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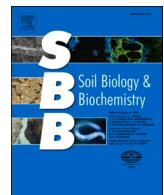
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## Nutrient (C, N and P) enrichment induces significant changes in the soil metabolite profile and microbial carbon partitioning

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### ABSTRACT

The cycling of soil organic matter (SOM) and carbon (C) within the soil is governed by the presence of key macronutrients, particularly nitrogen (N) and phosphorus (P). The relative ratio of these nutrients has a direct effect on the potential rates of microbial growth and nutrient processing in soil and thus is fundamental to ecosystem functioning. However, the effect of changing soil nutrient stoichiometry on the small organic molecule (i.e., metabolite) composition and cycling by the microbial community remains poorly understood. Here, we aimed to disentangle the effect of stoichiometrically balanced nutrient addition on the soil metabolomic profile and apparent microbial carbon use efficiency (CUE) by adding a labile C source (glucose) in combination with N and/or P. After incorporation of the added glucose into the microbial biomass (48 h), metabolite profiling was undertaken by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). 494 metabolites were identified across all treatments mainly consisting of lipids ( $n = 199$ ), amino acids ( $n = 118$ ) and carbohydrates ( $n = 43$ ),  $>97\%$  of which showed significant changes in concentration between at least one treatment. Overall, glucose-C addition generally increased the synthesis of other carbohydrates in soil, while addition of C and N together increased peptide synthesis, indicative of protein formation and turnover. The combination of C and P significantly increased the number of fatty acids synthesised. There was no significant change in the PLFA-derived microbial community structure or microbial biomass following C, N and P addition. Further, N addition led to an increase in glucose-C partitioning into anabolic processes (i.e., increased CUE), suggesting the microbial community was N, but not P limited. Based on the metabolomic profiles observed here, we conclude that inorganic nutrient enrichment causes substantial shifts in both primary and secondary metabolism within the microbial community, leading to changes in resource flow and thus soil functioning, however, the microbial community illustrated significant metabolic flexibility.

### 1. Introduction

A major portion of terrestrial carbon (C) cycling is mediated and driven through soil microorganisms (Gouguilas et al., 2014). Soil microbes and their ability to metabolise (i.e., catabolise and anabolise small molecules fundamental to biological function) is inherently governed by the stoichiometry of bioavailable nutrients present in the soil (Cleveland and Liptzin, 2007). In most soils, available C is the primary factor limiting microbial growth (Heuck et al., 2015), with the

availability of inorganic nutrients (N and P) being secondary regulators once C limitation is overcome (Creamer et al., 2016). It has been suggested that as the soil stoichiometric balance, here referring to the ratio of C:N:P, reaches the optimum for microbial cells, growth will lead to C storage, with no additional limitations (i.e., pH, oxygen and moisture status) (de Sosa et al., 2018; Mason-Jones et al., 2021). However, there is limited information on how the availability of these compounds will affect soil metabolomic processing and function, particularly at the individual metabolite level. Ultimately, it is the relative balance of these

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metabolites that determines key soil processes (e.g., the amount of C stored in the microbial biomass, the release of organic acids and mineral weathering rates, secondary metabolite production).

The metabolome is defined as the entirety of small molecules (<1500 Da) found within a biological sample (Klassen et al., 2017). Primary metabolism, concerning the small molecules directly involved in the growth, development and reproduction of an organism, is principal to normal organismal function (Rojas et al., 2014). Glucose, being a simple sugar, is considered a ubiquitous, labile, C substrate and is key in glycolysis, the major energy production pathway in most microorganisms (Sanchez and Demain, 2008). N and P are also potentially rate limiting nutrients, important in protein and amino acid synthesis, nucleic acid biosynthesis, and energy metabolism and the formation of various organic acids and phospholipids, respectively (Vitousek and Howarth, 1991; Kornberg, 1995; Kuyper et al., 2018; Y. S. Zhang et al., 2019).

