Final Report

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Student Project No. 21140029

Title: High throughput sequencing to measure changes in soil biology in response to long-term management practices

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Student Report No: 57

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1. Industry Summary

Biological communities have been described as an important factor in soil health, whether recycling nutrients, improving plant growth, suppressing plant pathogenic organisms or forming beneficial symbiotic associations with plants.

The present study aims to improve understanding of the diversity of soil biology and how it responds to common management practices within the agricultural sector so that soils can be managed to safeguard essential biological functions that ensure crop productivity as well as ecological and human sustainability.

This project aimed to expand the knowledge base on soil health by furthering understanding of the effects of common soil management practices (including organic and pH amendments, cultivations, crop rotations and cover cropping) on the soil microbiome. The project also aimed to improve grower understanding of the importance of the biological component of soil health and the practical approaches that can be used to sustain productive soils into the future across rotations that include grassland, arable and horticultural crops.

Soils sampled from various long-term soil management experiments have been analysed to assess the effects of routine management practices on the soil microbiome. A combination of six experimental sites within the AHDB Soil Biology and Soil Health (SBSH) Partnership were investigated to study long-term effects on soil biology of different organic soil amendments (at ADAS-Terrington, ADAS-Gleadthorpe and Harper Adams University), drainage treatments (at ADAS-Boxworth), tillage approaches (at GWCT-Loddington), pH levels and fertiliser applications (at SRUC-Craibstone). Soil biodiversity was analysed using metabarcoding procedures following PCR amplification of 16S and ITS rRNA gene markers to assess bacterial and fungal community diversity. Analyses were performed using the open-source software bioinformatics pipeline QIIME2 to assess the main changes in soil microbiome arising from differences in soil management practices. The data show a significant effect of pH on fungal and bacterial communities. Different fertiliser, cropping sequence, sampling season, organic amendments, soil compaction, drainage and tillage applications also affected soil microbes, but the sizes of these effects were much smaller than for pH. However, the striking observation was that agronomic soil managements had less impact on soil microbial diversity than the effects of geographical location or season. The main conclusion was therefore that natural variation in soil biology, either between soil types at different locations or between seasons at the same locations, was much greater than the variation attributed to agronomic soil management. The overall effects of agronomic practices on soil biology are therefore likely to be location and season specific, meaning that localised monitoring will be required over multiple seasons in order to establish best soil management practices for a given location.

2. Introduction

2.1. Effect of soil management on soil microbiology

Two conventional key soil chemistry management interventions revolve around manipulating crop nutrient availability, either indirectly by altering pH or directly via fertiliser addition. Soil pH, which defines nutrient availability (Binkley & Vitousek, 1989), is known to be one of the strongest abiotic factors driving bacterial and fungal community assemblages in multiple studies (Borneman & Hartin, 2000; Fierer *et al.*, 2005; Fierer & Jackson, 2006; Rousk *et al.*, 2010) including in agricultural soils (Wang *et al.*, 2019). Fertiliser application has also been shown to affect the composition of bacterial and certain groups of fungal communities depending on the type of phosphate fertilisation (Silva *et al.*, 2017). In addition, there is evidence for the inhibitory effect of triple superphosphate fertiliser on mycorrhizal formation (Peine *et al.*, 2019). However, to date, the general effect of fertiliser applications on soil bacterial and fungal diversity remains unclear.

Soil management practices have mostly developed with the aim of conserving soil physical structure and chemical fertility, but less is known of how they affect soil biological activity. Such practices include managing organic matter input amounts and types, minimizing soil structural disturbance (e.g. minimum or no tillage cultivations), and maintaining and diversifying plants through mixed cropping, crop rotations and/or cover cropping (Larkin, 2015). However, the relative effects of traditional and conservation agricultural soil management practices across different regions and soil types on biological communities and their functions remain unclear due to the lack of a standardized approach for accurately measuring soil biodiversity. As a result, the relationships between soil biodiversity, soil management and crop health and productivity remain poorly described.

2.2. Role of molecular biology in monitoring soil microbiology

Modern high-throughput DNA sequencing and associated bioinformatic tools have the potential to comprehensively characterize microbial communities (George *et al.*, 2019; Tedersoo *et al.*, 2020). Lauber *et al.* (2009) were one of the first to use such an approach to describe a correlation between soil bacterial community structure and pH from a wide array of ecosystem types. DNA sequencing studies have not achieved an agreement on the effect of fertiliser application on bacterial and fungal communities: some report fertiliser application increases richness and diversity (Pan *et al.*, 2020; Wang *et al.*, 2017) while others found no significant influence of fertilisers (Yao *et al.*, 2018). More generally, recent soil DNA sequencing studies have shown significant but small and inconsistent differences between fungal (Hannula *et al.*, 2021; Morrison-Whittle *et al.*, 2017) and bacterial (Hendgen *et al.*, 2018) diversity in soils under conventional versus conservation agricultural management approaches. These include simultaneous analyses of bacteria and fungi (Hartmann *et*

al., 2015), and total soil biology across time and space (Giraldo-Perez *et al.*, 2021), and include attempts to analyse the functions of these communities (Harkes *et al.*, 2019). Recent studies have also shown how fungal community structure and functionality (Hannula *et al.*, 2021), as well as bacterial diversity (Hartmann *et al.*, 2015), have been affected by different long-term agricultural practices such as tillage, cover cropping and organic amendments. Moreover, Hannula *et al.* (2021) and Giraldo-Perez *et al.* (2021) concluded that different components of soil biodiversity responded differentially to agricultural practices depending on geographic location and time of year. In general, studies to date have focused on different agricultural system types, and only evaluated soils in one or a few locations and timepoints and have lacked methodological and analytical standardisation making it very hard to cross-compare studies to evaluate any general effects on soil biology. There is an urgent need for a standardised approach for measuring soil biodiversity to allow meaningful comparisons and to quantify the effects of soil management practices across agricultural systems, climates, and soil types.

3. Materials and methods

3.1. Method optimisation

A DNA metabarcoding approach was optimised and trialled to test the extent to which changes in bacterial and fungal diversities in response to key soil management practices can be quantified by analysing total DNA extracted from the soil. For this, the hypothesis was tested that long-term differential pH and fertiliser managements have correspondingly applied varying selection pressures on the diversity of soil microbial communities. The standardised approach was then tested by comparing microbial communities in soil samples from two of the long-term field trials investigated in Project 4 of the SBSH Partnership, that have consistently manipulated pH and inorganic fertiliser input in different plots at the Craibstone Estate in Aberdeenshire over the last 50-100 years. In addition, new bioinformatic approaches were evaluated to determine whether observed diversity within the DNA of microbial communities may be used to predict shifts in their biological functions.

3.1.1. Soil sampling and DNA extraction

At the start of the project (July 2017) soil was sampled from 21 sites representing all cropping rotations and soil types at trial sites across the UK to be studied during the SBSH Partnership (Table 1; see also the report for Project 4 of the Partnership, 91140002-04). Composite samples of 2 kg of soil were collected from each site as multiple 1.5 cm diameter cores collected from the top 10-15 cm soil, collected in a ‘W’ pattern transect. Samples were thoroughly mixed to homogeneity after collection. Samples were kept refrigerated until required for DNA extraction.

Table 1. Soil sampled from different trial sites under various crop rotations and management conditions

Site	Soil texture (% clay)	Treatments & design
1.Terrington (Tebbs South)	Silty clay loam (28% cl)	1. Manufactured fertiliser (RB209 recommendations) 2. Green compost (@250 kg N/ha; *c.*25 t/ha) 3. Pig FYM (@250 kg N/ha; *c.*35 t/ha) 3 replicates (9 plots)
2.Harper Adams	Sandy loam (12% cl)	1. Manufactured fertiliser (RB209 recommendations) 2. Green compost (@250 kg N/ha; *c.*25 t/ha) 3. Cattle FYM (@250 kg N/ha; *c.*35 t/ha) 3 replicates (9 plots)
3.Gleadthorpe (Lamb field)	Loamy sand (6% cl)	**1. Manufactured fertiliser (RB209 recommendations)** **2. 10 t/ha Broiler litter** **3. Green compost (@250 kg N/ha; *c.*25 t/ha)** **4. Cattle FYM (@250 kg N/ha; *c.*35 t/ha)** **5. Cattle slurry (@250 kg N/ha; *c.*80 m_3/ha)** 3 replicates (15 plots)
4. Boxworth (40 Acres)	Clay (35% cl)	1. Improved drainage (moled autumn 2017) 2. Poor drainage 6 replicates
5.Loddington (GWCT)	Clay (40% cl)	1. Zero till (6+ yrs) 2. Conventional plough Tillage treatments to be introduced in autumn 2017; 3 and 6 replicate treatment areas in 2018 and 2020 respectively
6. Craibstone (Woodlands field)	Sandy loam (12% cl)	Large plots (45 x 5m) following the rotation: **Swede, Barley**, Hay, Pasture, **Pasture, Oats** (each crop present every year) 2 fertiliser subplots: *1. No fertiliser* *2. Complete fertiliser + superphosphate* *Soil health assessment to be undertaken in 4 crops & 2 fertiliser treatments (in bold)*
7. Craibstone (Woodlands field)	Sandy loam (12% cl)	Large plots (45 x 5m) following the rotation: **Potatoes**, **W. Wheat**, Hay, Pasture, **Pasture**, **Oats,** Swede, S. Barley (each crop present every year) 7 pH subplots 4.5-7.5 in 0.5 increments Soil health assessment to be undertaken in 4 crops (in bold type) & 4 pH levels (**4.5, 6.0, 6.5 & 7.5**)

Three replicate soil samples per plot were collected in October 2018 and 2019 from the pH and fertiliser trials at Scotland's Rural University College (SRUC), Craibstone Estate. Plots in the pH trial had been maintained for 50 years at pH gradients from 4.5 – 7.5 by annual application of lime or ferric sulphate, as required. Each plot had been annually rotated between potatoes, spring barley, swede, spring oats, 3 years of perennial ryegrass/white clover pasture followed by winter wheat, such that all rotation stages were grown every year at each pH. Three replicate samples were collected in each year after cropping with either wheat, potato, oat or second year pasture from pH treatments 4.5, 6.0, 6.5 and 7.5, making a total of 48 samples. The long-term fertiliser trial comprised

six plots annually rotated between barley, the same 3 years of pasture, oats and potatoes with all crops grown every year since 1922. Each plot was split with one half treated with complete fertiliser and other half remained untreated. The complete fertiliser comprised N as ammonium nitrate, P as triple super phosphate (TSP) and K as muriate of potash (MOP).

The soil samples were thoroughly mixed and maintained at 4 °C prior to analysis. Total DNA was extracted from 10g sub-samples, within two weeks of sampling, using the DNeasy PowerMax Soil Kit (Qiagen, Carlsbad, CA, United States) following the manufacturer's instructions. The same protocol was followed for the other five experimental sites.

Twelve soil samples were also taken from the STAR project (Sustainability Trial in Arable Rotations), a long-term rotational and cultivation study at Stanaway Farm, Otley, Suffolk, UK (52°08'17"N 1°12'49"E) on a Beccles/Hanslope Series (heavy) clay soil. The experimental plot was uniformly cropped with winter wheat in 2018. The objective of this investigation was to measure changes of soil biology at two different seasons by sampling soils from the same plots in spring and autumn the same year.

In addition, as part of the AHDB Horticulture FV 450/450a long-term asparagus field trial (Mašková *et al.*, 2021), 24 soil samples were collected and processed following the above mentioned protocol to evaluate the effect of soil disturbances on soil biology. The trial comprised 4 management practices: (1) companion crops - Rye (*Sereale cecale* L var. Protector.) and Mustard (*Sinapis alba* L. var. Severka), (2) interrow surface mulch (Straw and PAS 100 compost) applications in combination with shallow soil disturbance (SSD), (3) modifications of the conventional tillage practice by not re-ridging (NR) and applying SSD and (4) a zero-tillage option (Table 2).

Table 2. Treatments used in the 24 sampled soils from the project AHDB Horticulture FV 450/450a long-term asparagus field trial.

Treatment Group	Treatment	Re-ridge
M	PAS100	NR
M	PAS100	NR
M	PAS100	R
M	PAS100	R
M	StrawMulch	NR
M	StrawMulch	NR
M	StrawMulch	R
M	StrawMulch	R
CC	Mustard	NR
CC	Mustard	NR
CC	Mustard	R
CC	Mustard	R
CC	Rye	NR
CC	Rye	NR
CC	Rye	R
CC	Rye	R
BS	NoSSD	NR
BS	NoSSD	NR
BS	NoSSD	R
BS	NoSSD	R
BS	SSD	NR
BS	SSD	NR
BS	SSD	R
BS	SSD	R

3.1.2. Metabarcoding

Bacterial and fungal V4-16S and ITS1 rRNA barcodes were amplified from the total extracted DNA via PCR using 515F (Parada et al., 2016) and 806R 16S primers (Apprill *et al.*, 2015), and ITS1-F_KY02 ITS forward primer (Toju *et al.*, 2012) with a modified ITS2 (Table 3). For high throughput sequencing of the PCR products, indexed libraries were prepared using Nextera (Illumina) adapters following the manufacturer's protocol. A PhiX internal control was added to the library pool before sequencing with 600 cycles reagent kit v3 (2x300 PE) using a MiSeq sequencer (Illumina, San Diego, CA, USA). DNA from each trial was sequenced and analysed separately.

Table 3. Primer sequences used for amplifying the bacterial and fungal gene barcode regions that were subsequently sequenced using the metabarcoding approach.

16Sv4	F	515F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3'
	R	806R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3'
ITS1	F	ITS1-F_KY02	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAGAGGAAGTAAAAGTCGTAA-3'
	R	ITS2_Wobble	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCWGYGTTCTTCATCGATG-3'

3.1.3. Data analysis

Each of the two Craibstone trials was first analysed independently. Sequences of all PCR products from amplification of the total fungal and bacterial DNA in each soil sample were compared using the microbiome bioinformatics platform QIIME2 v. 2020.6 (Bolyen *et al.*, 2019) complemented with various R (R Core Team, 2021) statistics packages. Only 16S and ITS sequence reads >50 bp were compared. 16S reads were truncated to 253 bp but ITS reads were not trimmed. Tables of the numbers of various amplicon sequence variants (ASVs), with exact sequence matches, were compiled using DADA2 software (Callahan *et al.*, 2016).

Taxonomic assignment of each ASV was attempted using a naïve bayes classifier trained with either a 16S sequence database (release 138) from SILVA (Quast *et al.*, 2012) or a modified ITS database (v. 8.2) from Unite (Nilsson *et al.*, 2018). Only bacterial ASVs that could be recognised at phylum level with 70% confidence were further considered. Only fungal ASVs recognised with 95% confidence as belonging to the kingdoms Fungi or Stramenopila (including oomycetes) were further considered. A full account of the standardised bioinformatics procedures and subsequent statistical analyses used has been submitted for publication (Fernadez-Huarte et al, 2022).

3.2. Standardised pipeline for analysis of combined data across different trials

A standardised approach for comparison of ASV communities between soil samples was based on three main classes of soil community metrics, as defined by Morrison-Whittle *et al.* (2017), Morrison-Whittle and Goddard (2018) and Giraldo-Perez *et al.* (2021), and this comprises the core of the standardized analysis proposed here (Figure 1). These were:

1. **Numbers**: Differences in the total number (absolute richness) of different ASVs (representing different taxa) and their distribution across sample treatments.
2. **Types**: Differences in the presence/absence of different types of taxa (clusters of related sequences) between samples.
3. **Abundances**: Differences in the relative proportions of taxa between samples inferred by sequence read counts. Where significant differences in taxa abundances are revealed, then this is followed by Indicator taxa and functional prediction analyses to highlight which taxa are significantly overrepresented in particular treatments and what functions are ascribed to these.

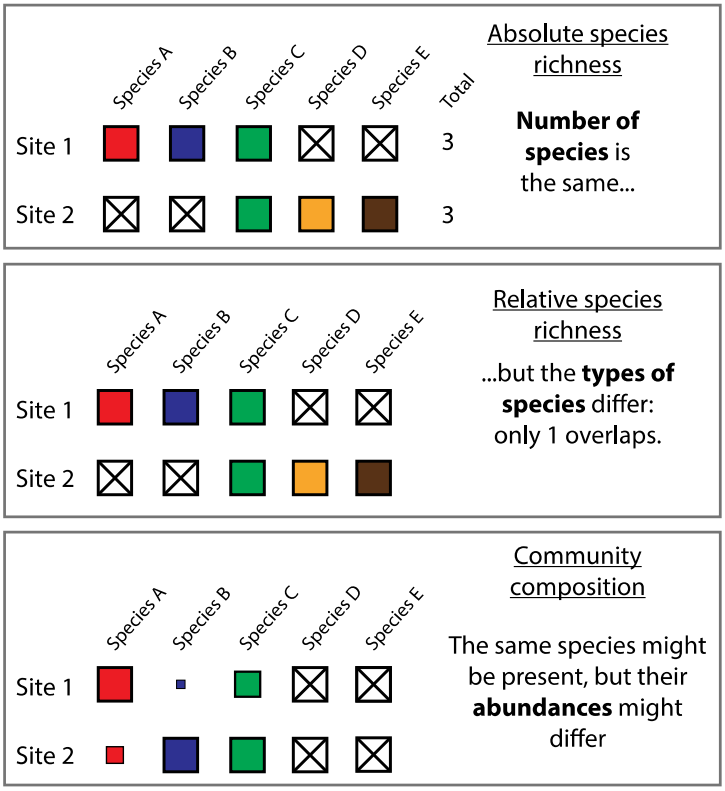


Figure 1. The three standard measures of biodiversity proposed for the soil DNA biodiversity standard analysis method following Morrison-Whittle *et al.* (2017), Morrison-Whittle and Goddard (2018).

Differences in absolute taxa numbers were evaluated with Kruskal-Wallis tests and effect sizes calculated with E2 = H/((n2-1)/(n+1)), where n = number of observations and H is the Kruskal-Wallis H-test statistic (Tomczak & Tomczak, 2014), and distributions of counts were analysed using Shannon (Shannon, 1948) diversities. In addition, phylogeny relatedness within different taxa was assessed using Faith's phylogenetic diversity analysis (Faith, 1992).

Differences in types and abundances of taxa were evaluated using weighted and unweighted Jaccard distance matrices respectively with PERMANOVA (Anderson, 2014) with 999 permutations to determine pseudo-F ratios to calculate P and R2 estimates of effect sizes. In addition, unweighted UniFrac was used to analyse phylogenetic differences between communities, and weighted UniFrac that also takes abundances into account giving more weight to most abundant taxa (Lozupone & Knight, 2005).

To identify those individual ASVs (potential biomarkers) that were most likely to explain observed differences in diversity between soil samples, an algorithm known as LEfSe (Linear discriminant analysis Effect Size) was tested (Guo & Gao, 2021; Segata *et al.*, 2011). Further software packages

were also evaluated for prediction of possible biological functions from identified ASVs. Bacterial functional predictions were estimated using the FAPROTAX tool (Louca *et al.*, 2016) on ASVs annotated using the SILVA database. For fungal functional predictions, the FunGuild tool (Nguyen *et al.*, 2016) was used on ASVs annotated using the UNITE database.

Analyses of the combined data from both trials were also performed. For comparing datasets from different trials, the sequence reads were first independently processed using DADA2, to account for potential differences between sequencing runs, prior to merging the datasets for analysis. Subsequently, the two generated tables were merged using the 'table merge' tool in Qiime2, which identifies identical ASVs between tables. To account for minor differences between ASVs from each trial, potentially resulting from differences in original orientation or trimmed length, ASVs in the merged table were subsequently clustered into groups of greater than 98% genetic identities and termed merged ASVs (mASVs) to delineate them from ASVs. mASV representative sequences for taxonomic identification were derived by VSEARCH (Rognes *et al.*, 2016) *de novo* clustering approach based on 98% identity threshold. A scaling with ranked subsampling (SRS) normalization method was then applied to the merged table (Beule & Karlovsky, 2020) to obtain an identical sample size for each data set as required in ecological analysis of taxa count. This SRS normalization method provides a more conservative approach than the traditional rarefying approach (random subsampling without replacement).

