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

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Abstract

Soil exoenzymes 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 on two historically managed soils maintained at either pH 5 or 7 from the Rothamsted Park Grass Long-term experiment. The pH optima for a range of exoenzymes involved in C, N, P cycling, differed 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 clearly distinct between pH 5 and soil pH 7 soils, possibly explaining differences in enzymatic responses. Furthermore, β-glucosidase gene sequences extracted from metagenomes revealed an increased abundance of Acidobacterial producers in the pH 5 soils, and Actinobacteria in pH 7 soils. Our findings demonstrate that the pH optimum of soil exoenzymes adapt to long term changes in soil pH, the direction being dependent on the soil pH shift; and we provide further evidence that changes in functional microbial communities may underpin this phenomena, though new research is now needed to directly link change in enzyme activity optima with microbial communities. More generally, our new findings have large implications for modelling the efficiency of different microbial enzymatic processes under changing environmental conditions.

Keywords: enzyme activity, adaptation, liming, carbon degradation, metagenomics, microbial community

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.

In soil systems, much research has focused on enzyme adaptation to temperature (Allison et al., 2018; Alvarez et al., 2018; Blagodatskaya et al., 2016; Razavi et al., 2017) due to concerns on the effects of future climate change on ecosystem processes. The molecular mechanisms underpinning these adaptations have been studied and are believed to be driven by conformational flexibility within the enzyme active site or protein surface, which affects efficiency in relation to enzyme activation energy (Åqvist et al., 2017; Lonhienne et al., 2000). However, these adaptations also result in various trade-offs between efficiency and enzyme stability (Åqvist et al., 2017; Zanphorlin et al., 2016); meaning both specific exoenzyme-catalyzed processes as well as other non-specific microbial processes may be affected by a changing environment. The assessment of soil enzymatic responses to change in temperature is an active area of research, with some studies suggesting that acclimation can be rapid and driven by changes in underlying microbial communities (Bradford, 2013; Nottingham et al.,

2019; Wei et al., 2014). Surprisingly there has been limited reporting of enzymatic 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 as acid rain (Goulding, 2016; Kirk et al., 2010; Slessarev et al., 2016; Tian and Niu, 2015; van Breemen et al., 1983; Wu et al., 2017). It is well established from laboratory assays that the rate of enzymatic catalytic reactions is dependent on the pH at which the reactions occur, with the point of maximal activity known as the pH optimum (Frankenberger & Johanson,1982, German et al., 2011). Previous studies have demonstrated different pH optima for the same enzyme across widely differing soil types (Niemi and Vepsäläinen, 2005; Turner, 2010), though the causal role of soil pH in predicting pH optimum has never been established. Additionally, pH is known to be one of the main factors affecting soil microbial diversity (Fierer et al., 2017; Griffiths et al., 2011), yet the relevance of reported changes in communities across pH gradients for soil enzymatic processes remains unknown. With enzymatic kinetics now being incorporated into recent C decomposition models (Allison, 2012; Davidson et al., 2012; Wang et al., 2013), we believe empirical data on the specific role of pH in affecting enzyme kinetic parameters is now required, since soil pH changes can occur rapidly with unknown acclimation responses. Furthermore, new understanding of the role of microbes in driving responses is essential to both increase understanding of acclimation mechanisms, but also potentially provide easily measurable indicators for model parameterization.

We therefore sought to test soil exoenzymatic adaptation to local pH, by conducting enzymatic assays at a range of buffered pH levels on soils from the Park Grass long-term experiment (Rothamsted) in which the same soil type had been maintained at either pH 5 or 7 for over 100 years. Hydrolytic exoenzymes corresponding to major enzymes involved in organic C, N and P cycling were selected to study. We hypothesize that enzyme pH optimum

will be affected by ancestral soil pH treatment, with soil exoenzymes from soil pH 5 being more adapted towards acidic conditions and exoenzymes from soil pH 7 adapted towards more alkaline conditions. To better understand the microbial community relationships underpinning exoenzyme activity and pH adaptation, we also sought to assess the change in microbial community composition (bacteria and fungi) with amplicon sequencing, and functional genes using a metagenomics sequencing approach. Specifically, we wished to determine whether change in enzyme activity is associated with change in specific microbial enzyme producers or adaptation of exoenzymes to environmental conditions.

2. Materials and methods

2.1 Soil sampling

We took advantage of the unique Park Grass Long-term experiment (Rothamsted, UK; Macdonald et al., 2018) in which soils have been maintained at either pH 5 or 7. The experiment originally started in 1856 on permanent pasture to investigate ways of improving hay yields, is managed with a range of fertilisers and pHs with the hay cut twice a year. Soils cores (0-15 cm depth, 4 cm Ø) were sampled on the 27th November 2015 in subplots 'a' (pH ~ 7) and 'c' (pH ~ 5) of the Nil plot 12, which has never received any fertilisers (Storkey et al., 2016). The soil pH is regularly monitored and controlled by liming, in subplot 'a' to reach pH~7 since 1903 (every 4 yr and then every 3 yr from 1976), in subplot 'c' to reach pH~5 since 1965 (every 3 yr). However, because the natural soil pH was 5.4-5.6, pH 5.5 plots have only received minimal liming across the experimental duration to combat natural acidification processes.

