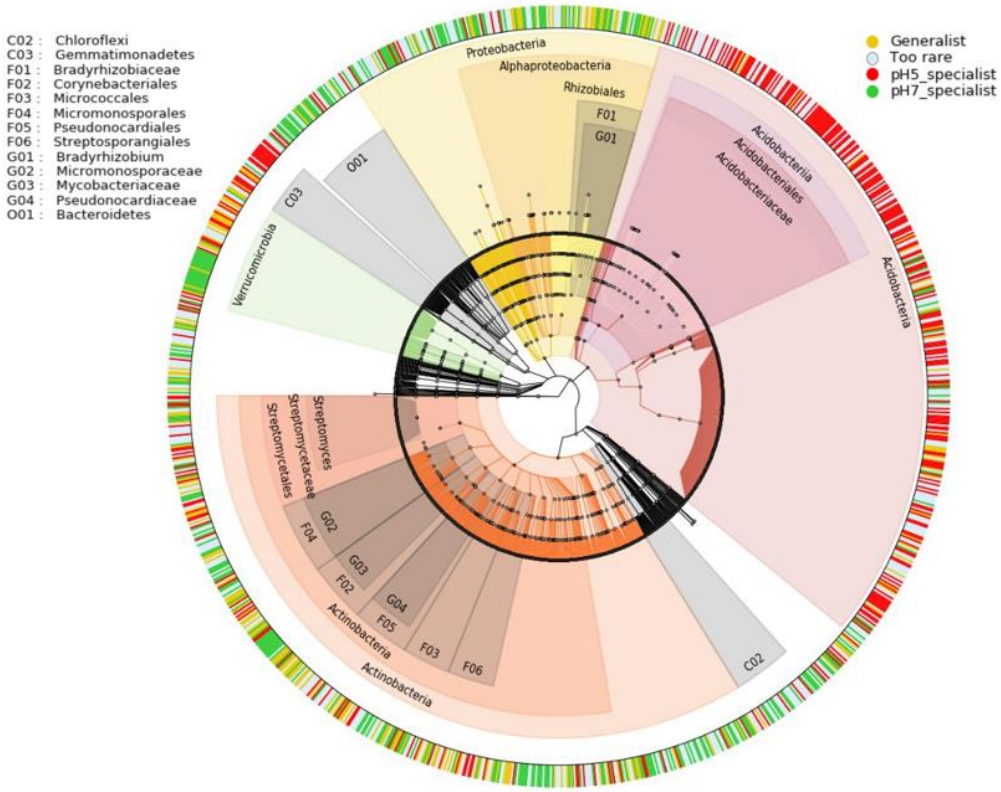


Fig 4. Taxonomy and pH associations of β-glucosidase sequences assembled from metagenomes. Inner tree and labels depict the taxonomy of β-glucosidase gene assemblies

constructed from pooled metagenomes from the pH 5 and pH 7 soils (n=4). Outer ring shows putative pH associations of each assembled gene, following tabulation of reads mapped to the contigs from each of the 8 soil metagenomes, and statistical classification using a multinomial model based on relative abundance across the two soils (CLAM).