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**A comparative *in situ* decomposition study using still born piglets and leaf litter from a deciduous forest**

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# **A comparative *in situ* decomposition study using still born piglets and leaf litter from a deciduous forest**

## **Abstract**

A cadaver and dead plant organic matter, or litter, are rich energy sources that undergo a complex decomposition process, which impact the surrounding environmental microbiota. Advances in molecular microbiology techniques, with study of the 16S RNA genes, in particular, have highlighted the potential of forensic ecogenomics in addressing key knowledge gaps. To investigate subsurface microbiome shifts as a novel tool to establish “postmortem microbial clock” and augment postmortem interval (PMI) and time-since-burial estimations, an *in situ* study with triplicate underground burials of piglets as human taphonomic proxies and *Quercus robur* leaf litter was monitored for 270 days. Changes in microbial community structure and composition were related directly to changes in seasonal temperature, with microbial shifts more pronounced during the summer. For example, Methylococcaceae could be used as seasonal bacterial indicators, from winter to summer, in establishing postmortem microbial clock for this site. Furthermore, Methylophilaceae (Methylophilales order) and Anaerolineaceae could be used to differentiate for the piglet and leaf litter soils, respectively.

## **Keywords**

*Cadaver, Ecogenomics, Piglets, Postmortem, Microbiota, Leaf litter*

## Introduction

A cadaver is an energy resource, which plays a role in nutrient cycling with the release of numerous compounds such as acetic acid, amino acids and propionic acid, into the surrounding soil [1,2]. In particular, its decomposition is often described as a complex process that is attributed to microbial, vertebrate and invertebrate scavenger metabolic activities, which impacts the surrounding environmental microbiota [1,3]. Advancements in molecular microbial ecology techniques have enabled researchers to study the epinecrotic, necrobiome and thanatomicrobiome communities in these complex interactions within the novel forensic ecogenomics discipline [4,5,6].

Microorganisms play crucial roles in both cadaver and plant litter decomposition with subsequent increases of soil particulate organic matter content and available substrates. These, in turn, effect successional dynamics in the occurring microbial community structure and composition [2,7,8,9]. So there is intense interest in elucidating the relationships between cadaver microbiota and soil microbial fauna as potential indicators in forensic applications. For example, the possible use of the epinecrotic community as a “postmortem microbial clock” was indicated by Metcalf *et al.* [4] and Pechal *et al.* [10]. In a recent study, Metcalf *et al.* [2] stated that approximately 40% of microbial decomposer communities were found at very low abundances in soils at the start of their experiments and recorded significant changes in microbial communities relative to seasonal shifts (spring and winter). Notwithstanding this, comparisons between soil microbial communities associated with subsurface cadaver and litter decomposition processes remain unexplored.

As a result, this *in situ* study was made with stillborn piglets and leaf litter from a deciduous oak (*Quercus robur*) forest to address the following research questions:

- (i) Do whole piglets and leaf litter decomposition illicit the same trends or shifts in biodiversity *in situ* compared to soil controls?;
- (ii) Do seasonal variations impact the microbial community compositions and structures as expressed by 16S taxa distributions?

## **Experimental design**

### **Carcasses and *in situ* site**

Frozen (-20°C) still-born piglets (~ 1.5 kg) were sourced from Northumbria Police (Ponteland, U.K.), transported on icepacks, re-frozen (-20°C) and thawed completely and immediately before the study burials. Prior to the start of the study, the site located at an undisclosed site in North Yorkshire, U.K. was cleared and mapped with a Leica GS15 global navigation satellite system (GNSS; Heerbrugg, Switzerland) with real-time kinematic (RTK) corrections typically providing typically 10 mm accuracy.

The soil was characterised as loam soil constituted by (w/w) 22% clay, 32% silt and 46% sand (Forestry Commission, Surrey, U.K.) and physicochemical characteristics of Al (20 g kg<sup>-1</sup>), Ca (25 g kg<sup>-1</sup>), K (4.7 g kg<sup>-1</sup>), Mg (8.2 g kg<sup>-1</sup>), Na (0.47 g kg<sup>-1</sup>), nitrate aqueous extract as NO<sub>3</sub> (3.5 mg l<sup>-1</sup>), total organic carbon (3.0%), total S (0.03%), pH (7.9), P (<0.10 mg kg<sup>-1</sup>), calorific value (<1.0 MJ kg<sup>-1</sup>) and electrical conductivity (250 μS cm<sup>-1</sup>).

The burial site (6 m by 6 m) was cleared and divided into three 1-metre sections, 1.5 m apart. Each of the three 1-metre sections (Figure 1) had three pits dug with dimensions of 50 cm (length) by 30 cm (width) by 40 cm (depth), 2 m apart for the control (C), indigenous oak leaf litter (*Quercus robur*) (L) and piglet (P). For the piglet burials, aluminium wire mesh cages (40 cm long x 25 cm wide x 15 cm height) were fabricated to prevent scavengers from gaining access. Soil sample cores (20-60 cm) were collected monthly from December 2014 (day 0) to September 2015 (day 270) with a gouge auger (Eijkelkamp, Netherland) from each side of each

pit. For the piglets, care was taken to avoid carcass disturbance. The four samples from each pit were combined prior to storage in 25 mL sterile universal bottles for transport to the laboratory. Composites of the homogenised (10 g) samples were stored (25 mL sterile universal bottles; Sarstedt, Germany) at -20°C until required for both pH and DNA extractions. The study was conducted from December 2014 (winter) to September 2015 (autumn).

**Environmental parameters** atmospheric temperatures for the site location were obtained from <http://www.metoffice.gov.uk/>, while pH and temperature were determined as described in Olakanye et al. [11].

### **Soil DNA extraction and purification**

Total soil community DNA was extracted as described previously Olakanye et al. [11] prior to purification using the PowerClean® DNA Clean-Up Kit (Mo Bio Laboratories, Inc., U.S.A.) according to the manufacturer's instructions.

