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Profiling of Successional Microbial Community Structure and Composition to Identify Exhumed Gravesoil—A Preliminary Study

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Abstract: Advancements in molecular microbial ecology techniques have enabled researchers to study the complex interactions of epinecrobiome, necrobiome, and thanatomicrobiome communities during cadaver decomposition within novel forensic disciplines. This preliminary study tracks shifts in subsurface soil necrobiome as indicators of time-since-exhumation where whole juvenile *Sus scrofa domesticus* was used as the human cadaver proxy. Principal component analysis of DGGE-based diversity index (Shannon-Weiner, H'; Simpson (D)) measurements showed more consistent delineation of pre- and post-exhumation periods of the fungal 18S gene with further clustering for days 270 and 300. More importantly, high-resolution metabarcoding of the 16S rRNA gene recorded temporal bacterial clock indicators at order and family levels. Specifically, Xanthomonadales (11.29%) and Xanthomonadaceae (4.27%), and Verrucomicrobiaceae (4.00%) were abundance-based season (spring–summer) and microbial clock indicators for post-burial interval (PBI) $\geq$ 150 days. Hydrogenophilales (7.13%) and Hydrogenophilaceae (7.56%), Clostridiales (4.57%) and Clostridiaceae_1 (3.13%), and Bacteroidales (3.33%) defined the impacts of 120 days since exhumation of *Sus scrofa domesticus*. They could, therefore, be tracked to identify grave emptying for the current soil type.

Keywords: sub-surface decomposition; soil necrobiome; metabarcoding; *Sus scrofa domesticus*; exhumation

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1. Introduction

Human cadavers and animal carrion are substantive energy resources with key roles in nutrient cycling. Their decomposition and subsequent influx of nutrients affect the surrounding environmental microbiota [1–3] and ecology directly; hence, Carter et al. (2007) [4] designated this unique and dynamic ecosystem as a cadaver decomposition island (CDI). Consequently, the forensic efficacies of biochemical and microbiological profiling, particularly in decomposition/burial soils, have been recognized. Thus, the applicability of a suite of techniques to study the postmortem microbiome and enhance postmortem interval (PMI) and time-since-burial/post-burial interval (PBI) determinations is evidenced with human cadavers [5] or mammalian taphonomic proxies such as pig (*Sus scrofa domesticus*; [6]), both the brown and laboratory rat (*Rattus norvegicus* Berkenhout; [7]), and mouse (*Mus musculus* Linnaeus; [8,9]).

Despite demonstrable achievements, there remains a significant scope for knowledge development on the impacts of cadaver or mammalian surrogate decomposition on subsurface soil ecology. In particular, there is a recognised paucity in understanding the long-term effects after the body has decomposed or when clandestine graves have been exhumed [6,10]. The detectable and measurable longevity of the biological CDI footprint can potentially provide further intelligence, generally, and at old crime scenes, in particular.

Additionally, emerging studies have identified the need to understand forensic ecology for soil provenance [11] and ecosystem services of exhumed gravesites before they are repurposed for other societal needs such as recreational spaces in densely populated urban areas [12,13]. Therefore, this preliminary study was designed to test the capacity of microbial community dynamics to determine time-since-burial and subsequent emptying of a clandestine grave. Whole still-born juvenile *S. s. domesticus* was buried in a soil mesocosm with subsequent exhumation to test the hypothesis that: shifts in the composition and structure of bacterial and fungal communities will differentiate between exhumed and non-exhumed gravesoil. To our knowledge, this is the first study of its type, with the exception of exhumation to measure total body score, total decomposition score [14] and stable isotope analysis of decompositional lipids (adipocere) [15].

2. Experimental Section

2.1. Mesocosms

Soil was sourced from a well-secured site at Framwellgate Moor, County Durham, UK (Lat. 53.15° N, Long. 1.59° W) and transported in sterile 25 *l* air-tight buckets. As published in a previous study [16], the soil was homogenised (Retsch SM 100, Retsch, Haan, Germany), sieved (ASTM-standard soil sieve N^{o} 10, 2 mm mesh; sterilized by autoclaving at 120 °C, 15 psi for 20 min), and analysed physiochemically (Forestry Commission, Surrey, UK; Derwentside Environmental Testing Services Ltd., Durham, UK). A still-born juvenile *Sus scrofa domesticus* used for this study to create an approximate carbon source to soil ratio of 1:20 (w/w) was from several that were sourced frozen from the Northumbria Police (Ponteland, UK). The juvenile pig (1.8 kg) was defrosted at ambient temperature prior to burial in the fine fraction of sieved soil (36 kg) and exhumed on day 180. A control mesocosm (C) contained 20 kg of the study soil without piglet cadaver. The lysimeters were maintained outdoors (Teesside University, Middlesbrough, UK; Lat. 54.5722° N, Long. 1.2349° W).

Soil samples (1 g) were collected from 10 random mesocosm positions on weeks 2 and 4 and then monthly for a total of 10 months (300 days) from November 2014 (late autumn–winter) to September 2015 (early autumn). Specifically, soil sampling proceeded for 6 months (180 days) pre-exhumation and then 4 months (120 days) post-exhumation. Composites of the homogenised (10 g) samples were used for pH and temperature measurement and the remainders stored (25 mL sterile universal bottles; Sarstedt, Germany) at −20 °C until required for DNA extraction [17].

2.2. Soil DNA Extraction, PCR-DGGE and DGGE Data Analysis

The protocols for total microbial soil DNA extraction, agarose gel electrophoresis, polymerase chain reaction, denaturing gradient gel electrophoresis (PCR-DGGE) of the 16S and 18S rRNA genes, Phoretix analysis and diversity analyses (Shannon-Wiener index $(H') = -\sum P_i \ln P_i$ and Simpson index $(D) = 1 - \sum Pi^2$) were as detailed previously [16,17]. The data were tested with Shapiro–Wilk W for normal distribution prior to analysis. All data for pH, temperature, and ecological indices were evaluated statistically by a univariate one-way ANOVA.

2.3. Next-Generation Sequencing and Data Analysis

Microbial community DNA extracts were analysed for purity (A_{260}:A_{280}) and concentration (Beer–Lambert equation; A = *εlc*;) using UV spectrophotometry (Synergy-HT microplate reader; Biotek, Bedfordshire, UK), and purified (PowerClean® DNA Clean-Up Kit, Mo Bio Laboratories, Inc., Carlsbad, CA, USA) prior to next generation sequencing. Sequencing of the V4 region of 16S bacterial gene was made with an Illumina Miseq platform (Northumbria University, Newcastle, UK) with the primer set SB701-702/SA501-508. The raw sequencing reads were processed in FASTQ format and analysed with Mothur software package (version 1.36.1) (University of Michigan, Ann Arbor, MI, USA). The FASTQ formatted sequences were quality checked and filtered with UCHIME. The sequences were

identified by Ribosomal Database Project (RDP) classifier as described by Kozich et al. (2013) [18]. Raw sequencing reads are available as operational taxonomic units (OTUs) (Table S1).

