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Volatile organic compounds (VOCs) allow sensitive differentiation of biological soil quality

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ABSTRACT

Understanding the change in function of the biological community under different soil conditions is key to effective soil quality monitoring and mitigation of soil degradation. Current measures of biological soil quality suffer from drawbacks with most techniques having high expense, low throughput or a narrow focus on one component of the community. The aim of this study was to assess the use of volatilomics as a method to profile the soil microbial community and compare the technique to phospholipid fatty acid (PLFA) profiling as a measure of biological soil quality. An agricultural grassland soil (Eutric Cambisol) was subjected to a range of stresses in replicate laboratory mesocosms. Treatments included the imposition of hypoxia/anoxia by flooding with freshwater or saltwater in the presence or absence of plant residues. The volatile organic compound (VOC) and PLFA profile of each treatment was then compared to unamended mesocosms. We hypothesized that the VOC fingerprint of soil would be highly responsive to changes in microbial metabolic status/functioning and thus provide a complementary approach to PLFAs for evaluating soil biological health. We also hypothesized that the VOC profile would have greater discriminatory power than PLFAs for determining differences between soil treatments. A headspace solid phase microextraction (HSSPME) method coupled with gas chromatography quadrupole-time of flight mass spectrometry (GC/Q-TOFMS) was used to analyse the broad spectrum of VOCs produced by each soil. Across all soil treatments 514 unique VOC peaks were detected. Overall, VOCs showed greater sensitivity than the PLFA analysis in separating soil quality treatments. Eighteen individual VOCs were identified which were primarily responsible for this separation (e.g. indole, α-ionone, isophorone, 3-octanone, *p*-cresol, 2-ethyl-phenol). Anaerobic soils amended with residues showed the greatest separation from other treatments, with most of this differentiation associated with ten individual VOCs. The anaerobic soils also showed a significant reduction in the number of VOCs emitted but an increase in total VOC emissions. In conclusion, our findings provide evidence that soil VOCs rapidly respond to changes in soil quality and therefore hold great potential as a novel functionally relevant diagnostic measure of biological soil quality.

Keywords: soil function, metabolomics, method, microbial communities, soil quality indicator

1. Introduction

Soils are key to providing a wide range of ecosystem services crucial for earth system function and stability (Adhikari and Hartemink, 2016). However, most of the ice-free soils on the planet have been exploited either directly or indirectly by humans. Anthropogenic activity has subsequently contributed to the global-scale degradation of around 6 million ha of agricultural land (UNFAO and ITPS, 2015). Projections estimate that 95% of the land area on Earth could become degraded by 2050 (Montanarella et al., 2018). Therefore, it is essential that we continually assess the quality of our soils so that the provision of ecosystem services (e.g. nutrient cycling, water purification, food provisioning, climate regulation) can be sustained. Effective soil monitoring is fundamental to understanding the causes of degradation, which in turn could decrease the economic burden of soil degradation, which is estimated to be $231 bn globally and $2 bn in the UK (Graves et al., 2015; Nkonya et al., 2016).

Soil quality is often broadly defined as the capacity of a soil to function (Karlen et al., 1997). Although a range of soil quality indicators have been proposed, these mainly focus on the measurement of chemical attributes of the soil (e.g. pH, plant macronutrients, organic matter) and the physical characteristics of the soil (e.g. texture, bulk density, aggregate stability, hydrophobicity) (Bünemann et al., 2018; Schloter et al., 2018). However, soil fertility and productivity are not merely a function of soil physical and chemical characteristics. Soil biology is a crucial mediator in many processes linked to nutrient cycling, plant health and soil productivity, and is highly responsive to changes in management and environmental conditions, often being correlated to functional change (Lal, 2016; Lemanceau et al., 2015). Common soil biological indicators include measures of microbial activity (e.g. basal or substrate-induced respiration, enzyme activity) and the size and composition of the microbial community (e.g. metagenomics-metabarcoding, mesofaunal counts, $CHCl_3$-fumigation-extraction) (Bending et al., 2004). However, these methods all suffer from major drawbacks, including: (i) problems defining critical thresholds of 'good' or 'bad' soil quality, (ii) low sample throughput, (iii) high labour or equipment costs, (iv) narrow focus on one component of the community, and (v) limited spatial resolution/integration.

Volatile organic compounds (VOCs) are relatively low-molecular weight organic compounds (typically <250 amu) with high vapour pressures that give the soil its odour and can be produced via both biotic and abiotic processes (though biological production of soil VOCs far exceeds the production of VOCs by abiotic processes) (Insam and Seewald, 2010). Farmers and land managers have, for centuries, used soil odour to infer soil quality (Semple, 1928). Study of soil odour first began to develop with the documentation of geosmin, the odour of moist soil in 1891 (Berthelot and André, 1891). Since then, studies have identified a large number of VOCs produced from soil but the full extent of the functional significance of these compounds still remains largely unknown (Peñuelas et al., 2014). Biological VOCs are secondary metabolites, therefore not directly involved in organismal growth, development or reproduction. However, soil VOC emissions are highly dynamic, responding rapidly to changes in soil conditions and thus giving the soil system a unique VOC emission profile depending on soil conditions, the taxa present in soil, and their metabolic activities (Insam and Seewald, 2010). The type and amount of VOC compounds emitted from soil are dependent on a range of factors including; nutrient availability (Wheatley et al., 1996), oxygen status (McNeal and Herbert, 2009), moisture availability (Asensio et al., 2007), organic matter inputs (Seewald et al., 2010), temperature (Schade and Custer, 2004), pH (Insam and Seewald, 2010) and interactions (sorption) with the solid phase (Serrano and Gallego, 2006). This sensitivity of VOCs to soil conditions makes them a good candidate as an indicator of soil biological quality.

Phospholipid fatty acid (PLFA) analysis has become a standard method for profiling the soil microbial community; giving a quantitative description of the microbial community within a sample (Frostegård et al., 2011). PLFA analysis provides information of the size of the microbial biomass, biomarkers of bacterial and fungal community structure, and an insight into the functional composition of the community (Willers et al., 2015). Microbial community composition and structure is responsive to management or naturally induced changes driven by soil physico-chemistry (Chang et al., 2017; Cobb et al., 2017; Hardy et al., 2019). For example, soil pH and organic matter content can greatly affect the PLFA composition of the microbial community, particularly in regard to fungal:bacterial ratios (Rousk et al., 2009; Welc et al., 2012). Despite the development of alternative

methods in soil biological analysis, for example metabarcoding (Orwin et al., 2018) or community level physiological profiling (CLPP; Ramsey et al., 2006), PLFA analysis remains a rapid, sensitive and reproducible method of detecting differences in community composition between treatments (Frostegård et al., 2011).