3.3. Correlations between microscopic observation and metabarcoding of mesofauna diversity

A second DNA metabarcoding run was performed with samples from the Craibstone pH trial to test the extent to which changes in mesofauna diversities in response to changes in soil pH levels. A comparison of results between sequencing and a direct microscopic assessment performed by Natural England was carried out to compare the two approaches.

3.3.1. Metabarcoding

An animal cytochrome oxidase (CO1) barcode was amplified from the total extracted DNA using PCR primers mlCOIintF (Leray *et al.*, 2013) and HCO2198 (Vrijenhoek, 1994) (Table 4). For high throughput sequencing of the PCR products, indexed libraries were prepared using Nextera (Illumina) adapters following the manufacturer's protocol. A PhiX internal control was added to the library pool before sequencing with 600 cycles reagent kit v3 (2x300 PE) using a MiSeq sequencer (Illumina, San Diego, CA, USA). DNA from each trial was sequenced and analysed separately.

Table 4. Primer sequences used for amplifying the arthropod gene barcode regions that were subsequently sequenced using the metabarcoding approach.

CO1	F	mlCOIintF	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TAAACTTCAGGGTGACCAAAAAATCA**-3'
	R	HCO2198R	5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGWACWGGWTGAACWGTWTAYCCYCC**-3'

3.3.2. Data analysis

Analyses were conducted following the standardize pipeline and ASVs were assigned using a naïve bayes classifier trained with a CO1 database developed at Fera Science Ltd. Only arthropod ASVs recognised at phylum level with 70% confidence were considered. Arthropod ASVs were subsequently clustered by VSEARCH (Rognes *et al.*, 2016) *de novo* clustering based on 97% identity threshold to emulate taxonomic assignment at species level.

Differences in taxa numbers were evaluated with Kruskal-Wallis tests and effect sizes calculated with E^2 = H/((n2-1)/(n+1)), where n = number of observations and H is the Kruskal-Wallis H-test statistic (Tomczak & Tomczak, 2014). Data from metabarcoding and microscopy approaches were compared according to (a) whether pH level or cropping stage of the rotation significantly affected arthropod richness, and (b) whether the results from pairwise tests showed similar patterns of differences in richness at species level. Correlations in types of taxa were evaluated with a two-sided Mantel test (Mantel, 1967) to identify any correlation between their binary Jaccard distance matrices.

4. Results

4.1. Effect of long-term pH manipulations on soil bacterial and fungal diversity

A total of 5,852,860 16S rRNA and 5,291,924 ITS sequence reads were generated from the 48 pH trial soil samples which clustered into 8,353 and 4,975 different ASVs assigned to bacteria and fungi-like organisms respectively. The 16S ASVs were allocated phylogenetically into 38 phyla, 120 classes, 274 orders, 414 families, and 684 genera. ITS ASVs represented 13 phyla (including the phylum Oomycota of the kingdom Stramenopila), 38 classes, 100 orders, 217 families and 394 genera.

Numbers: As expected, a significant effect of pH extremes on both 16S (P = 0.006) and ITS (P = 0.0005) ASV numbers was observed (Table 5, Figure 2). Kruskal-Wallis pairwise comparisons showed the effect of pH mainly manifested as lower numbers of ASVs at the lower pH of 4.5, with a minor significant difference also observed between pH 6 and 7.5 level for ITS ASVs (Figure 2). On average, there were 450 fewer 16S ASVs and 135 fewer ITS ASVs from pH 4.5 samples than from

samples at the other three pH levels. An average of 27% of the variance in ASV numbers was explained by the effect of pH (Table 5).

Table 5. Effect of long-term pH and fertiliser manipulations on soil microbial diversity.

Treatment		pH		Fertiliser		Differences
Barcode		16S	ITS	16S	ITS	
Numbers	P	**0.005**	**0.001**	0.267	0.644	Only pH sig.
	E^2	**0.230**	**0.338**	-	-	-
Type	P	**0.001**	**0.001**	**0.017**	0.083	pH sig. effect on both; fertiliser only on bacteria
	R^2	**0.387**	**0.276**	**0.056**	-	pH ~7x greater size effect on bacteria only
ASV relative abundance	P	**0.001**	**0.001**	**0.037**	**0.001**	pH greater sig.
	R^2	**0.446**	**0.348**	**0.06**	**0.149**	pH ~4x greater mean size effect

The effect of long-term pH and fertiliser manipulations on soil microbial diversity and a comparison of the effects. P values from Kruskal-Wallis tests for ASVs richness and PERMANOVA test on binary (types) and abundance based Jaccard distances assessing the effect of pH and fertiliser manipulations on bacterial (16S) and fungal (ITS) communities. Effect sizes (E^2 and R^2) for significant differences at P<0.05 (in bold) are shown.

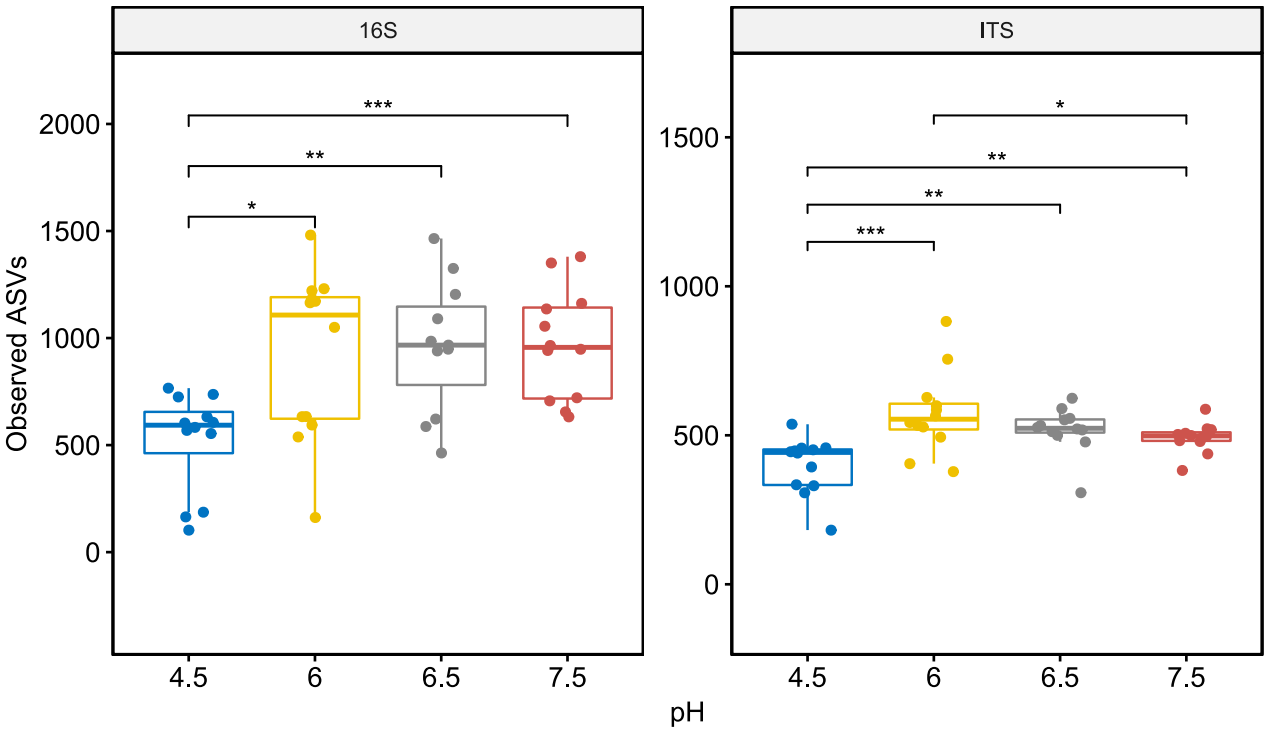


Figure 2. Boxplots comparing absolute ASV richness derived from bacterial (16S) and fungal (ITS) barcodes. Samples were grouped by soil pH level. Kruskal-Wallis pairwise results are indicated where adjusted p-value is significant (0 '***', 0.001 '**', 0.01 '*').

Types: PERMANOVA analyses revealed significant differences (P = 0.001) in the types of 16S and ITS ASVs present at each soil pH level (Table 5) and individual pairwise comparisons showed significant differences in the presences of 16S and ITS ASV types between all pH levels (P < 0.001; Table 5). Unweighted UniFrac analyses were also consistent with these findings (Table 6) and on average, from the mean of R^2 values across PERMANOVA and UniFrac analyses, pH explained 33% of the variance in the presence of ASV types.

Table 6. Kruskal-Wallis results for Shannon and Faith PD estimated diversities; along with PERMANOVA results for UniFrac distances assessing the effects of soil pH and fertiliser application on bacterial (16S) and fungal (ITS) communities. Bold font indicates significant values where p-value ≤ 0.05.

Treatment		**pH**		**Fertiliser**	
Barcode		**16S**	**ITS**	**16S**	**ITS**
Shannon	**P**	**0.001**	0.415	0.268	**0.050**
	E^2	**0.308**	-	-	**0.130**
Faith PD	**P**	**2.75E-05**	**0.045**	0.538	0.488
	E^2	**0.484**	**0.115**	-	-
Unweighted UniFrac	**P**	**0.001**	**0.001**	0.377	0.26
	R^2	**0.46**	**0.315**	-	-
Weighted UniFrac	**P**	**0.001**	**0.001**	0.979	**0.003**
	R^2	**0.754**	**0.559**	-	**0.153**

Abundances: Significant differences were also observed in the relative abundances of 16S and ITS ASVs at all pH levels (P = 0.001, Table 5) and pairwise analyses showed significant differences between all pH levels (P < 0.001) and the delineation of bacterial and fungal communities, especially at pH 4.5, could be clearly seen in PCoA plots (Figure 3). Again, UniFrac analyses were consistent with the signal from analyses of Jaccard dissimilarities, and collectively pH explained an average of 40% of the variance in ASV relative abundances.

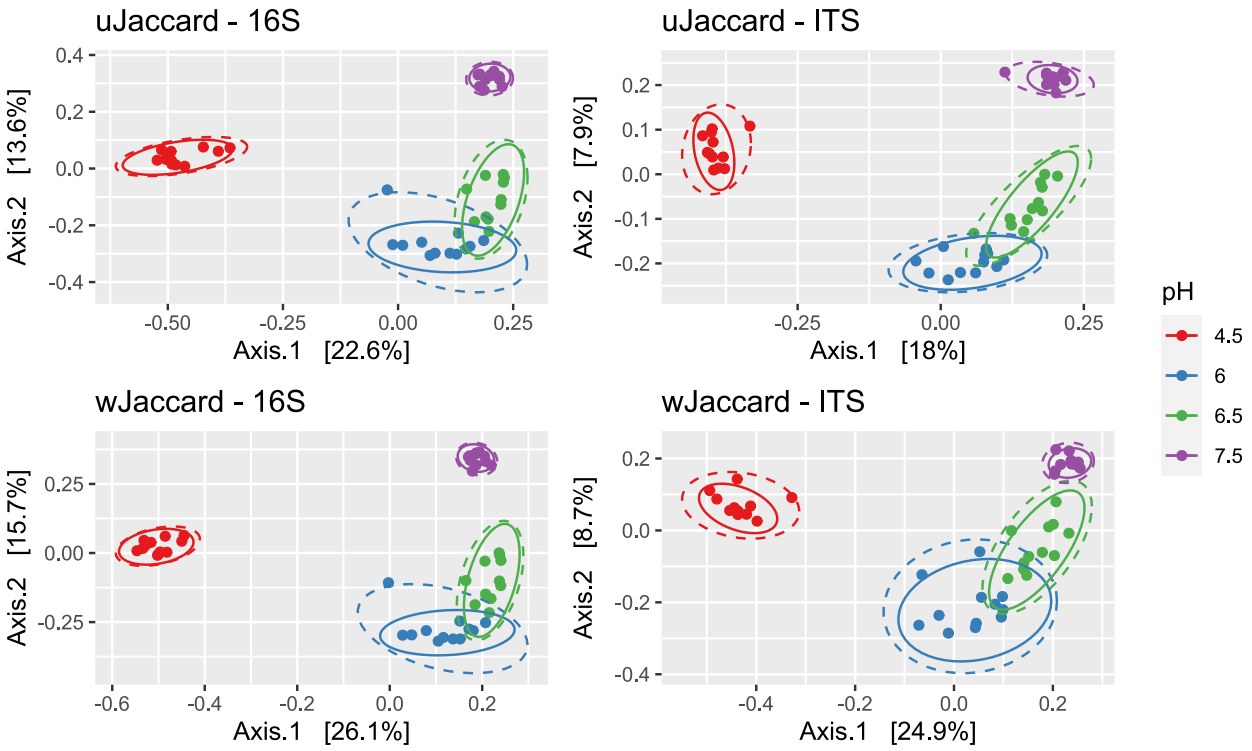
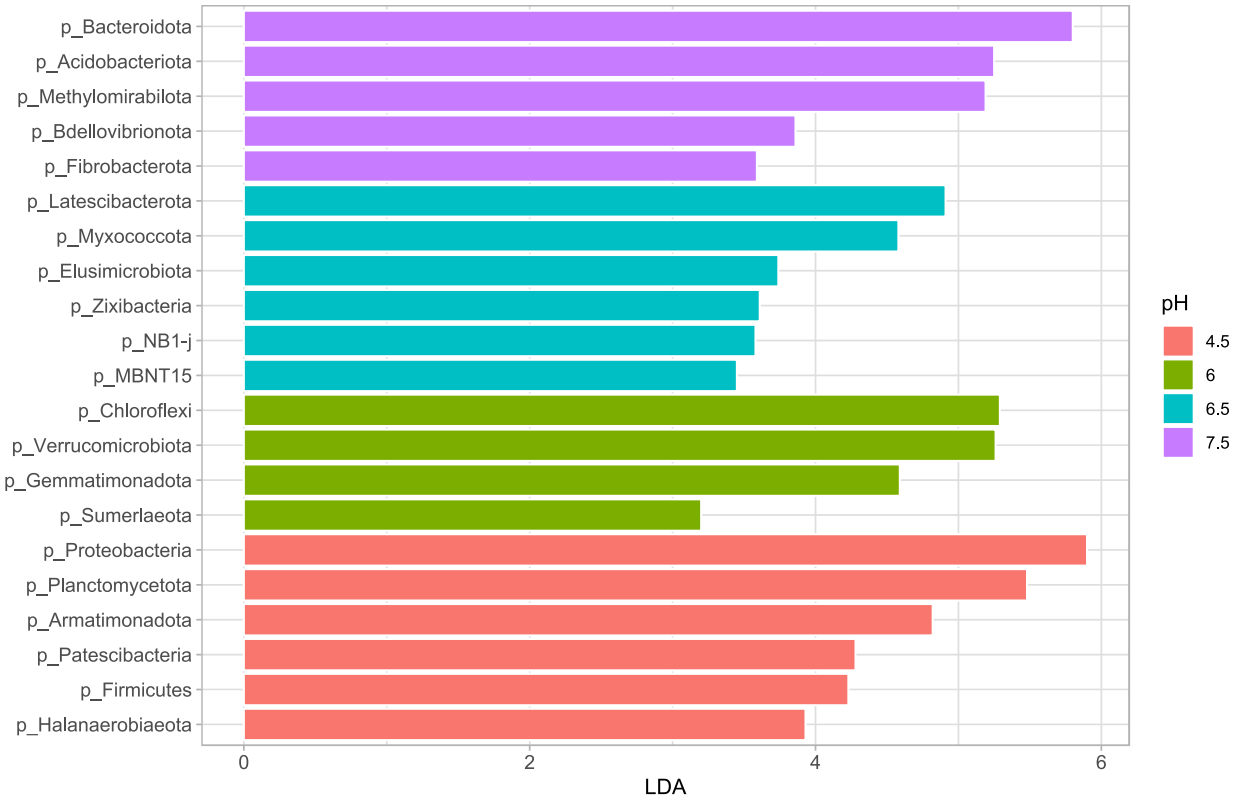


Figure 3. Principal Coordinate Analysis (PCoA) based on weighted and unweighted Jaccard distances matrices showing differences in relative abundances of 16S and ITS ASVs at each pH level.

Indicator taxa: LEfSe predictions suggested that a total of 1,583 16S and 583 ITS ASVs significantly (p.adj < 0.05) differed in abundance between pH levels spanning 21 assigned bacterial phyla (Figure 4a) and 8 fungal phyla (Figure 4b). The number of 16S ASVs overrepresented at pH 7.5 (671) was greater than those at other pH levels (mean = 395.75) and spanned ASVs assigned to ten different phyla (Figure 5a). The pattern for fungi was different with larger numbers of ITS ASVs overrepresented at both pH 4.5 (204) and 7.5 (181) compared to pH 6 and 6.5 (mean = 145.75, 95 and 103 at pH 6 and 6.5 respectively; Figure 5b), and the largest number of differentially abundant ASVs were assigned to Ascomycota.

(a)



(b)

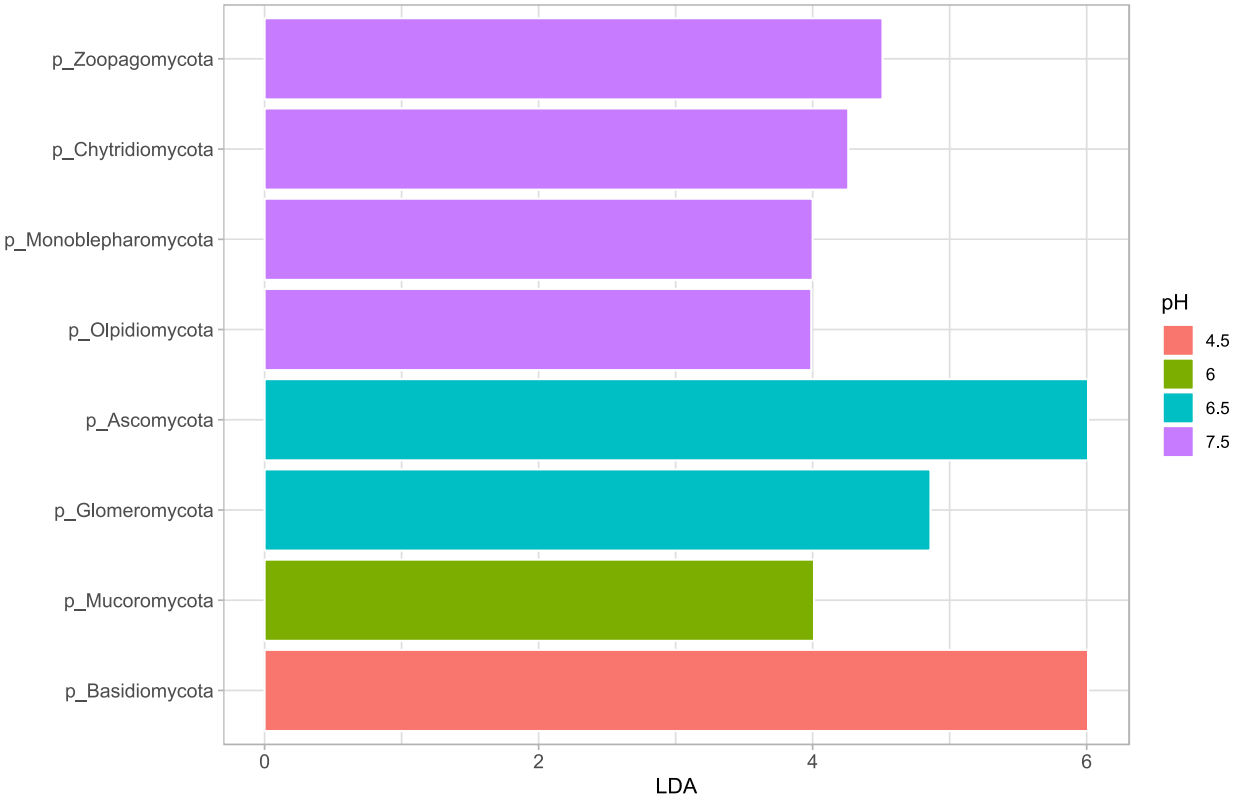


Figure 4. LEfSe prediction at phylum level of (a) 16S and (b) ITS ASVs as potential biomarkers of pH. Each predicted biomarker was ranked according to Linear Discriminant Analysis (LDA) effect size.