2.4. Statistical Analyses

For multivariate analysis, data were unit vector normalized, mean centered, and scaled to standard deviation. Principal component analysis (PCA) was then applied to demonstrate temporal clustering and the differences in fungal and bacterial diversity indices against pre- and post-exhumation phases. PC1, which represents the greatest variation, and PC2 were analysed for correlation by linear regression with $R^2 > 0.7$ judged to be significantly different. For the NGS data, the phylogenetic distance matrices were analysed using Bray–Curtis dissimilarity with nonmetric dimensional scaling (NMDS). Correlations between soil pH, temperature, and phyla relative abundance were analysed by Spearman's rank correlation coefficient (SCC) (Xlstats 2016.02.27313, New York, NY, USA) where R is the correlation coefficient. To reveal significant differences in OTU between the treatment, control, and season, pairwise multiple comparisons after a multi-way ANOVA with the Tukey (HSD) post hoc test ($p < 0.05$) was used (Xlstats 2016.02.27313, New York, NY, USA). Taxa with relative abundance >3% were plotted with Microsoft Excel (Microsoft 2019). To ensure accuracy, all the results were checked with the Palaeontological software package for education and data analysis (Past 3.10) and Xlstats (xlsta 2016.02.27313, New York, NY, USA) software.

3. Results

3.1. Soil Properties

The sandy clay loam was constituted by (*w*/*w*) 26% clay, 21% silt, and 53% sand and physicochemical characteristics of Al (13 g kg^{-1}), Ca (2.2 g kg^{-1}), K (1.8 g kg^{-1}), Mg (1.1 g kg^{-1}), Na (0.25 g kg^{-1}), nitrate aqueous extract as NO_3 (4.6 mg l^{-1}), total organic carbon (4.1%), total S (0.03%), pH (6.3), P (1.2 mg kg^{-1}), calorific value (2.5 MJ kg^{-1}), and electrical conductivity (1400 µS cm^{-1}).

3.2. Environmental Parameters Trends

The control and treatment soils recorded different pH values between days 0 (November 2014) and 300 (September 2015) with 6.69 on day 14 for the control and 6.80 for-exhumation (FE) mesocosms (Figure S1). A pH of 7.83 was recorded for the *S. s. domesticus* mesocosm on day 60, which was in contrast to pH = 6.98 for the control. pH decreases then occurred between days 120 and 270 to reach values around 6.09 in the decomposition mesocosm. Overall, temporal changes in soil pH showed no statistically significant differences ($p = 0.075$) between the control and treatment lysimeters.

Average temperatures of the ambient environment, control, and experimental mesocosms were determined 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 [19,20]. The averages of the maximum and minimum ambient temperatures were used to calculate the daily ADD [21,22], with a base temperature of 0 °C according to the work of Megyesi et al. (2005) [22]. The control and decomposition soils recorded similar trends where the temperature increased from day 14 (average ADD 91.5) to day 30 (average ADD 194.1), with an average mesocosm temperature of 10.7 °C (Table 1). This was followed by a shift from late autumn to winter when the average temperature was 5.6 °C. Therefore, decreases in average ADD were recorded from 310.6 on day 60 to 382.2 on day 90. A further seasonal change from spring (day 120, average ADD 564.4) to summer (day 240, average ADD 1 929) led to an increase in temperature to reach an average of 16.7 °C. Subsequent decreases on day 270 (average ADD 2 411.8) and day 300 (average ADD 2 848.3) resulted in an average of 13.6 °C.

Table 1. Decomposition temperature timeline as expressed by ADD.

Day	Season	Control	Piglet
0	Late autumn to Winter 2014	6.3	6.1
14		91.6	91.2
30		194.1	194.1
60		310.7	310.4
90		382.6	381.9
120	Spring 2015	564.8	564
150		808.6	806.5
180		1 066.6	1061.6
210	Summer 2015	1 466.9	1460.9
240		1 932.8	1926.4
270		2 415.4	2409.1
300	Autumn 2015	2 852.4	2845.3

3.3. Soil Ecological Analyses

The for-exhumation piglet decomposition microcosm (FE) was sampled for six months pre-exhumation (days 0–180) and four months post-exhumation (days 210–300). The *S. s. domesticus*–associated soil microbial community diversity was compared with the control using the Shannon–Wiener (H') index for number and distribution (dominance) of different species and the Simpson (D) parameter to assess their proportional abundance.

3.3.1. 16S Bacterial and 18S Fungal Diversity

Generally, diversity profiles recorded a marked decrease on day 150 (spring) for the bacterial components and on day 90 (winter) for the fungal fraction of the soil communities for both the control and *Sus scrofa* lysimeters.

The 16S bacterial Shannon–Wiener indices trends showed differences in bacterial community diversity between the control and treatment soils following exhumation on day 180. Thus, an increase in the Shannon–Wiener index was observed between days 180 and 210 to reach 2.78 in the juvenile pig mesocosm (Figure 1a). Additionally, in general, the exhumed lysimeter recorded higher Shannon–Wiener diversity indices between days 270 and 300 than the control with final values of 2.83 and 3.08, respectively. Nonetheless, these trends did not reveal statistically significant temporal differences ($p = 0.48$) between the control and treatment microcosms. Moreover, the trends for the treatment were not statistically significantly different for pre- and post-exhumation ($p = 0.27$). The 18S fungal (Figure 1b) H' profiles showed statistically significant temporal differences ($p = 0.005$) between the control and treatment soils overall. Post-exhumation differences in the diversity indices were exemplified by an increase between days 210 and 240 to reach 2.88 (Figure 1b). The control microcosm recorded a decrease in the Shannon–Wiener index between days 210 and 240 to 2.47 and a subsequent increase to 2.76 on day 300. Finally, the exhumed microcosm recorded an index of 2.85, which was closer to the control both at the beginning (2.86) and end (2.755) of the study. However, the trends for the control and *S. s. domesticus* soil after exhumation were not statistically significantly different ($p = 0.09$).

The Simpson indices for both the 16S bacterial (Figure 2a) and 18S fungal (Figure 2b) communities showed differences in diversity after exhumation when compared to the control microcosm. Although the 16S bacterial Simpson index showed no statistical differences ($p = 0.33$) when analysed by one-way ANOVA, trend differences were observed post-exhumation between days 180 and 240 with an increase to 0.93. A further increase to 0.94 was observed for the longer post-burial interval (PBI) between days 270 and 300. For the 18S fungal Simpson diversity indices, statistically significant ($p = 0.044$) temporal differences were apparent between the control and treatment lysimeters as also exemplified by trend changes on days 210 and 270, i.e., 30 and 90 days after exhumation.

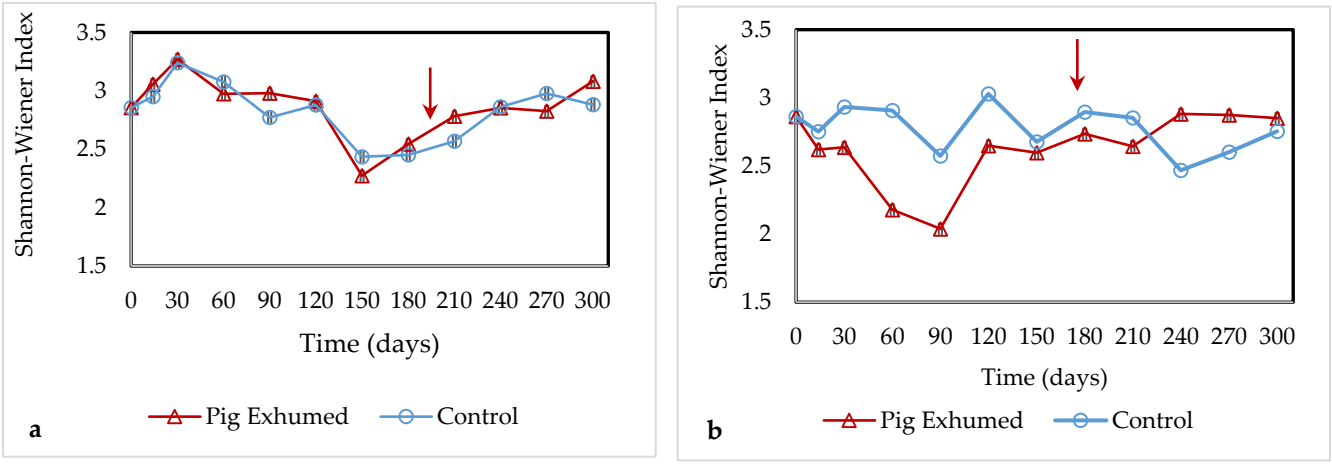

Figure 1. 16S bacterial (**a**) and 18S (**b**) fungal Shannon–Wiener (*H′*) indices of the control (○) and piglet-exhumed (Δ) microcosms during 300 days of study. Arrow identifies day of exhumation.