This laboratory mesocosm study aims to critically test the relationship between microbial community structure (PLFAs) and VOC production under a range of soil stresses (anoxia/waterlogging, salinity) and organic matter regimes (e.g. urine, plant residue addition). It tests the hypothesis that there will be significant differences in both the PLFA profile of the microbial community and the number and amount of VOCs emitted between treatments. However, as VOC production largely reflects the metabolism of the active microbial community, we hypothesize that it will have greater discriminatory power to resolve differences between treatments in comparison to PLFAs which reflects both the active, inactive and dead microbial biomass pools.

2. Materials and methods

2.1. Experimental setup

A Eutric Cambisol (n = 5, depth = 0 - 10 cm, Ah horizon) was collected from a post-harvest maize field located at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). The site is characterised by a temperate-oceanic climate regime with a mean annual temperature of 11 °C and annual rainfall of 960 mm. On collection, the soil was sieved to pass 2 mm to remove stones and plant material and to ensure sample homogeneity. Field-moist soil (200 g) was then placed in 300 cm^3 polypropylene sample containers and treatments applied. Treatment consisted of the following, i) aerobic - control, ii) aerobic – amended with lysed grass residue (5 % w/w), iii) aerobic – amended with sheep urine (equivalent of 5 L m^{-2}), iv) anaerobic – with river water, v) anaerobic – with river water, amended with lysed grass residue (5 % w/w), vi) anaerobic – with sea water, and vii) anaerobic – with sea water, amended with lysed grass residue (5 % w/w) (Fig. 1). There were five independent replicates of each treatment. Grass residues were lysed by freezing for 1 h at -80 °C before being incorporated. This ensured that the plant material was metabolically inactive

prior to addition (i.e. no *de novo* biotic plant emissions). Sheep urine was collected from Welsh Mountain ewes (*Ovis aries* L.) as described in Marsden et al. (2018) and was loaded onto soil at rates equivalent to a typical sheep urination event (Selbie et al., 2015). To induce anaerobiosis, treatments were saturated with either freshwater or saline water and the containers hermetically sealed. These conditions reflected recent coastal and river flooding events which have occurred close to the sampling site (Sánchez-Rodríguez et al., 2018). Aerobic treatments remained unsealed and kept at constant weight throughout the 10-day incubation through the daily addition of deionised water. All mesocosms were subsequently incubated at 24 °C to stimulate the soil microbial community. This reflected summer soil temperatures at the field site. Duplicate mesocosms of each treatment replicate were created to allow for half of the mesocosms to be destructively sampled for soil properties and PLFAs, and half to be analysed for VOC's.

2.2. Soil sampling and analysis

At the end of the 10-day incubation, excess water was drained from the anaerobic mesocosms and soil was homogenised thoroughly by hand with a spatula and analysis undertaken immediately. Soil moisture content was determined gravimetrically by oven drying (105 °C, 24 h) and soil organic matter was determined by loss-on-ignition (450 °C, 16 h) (Ball, 1964). Soil pH and electrical conductivity (EC) were measured using standard electrodes submerged in 1:5 (w/v) soil-to-deionised water suspensions. The oxidation–reduction potential (ORP) was measured directly in the soil using a SenTix® ORP-T 900 combination electrode (Xylem Analytics, Weilheim, Germany) connected to a mV reader. Total C and N was determined on oven-dried, ground soil using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI).

At the end of the incubation, 1:5 (w/v) soil-to-0.5 M K_2SO_4, 1:5 (w/v) soil-to-0.5 M AcOH (acetic acid) and 1:5 (w/v) soil-to-deionised (DI) H_2O extractions were performed. TOC (total organic carbon) and TN (total nitrogen) were determined on K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO_3-N) and ammonium (NH_4-N) concentrations within the K_2SO_4 extracts were determined by the colorimetric VCl_3 method of Miranda et al. (2001) and the salicylic acid method of Mulvaney (1996), respectively. Available P was measured on the DI

157 H_2O extracts using the molybdate blue colorimetric method of Murphy and Riley (1962). Cations (Na,
158 K and Ca) were determined in the AcOH extracts using a Sherwood Model 410 Flame Photometer
159 (Sherwood Scientific Ltd, Cambridge, UK). Soil characteristics are summarised in Table 1.

160 Characterisation of the soil microbial community was performed by PLFA analysis.
161 Homogenised soil was stored at -20 °C prior to, and post lyophilisation using a Modulyo Freeze Dryer
162 with RV pump (Edwards, Crawley, UK). 15 g samples were shipped, on dry ice, to Microbial ID Inc.
163 (Newark, DE, USA), extracted, fractionated, and transesterified using the high throughput method of
164 Buyer and Sasser (2012). Subsequently, samples were analysed using an Agilent (Agilent
165 Technologies, Wilmington, DE, USA) 6890 gas chromatograph (GC) equipped with autosampler,
166 split–splitless inlet, and flame ionization detector. The system was controlled by MIS Sherlock®
167 (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis
168 parameters and standards can be found in Buyer and Sasser (2012).

169 *2.3. VOC extraction, collection, analysis and data processing*

170 Duplicate soil samples, as aforementioned in section 2.1, were stored at 4 °C before analysis. VOCs
171 were collected using headspace solid-phase microextraction (HSSPME) due to the method's
172 suitability for evaluating complex sample matrices. A multi-component solid-phase microextraction
173 (SPME) fibre (50/30 µm DVB/CAR/PDMS; Supelco, Bellefonte, USA), was selected due to both its
174 sensitivity to a wide range of non-polar compounds and the thickness of the fibre (Cserháti 2010),
175 which allowed transfer of the fibre from the extraction vial into the GC without the likelihood of
176 significant loss of adsorbed volatile compounds. This was attached to a manual SPME holder
177 (Supelco, Bellefonte, USA) for sampling.