### **Next-generation sequencing and data analysis**

The purified microbial community DNA extracts were sequenced with an Illumina Miseq platform (NU-OMICS, Northumbria University, Newcastle Upon Tyne, U.K.) with a primer set targeting the V4 region of the bacterial 16S rRNA gene as described previously [12]. The raw sequencing reads were processed in FASTQ format and were analysed with Mothur software package (version 1.36.1) (University of Michigan, U.S.A.). The FASTA formatted sequences were quality checked and filtered with UCHIME. The sequences were aligned to the SILVA reference and taxonomic identification of the reads were assessed by assigning sequences to OTUs with Ribosomal Database Project (RDP) classifier. PCR negative controls were run and sequenced in parallel to the samples with OTUs present in negative controls and samples excluded from any further analysis. Non-bacterial sequences (e.g. archaea) were discarded and reads rarified at 6 750 sequences per sample (S1). OTUs less than 3% were



classified as rare taxa. Both the rare taxa and the unclassified OTUs were omitted from the plots.

**Data analysis.** All data were evaluated statistically by a univariate two-way ANOVA with repeated measure (RMA). Taxa similarities between the controls and treatments were analysed with the Bray-Curtis (BC) distance un-weighted pair-group using the arithmetic average (UPGMA) clustering algorithm. The phylogenetic distance matrices were analysed using Bray-Curtis dissimilarity with nonmetric dimensional scaling (NMDS) by the paleontological statistics software package for education and data analysis (PAST 3.10, 2015). Alpha diversity was estimated with Shannon diversity (S2), which was expressed by boxplot with xlstats. Relationships between soil pH, temperature and phyla relative abundance were analysed using Spearman's rank correlation coefficient (SCC) (S3) (xlstats 2016.02.27313, New York, U.S.A.).

## Results

### *pH trends*

The average pH values for the control and treatments (*Sus scrofa domesticus* and *Quercus robur* litter) soils were compared between day 0 (winter, December 2015) and 270 (autumn, September 2015) (Figure 2) and showed an increase for the *Sus scrofa domesticus* ( $7.84 \pm 0.07$ ) from day 0 to day 30 compared to control ( $7.86 \pm 0.14$ ) and leaf litter ( $7.80 \pm 0.03$ ). While increases in pH between days 30 and 60 were recorded for the control ( $8.03 \pm 0.08$ ) and leaf litter ( $8.05 \pm 0.02$ ), the *S. scrofa domesticus* soil decreased ( $7.92 \pm 0.08$ ). Both the control and *Quercus robur* litter soils recorded pH decreases between days 60 (control,  $8.03 \pm 0.08$ ; leaf litter,  $8.05 \pm 0.02$ ) and 150 (control,  $7.61 \pm 0.05$ ; leaf litter,  $7.70 \pm 0.05$ ) while the piglet soil showed an earlier fall between days 90 ( $7.96 \pm 0.09$ ) and 120 ( $7.66 \pm 0.04$ ) before increasing again. Likewise, both the control and leaf litter then recorded pH increases between days 150 to 210. The highest pH values for the control ( $8.25 \pm 0.14$ ) and *Quercus robur* leaf litter ( $8.44$

$\pm 0.24$ ) were both recorded on day 210, while the highest pH value ( $8.02 \pm 0.01$ ) for the *Sus scrofa domesticus* was recorded on day 30. All three soils recorded pH decreases between days 210 and 240 with subsequent increases to final values of  $8.14 \pm 0.19$  (control),  $8.09 \pm 0.02$  (leaf litter) and  $7.97 \pm 0.04$  (piglet) on day 270. Two-way repeated measure ANOVA (RMA) showed no statistically significant temporal differences ( $p = 0.064$ ) between the control and experimental graves over the course of the study.

### *Temperature*

The average soil temperatures for the control, piglet and *Quercus robur* leaf litter burials were recorded on each sampling day. For accurate PMI estimation, the temperature data were further expressed in accumulated degree days (ADD), which is a model that measures the heat required for biological processes [13,14]. The averages of the maximum and minimum ambient temperatures were used to calculate the daily ADD [14,15], with a base temperature of  $0^{\circ}\text{C}$  according to earlier work of Megyesi *et al.* [16] (Table 1). Decreases were recorded from day 0 (ADD 12.3) for the control ( $12.2 \pm 0.09$ ), *Sus scrofa domesticus* ( $12.3 \pm 0.12$ ) and *Quercus robur* leaf litter ( $12.4 \pm 0.19$ ) soils to day 60 (ADD 243.3: control,  $2.9 \pm 0.28$ ; *Sus scrofa domesticus*,  $2.9 \pm 0.27$ ; leaf litter,  $2.9 \pm 0.17$ ). Seasonal change from late winter (March 2014) to summer (July 2015) resulted in temperature increases from days 90 (ADD 422.8) to 210 (ADD 1770.7) for the control ( $6.5 \pm 0.15$ ;  $21.7 \pm 0.78$ ), *Sus scrofa domesticus* ( $6.3 \pm 0.03$ ;  $22.1 \pm 0.65$ ) and leaf litter ( $21.9 \pm 0.32$ ) soils while falls, due to seasonal weather change from late summer (August 2015) to early autumn (September 2015), were observed between days 240 (ADD 2232) to 270 (ADD 2723) for the control ( $17.8 \pm 0.47$  to  $15.4 \pm 0.56$ ), *Sus scrofa domesticus* ( $17.2 \pm 0.47$  to  $14.8 \pm 0.18$ ) and leaf litter ( $18.5 \pm 0.55$  to  $15.4 \pm 0.34$ ) soils (Figure 3). Since similar trends were recorded for the three soils during the study, two-way RMA showed no statistically significant temporal differences ( $p = 0.085$ ) between the control and experimental soils.

### *16S bacterial community taxonomic resolution*

Overall, the dominant phyla included Proteobacteria (28.70 – 40.85%), Acidobacteria (15.04 – 32.53%), Verrucomicrobia (4.70 – 11.10%), Bacteroidetes (6.43 – 15.92%) and Actinobacteria (8.60 – 14.66%). Taxonomic comparisons (Figure 4) of the control and treatments showed 97% similarity on days 90 and 150 for the controls, 96% similarity on days 60 and 150 for the leaf litter and 98% similarity between days 60 and 120 for the piglet graves. Further comparison revealed 97% similarity between the control, leaf litter and piglet graves between days 60 and 150, and 98% similarity between the leaf litter and piglet graves on days 60 and 90. The NMDS showed taxa similarities between the control and experimental soils from day 60 to 150 while differences between the control and experimental gravesoils were observed from day 180 to 270 (Figure 5). Chlorobi, Chloroflexi, Gemmatimonadetes and Nitrospira correlated positively with temperature measures while Actinobacteria, Bacteroidetes, Planctomycetes and Verrucomicrobia correlated negatively (Table 2). In contrast, pH measures did not record any correlation with the phyla during the study.

