Shifts in soil biodiversity - a forensic comparison between *Sus scrofa domesticus* and vegetation decomposition

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ABSTRACT

In a forensic context, microbial-mediated cadaver decomposition and nutrient recycling cannot be overlooked. As a result, forensic ecogenomics research has intensified to gain a better understanding of cadaver/soil ecology interactions as a powerful potential tool for forensic practitioners. For this study, domestic pig (*Sus scrofa domesticus*) (4 g) and grass (*Agrostis*/*Festuca* spp) cuttings (4 g) were buried (July 2013 to July 2014) in sandy clay loam (80 g) triplicates in sealed microcosms (127 m*l*; 50 x 70 cm) with parallel soil only controls. The effects of the two carbon sources were determined by monitoring key environmental factors and changes in soil bacterial (16S rRNA gene) and fungal (18S rRNA gene) biodiversity. Soil pH changes showed statistically significant differences ($p < 0.05$) between the treatments. The measured ecological diversity indices (Shannon-Wiener, H'; Simpson, D; and richness, S) of the 16S rRNA and 18S rRNA gene profiles also revealed differences between the treatments, with bacterial and fungal community dominance recorded in the presence of *Sus scrofa domesticus* and grass trimming decomposition, respectively. In contrast, no statistically significant difference in evenness ($p > 0.05$) was observed between the treatments.

Keywords: Cadaver, Forensic ecogenomics, Soil microbial communities, *Sus scrofa domesticus*, *Agrostis*/*Festuca* spp

1. Introduction

Cadaver decomposition is a complex process that begins with post-mortem changes such as algor mortis, rigor mortis and livor mortis followed by soft tissue decomposition through the processes of autolysis, putrefaction, decay and skeletonization [1–6]. The soft tissue decomposition stages are characterised by protein, carbohydrate and lipid catabolisms in the body [1, 2, 5]. The rate of cadaver decomposition can be influenced by both biotic and abiotic factors, which can vary between above- and underground situations [2, 3]. Studies which compared above- [7–9] and underground [10–12] scenarios have shown that cadaver decomposition rate is slower in the latter.

The application of fatty acid-based techniques, such as phospholipid fatty acid and fatty acid methyl ester analyses, and molecular techniques, such as polymerase chain reaction (PCR), denaturing/temperature gradient gel electrophoresis (T/DGGE) and terminal restriction length polymorphism (T-RFLP) with next generation sequencing for microbial community profiling, are beginning to elucidate the complex relationships between cadaver decomposition, nutrient cycling and soil microbial community dynamics in a forensic context [6, 13–18]. For example, some sub-surface studies by Bergmann et al [19] and Olakanye et al [20] recorded spatial and temporal changes in soil bacterial diversity relative to burial depth and decomposition time, respectively. Also, aboveground studies by Lauber et al [21] that investigated the roles of microorganisms in cadaver ecogenomics recorded changes in 16S rRNA bacterial, 16S rRNA archaeal, and 18S rRNA fungal communities in sterile and non-sterile soils with differences between skin-associated and grave soil during the active and advanced decay stages. Thus, several researchers have illustrated and suggested that changes in epinecrotic and burial soil microbial diversity can be a potential "post-mortem microbial clock" tool for PMI estimation [15, 16].

Although the possible use of forensic ecogenomics as a novel suite of techniques for crime scene investigation is gaining momentum [15-17, 19, 22, 23], more detailed studies are required to elucidate fully the interactions between soil ecology and cadaver decomposition (cadaver decomposition-mediated soil ecology changes). Consequently, the values of different microbial ecology tools in this novel context, including profiling platforms that are accessible to most researchers and practitioners, must be considered while taking full cognizance of their limitations. To

explore this, two different carbon sources (*Sus scrofa domesticus* and *Agrostis*/*Festuca* spp) were buried and studied over a one-year period (July 2013 to July 2014). The specific research questions were: (i) What are the effects of abiotic factors such as temperature/pH on decompositional soil biodiversity?; (ii) Will *Sus scrofa domesticus* and plant matter decompositions illicit the same trends or shifts in biodiversity compared to the soil only controls?; and (iii) Which biochemical and molecular markers (microbial community indices) can be employed reliably during *Sus scrofa domesticus* decomposition? Thus, DGGE profiling was complemented by ecological community indices measurements of Shannon-Wiener and Simpson diversity, taxa richness and evenness.

2. Materials and methods

2.1 Soil collection and characterization

Twenty kg of sandy clay loam soil were dug from a well secured site at Bishop Burton College of Agriculture, Lincolnshire, U.K. (Lat. 53.27°N, Long. 0.52°W) [24] and stored in a sterilized 25 *l* airtight bucket. To ensure homogeneity, the soil was milled thoroughly (Retsch SM 100, Retsch, Haan, Germany) and sieved (ASTM - standard soil sieve No 10; 2 mm mesh). The sandy clay loam soil was constituted (w/w) by 21% clay, 21% silt and 58% sand (Forestry Commission, Surrey, U.K.) and physicochemical charateristics of Al (25 g kg^{-1}), Ca (5.4 g kg^{-1}), K (4.6 g kg^{-1}), Mg (2.8 g kg^{-1}), Na (0.28 g kg^{-1}), nitrate aqueous extract as NO_3 (76 mg l^{-1}), total organic carbon (5.9 %), total S (0.05%), pH (5.8), P (1.0 mg kg^{-1}), calorific value (2.0 MJ kg^{-1}) and electrical conductivity (590 µS cm^{-1}) (Derwentside Environmental Testing Services Ltd, County Durham, U.K.).