178 Briefly, the SPME fibre was conditioned in the GC injection port at 270 °C prior to each soil
179 being sampled, until no interfering peaks were obtained in blank injections. Fresh soil (3.5 g) was
180 deposited into a glass vial (10 mL) (Supelco, Bellefonte, USA) alongside a magnetic stirrer bar for
181 agitation, and subsequently capped with a SPME compatible PTFE/silicone septum (Supelco,
182 Bellefonte, USA). To equilibrate the samples, they were agitated in a thermostat bath (80 °C) for 60

min, to decrease the partition coefficient between the sample and the headspace, and increase the vapour pressure (Zhang and Pawliszyn, 1993). A preconditioned SPME fibre was then manually inserted through the septum into the vial and exposed to the headspace for 20 min. The SPME fibre was then withdrawn into the holding sheath, removed from the vial and directly (< 30 s) desorbed into a GC-MS injection port at 250 °C in split mode (1:10 split ratio) for 2 min. A gas chromatograph (7890B; Agilent Technologies, Palo Alto, USA) interfaced to a quadrupole time-of-flight mass spectrometer (7200B; Agilent Technologies, Palo Alto, USA) (GC/Q-TOFMS) was used for compound identification. Chromatographic separation was obtained on a non-polar column (HP-1 50 m length x 0.32 mm id x 0.17 µm D_f; Agilent Technologies, Palo Alto, USA) using a He carrier gas with a flow of 2 mL min^{-1}. The oven temperature was programmed from 60 °C to 250 °C at 4 °C min^{-1}. The transfer line and ion source temperatures were both set to 300 °C. Using the electron ionisation (EI) mode at 70 eV, mass spectra were acquired across an *m/z* range of 25-400. Compound identification was attained using the NIST Mass Spectrometry library (Stein et al., 2014).

2.4. Treatment analysis

The lysed grass treatments' moisture content was determined gravimetrically by oven drying (80 °C, 24 h). Subsequently, grass C:N ratios were determined on the oven-dried and ground samples using a TruSpec® Analyzer. The sheep urine treatment was analysed for dissolved organic C (DOC) and total dissolved N (TDN) using a Multi N/C 2100S Analyzer. River and sea water samples were analysed for pH and EC using standard electrodes in addition to colorimetric determination of NO_3-N, NH_4-N, and P as per the methods described above, TOC and TN were also measured using the Multi N/C 2100S Analyzer. Treatment characteristics are summarised in Table S1.

2.5. Data and statistical analysis

The raw VOC data files were processed using MassHunter Workstation Profinder version B.08.00 (Agilent Technologies, Palo Alto, CA, USA). Feature extraction was achieved using the Batch Feature Extraction algorithm with the subsequent processing parameters: peak filters = 500, ion count threshold = 5, retention time tolerance = 0.3 min, absolute height = 10000 counts, *m/z* range = 25-250,

retention time range = 5-35 min. This processing step produced a data output for each independent entity in the form of [intensity × retention time × mass]. These data were then input into MassHunter Mass Profiler Professional version B.14.5 (Agilent Technologies, Palo Alto, CA, USA) and $\log_{10}$ transformed and normalised using an external scalar. To moderate the number of features subjected to statistical analysis, data filtering was executed. Briefly, a frequency filter (entities present in >90% of samples in at least one sample group), fold change filter (14.0) and sample variability filter (coefficient variable < 25%) were applied. This approach identified 18 discriminatory compounds, summarised in Figure 2. Total relative VOC production was also quantified by summing the areas of all peaks considered from each sample's chromatogram, examples of which are shown in Figure S2.

All of the following statistical analysis was performed in R v3.5.3 (R Core Team, 2019). In the analysis of discriminatory VOCs, relative peak heights were used to effectively compare samples based on the chemical diversity of compounds. A non-metric dimensional scaling (NMDS) approach was used to condense the multivariate VOC data in a comprehensible number of dimensions and visualize the relative degree of similarity among samples using the whole VOC dataset, which was performed using the 'vegan' package. NMDS was also used to analyse PLFA data. All PLFAs detected were used in the analysis, to represent the whole microbial community. Heatmap analysis and an ANOVA with Tukey post-hoc test was performed on $\log_{10}$ transformed and pareto-scaled discriminatory VOC compound data in 'metaboanalyst 4.0' (Chong et al., 2018). This package was also used to perform hierarchical cluster analysis on $\log_{10}$ transformed and pareto-scaled PLFA and VOC data. An ANOVA was also used to test $\log_{10}$ transformed total relative VOC production as well as total number of peaks identified between treatments. Significant differences were further explored using a Tukey HSD post-hoc test. The relationships between total VOCs and number of VOCs to key soil parameters were tested using Spearman correlation analysis, using the 'corrplot' R package (Wei and Simko, 2017). For all analyses the significance threshold was set at $p \leq 0.05$.

3. Results

3.1. Treatment driven changes in VOCs and PLFAs

NMDS analysis was used to show the clustering for all VOC and PLFA compounds, respectively, produced under the soil treatments. Both, VOC (Fig. 3A) and PLFA (Fig. 3B) analysis show separation between anaerobic + residues treatments compared to other treatments, implying a different microbial community and production of VOCs under these conditions. Using VOC analysis, anaerobic treatments without residues also grouped closely. Hierarchical clustering analysis (Fig. 4A and 4B) further illustrated the relationships between treatment levels, with results largely supporting NMDS findings. Overall, VOC analysis was more able to differentiate between anaerobic + residues treatments and aerobic treatments, with anaerobic treatments without residues and urine treatments being more closely related than they appeared using NMDS analysis. PLFA analysis was less able to separate treatments from one another.

3.2. Relative VOC production between treatments

Total relative VOC production was significantly higher from anaerobic + residues treatments ($p < 0.001$) compared to other treatments. Aerobic residue treatments also produced significantly more VOCs than anaerobic without residues and urine treatments ($p < 0.001$). Across all soil treatments 514 unique VOC peaks were detected. The total number of VOC compounds detected was significantly lower for both anaerobic + residues treatments ($p < 0.05$). Control and urine treatments had a significantly higher number of VOC compounds detected ($p < 0.05$).

3.3. Soil factors affecting VOC production

Several key soil properties were found to be significant predictors of both, the relative total concentration of VOCs and the number of VOC compounds emitted from soil samples. Particularly, organic matter content, dissolved organic carbon and PLFA microbial biomass were significantly correlated with total VOC concentration and number of VOCs emitted (summarised in Table 2). ORP and soil moisture were also significantly correlated to the number of VOCs emitted from soil.

3.4. Identities of discriminatory VOCs produced

Across the 7 treatments, software was able to identify 18 VOC compounds that were able to differentiate between the control and treated samples; these compounds are subsequently referred to

as discriminatory compounds (Fig. 2). Of the 18 compounds, 16 were found to have significant differences between treatments when tested statistically (*p*-cresol and hexanoic acid were not significantly different between treatments). Additionally, 10 compounds were found in significantly higher concentrations in anaerobic + residues treatments compared to the other treatments ($p < 0.001$), as highlighted in Figure 2.