It has been suggested that N and P cycling rates are intrinsically linked due to the potential of P limitation to develop under high N availability, as well as in terms of their impact on SOC processing under different stoichiometric balances (Brailsford et al., 2019). Thus, nutrient inputs will shift the underlying stoichiometry of SOC and ultimately the soil organic matter (SOM) pools, which are key in both, soil health and ecosystem service provisioning (i.e., climate regulation, crop production and water management) (Garratt et al., 2018). However, based on the current literature, it is unclear how changes to soil nutrient stoichiometry impact the soil biological community's metabolism and SOC cycling. Generally, inference of changes in SOM/C cycling have been made through direct measurement of soil chemistry (Abrar et al., 2020), soil processes (e.g., CO<sub>2</sub> flux, exoenzyme activity; Hartman and Richardson, 2013), shifts in microbial community structure (Aanderud et al., 2018) or functional gene assays (Schleuss et al., 2019). However, high-resolution metabolomic approaches to C cycling have been shown to be very sensitive to changes in soil conditions (Withers et al., 2020; Overy et al., 2021), yet are rarely applied.

It is estimated that only a small proportion (0.1–2%) of the total microbial biomass is active in soil (Blagodatskaya and Kuzyakov, 2013). However, the quantity of potentially active microorganisms (that can rapidly respond to increased availability of labile substrates) is suggested to be significantly higher, contributing between 10 and 40% of the total microbial biomass (Blagodatskaya and Kuzyakov, 2013). Thus, on the loading of labile substrates the soil microbial community (particularly the membrane-associated phospholipid fatty acid (PLFA) fingerprint) is likely to respond rapidly (Fanin et al., 2019). With fast-growing (copiotrophic) organisms (likely *r*-strategists e.g., Gram-negative bacteria), likely to react more quickly to the addition of labile nutrients than slower growing organisms (*K*-strategists, e.g., fungi and Gram-positive bacteria) (Fierer et al., 2007).

The laboratory-based mesocosm study reported here investigates the effect of changing nutrient stoichiometry on primary and secondary metabolism of the soil microbial community, with the aim of providing a mechanistic understanding of the microbial breakdown and metabolomic processing of labile C, N and P substrates. We used a combination of (i) liquid chromatography-mass spectrometry (LC-MS) based untargeted primary metabolomics assay, (ii) a PLFA assay to assess cell growth and structural community change, and (iii) a <sup>14</sup>C-labelled glucose assay to assess the temporal uptake and transformation of labile nutrient substrates.

Within the context of a typical agricultural soil this study aims to provide a better mechanistic understanding of soil microbial nutrient processing. We hypothesise that: 1) nutrient (C:N:P) addition will cause a large shift in the whole (intercellular and extracellular) metabolic profile of soil, but that the direction of these responses will be compound-specific reflecting changes in resource partitioning into catabolic and anabolic processes, 2) glucose addition would lead to an increase in the Gram-negative-to-Gram-positive bacterial ratio and a decrease in the fungal-to-bacterial ratio due to preferential bacterial

growth in the short-term (48 h), and 3) eliminating nutrient limitation will increase the microbial removal of low-molecular weight C from a high C, low inorganic N and P environment.

## 2. Materials and methods

### 2.1. Soil characteristics and analysis

Independent replicate samples (0–10 cm) of a sandy clay loam textured Eutric Cambisol soil were collected from a postharvest maize (*Zea mays* L.) field located at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14'N, 4°01'W). Following collection, the field-moist soil was sieved through a 2 mm mesh to remove stones and plant material and ensure sample homogeneity. Soil characteristics are summarised in Table 1. Briefly, gravimetric soil moisture was determined by oven drying (105 °C, 24 h), organic matter was quantified by loss-on-ignition in a muffle furnace (450 °C, 16 h) (Ball, 1964). Soil C:N ratio was determined on oven-dried, ground soil using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI, USA). pH and electrical conductivity were determined on 1:5 (w/v) soil-to-DI H<sub>2</sub>O suspensions using standard electrodes. Bioavailable N and P levels in soil were determined using 1:5 (w/v) soil-to-0.5 M K<sub>2</sub>SO<sub>4</sub> and 1:5 (w/v) soil-to-0.5 M AcOH (acetic acid) extracts, respectively. Total dissolved organic carbon (TOC) and dissolved N (TN) were determined in the K<sub>2</sub>SO<sub>4</sub> extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO<sub>3</sub>-N) and ammonium (NH<sub>4</sub>-N) in the K<sub>2</sub>SO<sub>4</sub> extracts were measured by the colorimetric methods of Miranda et al. (2001) and Mulvaney (1996), respectively. Phosphate (PO<sub>4</sub>-P) was measured in the AcOH extracts using the colorimetric method of Murphy and Riley (1962). Cations (Na, K and Ca) were measured in the AcOH extracts using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK).