Taxa resolution at the order level (Figure 6) revealed temporal taxonomic changes of the control and treatment soils between days 180 and 270. NMDS analysis identified similar dominant taxa, such as Acidobacteria\_Gp6\_order (15.37 – 23.23%), Planctomycetales (7.55 – 12.57%), Subdivision3\_order (2.42 – 7.66%), Rhizobiales (6.50 – 8.62%), Sphigobacteriales (3.93 – 7.33%) and Actinomycetales (6.06 – 8.54%) between days 0 and 150, which represented the winter to spring interval. A shift in bacterial community structure was recorded on day 180 with a decrease in relative abundance of Acidobacteria\_Gp6\_order (7.02 – 14.44%) between the control and treatments with Planctomycetales (7.43 – 10.95%) becoming the predominant taxon in the latter. Also, increases in the relative abundances of Anaerolineales (5.58%) and Acidobacteria\_Gp7\_order (3.15%) were observed for the *Quercus robur* leaf litter soil. In contrast, the piglet soil recorded relative abundance increases of Methylophilales

(4.01%), Methylococcales (4.97%) and Flavobacteriales (3.85%). For all samples, shifts in taxa dominances were observed on day 210 with increases in Acidobacteria\_Gp6\_order (most dominant) and Acidobacteria\_Gp16\_order, and a decrease in Planctomycetales relative abundance. Increases in the relative abundances of Methylococcales (3.47%) and Anaerolineales (2.63%) were observed on day 240 for the leaf litter soil. For the control, further taxa changes were observed on day 270 with an increase in relative abundance of Xanthomonadales (7.50%) and a decrease of Acidobacteria\_Gp6\_order (15.72%).

The alpha Shannon-Wiener diversity box plot showed no statistically significant differences ( $p = 0.41$ ) neither between the control and treatments, nor with season (Figure 7).

## **Discussion**

Postmortem microbial changes are gaining considerable attention with studies of cadaver decomposition epinecrotic communities [4,7,17]. Ethical guidelines in different countries dictate whether human cadavers, organs and tissue can be used hence different mammalian surrogates, particularly pig (*Sus scrofa*), are often adopted as human taphonomic proxies for investigative purposes.

An increase in *Sus scrofa domesticus* gravesoil pH between days 0 to 30 was in agreement with earlier reports where increases were attributed to release of ammonia-rich fluid; mineralisation of base-forming cations ( $\text{Ca}^{2+}$ ,  $\text{K}^+$  and  $\text{Mg}^{2+}$ ); and ammonification of organic nitrogen (protein and peptide) [2,18,19]. Similarly, the subsequent pH decrease was supported by the work of Meyer *et al.* [19] who suggested that nitrate accumulation reduced pH. The pH differences recorded for the *Sus scrofa domesticus* and *Quercus robur* leaf litter soils can possibly be related to their nutrient contents [20,21] and decomposition rates, which have been reported as slower for plants [2].

The potential use of epinecrotic communities as the “postmortem microbial clock” [4,5,22] highlighted the need for more comprehensive decomposition studies. While characteristically predominant in soils [23,24,25], the numerical abundances of Proteobacteria, Acidobacteria and Actinobacteria specifically in decomposition-impacted soils have been reported by various researchers [26,27,28,29]. As observed in this experiment, Proteobacteria was found to be the most abundant phylum throughout. Although Acidobacteria was expected to correlate negatively with pH [4] in piglets samples, this was not apparent in the study. Overall, phylum-level differentiation between the control and treatment soils was achieved in the summer months with Proteobacteria as the potential seasonal community structure-based PMI and time-since-burial indicator.

Microbial activities are influenced by ambient temperature with higher activity recorded between 21°C and 38°C and a lower activity resulting below 4°C [30,31]. During the study, an ambient temperature decrease occurred from days 0 (8°C) to 60 (3°C) (autumn 2014) while a seasonal change from spring to summer (days 90 to 210) resulted in an increase to 14°C before the change from summer to early autumn (days 240 to 270) resulted in a decrease to 12°C. The control and treatments phyla were comparable between winter (day 0, December 2014) and spring (day 150, May 2015) but family-level resolutions (Fig 8) revealed more detailed phylogenetic variations between the soils with pronounced taxa community shifts recorded during the summer season (days 180 to 270).

Increases in Methylococcaceae, aerobic Gram-negative methane-oxidising bacteria [32], and decreases in Acidobacteria\_Gp6\_family were recorded in all soils on day 180. The predominance of obligate anaerobes, such as Anaerolineaceae, which are associated with anaerobic degradation of crude oil related compounds [33], and Gram-negative sulphur-oxidising Hydrogenophilaceae [34] were recorded for the leaf litter soil on day 180 while aerobic Gram-negative methanol-oxidising [34] and dimethylsulphide-degrading [35]

Methylophilaceae [34] dominated the piglet soil. The former contrasted an earlier report by Purahong *et al.* [8], where genera such as *Frigoribacterium* and *Sphingomonas*, known for their proteolytic and cellulolytic enzymes, were recorded during the early stages of leaf litter decomposition. These contrasting trends may be attributable to different leaf litter types – plant species and respective cellulose, lignin and hemicellulose content – and experimental designs (litter bags vs. *in situ*).

The particular occurrence and numerical abundance of Methylophilaceae on day 180 (early summer) in the presence of *Sus scrofa domesticus* identified this family as a likely community composition- and structure-based “microbial clock” indicator, and suggested an influx of methanol. Therefore, although outwith the scope of the current study, this molecule could also be a potential biochemical signal to be targeted in complementary forensic chemistry and forensic ecogenomic analyses. Furthermore, the role of chemical signals, including methanol, is well recognized in forensic entomology and justifies adoption of this discipline along with the two above to address, comprehensively, key knowledge gaps in forensic subsurface decomposition work.