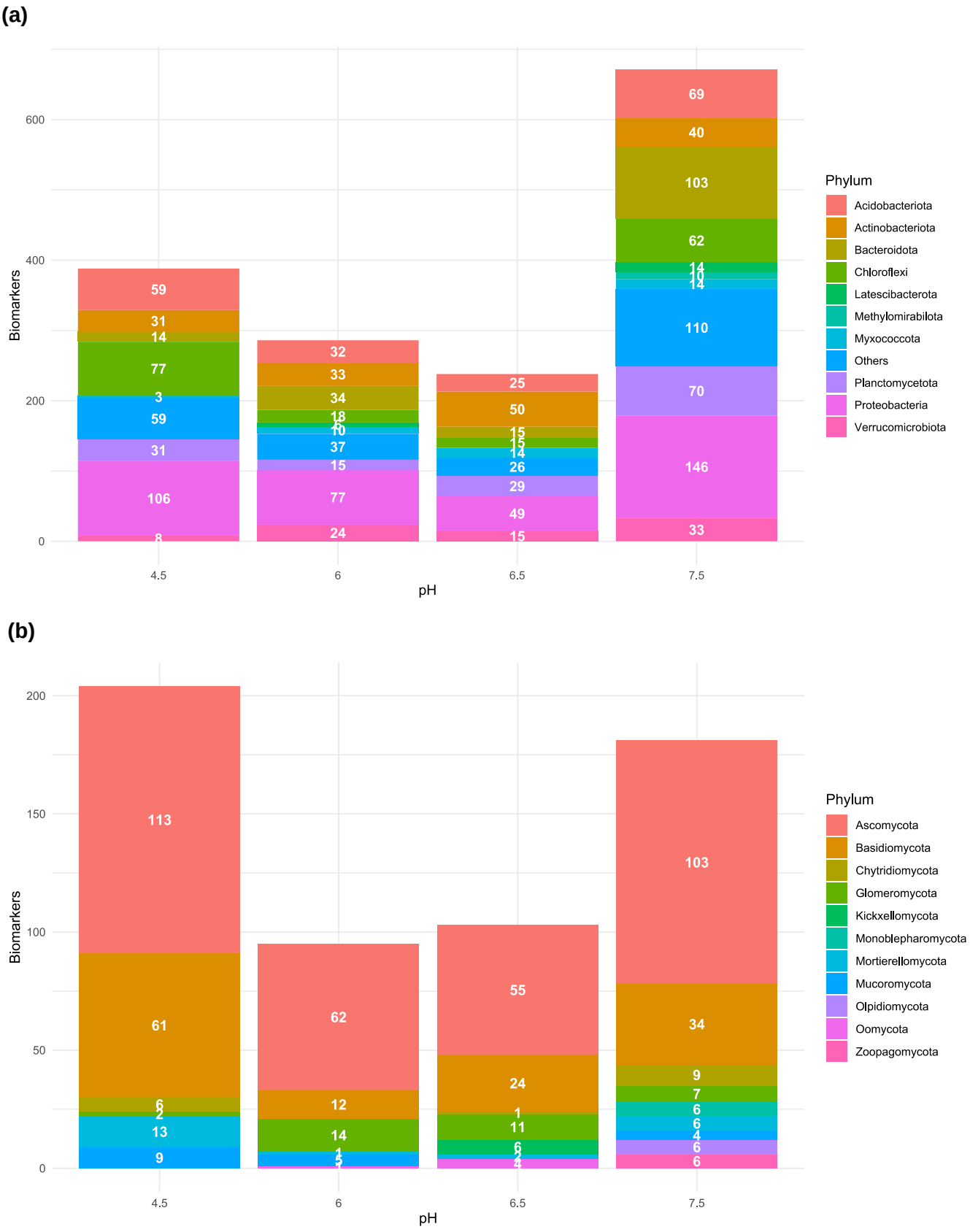


Figure 5. Histograms representing the number of total (a) 16S and (b) ITS ASVs per phylum predicted by LEfSe analysis as biomarkers of the different pH levels. “Others” includes a cluster of bacterial phyla, each with less than 40 total biomarkers.

Functional predictions: Due to the low resolution at species and genus levels using the SILVA database, only 12.7% of 16S ASVs could be assigned to a functional group by FAPROTAX. The largest assigned groups recognised were aerobic-chemoheterotrophs, animal parasites or symbionts, and chemoheterotrophic bacteria (Figure 6a). A greater taxonomic resolution at family, genus and species levels allowed 65% of ITS ASVs to be assigned to a trophic mode and guild (Figure 6b).

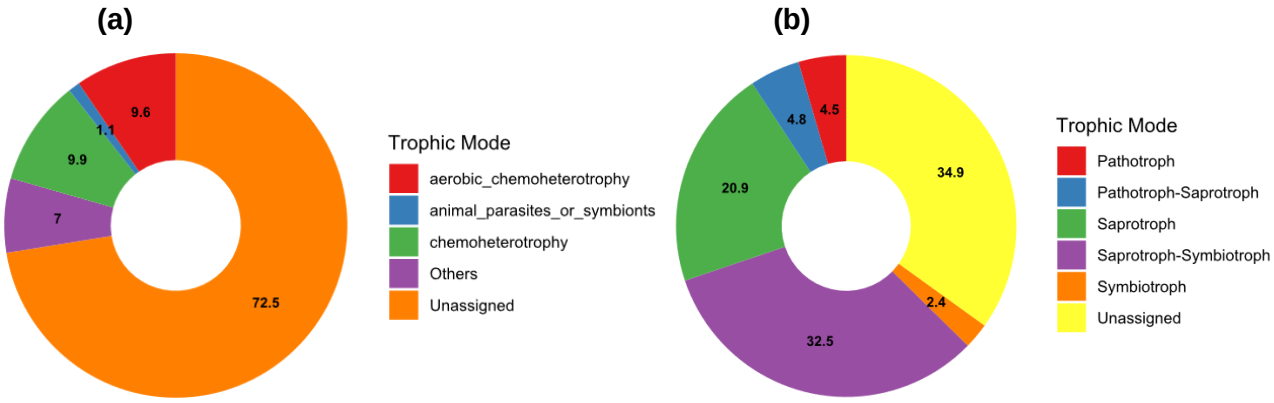


Figure 6. Proportion of (a) 16S and (b) ITS ASVs from the pH trial, assigned to functional groups and trophic modes. Bacterial functional groups with less than 0.01 abundance were clustered into the group “Others”. Fungal group “Unassigned” also contains ambiguous trophic modes assigned to “Pathotroph-Symbiotroph” and “Pathotroph-Saprotroph-Symbiotroph”.

Differential abundance analyses showed that fungal symbiotrophs were significantly ($p.adj < 0.05$) less abundant at pH 4.5 (Figure 7a), and pH 7.5 contained significantly ($p.adj < 0.05$) more fungal pathotrophs than pH 4.5 and 6 (Figure 7b).

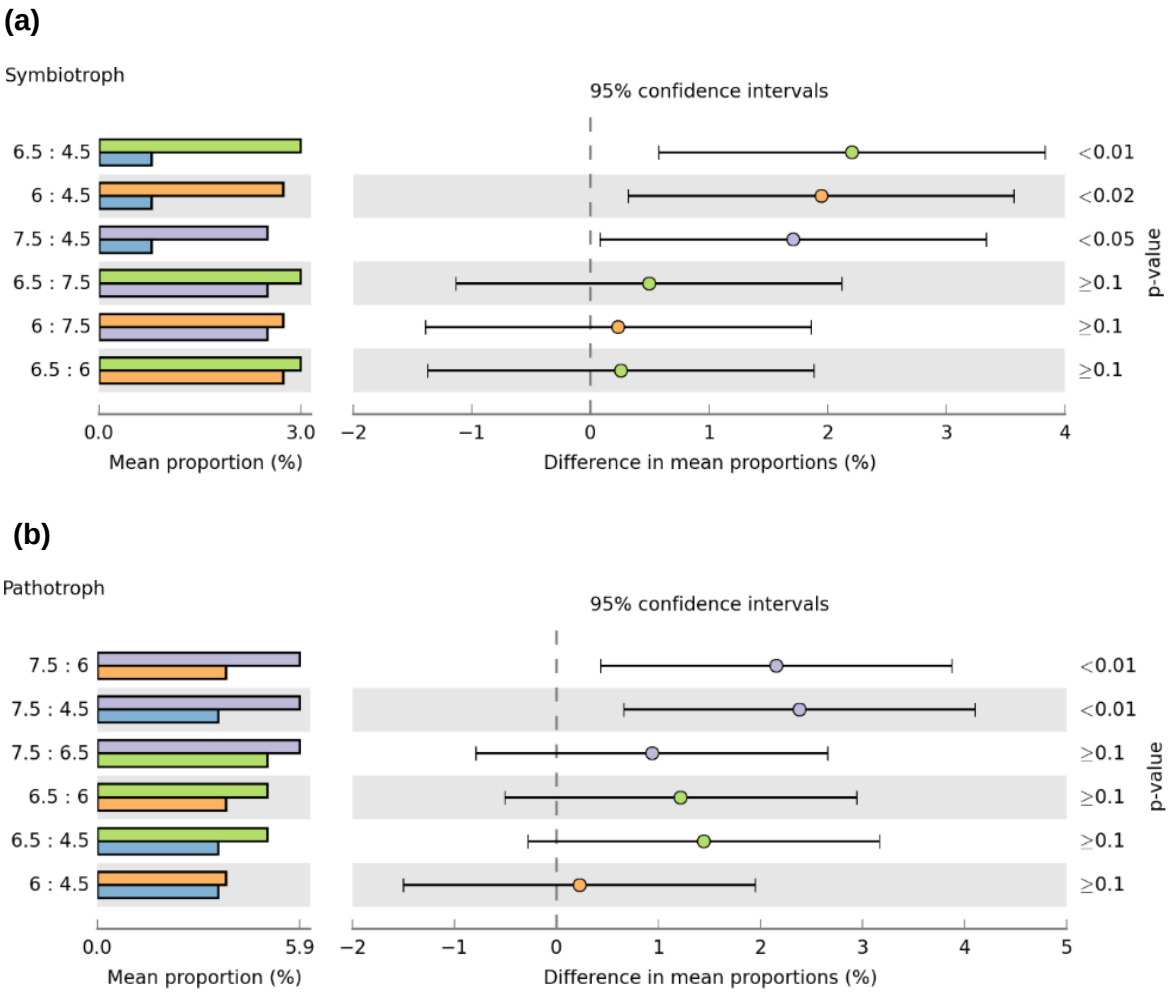


Figure 7. STAMP plot representing statistical pairwise comparing relative abundances of the trophic modes symbiotrophs (a) and pathotrophs (b) at different pH levels. Top bar of each pairwise comparison represent the treatment on the left while bottom bars represent the treatment on the right.

4.2. Effect of long-term fertiliser application on soil bacterial and fungal diversity

The 24 fertiliser trial samples yielded 2,596,928 16S and 2,347,133 ITS sequence reads which clustered into 5,188 16S and 3,458 ITS ASVs. 16S ASVs were phylogenetically allocated into 38 phyla (of which 9 were candidate phyla), 119 classes, 244 orders, 365 families and 568 genera. ITS ASVs represented 12 phyla (including the phylum Oomycota of the kingdom Stramenopila), 34 classes, 87 orders, 191 families and 336 genera.

Numbers: Analyses revealed no significant effect of fertiliser application on 16S or ITS ASV richness (P >0.25; Table 5) and this was consistent with Faith-PD analysis.

Types: PERMANOVA analyses revealed weakly significant differences between fertiliser treatments in the types of 16S ASVs (P = 0.017), which only explained approximately 6% of the variance in assigned bacterial types (Table 5). There were no differences in the types of ITS ASV between fertiliser treatments (P = 0.083), and unweighted UniFrac analyses revealed no effect of fertiliser treatment on either bacteria or fungi (Table 6).

Abundances: There were significant differences between fertiliser treatments in the relative abundance of types of both 16S (P = 0.037) and ITS (P = 0.001) ASVs which explained 6% and 15% of variance in ASV abundances respectively (Table 4, Figure 8). These analyses were in line with weighted UniFrac (P = 0.003) for fungi but not bacteria (P = 0.98) (Table 6).

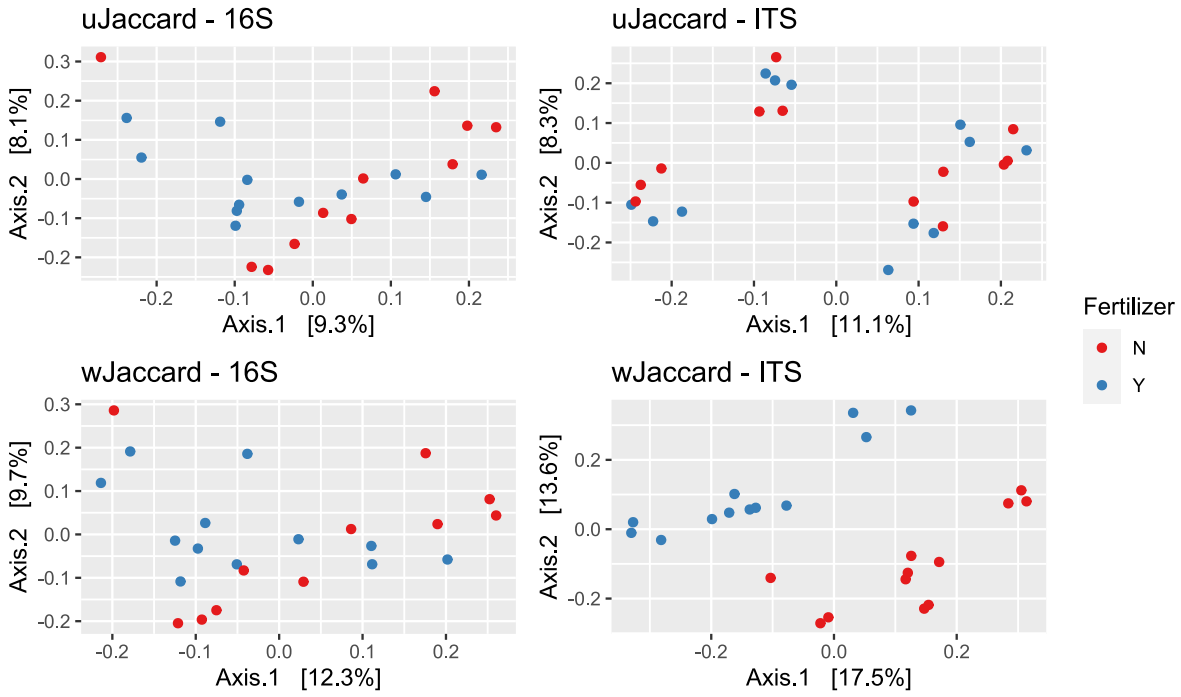


Figure 8. Principal Coordinate Analysis (PCoA) based on weighted and unweighted Jaccard distances matrices showing differences in relative abundances of 16S and ITS ASVs. Colours represent soil from plots treated with (Blue) or without (Red) fertiliser.

Indicator taxa: No 16S ASVs were identified as significantly overrepresented in one or other fertiliser treatment. Only 4 fungal Ascomycota ASVs, and one assigned to *Mortierella minutissima* (Mortierellomycota), were significantly associated with the no fertiliser control treatment.

Functional predictions: No bacterial or fungal function groups were inferred to be overrepresented between fertiliser treatments.

4.3. Standardised approach for combined analyses of the relative effects of pH and fertiliser across both trials

The newly proposed standard bioinformatics pipeline was used to compare the diversity of bacteria and fungi across both Craibstone field trials. To understand which highly similar ASVs were common across the two independent trials, ASVs from both trials were merged and normalised together and those within 98% similarity were then clustered to form a new data set of merged ASVs, hereafter referred to as mASVs. Those mASVs common to both trials could then be selected and the relative effects of pH and fertilisation on their numbers, types and relative abundances could be compared using this standardised approach. When the distribution of individual mASVs (roughly equivalent to species) was considered, all diversity metrics were significantly affected by pH, fertilisation, and trial location (Table 7).

Table 7. The effects of long-term pH and fertiliser manipulations and trial site on soil microbial diversity using merged mASV datasets.

Treatment		All treatments		pH		Fertiliser		Trial site	
Barcode		16S	ITS	16S	ITS	16S	ITS	16S	ITS
Taxa richness	P	**1.44E-05**	**0.0003**	**0.001**	**0.001**	0.424	0.644	**0.003**	**0.003**
	E^2	**0.391**	**0.286**	**0.315**	**0.331**	-	-	**0.114**	**0.116**
Types of taxa	P	**0.001**	**0.001**	**0.001**	**0.001**	**0.032**	0.087	**0.001**	**0.001**
	R^2	**0.397**	**0.302**	**0.411**	**0.305**	**0.056**	**-**	**0.100**	**0.088**
Abundances of taxa	P	**0.001**	**0.001**	**0.001**	**0.001**	**0.033**	**0.002**	**0.001**	**0.001**
	R^2	**0.514**	**0.362**	**0.520**	**0.357**	**0.063**	**0.114**	**0.131**	**0.097**

The effect of long-term pH and fertiliser manipulations on soil bacterial (16S) and fungal (ITS) communities and the differences between pH and fertiliser trials overall and between all treatments from both trials using >98% mASVs derived from a merged dataset. P values from Kruskal-Wallis (taxa richness) and PERMANOVA tests for taxa types and abundance based Jaccard distances. Significant test statistics at $P<0.05$ are shown in bold and include effect sizes (E^2 and R^2).

Numbers: Analyses of the merged dataset indicated significant differences in total numbers of both 16S and ITS mASVs between all treatments and across both trial sites ($P < 0.00026$, Figure 9). Similar differences were also observed following pairwise comparisons of all treatments when the two trials had been analysed separately. As in the previous analyses, no effect of fertiliser treatments on 16S or ITS number of mASVs was found. Additional significant differences between 16S number of mASVs in the fertiliser treated plots with all pH level groups except 4.5 were observed. However, in the case of ITS number of mASVs, fertiliser treated soil differed only with soil from the most extreme pH levels (4.5 and 7.5: Figure 9).