### 2.2. Soil treatment

To stimulate microbial metabolism and metabolite production, a nutrient solution was added to the soil. In general, the most common factor limiting microbial activity is the availability and quality of C (Demoling et al., 2007). As such, glucose was chosen as the primary nutrient to be added, as it represents a major input of C, in both monomeric and polymeric form, into soil systems and is utilised by almost all organisms within the microbial community (Gunina and Kuzyakov, 2015; Reischke et al., 2015). As N and P also have the potential to be microbial growth limiting, glucose was either added alone or in the presence of N, or P, or N + P at a stoichiometric ratio of 60:7:1 (C:N:P), based on the ratio of the microbial biomass (Cleveland and Liptzin, 2007; Brailsford et al., 2019). The concentration at which

Table 1

Characteristics of the soil used in the study. Values represent mean ± SEM (*n* = 4), reported on a dry weight basis.

Soil characteristics	
Gravimetric moisture content (%)	32.6 ± 1.5
Organic matter (%)	6.57 ± 0.26
pH	5.7 ± 0.1
EC (µS cm <sup>-1</sup> )	64 ± 4
Total C (%)	2.62 ± 0.06
Total N (%)	0.30 ± 0.01
C:N ratio	8.7 ± 0.1
Dissolved organic C (mg C kg <sup>-1</sup> )	54.7 ± 3.0
Total N (mg N kg <sup>-1</sup> )	39.0 ± 3.8
Extractable NO <sub>3</sub> <sup>-</sup> (mg N kg <sup>-1</sup> )	6.3 ± 0.3
Extractable NH <sub>4</sub> <sup>+</sup> (mg N kg <sup>-1</sup> )	1.0 ± 0.2
Extractable P (mg P kg <sup>-1</sup> )	14.1 ± 0.6
Exchangeable Na (mg kg <sup>-1</sup> )	13.5 ± 1.3
Exchangeable K (mg kg <sup>-1</sup> )	219.2 ± 63.9
Exchangeable Ca (mg kg <sup>-1</sup> )	134.9 ± 12.7

glucose was added to the soil was based on the likely amount released on plant cell death (50 mM; [Jones and Darrah, 1996](#); [Teusink et al., 1998](#)). This concentration was chosen as it provided an excess of C (30 mmol C kg<sup>-1</sup>) relative to the size of the native microbial biomass (18 mmol C kg<sup>-1</sup>, of which ca. 10% is active; [Wang et al., 2014](#)) and of the DOC pool (4.6 mmol C kg<sup>-1</sup>), which should therefore promote microbial growth but remain in a realistic range. Preliminary experiments showed that this level of glucose induces exponential microbial growth after ca. 16 h (data not presented). The N was added as NH<sub>4</sub>NO<sub>3</sub> (3.5 mmol N kg<sup>-1</sup>) and P was added as NaH<sub>2</sub>PO<sub>4</sub> (0.5 mmol P kg<sup>-1</sup>), the compounds were selected as they are commonly used as inorganic fertilisers. A No-addition (unamended sieved field soil) and Control treatment (HPLC-plus grade water only, applied to soil at equal rates to nutrient treatments) were also included to determine background soil metabolite concentrations and the effect of adding liquid to the sample. Following treatment, soil samples were incubated at 25 °C (reflecting a summer period) for 48 h (the time at which exponential growth was ending, as illustrated in [Fig. 6](#)), in the dark to stimulate microbial growth and substrate utilisation.