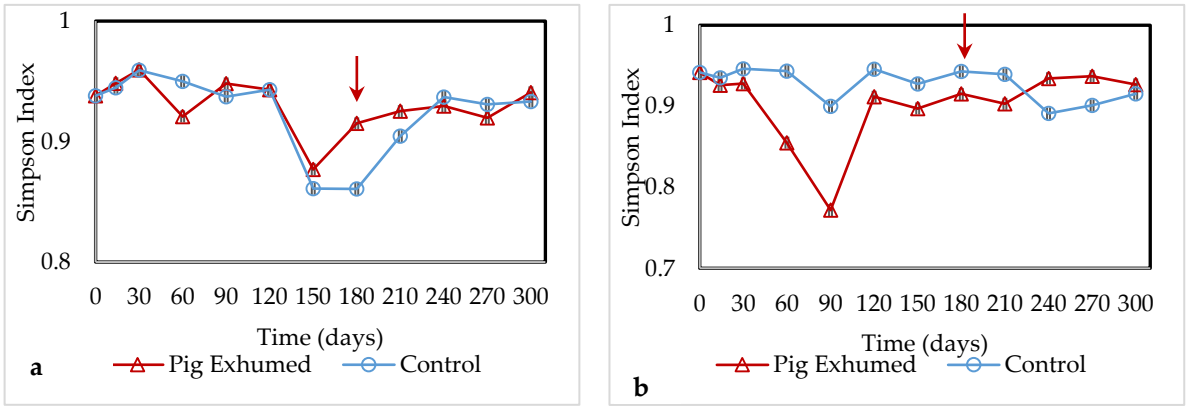

Figure 2. 16S bacterial (**a**) and 18S fungal (**b**) Simpson (*D*) indices of the control (○) and piglet-exhumed (Δ) microcosms during 300 days of study. Arrow identifies day of exhumation.

3.3.2. Principal Component Analysis

The principal component analysis (PCA) of the bacterial (Figure 3a) and fungal (Figure 3b) Shannon–Weiner (*H′*) and Simpson (*D*) diversity indices showed clustering of the control separate from the piglet soil. Although there was no consistent temporal delineation for pre- and post-exhumation phases for the 16S gene diversity, F1 accounted for 68.49% while F2 accounted for 15.33% of the bacterial communities. For the fungal 18S gene diversity, F1 and F2 accounted for 33.83% and 27.57%, respectively, especially for sampling days 270 and 300 which equated clustering at 90- and 120-days post-exhumation.

3.4. Taxonomic Resolution of Post-Exhumation Samples

To determine the effect of exhumation on soil necrobiome structure and composition, taxonomic comparisons were made at phylum, order, and family levels before and after *Sus scrofa* exhumation. The overall dominant phyla both pre- and post-exhumation included Proteobacteria (22.30–52.98%), Acidobacteria (5.29–33.83%), Bacteroidetes (5.17–20.96%), Verrucomicrobia (4.46–12.85%), Actinobacteria (7.51–12.18%), and Planctomycetes (2.02–11.03%) (Figure 4). NMDS analysis of the phylum-level community composition showed that the control samples were similar throughout while differences were observed in the for-exhumation mesocosm (Figure 5) with the shifts along coordinate 1 and distinct clustering for days 210–300 (30 to 120 days since exhumation) possibly due to seasonal temperature change. Three bacterial phyla correlated with mesocosm soil temperature where Firmicutes and Planctomycetes correlated negatively while Gemmatimonadetes recorded a

positive correlation. Similarly, these bacterial phyla correlated with soil pH, but Firmicutes and Planctomycetes showed a positive correlation while Gemmatimonadetes correlated negatively (Table 2).

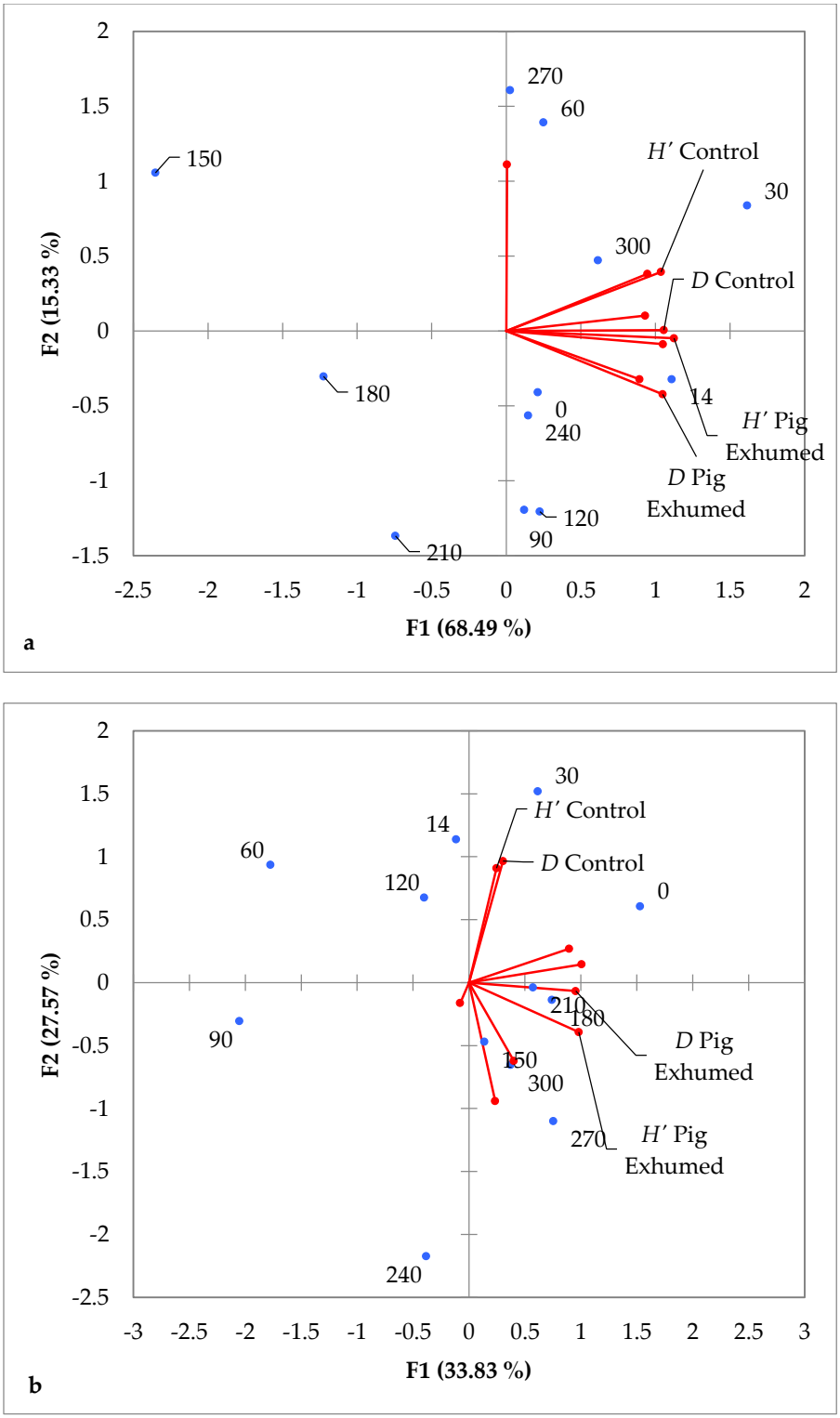

Figure 3. 16S bacterial (**a**) 18S Fungal (**b**) PCA biplot for DGGE-based diversity ecological measures (Shannon–Wiener, H'; Simpson, D) at specific sampling times of the control and piglet-exhumed microcosms. Numbers identify sampling times.