2.2 Experimental design and sampling

Triplicate polyethylene microcosms (127 m*l*, 50 x 70 cm; VWR, Lutterworth, U.K.) for the control and each treatment were perforated at equal distances and heights to facilitate aeration, moisture migration, sampling and hygiene maintenance. In total, 24 microcosms were established for the control and each of the two treatments to enable triplicate destructive sampling on days 7, 14, 28, 60, 120, 180, 300 and 365. Freshly-butchered domestic pig (*Sus scrofa domesticus)* was bought from a local butcher (Middlebrough, U.K.), washed thoroughly with sterile

deionised water and cut into similar 4 g cubes while freshly cut grass (*Agrostis*/*Festuca* spp) was collected from a domestic garden (Wynyard, U.K.). The *Sus scrofa domesticus* and grass cuttings (4 g) were buried individually in 80 g (fresh weight) of homogenized soil. In parallel, an 80 g soil sample was included as a control. All microcosms were stored outside (Teesside University, Middlesbrough, U.K.; Lat. 54.5722° N, Long. 1.2349° W) but within perforated plastic boxes.

2.3 pH measurement

The average pH of each treatment and control (n = 3) soil was determined by mixing thoroughly samples with deionised water in a ratio (w/v) of 1:5 prior to measurement with a pH 213 microprocessor (Hanna Instruments, Bedfordshire, U.K.) fitted with a Fisher electrode.

2.4 Temperature measurement

The average soil temperatures (n = 3) were measured at every sampling time with a hand-held Hanna thermometer (Hanna Instruments, Bedfordshire, U.K.) while daily atmospheric temperatures for Middlesbrough, U.K. were obtained from http://www.metoffice.gov.uk/.

2.5 DNA extraction

DNA extraction and PCR-DGGE of each sample were as described by Olakanye et al [20]. The DNA extractions were made with FastDNA SPIN Kit for Soil™ (MP Biomedicals, U.K.) according to the manufacturer's instructions and stored at -20 °C until needed. Triplicate DNA extracts (5 µ*l*), each mixed with 1 µ*l* 6X loading buffer, were analysed on 1.5% (w/v) agarose gels which contained 6 µ*l* SYBR Safe (Invitrogen, U.S.A.). The gels were electrophoresed in 1X TBE buffer for 90 min at 150 V and viewed (AlphaImager HP®, Alpha Innotech, Braintree, U.K.) under UV light.

2.6 PCR – DGGE analysis

Triplicate PCR amplifications used GC 388F – 530R to target the 16S rRNA gene [25] and the nested primer sets NS1/NS8 and NS1/NS210-GC for the 18S rRNA gene [26]. The 25 µ*l* PCR mixtures contained 12.5 µ*l* of 2X PCR master mix (Promega, Southampton, U.K.), 8.25 µ*l* molecular grade water (Promega,

Southampton, U.K.), 1.25 µ*l* BSA (0.5 mg m*l*$^{-1}$), 0.5 µ*l* of both the forward and reverse primers (0.2 µM), and 2 µ*l* of DNA templates. The thermo-cycling programme (Primus 96 Plus, MWG-Biotech, Ebersberg, Germany) for the 16S rRNA consisted of initial denaturation at 95 °C for 2 min; 35 cycles of: denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1.5 min; and final extension at 72 °C for 30 min. The first 18S rRNA gene step (NS1/NS8) consisted of initial denaturation at 94 °C for 2 min; 30 cycles of: denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 2 min; and final extension at 72 °C for 5 min, while the second step (NS1/NS210-GC) entailed initial denaturation at 94 °C for 2 min; 35 cycles of: denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1.15 min; and final extension at 72 °C for 5 min.

Subsequent to checking on 1.5% (w/v) agarose gels as described above, the amplicons (20 µ*l*) were separated on 10% (w/v) polyacrylamide gels (acrylamide/bis-acrylamide gel 37.5:1) with a 30% to 70% denaturing gradient for the 16S rRNA, and 6% (w/v) polyacrylamide gels with a 25% to 45% denaturing gradient for the 18S rRNA with a PHOR-U (X2) Ingeny system (Leiden, Netherlands) at 60 °C and 110 V for 18 hours. The gels were stained with SYBR Gold (Invitrogen, U.S.A.) and viewed under UV light (AlphaImager HP®; Alpha Innotech, Braintree, U.K).

2.7 Statistical analyses

The DGGE images were quantified by Phoretix 1D software (TotalLab, Newcastle, U.K.) with the gel bands used to infer bacterial and fungal 'species' or operational taxonomic units (OTUs). Similarities between microbial community fingerprints were determined by cluster analysis, which was made with un-weighted pair-group using arithmetic average (UPGMA). The number, intensity and rank of the $\log_{10}$ of abundance of detectable OTUs were used to determine the ecological indices for richness (S), diversity (Shannon-Wiener, H' (Equation 1); Simpson, D (Equation 2)) and evenness/equatibility (E), respectively. All data were evaluated statistically by a two-way ANOVA with repeated measures (xlstats 2014.5.02, New York, U.S.A.). To ensure robustness beyond the overall mean used in ANOVA, specific temporal statistical differences between the treatments and control were determined by further analysis with the Tukey (HSD) and Bonferroni *post hoc* tests (xlstats 2014.5.02, New York, U.S.A.).