Taxa shifts with increases in relative abundance of the Acidobacteria\_Gp4, Gp7 and Gp16\_family were recorded on day 210 for all soils. For the *Quercus robur* leaf litter soil, decreased Anaerolineaceae relative abundance was recorded on day 240 while increases in Hydrogenophilaceae and Xanthomonadaceae were found for the control on day 270. As reported by Purahong *et al.* [36], microbial communities involved in litter decomposition undergo temporal season-dependent changes, which was also observed during this study. The presence of nitrogen-fixing Rhizobiales recorded for the control and treatments throughout the decomposition timeline is in contrast to the work of Hoppe *et al.* [29], who reported these as mutualistic bacteria that are associated with fungi in *Picea abies* and *Fagus sylvatica* deadwood log decomposition and whose abundance increased in late intermediate to advanced decay

stages. Notwithstanding this, some similarities occurred thus Hoppe *et al.* [29] recorded the presence of methanotrophic bacteria in deadwood logs, with the most abundant belonging to the *Methylovirgula* genus, while the current investigation showed the presence of Methylococcaceae in both the control and treatments, and Methylophilaceae only in the piglet gravesoil.

Overall, the order-level resolution matched phylum-based profiling where the shift in microbial community structure was seemingly season-dependent and occurred between early summer (day 180; June, 2015) and early autumn (day 270; September, 2015) when ADD maxima between 1314.9 and 2755.8 were also recorded. This was evidenced, by increased abundances of bacterial orders Methylophilales, Methylococcales, and Flavobacteriales for the mammalian surrogate gravesoils in particular. Specifically, the Methylophilales recorded considerably low relative abundances at most sampling times. Therefore, the differential presence and absence identified this as a potential order-level “microbial clock” indicator relative to community composition, in particular.

Generally, the abundance of Methylophilales and Methylococcales confirmed anaerobic conditions *in situ* and the sensitivity of the rare methylotrophic denitrifiers and methanogenic orders, respectively, as biomarkers for decomposition. Notwithstanding this, the increased abundance of Methylococcales in the control and treatment soils on day 180, and on day 240 in the presence of plant litter, indicated that it would, potentially, not be an optimum differentiating taxon for *Sus scrofa domesticus*, as the mammalian proxy, versus plant matter-based decomposition. The increased abundance of the order Flavobacteriales suggested a piglet decomposition-based enrichment of commensal opportunistic pathogenic strains that are typically found in marine (animals), contaminated or waste treatment ecosystems [e.g. 37,38].

A report by Weiss *et al.* [7] identified a marginal difference in the epinecrotic community of a 1 kg swine when compared with 20 – 50 kg carcasses but recommended the use of the latter weight range as human surrogates in postmortem microbial investigations. Nevertheless, this programme used ~1.5 kg piglets, which are recognized as good models for research purposes [48–39], even if they may not have effected decomposition and associated microbial communities of adult pigs. Although it was assumed that the stillborn piglet carcasses used in this study would have relatively high lipid contents, Charneca *et al.* [49–40] reported a higher protein to lipid ratio in newborn piglets. Therefore, future studies should investigate the effects of age and nutrient composition on the environmental/soil microbial community dynamics.

## **Conclusions**

Determinations of the effects of abiotic factors on cadaver decomposition activity rates are essential in postmortem and time-since-burial investigations. Since dead plant organic matter, or litter, is a unique and considerable energy source, its decompositional impacts on occurring soil microbial community dynamics must be explored specifically for direct comparisons to carcass-based influences within forensic ecogenomics. As observed in this study, temperature differences were related directly to seasonal differences in decomposition activities, as expressed by taxa relative abundance, identified by next-generation sequencing. NGS revealed that microbial community shifts were more evident through the summer season (from day 180, June 2015). Also, some unique taxa such as Methylococcaceae could be used generally, as community structure-based seasonal indicators as observed from day 180. In particular, Anaerolineaceae, recorded for the *Quercus robur* leaf litter soil, and Methylophilaceae especially the Methylophilales order, recorded for the piglet, could be used in the “postmortem microbial clock” and time-since-burial determinations for the two different substrates in the summer for graves associated with similar soils or sites.



This study was carried out with pigs as surrogates for human cadavers. Since piglets were used, the current findings may start to provide information relevant to suspected clandestine child burial investigations in contrast to those of adults. Also, the piglets were frozen prior to being thawed on site immediately before setting up the study hence an assumption was made that the impacts of their indigenous microbiome would be minimal. Therefore, future decomposition investigations should include comprehensive study designs and analyses and consider: various animal models of different sizes and ages relative to nutrient load and composition; study animals of similar age and size in different *in situ* soils and local climates; effects of cadaver/surrogate storage (freezing); parallel profiling of the cadaver/taphonomic proxy microbiome and gravesoil microbiota; soil nutrient analysis; gaseous and volatile organic carbon emissions; and volatile fatty acid profiling relative to soil pH. All of these must be implemented parallel to both affordable microbial community profiling techniques and more high-throughput platforms such as NGS.

Also, although not implemented in the current study, our future subsurface work entails analysis of the decomposition stages by monitoring physical changes of [41], and total body score [42] determinations for, decomposing mammalian taphonomic proxies. Furthermore, as illustrated by some emerging but aboveground studies [43], innovative experimental designs are required to elucidate microbiome-insect interactions (*re* chemical signals) in belowground decomposition scenarios, including in the presence of plant litter [44]. These complementary approaches at the interface of forensic ecogenomics, forensic entomology and forensic chemistry should provide additional metadata for enhanced subsurface postmortem interval and time-since-burial estimations.