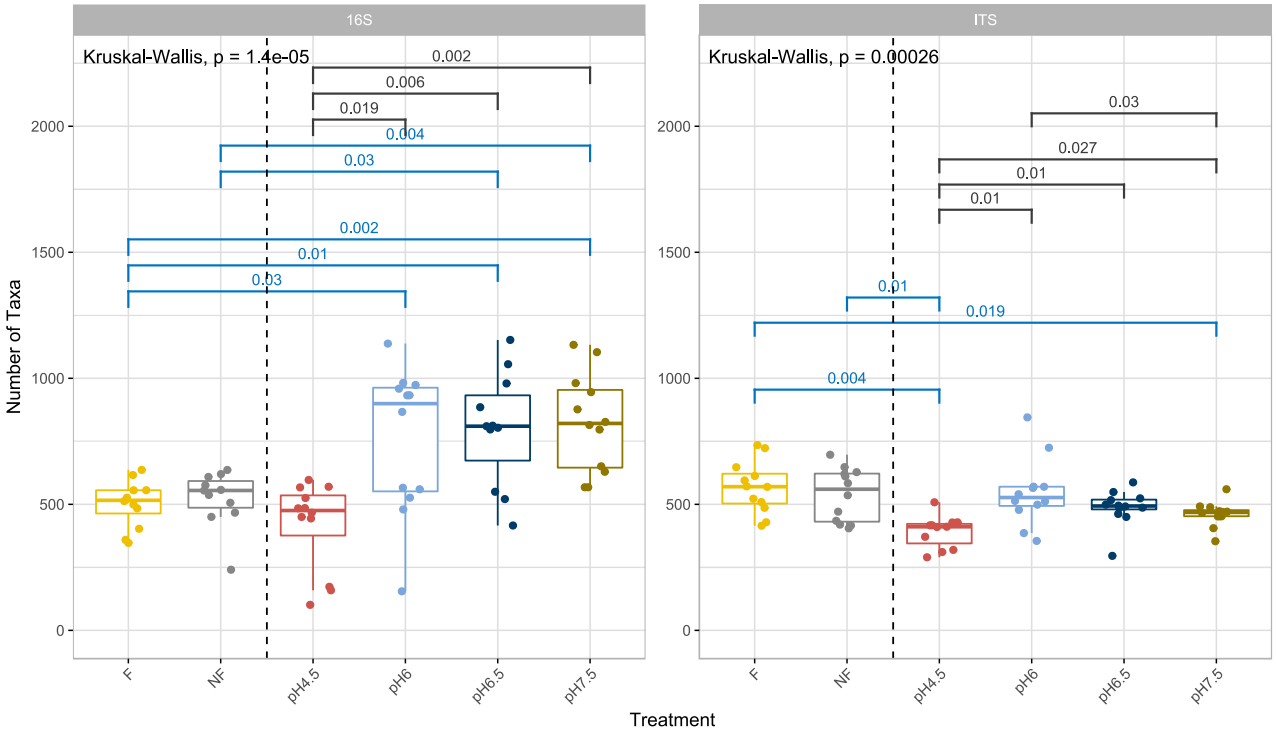
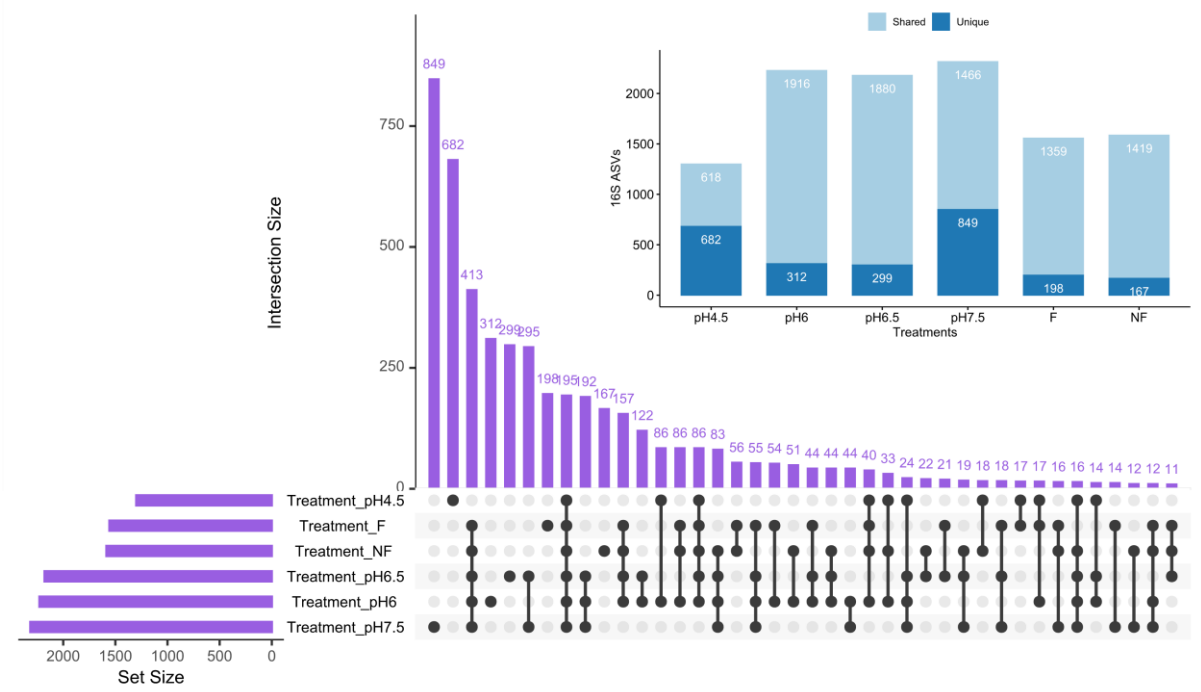


Figure 9. Boxplots comparing mASV richness for 16S and ITS barcodes from the merged dataset across all treatments. Significant differences in numbers of mASVs between treatments as revealed by Wilcoxon pairwise tests are shown in blue for between trial and in black for within trial comparisons (p-values have been adjusted using the Benjamini-Hochberg method).

Types: PERMANOVA analyses revealed significant differences ($P < 0.001$) in the types of 16S and ITS mASVs present in soil following all treatments, except for the fertiliser treatments which (as in the seperate analysis of the fertiliser trial) did not significantly affect the types of ITS mASVs (Table 7). Approximately 40% of the variance in bacterial types and 30% in fungal types was explained overall. These results compare similarly to those obtained from the unmerged analyses of the separate pH and fertiliser trials, except that the size of the pH effect was even greater than previously estimated. Figure 10 displays the proportion of mASVs overlapping between each of the 6 treatments, where most unique taxa were found in soil samples with extreme pH levels (4.5 and 7.5).

a)



b)

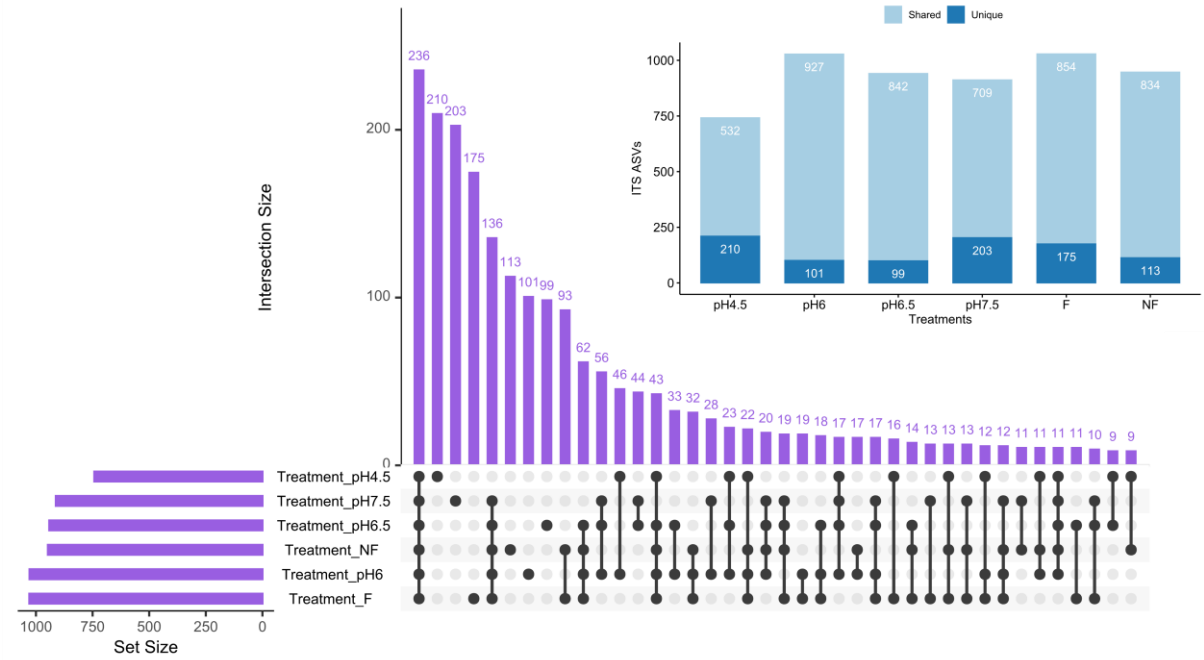


Figure 10. UpSet plots representing the proportion of different 16S (a) and ITS (b) mASVs across two field trials as influenced by six different soil management treatments: No fertiliser (NF), complete fertiliser (F), pH 4.5, pH 6.0, pH 6.5 and pH 7.5. Intersection Size bars show the number of mASVs in common between treatments indicated by the dots below. Set Size bars represent the number of observed different mASV associated with each soil treatment.

Abundances: As with the previous results of analyses of the individual trials, PERMANOVA analyses of the merged data set revealed significant differences ($P < 0.001$) in the relative abundances of 16S and ITS mASVs present in soil following all treatments, explaining approximately 51% of the variance in the relative abundance of bacteria and 36% of fungi-type organisms (Table 7). These differences were again manifest in PCoA ordination plots (Figure 11) where all pairwise analyses revealed significant differences between all treatments ($P < 0.033$), although pH 4.5 clearly had a greater effect than any of the other treatments. The relative abundance of both 16S and ITS mASVs from the fertiliser treatments aligned most closely with those from the pH 6.0 treatments, reflecting the fact that soil in the fertiliser trial had also measured pH 6.0; again highlighting the importance of pH influencing the microbial diversity. Nevertheless, a significant difference in both 16S and ITS mASV abundances was observed between the two trials at pH 6.0. The effect of fertiliser/no fertiliser on relative mASV abundances, was more difficult to distinguish.

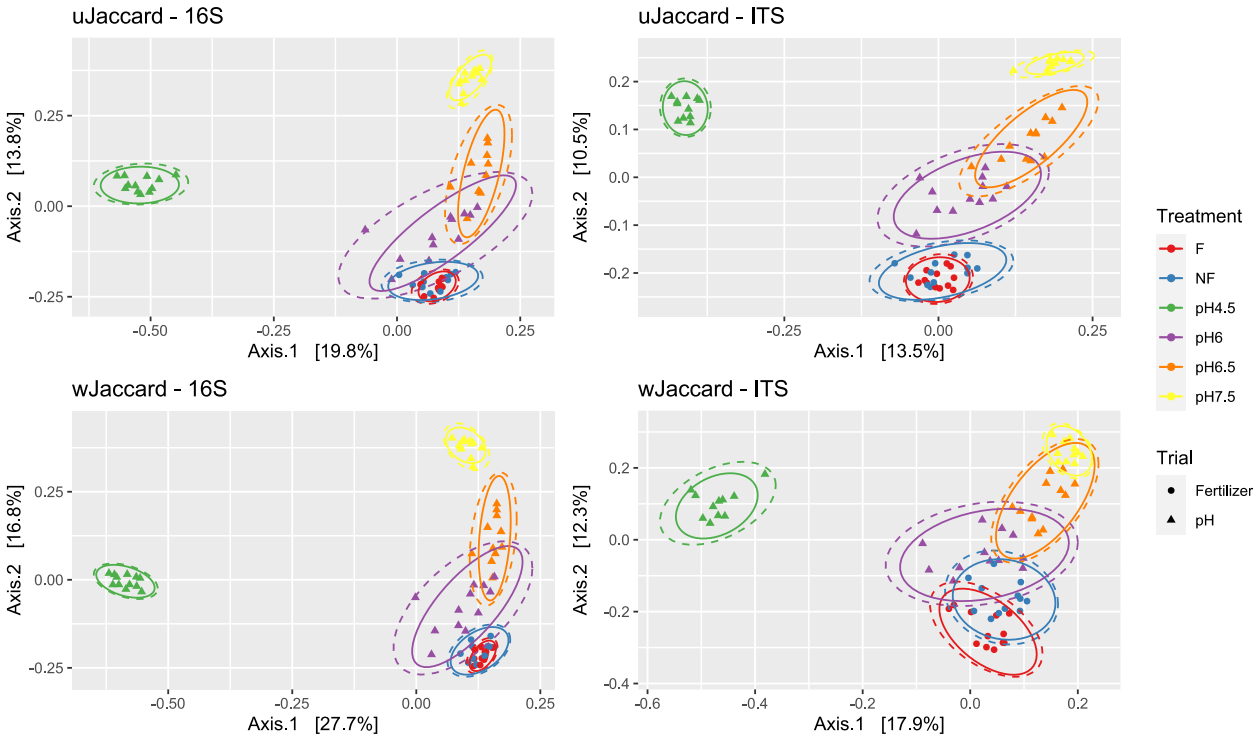


Figure 11. Principal Coordinate Analysis (PCoA) of the relative abundance of 16S and ITS mASVs based on weighted and unweighted Jaccard distance matrices the merged datasets from the two trials. No supplemental fertiliser (NF), supplemental fertiliser (F), pH 4.5, pH 6.0, pH 6.5 and pH 7.5.

Figure 12 shows the community composition of the bacterial and fungal microbiomes of the soils in both trials to be roughly similar when mASVs were clustered at phylum level, as may be expected from trials in close proximation to each other with similar soil types and conditions. Similarly, at this taxonomic level, the relative contribution of each phylum to the soil community was similar irrespective of pH or fertiliser regime (Figure 13).

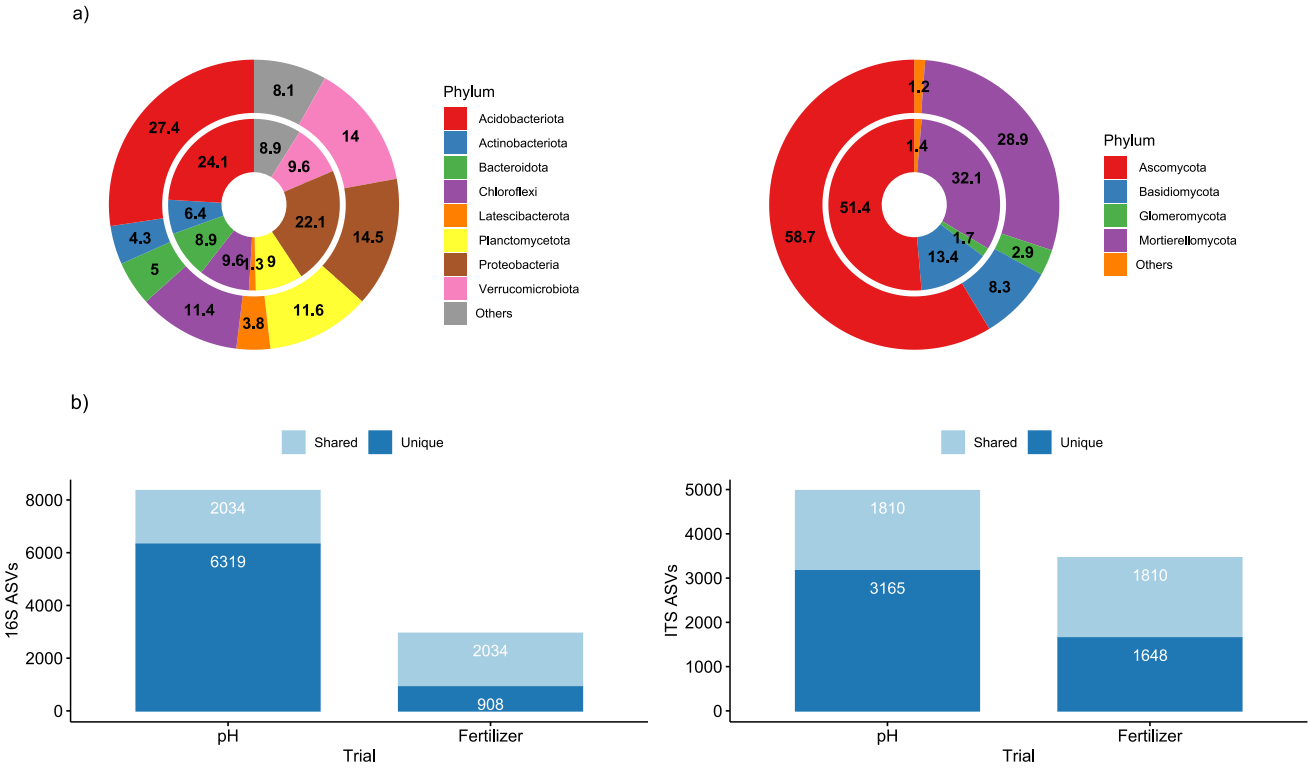


Figure 12. Comparison of bacterial and fungal diversity across two Craibstone field trials.
(a) bacterial (left) and fungal (right) community composition: Relative abundance of indicated phyla in all samples from the pH trial (inner plot) and fertiliser trial (outer plot)
(b) the numbers of observed ASVs: Bar-plots represent the number of ASVs common to both trials (light blue) and the number of ASVs exclusive to each (dark blue).

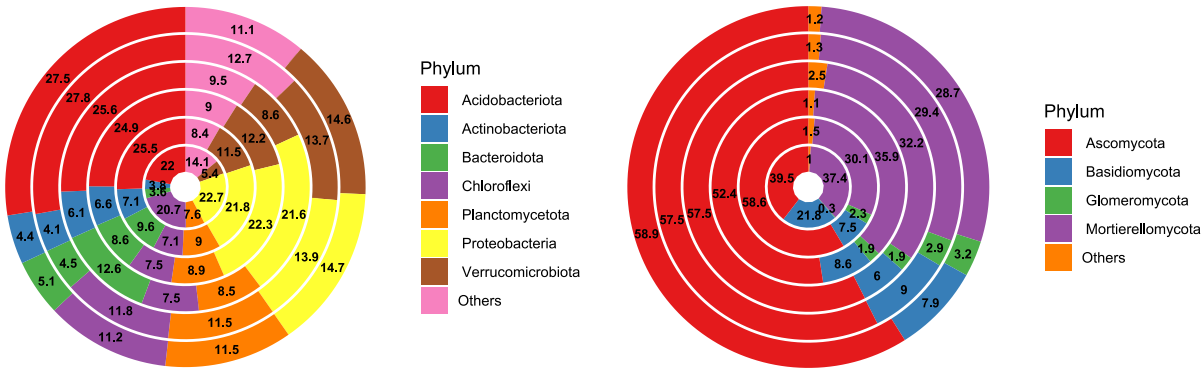


Figure 13. Comparison of bacterial (left) and fungal (right) diversity at phylum level across two field trials as influenced by different soil management treatments. Treatments represented by inner to outer plots are: (a) no fertiliser, (b) complete fertiliser, (c) pH 4.5, (d) pH 6.0, (e) pH 6.5 and (f) pH 7.5.

4.4. Long-term effects of organic amendments on soil bacterial and fungal diversity

Estimated diversity of both bacteria and fungi were independently compared across three field trials, enabling comparison of any long-term effects of organic amendment treatments on the microbiome. Figure 14 shows the community composition of the bacterial and fungal microbiomes of these soils to be roughly similar when ASVs were assigned at phylum level, despite the geographical distance from each other. However, Figure 15 indicates significant numbers of both 16S and ITS ASVs and mASVs that are unique to each site, with only around 5% of ASVs being common to all three sites in each case (Figure 15a) but higher proportions of mASVs common to more than one site (Figure 15b). The highest numbers of unique taxa were found in Gleadthorpe from where a larger number of soil samples were analysed compared with the other sites.

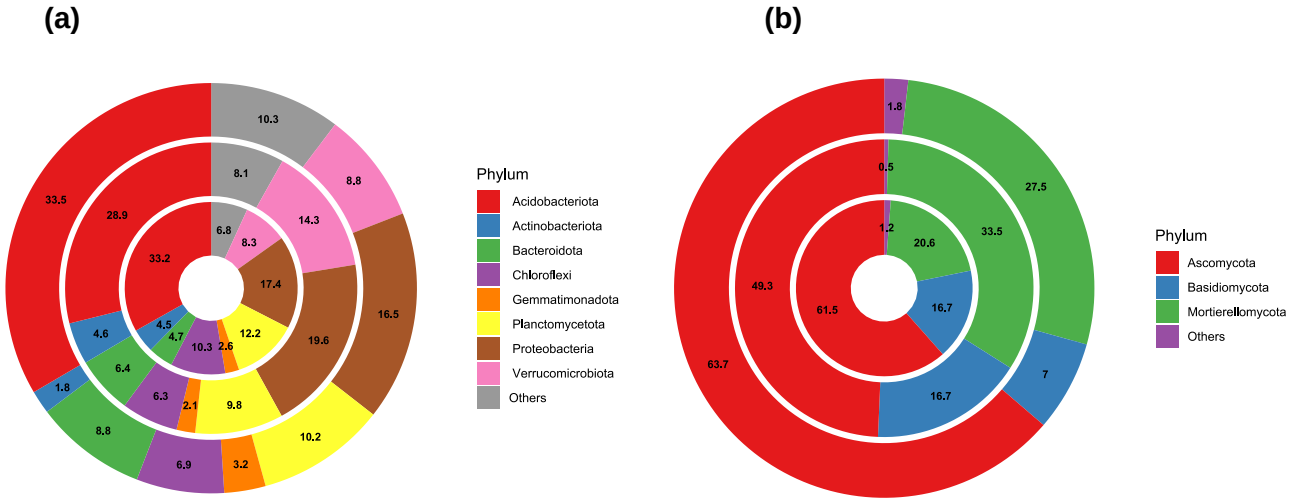


Figure 14. Comparison of bacterial and fungal soil community compositions across three long-term organic-amendment trials. Relative abundance of assigned phyla to (a)16S and (b) ITS ASVs from all samples collected from trials at ADAS Terrington (outer), Harper Adams University (middle) and ADAS Gleadthorpe (inner).

