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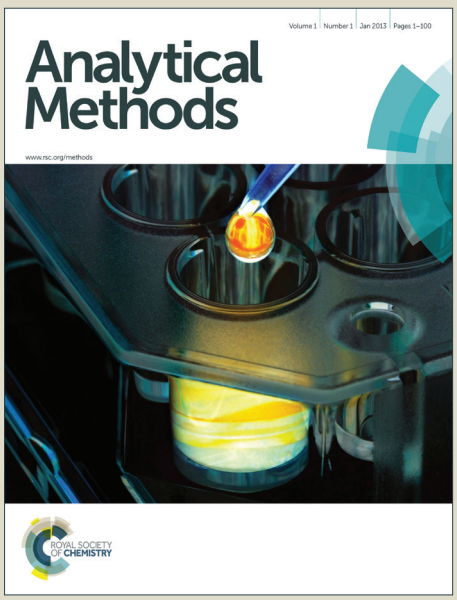

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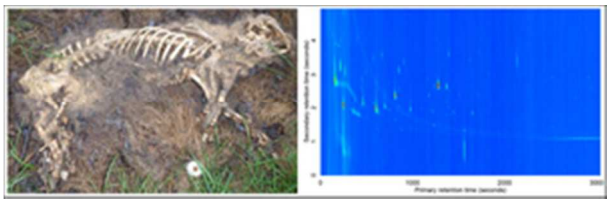

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ARTICLE

Seasonal comparison of carrion volatiles in decomposition soil using comprehensive two-dimensional gas chromatography – time of flight mass spectrometry

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Katelynn A. Perrault,[a*] Tapan Rai,[b] Barbara H. Stuart[a] and Shari L. Forbes[a]

Increased characterisation of decomposition odour has improved existing knowledge regarding the decomposition volatile organic compound (VOC) profile of carrion. Validation of this dynamic decomposition VOC profile is required in order to characterise the variables that affect their production. This study was performed to determine whether the decomposition VOC profile produced under field conditions differed between summer and winter in an Australian environment. Outdoor studies were conducted using pig carcasses as human analogues in order to assess seasonal variation in the decomposition process. Common decomposition VOCs were identified using comprehensive two-dimensional gas chromatography – time of flight mass spectrometry (GC×GC-TOFMS). Fewer compounds and reduced abundance of VOCs was observed during winter. Relationships between the levels of detected decomposition VOCs and weather variables were established to be stronger in winter. Weak relationships during summer suggested the potential that an underlying variable (*e.g.* microbial activity, insect activity) had a stronger relationship to the abundance of decomposition VOCs. The seasonal robustness of the decomposition VOC profile is important to fields relying on the presence of a decomposition odour, *i.e.* search and recovery of victims in mass disasters, homicides, and missing persons cases.

Introduction

Post-mortem degradation of soft tissue produces numerous volatile organic compounds (VOCs) which in combination give rise to a characteristic odour of decomposition. Chemical profiling and characterisation of VOCs produced during cadaver and carrion decomposition has increased considerably in recent years.[1–14] Characterising trends throughout the decomposition process is fundamental in a variety of forensic applications that rely on the presence of decomposition odour. Cadaver dogs (used to search for human remains) require interaction of the VOCs from decomposition odour with their olfactory system to register an alert. Carrion insects orient their colonisation towards remains based on olfaction of VOCs in decomposition odour, and the pattern of insect arrival is used to estimate post-mortem interval of discovered remains. In addition, the development of hand-held instrumentation for disaster victim, homicide victim, or missing persons searches require knowledge of VOC production during decomposition if these devices are to be sufficiently sensitive and selective for routine use. Despite these important societal implications, knowledge of the reproducibility of the decomposition VOC profile under variable conditions remains poorly documented.

Decomposition is affected by a large number of extrinsic factors (*e.g.* temperature, humidity, rainfall, wind, soil microbial community, insect colonisation, etc.) and intrinsic factors relating to the remains (*e.g.* biomass, fat distribution, enteric microbial activity, etc.). Trends in the decomposition VOC profile produced at different geographical regions have provided information regarding a subset of the decomposition VOC profile that is stable between different climates. However, decomposition VOC production exhibited in lower temperature ranges remains largely undocumented in the literature with only a few studies investigating decomposition in cooler temperatures (*i.e.* 0-10°C).[2,3] Outdoor research facilities are often located in warm climates globally, and where they are not, research studies are often conducted during spring and summer months to guarantee visual decomposition occurs. It has been proven under controlled laboratory conditions that decomposition VOCs are highly affected by changes in temperature and humidity.[2]

Recent studies have highlighted the lack of validation in the area of decomposition odour research.[2,10,11] Validating analytical instrumentation and the robustness of the decomposition VOC profile under various conditions can confirm proposed trends and provide confidence in the decomposition VOC profile that has been proposed in previous studies. The inter-year reproducibility of core decomposition VOCs,[10] comparison of VOC collection techniques for decomposition odour[14] and method validation of sorbent tubes and thermal desorption – gas chromatography mass spectrometry (TD-GC-MS) for decomposition VOC analysis[11] have been previously documented. However, a study has yet to

be performed reflecting the stability of these established trends when comparing directly between seasons.