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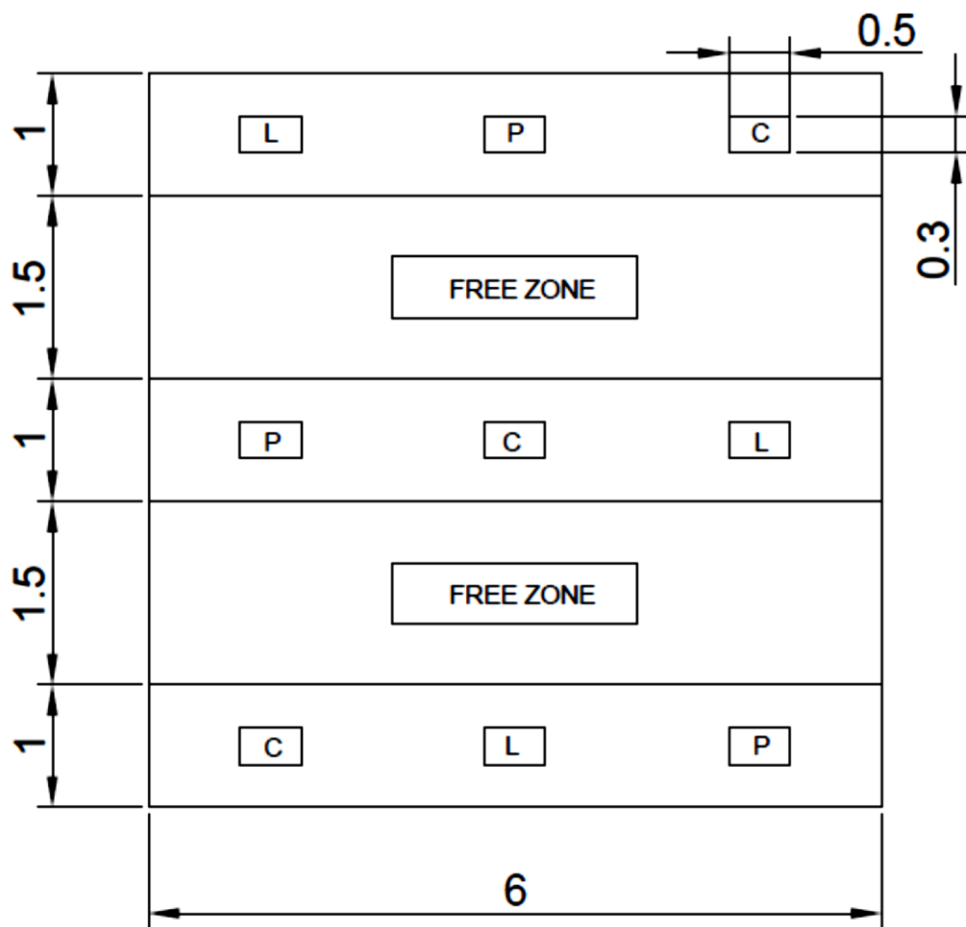


Figure 1: Site configuration of the control (C), leaf litter (L) and piglet (P) burials with all dimensions in metres. All pits were dug with dimensions of 50 cm (length) by 30 cm (width) by 40 cm (depth).

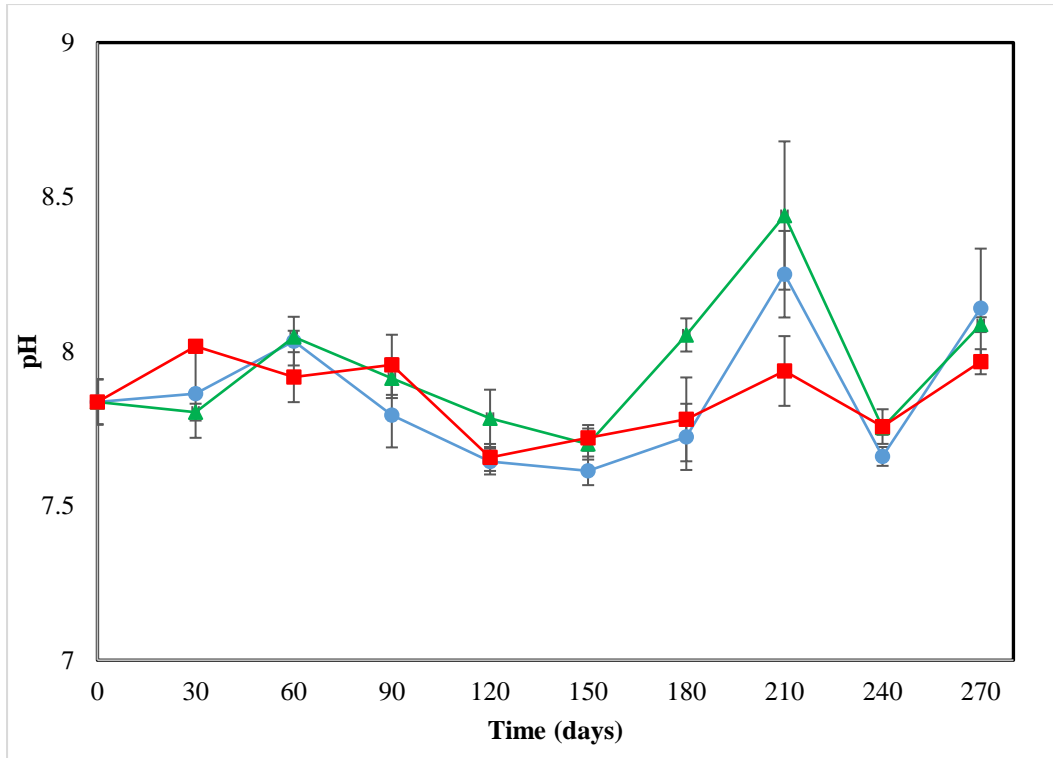


Figure 2: Average ( $n = 3$ ) pH values of the control (●), *Sus scrofa domestica* (■) and leaf litter (▲) soils during a 270-day *in situ* study where day 0 was December 2014. Error bars represent standard error calculated from means of three replicates.

Table 1: Average decomposition temperature timeline as expressed by accumulated degree days (ADD).

Day	Control	Leaf litter	Piglet
0	12.2	12.4	12.3
30	170.5	170.2	170.3
60	243.4	243.1	243.2
90	423.0	422.3	422.5
120	718.3	718.1	718.0
150	902.8	903.4	902.7
180	1315.6	1316.9	1314.9
210	1770.3	1771.9	1770.0
240	2259.1	2261.4	2258.2
270	2753.5	2755.8	2752.0