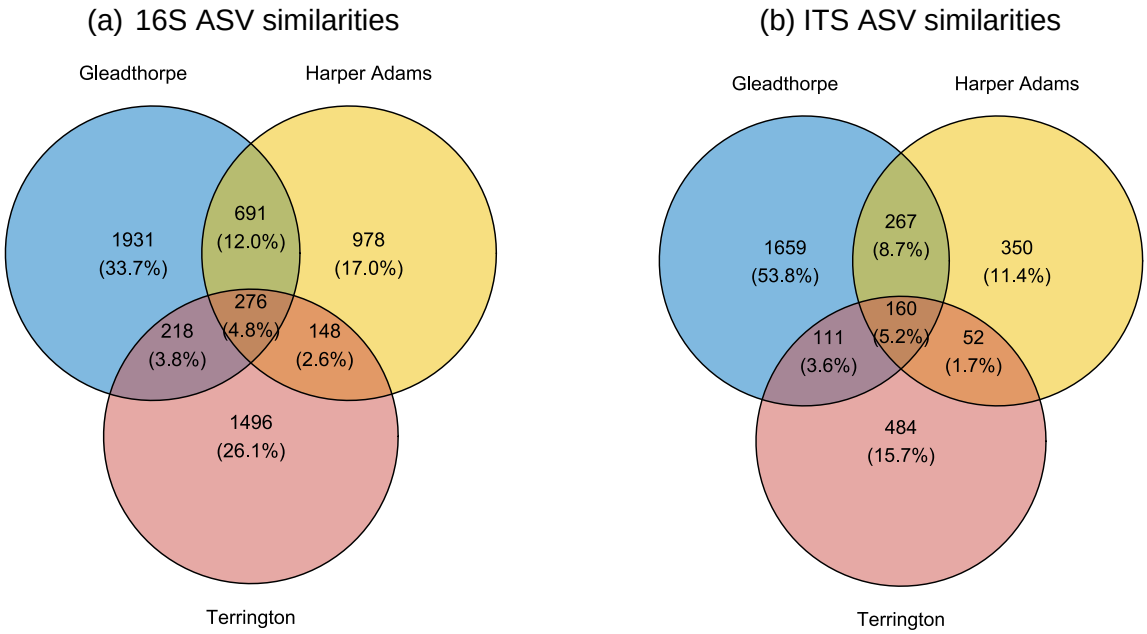


Figure 15a. Commonality of (a) 16S and (b) ITS ASVs across three long-term organic amendment trials.

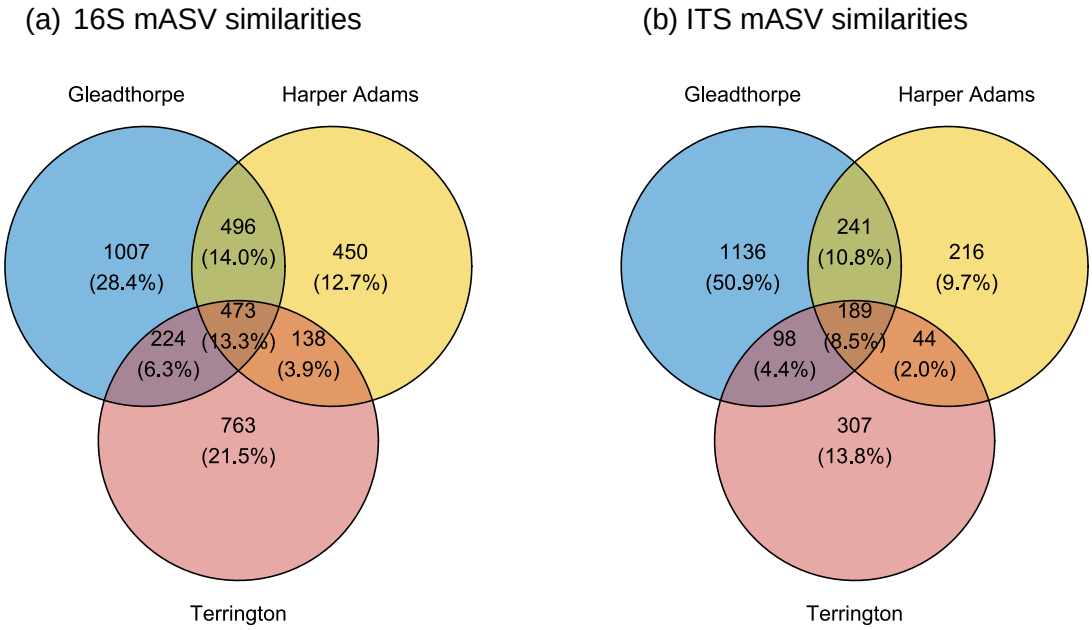


Figure 15b. Commonality of (a) 16S and (b) ITS mASVs across three long-term organic amendment trials.

Numbers: Analyses revealed no significant effect of organic amendment application on 16S richness (P >0.2; Table 8) in any of the trials. However, a slightly significant effect was observed on ITS ASV richness only in the Gleadthorpe trial.

Table 8. Kruskal-Wallis results for Observed ASVs along with PERMANOVA results for Jaccard distances assessing the effect of the different organic amendment treatments in the different experimental sites separately over bacterial (16S) and fungal (ITS) communities. Values at P < 0.05 are shown in bold.

Site		Gleadthorpe		Harper Adams		Terrington	
Barcode		16S	ITS	16S	ITS	16S	ITS
Numbers	P	0.634	**0.045**	0.202	0.430	0.561	0.561
	E^2	-	**0.573**	-	-	-	-
Types	P	**0.007**	**0.001**	0.192	**0.015**	0.158	0.129
	R^2	**0.379**	**0.360**	-	**0.309**	-	-
Abundances	P	**0.004**	**0.001**	0.247	**0.035**	0.305	**0.015**
	R^2	**0.406**	**0.463**	-	**0.359**	-	**0.309**

When the ASV data from all three sites were merged and normalised (as described previously), again no effect of any of the organic amendments on numbers of the 16S mASVs was observed compared with the unamended controls or one each other (Figure 16). However, there were again indications that the cattle slurry treatment may have increased fungal ITS mASVs compared with unamended control, broiler litter and pig manure treatments.

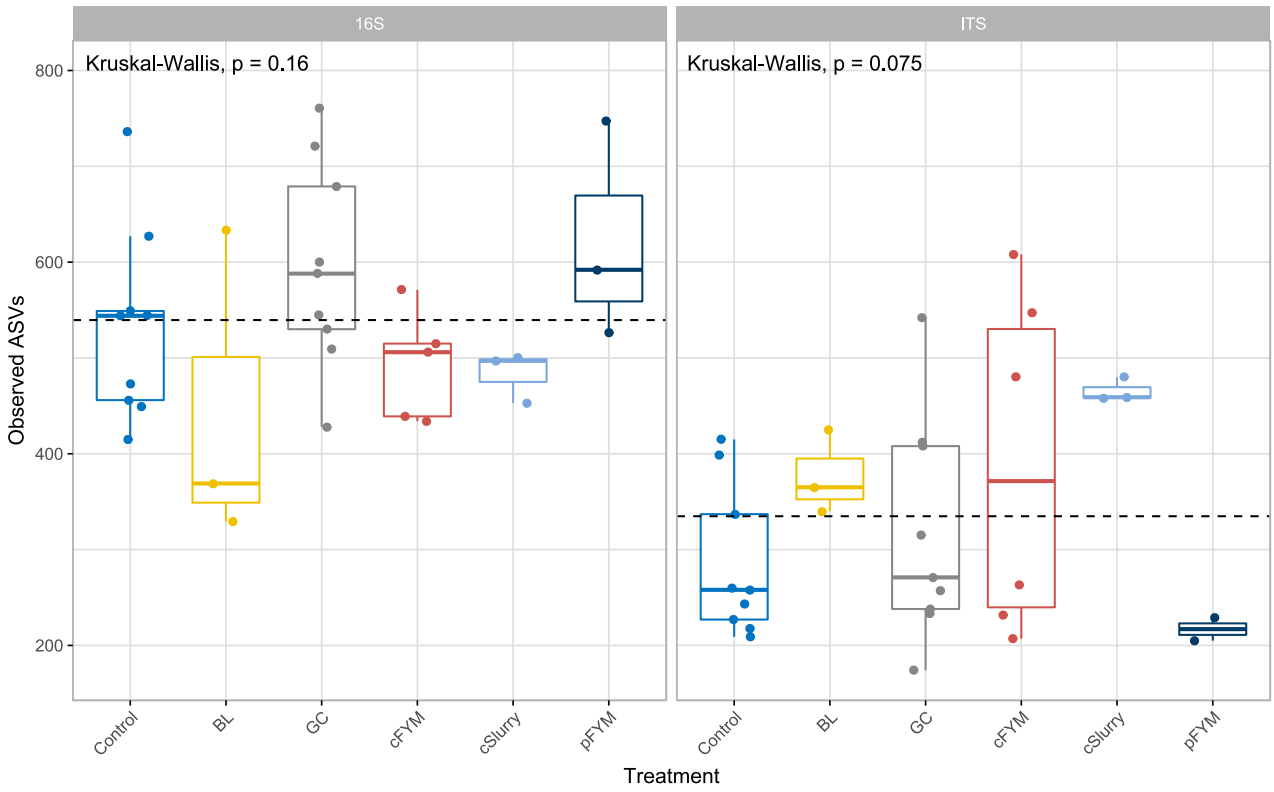


Figure 16. Numbers of 16S and ITS mASVs as affected by soil amendments with broiler litter (BL), cattle farmyard manure (cFYM), pig farmyard manure (pFYM), cattle clurry (cSlurry) or green compost (GC), compared with untreated controls.

By far the largest effect on both 16S and ITS mASV richness was due to location of the trial sites (Table 9). Pairwise analyses showed that 16S mASV richness was significantly higher at Terrington than at Gleadthorpe and Harper Adams. ITS mASV richness in Gleadthorpe was higher than at Harper Adams and Terrington (Figure 17).

Table 9. Kruskal-Wallis results for Observed ASVs along with PERMANOVA results for Jaccard distances assessing the effect of spatial over bacterial (16S) and fungal (ITS) communities. Values at P < 0.05 are shown in bold.

		Spatial Variation	
Barcode		**16S**	**ITS**
mASV richness	**P**	**0.002**	**7.4E-6**
	E^2	**0.355**	**0.746**
Types of mASVs	**P**	**0.001**	**0.001**
	R^2	**0.317**	**0.353**
Abundances of mASVs	**P**	**0.001**	**0.001**
	R^2	**0.389**	**0.421**

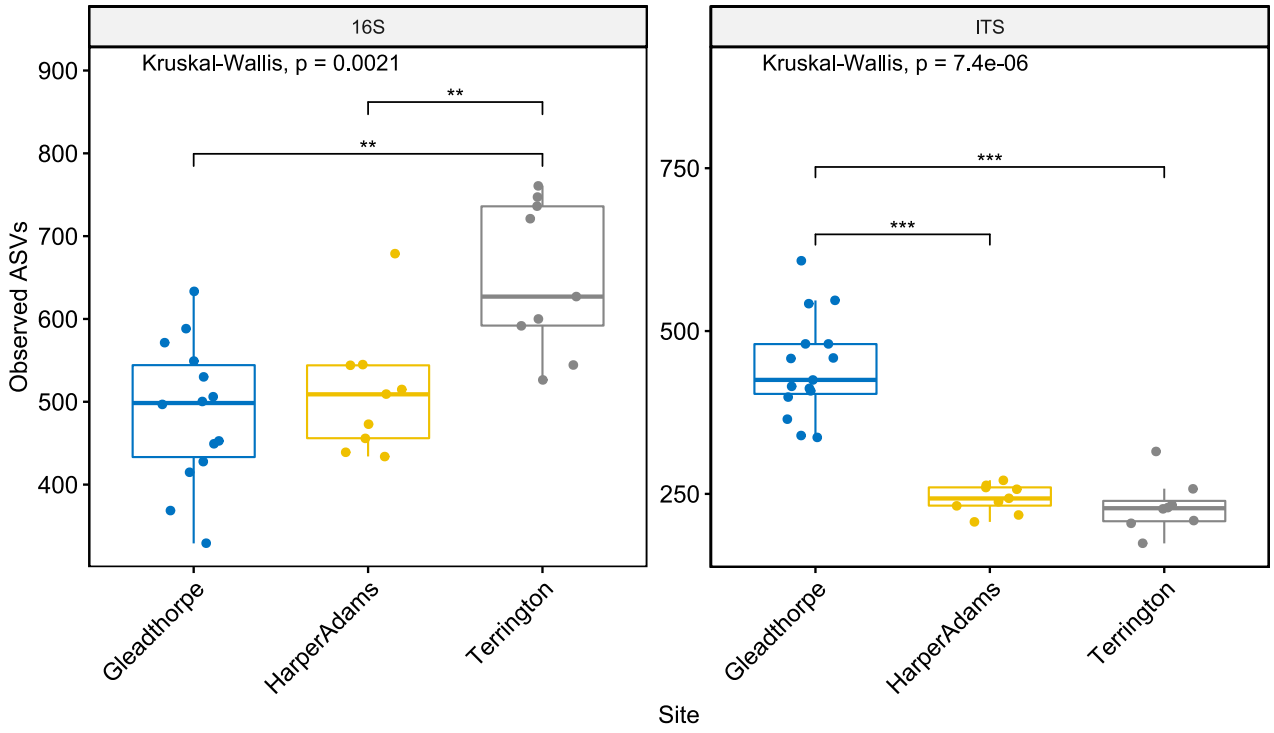


Figure 17. Absolute 16S and ITS mASV richness across all three trials. Kruskal-Wallis general and pairwise results are indicated where adjusted p-value was significant (0 '***', 0.001 '**', 0.01 '*').

Types: PERMANOVA analyses revealed significant differences between organic amendment treatments in the types of 16S and ITS ASVs present in Gleadthorpe, explaining approximately 37% of the variance in assigned bacterial types. Differences in the types of ITS mASV present were also significant between treatments at Harper Adams, explaining 31% of the variance in assigned fungal types ($p<0.015$; Table 8). No significant effects of organic amendments on types of 16S or ITS ASVs were observed at Terrington.

Further PERMANOVA analyses revealed larger significant differences ($P<0.001$) between the types of 16S and ITS mASVs present in soil from each trial site (Table 9, Figure 18). Approximately 32% of the variance in bacterial types and 35% in fungal types was explained overall by the site location.

Abundances: Significant differences were also observed between the treatments in relative abundances of types of ITS ASVs at all three sites and of types of 16S ASVs at Gleadthorpe only ($P<0.035$, Table 8). When the data were merged, it was clearly apparent that the location of the trial sites had a much greater effect on both types and relative abundances of both 16S and ITS mASVs than did the organic amendment treatments (Figure 18).

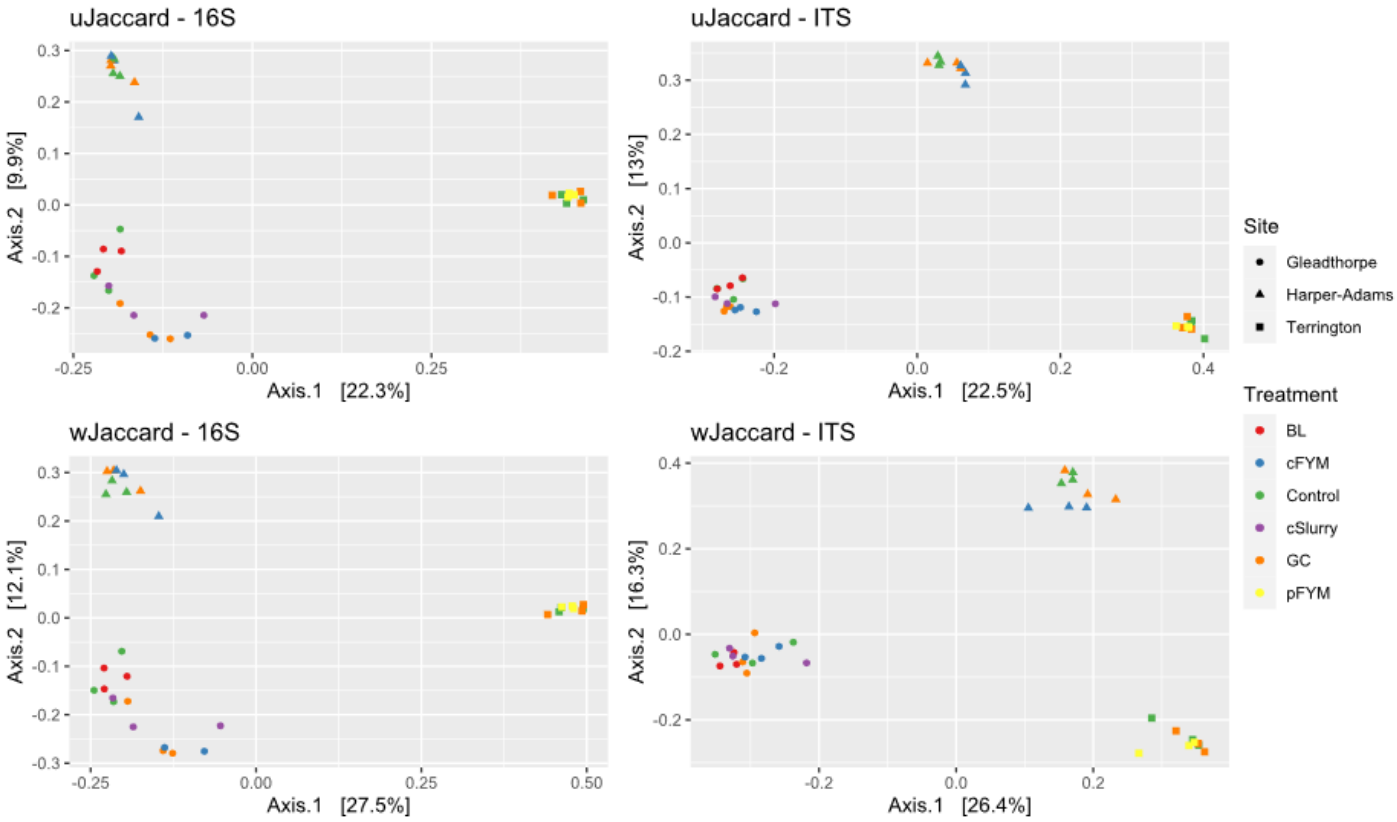


Figure 18. Principal Coordinate Analysis (PCoA) based on weighted and unweighted Jaccard distances matrices showing differences in relative abundances of 16S and ITS mASVs.

4.5. Long-term effects of different tillage approaches on soil bacterial and fungal diversity, in two sampling years

Bacterial and fungal diversity analyses were carried out on soil samples from field trials at the Game and Wildlife Conservation Trust (GWCT) at Loddington in Leicestershire. Samples were collected in 2018 (6 samples) and 2020 (12 samples) from plots with two different tillage approaches: direct-drill and ploughing. During the second sampling, twice as many samples were collected to intensify the statistical power and thus obtain more reliable results on the effect of tillage treatment on the microbiome. In addition, microbial diversities were compared between the two years to obtain a glimpse of temporal fluctuations on the microbial communities.