2.2 Basic characterization of bulk soil samples

Gravimetric soil moisture content was determined by drying 15 g of soil at 105 °C for 48 h. All other chemical analyses were performed using sieved (2 mm), air-dried (40 °C) soil. Soil

pH was measured in H_2O (1:5 weight: vol) according to the protocol NF ISO 10390 (2005). Soil organic carbon C, total N and total P were measured according to CS Technical report No. 3/07 (Emmett et al., 2008). The fingerprint of soil mineralogy 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 exoenzyme activities of phosphatase (PHO, EC number: 3.1.3.1, substrate: 4-MUB-phosphate), 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) 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 (2010) and following German et al. (2011) recommendations for measuring enzyme activity in soil solution. A range of buffered pH solutions (from 2.5 to 10, in increments of 0.5) was prepared by adjusting 50 mL of modified universal buffer with 1.0 M HCl and 1.0 M NaOH, at 20°C, then diluting to 100 mL with deionized water. The corresponding composition for one litter of modified universal buffer was: 12.6g of boric acid, 28g of citric acid, 23.2 g of maleic acid, 24.2 of Trizma base and 39g of NaOH. Note that the

buffered pH solution was diluted 4-fold in the final assay solution giving a concentration of each chemical of 25mM. Turner (2010) showed that such a concentration was necessary to maintain the required pH during the assay. For each sample, a soil slurry was prepared by adding 20 mL deionized water to 0.5 g of soil (fresh weight), then rotary shaking on a magnetic plate for 20 min at 28 °C. 10 mL of this soil solution was diluted to 25 mL with deionized water to give a 1:100 (w/v) soil-to-water ratio. Enzyme reactions were measured in 96-well microplates containing 50 µL of the specific buffer (25mM), 50 µL of soil slurry (1:400 (w/v) soil-to-water ratio) and 100 µL of substrate solution (saturated concentration, 200 µM). Microplates were then incubated in the dark for 3 h at 28 °C, with one fluorometric measurement every 30 min (BioSpa 8 Automated Incubator) to follow the kinetics of the reaction. Soil pH values were checked before and after incubation and a small drop of 0.1 to 0.2 pH unit was observed after incubation (3h) which we consider being negligible compared to the entire pH range evaluated (2.5 to 10).

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 amounts of fluorophore in the well (3000, 2000, 1000 pmol) (Puissant et al., 2015). 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 expressed as a percentage of the total activity measured across the entire pH range (from pH 2.5 to pH 10).

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 2 g 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 x 150 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 (<20 bp). Preliminary analysis was conducted using MGRAST to functionally annotate with SEED subsystems and taxonomically annotate with refseq. We focused our analyses on bacterial β-glucosidases, since the bacteria dominate soil metagenomics gene libraries (Malik et al., 2017) and the β-glucosidases are genetically well characterized enzymes, known to be important for soil C transformations. 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 from individual samples were mapped back to assembled contigs using BlastX, and mappings with an identity percentage of < 97% and/or an e-value of > 0.001 were discarded. Mapping outputs were used to tabulate the abundance of individual reads from the pH 5 and pH 7 samples forming each contig, and then the multinomial species classification method (CLAM) (Chazdon et al., 2011) was used to classify contigs with respect to soil pH designation: generalist- the contig is made up of sequences from both pH 5 and 7 soils; pH specialist- reads making up a contig are predominantly from either pH5 or pH7 soil; or "too rare" whereby the number of reads is too low to reliably classify.

2.6 Statistical analysis

The effects of assay pH, soil field pH treatment and their interactions on enzyme kinetics were assessed by repeated-measures ANOVA. Fixed factors were sampling "assay pH" and "soil 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 activity at each pH step (from 2.5 to 10). Differences in relative abundances of microbial taxa between soil pH 5 and soil pH 7 were 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 3.6.0 (R Development Core Team, 2011), using the R packages vegan (Oksanen et al., 2013), ade4 (Dray and Dufour, 2007) and NLME (Pinheiro et al.,2014). 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. Liming soil from pH 5 to pH 7 significantly increased by ~20% the total C and N contents (Table 1). Soil moisture, total P and C: N were not significantly different between soil 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 materials, Fig.1). 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: $R^2 = 0.82$, $p<0.001$ 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 the 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. 2). Basidiomycota was significantly more abundant at soil pH 5 (83%, $p<0.001$, Fig. 2) 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, $p<0.01$, respectively, Fig. 2). Concerning the bacterial community, higher relative abundances of the phyla Acidobacteria and Verrucomicrobia were observed at pH 5 versus pH 7 (22% vs 16%, $p=0.02$; 26% vs 18%, $p<0.01$, respectively Fig.

2). In contrast, a higher relative abundance of Proteobacteria and Actinobacteria phylum was observed at pH 7 versus pH 5 (33% vs 27%, p=0.01; 11% vs 7%, p:<0.01, respectively Fig. 2).

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. 3, Table 2). At extremely low pH (2.5), activity was low or could not be detected for leucine aminopeptidase and acetate esterase. For each enzyme, changes in the assay pH strongly impacted the relative enzyme activity with a 15-fold increase between lowest and highest activity at the pH optimum (Fig. 3). After reaching the optima, the activity decreased more or less rapidly depending on the assay. Regardless of the initial pH of the soil, pH optima appeared to be specific to the enzyme studied (Fig. 3). The pH optimum of leucine aminopeptidase and acetyl esterase enzymes were close to neutrality, with an average pH optimum at 7.2 and 6.7, respectively (Fig. 3). The pH optima for β-glucosidase enzyme was acidic with an average of pH 4.3 (Fig. 3). Two pH optima were observed for phosphomonoesterase, one acidic (pH 5.7) and the other alkaline (pH 10), although the alkaline optima may not have been fully reached.