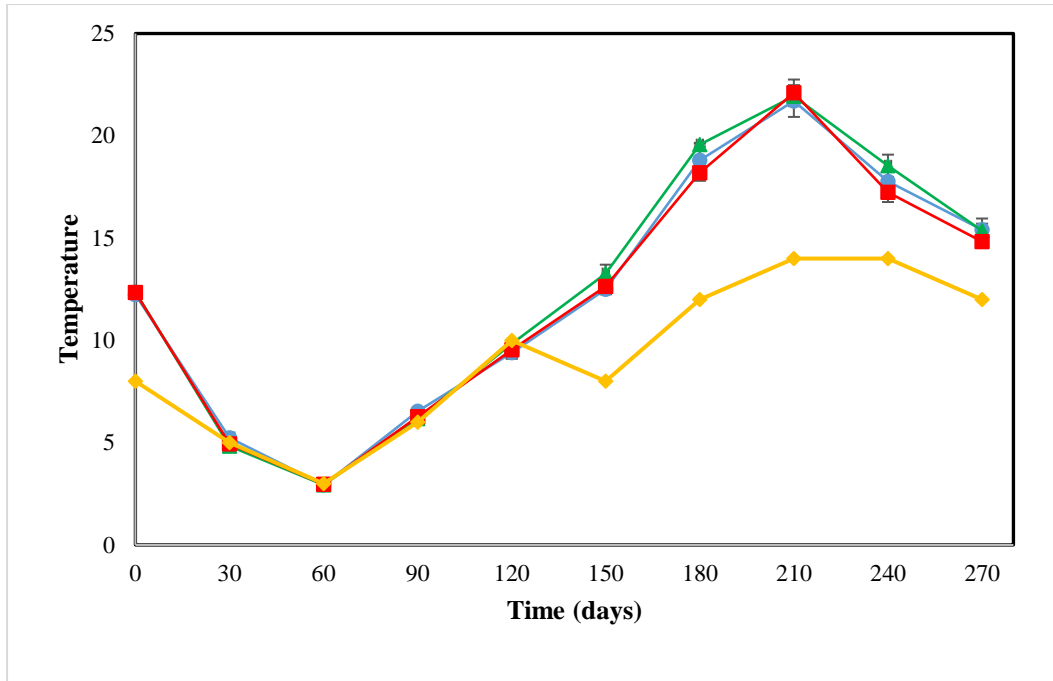


Figure 3: Average ( $n = 3$ ) temperatures of the control (●), leaf litter (▲) and piglet (■) soils, and ambient temperatures (◆) in North Yorkshire, U.K., during a 270-day *in situ* study. Error bars represent standard error calculated from means of three replicates.

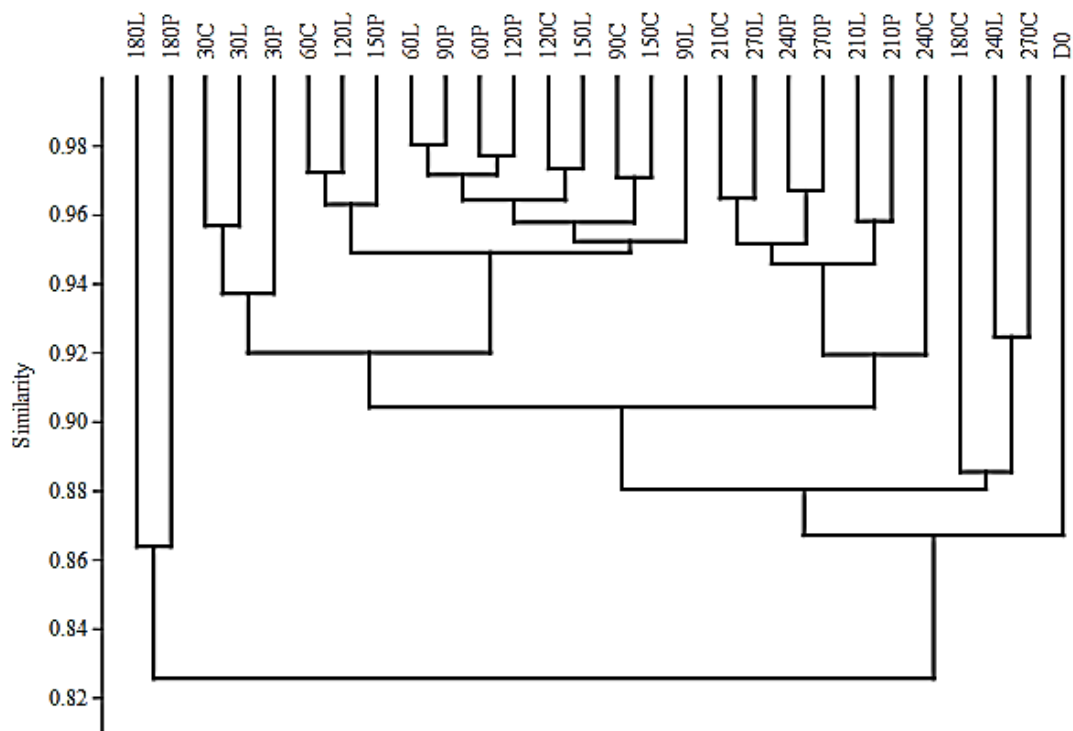


Figure 4: Average ( $n = 3$ ) 16S bacterial phyla distance un-weighted pair-group using the arithmetic average (UPGMA) cluster analysis of the control (C), leaf litter (L) and piglet (P) soils.