4. Discussion

4.1. Identification of VOC profile trends

NMDS and hierarchical clustering of VOC data (Fig. 3A and 4A) illustrate the clustering of anaerobic + residues treatments, suggesting that nutrient-rich anaerobic conditions had the greatest impact upon the VOC profile of the soils relative to the control soils. Anaerobic + residues treatments also had the highest levels of total VOCs, but the lowest number of individual compounds detected. This suggests that addition of plant residues under anaerobic conditions caused a large increase in the concentration, but not number, of VOCs detected. It is generally understood that VOCs are found in greater concentrations under anaerobic conditions, due to the production of metabolic end-products by anaerobic fermentation and extracellular degradation of complex organic metabolites (Insam and Seewald, 2010; Seewald et al., 2010; Stotzky et al., 1976). However, the results of this study show this to be conditional on the amount of available nutrients and C under anaerobic conditions, as soils under anaerobic conditions but with no plant residues produced significantly less total VOCs than anaerobic soils with plant residues. This is likely due to a lack of easily assimilable C in non-residue amended anaerobic samples, as well as lower levels of microbial activity and thus VOC production. However, although anaerobic + residues treatments resulted in a greater total VOC concentration compared to other treatments, the number of compounds contributing to the signal produced was significantly less. This suggests a limited range of metabolic reactions are responsible for producing the majority of the soil VOC profile, as microbial metabolism is the dominant source of VOC emissions from soil (Leff and Fierer, 2008).

Control and urine amended soils produced a significantly greater number of VOC compounds compared to other treatments. Under the control treatment, it is likely that the soil microbial community will be highly adapted to the prevailing conditions (i.e. low stress) and thus more likely to have a streamlined metabolism and high C use efficiency. Most of the C used for energy production will produce only CO_2 as an end-product rather than respiratory-derived VOCs (e.g. ethanol, volatile organic acids) or those used in secondary metabolism for stress alleviation (Insam and Seewald, 2010). Under these low stress conditions, the large number of different VOC compounds are likely to be characteristic of a diverse soil microbial community (McNeal and Herbert, 2009). Conversely, under the urine treatment, the input of nutrients into the system, particularly K and soluble N (e.g. urea NH_4^+), is likely to induce a rapid change in soil microbial metabolism (Waldrop and Firestone, 2004; Williams et al., 2000). However, it is also likely that the urine itself will contain some unique VOCs (Deev et al., 2020; Mozdiak et al., 2019). The experimental set up used here, however, was unable to distinguish between the direct and indirect effects of urine on the soil VOC profile.

4.2. Sources of discriminatory VOC compounds

Following the positive identification of 18 discriminatory VOCs, the abundance of which could be used to determine several soil characteristics key in determining a soils quality (e.g. SOM, levels of oxygen content), prospective sources and functions of the compounds were examined using the existing literature. Notably, of these compounds, 10 were found in significantly higher relative concentrations in the anaerobic + residues treatment, several of which can be identified as breakdown products of the residues.

For example, the biodegradation of carotenoids is the most likely source of β-ionone and α-ionone. Carotenoids form part of a group of terpenoid organic pigments that play a major role in photosynthesis in addition to the photoprotection of photosystems in plants and photosynthetic bacteria (Lobo et al., 2012). These compounds were only produced under anaerobic + residues treatments suggesting that either under aerobic conditions these molecules were further catabolised or were only produced under nutrient-rich anaerobic conditions.

Benzeneacetic acid, an auxin, was solely identified in anaerobic soils with added grass residues. This suggests that under aerobic conditions all the benzeneacetic acid was mineralised. Alternatively, benzeneacetic acid may only be produced by anaerobic bacteria. For example, it has been reported that *Azoarcus evansii*, an endophytic facultative anaerobic denitrifying bacterium found in several grass species, is a significant producer of benzeneacetic acid (Schulz and Dickschat, 2007; Sun et al., 2019).

Despite many possible sources of the discriminatory compounds, linking a VOC to specific processes, functions or microorganisms is challenging due to the variety of degradation pathways which a VOC can take within the soil, dependant on environmental conditions (i.e. oxygen and nutrient status). Furthermore, the necessity to increase the temperature of the sample during the extraction procedure in order to increase the partition coefficient, generates a degree of ambiguity as to the true source of the compounds in the headspace, as under such conditions there is a possibility of the breakdown of thermolabile compounds within the sample (Kaspar et al., 2008). Accordingly, it is uncertain whether the compounds extracted are a samples' intrinsic VOCs; released by the microorganisms within the soil or as a direct result of an amendment (e.g. urine), or VOCs produced during the extraction procedure due to thermal degradation of the sample. For example, lignin is widely described as stable below temperatures of 100 °C, however, the thermal stability of many soil components is poorly reported within the literature (Brebu and Vasile, 2010).

Additionally, there is potential discrepancy between the production of a volatile compound and its emission from matrix. The emission of VOCs from the soil matrix, is highly dependent on the soil's structure and moisture as well as the rate at which the VOCs are being emitted. These factors ultimately control the rate of VOC production, dispersal and consumption (Aochi and Farmer, 2005). Similarly to greenhouse gases, it is likely that soils act as sources or sinks for VOCs depending on environmental conditions (Insam and Seewald, 2010; Oertel et al., 2016). For example, VOCs produced further down the soil profile could be consumed or degraded before reaching the soil surface. Particularly in aerobic systems, when in a steady state equilibrium these production-consumption systems may cause emission of very few VOCs, however, on disturbance, emissions

may increase. Heating during analysis, to improve the partition co-efficient (the ratio of analyte in matrix-to-analyte in headspace) and drive VOCs into the headspace, is likely to increase the emissions from the soil sample (Turner et al., 2019).

4.3. Relationship between soil properties and VOC emission

Overall, levels of soil moisture seemed to be the best predictor of the number of VOCs produced from samples, with higher levels of soil moisture reducing the number of VOCs emitted. Similarly, ORP was positively correlated to the number of VOCs produced. Under anaerobic, reducing conditions it is likely that alcoholic sugar fermentation predominates (Pezeshki and DeLaune, 2012). Previous studies, e.g. Stotzky et al. (1976) and Seewald et al. (2010), have shown that anaerobic conditions increase the diversity and amount of VOCs emitted. However, this study showed that this was only the case if conditions were not nutrient limited. Both anaerobic treatments + residues additions resulted in an increase in total VOCs emitted but in a reduction in the number of VOC compounds emitted. As shown previously, substrate availability and quality are key in the production and emission of VOCs (Wheatley et al., 1996). However, there was no correlation between total dissolved nitrogen and total VOCs or number of VOCs produced, and only a weak correlation between available inorganic N (NO_3 and NH_4) and total VOCs emitted.