### 2.3. Untargeted soil metabolomic sample preparation, extraction, and analysis

Prior to use, all glassware was acid-washed (5% HCl, > 24 h) to remove chemical residues, rinsed in HPLC-grade water three times, and subsequently dry heat sterilised (150 °C, 2.5 h) ([Jain et al., 2020](#)). All equipment used to process the soil (e.g., tweezers and spatulas) were thoroughly cleaned both before and between samples with deionised water and 70% industrial methylated spirit (IMS) to prevent cross-contamination. Nutrients in the concentrations described in section 2.2 were added by pipette in 1 ml of HPLC-plus grade water (Sigma-Aldrich, Munich, Germany) evenly distributed across the surface of 10 g fresh weight of soil in 240 ml polypropylene sample containers (Snap-Seal®; Corning, NY, USA). This type of container was used to ensure even coverage of the soil surface with the nutrient solution (soil depth ca. 10 mm). Five independent soil replicates ( $n = 5$ ) were set up per treatment. Samples were subsequently incubated as described in Section 2.2. At the end of the incubation period, the soil samples were immediately transferred to 20 ml glass vials and fumigated with ethanol-free chloroform for 24 h to lyse microbial cells, to increase metabolite yield ([Swenson et al., 2015](#)) and limit microbial processing of metabolites, using the method of [Vance et al. \(1987\)](#). After fumigation, samples were immediately frozen (−80 °C) to quench any residual metabolic and enzymatic activity ([Wellerdiek et al., 2009](#)). From this point onwards, all samples were stored at −80 °C, unless otherwise stated and while being processed (i.e. out of the freezer), samples were kept on ice (4 °C). Lyophilisation of samples (>24 h) was then performed using a Modulyo Freeze Dryer (ThermoFisher Corp, Waltham, MA) equipped with an RV vacuum pump (Edwards Ltd., Crawley, UK). Samples were then mechanically ground using a Retsch MM200 stainless steel ball mill (Retsch GmbH, Haan, Germany) for 60 s at a frequency of 20 Hz to aid in cell lysis and metabolite recovery and to increase sample homogeneity ([Wang et al., 2015](#); [Withers et al., 2020](#)). The ball mill was cleaned thoroughly between samples with HPLC-grade water followed by 70% IMS, to avoid contamination.

The following extraction method is based on the hybridised methods of [Swenson et al. \(2015\)](#) and [Fiehn et al. \(2008\)](#). Briefly, we used the extraction method from [Swenson et al. \(2015\)](#) and the solvent makeup (3:3:2) from [Fiehn et al. \(2008\)](#), to ensure broad metabolite coverage, with the recovery of both polar and non-polar metabolite classes. Further, the weight of soil extracted was increased in order to maximise the volume of supernatant available for lyophilisation and subsequent analysis. A pre-experiment was used to compare the efficiency of the metabolite extraction method proposed here to that of the [Swenson et al. \(2015\)](#) method (the results are summarised in [supplementary information Section S1](#)).

Upon soil extraction, 6 g of each fumigated, lyophilised, ground soil sample was weighed into a 35 ml glass centrifuge tube (Kimax®; DWK Life Sciences, Stoke-on-Trent, UK), to which 24 ml of pre-cooled (−20 °C) acetonitrile (MeCN)/isopropyl alcohol (IPA)/HPLC-plus grade water (H<sub>2</sub>O) (3:3:2 v/v/v) extractant was added, using a glass pipette. Samples were then horizontally shaken on ice (4 °C) at a frequency of 200 Hz for 1 h, and then centrifuged at 3320×g for 15 min ([Swenson et al., 2015](#)). Supernatants were then transferred, using glass pipettes, into 20 ml glass vials and stored at −20 °C (to ensure metabolic activity was quenched but the supernatant was not frozen). Samples were left unfiltered due to the potential of contamination from dissolution of contaminants from the filter paper and plastic housing. The supernatant was lyophilised in 2 ml glass vials using a Modulyo Freeze Dryer with RV pump attached to a SpeedVac vacuum concentrator (Savant; ThermoFisher Corp.). The vials were periodically topped up with the supernatant, taking note of the quantity added (~15 ml total) and lyophilised to complete dryness. Samples were then shipped on dry ice (−78.5 °C) to Metabolon Inc. (Morrisville, North Carolina, USA) for untargeted LC-MS metabolomic analysis. Upon analysis, samples were dissolved in methanol:water (4:1 v/v) and subjected to the standard Metabolon sample preparation procedure. Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) analysis parameters, bioinformatics, compound ID and data curation are summarised in [Supplementary information Section S2](#).

### 2.4. <sup>14</sup>C-glucose labelled nutrient metabolism assays

Soil glucose metabolism was measured by nutrient depletion following a protocol similar to that described for freshwater sediments in [Brailsford et al. \(2019\)](#). Briefly, 2 g fresh weight of soil (as described in Section 2.1) was added to a sterile 50 ml polypropylene centrifuge tube (Falcon®, Corning Inc., Corning, NY), and 200 µl of <sup>14</sup>C-[U]-glucose (Lot 3,632,475; PerkinElmer Inc., Waltham, MA) was added to the soil surface to a final C concentration of 3.7 kBq ml<sup>-1</sup>. Five independent soil replicates ( $n = 5$ ) were used per treatment. To measure glucose depletion in each of the C, N and P amended treatments (as described in section 2.2), <sup>14</sup>C-labelled glucose solution (50 mM) was added alone or in the presence of N, or P, or N + P in the concentrations described in Section 2.2. In this assay, the Control treatment (HPLC-plus grade water) was also spiked with <sup>14</sup>C-glucose to assess the soil biological community's ability to respond to small inputs of labile C (i.e., trace concentration to label the intrinsic glucose pool). This unamended control reflects the natural background level of glucose in soil solution in this soil (ca. 50 µM; [Boddy et al., 2007](#)).