Shannon-Wiener index (H') = $-\sum \boldsymbol{P}_i \ln \boldsymbol{P}_i$ Equation 1

Simpson index (D) = $1-\frac{\sum \boldsymbol{n}_i(\boldsymbol{n}_i-\mathbf{1})}{\mathbf{N}(\mathbf{N}-\mathbf{1})}$ Equation 2

3. Results

3.1 Temperature

Between days 0 and 60, the average microcosm temperature was 25.1 °C ± 0.31 while the atmosphere was 19.2 °C ± 0.19. The seasonal weather change then resulted in a temperature decrease that was marked from days 120 to 180 with an average of 8 °C ± 0.26. For days 300 to 365 temperature increases were apparent with an average of 14.5 °C ± 0.14. The two-way ANOVA showed a statistically significant difference ($p < 0.001$) between the atmospheric and microcosm temperatures but no difference ($p > 0.05$) between the *Sus scrofa domesticus* and grass burial soils (Fig. 1).

3.2 pH

Compared to the control (pH 6.08 ± 0.04), increased average pH values were recorded for the *Sus scrofa domesticus* (6.83 ± 0.1) and grass (6.33 ± 0.07) microcosms between days 0 and 28. Subsequently, decreased values were recorded for all of the microcosms between days 60 and 180 before each reached its highest value on day 300 (control, 6.44 ± 0.09; *Sus scrofa domesticus,* 8.43 ± 0.01; grass, 7.58 ± 0.03) (Fig. 2). Two-way ANOVA showed statistically significant temporal differences ($p < 0.001$) between the control and experimental microcosms.

3.3 Ecological indices measurement

Species richness was calculated by the numbers of visualized DGGE bands (OTUs). For the 16S rRNA gene profiles, two-way ANOVA showed statistically significant temporal differences ($p < 0.001$) in response to the presence of *Sus scrofa domesticus* and grass cuttings (Fig. 3a & 3b). These observations were most pronounced on days 28 (9 ± 1.5, 20 ± 0.9, 19 ± 1.2) and 300 (8 ± 1.2, 19 ± 1.9, 13 ± 0.6) for the control, *Sus scrofa domesticus* and grass cuttings microcosms, respectively. In contrast to the bacterial community trends, the 18S fungal profiles did not show any statistically significant differences ($p = 0.27$) in response to the burials. The Tukey (HSD) and Bonferroni *post hoc* tests did, however, identify

differences ($p < 0.05$) between the grass trimmings (8 ± 1.2, 19 ± 0.7) and *Sus scrofa domesticus* (16 ± 0.9, 11 ± 0.8) microcosms on days 28 and 180, respectively (Fig. 4a & 4b).

The Shannon-Wiener index, which is a function of evenness and species relative abundance, showed statistically significant temporal differences ($p = 0.034$) in diversity between the control and the *Sus scrofa domesticus* and grass microcosms with the divergence more pronounced on days 28 (1.94 ± 0.19, 2.72 ± 0.10, 2.36 ± 0.04) and 300 (1.94 ± 0.14, 2.75 ± 0.12, 2.30 ± 0.06), respectively (Fig. 3c & 3d). In contrast to the 16S profiles, the 18S fungal communities recorded no statistically significant differences ($p = 0.41$) between the control and experimental microcosms (Fig. 4c & 4d). Nevertheless, both the Tukey (HSD) and Bonferroni *post hoc* tests showed statistically significant differences ($p < 0.05$) between the *Sus scrofa domesticus* and grass cuttings microcosms on days 28 (2.41 ± 0.10, 1.79 ± 0.03), 60 (1.84 ± 0.19, 2.33 ± 0.07) and 180 (2.09 ± 0.08, 2.53 ± 0.07), respectively.

The Simpson diversity index, which exemplifies species richness and relative abundance, showed no statistically significant temporal differences ($p = 0.89$) for the 16S bacterial communities between the control and experimental microcosms. Nonetheless, Tukey (HSD) and Bonferroni *post hoc* analyses recorded differences ($p < 0.05$) on days 28 (control, 0.82 ± 0.03; *Sus scrofa domesticus*, 0.92 ± 0.01), 180 (control, 0.69 ± 0.01; *Sus scrofa domesticus*, 0.64 ± 0.02; grass, 0.82 ± 0.03) and 300 (control, 0.83 ± 0.02; *Sus scrofa domesticus*, 0.92 ± 0.01) (Fig. 3e & 3f). For the fungal 18S rRNA gene profiles no temporal differences were recorded ($p = 0.49$). As shown earlier, the Tukey (HSD) and Bonferroni *post hoc* tests recorded statistically significant differences ($p < 0.05$) between the grass and *Sus scrofa domesticus* microcosms on days 28 (0.81 ± 0.02, 0.88 ± 0.04), 60 (0.89 ± 0.02, 0.81 ± 0.01) and 80 (0.89 ± 0.01, 0.84 ± 0.01), and between the control and *Sus scrofa domesticus* microcosms on day 180 (0.89 ± 0.01, 0.84 ± 0.01) (Fig. 4e & 4f).

Since changes in microbial diversity can sometimes result in an imbalanced community, evenness is measured to assess the distribution of microorganisms over time [27-30]. Evenness was calculated using the rank of the $\log_{10}$ of species relative abundance, with the actual value determined from the inverse log of the gradient. Calculations for the bacterial 16S and fungal 18S rRNA gene profiles (Table 1) showed that the biodiversities of both microbial community types were distributed

evenly in the control, *Sus scrofa domesticus* and grass soils independent of both the decomposition time and the presence or absence of decomposing matter.