4.4. Critical analysis of VOCs and PLFAs as soil quality indicators

Whilst both VOCs and PLFAs can be related directly to the soil biological community, each offer very different types of insight. PLFAs focus on the membrane lipid composition, from which links can be made to microbial community composition (Mann et al., 2019). However, use of isotopic substrates can enable PLFA to be used as indicators of soil function, i.e. Bull et al. (2000). VOCs focus on secondary metabolism of the soil biological community, which relates to community function. Advantages and disadvantages of each method are summarised in Table 3.

Sensitivity is a key attribute in biological quality monitoring, as methods must be able to detect subtle changes in soil biochemistry to give an accurate representation of soil quality. Both PLFA and VOC analysis are, in theory, sensitive, as both sets of compounds degrade rapidly under

environmental conditions (Li et al., 2019; Zhang et al., 2019). However, this experiment has demonstrated that, in terms of discriminatory power, VOCs can more robustly separate the impact of short-term soil treatments, which correspond to different soil qualities (Fig. 3 and 4).

Both PLFAs and VOCs relate to the biological function of soil. However, the turnover of VOCs is more rapid and more functionally relevant than PLFAs. The impact of many environmental factors e.g. pH, heat and moisture content are similar between both types of analysis. One advantage of VOC analysis over PLFA analysis is the lack of pre-treatment and multi-stage chemical extraction required, reducing the amount of inherent bias within the method. However, identification of individual VOCs is difficult; while databases exist, they are by no means extensive and identification may not be absolute without the use of confirmatory standards.

4.5. Future research direction

This study highlights several potential future research areas within the soil and environmental sciences. Specifically: Is it possible to increase the rapidity of VOC analysis, by reducing extraction and/ or analysis time, while retaining the resolution and sensitivity to enable compound identification? How do *ex-situ* sampling techniques demonstrated in this study compare to *in-situ*, non-destructive, methods, for example, sorbent tube sampling? To what extent are abiotic VOCs contributing to the overall soil VOC profile compared to biotic VOCs? Under different soil types and cropping regimes, does the core microbiome have similar metabolism and therefore produce similar VOCs, i.e. is analysis of VOCs applicable over large spatial scales? And what is the temporal frequency of analysis required to accurately assess a soils quality? Addressing these questions will further advance sampling and analysis of VOCs in soil and elucidate the role of VOCs as indicators of changes in soil quality. Another key aspect is defining the boundary values for VOC concentrations and profiles for ‘healthy’ soils. Arguably, the control and aerobic residues treatment in this study have started to provide some data to represent healthy soils.

5. Conclusions

This study applied a HSSPME extraction and analysis method for the determination of VOCs in soils. The method was applied to seven soil treatments representing a range of soil qualities and a number of discriminatory compounds were identified. When compared to PLFA analysis, VOC analysis was better able to differentiate between soil treatments. 18 discriminatory compounds were identified, 10 of which were associated with anaerobic treatments with residues. Contrary to previous findings, VOCs under anaerobic treatments with residue additions exhibited a relative increase in concentration of total VOCs emitted but a reduction in the abundance of specific compounds emitted. These results demonstrate the potential of secondary metabolites as an indicator of soil quality and highlights the need for further research into soil VOC analysis to understand nutrient cycling and metabolism as well as the effect of *in-situ* vs. *ex-situ* sampling.

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584

585 **Table and figure captions**

586 **Fig. 1.** Flow diagram illustrating the seven treatments applied in the study.

587 **Fig. 2.** Heatmap of eighteen VOC compounds identified as driving changes between soil treatments.
588 Compounds highlighted in purple are found in significantly higher concentrations in anaerobic +
589 residues treatments. Compounds highlighted with † were not significantly different between
590 treatments ($p > 0.05$). Darker red = large positive relative difference between treatments, darker blue
591 = large negative relative difference between treatments. Samples are ordered using group averages
592 and clustered using a Ward algorithm. Compound structures are shown in Fig. S1.

593 **Fig. 3.** A) NMDS plot of VOC profiles of each soil treatment. B) NMDS plot of PLFA profile for
594 each soil treatment. Ellipses represent 95% confidence intervals for each treatment. The legend is the
595 same for both panels.

596 **Fig. 4.** A) Dendrogram, using Euclidean distance measure and a ward clustering algorithm, of VOC
597 profiles of each soil treatment ($n = 5$). B) Dendrogram, using Euclidean distance measure and a ward
598 clustering algorithm, of PLFA profiles of each soil treatment replicate ($n = 5$).

599 **Table 1.** Characteristics of treated soils used in this study. Values are expressed on a mean dry soil
600 weight basis ± SEM ($n = 5$). Letters denote significant differences between treatments using a
601 Kruskall-Wallis test with Dunn Post-hoc test and Bonferroni correction ($p < 0.05$).

602 **Table 2.** Pearson's correlation coefficients for key soil properties in relation to both total amount of
603 VOCs emitted and the number of VOCs emitted from samples. *NS* signifies not significant (i.e. $p >$
604 0.05).

605 **Table 3.** Summary of the advantages and disadvantages of VOC and PLFA analysis in the analysis of
606 soil quality.