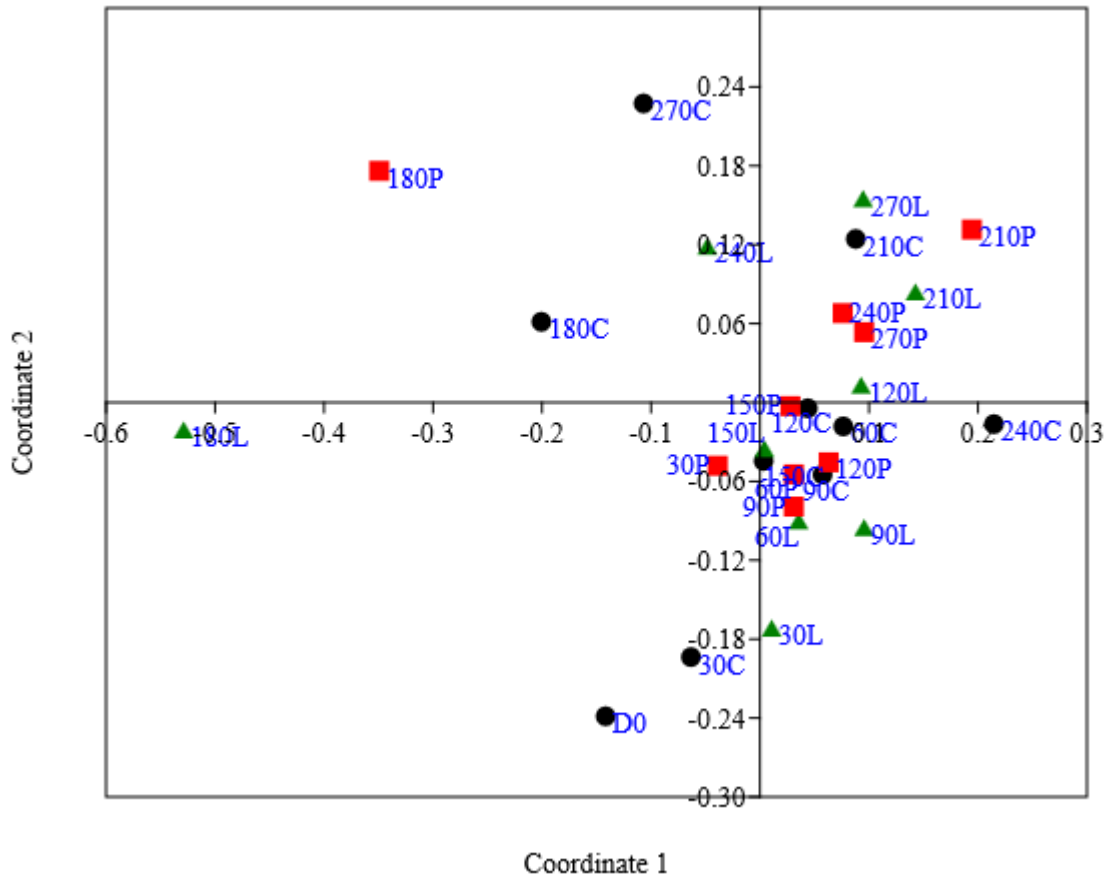


Figure 5: Average ( $n = 3$ ) nonmetric dimensional scaling (NMDS) plot ( $R^2 = 0.65$ , stress = 0.11) for phylum level 16S bacteria community of the control (C, ●), leaf litter (L, ▲) and piglet (P, ■) soils.

Table 2: Spearman's rank correlation coefficient showing significant correlation ( $p < 0.05$ ) between phyla and temperature.

Positive			Negative		
OTUs (phylum)	R	P	OTUs (phylum)	R	P
<b>Chlorobi</b>	0.729	<0.0001	Actinobacteria	-0.563	0.002
<b>Chloroflexi</b>	0.422	0.026	Bacteroidetes	-0.583	0.001
<b>Gemmatimonadetes</b>	0.748	<0.0001	Planctomycetes	-0.673	0.0001
<b>Nitrospira</b>	0.573	0.002	Verrucomicrobia	-0.697	<0.0001

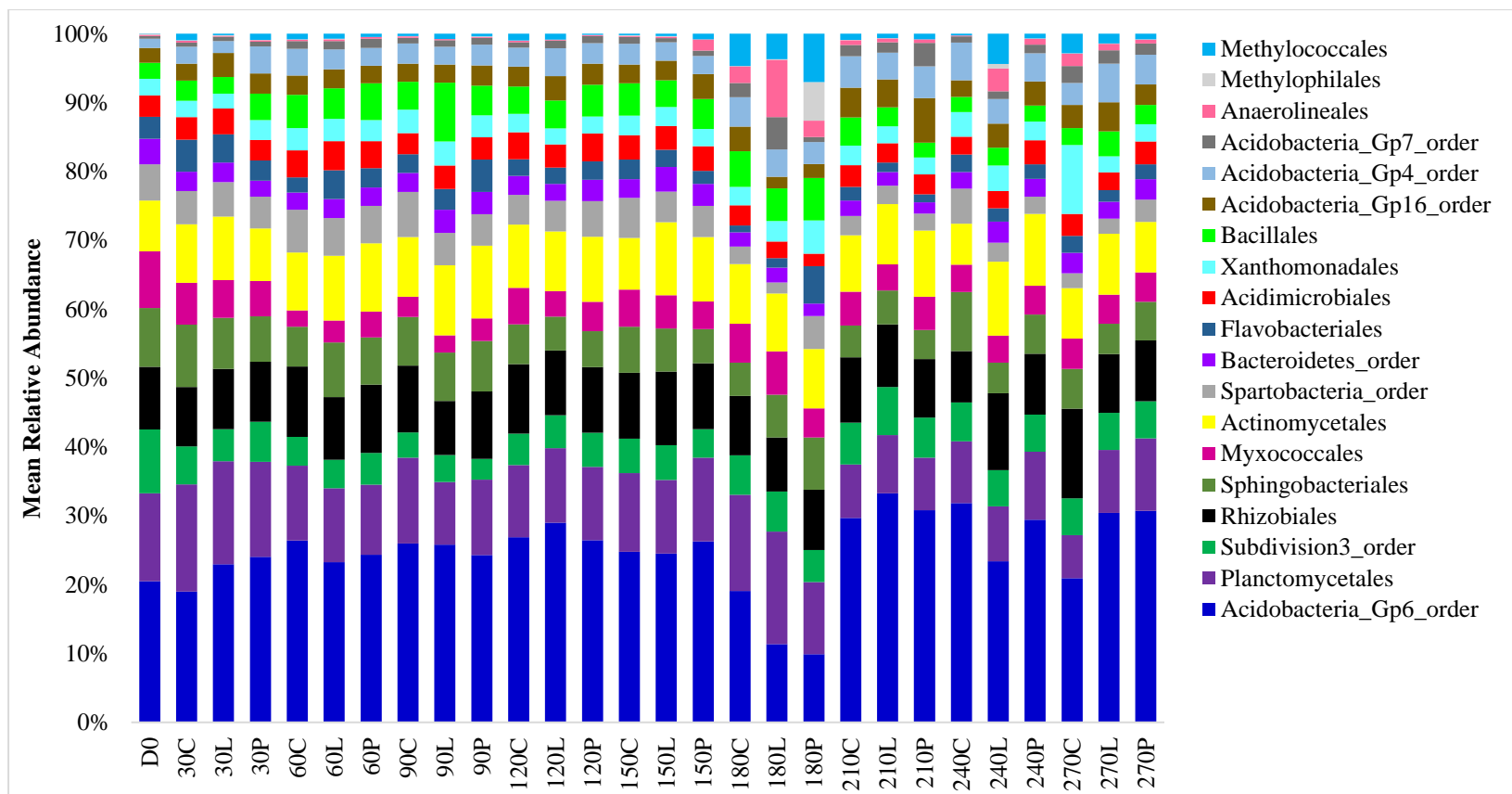


Figure 6: Average (n = 3) order taxa resolution of the control (C), leaf litter (L) and piglet (P) soils.