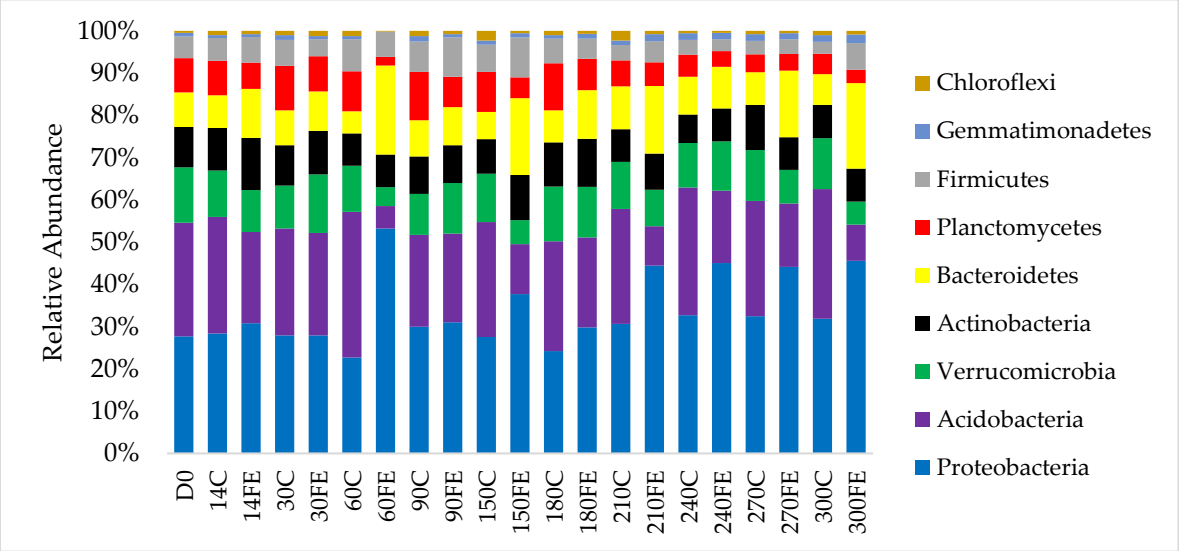

Figure 4. Phylum-level resolution for soil only control and juvenile *Sus scrofa domesticus* (FE) mesocosms over a 300-day incubation period. Arrow with numbers designate days post-exhumation.

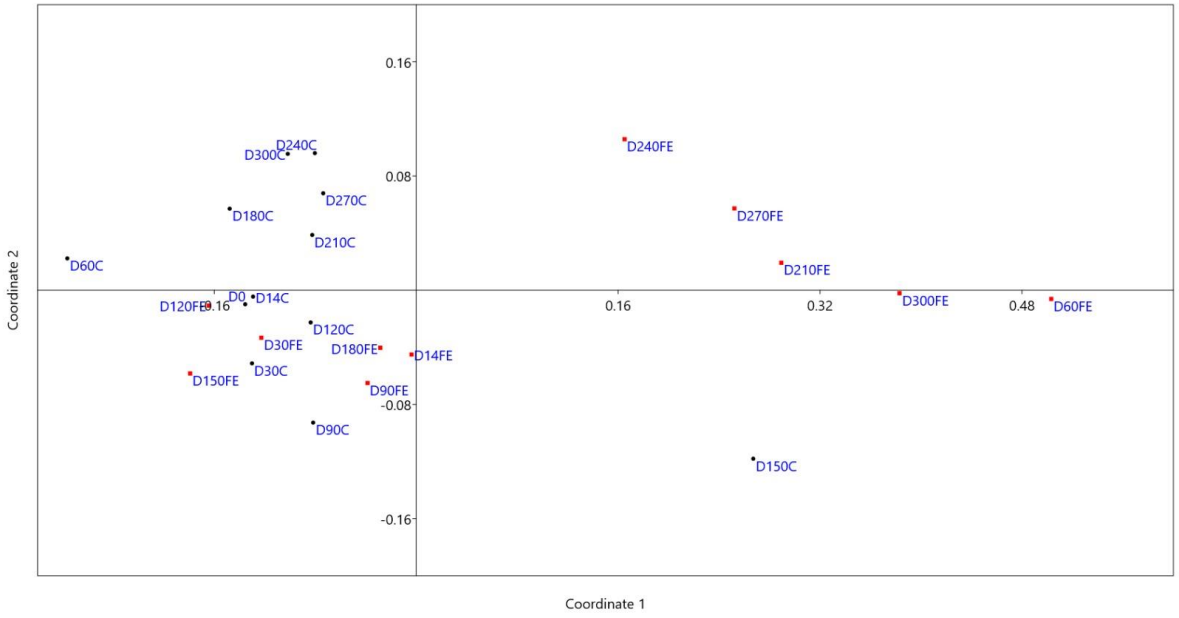

Figure 5. NMDS plot (R^2 = 0.94; stress = 0.092) of phylum-level resolution of the control (C, ●) and for-exhumation (FE, ■) microcosms.

Table 2. Post-exhumation OTUs recording statistically significant correlations ($p < 0.05$) with soil temperature and pH.

Temperature					
Positive			Negative		
OTUs (phylum)	R	*p*	OTUs (phylum)	R	*p*
Gemmatimonadetes	0.595	0.001	Firmicutes	−0.656	0.0001
			Planctomycetes	−0.471	0.009
pH					
Firmicutes	0.505	0.005	Gemmatimonadetes	−0.512	0.004
Planctomycetes	0.445	0.014			

Order-level comparisons for the pre-exhumation phase (days 0–180) revealed increases in the relative abundances of Pseudomonadales (3.70%) and Flavobacteriales (3.41%) on day 14 (Figure 6). A subsequent prominent shift in community composition was recorded on day 60 with predominances of Pseudomonadales (28.20%), Flavobacteriales (11.90%), Burkholderiales (8.43%), and Campylobacterales (3.05%) in the FE treatment. This contrasted the control mesocosm where predominances of Acidobacteria_Gp6_order (17.43%), Spartobacteria_order (8.92%), Planctomycetales (8.05%), and Rhizobiales (6.56%) resulted. Further changes were recorded on day 150 (spring) with increased relative abundances of Xanthomonadales (9.85%), Burkholderiales (5.60%), Nitrosomonadales (3.38%), Sphingobacteriales (7.22%), and Flavobacteriales (8.34%) in the piglet mesocosm. At- and post-exhumation (days 180–300) profiles showed temporal changes with the exhumed gravesoil recording marginally decreased dominances of Xanthomonadales (8.47%) and Burkholderiales (4.10%) 30 days after grave emptying (sampling day 210) while Sphingobacteriales (9.85%), Verrucomicrobiales (3.83%), and Sphingomonadales (2.61%) increased in relative abundance. Xanthomonadales subsequently showed its highest abundance of 11.29% on day 240, which was 60 days after the removal of the juvenile *Sus scrofa*. Further taxa shifts were observed on day 270 with increases in the relative abundances of Flavobacteriales (5.99%) and Alphaproteobacteria_order (2.14%) but decreased abundance of Xanthomonadales (9.45%) in the exhumed treatment. For the final stage of the study in early autumn 2015, increases in the relative abundances of Hydrogenophilales (7.13%), Clostridiales (4.57%), Bacteroidales (3.33%), and Flavobacteriales (6.37%) were recorded for the exhumed treatment (FE) on PBI = 300 or 120 days since exhumation.