Advancements in the analytical instrumentation used to identify decomposition VOCs have improved the overall characterisation of the matrix. Comprehensive two dimensional gas chromatography – time of flight mass spectrometry (GC×GC-TOFMS) has been an emerging technology in this field due to the improved peak capacity and sensitivity offered by the instrument.[3,6,8,13,15,16] Due to these benefits, GC×GC-TOFMS is capable of detecting and accurately identifying many more compounds in a complex sample, especially in mixtures demonstrating dynamic compound range. Trace compounds may be masked by co-elution with highly abundant compounds when using traditional gas chromatography – mass spectrometry (GC-MS) to analyse complex samples. Although these benefits are now realised in this research area, there are still very few published studies that use GC×GC-TOFMS to characterise the suite of VOCs produced throughout the entire decomposition process.[3,6,8] GC×GC-TOFMS was therefore considered to be extremely valuable for this study to provide seasonal comparison of decomposition VOCs. Different ranges in VOC abundance were anticipated between seasons. Using GC×GC-TOFMS allows for the same method to be applied for the analysis of both low-level and high-level VOC production.

This study arose from the requirement for further characterisation of the pattern of the decomposition VOC profile in contrasting seasons. The outdoor taphonomic research facility located near Sydney, Australia experiences variable weather conditions depending on the season. During winter (June-August), average daily temperatures can range between 10-15 °C while declining close to 0 °C overnight. During the summer (December to February), daily average temperatures range from 20-30 °C and often surpass 40 °C during heat waves. Although preliminary laboratory studies have demonstrated effects of temperature and humidity on decomposition VOC production, field research introduces many additional variables (*i.e.* wind, rain, solar radiation, etc.) that can impact decomposition. The objective of this study was to profile decomposition VOCs from soil surrounding human analogue decomposition during the winter and summer months in Sydney, Australia. Correlation analysis was used to establish the nature and strength of the relationships between weather variables and the resultant decomposition VOC profiles, thereby facilitating an improved understanding regarding decomposition VOC production under different conditions.

Experimental

Field Setup

Two trials were performed at an outdoor research facility near Sydney, Australia. Each trial used four pig carcasses (*Sus scrofa domesticus*) as human decomposition analogues and to provide four replicate measurements. The pigs were placed on the soil surface at the research facility. Four control sites (absent of pig remains) were analysed throughout the trials to establish the background VOC profile at the research location. Control sites used in the winter trial were reused for the summer trial. A distance of approximately 4 m separated each control or experimental site from each other, and a distance of approximately 20 m separated control sites from experimental sites. Each pig carcass was covered with a stainless steel cage to prevent animal scavenging (130 cm length × 90 cm width x 60 cm height, 1 cm wire mesh) which was removed during sample collection. A schematic of the field research facility is available in the electronic supplementary information, Figure S1. Two trials were performed using the same experimental setup; a winter trial between July 2013-November 2013 (106 days post-mortem) and a summer trial between January 2014-March 2014 (73 days post-mortem). Monitoring of decomposition stage and VOCs was performed periodically for each study. Sample collection was performed on days 0, 1, 6, 9, 16, 22, 30, 37, 44, 51, 65, 79, and 106 for the winter trial and days 1, 3, 6, 8, 10, 14, 17, 24, 31, 44, 59, and 73 for the summer trial. Frequent monitoring was performed in the first month post-mortem and then subsequently decreased in frequency as the decomposition process slowed. The stage of decomposition was classified during each monitoring period as fresh, bloat, active decay, advanced decay, or dry/remains based on the stages developed by Payne.[17] These stages were ranked ordinally (1 through 5) for statistical analyses.

Ambient temperature (°C), relative humidity (%), solar radiation (W/m^2), wind speed (m/s), gust speed (m/s), wind direction (ø) and rainfall (mm) were measured hourly using a HOBO® No Remote Communication (NRC) weather station base and respective sensors (OneTemp, Marleston). The weather station was placed midway between all sites used. Soil pH was measured at each site during VOC sampling using a direct soil pH measurement kit (Hanna Instruments, Australia). Volumetric water content (VWC, percentage of water in the soil by volume) was measured using a soil moisture sensor with LabQuest 2 interface (Vernier Software and Technology, Scientrific Pty Ltd., Australia) from each site during sample collection. Accumulated degree days (ADD) was used to monitor the duration of stages for each trial. Hourly temperature measurements were averaged for each day. The sum of the average daily temperatures produced the ADD which was used to account for differences in the rate of decomposition based on temperature effects.[18]

Sample collection and analysis

Decomposition VOC collection was performed from experimental and control soil using pumped sampling via a 30 cm VOC-Mole™ Soil Probe (Markes International Ltd., UK) onto Tenax TA/Carbograph 5TD sorbent tubes (Markes International Ltd., UK) described in previous studies.[12,14] The VOC-Mole™ Soil Probe was probed into the soil directly in front of the torso of each pig carcass so that VOCs were collected from the within the cadaver decomposition island (CDI). Collecting decomposition VOCs from soil reflects the wider range of microbial VOCs in the surrounding environment that can provide a more comprehensive overall profile.[12] Samples were collected for 15 min at a rate of 100 mL/min from the soil probe at each experimental and control site using an ACTI-VOC constant air flow sampling pump (Markes International Ltd., UK). Field blanks were collected passively by exposing an unsealed sorbent tube to ambient air for 10 seconds prior to and following VOC sample collection. Sorbent tubes were wrapped in aluminium foil and transported to the laboratory in sealed jars and stored in a refrigerator until analysis (typically the same day or on the following day after sample collection).