607

608

Figure 1

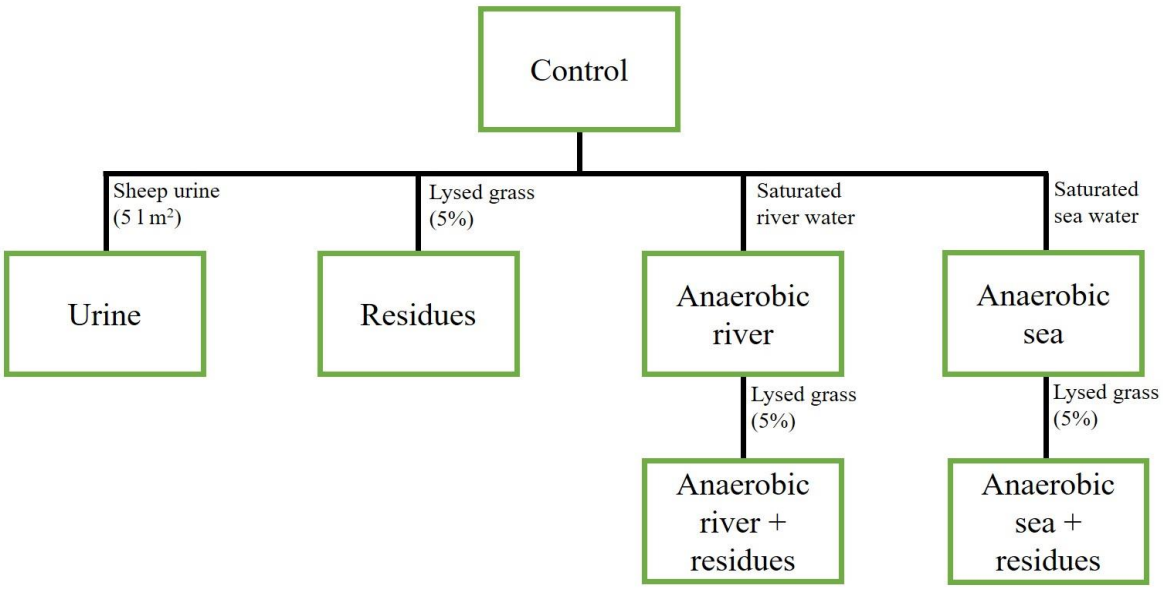
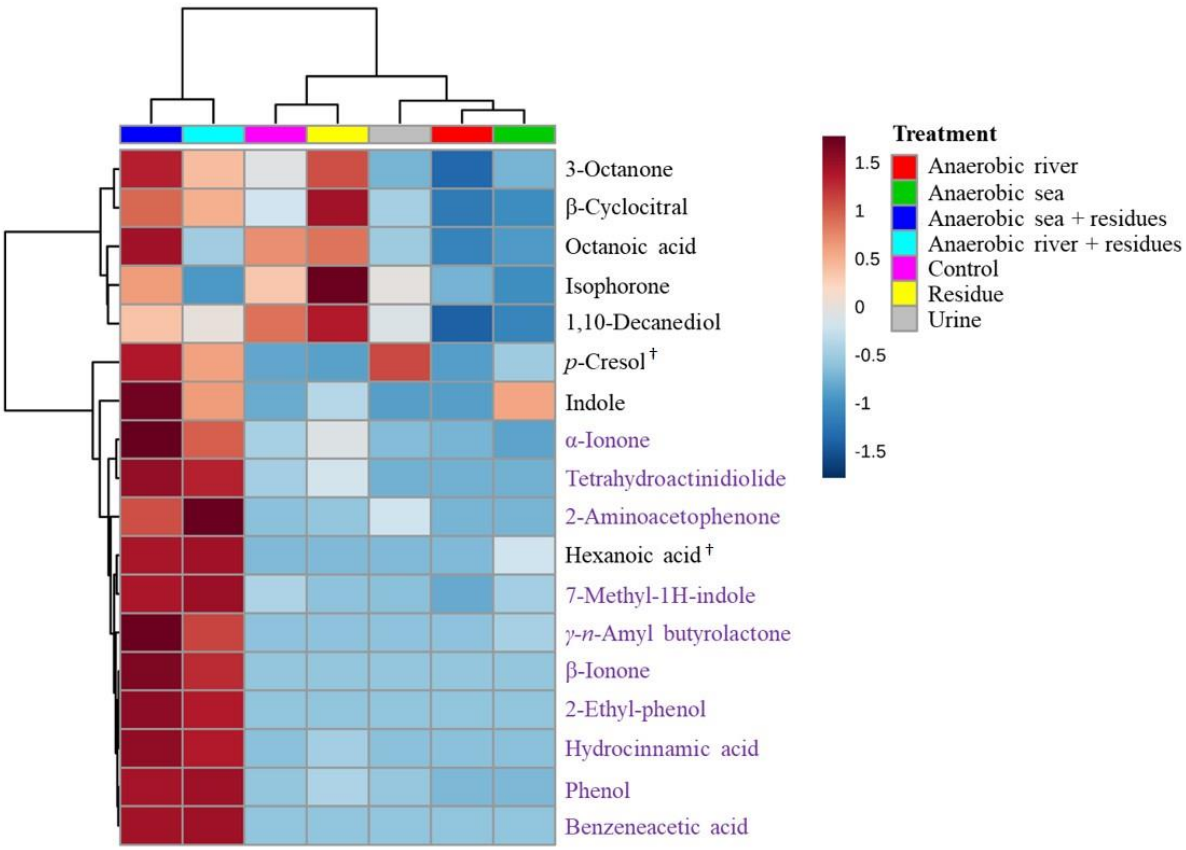
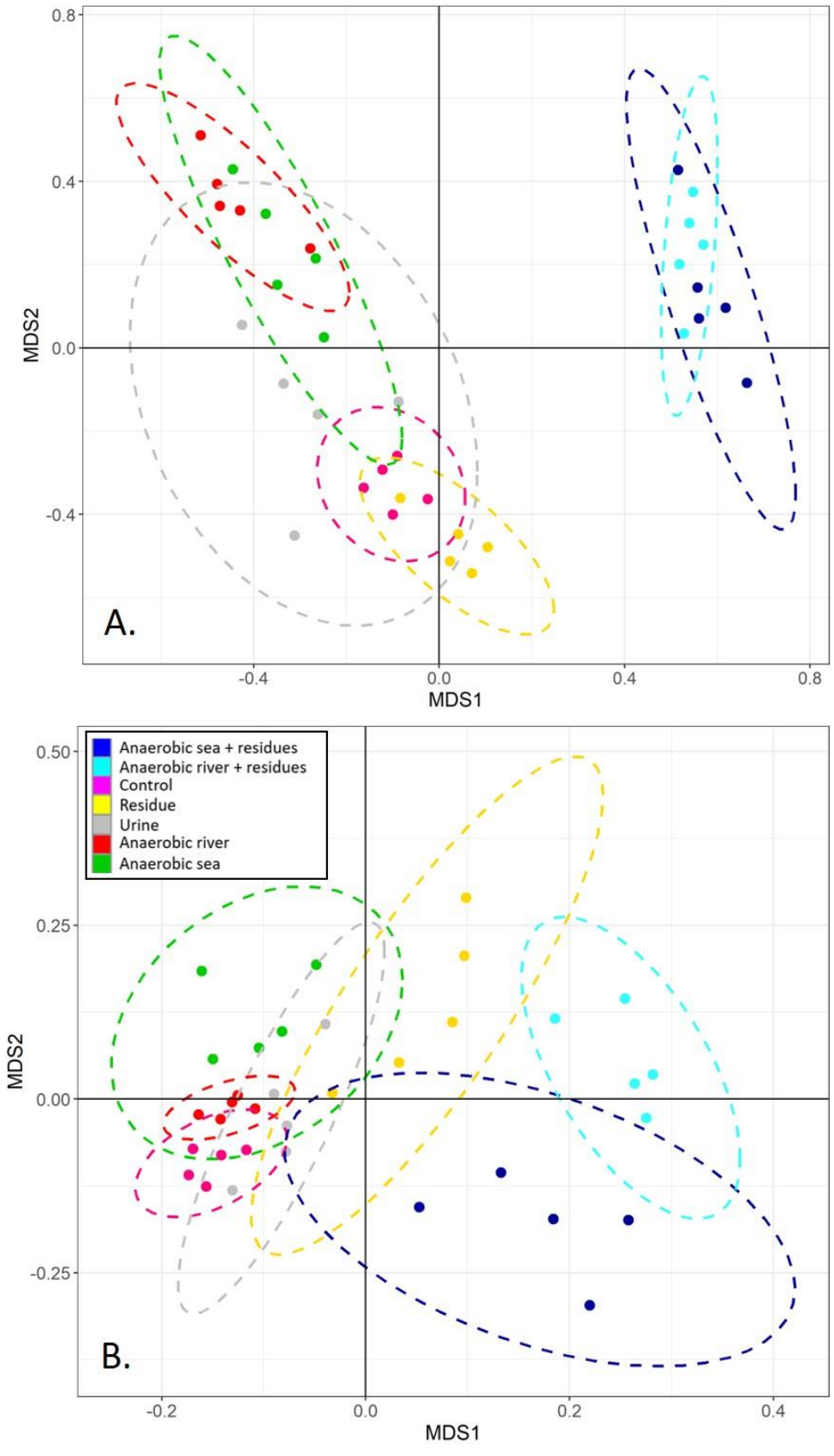