Maintaining field soil at either pH 5 or pH 7 for over 100 years had a strong significant impact on the pH optimum of all enzymes (Table 2). Enzyme pH preference and optima shifted between acidic and alkaline soil whatever the enzyme considered, though this was more pronounced for phosphatase, β-glucosidase and acetate esterase compared to leucine-aminopeptidase (mixed model, Table 2). For each enzyme, the optimum pH differed between the two soils by 0.5 pH units (Fig.3). The interaction between enzymatic assay pH and field soil pH was significant for each enzyme assayed, indicating that the magnitude of the difference in enzyme activity between pH 5 and pH 7 soil is dependent upon assay reaction pH (Table 2). A second optimum at pH 10 was observed for phosphatase and acetyl esterase from pH 7 soil,

in contrast to little or no activity of these enzymes from pH 5 soil (Fig. 3A, 3D). Similarly, the relative activity of enzymes from pH 5 soil was always higher to enzymes from pH 7 in acidic assay conditions (< pH 5.5), while the relative activity of enzymes from pH 7 soil was always higher than enzymes from pH 5 soil in more alkaline conditions (> pH 7).

3.4. Soil metagenomics

The amplicon sequencing results revealed large differences in broad taxa between the two soils of different pH. To determine whether similar shifts were also observed in associated enzymatic gene sequences, shotgun metagenomes datasets generated from the same soils were utilized. Analyses of the functional and taxonomic annotations of β-glucosidase related genes using subsystems annotation revealed greater abundance of sequences from Acidobacteria in the pH 5 compared to pH 7 soil (15.9% vs 1.9%, p-value: 7.4×10^{-5}; Fig.4); and conversely more Actinobacterial β-glucosidase genes in pH 7 soils (34.6% vs 43.4%, p-value: 6×10^{-3}; Fig.4). We further tested differences in abundance by normalizing to a housekeeping gene (gyrB), and found significant differences only for Acidobacterial β-glucosidase genes, which were significantly enriched at pH 5 soil compared with the pH 7 soil, being on average twice as abundant (Supplementary materials, Fig.2) .

It is, therefore, apparent at the level of broad phyla, large increases of Acidobacterial β-glucosidases in acid soils are associated with the shift in exoenzyme pH optimum . However, this does not rule out that other phyla may have distinct pH responsive sub clades. To assess this, we assembled pooled metagenomic sequence reads and extracted contigs containing β-glucosidases following functional classification using CAZY and taxonomic annotation to RefSeq. β-glucosidase contigs were then classified as pH specialist (pH 5 or 7) or generalist using a multinomial classification method (CLAM) typically used to classify species' habitat preference based on surveyed counts, but here used on the number of reads per individual

sample from the two treatments mapping to each β-glucosidase contig. The majority of Acidobacteria sequences were classed as pH 5 specialists, suggesting that not only is there 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. 5). Sequences annotated as other dominant phyla such as Actinobacteria and Proteobacteria appeared to have a higher proportion of pH 7 specialist and generalist sequences (supplementary materials, Table 2), whilst Verrucomicrobia possessed a distinct sub-clade of pH 7 specialist sequences (Fig. 5).

4. Discussion

4.1 Soil exoenzyme pH optima are adapted toward local pH

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 a neutral 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; Humberstone and Briggs, 2000). 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. 3). 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 et 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 underlying causes responsible for this were not identified. 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 et 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 materials, Fig.1). Moreover, the dilution factor used to perform enzyme assays (1:400 soil-to-water ratio) helped to reduce potential effect of small increases in soil total C content and total N 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 isoenzymes.

4.2 Potential microbial mechanisms governing exoenzyme local adaptation to pH

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 may be responsible for the production of different versions of the same enzyme

(Fig. 3). 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). To explore this further, we performed metagenomic sequencing to examine whether the change in enzyme pH preference in the two soils was associated with differences in functional diversity. Focusing specifically on the β-glucosidase exoenzyme, our 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).

Our results highlight the utility in linking metagenomics approaches to measures of specific enzymatic functional traits (pH optimum), with the demonstration of both biodiversity and functional differentiation caused by manipulated soil pH change. In addition the use of molecular approaches here adds to the emerging molecular understanding of the biodiversity of soil enzymes (Berlemont et al., 2013; Heath et al., 2009; Lidbury et al., 2017), and provides new information on the functional capacity of previously undiscovered soil microbial biodiversity. However, we cannot empirically prove that differentially abundant enzyme producers are directly responsible for altered efficiency, since it is currently not possible to assess the diversity of enzymes functionally active within the laboratory-based assays, or

indeed the soil. New advanced research is required to determine the relevance of alterations in enzyme producing organisms for soil processes. With respect to pH effects, further insight could be achieved through new computational approaches predicting the pH optima based on amino acid sequence composition (Yan and Wu, 2012; Lin et al., 2013), or in vitro enzyme testing of novel cultured isolates or expressed metagenomic sequences. We also cannot discount evolutionary processes acting within non pH responsive taxa contribute to altered soil pH optima, e.g. through discrete 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) there is little information in the literature on specific exoenzyme adaptations; and whether these result in wider trade-offs with respect to resource acquisition also remains an open question. Addressing these important questions will bring new understanding of the microbial ecological mechanisms governing soil biochemical function under conditions of environmental change; and advances could allow better model parameterization. Specifically, we highlight that incorporation of enzymatic temperature acclimation into models has widely been discussed despite many mechanistic uncertainties (Bradford, 2013; Nottingham et al., 2019; Allison et al., 2018). Our results revealing strong pH adaptation of both enzymatic optimum activity and producer diversity therefore offers an important area for further study within a modelling context, since microbial pH responses are largely predictable (Fierer et al., 2017; Griffiths et al., 2011), and soil pH is highly sensitive to land use and climatic change.