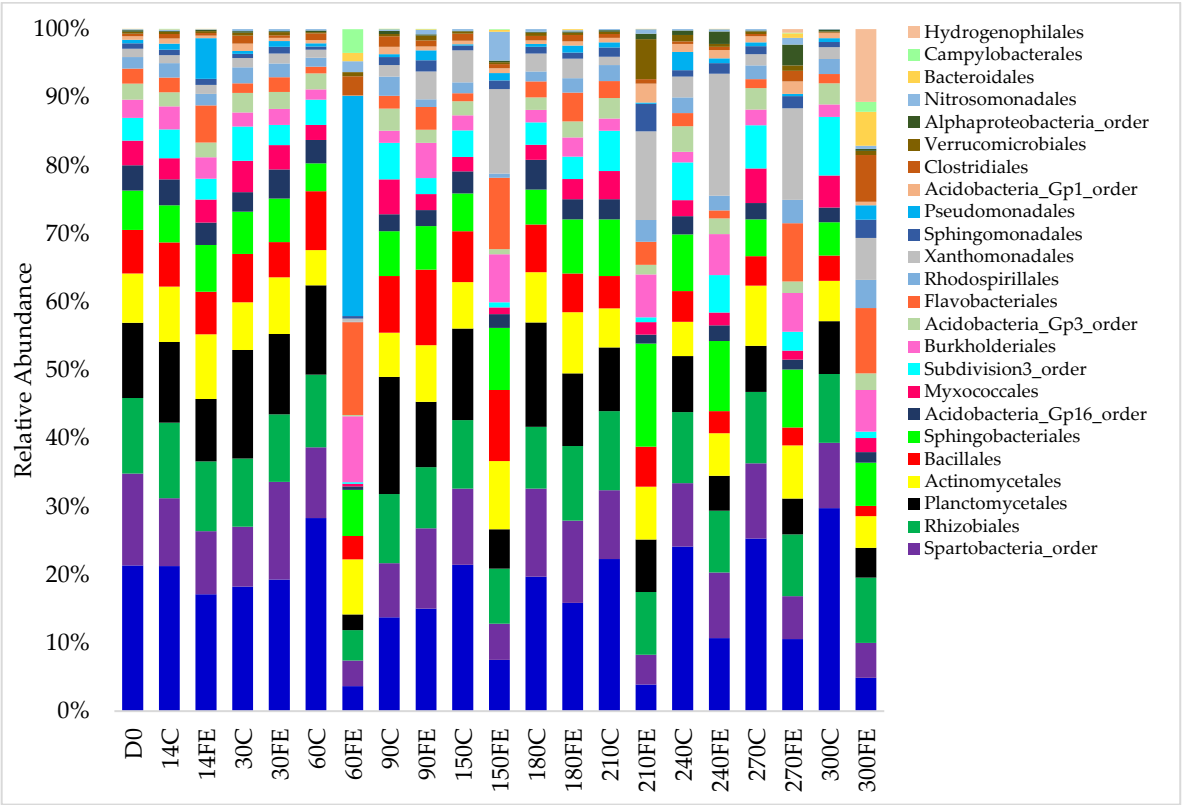

Figure 6. Order-level resolution for soil only control and juvenile *Sus scrofa domesticus* (FE) mesocosms over a 300-day incubation period. Arrow with numbers designate days post-exhumation.

Acidobacteria_Gp6_family, Spartobacteria_family, Planctomycetaceae, Chitinophagaceae, and Acidobacteria_Gp4_family were the predominant 16S bacterial taxa observed for the control and *S. s. domesticus* treatments, both pre- and post-exhumation (Figure 7). Nevertheless, some taxa differences at family level, such as Pseudomonadaceae (3.36%

at PBI = 14; and 30.67% at PBI = 60), aerobic Gram-negative chemoorganotrophic flagellate bacteria [23], and Campylobacteraceae (3.35% at PBI = 60), Gram-negative aerobic spiral rod-shaped bacteria [24], were observed at specific intervals of the pre-exhumation phase. A seasonal shift from winter to spring resulted in the predominance of Xanthomonadaceae (10.48%) on day 150 for the treatment lysimeter. This was similar for Alcaligenaceae (4.29%), Gram-negative lithoautotrophic ammonia-oxidising Nitrosomonadaceae (3.64%) [24] and Sphingobacteriaceae (3.39%). Post-exhumation microbial community characterisation revealed the dominance of Gram-negative Verrucomicrobiaceae (4%) [25] on day 210. Day 300 (early autumn 2015), which was 120 days after the removal of the decomposing material, was characterised by increased abundances of the Gram-negative sulphur-oxidising [24,26] Hydrogenophilaceae (*Thiobacillus* sp.) (7.56%), and aerobic Gram-negative Cryomorphaceae (1.75%) whose dominance of 3.63% on day 60 was followed by subsequent decrease close to the detection limit.

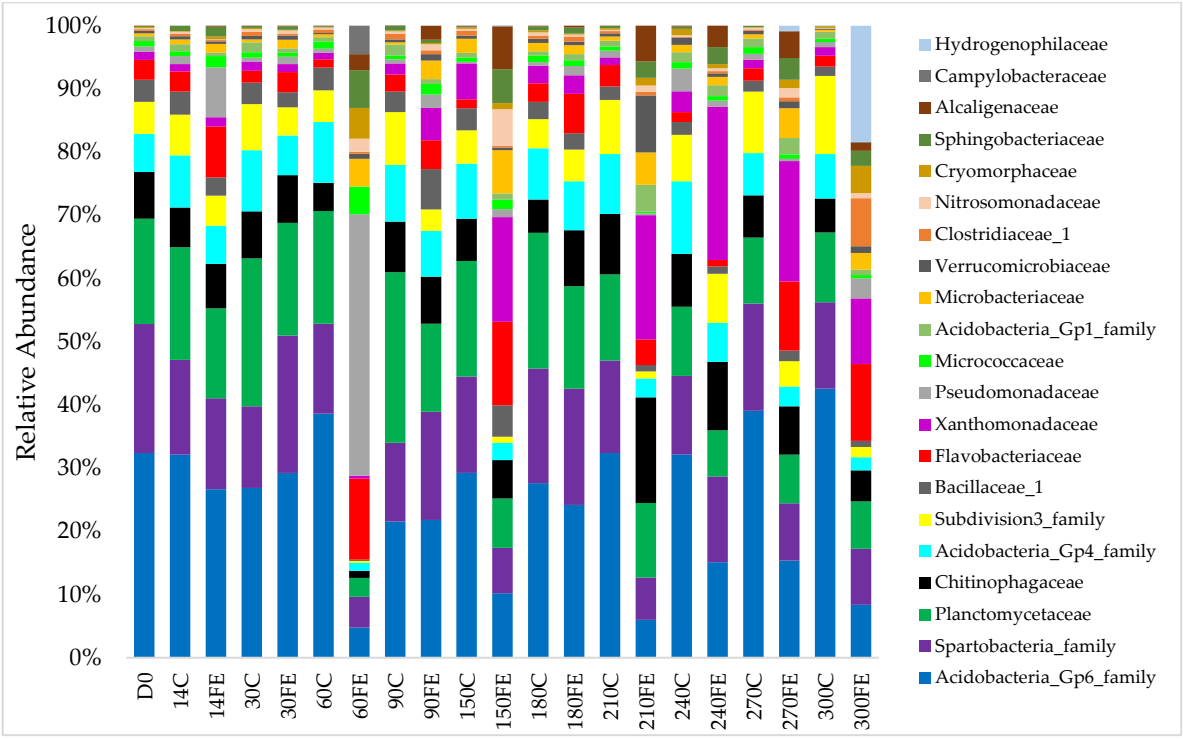

Figure 7. Family-level resolution for soil only control and juvenile *Sus scrofa domesticus* (FE) mesocosms over a 300-day incubation period. Arrow with numbers designate days post-exhumation.