Numbers: Analyses revealed no significant effect of tillage approach on total numbers of 16S or ITS ASVs (P >0.27; Table 10). In addition, no significant differences were observed in 16S number of ASVs when samples from the same plots were analysed between the two years. In contrast, large differences were detected in ITS mASV richness between the two years explaining approximately 65% of the variance (Table 10, Figure 19).

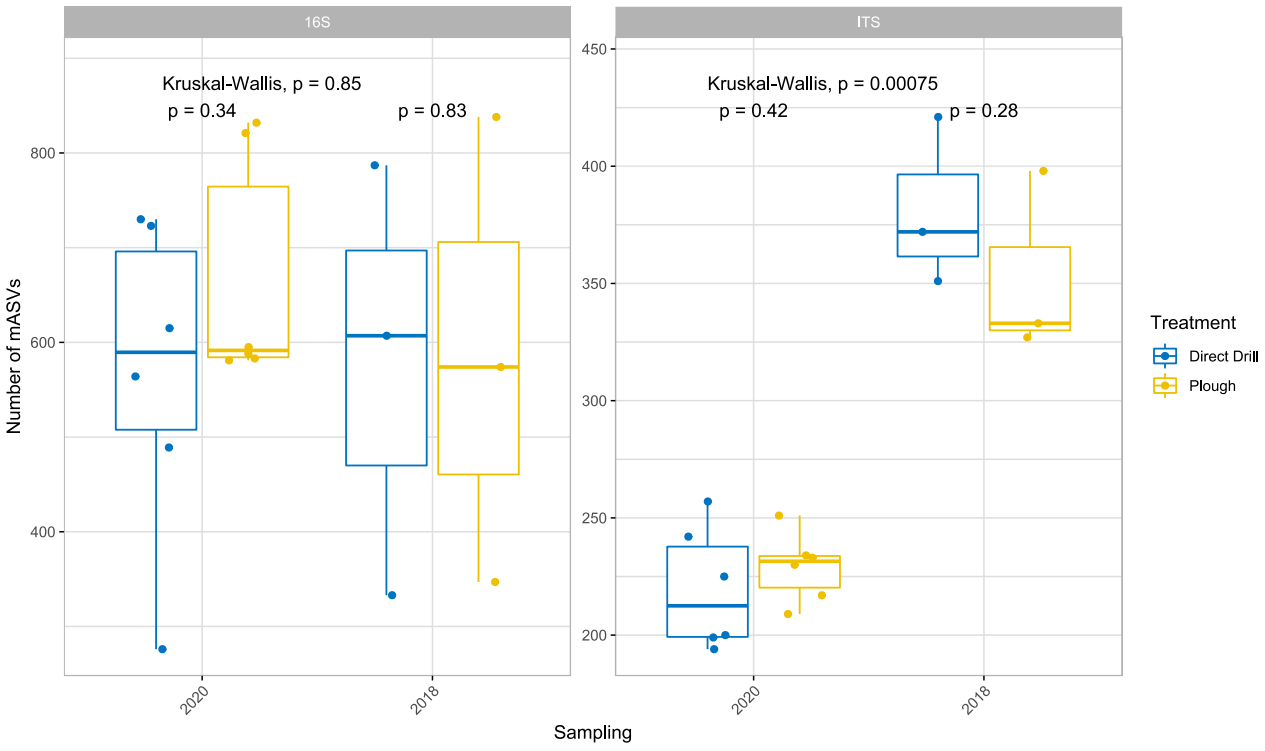


Figure 19. Number of 16S and ITS mASVs at two sampling times. Kruskal-Wallis general and pairwise results are indicated at the top of each boxplot.

Types: PERMANOVA analyses revealed significant differences between tillage approaches in the types of ITS ASVs which explained approximately 14% of the variance, but only in samples collected in 2020. No similar effect of these treatment was observed in samples collected two years before (Table 10). No significant effects were observed on the types of 16S ASVs. In terms of temporal variation, analyses revealed significant differences in the types of both 16S and ITS ASVs between the two years (Table 10, Figure 20).

Table 10. Kruskal-Wallis results for observed ASVs and PERMANOVA results for Jaccard distances assessing the effect of two tillage approaches and year of sampling on bacterial (16S) and fungal (ITS) communities. Values at P < 0.05 are shown in bold.

		Tillage – 2018		Tillage – 2020		Sampling year	
Barcode		**16S**	**ITS**	**16S**	**ITS**	**16S**	**ITS**
Numbers*	**P**	0.827	0.275	0.337	0.423	0.851	**0.001**
	E^2	-	-	-	-	-	**0.648**
Types of ASV/mASV*	**P**	0.9	0.3	0.082	**0.005**	**0.035**	**0.001**
	R^2	-	-	-	**0.136**	**0.080**	**0.149**
Abundances of ASVs*	**P**	0.8	0.2	**0.049**	**0.015**	**0.001**	**0.001**
	R^2	-	-	**0.136**	**0.143**	**0.143**	**0.169**

*****Results assessing the effect of sampling time were obtained using mAVS

Abundances: Similarly, significant differences were observed between samples collected in different years on both 16S and ITS ASVs abundances. Also, a significant effect of tillage on both 16S and ITS ASVs relative abundances was observed amongst samples collected in 2020 (Table 10, Figure 20).

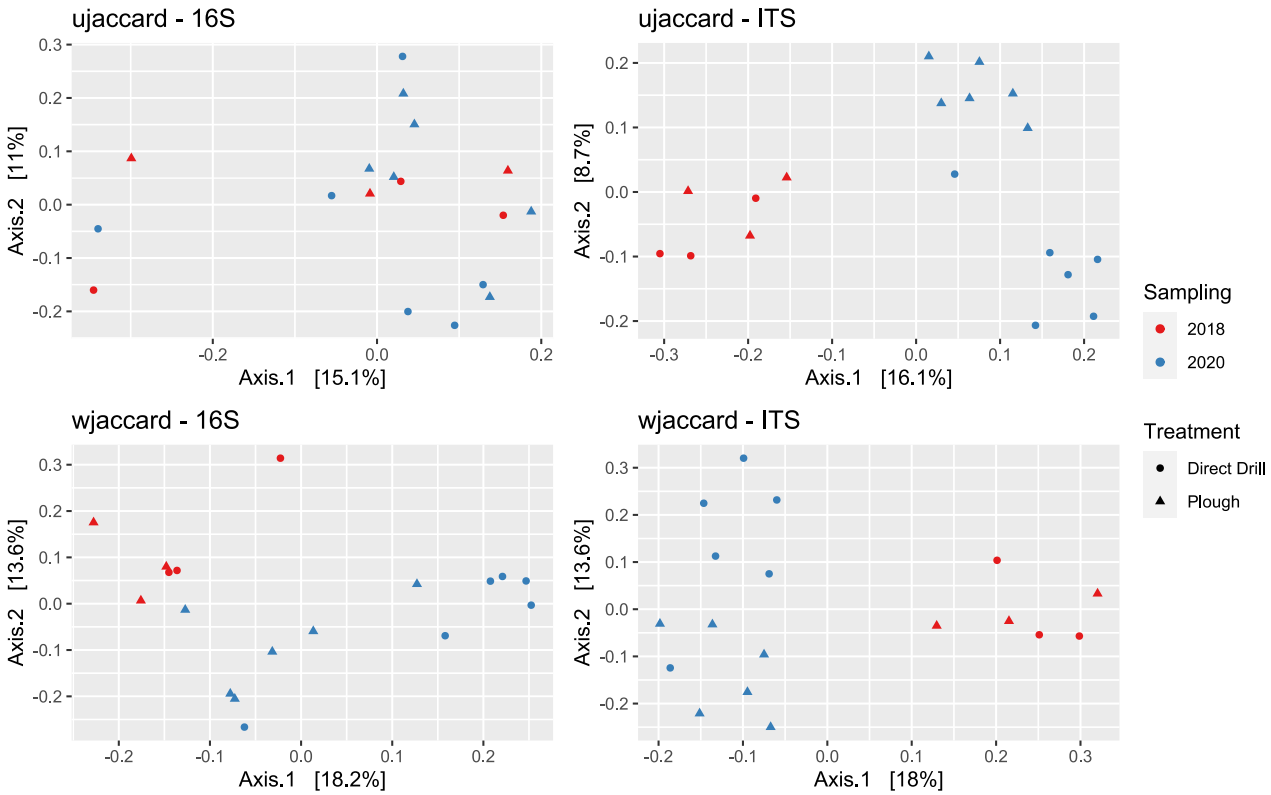


Figure 20. Principal Coordinate Analysis (PCoA) based on weighted and unweighted Jaccard distances matrices showing differences in relative abundances of 16S and ITS mASVs.

4.6. Effects of sampling season on soil bacterial and fungal diversity

As an additional study conducted as part of a MSc degree (Briggs, 2018), soil was sampled from the long-term STAR (Sustainability Trial in Arable Rotations) trial conducted by NIAB at Stanaway Farm, Suffolk on a heavy clay soil. This trial aimed to assess the effects of rotation and cultivation treatments on soil health, agronomy and production (Stobart & Morris, 2011). Wheat plots were sampled during spring and autumn 2018. Bacterial and fungal diversities were compared in both seasons to evaluate temporal changes across two different seasons/cropping stage.

Numbers: Analyses revealed no significant effect of cultivation on 16S or ITS ASV richness ($P > 0.23$; Table 11) in either season. However, large significant differences were detected in both 16S and ITS mASV richness between the two sampling occasions, explaining approximately 70% of the variance ($P < 0.00003$, Table 11). The total numbers of 16S or ITS mASVs were both higher when soil was sampled in Autumn (Figure 21).

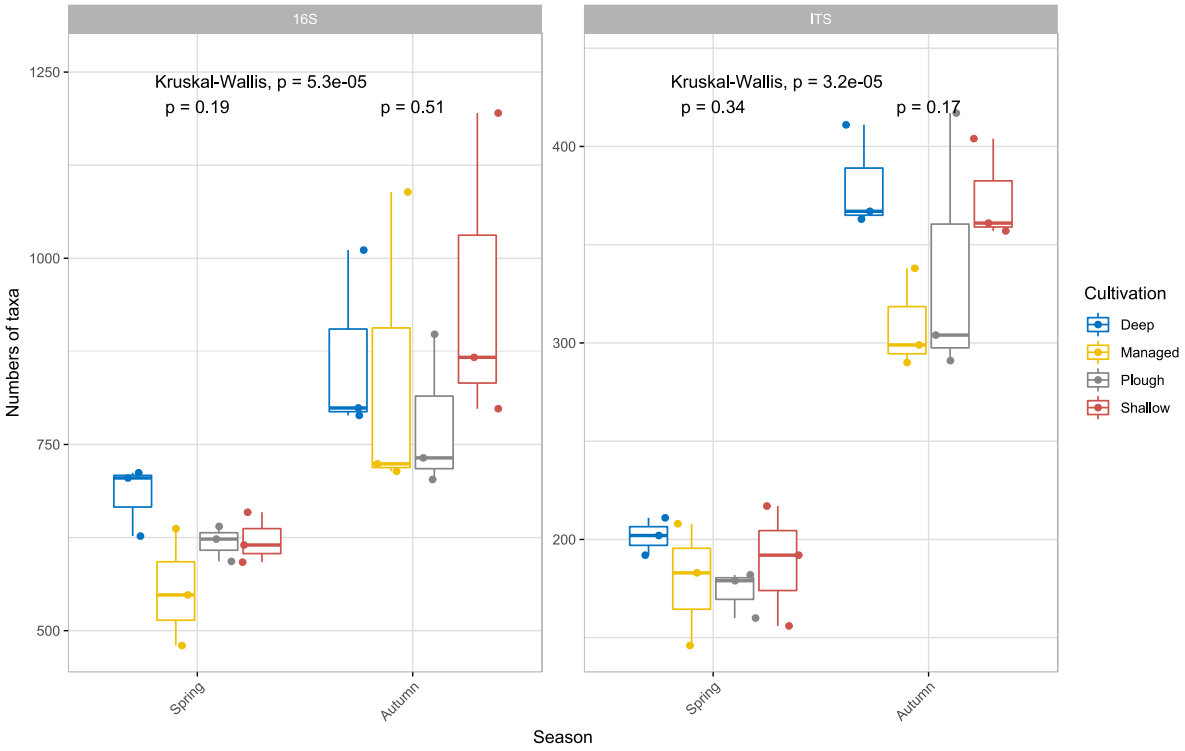


Figure 21. Absolute 16S and ITS mASV richness at two sampling times. Kruskal-Wallis general and pairwise results are indicated where the adjusted p-value was significant (0 '***', 0.001 '**', 0.01 '*').

Types: PERMANOVA analyses revealed significant differences between cultivation treatments on the types of ITS ASVs, which explained 30% of the overall variation, but only in samples collected in spring. However, this effect of these treatments was not observed in samples collected in the autumn (Table 11, Figure 22). Analyses showed large differences in the types of both 16S and ITS ASVs between the two sampling times.

Table 11. Kruskal-Wallis results for observed ASVs and PERMANOVA results for Jaccard distances assessing the effect of the different tillage approaches and sampling times on bacterial (16S) and fungal (ITS) communities. Values at P < 0.05 are shown in bold.

		Cultivation Spring		Cultivation Autumn		Sampling season	
Barcode		**16S**	**ITS**	**16S**	**ITS**	**16S**	**ITS**
ASV richness*	**P**	0.238	0.379	0.468	0.287	**5.31E-05**	**3.21E-05**
	E^2	-	-	-	-	**0.696**	**0.740**
Types of ASVs*	**P**	0.153	**0.010**	0.331	0.155	**0.001**	**0.001**
	R^2	-	**0.311**	-	-	**0.105**	**0.194**
Abundances of ASVs*	**P**	0.171	0.349	0.284	0.093	**0.001**	**0.001**
	R^2	-	-	-	-	**0.120**	**0.138**

***Results assessing the effect of sampling time were obtained using mAVS

Abundances: there were significant effects of cultivation on the relative abundances of both 16S and ITS ASVs. Large differences were also observed between the two sampling occasions in both 16S and ITS ASV abundances (Table 11, Figure 22).

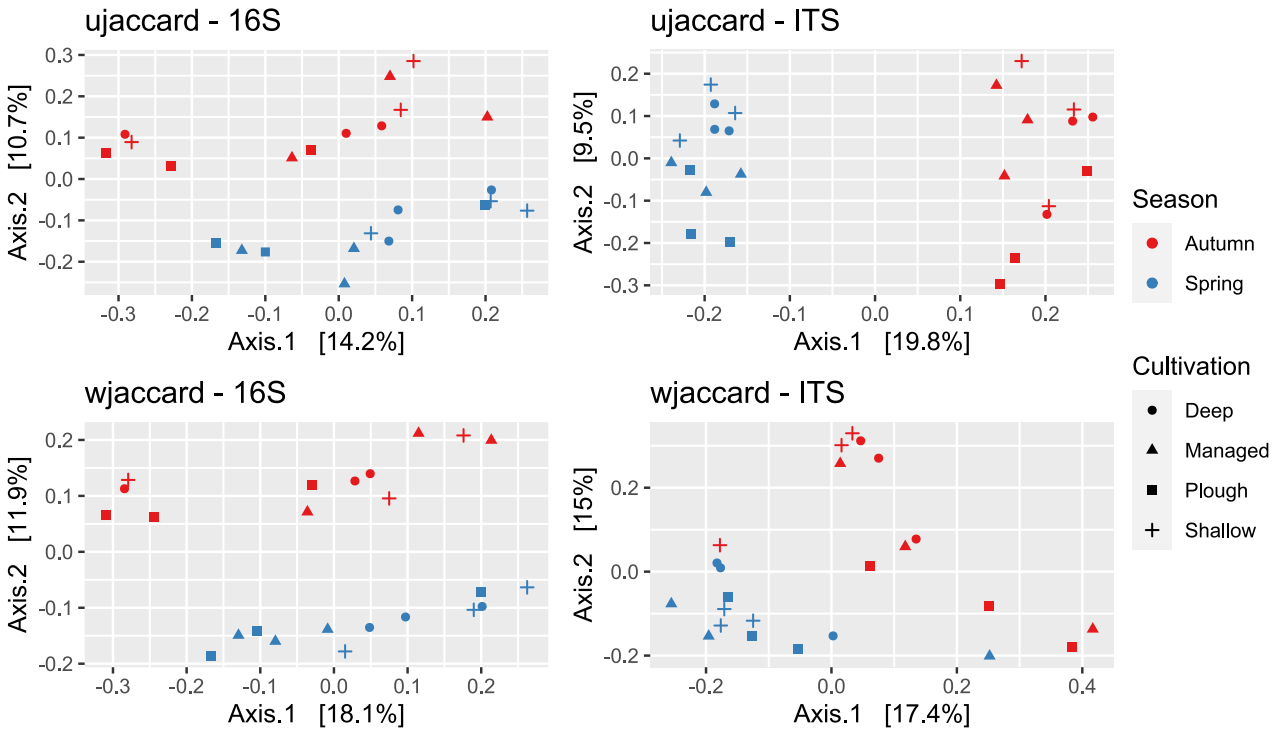


Figure 22. Principal Coordinate Analysis (PCoA) based on weighted and unweighted Jaccard distances matrices showing differences in relative abundances of 16S and ITS mASVs.

4.7. Long-term effects of re-ridging and inter-row companion crops or mulching on soil bacterial and fungal diversity in asparagus production

Estimated diversity of both bacteria and fungi were independently compared across the long-term field trial conducted by Cranfield University to investigate the effects of companion crops, mulching with either straw or PAS100 green compost, re-ridging and shallow soil disturbance between seasons on inter-row soil compaction during asparagus cultivation (Mašková *et al.*, 2021). Metabarcoding was used to compare any effects on the microbiome resulting from re-ridging between crops (Table 12) and any secondary effects of using inter-row companion crops or mulching with PAS-100 green compost compared with leaving bare soil between the asparagus beds (Table 13).

Table 12. Kruskal-Wallis results and PERMANOVA results for Jaccard distances comparing the effects of re-ridging and other secondary soil treatments on the diversity of bacterial (16S) and fungal (ITS) ASVs (values where P<0.05 shown in bold).