Conclusion

We have specifically demonstrated that the pH optimum of soil exoenzymes adapt towards source soil pH, using soils from a long-term pH manipulation experiment. This was found for all enzymes tested with implications for understanding the resilience of biochemical

transformations of carbon, nitrogen and phosphorous across soil systems. Amplicon sequencing and metagenomic data also demonstrated concurrent shifts in taxonomic and functional communities with pH governed shifts in pH optima, providing further evidence that changes in functional microbial communities may underpin pH related change in enzyme kinetic efficiency. These findings call for more research into the underlying genetic controls of enzymatic efficiency in relation to pH, as well as deeper ecological understanding of adaptation mechanisms. More generally, our findings have implications for modelling the efficiency of different microbial enzymatic processes under changing environmental conditions; and soil pH change should be considered, alongside previously documented temperature acclimation, in new carbon models incorporating enzymatic responses to climate change.

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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 ± 0.0 a**	**7.3 ± 0.1 b**
Soil moisture	%	30.2 ± 1.1	31.5 ± 1.2
Total carbon content	%	**3.0 ± 0.1 b**	**3.9 ± 0.3 a**
CN ratio	-	10.7 ± 0.1	11.0 ± 0.1
Total nitrogen	%	**2.8 ± 0.1 b**	**3.5 ± 0.2 a**
Total phosphorus	mg/kg	54.0 ± 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**

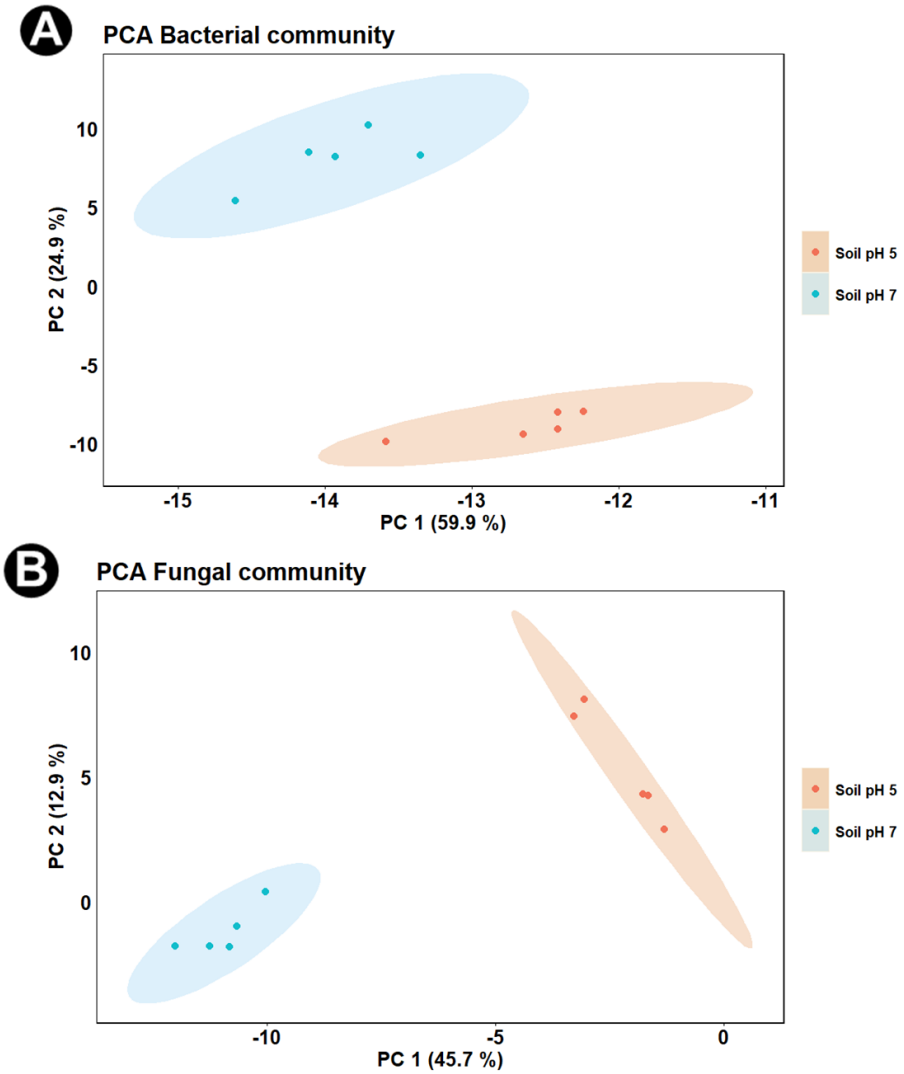


Fig. 1. Principal component analysis (PCA) ordination of soil bacterial (A) and fungal (B) communities from grassland soil at either pH 5 or 7. The orange and blue colors correspond to pH 5 and pH 7 soils, respectively and ellipses indicate 95% confidence interval.