The similarities between the control and treatments were measured with the un-weighted pair-group using arithmetic average (UPGMA) for cluster analysis of the PCR-DGGE image fingerprints. The 16S bacterial communities showed 75% similarities between the microcosms on day 0, 35% on day 7, 8% on day 14, 22% on day 28, 38% on day 60, 24% on days 120, 180 and 300, and 35% on day 365 (Fig. 5a). For the 18S fungal communities, 63% similarities were recorded for the microcosms on day 0, 58% on day 7, 31% on day 14, 49% on day 28, 44% on day 60, 48% on day 120, 24 % on day 180, 43 % on day 300 and 28% on day 365 (Fig. 5b).

4. Discussion

Studies of cadaver decomposition and its interactions with, and effects on, soil ecology have highlighted the potential of forensic ecogenomics as a powerful tool to estimate PMI and identify clandestine graves through changes in microbial communities [14-16, 20-23]. Although this tool has potential advantages compared with conventional methods for estimating PMI, most studies have, however, only considered a single carbon source (the cadaver) while dual sources can provide useful information for forensic practitioners to identify and differentiate gravesites in difficult cases such as transit or clandestine scenarios. This study examined the effects of two carbon sources, *Sus scrofa domesticus* and grass (*Agrostis*/*Festuca* spp) on soil diversity.

Ambient temperature has been shown to have one of the greatest effects on cadaver decomposition rate. According to various workers [2, 9, 12, 31], the rate is enhanced by high temperature while the process slows or stops at cold temperatures. For cadaver decomposition under anaerobic conditions the optimum temperature range is 21°C to 38°C with very limited activity at temperatures below 4°C [2, 3, 32]. In our study, seasonal temperature changes were accompanied by increases and decreases in diversity of both the 16S bacterial and 18S fungal communities. On day 14 a microcosm temperature of 38°C, indicative of exothermic microbial activity, contrasted the air temperature of 22°C and preceded an increase in 16S bacterial biodiversity as expressed by ecological measures of richness (20.3; 19.3) (Fig. 3a & 3b), Shannon-Wiener index (2.72; 2.36) (Fig. 3c & 3d) and Simpson index (0.92; 0.84) (Fig. 3e & 3f) for *Sus scrofa domesticus* and grass, respectively on day 28.

From day 0 to 28, the 18S fungal communities (Fig. 4b, 4d & 4f) did not follow the same pattern as the bacterial species. Although different platforms were used, i.e. DGGE vs. next generation sequencing, Lauber et al [21] stated that initial decomposition does not provide robust 18S fungal community analysis data until after 20 days of carcass rupture. This supports earlier research [2, 8] where approximately 90% of the microorganisms isolated during initial decomposition were bacteria with *Staphylococcus*, *Malasseria*, *Bacillus* and *Streptococcus* species predominant. Other documented genera included *Proteus, Salmonella, Klebsiella, Serratia, Flavobacterium* and *Pseudomonas* [2, 8].

Changes in soil pH due to cadaver decomposition have been reported [12, 33-35]. The results of our study showed differences in pH in response to *Sus scrofa domesticus* and grass decomposition due to their chemical compositions of protein, lipid and carbohydrate [2, 5] and cellulose and lignin [36-38], respectively. Hopkins et al [35] reported an accumulation of $NH4^+$ and mineralisation of C and N at a gravesite which resulted in a soil pH increase similar to the one recorded for the *Sus scrofa domesticus* microcosm. Thus, while $NH4^+$, PO_4^-, K^+ and NO_3^- were not analysed in the current study, the recorded pH trends can still be related to earlier research [e.g. 5, 35] which entailed physico-chemical characterization of the gravesoil. Consequently, changes in pH can result in microbial population shifts with bacteria more dorminant in pH 5.5 – 7.5 grave soil [23].

The use of some ecological indices, as employed by other researchers [20, 29, 30], revealed interesting data that could be used to differentiate between *Sus scrofa domesticus* and grass decompositions. As suggested by McGuire and Treseder [38], bacterial and fungal richness might be useful in calculating decomposition time. One noticeable result from this study was that ecological indices determined for 16S rRNA bacteria communities seemed to be the most useful for estimating PMI for *Sus scrofa domesticus*. For example, bacterial diversity and richness indices highlighted some temporal differences particularly on days 28 (summer; July 2013), 180 (winter; January 2014) and 300 (summer; July 2014) (Fig. 3a-3f). In contrast, 18S rRNA fungal determinations were the best estimates of grass decomposition (Fig. 4a-4f). As reported by Voříšková and Baldrian [39], fungi tend to dominate the later stage of plant decomposition and this was evidenced by the grass microcosms diversity measurements (Fig. 4c & 4e) although equatibility between the species diversities showed no significant difference ($p > 0.05$).

5. Conclusion

Abiotic factors such as temperature and pH are key variables in taphonomic studies. This research identified interesting trends, as revealed by an accessible ecogenomics technique and multiple and robust statistical analyses, that could be useful in identifying a cadaver grave by targeting specific microbial community diversity changes. In this study microcosms were used but an extensive whole carcass programme is in progress and should result in more comprehensive data by targeting specific microbial communities with next generation sequencing.