After addition of the <sup>14</sup>C-labelled substrate, a 1 M NaOH trap (1 ml) was inserted above the soil to catch any <sup>14</sup>CO<sub>2</sub> produced from the breakdown of <sup>14</sup>C-labelled glucose. The tubes were then hermetically sealed and incubated at room temperature (25 ± 1 °C) in the dark. The NaOH traps were replaced after 0, 1, 3, 6, 9, 24, 36, 48, 60, 72, 80, 96, 103, 122, 148 and 168 h, post-glucose application. The efficiency of the NaOH traps was >98% (as determined by collecting <sup>14</sup>CO<sub>2</sub> generated from adding excess 0.1 M HCl to 0.001 M NaH<sup>14</sup>CO<sub>3</sub>). The amount of <sup>14</sup>C in the NaOH traps was measured by mixing with Optiphase HiSafe 3 liquid scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and placing on a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK) with automated quench correction.

The amount of <sup>14</sup>C-labelled glucose remaining in the soil was determined after 7 d (168 h) by extracting each sample with 1:5 (w/v) ice-cold (4 °C) 1 M KCl to halt any further glucose turnover ([Rousk and Jones, 2010](#)). Samples were shaken (200 rev min<sup>-1</sup>, 30 min) and centrifuged (33,000 g, 5 min). Subsequently, 1 ml of supernatant was mixed with Optiphase HiSafe 3 liquid scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and the <sup>14</sup>C content measured by liquid scintillation counting as described above.

## 2.5. Soil PLFA analysis

Nutrients in the concentrations described in section 2.2 were added in 2 ml of HPLC-plus grade water (Sigma-Aldrich, Munich, Germany) to 20 g fresh weight of soil in 240 ml polypropylene sample containers (Snap-Seal®; Corning, NY, USA). Four independent soil replicates ( $n = 4$ ) were used per treatment. Samples were subsequently incubated for 48 h as described in section 2.2. At the end of the incubation period, samples were immediately transferred to sterile 50 ml polypropylene centrifuge tubes (Falcon®, Corning, NY, USA) and immediately frozen ( $-80^{\circ}\text{C}$ ) to quench lipid turnover. Lyophilisation ( $>24$  h) was then performed using a Modulyo Freeze Dryer with RV pump. Samples were then shipped on dry ice ( $-78.5^{\circ}\text{C}$ ) to Microbial ID (Newark, DE, USA) for extraction, fractionation and transesterification using the high throughput method of Buyer and Sasser (2012). Subsequently, samples were analysed using an Agilent (Agilent Technologies, Wilmington, DE, USA) 6890 gas chromatograph (GC) equipped with autosampler, split-splitless inlet, and flame ionization detector (FID). The system was controlled by MIS Sherlock® (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis parameters and standards can be found in Buyer and Sasser (2012). Microbial biomass was calculated as the sum of all PLFAs detected in the sample. The PLFAs detected are summarised in Table S1.

## 2.6. Statistical analysis

All statistical and graphical analyses were performed in the R environment (v 4.1.1; R Core Team, 2021), and graphical analysis was constructed using the 'ggplot2' package (Wickham, 2016), unless otherwise stated. Analyses were deemed significant if  $p < 0.05$ . All metabolomic statistical analysis was performed using natural log (ln) transformed median scaled imputed data. A principal component analysis (PCA) was constructed using the 'vegan' package (Oksanen et al., 2020) to reduce the dimensionality of the dataset and provide a visual representation of data variance. An analysis of similarity (ANOSIM) was subsequently performed using 'vegan' to test treatment separation statistically.