Sorbent tubes were injected with 2 µL of an internal standard containing 150 ppm bromobenzene (GC grade, Sigma Aldrich, Australia) in methanol (HPLC grade, Sigma Aldrich, Australia) using an eVol® XR handheld automated analytical syringe (SGE Analytical Science, Australia). VOCs were thermally desorbed from sorbent tubes using a Unity 2 Thermal Desorber equipped with a Series 2 ULTRA™ multi-tube autosampler (Markes International

Ltd., UK). The thermal desorption parameters were specified for the product by the manufacturer based on the sorbent combination and can be found in previous studies.[12,14] Injection occurred via an uncoated fused silica transfer line onto the Pegasus® 4D GC×GC-TOFMS (LECO, Australia). The first dimension (^{1}D) column was a Rxi®-624Sil MS (30 m × 0.250 mm ID, 1.40 µm film thickness, Restek Corporation, Australia) connected to a second dimension (^{2}D) Stabilwax® column (2 m × 0.250 mm ID, 0.50 µm film thickness, Restek Corporation, Australia). Column connections were made with an Ultimate Union kit (TD transfer line to primary column, Agilent, Australia), and a SilTite™ µ-Union (primary to secondary column connection, SGE Analytical Science, Australia). Helium carrier gas flow was held at a constant rate of 1.00 mL/min throughout the run. The primary oven was held initially at 35°C for 5 minutes, followed by an increase to 240°C at a rate of 5°C/min, and was then held for 5 minutes (total 51 minutes). The modulator offset was 5°C and the secondary oven temperature offset was 15°C. A 5 second modulation period was used with a 1 second hot pulse time. The MS transfer line was held at 250°C. The mass range was 29-450 amu at an acquisition rate of 100 spectra/second. The ion source temperature was 200°C and the electron ionization energy was -70 eV. A 200V offset was used above the optimised detector voltage.

Data processing and correlation analysis

Data processing was performed using ChromaTOF® (version 4.50.8.0, LECO, Australia). Peak alignment and normalisation was performed using Statistical Compare (LECO, Australia) on experimental and control sample classes. Baseline tracking was performed (80% offset) with automatic baseline smoothing. A 30 second peak width in ^{1}D and 0.15 second peak width in ^{2}D was used. A signal-to-noise ratio (S/N) of 150 was used with a minimum similarity match > 700 to the NIST (2011) mass spectral library database. Peak identification was also confirmed by retention indices and using a range of 84 chemical standards (alkanes, alkylbenzenes, aromatic hydrocarbons, heterocyclic aromatics, chlorinated hydrocarbons, ketones, aldehydes, sulphur-containing compounds, phthalates, primary alcohols, secondary alcohols, fatty acid methyl esters, phthalates and Grob test mix compounds). Peak area calculations by unique mass were normalised using the internal standard. A 50% filter was applied for each component and peaks >20 S/N were included by Statistical Compare for analytes that were not identified using the initial peak find algorithm.

Aligned compound tables from Statistical Compare were transferred to Microsoft Excel where background compounds (*i.e.* column bleed, internal standard artefacts, and field blank compounds) were removed. The normalised peak areas were log-transformed and an independent samples t-test was used to compare the experimental replicates to the control replicates on each individual sampling day (using p-value < 0.05). This data handling approach has been previously demonstrated to focus on the most discriminating compounds in the overall decomposition VOC profile.[19] Significant compounds were then classified into one or more of the following compound classes: sulphur-containing, nitrogen-containing, aromatic, carboxylic acid, ester, aldehyde, ketone, alcohol, ether, halogenated, or hydrocarbon.

Excel files of the significant compounds were imported into SPSS® Statistics (version 21, IBM®, Australia) to provide a database including the response variable (cumulative normalised decomposition VOC abundance for each carcass) and VOC profile predictor variables (*i.e.* weather and soil variables). VOC profile predictor variables analysed were day, stage, soil VWC, soil pH, daily average temperature, relative humidity, rainfall, and solar radiation. Although predictive modelling was not used, "predictor" describes variables that were investigated for a potential relationship with the resultant decomposition VOC profile. Wind speed and gust speed were measured at or slightly above 0 m/s throughout both trials and were therefore removed from the list of potential predictor variables. Wind direction varied only slightly throughout both trials, and therefore was also removed from the predictor list. To assess the cumulative effects of environmental conditions on the resultant decomposition VOC profile, each of these variables was aggregated over all sampling days between observation periods.

Correlations between response and each predictor were calculated using the Spearman's correlation coefficient (rho, ρ), which can be used to examine the strength of the relationship between non-linear variables. Negative ρ values indicated a relationship with a negative slope and positive ρ values indicated a relationship with a positive slope. A value of $|\rho| < 0.3$ indicated a weak relationship between response and predictor variable(s). When $0.3 < |\rho| < 0.7$, a medium-strength relationship existed between the variables. A value of $|\rho| > 0.7$ indicated a strong relationship between variables. Values of ρ were only considered when significant (p-value < 0.05).