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Novelty Statement

Recent cadaver decomposition studies focussing on soil ecology impacts have highlighted the potential of forensic ecogenomics to estimate PMI and identify clandestine graves through microbial community changes. Most studies have, however, only considered a single carbon source (the cadaver) while dual sources (Sus scrofa domesticus and plant matter) can provide information for academics and forensic practitioners to identify and differentiate gravesites in difficult cases such as transit or clandestine scenarios.

Highlights

- Significant differences in 16S Shannon-Wiener and Simpson diversity recorded for the control vs. two burial soils.
- Distinct bacterial and fungal rRNA gene profile shifts for pig and grass soils, respectively.
- Temporal divergence in burial soils observed after Tukey (HSD)/Bonferroni *post hoc* tests.
- Potential applicability determined by sequencing and *in situ* studies.

Table 1: Evenness values of the 16S bacterial and 18S fungal communities of control, *Sus scrofa domesticus* and grass soil microcosms during 365 days of study.

Treatment	16S bacterial communities	18S fungal communities
Control	0.87 ± 0.06	0.90 ± 0.04
Sus scrofa domesticus	0.84 ± 0.08	0.89 ± 0.03
Grass (*Agrostis*/*Festuca* spp)	0.86 ± 0.02	0.90 ± 0.03

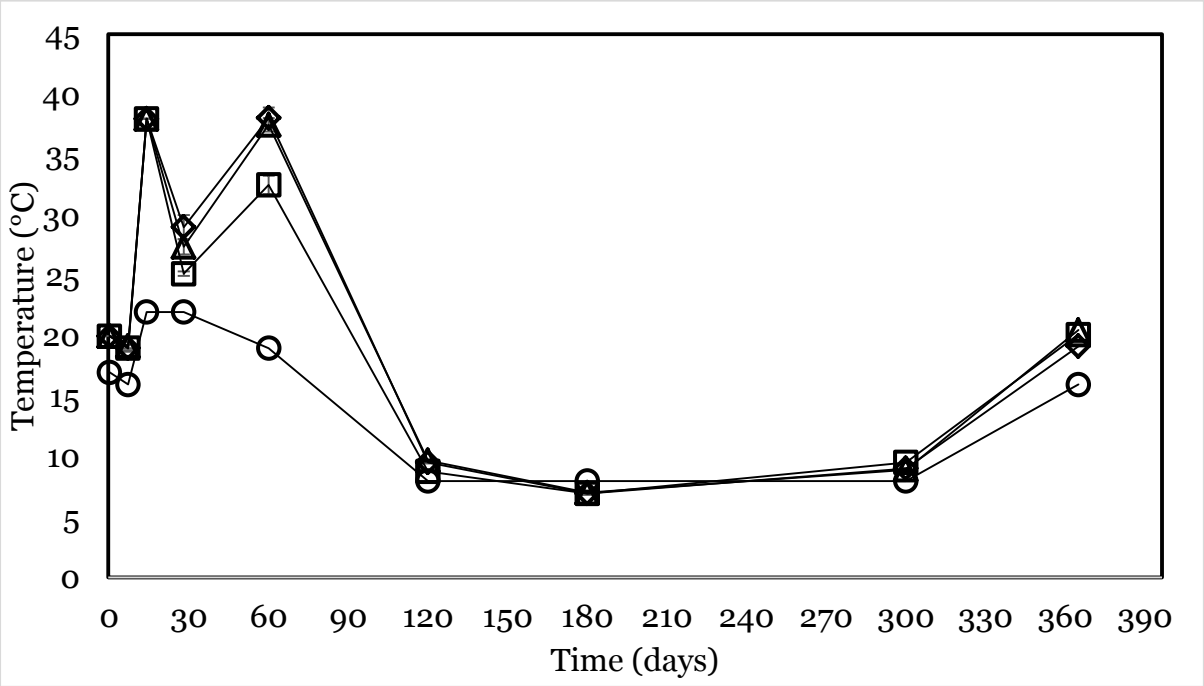

Fig. 1: Atmospheric (○) and microcosm (control (◇), *Sus scrofa domesticus* (□) and grass (Δ)) soil temperatures during 365 days of study.