To measure the magnitude of the effect of nutrient (C, N and P) addition, the number of compounds with significant differences using pairwise treatment comparisons were assessed using the 'stats' package (R Core Team, 2021), and for compounds deemed significantly different between treatments the direction of change was summarised. Fatty acids (FAs) were also examined in greater detail; a statistical heatmap of a number of exemplary short chain, medium and long chain saturated and unsaturated FAs, with pairwise treatment comparisons was carried out as above and then graphically represented.

Specific examples of representative metabolites (carbohydrates, amino acids, peptides and FAs) and compounds associated with specific metabolic pathways (TCA cycle and glutamate pathway) were further explored graphically and statistically using ANOVA models in the 'stats' package, significant results being additionally tested using Tukey post-hoc tests using the 'agricolae' package (de Mendiburu, 2019).

A non-metric dimensional scaling (NMDS) approach was used to condense the multivariate PLFA data in a comprehensible number of dimensions and visualize the relative degree of similarity among samples using the whole PLFA dataset, this was performed using the 'vegan' package. All PLFAs detected were used in the analysis, to represent the structure of the whole microbial community. An analysis of similarity (ANOSIM) was subsequently performed using 'vegan' to test separation statistically. PLFA derived microbial biomass was also tested using ANOVA, as above.

The cumulative mineralisation of  $^{14}\text{C}$ -labelled glucose over time was calculated for each replicate. The final percentage (7 d post glucose application) of respired  $^{14}\text{CO}_2$  was used to calculate apparent microbial C use efficiency (Jones et al., 2018a,b). The final concentration of  $^{14}\text{C}$  labelled compounds in the soil (as determined by ice-cold 1 M KCl

extraction on day 7) was not subtracted from this, as it was assumed that these compounds were the result of glucose turnover in the soil, i.e., either metabolic by-products or end-products (Glanville et al., 2016). Differences in total  $^{14}\text{CO}_2$  respiration were assessed using a Kruskall-Wallis test, followed by a pairwise Wilcoxon posthoc test, as data did not conform to parametric assumptions, and the final concentration of  $^{14}\text{C}$ -labelled compounds in the soil was assessed by ANOVA, as above.

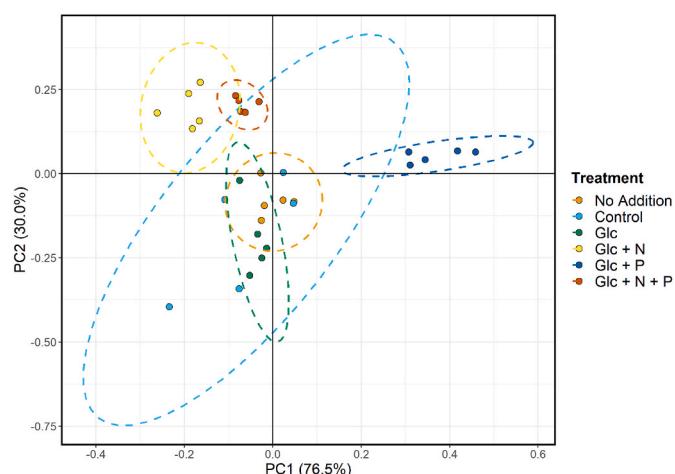
## 3. Results

### 3.1. Soil primary metabolite profile

In total, 494 individual metabolite compounds were identified across all treatments. This included, 199 fatty acids, 118 amino acids, 43 carbohydrates, 41 nucleotides, 21 peptides and 72 other compounds, including xenobiotics, secondary metabolites and cofactors and electron carriers.

PCA (Fig. 1) was used to gain a high-level overview of data variance and sampling grouping. The ANOSIM confirmed significant similarity between treatment groups ( $R = 0.147, p = 0.002$ ). However, qualitative interpretation of the PCA showed that the Glc treatment was not strongly separated from the no-addition and control treatment groups, possibly because these groups appeared to exhibit more variation between replicates than the mineral (+N/+ P/+ N + P) treated groups (Supplementary Fig. S1). The addition of Glc + N and Glc + P led to extensive shifts in the soil metabolome, however, the changes in the overall metabolic profile of the soil were distinctly different for the two elements. As noted above, P addition in the absence of N resulted in a significant accumulation of many lipids, but if N was also present, this accumulation was much less, or reversed, as evidenced by the proximity of the Glc + N and Glc + N + P groups in Fig. 1 and shown in Table 2.