Results and Discussion

Weather

Weather patterns during the trials were distinct (Figure 1). In the winter trial, the absolute minimum temperature was 1.64°C and the absolute maximum was 37.32°C. Although the absolute maximum temperature was high, it was reached late in the trial (Day 100) during the transition from winter to spring. Very low rainfall was experienced throughout the duration of the winter trial. In the summer trial, higher temperatures and abundant rainfall were experienced. During this time, rainfall was concentrated in short intervals when it occurred. The absolute temperature minimum was 11.49°C. The absolute maximum temperature was 42.92°C. The average relative humidity between the trials was comparable for winter (71 ± 15%) and summer (79 ± 12%). Solar radiation measurements were 15.00 - 130.10 W/m^2 in winter and 12.38 - 208.43 W/m^2 in summer. Wind speed, gust speed and wind direction were negligible and varied only slightly above zero.

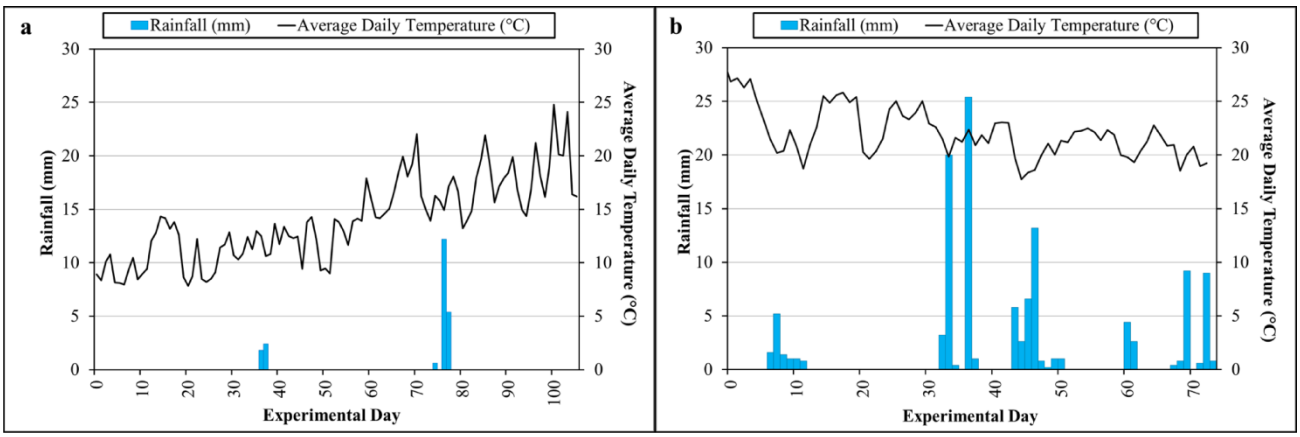

Figure 1. Average daily temperature and rainfall plots for monitoring periods during the a) Winter 2013 trial and b) Summer 2014 trial.

Decomposition rates

The carcass decomposition rate was monitored throughout both trials and differed between seasons (Figure 2). Similar rates were experienced by all carcasses within trials, although minor variations were observed in the onset of some stages for each carcass (Figure 2). Differential decomposition was represented by choosing the predominant stage of decomposition exhibited by the carcass, as in previous research.[10] Decomposition progressed at a faster rate during the summer trial than in the winter trial. The ADD standardization for time is used to account for this variation in decomposition rate due to temperature differences in the two trials.[18] In theory, once ADD calculations are performed, the onset of each stage should occur at roughly the same ADD between both trials, however, this is not always the case.[10] Figure 2 demonstrates that although temperature is accounted for, there are additional factors that affected the decomposition rate. The major differences in stage duration were the elongation of the fresh, bloat and advanced decay stages in the winter trial.

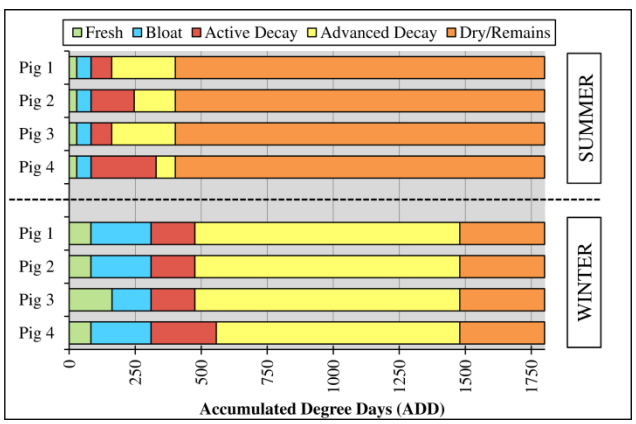

Figure 2. Onset of decomposition stages in the winter and summer trials for each pig carcass.