Figure 2



616 **Figure 3**



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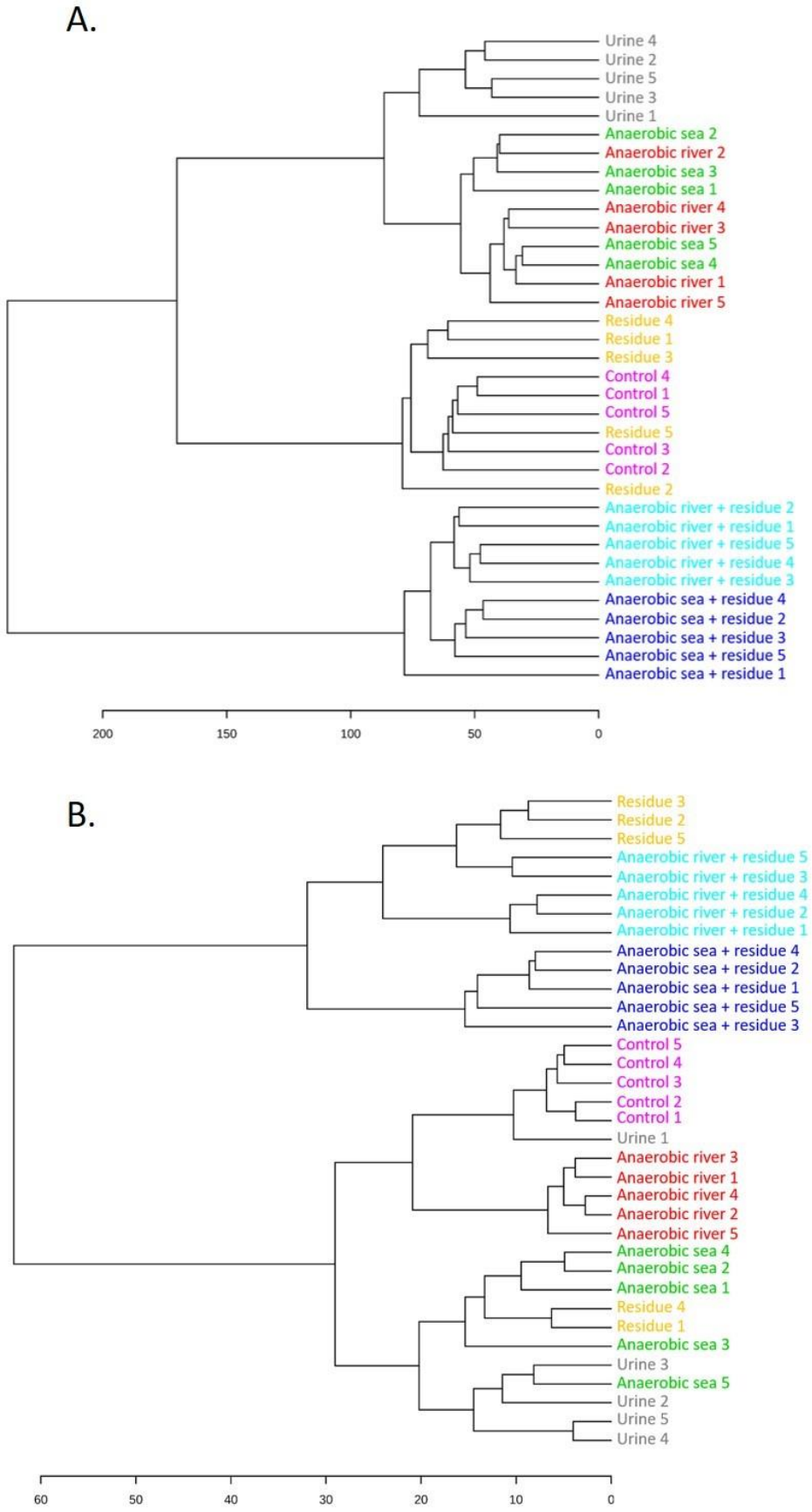
619 **Figure 4**



620

621 **Table 1.** Characteristics of treated soils used in this study. Values are expressed on a mean dry soil weight basis ± SEM (n = 5). Letters denote significant differences
622 between treatments using a Kruskall-Wallis with Dunn Post-hoc test and Bonferroni correction ($p < 0.05$).