Treatment pairwise comparison (number of compounds significant and direction of change) are summarised in Table 3. Overall, the number of metabolites significantly affected and/or produced in the soil following the addition of water (Control) to the untreated soil (No addition) was minimal ( $n = 25$ ; 5% of the total number of metabolites detected). However, all the compounds affected were lower in the Control treatment, possibly representing a dilution effect compared to the No-addition treatment. Glc addition alone (relative to the water-only Control) produced a number of significantly different compounds ( $n = 55$ ; 11% of total detected), generally resulting in higher concentrations



**Fig. 1.** Influence of nutrient (C, N and P) addition on the primary metabolite profile of an agricultural soil. 2D principal component analysis (PCA) of soil metabolite composition (all compounds with a positive identification;  $n = 494$ ). Principal component 1 (PC1) explains 76.5% of the total variance, and principal component 2 (PC2) explains 30.0% of the total variance. Plotting was performed on natural log (ln) transformed median scaled data.

Table 2

Summary of changes in fatty acids (FAs) between nutrient addition treatments highlighted by a statistical heatmap. Numbers in the table indicate the ratio of the mean scaled intensity for a metabolite between two experimental groups being compared. Red and green filled cells indicate a significant decrease in metabolite concentration and a significant increase in metabolite concentration, respectively, using a Welch's two sample *t*-test ( $p \leq 0.05$ ). Statistical analysis was performed on natural log-transformed data.

	Biochemical name	Effect of N			Effect of P			Effect of N + P					
		Glc + N / No addition	Glc + N / Control	Glc + N / Glu	Glc + P / No Addition	Glc + P / Control	Glc + P / Glu	Glc + P / Glc + N	Glc + N + P / No addition	Glc + N + P / Control	Glc + N + P / Glu	Glc + N + P / Glc + N	Glc + N + P / Glc + P
Short chain	butyrate/isobutyrate (4:0)	0.48	0.8	0.83	0.12	0.19	0.2	0.24	0.19	0.31	0.32	0.39	1.61
	isovalerate (15:0)	0.74	0.69	0.84	0.42	0.4	0.48	0.57	0.28	0.26	0.32	0.38	0.66
	Valerate (5:0)	0.66	0.85	0.87	0.21	0.27	0.27	0.31	0.25	0.32	0.33	0.37	1.2
	Isocaproate (6:0)	0.81	0.52	1.03	0.15	0.1	0.19	0.18	0.23	0.15	0.29	0.28	1.53
	Caproate (6:0)	1.06	1.03	1.44	0.33	0.33	0.45	0.32	0.59	0.57	0.8	0.56	1.76
Medium and long chain saturated	Caprate (10:0)	0.99	1.02	1.12	1.13	1.16	1.28	1.14	1	1.03	1.13	1.01	0.89
	Myristate (14:0)	0.68	0.64	0.76	4	3.8	4.5	5.92	0.84	0.8	0.95	1.25	0.21
	(12 or 13)-methylmyristate (a15:0 or i15:0)	0.38	0.34	0.44	7.87	7.05	9.11	20.81	0.56	0.51	0.65	1.49	0.07
	Palmitate (16:0)	0.82	0.7	0.88	4.81	4.09	5.13	5.85	0.97	0.83	1.04	1.18	0.2
	Margarate (17:0)	0.52	0.42	0.6	5.71	4.62	6.59	10.98	0.8	0.65	0.92	1.54	0.14
	Stearate (18:0)	0.84	0.78	0.86	2.98	2.65	2.96	3.42	1	0.92	1.02	1.18	0.35
	(16 or 17)-methylstearate (a19:0 or i19:0)	0.28	0.24	0.31	6.72	5.8	7.51	24.08	0.49	0.43	0.55	1.77	0.07
	Arachidate (20:0)	0.67	0.48	0.76	5	3.58	5.67	7.46	1.19	0.85	1.35	1.78	0.24
Unsaturated	Palmitoleate (16:1n7)	0.34	0.31	0.34	9.38	8.58	9.29	27.57	0.51	0.47	0.51	1.5	0.05
	Oleate/vaccinate (18:1)	0.36	0.31	0.34	12.65	11.01	12.05	35.55	0.57	0.5	0.54	1.6	0.05
	Eicosenoate (20:1)	0.26	0.25	0.29	5.84	5.5	6.42	22.09	0.5	0.47	0.55	1.9	0.09
	Erucate (22:1n9)	0.28	0.25	0.3	5.62	5.09	6.15	20.22	0.52	0.47	0.57	1.86	0.09
	Hexadecadienoate (16:2n6)	0.44	0.43	0.35	1.69	4.67	3.76	10.76	0.51	0.51	0.41	1.18	0.11
	Linoleate (18:2n6)	0.23	0.31	0.26	9.14	12.64	10.36	40.43	0.37	0.51	0.42	1.63	0.04
	Dihomo-linoleate (20:2n6)	0.36	0.34	0.4	8.23	7.75	9.15	23.06	0.61	0.57	0.67	1.7	0.07
	Hexadecatrienoate (16:3n3)	0.6	0.61	0.48	2.57	2.65	2.08	4.32	0.64	0.66	0.52	1.07	0.25
Unsaturated	Linolenate (alpha or gamma; (18:3n3 or 6)	0.43	0.5	0.33	11.09	13.1	8.46	25.94	0.63	0.74	0.48	1.47	0.06
	Stearidonate (18:4n3)	0.52	0.56	0.38	5.26	5.69	3.83	10.2	0.64	0.69	0.46	1.24	0.12
	Dihomo-linolenate (20:3n3 or 6)	0.23	0.24	0.26	11.77	12.76	13.42	52.25	0.49	0.53	0.56	2.17	0.04
	Arachidonate (20:4n6)	0.2	0.21	0.21	15.35	16.48	16.49	78.46	0.46	0.5	0.5	2.37	0.03
	Eicosapentaenoate (20:5n3)	0.8	0.82	0.67	10.48	10.65	8.66	13.02	0.72	0.73	0.59	0.89	0.07
	Docosapentaenoate (22:5n3)	0.6	0.81	0.72	11.64	15.82	13.97	19.51	0.6	0.81	0.72	1	0.05
	Docosapentaenoate (22:5n6)	0.11	0.12	0.13	12.04	13.31	14.46	113.56	0.37	0.41	0.44	3.49	0.03
	Docosahexaenoate (22:6n3)	0.23	0.22	0.3	16.75	16.38	21.59	73.01	0.48	0.47	0.61	2.08	0.03