Pairwise comparison with Tukey post hoc identified OTUs with statistically significant differences at family-level resolution between the control and treatment soils (Table 3) and relative to season (Table 4). For example, Alcaligenaceae, Flavobacteriaceae, Microbacteriaceae, and Xanthomondaceae recorded a significant difference ($p < 0.05$) between the control and the pig treatment. Likewise, significant seasonal differences ($p < 0.05$) were observed with OTUs such as Clostridiaceae_1 and Hydrogenophilaceae recorded during autumn 2015. Furthermore, Planctomycetaceae was observed to be significantly different ($p < 0.05$) between winter 2014 and summer 2015.

Table 3. Family-level OTUs that are statistically significantly different between the control and treatments soils according to the least squares means (LS-means). Combinations sharing the same letter (a, b) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) post hoc test.

OTU Family	Control	Pig Exhumed	*p*
Acidobacteria_Gp4_family	407.708 a	251.526 b	0.002
Acidobacteria_Gp6_family	1598.503 a	1002.230 b	0.004
Alcaligenaceae	−4.838 b	151.071 a	0.007
Flavobacteriaceae	123.633 b	479.633 a	0.010
Microbacteriaceae	35.361 b	188.906 a	0.008
Planctomycetaceae	829.364 a	615.636 b	0.013
Sphingobacteriaceae	12.788 b	158.879 a	0.021
Subdivision3_family	353.344 a	197.889 b	0.004
Xanthomonadaceae	91.445 b	547.355 a	0.006

Table 4. Family-level OTUs that are statistically significantly different between seasons according to LS-means. Combinations sharing the same letter (a, b) are not significantly different while those with no letter in common are significantly different as calculated by multi-way ANOVA with Tukey (HSD) post hoc test.

OTU Family	Autumn 2014	Winter 2014	Spring 2015	Summer 2015	Autumn 2015	*p*
Clostridiaceae_1	14.500 b	30.500 b	34.500 ab	20.000 b	195.500 a	0.044
Hydrogenophilaceae	0.000 b	0.000 b	0.000 b	8.167 b	469.500 a	0.020
Planctomycetaceae	853.750 ab	880.833 a	918.250 a	519.167 b	853.750 ab	0.004

4. Discussion

Recognition for the potential of forensic microbiology in crime scene investigation is gaining momentum. As a result, postmortem successional changes in microbial consortia structure and composition have been illustrated by multiple research groups with profiling of communities of decomposing cadaver/mammalian taphonomic proxies. Additionally, key variables including environmental parameters or abiotic factors such as temperature and pH, e.g., [9,27], must complement microbial community profiles to establish and validate novel postmortem interval estimation tools. The current preliminary forensic ecology study was, therefore, designed to test the hypothesis that: shifts in the composition and structure of bacterial and fungal communities will differentiate between exhumed and non-exhumed gravesoil. This was proven with next-generation sequencing of the bacterial 16S rRNA gene. The decomposition mesocosm (FE) was sampled for six months before (days 0–180) and four months after (days 210–300) exhumation, parallel to the soil-only control mesocosm. Exhumation of juvenile *Sus scrofa domesticus* effected soil microbial community changes starting from late spring (day 210) to early autumn (day 300) and so simulated temporal seasonal shifts that would occur 30–120 days after body removal from a clandestine grave.

An underpinning objective was to profile the necrobiome following exhumation of the decomposing material and link this to key edaphic factors and seasonal shifts. Changes in pH that resulted for the experimental lysimeter can be attributed to shifts in the metabolic and activity rates of the gravesoil microbial communities relative to decompositional or catabolic products where the *S. scrofa domesticus* contributed proteins, lipids, and carbohydrates [28]. An overall increase in pH for the piglet gravesoil occurred during early decomposition between days 14 and 60. Although we did not measure these nutrients, the trend was consistent with findings by Haslam and Tibbett (2009) [29] and Meyer et al. (2013) [30] who attributed initial pH increases to the ammonification of organic nitrogen in proteins and peptides, and mineralisation of base-forming cations such as Ca^{2+}, K^{+} and Mg^{2+}. As also reported by Meyer et al. (2013) [30], the subsequent drop was possibly due to increased nitrate concentration.

According to Meyer et al. (2013) [30], seasonal differences between winter and summer can impact microbial catabolic rates considerably, with reduced rates resulting in winter and increased rates a consequence of summer. Moreover, lysimeter temperature differences can be attributed directly to seasonal weather changes and exothermic microbial catabolism. For this study, shifts in seasons occurred for late autumn 2014 (day 0 to 30), winter (day 30 to 120), spring (day 120 to 210), summer (day 210 to 270), and early autumn 2015 (day 270 to300). Despite these changes in the temperature and ADD trends, the one way-ANOVA showed no statistically significant temporal differences (p = 0.99) between the control and treatment mesocosms. This suggested that any increases in temperature due to exothermic microbial catabolism were counterbalanced by the ambient temperature. Therefore, microbial community composition and structure were also determined relative to seasonal changes.

Overall, ecological indices measurements indicated that seasonal temperature shifts, even if not supported by statistically significant temperature changes, resulted in microbial community structure changes. Bacterial 16S gene ecological measures of Shannon–Wiener and Simpson diversity recorded no statistically significant differences between the control and burial soil before and following exhumation. In contrast, measures of both ecological indices revealed statistically significant different differences in the fungal communities between the control and burial soils, but not strictly as a result of the removal of the decomposing material. Therefore, statistically relevant differentiation of non-burial and gravesoil in the presence of juvenile *Sus scrofa domesticus* was recorded only when 18S rRNA gene was targeted with the two diversity tools. These, however, were not conclusive on their own to distinguish between when the decomposing material was in-place or had been exhumed for the current soil type.

Comparisons of DGGE profile data from Shannon–Weiner (H), Simpson (*D*), sampling time, non-exhumation, and pig exhumation were co-analysed. In general, PCA for the bacterial 16S rRNA gene showed no differentiation between the control and treatment soil, or between the pre- and post-exhumation phases. In contrast, some clustering resulted for the fungal 18S rRNA gene especially for the late summer to early autumn period of PBI = 270 and PBI = 300. To address the known limitations of DGGE-based profiling, and augment the subsequent multivariate analysis, next-generation sequencing of the 16S rRNA gene was applied for more robust analysis of community structure and composition. Fungi have a different role and response in decomposition and, hence, post-burial interval calculations. Therefore, future research on identifying exhumed gravesoils should apply parallel sequencing of 18S fungal gene as illustrated in a similar burial context [31].