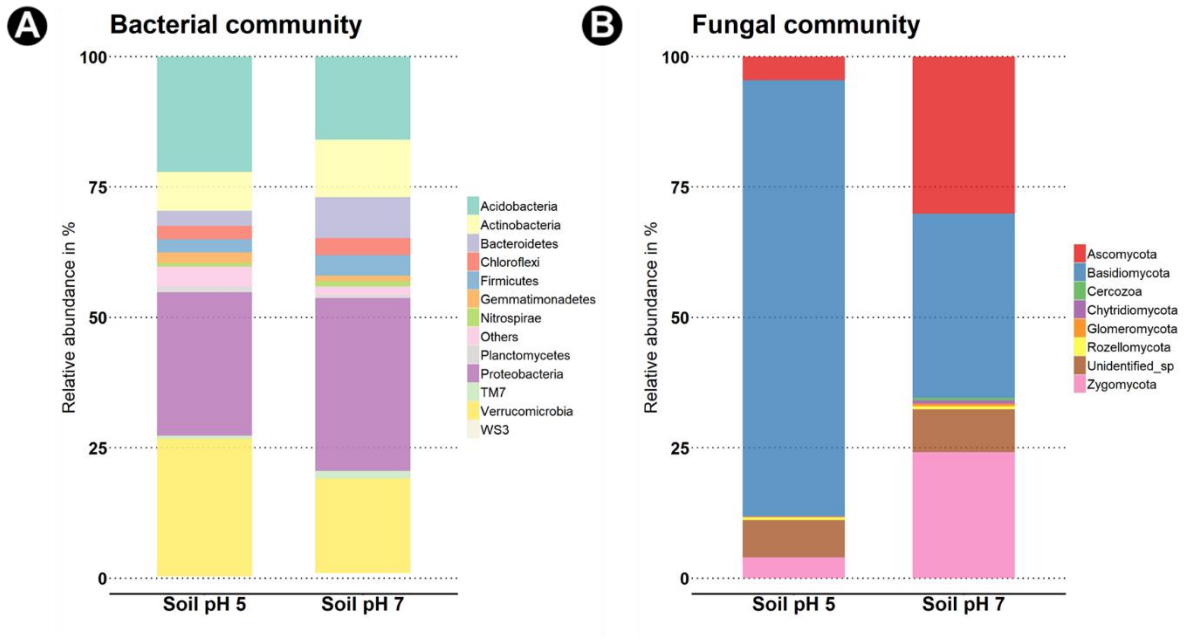


Fig. 2. Stacked bar plots showing the mean relative proportion of abundant phyla (>0.5 %) for bacterial (A), and fungal communities (B), in grassland soils maintained long-term at either pH 5 or 7.

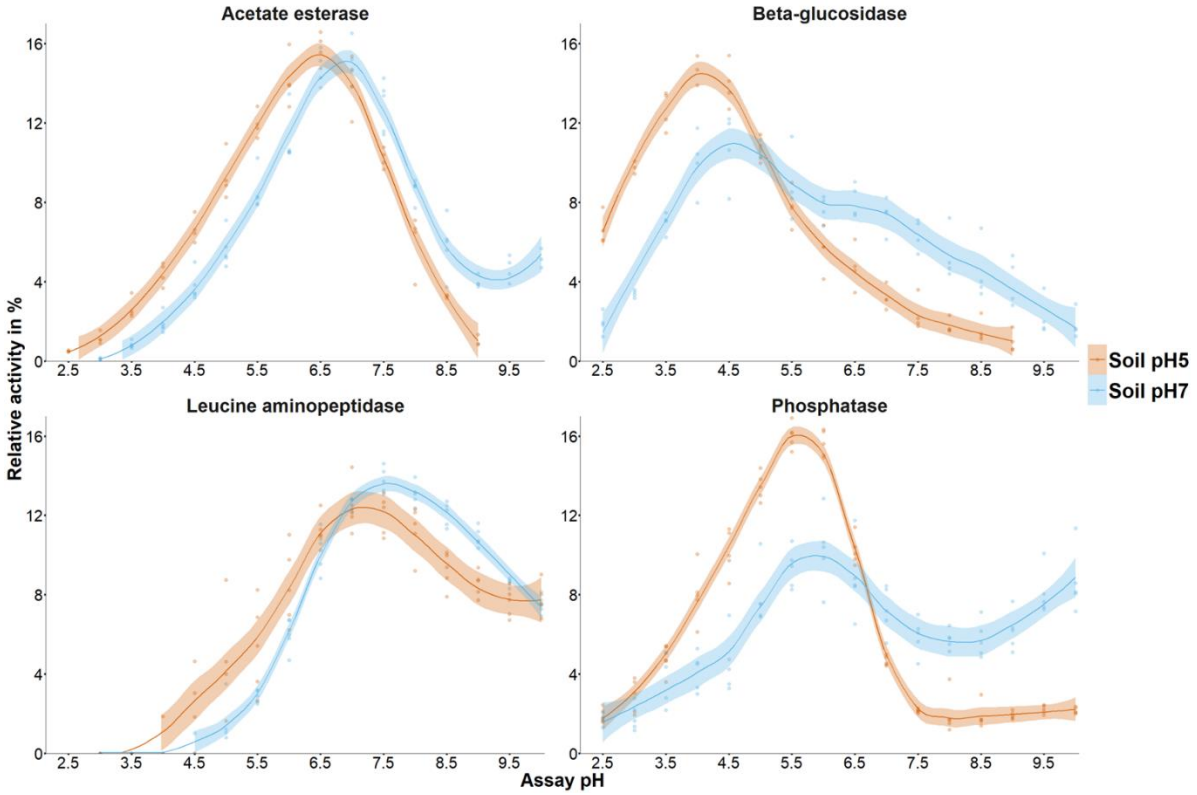


Fig. 3. pH optima of acetylesterase (A), beta-glucosidase (B), leucine aminopeptidase (C), phosphomonoesterase (D) from grassland soils maintained at either pH 5 or 7. Activity is expressed as a percentage of the total activity measured across the entire pH range assayed (from pH 2.5 to pH 10). The orange and blue lines correspond to pH 5 and soil pH 7 soils, respectively. Shaded area represents 95% confidence intervals around the trend line using a t-based approximation (LOESS smoothing).