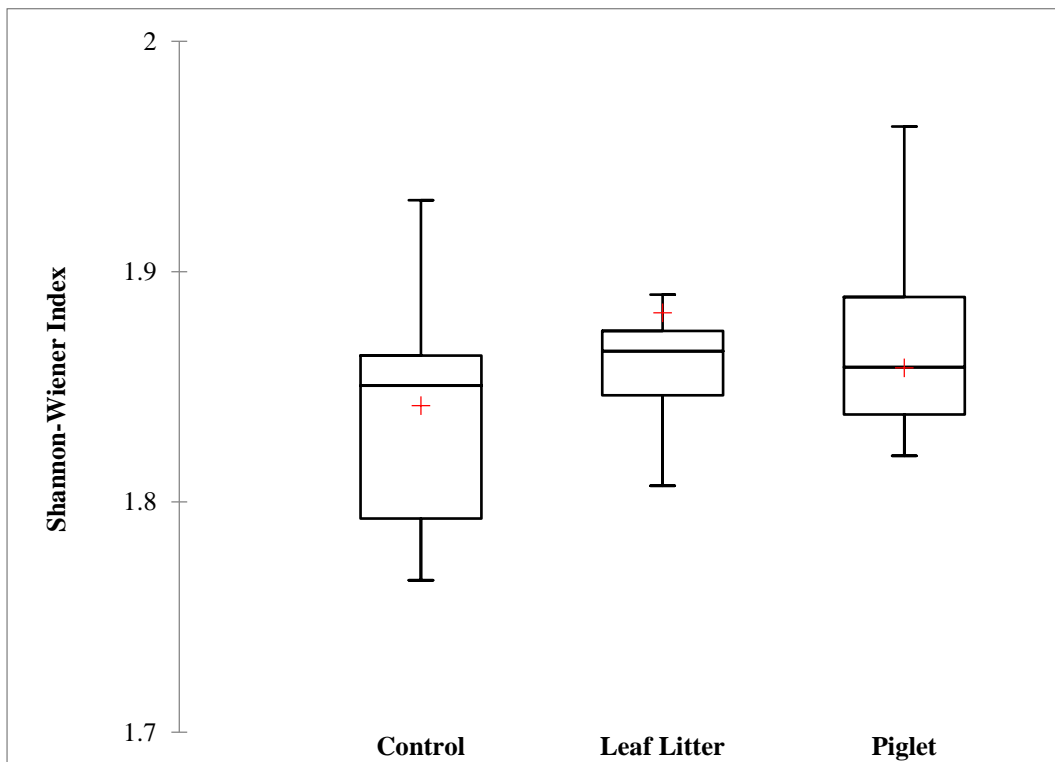
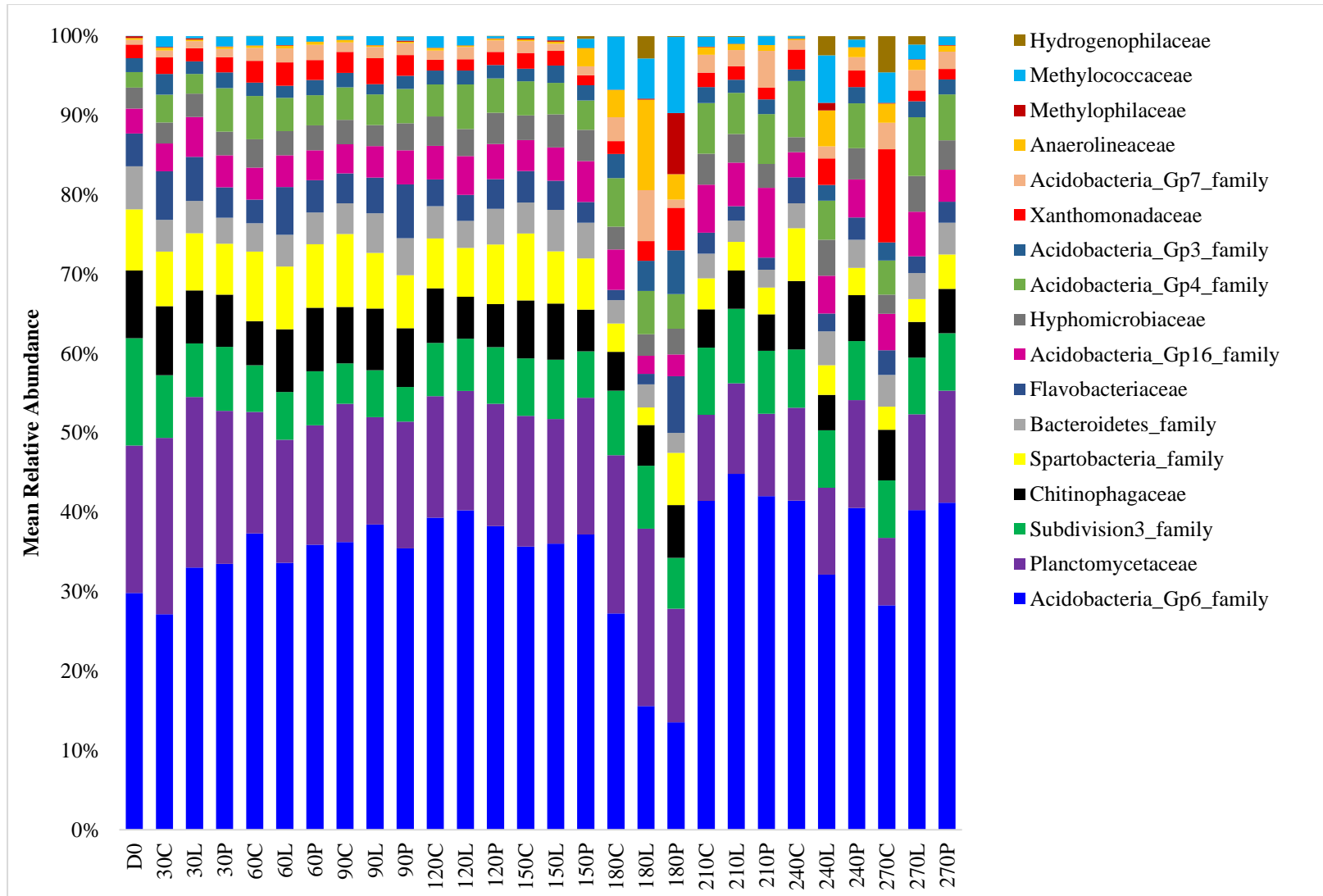


Figure 7: 16S bacterial taxa alpha Shannon–Wiener diversity box plot of the control, leaf litter and piglet soils



2 Figure 8: Average (n = 3) family taxa resolution of the control (C), leaf litter (L) and piglet (P) soils.