During the fresh stage of decomposition, the primary drivers of biochemical processes are enzymes and bacteria that are native in the body. Native enzymes and bacteria can have continued effects after death when temperature is maintained between an optimal 21-37 °C.[20] During the summer, ambient temperature would favour these processes in their optimum range allowing an acceleration of decomposition during early stages. In winter, the cooling of the carcasses to equilibrate with ambient temperature would considerably impede these reactions. Temperatures dropped to approximately 2 °C overnight for the first 72 hours post-mortem in the winter trial. Delay in the fresh and bloat stages from this trial were likely impacted by the lower temperatures experienced, causing a slow initiation of post-mortem biochemical reactions within the body. During the bloat stage, carcass distention was observed to a much lesser extent than in comparison to the summer trial, further supporting the theory of reduced intrinsic bacterial activity in the soft tissues. Delayed decomposition in the winter trial would have subsequently impacted the transference of decomposition VOCs into the soil.

The active decay stage was comparable during both trials, but with some minor variation. Active decay is largely impacted by insect consumption of the carcasses. Temperatures below 6 °C can cause insect activity to cease.[21] The temperatures in the winter trial were, on average, above this value, allowing sufficient invertebrate colonisation during the day, although species assemblage differed slightly between seasons.[22] Once colonisation occurred, larval mass movement maintained higher temperatures within the larval mass even though cooler temperatures (below 6 °C) were experienced. Lower temperatures would, however, impede the metabolic and developmental rate of insects considerably, thereby slowing soft tissue consumption.[21] Continued (although minimal) larval feeding and secondary minor colonisation events were observed throughout advanced decay in winter. Previous studies have shown that interruption or decrease in larval activity during active decay can impact the duration of advanced decay even in warm weather.[10]

Decomposition VOC detection

Figure 3 shows the difference in the overall VOC detection in the two trials. In the summer trial, a large peak in the cumulative abundance of detected VOCs in soil occurred during the first four stages of decomposition (fresh to advanced decay), and subsided as the carcasses entered the dry/remains stage. The peak maximum corresponded to active decay when putrefactive processes were highly stimulated and liquefactive products moved into the soil of the CDI. This large peak in VOC production did not occur in the winter trial, but rather a slow approach to peak VOC production was observed. This trend was reflected in both number and abundance of VOCs detected in the soil during the respective trials.

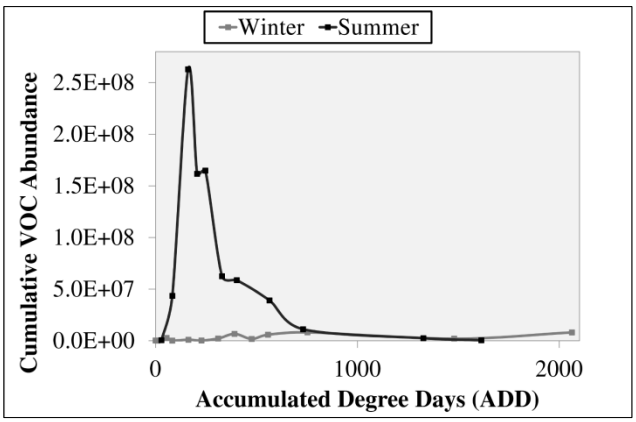

Figure 3. Cumulative normalised abundance of significant VOCs averaged for each carcass (n=4) detected in soil throughout Winter 2013 and Summer 2014 trials.

A list of the significant decomposition VOCs identified in each stage for both trials can be found in the electronic supplementary information, Table S1 and Table S2. A total of 41 decomposition VOCs were detected in the soil during the winter trial whereas 157 decomposition VOCs were detected in the soil during the summer trial (above the background VOC profile of the environment). Although far fewer VOCs were identified in winter, the majority of VOCs in the winter trial (71%) were also identified in the summer trial, suggesting they may be considered as part of the core decomposition VOC profile reproducible under variable conditions. Many additional compounds (80% of those identified in the summer trial) were not detected in the winter trial.

Based on laboratory research,[2] it was anticipated that fewer compounds would be detected in soil during the winter trial, but that they would overlap strongly with VOCs detected in the summer trial. However, the extent of the decline in number and abundance of VOCs detected during winter was unexpected and has been previously unreported (Figure 3). Upon visual inspection of the contour plots for soil collected beneath remains in the same stage of decomposition, a very clear difference can be seen in the soil samples. Figure 4 depicts the contour plot for soil VOC samples during the active decay stage for the summer and the winter trials, respectively. Although decomposition occurred in the same environment and the remains reached the same stage of decomposition, the profiles of these two samples differ considerably. A number of reasons are proposed to describe this phenomenon, which likely occur simultaneously and/or interact with each other. Reduced intrinsic microbial activity in cooler temperatures can result in reduced or altered volatile metabolic by-products.[23] Lower temperatures also impacts autolytic processes in the decomposing tissue.[20] Compounds present during summer may have been present in winter in lower quantities, reducing their concentrations in soil below instrumental detection limits. In addition, VOC partitioning in soil between gaseous and absorbed phases is temperature-dependent.[24] Volatilization from soil and the ability to collect VOCs onto sorbent tubes may have been impeded by cooler temperatures during sample collection in the winter trial. A previous study employed the use of resistive heating of a collection hood placed over the remains in order to maintain optimal collection temperature.[25] However, the VOC-Mole™ soil probes did not exhibit this feature, and ambient temperature was desired in an attempt to mimic realistic VOC mobility in soils.