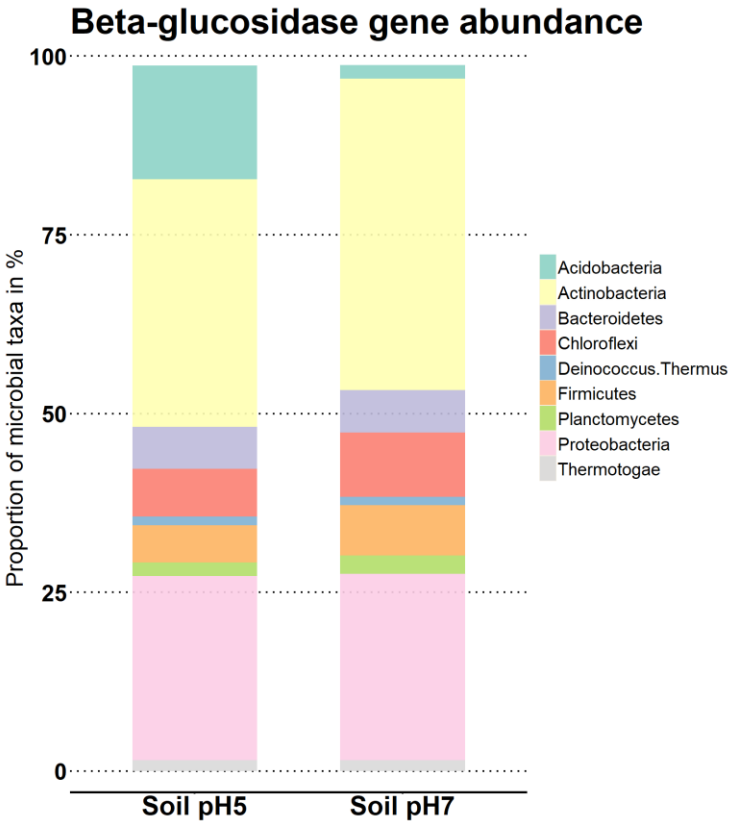
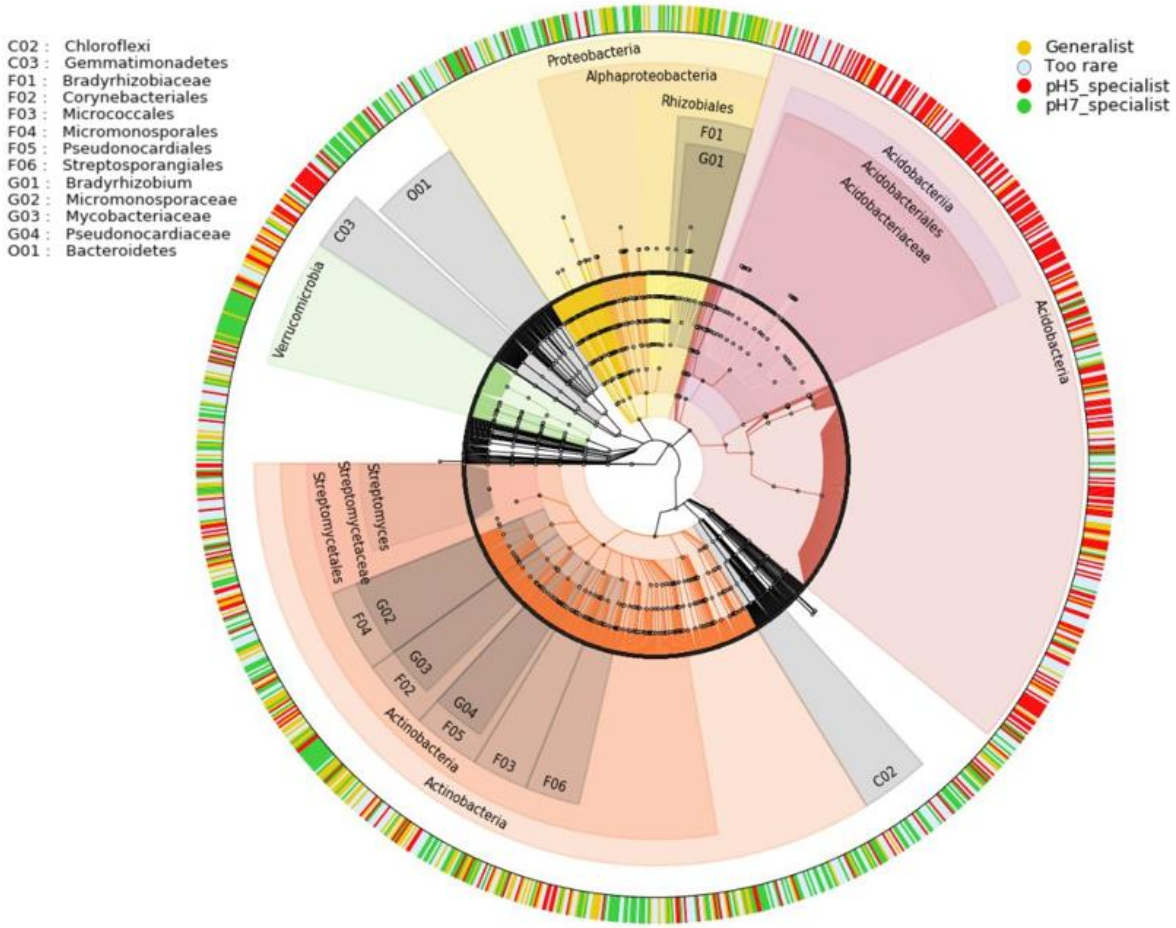


Fig. 4. Mean abundances of beta-glucosidase genes from different microbial phyla, from MG-RAST annotated metagenomes (SEED Subsystems) from grassland soils maintained at either pH 5 or 7.