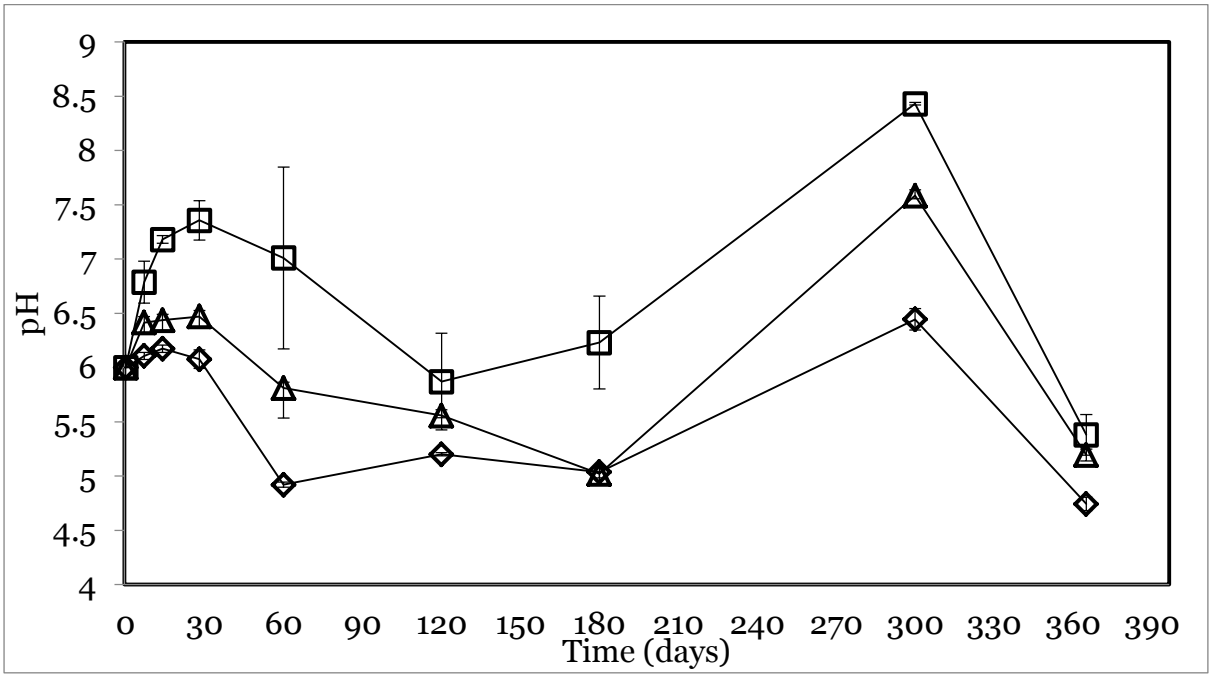

Fig. 2: Average (n=3) pH values of control (◇), *Sus scrofa domesticus* (□) and grass (Δ) soil microcosms during 365 days of study.

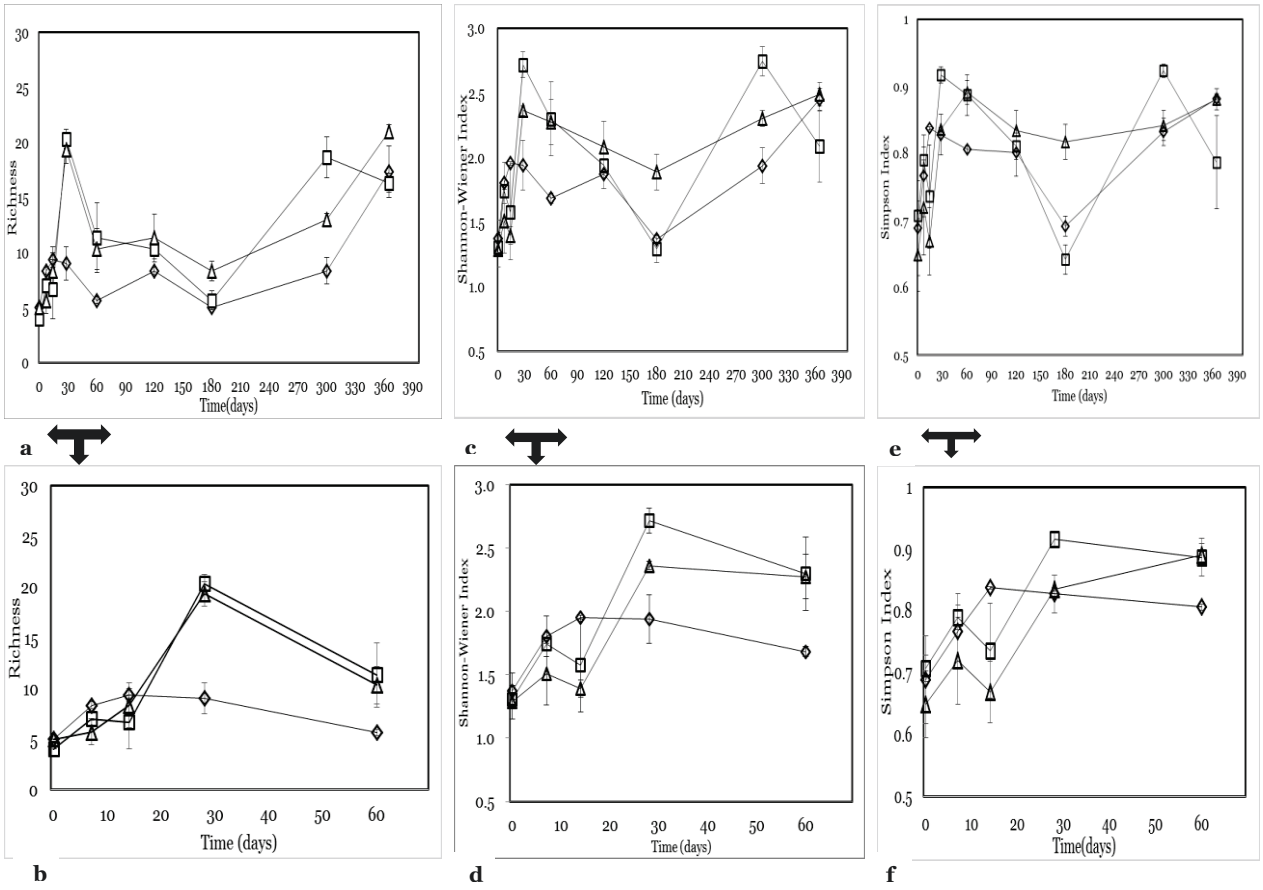

Figure 3a, 3c and 3e: Average (n=3) richness, Shannon-Wiener diversity index and Simpson diversity index for 16S bacterial communities of control (◇), *Sus scrofa domesticus* (□) and grass (Δ) soil microcosms during 365 days of study.