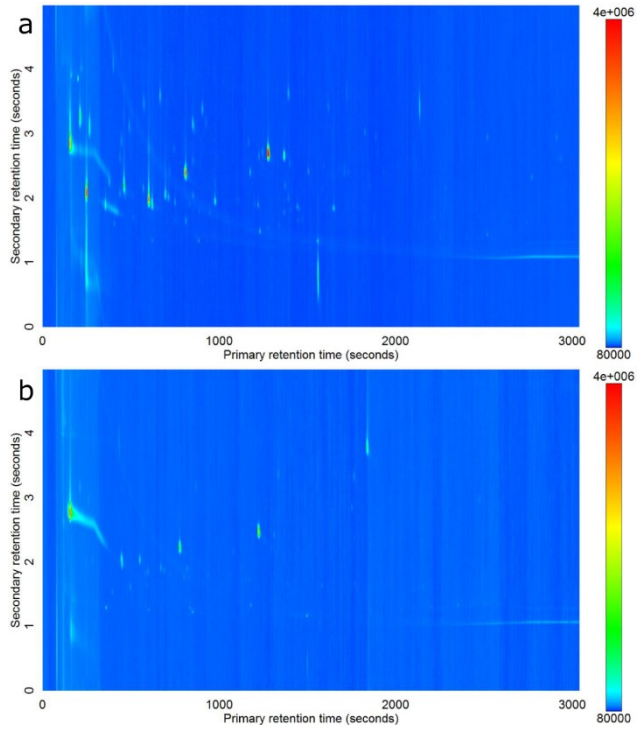

Figure 4. Comparison of contour plots obtained from soil VOC samples collected from beneath pig remains during the same stage of decomposition. a) Decomposition soil from the summer trial on day 6 during the active decay stage (161 ADD). b) Decomposition soil from the winter trial on day 37 during the active decay stage (391 ADD).

Sulphur-containing compounds tend to be the most abundant and widely-reported compounds in the decomposition VOC profile.[3,8,10,12,26–29] In winter, the largest contributor to the decomposition VOC soil profile was dimethyl disulphide (DMDS). However, dimethyl trisulfide (DMTS) was not identified during the winter trial, although it was detected at relatively high levels in the summer trial (Table S1). Fewer sulphur-containing compounds were detected in the winter trial and were not detected until active decay. Sulphur-containing compounds are produced during degradation of sulphur-containing amino acids during vertebrate decomposition.[25,26,28–31] They are also produced as metabolic by-products of many enteric and soil anaerobic bacteria.[23,32] Thus, the proliferation of enteric and soil bacteria (resulting from increased nutrient availability in the soil) can result in increased levels of sulphur-containing compounds above levels seen solely from macromolecule breakdown in soft tissues. During the winter trial, sulphur-containing compounds were decreased in number and abundance indicating that decreased bacterial activity may have taken place.

Oxygenated compounds such as alcohols, aldehydes, and ketones followed established trends in the summer trial whereby they were less prominent during early decomposition (Table S2).[1,3,8,10,30] In contrast, during the winter trial oxygenated compounds were only detected prior to advanced decay (Table S1). The presence of oxygenated compounds results from macromolecule breakdown and demonstrates that chemical breakdown of soft tissues was not only slower in winter, but that some biochemical reactions occurred during different stages of decomposition than previously reported. In the summer trial, degradation of macromolecules was likely accelerated and highly affected by extrinsic microbial

processes. Few aldehydes were detected in the soil during the winter trial, providing further evidence to support this theory. Previous work has suggested that the leaching of fatty acids into soil beneath decomposing remains coincides with the detection of volatile aldehydes.[10] Cool temperatures experienced in winter may have inhibited fatty acid oxidation by fungi and bacteria in the soil and thus decreased aldehyde production. This supports a model of intrinsically driven decomposition processes during the winter trial and extrinsically driven decomposition processes during the summer trial.

The highest variability in decomposition VOCs between the two trials was seen during active and advanced decay. These same trends have been observed during inter-year comparisons.[10] High decomposition VOC profile complexity was exhibited during these stages in the summer trial. While there were still a very large number of compounds present (Table S2), VOCs had decreased considerably in abundance in late decomposition (Figure 3). The VOC profile of late decomposition in the winter trial had comparable cumulative abundance to the summer trial, yet considerably fewer compounds were detected.

Figure 4 demonstrates the chromatographic separation using the GC×GC-TOFMS instrumentation. The benefits of improved peak capacity are apparent in Figure 4a for a soil VOC sample obtained during the active decay stage in the summer trial, which exhibited a large number of components. Components that eluted in the same vertical line would have been challenging to separate using GC-MS due to potential co-elution, but have been separated in the second dimension by GC×GC-TOFMS as shown in Figure 4. The dynamic range exhibited in this sample further confounds the ability to separate components using traditional GC-MS because compounds present in higher concentrations would have masked trace level compounds. Many of the trace level compounds were also present in similar levels to column bleed artefacts which can further mask these compounds in GC-MS and cause misinterpretation. The use of GC×GC-TOFMS allowed for these trace VOCs to be adequately separated providing improved identifications.