For real crime scene purposes, the differentiation between the control and gravesoils based on microbial community profiling remains a challenge due to the presence of several common dominant taxa such as Proteobacteria, Acidobacteria, and Actinobacteria [32–34]. The dominance of Proteobacteria in the piglet lysimeter aligned the current investigation to the work of Finley et al. (2016) [34] where the phylum was numerically abundant in an underground decomposition study when compared to its aboveground equivalent. Nonetheless, phylum-level profiling gave no clear resolution between control and piglet nor pre- and post-exhumation intervals. Further to differentiations recorded at order level, taxa resolutions at family level identified more detailed temporal phylogenetic variations between the mesocosm soils. This was exemplified by the additional resolution of the orders Xanthomonadales, Hydrogenophilales, Verrucomicrobiales, and Clostridiales to their respective family members of Xanthomonadaceae, Hydrogenophilaceae, Verrucomicrobiaceae, and Clostridiaceae_1. All could be microbial clock indicators for excavated clandestine graves in the summer for the current study soil.

For this study, some differences in microbial community profiles were more pronounced due to a seasonal temperature change from spring to summer than treatment, per se. For example, the control mesocosm was characterised by an increase in the relative abundance of Xanthomonadaceae (*Rhodanobacter* sp.) on day 120 (late winter to early spring). This Gram-negative denitrifying bacterium [35] was also the most abundant taxon

on day 270 (summer, August 2015) for the non-exhumation treatment. It will, therefore, be an appropriate indicator of seasonal change for this soil as well as a time-specific indicator in time-since-burial determinations. Potential family-level taxonomic indicators of exhumed gravesoils for the current soil type included Hydrogenophilaceae (*Thiobacillus* sp.), Gram-negative sulphur-oxidising bacteria, and aerobic Gram-negative Cryomorphaceae, which were dominant in that treatment on day 300 (autumn 2015).

Overall, a distinct shift on day 60 in the experimental lysimeter, which might have been indicative of purge, was evidenced with specific increases at phylum (Protoebacteria; Bacteroidetes), order (Pseudomonadales; Flavobacteriales), and family (Pseudomonadaceae; Flavobacteriaceae; Sphingobacteriacea) taxonomic resolutions. Unlike for the DGGE results, and with the exception of 18S fungal Simpson diversity (*D*) on day 240, multivariate analysis of the next-generation sequencing data showed clustering of the pre- and post-exhumation soils which was pronounced for those from 30 to 120 days after the removal of remains. This suggested that, for this soil, 16S-based next-generation sequencing was a better tool for determining the impact of exhumation. Future work should incorporate NGS profiling of the fungal 18S gene.

5. Conclusions

The relevance and applicability of studying the decompositional microbial communities for post-burial interval determinations, when the body has completely decomposed and/or removed from clandestine graves, is acknowledged [6,10] but has been investigated very little. Our preliminary study, the first to our knowledge, was designed to address this knowledge gap by comparing changes in microbial community structure before and after the exhumation of juvenile *Sus scrofa domesticus*. Temporal differences recorded with the DGGE-dependent ecological measures of Shannon–Wiener and Simpson diversity, especially on day 150, were reflected in the NGS-based taxonomic classification with a seasonal change to spring. Direct alignment of these was not made; however, the sequencing was not derived from DGGE-excised bands. Metabarcoding of the bacterial 16S gene indicated the possible use of Verrucomicrobiaceae (spring; day 210 = 60 days post-exhumation) and Hydrogenophilaceae and Clostridiaceae_1 (summer; day 300 = 120 days post-exhumation) as season and long PBI family taxa indicators for exhumed clandestine graves for the current soil type. They provided the highest potential to differentiate between control and burial soil, and between pre- and post-exhumation phases.

A strength of this work is the measurable shifts in bacterial and fungal community structures that differentiated, to different degrees, between pre- and post-exhumation gravesoils. For example, PCA of fungal-based DGGE analysis showed clustering due to remains removal at late PBI. The same was proven for bacterial components using NGS but not with DGGE. These findings mean that the study hypothesis was accepted albeit from two different profiling approaches for the two microbial clades. To address this imbalance, future work should incorporate parallel NGS profiling of the fungal 18S gene as illustrated by Procopio et al. (2020) [31].

Protracted replicated future studies should also consider the analysis of soil ecology after exhumation until skeletonisation. These can include comparisons of removal of remains/taphonomic proxies at different stages of decomposition. Additionally, profiling of the inherent mammalian-associated epinecrobiome/thanatomicrobiome could be made parallel to soil necrobiome characterisation. Thus, the longevity of the cadaver decomposition island and cadaver-derived microbial footprints could be determined simultaneously. This would facilitate assessments of their individual and combined applicability in (long-term) time-since-burial or time-since-exhumation estimations.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/forensicsci2010010/s1, Figure S1: Changes in pH values of the control (•) and piglet-exhumed (•) mesocosms during 300 days of study. Arrow identifies day of exhumation, Table S1: Next-generation sequencing data presented in this study.

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References

1. Pechal, J.L.; Benbow, E.M. Community Ecology. In *Forensic Entomology: International Dimensions and Frontiers*; Tomberlin, J.K., Benbow, E.M., Eds.; CRC Press: Boca Raton, FL, USA, 2015; pp. 347–360.
2. Metcalf, J.L.; Xu, Z.Z.; Weiss, S.; Lax, S.; Van Treuren, W.; Hyde, E.R.; Song, S.J.; Amir, A.; Larsen, P.; Sangwan, N.; et al. Microbial community assembly and metabolic function during mammalian corpse decomposition. *Science* **2016**, *351*, 158–162. [CrossRef] [PubMed]
3. Olakanye, A.O.; Nelson, A.; Ralebitso-Senior, T.K. A comparative in situ decomposition study using still born piglets and leaf litter from a deciduous forest. *Forensic Sci. Int.* **2017**, *276*, 85–92. [CrossRef] [PubMed]
4. Carter, D.O.; Yellowlees, D.; Tibbett, M. Cadaver decomposition in terrestrial ecosystems. *Die Nat.* **2007**, *94*, 12–24. [CrossRef] [PubMed]
5. Hyde, E.R.; Haarmann, D.P.; Petrosino, J.F.; Lynne, A.M.; Bucheli, S.R. Initial insights into bacterial succession during human decomposition. *Int. J. Leg. Med.* **2015**, *129*, 661–671. [CrossRef] [PubMed]
6. Procopio, N.; Ghignone, S.; Williams, A.; Chamberlain, A.; Mello, A.; Buckley, M. Metabarcoding to investigate changes in soil microbial communities within forensic burial contexts. *Forensic Sci. Int. Genet.* **2019**, *39*, 73–85. [CrossRef] [PubMed]
7. Guo, J.; Fu, X.; Liao, H.; Hu, Z.; Long, L.; Yan, W.; Ding, Y.; Zha, L.; Guo, Y.; Yan, J.; et al. Potential use of bacterial community succession for estimating post-mortem interval as revealed by high-throughput sequencing. *Sci. Rep.* **2016**, *6*, 24197. [CrossRef]
8. Metcalf, J.L.; Parfrey, L.W.; Gonzalez, A.; Lauber, C.L.; Knights, D.; Ackermann, G.; Humphrey, G.C.; Gebert, M.J.; Van Treuren, W.; Berg-Lyons, D.; et al. A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system. *eLife* **2013**, *2*, e01104. [CrossRef]
9. Lauber, C.L.; Metcalf, J.L.; Keepers, K.; Ackermann, G.; Carter, D.O.; Knight, R. Vertebrate Decomposition Is Accelerated by Soil Microbes. *Appl. Environ. Microbiol.* **2014**, *80*, 4920–4929. [CrossRef]
10. Ralebitso-Senior, T.K. Summary: An Assessment of Achievements, Limitations, and Potential of Forensic Ecogenomics. In *Forensic Ecogenomics: The Application of Microbial Ecology Analyses in Forensic Contexts*; Ralebitso-Senior, T.K., Ed.; Academic Press: London, UK, 2018; pp. 211–234.
11. Fløjgaard, C.; Frøslev, T.G.; Brunbjerg, A.K.; Bruun, H.H.; Moeslund, J.; Hansen, A.J.; Ejrnæs, R. Predicting provenance of forensic soil samples: Linking soil to ecological habitats by metabarcoding and supervised classification. *PLoS ONE* **2019**, *14*, e0202844. [CrossRef]
12. Massas, I.; Kefalogianni, I.; Chatzipavlidis, I. Is the ground of an old cemetery suitable for the establishment of an urban park? A critical assessment based on soil and microbiological data. *J. Soils Sediments* **2018**, *18*, 94–108. [CrossRef]
13. Ghosh, S.; Deb, S.; Ow, L.F.; Deb, D.; Yusof, M.L. Soil characteristics in an exhumed cemetery land in Central Singapore. *Environ. Monit. Assess.* **2019**, *191*, 174. [CrossRef] [PubMed]
14. Pittner, S.; Bugelli, V.; Benbow, M.E.; Ehrenfellner, B.; Zissler, A.; Campobasso, C.P.; Oostra, R.-J.; Aalders, M.C.G.; Zehner, R.; Lutz, L.; et al. The applicability of forensic time since death estimation methods for buried bodies in advanced decomposition stages. *PLoS ONE* **2020**, *15*, e0243395. [CrossRef] [PubMed]
15. Bull, I.D.; Berstan, R.; Vass, A.; Evershed, R.P. Identification of a disinterred grave by molecular and stable isotope analysis. *Sci. Justice* **2009**, *49*, 142–149. [CrossRef] [PubMed]
16. Olakanye, A.O.; Thompson, T.; Ralebitso-Senior, T.K. Shifts in soil biodiversity—A forensic comparison between Sus scrofa domesticus and vegetation decomposition. *Sci. Justice* **2015**, *55*, 402–407. [CrossRef]