Figure 3b, 3d and 3f: Average (n=3) richness, Shannon-Wiener diversity index and Simpson diversity index for 16S bacterial communities of control (◇), *Sus scrofa domesticus* (□) and grass (Δ) soil microcosms from day 0 to day 60.

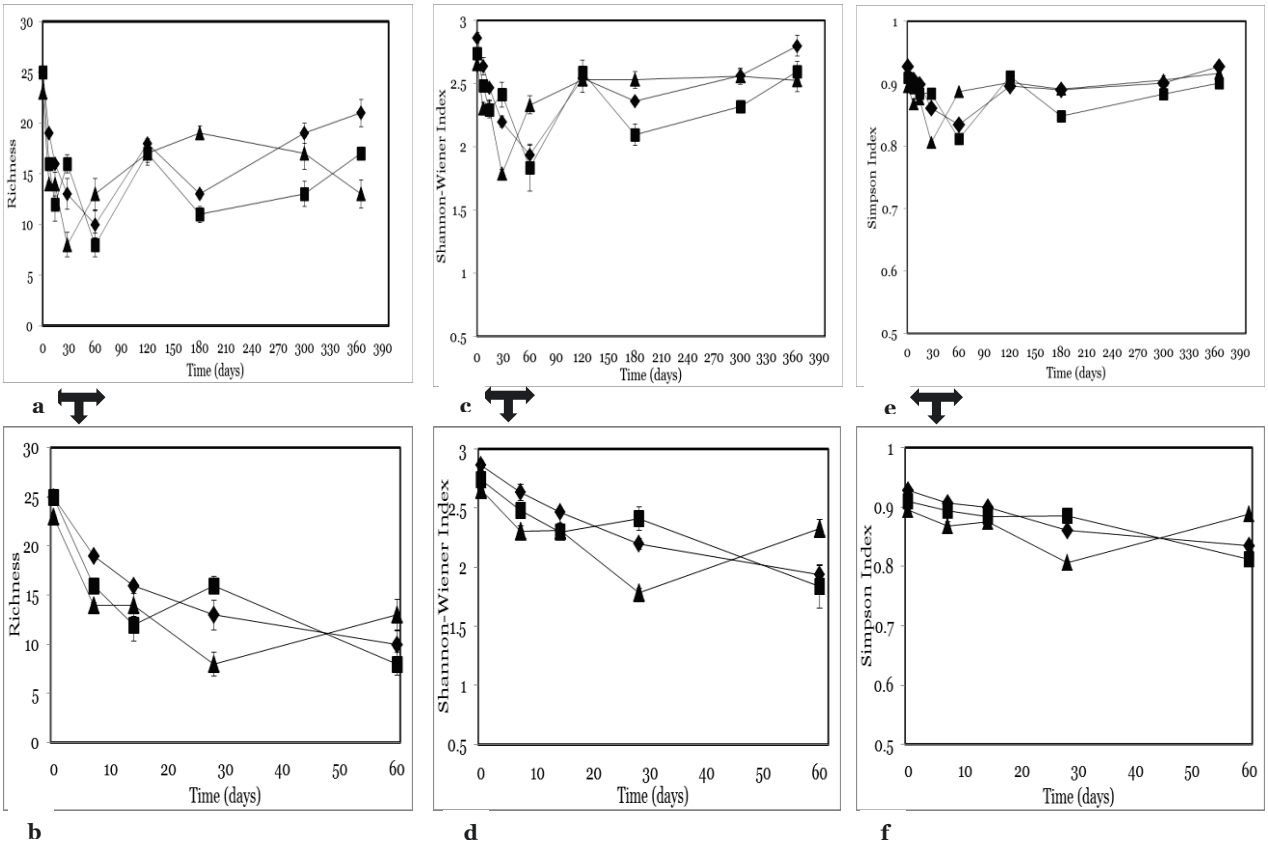

Figure 4a, 4c and 4e: Average (n=3) richness, Shannon-Wiener diversity index and Simpson diversity index for 18S fungal communities for control (♦), *Sus scrofa domesticus* (■) and grass (▲) soil microcosms during 365 days of study.

Figure 4b, 4d and 4f: Average (n=3) richness, Shannon-Wiener diversity index and Simpson diversity index for 18S fungal communities for control (♦), *Sus scrofa domesticus* (■) and grass (▲) soil microcosms from day 0 to day 60.