Figure 4b demonstrates a soil VOC sample collected during the active decay stage in the winter trial. Although reduced sample complexity was seen in the winter trial, GC×GC-TOFMS was still beneficial for its analysis as it provides a one-to-one comparison of the seasons, and also provided the sensitivity required for trace detection of compounds observed during both trials. Although fewer compounds were identified during the winter study, there are several areas of the contour plot that would have been challenging to resolve independently in the first dimension. Due to the fact that the decomposition process is dynamic and VOCs can appear, disappear and fluctuate in concentration as time progresses, the use of GC×GC-TOFMS in this study was undoubtedly advantageous for allowing standardised interpretation across the trials. The use of GC×GC-TOFMS in future decomposition odour studies that investigate taphonomic variables will improve the value of work in this area, especially where a non-target approach is taken and results cannot be anticipated before sample collection. Future work providing full validation of instrumental parameters and data handling procedures will be paramount to the development of this analytical instrumentation as a tool in decomposition odour analysis.

Correlation analysis

Factors affecting the progression and rate of decomposition are often implied to subsequently effect decomposition VOC production. Although this is generally accepted, whether certain variables impact the decomposition VOC profile, and the extent to which they do so, still remains largely speculative. Investigations of relationships using correlation can assist in establishing the strength of relationships between predictor variables (*i.e.* rainfall, temperature, soil pH, etc.) and response variables (*i.e.* level of VOCs detected). There are several benefits to examining correlations that make it suitable for this study. Based on the trends in Figure 3, linear regression approaches could not be followed since trends in the two seasons were distinct from each other in addition to being non-linear. Using Spearman's correlation coefficient (ρ), the relationship strength could be determined regardless of the linearity of the response. In addition, replicate carcasses allowed for multiple data points to be available at each predictor variable measurement, strengthening the significance of the relationships established. Correlation involves relatively simplistic statistical models that do not require intensive computing power and/or complex interpretation. Since GC×GC-TOFMS data increases the complexity of the decomposition VOC profile detected, providing a simplistic means of interpretation is desirable.

As this was one of the first studies to investigate effects of extrinsic decomposition variables on the resultant decomposition VOC profile, a preliminary attempt was made at characterising the strength of these relationships. Values of ρ were calculated for total VOCs detected in relation to each predictor variable (Table 1). Values of ρ were also calculated between predictor variables to see if strong relationships existed between measured predictors, therefore reducing the number of predictors that must be collected in future studies (values of ρ between predictors are given in text, all values available in electronic supplementary information, Table S3 and Table S4).

Decreasing relationships ($\rho < 0$) between total VOC abundance and predictor variables were dominant in summer while increasing relationships ($\rho > 0$) were dominant in winter. During summer, a sharp initial peak in compounds was observed (Figure 3) and therefore as compounds decreased from this maximum a declining relationship was measured over time (Table 1). In the winter trial, a sharp peak in decomposition VOCs during early decomposition did not occur. The number of VOCs produced over time appeared to rise and plateau, describing an increasing relationship between experimental day and total VOCs. Similar trends were observed for decomposition stage and experimental day. These predictors were highly correlated to each other ($\rho > 0.9$) because stages were ranked ordinally (1 through 5) and thus, a higher value for stage indicated a later experimental day.

Table 1. Summary of Spearman's correlation coefficients (ρ) used to assess relationship strength between the cumulative abundance of significant decomposition VOCs for both trials and predictor variables. Significant correlation coefficients are represented with *(p-value < 0.05) or **(p-value < 0.01). Highlighted values exceeded a moderate relationship and were significant (|ρ| > 0.3, p-value < 0.05).

	Summer Trial Total VOCs	Winter trial Total VOCs
Day	-.312*	.677**
Decomposition stage	-.188	.645**
pH	-.218	.622**
VWC (%)	.139	.175
Rainfall (mm)	-.188	.462**
Relative Humidity (%)	.098	-.422**
Solar Radiation (W/m^2)	-.372*	.637**
Temperature (°C)	-.316*	.688**

Soil pH had a medium-strength relationship with total VOC production in the winter trial yet did not exhibit a significant relationship in the summer trial (Table 1). Soil pH did not exhibit any strong correlations with other predictors in either trial (Table S3 and Table S4). Therefore, pH may be an influential variable on total VOCs in cool weather conditions, but its effect may be minimal in warmer conditions. Soil VWC did not have a significant relationship with total decomposition VOC production in either season (Table 1), yet relative humidity and rainfall (also moisture-related variables) exhibited a moderate relationship to detected VOCs in the winter trial. Water existing in the gas phase (associated with higher relative humidity and thus, with the presence of rainfall) has been shown to impact VOC adsorption to soil particles when VOCs are present in low concentrations.[33] This occurs because water molecules compete for adsorptive sites on soil particles, the effects of which are considerable when few VOC molecules are present. However, when VOCs are present at high concentrations this competition for adsorptive sites does not occur. This phenomenon explains the lack of relationship between detected VOCs to relative humidity and rainfall in the summer trial when decomposition VOCs were very concentrated in decomposition soil, while a moderate relationship was seen to these predictors in the winter trial.