17. Olakanye, A.O.; Thompson, T.; Ralebitso-Senior, T.K. Changes to soil bacterial profiles as a result of Sus scrofa domesticus decomposition. *Forensic Sci. Int.* **2014**, *245*, 101–106. [CrossRef]
18. Kozich, J.J.; Westcott, S.L.; Baxter, N.T.; Highlander, S.K.; Schloss, P.D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* **2013**, *79*, 5112–5120. [CrossRef]
19. Schoenly, K.G.; Michaud, J.-P.; Moreau, G. Design and Analysis of Field Studies in Carrion Ecology. In *Carrion Ecology, Evolution, and Their Applications*; Benbow, M.E., Tomberlin, J.K., Tarone, A.M., Eds.; CRC Press: Boca Raton, FL, USA, 2015; pp. 129–148.
20. Suckling, J.K.; Spradley, M.K.; Godde, K. A Longitudinal Study on Human Outdoor Decomposition in Central Texas. *J. Forensic Sci.* **2015**, *61*, 19–25. [CrossRef]
21. Vass, A.A.; Bass, W.M.; Wolt, J.; Foss, J.E.; Ammons, J.T. Time since death determinations of human cadavers using soil solution. *J. Forensic Sci.* **1992**, *37*, 1236–1253. [CrossRef]
22. Megyesi, M.S.; Nawrocki, S.P.; Haskell, N.H. Using Accumulated Degree-Days to Estimate the Postmortem Interval from Decomposed Human Remains. *J. Forensic Sci.* **2005**, *50*, 618–626. [CrossRef]
23. Brenner, D.J.; Krieg, N.R.; Staley, J.R.; Garrity, G. (Eds.) *Bergey's Manual of Systematic Bacteriology (The Proteobacteria; The Gammaproteobacteria)*, 2nd ed.; Springer: New York, NY, USA, 2009; Volume 2.
24. Brenner, D.J.; Krieg, N.R.; Staley, J.R.; Garrity, G. (Eds.) *Bergey's Manual of Systematic Bacteriology (The Proteobacteria; The Alpha-, Beta-, Delta-, and Epsilonproteobacteria)*, 2nd ed.; Springer: New York, NY, USA, 2005; Volume 2.
25. Krieg, N.R.; Staley, J.T.; Brown, D.R.; Hedlund, B.P.; Paster, B.J.; Ward, N.L.; Wolfgang, L.; Whitman, W.B. (Eds.) *Bergey's Manual of Systematic Bacteriology (The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Clamydiae, and Planctomycetes*, 2nd ed.; Springer: New York, NY, USA, 2010; Volume 4.
26. Forbes, S.L. Decomposition Chemistry in a Burial Environment. In *Soil Analysis in Forensic Taphonomy: Chemical and Biological Effects of Buried Human Remains*; Tibbett, M., Carter, D.O., Eds.; CRC Press: Boca Raton, FL, USA, 2008; pp. 203–223.
27. Weiss, S.; Carter, D.O.; Metcalf, J.L.; Knight, R.T. Carcass mass has little influence on the structure of gravesoil microbial communities. *Int. J. Leg. Med.* **2016**, *130*, 253–263. [CrossRef]
28. Janaway, R.C.; Percival, S.L.; Wilson, A.S. Decomposition of Human Remains. In *Microbiology and Aging: Clinical Manifestations*; Percival, S.L., Ed.; Springer: New York, NY, USA, 2009; pp. 313–334.
29. Haslam, T.C.F.; Tibbett, M. Soils of Contrasting pH Affect the Decomposition of Buried Mammalian (Ovis aries) Skeletal Muscle Tissue. *J. Forensic Sci.* **2009**, *54*, 900–904. [CrossRef] [PubMed]
30. Meyer, J.; Anderson, B.; Carter, D.O. Seasonal Variation of Carcass Decomposition and Gravesoil Chemistry in a Cold (Dfa) Climate. *J. Forensic Sci.* **2013**, *58*, 1175–1182. [CrossRef] [PubMed]
31. Procopio, N.; Ghignone, S.; Voyron, S.; Chiapello, M.; Williams, A.; Chamberlain, A.; Mello, A.; Buckley, M. Soil Fungal Communities Investigated by Metabarcoding Within Simulated Forensic Burial Contexts. *Front. Microbiol.* **2020**, *11*, 1686. [CrossRef] [PubMed]
32. Damann, F.E.; Williams, D.E.; Layton, A.C. Potential use of bacterial community succession in decaying human bone for estimating post-mortem interval. *J. Forensic Sci.* **2015**, *60*, 844–850. [CrossRef]
33. Carter, D.O.; Metcalf, J.L.; Bibat, A.; Knight, R. Seasonal variation of postmortem microbial communities. *Forensic Sci. Med. Pathol.* **2015**, *11*, 202–207. [CrossRef]
34. Finley, S.J.; Pechal, J.; Benbow, M.E.; Robertson, B.K.; Javan, G.T. Microbial Signatures of Cadaver Gravesoil During Decomposition. *Microb. Ecol.* **2016**, *71*, 524–529. [CrossRef]
35. Green, S.; Prakash, O.; Jasrotia, P.; Overholt, W.A.; Cardenas, E.; Hubbard, D.; Tiedje, J.M.; Watson, D.B.; Schadt, C.W.; Brooks, S.; et al. Denitrifying Bacteria from the Genus Rhodanobacter Dominate Bacterial Communities in the Highly Contaminated Subsurface of a Nuclear Legacy Waste Site. *Appl. Environ. Microbiol.* **2012**, *78*, 1039–1047. [CrossRef]