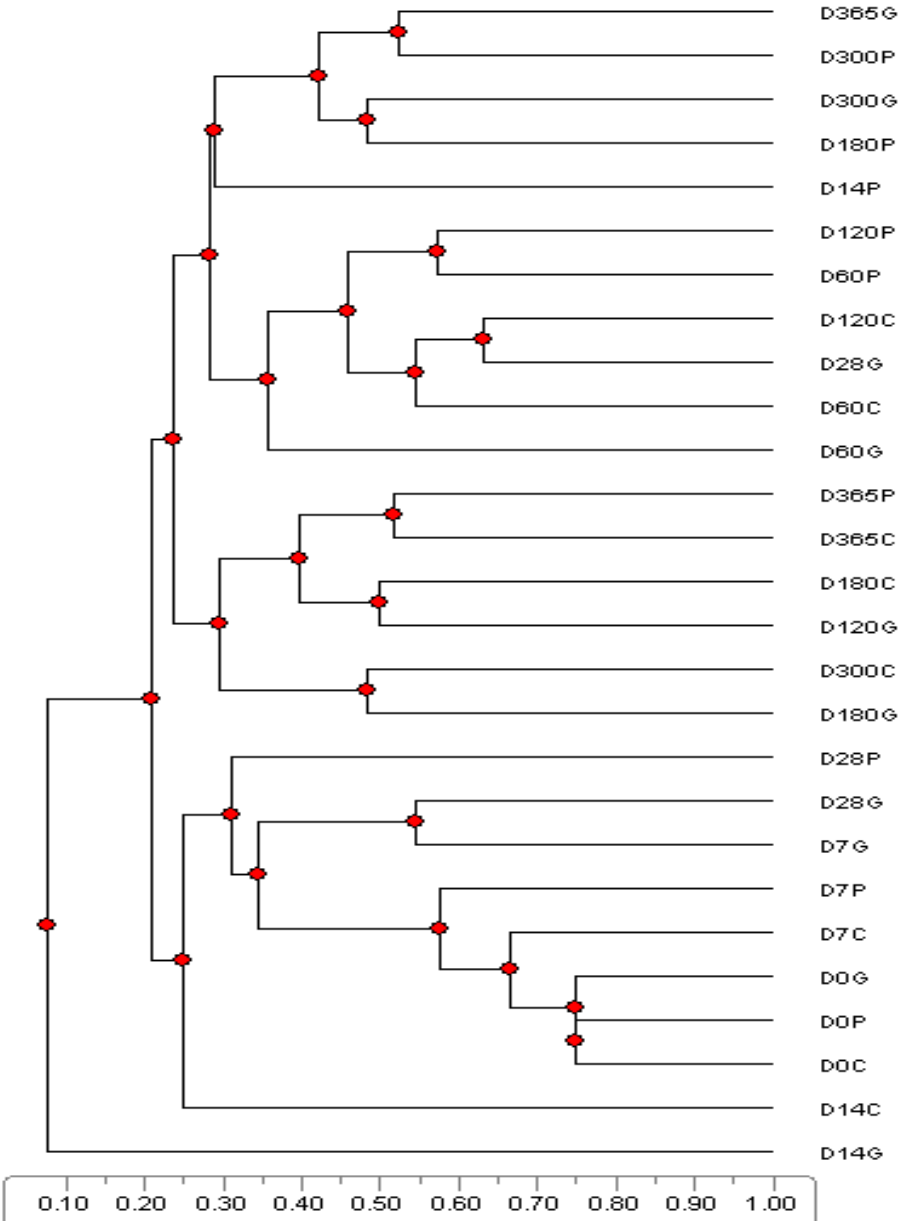

Fig. 5a: 16S bacteria community UPGMA cluster analysis (where: C, control*;* P, *Sus scrofa domesticus;* G, grass).

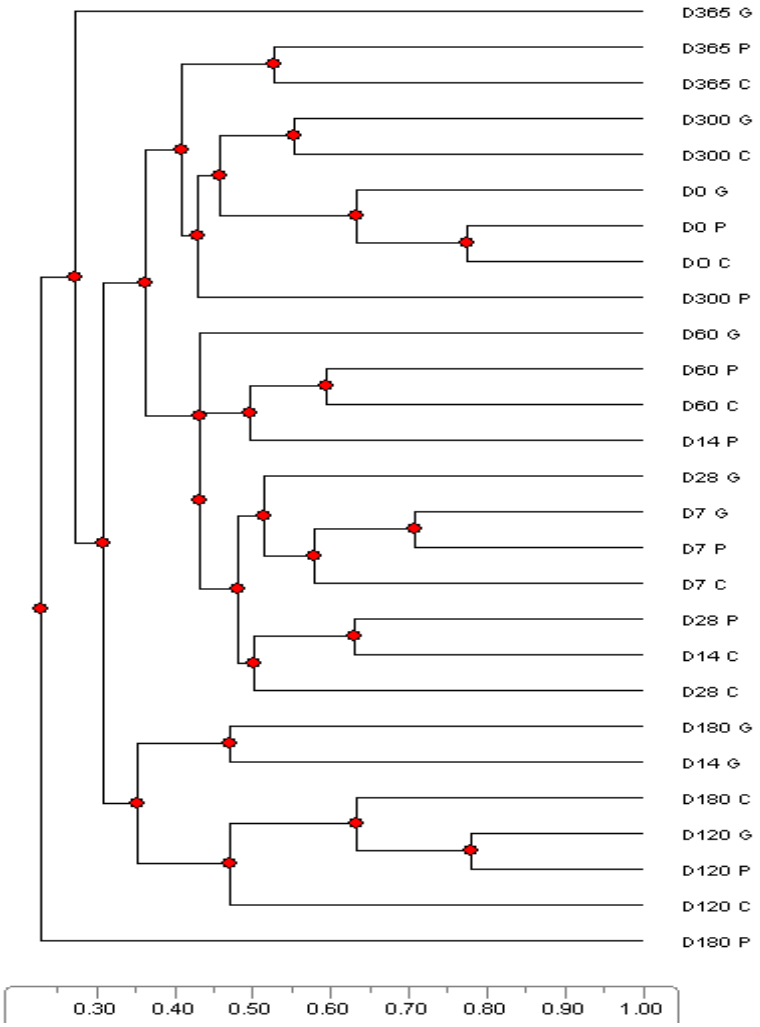

Fig. 5b: 18S fungal community UPGMA cluster analysis (where: C, control*;* P, *Sus scrofa domesticus;* G, grass).