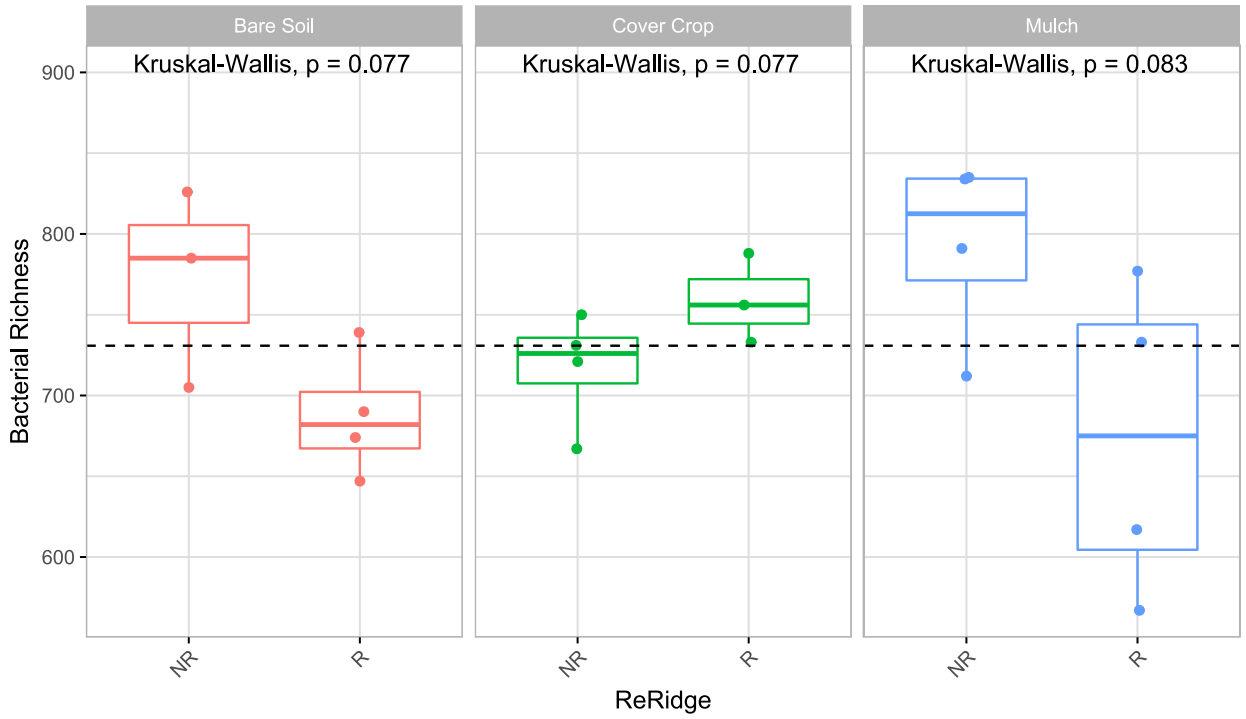
Treatment Groups		**+ or - re-ridging Bare soil**		**+ or - re-ridging Companion crop**		**+ or - re-ridging Mulch**		**+ or - re-ridging All treatments**	
Barcode		**16S**	**ITS**	**16S**	**ITS**	**16S**	**ITS**	**16S**	**ITS**
ASV richness	**P**	0.077	0.564	0.077	**0.043**	0.083	0.773	0.108	0.312
	E^2	-	-	-	**0.514**	-	-	-	-
Types of ASVs	**P**	0.21	0.259	0.119	0.304	0.205	0.343	**0.002**	**0.022**
	R^2	-	-	-	-	-	-	**0.067**	**0.055**
Abundances of ASVs	**P**	0.379	0.398	0.289	0.199	0.245	0.611	**0.012**	**0.031**
	R^2	-	-	-	-	-	-	**0.068**	**0.063**

Table 13. Kruskal-Wallis results and PERMANOVA results for Jaccard distances comparing the effects of secondary soil treatments on diversity of bacterial (16S) and fungal (ITS) ASVs (values where P<0.05 shown in bold).

Treatment Groups		**Bare soil (+ or - shallow disturbance)**		**Companion crop (Mustard or Rye)**		**Mulch (PAS100 or Straw)**	
Barcode		**16S**	**ITS**	**16S**	**ITS**	**16S**	**ITS**
ASV richness	**P**	0.157	**0.021**	0.289	0.564	0.564	0.773
	E^2	-	**0.722**	-	-	-	-
Types of ASVs	**P**	0.181	**0.036**	0.567	0.599	0.149	**0.049**
	R^2	-	**0.182**	-	-	-	**0.174**
Abundances of ASVs	**P**	0.117	0.061	0.572	0.329	0.203	0.362
	R^2	-	0.175	-	-	-	-

Numbers: No significant effect of re-ridging was observed on the total number of 16S ASVs (P >0.1; Table 12). However, re-ridging significantly increased the total number of ITS ASVs, but only in samples where a companion crop had been grown between the rows (Figure 23). Shallow soil disturbance within the bare soil treatment also significantly reduced ITS ASV richness (Table 13). No effect of growing companion crops on the types of ITS ASV was observed.

(a)



(b)

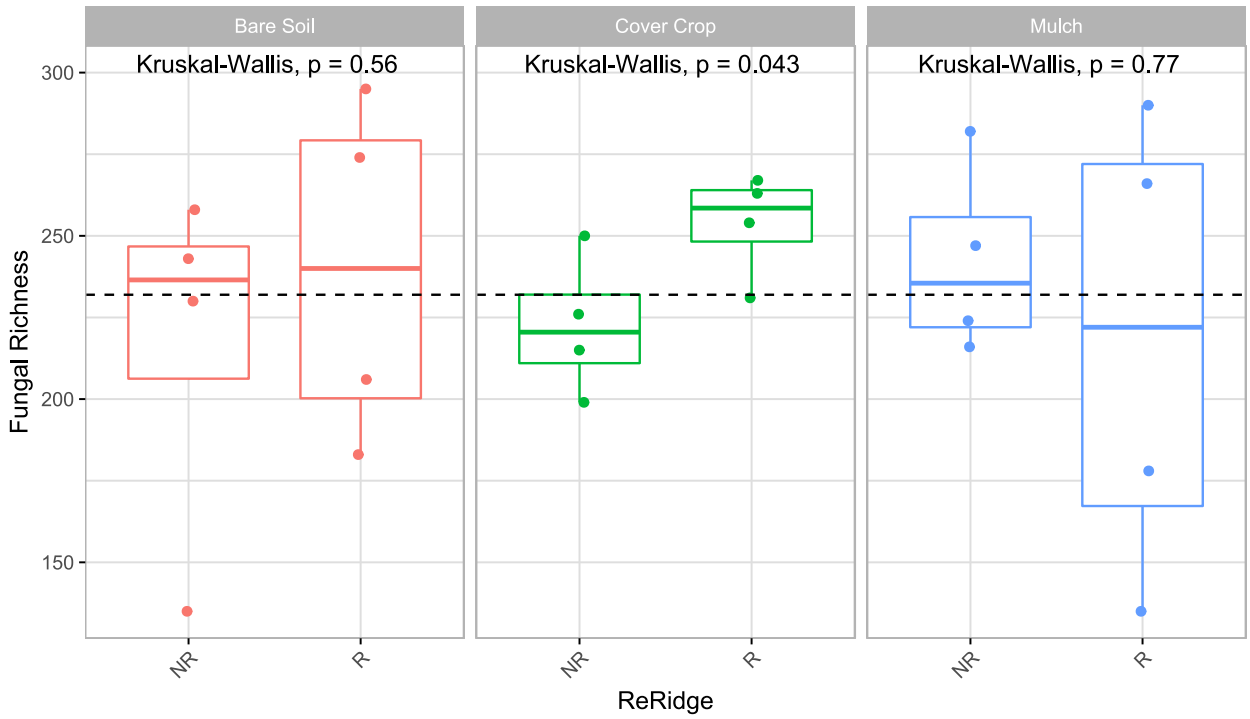


Figure 23. Numbers of (a) 16S and (b) ITS ASVs as affected by inter-row treatments of bare soil, companion crops or mulch, with (R) or without (NR) annual re-ridging of the asparagus beds.

Types: No significant effect of re-ridging on the types of 16S and ITS ASVs was observed in any specific treatment group (Table 12). However, when all sample were analysed together a significant effect was observed in types of both 16S and ITS ASVs with a marginal effect size of approximately 7% and 6%, respectively.

Abundances: Significant differences in relative abundances of both 16S and ITS ASV types, between both the re-ridging treatments and the secondary treatment groups, were also observed only when all samples were analysed together ($p<0.031$; Tables 11 and12).

The standardised approach was used to compare bacterial and fungal diversity between treatment groups: Bare soil, companion crop and mulch. There were no significant effects of treatments on the number of 16S or ITS ASVs, whereas the effects on types and abundances of both were highly significant (Table 14). Figure 24 shows the community composition of bacterial and fungal microbiomes in these soils to be roughly similar when ASVs were identified at phylum level. However, Figure 25 shows that a high proportion of unique ASVs were associated with bare soil, mulch and companion crop treatments, with only around one quarter of bacteria and fungi unaffected by any of the three soil treatments.

Table 14. Kruskal-Wallis results and PERMANOVA results for Jaccard distances comparing the effects of all treatment groups on bacterial (16S) and fungal (ITS) ASV diversity (values at $P<0.05$ shown in bold).

		Treatment Groups	
Barcode		**16S**	**ITS**
ASV richness	**P**	0.77	0.981
	E^2	-	-
Types of ASVs	**P**	**0.005**	**0.006**
	R^2	**0.119**	**0.108**
Abundances of ASVs	**P**	**0.005**	**0.018**
	R^2	**0.122**	**0.119**

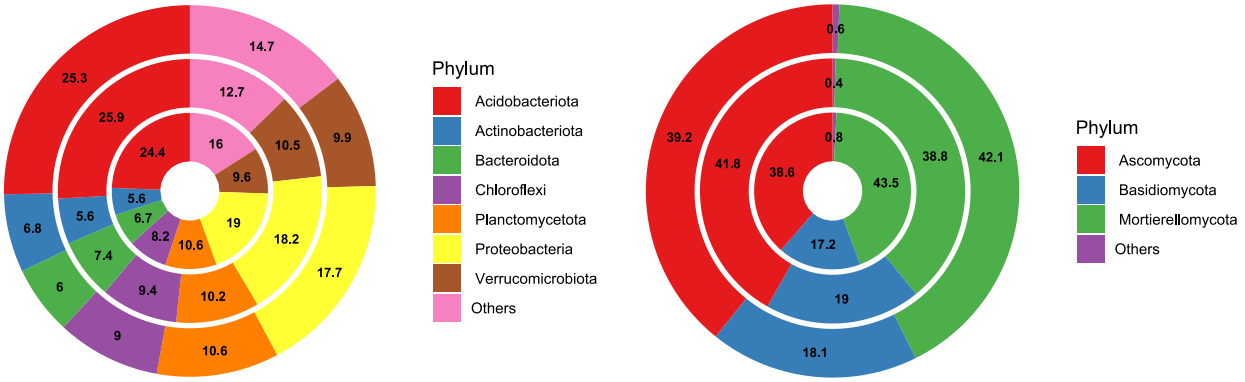


Figure 24. Comparison of bacterial and fungal soil community compositions across three treatment groups. Relative abundance of assigned phyla to (a)16S and (b) ITS ASVs across all samples from soils treated with mulch (outer), companion crops (middle) and bare soil (inner).

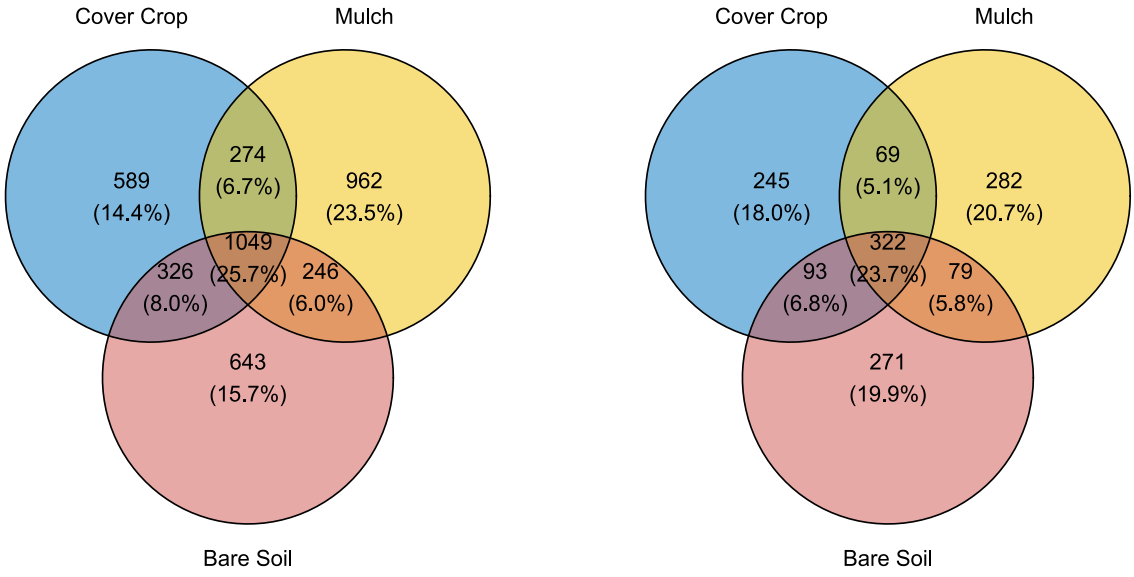


Figure 25. Commonality of (a) 16S and (b) ITS ASVs across three treatment groups.

4.8. Long-term effects of drainage on soil bacterial and fungal diversity

Estimated diversity of both bacteria and fungi were independently compared across a field trial conducted at ADAS Boxworth in Cambridgeshire, to compare the effects of drainage in a heavy clay soil on the microbiome. Two treatments were compared: improved drainage (mole drains) and poor drainage (undrained).

Numbers: Analyses revealed no significant effect of drainage approaches on 16S ASV richness. However, Kruskal-Wallis analysis detected a large significant effect over the absolute richness of ITS ASVs, explaining approximately 55% of the variance due to the drainage treatment (Table 15) and this was also consistent with the effect on ITS phylogenetic relatedness (Table 16).

Table 15. Kruskal-Wallis and PERMANOVA results for ASV richness and Jaccard distances comparing the effects of both drainage approaches on bacterial (16S) and fungal (ITS) ASV diversity (values at P<0.05 shown in bold).

		Drainage approach	
Barcode		**16S**	**ITS**
ASV richness	**P**	0.749	**0.010**
	E^2	-	**0.556**
Types of ASVs	**P**	0.678	0.393
	R^2	-	-
Abundances of ASVs	**P**	0.805	**0.01**
	R^2	-	**0.149**

Types: no significant differences were observed between drainage approaches in the types of 16S or ITS ASVs (P > 0.4, Table 15). However, a small effect was detected in UniFrac distances explaining approximately 14% of the variance (Table 16).

Table 16. Kruskal-Wallis and PERMANOVA results for Faith-PD and Unifrac distances comparing the effects of both drainage approaches on bacterial (16S) and fungal (ITS) ASV diversity with phylogenetic relatedness into account (values at P<0.05 shown in bold).

		Drainage approach	
Barcode		**16S**	**ITS**
Faith PD	**P**	0.631	**0.010**
	E^2	-	**0.556**
Unweighted UniFrac	**P**	0.527	**0.009**
	R^2	-	**0.138**
Weighted UniFrac	**P**	0.565	**0.012**
	R^2	-	**0.332**

Abundances: There were significant differences between drainage treatments in the types of ITS (P = 0.01) ASVs which explained 15% of variance in ASV abundances and this was consistent with the weighted UniFrac distances, however, more than double the variance in ASV relative abundances was explained in this case (Tables 14 and 15). No significant effects of drainage on types or relative abundances of 16S ASVs (p > 0.5, Table 15 and 16) were observed.

4.9. Correlations between microscopic observation and metabarcoding of mesofauna diversity

Estimated diversity of arthropodal communities were compared across the pH field trial, enabling comparison of any long-term effects on the mesofauna of soil pH gradients and crops stages in a rotation. In addition, this dataset was used for evaluating correlation between sequencing and microscopy data following the procedures described in 3.3.2.

Numbers: Analyses revealed significant effects of the pH gradient and crop stages on CO1 ASVs richness and Shannon entropy (P <0.041; Table 17). No effect of pH levels was observed on species from the microscopy datasets. However, the effect of crop stages was significant over species richness and Shannon entropy in this dataset. Although no significant effect, pairwise comparison showed a similar trend between the different pH level on both datasets and opposite patterns on crop stages when results from sequencing and microscopy data were compared (Figure 26).

Table 17. Kruskal-Wallis results for richness and Shannon entropy assessing the effect of pH gradient and crops different stages over arthropodal mASVs and species from sequencing and microscopy datasets, respectively. Values at P < 0.05 are shown in bold.

Method		**Sequencing**		**Microscopy**	
Treatment		**pH**	**Crop**	**pH**	**Crop**
Richness	**P**	**0.023**	**0.003**	0.248	**0.009**
	E^2	**0.148**	**0.258**	-	**0.194**
Shannon	**P**	**0.002**	**0.041**	0.152	**0.004**
	E^2	**0.267**	**0.120**	-	**0.229**

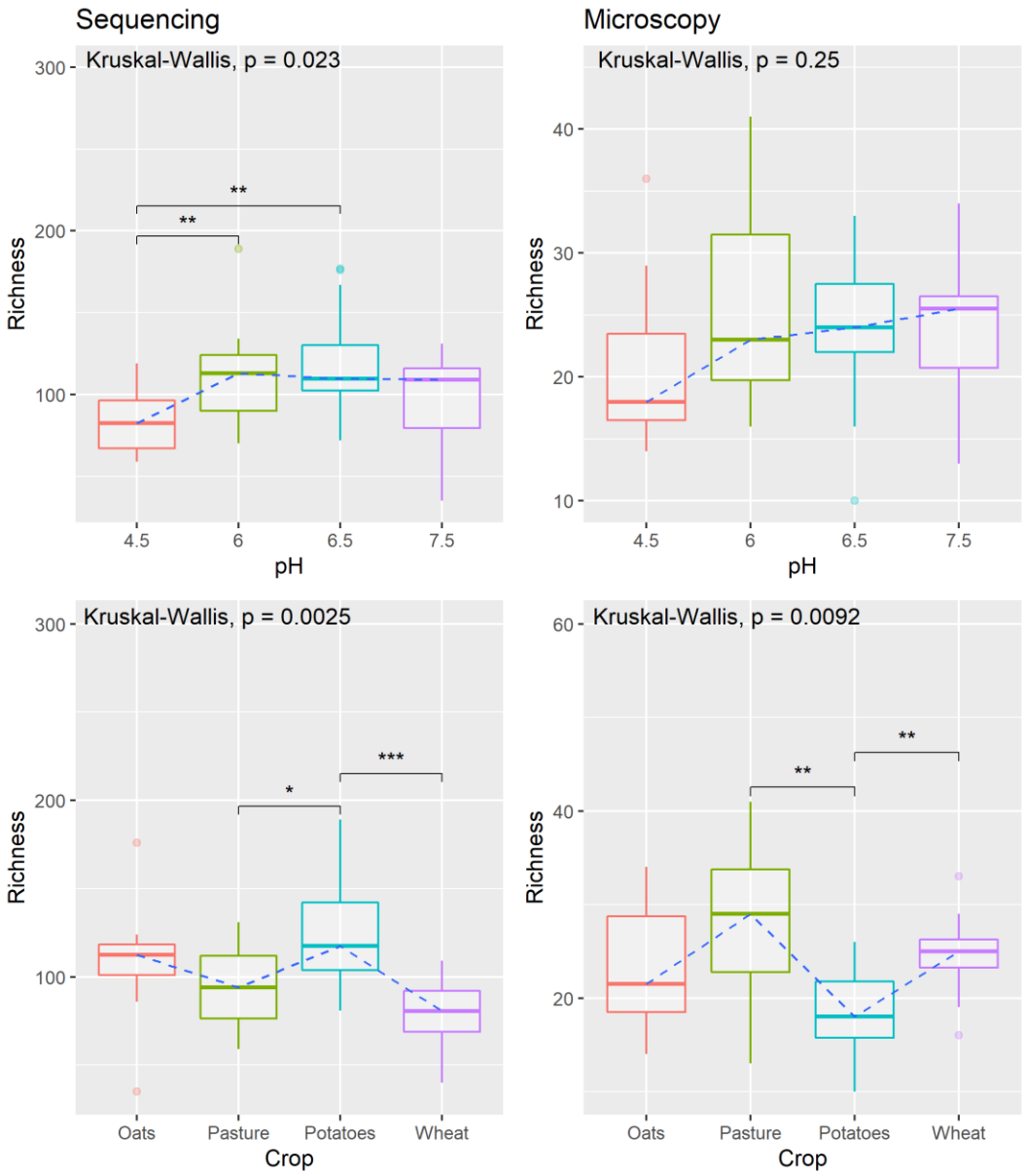


Figure 26. Numbers of arthropodal mAVS (Sequencing) and species (Microscopy) affected by different pH levels (top) and crop stages (bottom).

Correlation analysis: analyses revealed no correlation between datasets for both observed features and Shannon entropies (Figure 27; Table 18). However, when both unweighted Jaccard distances were tested with Mantel, a significant but weak correlation was observed (Figure 28).