The ρ values obtained for solar radiation and temperature were very similar (Table 1). These two variables were found to have a strong significant correlation with each other (*i.e.* $\rho > 0.9$ and $p < 0.01$). This means that the use of both predictors was redundant because they exhibited similar relationships with total VOCs. This is likely because the strength of solar radiation on a given experimental day also caused an increase in ambient temperature. For this reason, temperature was preferred to examine relationship strength, since it incorporated solar radiation in addition to other temperature-impacting variables (*i.e.* ground cover, wind speed). Although the use of both predictors were redundant for this study, in the case of extreme solar radiation (such as the Australian outback or a desert environment), the relationship between these two predictors should be further investigated, including their impact on factors such as mummification and the resultant decomposition VOC profile.

Temperature had a moderate-strong relationship to total VOCs in the winter trial which was not reflected during the summer trial. The lower end of the temperature range in winter (1.64-37.32 °C) would have largely inhibited microbial and insect activity, while the higher end of the range reaching warmer temperatures during the afternoon would have caused these organisms to be in an optimal range for metabolism and proliferation. Therefore, an increasing relationship between temperature and VOC production is logical because higher VOC production was expected with higher temperature. During the summer trial, warmer temperatures would have allowed microorganisms and insects to be in an optimal range for the majority of the study, decreasing the strength of the relationship between temperature and total VOCs detected. On occasion, high temperatures above 35 °C may have inhibited microbial and insect activity due to overheating, thereby reducing their impact on the total VOC production. Therefore, a negative moderate relationship between total VOC production and temperature was expected. Contrasting relationships between the two seasons highlights the need to analyse their correlations independently. This was the preferred approach rather than combining the two databases, which generally resulted in insignificant and low-strength relationships. The complexity of these trends and their specificity to the conditions demonstrates the need for increased characterisation of decomposition VOCs in relation to weather trends.

The strength of relationships between total VOCs and predictor variables tended to be higher overall in the winter trial than in the summer trial (Table 1). The production of VOCs in the summer trial was not strongly related to any of the measured variables. When a relationship cannot be described by measured variables, an unknown (lurking) variable is often responsible for the relationship. In this case, soil microbial activity, enteric microbial activity and insect activity could be highly influencing the resultant decomposition VOC profile in the summer trial. Future studies should collect data based on these variables to investigate the thanatomicrobiome of the decomposing tissue in addition to soil microbiome interactions. Stronger relationships in the winter trial suggest the potential that these measured predictors may have had a more influential relationship to total VOCs, and that the unknown variable mentioned in the summer trial may not have had a relationship to total VOCs in the winter trial.

Overall, the strength of most relationships examined was towards the low end of moderate. This highlights one of the challenges in decomposition VOC analysis. Many factors are moderately correlated to the decomposition VOC profile, yet no known variable has been statistically shown to exhibit a very strong relationship. As such, predictive modelling of the presence and amount of decomposition VOCs based on the measurement of all possible variables would be extremely challenging. Future modelling approaches investigating which compounds appear under certain conditions (and trends over time) may benefit from a target analysis approach. As the core decomposition VOCs in the profile becomes increasingly apparent through validation studies in the field, this will facilitate deeper investigations of the impact of varying conditions on target decomposition VOCs. In addition, based on Figure 1, it was apparent that very different trends in detected decomposition VOCs occurred in the various stages of decomposition. As such, further investigation by sub-sectioning data by time or stage would be beneficial. This type of approach would benefit from an increased number of replicates to provide sufficient replicates within data subsections to determine relationship significance.

Conclusions

The validation of the VOC profile produced by decomposing remains has only recently been highlighted by researchers in the field. As such, this study investigated trends in the decomposition VOC profile during two different seasons in an

Australian environment. Trends in the decomposition VOC profile were distinct between the two seasons, with compound classes for decomposition stages in the winter trial differing from trends documented in literature. Fewer decomposition VOCs were detected at lower abundance during the winter trial. A notable maximum in decomposition VOCs was observed during active decay in the summer trial that was not exhibited during winter. GC×GC-TOFMS was valuable for providing a direct comparison between the results of the two seasons. In future studies of taphonomic variables, this tool will undoubtedly provide more confidence in the characterisation of the decomposition VOC profile. A preliminary attempt was made at providing relationships between the decomposition VOC profile and weather variables that were recorded. This is the first reported study that provides a statistical magnitude of the relationship of these variables to the decomposition VOC profile produced. While the results of correlation analysis are preliminary, they provide a foundation for future investigation into factors affecting the decomposition VOC profile. The interaction of these factors with each other complicates the ability to determine exact mechanisms for decomposition VOC production, yet further studies involving mathematical modelling may reveal more information to deconvolute these elusive processes.

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Notes and references

[a] Centre for Forensic Science, University of Technology, Sydney, PO Box 123, Broadway, NSW, 2007, Australia

[b] School of Mathematical Sciences, University of Technology, Sydney, PO Box 123, Broadway, NSW, 2007, Australia

*Corresponding author: Katelynn.Perrault@uts.edu.au

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Electronic Supplementary Information (ESI) available: Schematic of field research facility (Figure S1); Presence of significant VOCs detected during Winter 2013 trial (Table S1) and Summer 2014 trial (Table S2) according to compound class and stage of decomposition; Spearman's correlation coefficients (ρ) used to assess relationship strength between measured variables in the Winter 2013 (Table S3) and Summer 2013 (Table S4) trials. See DOI: 10.1039/b000000x/

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