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734 **Fig 5.** Detailed taxonomy and pH associations of β-glucosidase sequences assembled from
735 metagenomes, showing Acidobacterial β-glucosidases are predominantly associated with the
736 more acid soil. Inner tree and labels depict the taxonomy (from phylum to genus) of β-
737 glucosidase gene assemblies constructed from pooled metagenomes from the pH 5 and pH 7
738 soils (n=4). Outer ring shows putative pH associations of each assembled gene, following
739 tabulation of reads mapped to the contigs from each of the 8 soil metagenomes, and statistical
740 classification using a multinomial model based on relative abundance across the two soils.

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Supplementary Materials

Table. S1: Relative proportion of the main abundant phyla (>0.5 % proportion) for bacterial and fungal phyla at soil pH 5 and soil pH 7.

Bacterial phyla	Soil pH 5		Soil pH 7	
	mean	se	mean	se
Acidobacteria	22.15	1.87	15.95	1.15
Actinobacteria	7.43	0.54	11.02	0.92
Bacteroidetes	2.87	0.66	7.83	0.96
Chloroflexi	2.54	0.20	3.24	0.42
Firmicutes	2.60	0.35	3.94	0.69
Gemmatimonadetes	2.02	0.54	1.10	0.24
Nitrospirae	0.64	0.16	0.94	0.17
Planctomycetes	1.08	0.15	0.55	0.08
Proteobacteria	27.48	1.11	33.20	1.34
TM7	0.65	0.08	1.42	0.23
Verrucomicrobia	26.31	1.73	18.13	1.41
WS3	0.37	0.06	0.98	0.20

Fungal phyla	Soil pH 5		Soil pH 7	
	mean	se	mean	se
Ascomycota	4.54	1.11	30.13	6.38
Basidiomycota	83.56	3.31	35.27	2.08
Cercozoa	0.04	0.02	0.51	0.11
Chytridiomycota	0.05	0.02	0.63	0.20
Glomeromycota	0.16	0.09	0.52	0.17
Rozellomycota	0.50	0.16	0.53	0.24
Zygomycota	4.03	2.00	24.12	4.53
Unidentified_sp	7.13	2.29	8.28	0.80

Table. S2: Percentage of beta-glucosidase gene sequences per bacterial phylum and found only at pH 7 soil (Specialist pH7), only at pH 5 soil (Specialist pH5), in both soils (Generalist) or too rare.

Phyla	Generalist	Specialist_pH7	Specialist_pH5	Too_rare
Unclassified Bacteria	25,0	8,3	8,3	58,3
Acidobacteria	6,9	4,7	48,3	40,1
Actinobacteria	20,3	28,5	14,6	36,7
Armatimonadetes	0,0	33,3	0,0	66,7
Bacteroidetes	5,9	47,1	7,8	39,2
Calditrichaeota	0,0	50,0	0,0	50,0
Zixibacteria	0,0	0,0	0,0	100,0
Candidatus Melainabacteria	0,0	0,0	100,0	0,0
Chloroflexi	2,8	30,6	11,1	55,6
Cyanobacteria	100,0	0,0	0,0	0,0
Deinococcus-Thermus	50,0	25,0	0,0	25,0
environmental samples	10,0	40,0	0,0	50,0
Euryarchaeota	25,0	25,0	0,0	50,0
Firmicutes	4,5	36,4	22,7	36,4
Gemmatimonadetes	9,7	3,2	58,1	29,0
Ignavibacteriae	0,0	33,3	33,3	33,3
Lentisphaerae	0,0	0,0	0,0	100,0
Planctomycetes	0,0	33,3	50,0	16,7
Proteobacteria	19,2	36,2	5,6	39,0
Spirochaetes	25,0	50,0	0,0	25,0
unclassified Bacteria	25,0	16,7	8,3	50,0
Verrucomicrobia	27,3	34,8	16,7	21,2