	Aerobic			**Anaerobic**			
	Control	**Residues**	**Urine**	**River water**	**River water + residues**	**Sea water**	**Sea water + residues**
Texture				Sandy clay loam			
Soil moisture (%)	$29.3^{A} \pm 1.2$	36.2 ± 1.3	$29.8^{B,C} \pm 0.5$	74.1 ± 1.8	$83.1^{A,B} \pm 1.5$	68.2 ± 1.1	$86.5^{C} \pm 1.3$
pH	$6.14^{A} \pm 0.15$	$5.71^{B,C} \pm 0.06$	$7.53^{A,B,D} \pm 0.11$	6.51 ± 0.08	$6.90^{C} \pm 0.05$	$6.23^{D} \pm 0.09$	6.88 ± 0.06
EC (µS cm^{-1})	27.1 ± 11.7	54.0 ± 6.3	72.3 ± 2.0	$3.3^{A,B} \pm 0.1$	21.0 ± 1.8	$563.6^{A} \pm 7.1$	$661.6^{B} \pm 18.9$
ORP (mV)	$413.5^{A,C} \pm 4.9$	325.6 ± 9.8	216.5 ± 2.8	$24.5^{B,D} \pm 4.7$	$-73.7^{A,B} \pm 3.6$	141.5 ± 11.3	$-144.9^{C,D} \pm 16.2$
Total C (%)	2.63 ± 0.05	2.97 ± 0.18	2.44 ± 0.05	2.44 ± 0.11	2.90 ± 0.12	2.59 ± 0.08	2.6 ± 0.10
Total N (%)	0.28 ± 0.01	$0.36^{A,B} \pm 0.02$	$0.32^{C} \pm 0.01$	$0.25^{A} \pm 0.01$	0.3 ± 0.01	$0.25^{B,C} \pm 0.01$	0.3 ± 0.01
C:N ratio	9.40 ± 0.31	$8.22^{C} \pm 0.15$	$7.74^{A,B,D} \pm 0.17$	$9.21^{A} \pm 0.57$	$9.53^{B} \pm 0.16$	$9.97^{C,D,E} \pm 0.22$	$9.42^{E} \pm 0.44$
Dissolved organic C (mg C kg^{-1})	$51.0^{A,C} \pm 2.1$	87.3 ± 14.3	171.6 ± 10.5	67.1 ± 4.8	$240.3^{A,B} \pm 8.6$	$58.4^{B,D} \pm 4.7$	$303.3^{C,D} \pm 45.1$
Extractable NO_3^- (mg N kg^{-1})	36.3 ± 1.7	$162.8^{A,B,D} \pm 17.0$	$70.8^{C} \pm 6.4$	$0.65^{A} \pm 0.16$	1.02 ± 0.23	$0.37^{B,C} \pm 0.24$	$0.77^{D} \pm 0.18$
Extractable NH_4^+ (mg N kg^{-1})	$3.33^{A,C} \pm 1.24$	51.32 ± 6.57	$487.04^{A,B} \pm 31.59$	$2.70^{B} \pm 0.50$	81.97 ± 4.66	3.28 ± 0.56	$98.72^{C} \pm 5.78$
Extractable P (mg P kg^{-1})	2.61 ± 0.33	2.47 ± 0.24	$3.12^{C} \pm 0.11$	$4.21^{A,B,D} \pm 0.27$	$1.90^{A} \pm 0.08$	$2.05^{B} \pm 0.16$	$1.74^{C.D} \pm 0.04$
Exchangeable Na (mg Na kg^{-1})	$13.8^{A,C} \pm 1.5$	28.3 ± 2.2	80.2 ± 5.9	$17.9^{B,D} \pm 1.1$	22.3 ± 2.5	$5477^{A.B} \pm 344$	$6490^{C,D} \pm 99$
Exchangeable K (mg K kg^{-1})	$89.5^{A,C} \pm 11.1$	406.8 ± 33.9	$646.9^{A,B} \pm 35.9$	$121.2^{B,D} \pm 8.6$	321.7 ± 74.3	376.2 ± 25.1	$671.8^{C,D} \pm 20.0$
Exchangeable Ca (mg Ca kg^{-1})	$1099^{A} \pm 77$	1083 ± 90	$1169^{B} \pm 133$	1455 ± 117	$1689^{A,B} \pm 116$	1262 ± 61	1587 ± 1028
Bacterial/Fungal PLFA ratio	0.06 ± 0.00	0.10 ± 0.01	$0.07^{A} \pm 0.01$	0.07 ± 0.00	0.07 ± 0.01	$0.13^{B} \pm 0.06$	$0.04^{A,B} \pm 0.01$
Microbial biomass (µmol PLFA kg^{-1})	$110.2^{A,B,D} \pm 2.6$	$177.6^{A} \pm 11.0$	145.0 ± 6.1	$128.7^{C} \pm 3.6$	$215.6^{B,C} \pm 14.1$	158.7 ± 20.0	$209.4^{D} \pm 27.3$

EC - electrical conductivity, ORP - redox potential.

Table 2. Pearson's correlation coefficients for key soil properties in relation to both total amount of VOCs emitted, and the number of VOCs emitted from samples. *NS* signifies not significant (i.e. $p > 0.05$).

	Total VOCs	No. VOCs
pH	*NS*	*NS*
ORP (mV)	*NS*	0.576
Soil moisture (%)	*NS*	-0.64
Organic matter (%)	0.361	-0.425
Total dissolved N (mg kg^{-1})	*NS*	*NS*
Total dissolved organic C (mg kg^{-1})	0.385	-0.391
Microbial biomass (μmol PLFA kg^{-1})	0.388	-0.489

634 **Table 3.** Summary of the advantages and disadvantages of VOC and PLFA analysis in the analysis of
635 soil quality.

	VOC analysis	PLFA analysis
Advantages	Functionally relevant Rapid extraction and analysis. Microbial culture not required. Rapid degradation - offers snapshot of current state of biological activity. Possibility of identifying characteristic biomarkers for specific conditions. Non-destructive.	Sensitive and reproducible. Measure of biomass and community structure. Microbial culture not required. Rapid degradation - offers snapshot of current state of biology. Lack of group or species-specific FA's
Disadvantages	Difficult to separate plant and microbial derived VOCs in soil. Lack of fundamental understanding of VOCs in soil. SPME requires destructive sampling. Most relationships have been investigated using laboratory/ pure culture studies – may not reflect diverse soil community response. Medium sample throughput with possibility of automation. High analytical capital costs.	Time consuming and complex extraction required. Little functional relevance. Extraction of entire microbial community – not only the active fraction. Most relationships have been investigated using laboratory/ pure culture studies – may not reflect diverse soil community response. Low sample throughput. High capital and labour costs. Destructive.

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