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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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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 application 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 would differentiate for the piglet and leaf litter soils, respectively, 180 days after internment.

1. 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 indicators or predictors 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 decidous 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?

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2. Experimental design

2.1. 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^{-1}), Ca (25 g kg^{-1}), K (4.7 g kg^{-1}), Mg (8.2 g kg^{-1}), Na (0.47 g kg^{-1}), nitrate aqueous extract as NO_3 (3.5 mg L^{-1}), total organic carbon (3.0%), total S (0.03%), pH (7.9), P (<0.10 mg kg^{-1}), calorific value (<1.0 MJ kg^{-1}) and electrical conductivity (250 μS cm^{-1}).

The burial site (6 m by 6 m) was cleared and divided into three 1-m sections, 1.5 m apart. Each of the three 1-m sections (Fig. 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 (*Q. robur*) (L) and piglet (P). For the piglet burials, aluminium wire mesh cages (40 cm long × 25 cm wide × 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; winter) to September 2015 (day 270; autumn) 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.

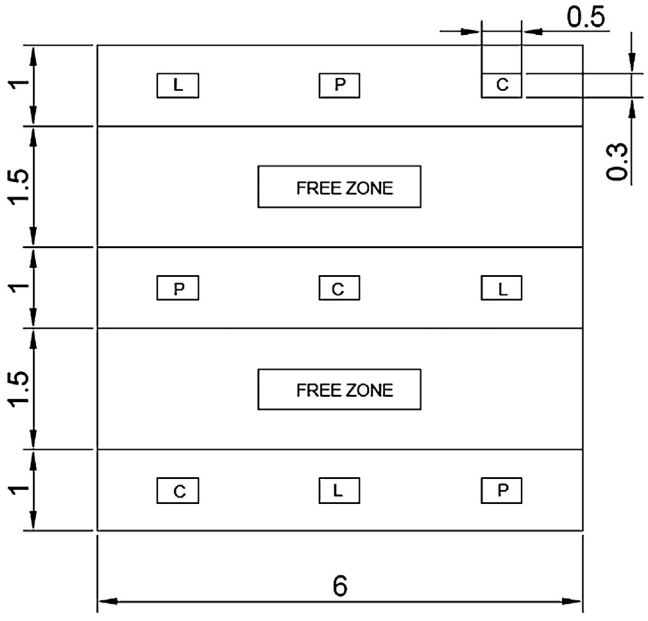

Fig. 1. Site configuration of the control (C), leaf litter (L) and piglet (P) burials with all dimensions in metres. All pits were dug with dimensions of 50 cm (length) by 30 cm (width) by 40 cm (depth).

2.2. 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].

2.3. 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.

2.4. 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.

2.5. 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.).

3. Results

3.1. pH trends

The average pH values for the control and treatments (*Sus scrofa domesticus* and *Q. robur* litter) soils were compared between day 0 (winter, December 17, 2014) and 270 (autumn, September 2015) (Fig. 2) and showed an increase for the *S. scrofa domesticus* (7.84 ± 0.07) from day 0 to day 30 compared to control (7.86 ± 0.14) and leaf litter (7.80 ± 0.03). While increases in pH between days 30 and 60 were recorded for the control (8.03 ± 0.08) and leaf litter (8.05 ± 0.02), the *S. scrofa domesticus* soil decreased (7.92 ± 0.08). Both the control and *Q. robur* litter soils recorded pH decreases between days 60 (control, 8.03 ± 0.08; leaf litter, 8.05 ± 0.02) and 150 (control, 7.61 ± 0.05; leaf litter, 7.70 ± 0.05) while the piglet soil showed an earlier fall between days 90 (7.96 ± 0.09) and 120