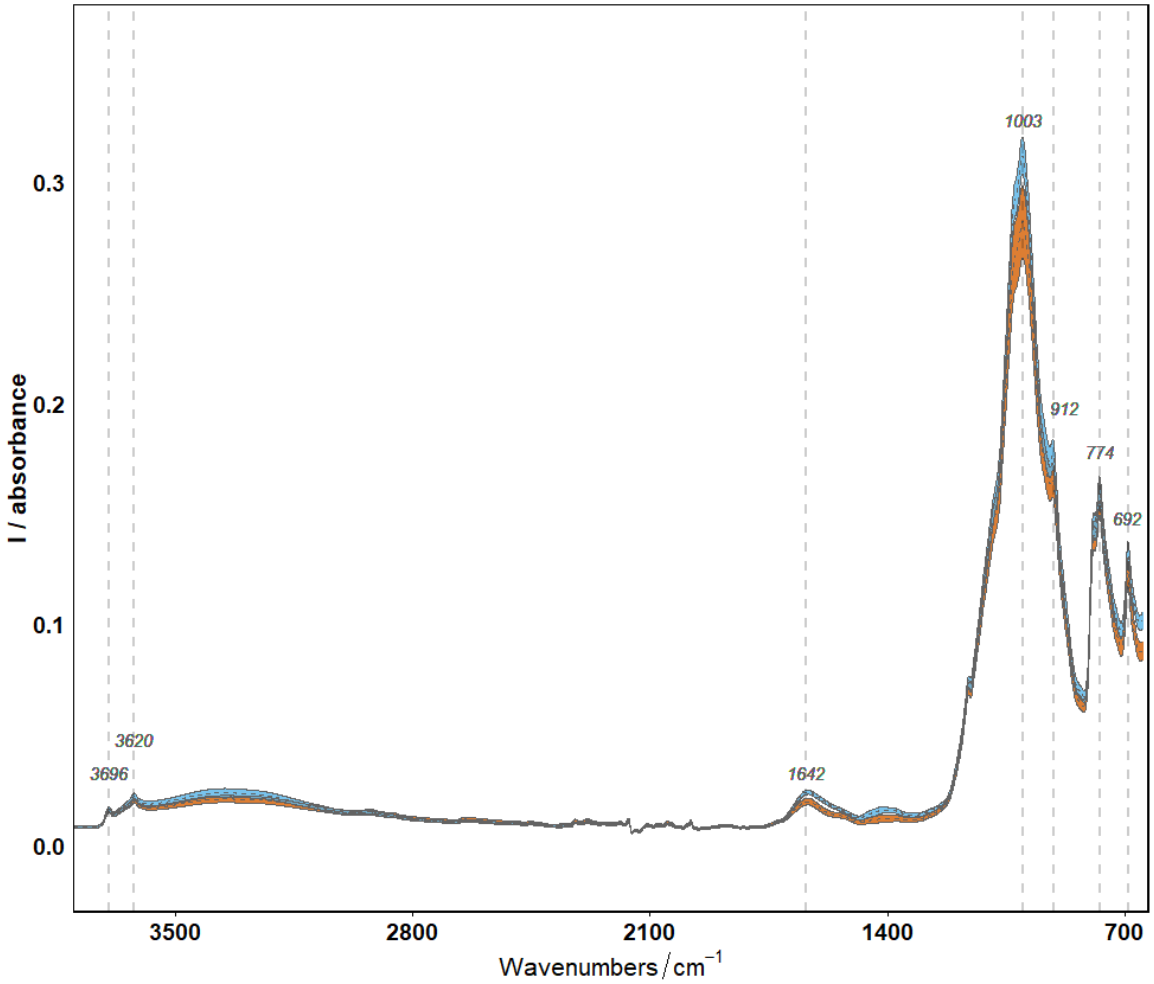

Fig. S1. Soil mid-infrared spectra for soils Nil plot pH 5 and Nil plot pH 7. Orange spectra correspond to soil pH 5 and blue spectra correspond to soil pH 7. The mid line indicates the mean spectrum (n=5) and the upper and lower lines indicate +/- standard deviation. Numbers written above spectra peaks indicate the wavelength for the main mid-infrared peaks observed.

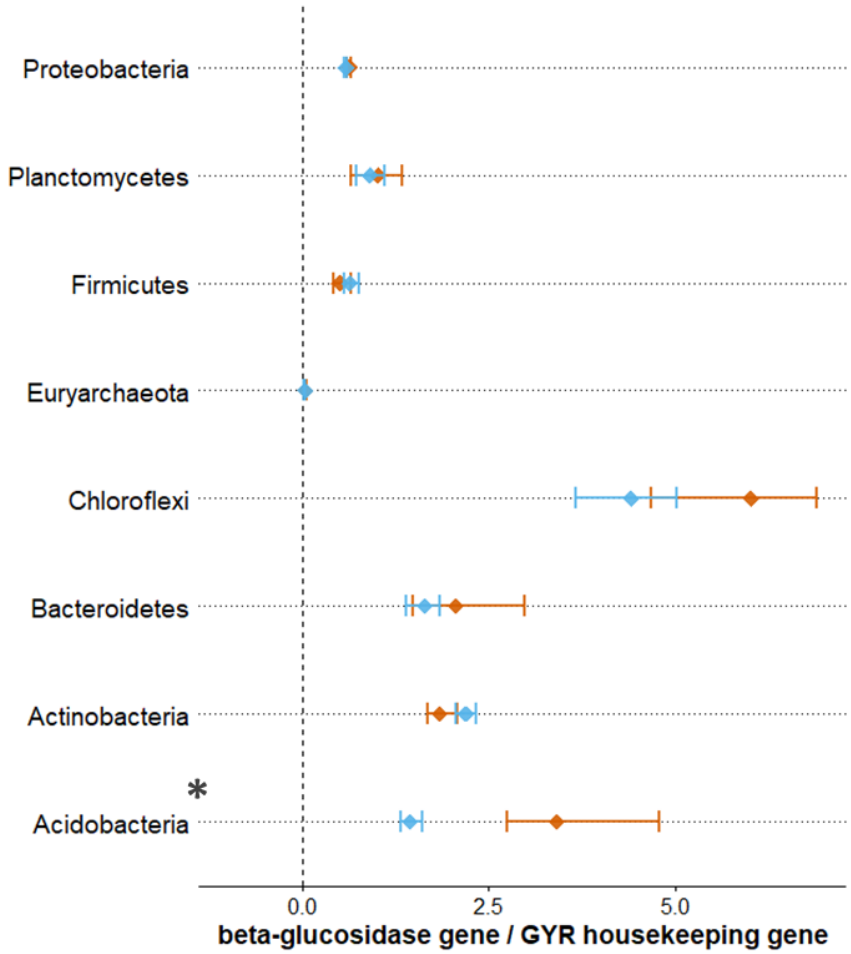

Fig. S2. The proportional change of beta-glucosidase gene abundance from different phyla, normalised to a housekeeping gene (DNA gyrase subunit B). Normalizing by housekeeping gene copy number allow evaluation of change in beta-glucosidase gene abundance regardless change in taxa abundance. Orange and blue colors correspond to pH 5 and pH 7 soil respectively. The x-axis shows the 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 $p<0.05$).