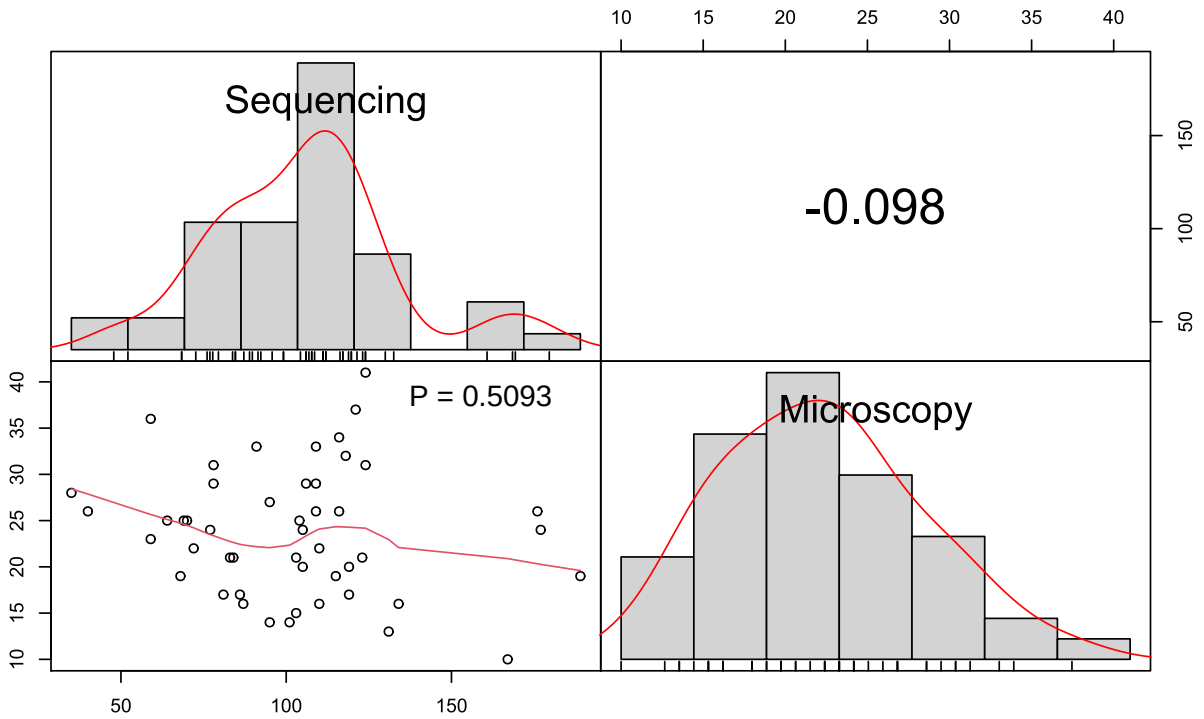


Figure 27. Spearman correlation chart between sequencing and microscopy richness data. Bar-plots show a normal sample distribution for both datasets. The scatter plot showed the low level of correlation between the richness values of both datasets.

Table 18. Spearman and Pearson correlation results between sequencing and microscopy richness and Shannon entropy data.

Method		Spearman	Pearson
Richness	**P**	0.509	0.315
	cor	-0.1	-0.15
Shannon	**P**	0.130	0.173
	cor	0.267	-0.2

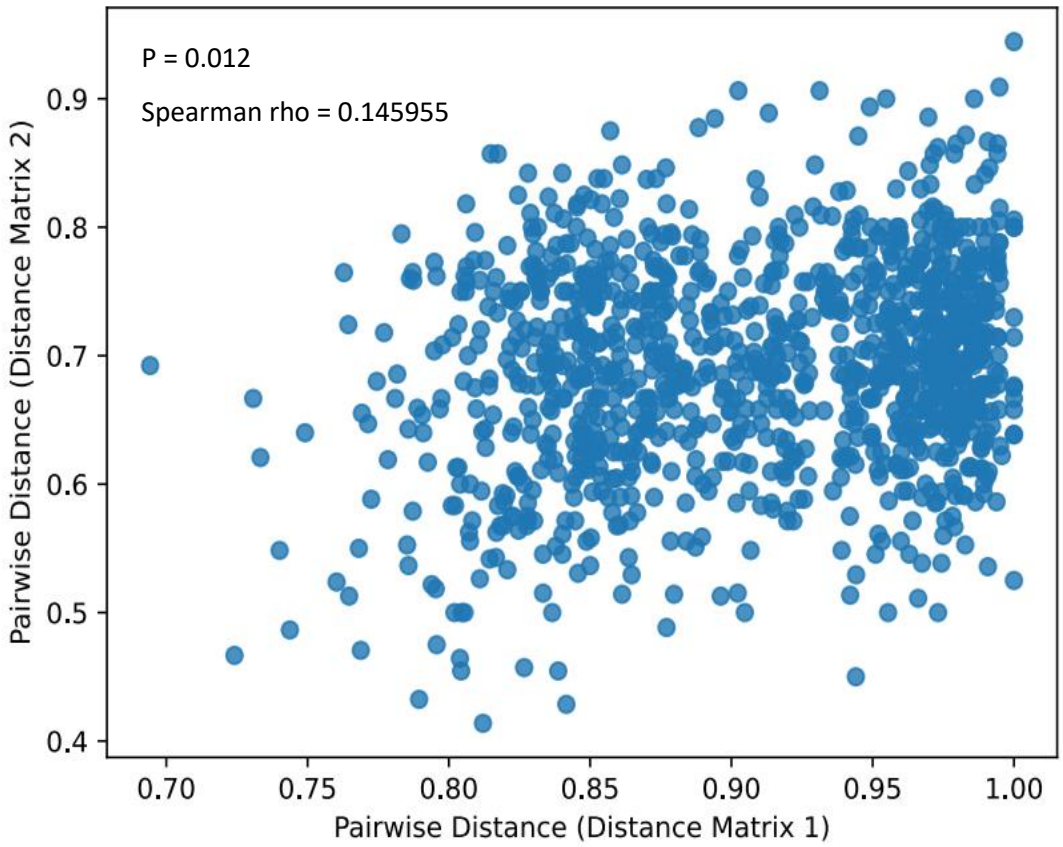


Figure 28. correlation plot representing unweighted Jaccard pairwise distances from sequencing datasets.

5. Discussion

5.1. Effect of long-term pH management on the soil microbiome

Much is already known of the effect of soil pH gradients on bacterial and fungal community structures (Fierer & Jackson, 2006; Rousk *et al.*, 2010). Bacterial correlations are usually stronger than fungal, corresponding with wider pH ranges observed for optimal growth of the fungal community. A recent metabarcoding analysis (George *et al.*, 2019) showed fluctuations of microbial diversity in 436 locations in 7 different temperate ecosystems across Wales, UK. In their analysis, pH was the best predictor for bacterial and second best for fungal diversity. Here, these findings were confirmed using the standardised metabarcoding approach at the controlled experimental site at Craibstone, which included pH levels within a range common to most agricultural systems (Msimbira & Smith, 2020). In response to pH, strong divergent trends in all three measured diversity metrics (numbers, types and abundances) were confirmed for both bacteria and fungi/oomycetes. These findings confirmed that small changes in soil pH can produce strong changes in community compositions.

The sequence data obtained was also successfully used to predict potential biomarkers within the bacterial and fungal communities, that appeared to fluctuate most in response to pH levels. A larger number of bacterial phyla were associated with pH 4.5, but more fungal phyla biomarkers were found at pH 7.5. However, when the total number of biomarkers across all taxonomic levels was analysed, significantly more bacterial biomarkers were found at pH 7.5 than at the other pH levels, whereas similar number of fungal biomarkers were found at each pH level. It is proposed that the total number of biomarkers is a good indicator of the strength of selection pressure exerted by a given treatment on biological communities.

It was also possible to some degree to use the sequence data obtained, to predict functional divergence within soil microbial communities as they diversify in response to different pH conditions. This represents one of the first attempts to use this kind of approach in the diagnosis of soil health. The FUNGUILD bioinformatics tool was demonstrated to predict the functions of biomarkers, at family, genus and species levels, although around one-third of these remained “unassigned” to any functional group. The distribution of different assigned fungal guilds was found to differ with soil pH, with symbiotrophs significantly more abundant at more neutral pH (6.0 and 6.5), while pathotrophs were more abundant at pH 7.5. Bacterial functionality predictions were as yet too inconclusive to reach any reliable conclusions due to the lack of taxonomic annotation at species level amongst the available databases. This approach to prediction of microbial functional in soil communities is expected to increase with the future development and combination of taxonomic and functional sequence databases. In the interim, data have been provided to support the hypothesis that fluctuation in diversity also translates into functional changes in agricultural soils.

5.2. Effects of long-term inorganic fertiliser application on the soil microbiome

There is a lack of precise information on the long-term impact of fertilisers on microbial communities. Previous studies have attempted to assess microbial fluctuation with different fertiliser treatments, but their conclusions are conflicting (Zhong *et al.*, 2010). Positive effects are related to increased microbial nutrient availability from fertilisers sustaining increased populations, also promoted by increased exudates from crop roots (Lehman *et al.*, 2015). The current study evaluated the long-term effect of fertiliser application over almost a century on soil bacterial and fungal communities. Results suggest that the fungal community may have been affected in terms of the relative abundance of its species rather than in the richness or types of species present. No particular phylum was significantly affected by the regular use of fertiliser, in disagreement with previous observations (Peine *et al.*, 2019; Silva *et al.*, 2017). Discrepancies between these findings may be due to methodological differences, including sampling depth, molecular and bioinformatic approaches, and sequence databases used in the analyses, highlighting the necessity for a standardized pipeline that can be used to compare diverse studies of soil microbial communities from different locations.

5.3. Effect of long-term organic amendments on the soil microbiome

Studies on the effects of organic soil amendments on bacterial and fungal communities have reported contradictory results (Li *et al.*, 2019). This may be due to the wide spatial variation in richness and diversity of microbial communities in soils. In a recent study across 12 European long-term experiments, Hannula *et al.* (2021) reported over two thirds of the fungal species described were unique for each of the countries involved. Moreover, some soil management practices had a variable effect on the diversity of fungal communities depending on the site location and no significant effects of different sources of organic amendment was observed in this case. This study evaluated the effect of organic amendments in three long-term trial sites on soil bacterial and fungal communities. As with results of the effects of long-term inorganic fertiliser application, the organic amendments results appeared to affect Numbers of the fungal rather than the bacterial community. These results suggest a higher sensitivity of fungal communities to organic/synthetic fertilisers application, at least at the time of sampling after harvest. However, the most relevant factor driving the composition and structure of both bacterial and fungal communities was the geographical location. The three experimental sites are distantly located, and their soil types differ with loamy sand (6% clay), sandy loam (12% clay) and silty clay loam (28% clay) at Gleadthorpe, Harper Adams and Terrington, respectively. A high clay content is thought to negatively affect DNA extraction from the soil matrix (Högfors-Rönnholm *et al.*, 2018). The average DNA yield obtained from Terrington samples was the lowest (42 ng/µL), although average DNA yield from Harper Adams (51 ng/µL) and Gleadthope (48.6 ng/µL) were similar even though soils from the former contained double the clay

content. Clay content did not, however, appear to affect the diversity metrics where, for example, Terrington samples showed the highest bacterial richness. The conclusion of microbial communities being mostly affected by their geographical location not only matches with the findings of Hannula *et al.* (2021) but can also explain the different results observed in the three experimental sites despite similar organic amendment treatments at each site. Interestingly, the largest effect of organic amendments on microbial diversity was observed at the Gleadthorpe site, suggesting that low clay soils may be more sensitive to these treatments.

5.4. Effect of sampling time and management practices on the soil microbiome

The analyses carried out on the experimental sites sampled in different years showed that the differences both in numbers and in types and abundance are significant for bacterial and fungal communities. Considering the effect size explained by the sampling time, it is suggested that the differences between numbers of both 16S and ITS ASVs are especially pronounced between different seasons.

These results in conjunction with those obtained from the experimental sites of organic amendments suggest that the effects of management practices may vary depending on place, time and season of the year.

5.5. Long-term effects of re-ridging and inter-row companion crops or mulching on soil bacterial and fungal diversity in asparagus production

Soil treatments in the asparagus trial were mainly designed to alleviate the effects of compaction, a common problem during highly mechanised asparagus production. Results from the current study have also indicated some influences of these treatments on the soil microbiology. Although the re-ridging treatment appeared to have no effect on number of bacterial or fungal ASVs, there was some evidence that shallow soil disturbance had some effect on reducing fungal richness at the time of sampling. Other interacting factors such as the use of companion crops and mulches appeared to have variable effects on the types and relative abundances of different bacterial and fungal taxa.

5.6. Standardised approach for combined analyses of the microbiome across different studies

A standardised approach was formulated and tested here for the first time that allows data from multiple studies to be merged and normalised in such a way that the effects of multiple factors can be evaluated on members of the soil community that are common to each study. Many studies on soil biology have reported the effect of agricultural management practices, however the

implementation of available diversity indexes has differed between studies. For that reason, a standardised analysis approach was proposed here and demonstrated that is viable to use it to test the extent to which bacterial and fungal diversities change in response to key soil managements across time and spatially dispersed datasets.

The advantage of merging ASV tables lies in being able to control for the effects of normalisation (i.e. all data are normalised together in the same way), and that more holistic analyses can be conducted, including being able to identify any differences in the direction of effects and to identify clusters of highly similar ASVs between studies. Together this allows comprehensive comparisons of the nature of effects and the types of taxa that are in common or different between different treatments in different studies. The identification of matching ASVs is possible because identical DNA sequences underlying ASVs produce the same MD5 identification tag between sequence runs with dada2 making ASVs from independent trials directly comparable, but only if sequences are in the same orientation and trimmed/truncated to the same length and sites. However, it is highly likely that independent studies will not have trimmed/truncated ASV reads to the same length and sites. To understand which highly similar ASVs are common across independent studies, ASVs in the merged table were clustered and analysed into mASVs, each of greater than 98% identity. Crucially, the analyses of mASVs then allow the hypothesis [that long-term independent pH and fertiliser managements have applied differential sustained selection pressures that have resulted in diverged and differentially adapted soil microbial communities] to be tested, by comparing the differences and similarities of communities between studies.

In the first instance, the utility of a standardised DNA metabarcoding method to assess agricultural soil microbial diversity was presented and validated, by evaluating the effect of two long-term agricultural trials that have consistently manipulated pH and fertiliser input for over 60 years. Comparisons of the analyses from both separate and then merged data have provided support for the hypothesis that long-term independent pH and fertiliser managements have applied differential sustained selection pressures that have resulted in diverged and differentially adapted soil microbial communities. Further, the DNA sequence data rejected the hypothesis that the magnitude of differences in soil biology due to sustained differential pH and fertiliser manipulations were approximately similar. Both individual and merged analyses showed the effect size of pH to be approximately 6-times larger than the effect of fertiliser additions.

Analysis of 16S and ITS ASVs in this study showed that the composition of types of bacterial and fungal species identified as ASVs differed between the two trials. A majority of both 16S and ITS ASVs were unique to the pH trial and almost half of the ITS ASVs were unique to the fertiliser trial. There could be methodological reasons for these dissimilarities between trials including differences in sampling times and differences in performance between different sequencing runs. The latter was addressed to some extent by the implementation of the SRS normalization method. This performs a

similar approach to traditional rarefaction (reducing all sample reads to a given sampling depth) but with a more conservative approach in terms of preserving ASVs richness (Beule & Karlovsky, 2020). Nonetheless, the ideal normalization method with different ASVs library size continues to be a topic of discussion (McMurdie & Holmes, 2014; Weiss *et al.*, 2017). The approach taken in this study was to first merge datasets from both trials and normalize the merged dataset by SRS. Differences between the trials was then analysed using the standardized pipeline to compare the effects of the pH and fertiliser treatments with the results obtained from the separated datasets.

Analyses of the effects of pH gradient and fertiliser application using the merged datasets showed similar results to those obtained using the two individual datasets except for the effect of fertilisers on the types of fungal ASVs, which was only found significant when the merged data were analysed.

In the case of the two Craibstone trials, identical methodology was applied in the DNA extraction, metabarcoding and analysis of both trials. Moreover, the soil conditions were very similar with the two trials being located less than 100m apart. It is therefore unsurprising that the analyses of individual and merged datasets gave rise to very similar findings. Both approaches showed that pH but not fertiliser significantly affected fungal and bacterial taxa and both pH and fertiliser significantly affected the types of bacterial and fungal taxa and their relative abundances. This indicates a possible sorting effect of differential natural selection variously imposed by different pHs and fertilisers. Furthermore, both approaches showed the relative effect of pH was greater than that of fertiliser. Inferences of the sizes of effects between studies will be particularly useful to increase an understanding of the relative size of effects of specific agricultural and soil treatment on soil biological communities.

The same standardised approach was used to compare data from a further three geographically remote trials (at ADAS Gleadthorpe, ADAS Terrington and Harper Adams University) that were designed to investigate the long-term effects of repeated soil amendments with organic matter from various sources. In this case, analysis of the merged data clearly showed that, although significant differences were found in the types and relative abundances of 16S and ITS mASVs between amended and non-amended soils, these effects were much smaller than the differences observed between locations of each trial (see Figure18). It was therefore concluded that whilst long-term independent organic amendments had applied differential selection pressures that resulted in diverged soil microbial communities, the magnitudes of these differences in soil biology created as a result of agronomic managements were much less than those observed between the natural biological communities adapted at each of the locations. It is assumed that differences in both soil types and conditions at sampling had contributed to the large effects observed between locations. These results indicate the true value of being able to merge datasets from multiple studies, rather than to extrapolate findings from any individual study, when forming general recommendations on the effects of soil management on soil microbiology.

The importance of the standardized approach to DNA analysis proposed here therefore roots in the necessity to implement the same metric across many different studies with greater expected variation than that observed between only the few sites studied here. Many individual studies on soil biology have reported the effect of agricultural management practices, however the implementation of available diversity indexes has differed between studies. For that reason, a standardised combined metabarcoding approach is presented here: it has been demonstrated that it is viable to use it to test the extent to which bacterial and fungal diversities change in response to key soil managements and the need to assess the magnitude of those changes across multiple datasets.

5.7. Potential for a molecular soil health testing service for UK growers and agronomists.

Standardised methods evaluated within this project have demonstrated that it is possible to use metabarcoding to compare the diversity of bacteria, fungi and, to a more limited extent, mesofauna across samples of soil. As a trial venture, these approaches have already been used to assess samples of arable soils submitted from growers (including samples from the field sites selected in this project) through the Big Soil Community initiative at Fera Science Ltd. (https://www.fera.co.uk/news/big-soil-community). This involves a community effort between growers, agronomists and Fera scientists to investigate the diversity of UK soils and understand how it can affect crop production and long-term soil health. Since the launch in 2018, over 400 samples have been analysed for fungal and bacterial diversity and an additional nematode screen was also introduced in 2021. Samples have mostly come from country-wide arable growers, enabling comparisons to be made between different variables, including locations, soil types, organic and conventional systems and crop yields.

Participating farmers have submitted samples in October/November and received results in January as two reports; the first detailing the diversity in each soil sample and highlighting the most abundant taxa of interest (including beneficial organisms and pathogens), and the second anonymously benchmarking each result against the wider community picture. Initially costed at £250 per sample, participating growers are aware that they are contributing to the development and interpretation of the tests as well as understanding the diversity of their soils. It is anticipated that the cost per sample will fall as interest in the scheme increases. Sustainability of the scheme is dependent on the usefulness of the information to each grower. Automated methods that identify key taxa and their relative abundances from the soil DNA, developed in this project, are key to increasing the value of information that can be fed back to growers. For example, methods that can automatically predict functions associated with taxa identified within the soil microbial communities will help to add practical value to the results by not only identifying the most abundant organisms but also estimating whether they are providing key ecological services and whether they may be harmful or beneficial to

crops. It is expected that this approach will facilitate the identification of bioindicators common to all samples, allowing the development of more targeted field tests that could monitor changes in the behaviour of these indicators as influenced by factors such as cropping practices, soil management, soil types and climate. Furthermore, standardised analytical procedures developed in this project, that allow analysis of merged DNA sequencing data, will permit comparisons of field assessments from multiple sources, even when there is variation in the methods used to collect the data. This will open the investigation of factors contributing to biological soil health to a much wider breadth of soils and agroecological systems both across the UK and internationally.

6. References

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