of carbohydrate molecules. This effect was noted in all comparisons involving Glc relative to the Control group, whether or not N and/or P was present. The additional effects of N and/or P caused the generation of a large number of further compounds (in some treatments up to half of all compounds), when compared to the Control or the Glc treatment groups. Additionally, N and/or P addition led to a larger number of changes relative to the Glc group than were seen relative to the Control group.

In terms of specific molecules, glucose itself was similar in all treated groups, and significantly higher than No addition and the Control, as would be expected (Figs. 2 and 6). With regard to compounds associated with the TCA cycle, the presence of P was a key factor in compound (and intermediate) synthesis, with notable increases in alpha-ketoglutarate, succinate and fumarate, under Glc + P relative to control and no treatment (Fig. 3). Relative N deficit (and C excess) led to lower levels of amino acids and other nitrogenous compounds, while the amendment of the C:N balance generally increased the concentration of these compounds. However, 14 (52%) aromatic proteinogenic compounds (e.g., phenylalanine and tryptophan) were found at similar levels in all groups, regardless of treatment with the exception of phenylacetate and kynureneate (Fig. 4A).

Lipid metabolism was dramatically and consistently altered by supplementation of just Glc + P, but not by the combination of Glc + N or Glc + N + P (Table 2). This was particularly characterised by a strong accumulation of long chain FAs in Glc + P treated samples. This effect was reversed for the short chain fatty acids (C<sub>4</sub>–C<sub>8</sub>; SCFAs). While medium chain length FAs were found at increased levels, the observed increases were less than seen for long chain FAs, especially polyunsaturated fatty acids (PUFAs), as illustrated in Table 2.

### 3.2. PLFA profile

The size of the microbial biomass showed significant differences between treatments, as tested by ANOVA ( $F_{(5, 18)} = 2.82, p = 0.04$ ) (Fig. S2). However, further exploration using a Tukey HSD posthoc test showed no significant pairwise differences. NMDS ordination analysis was used to visually explore the clustering of the PLFA compounds due to the different soil treatments (Fig. 5). Generally, there was little separation, with all 95% confidence intervals showing significant overlap, suggesting that the PLFA derived microbial community structure was similar across all samples. This was further tested using an ANOSIM, which confirmed similarity between groups ( $R = 0.192, p = 0.02$ ).

Table 3

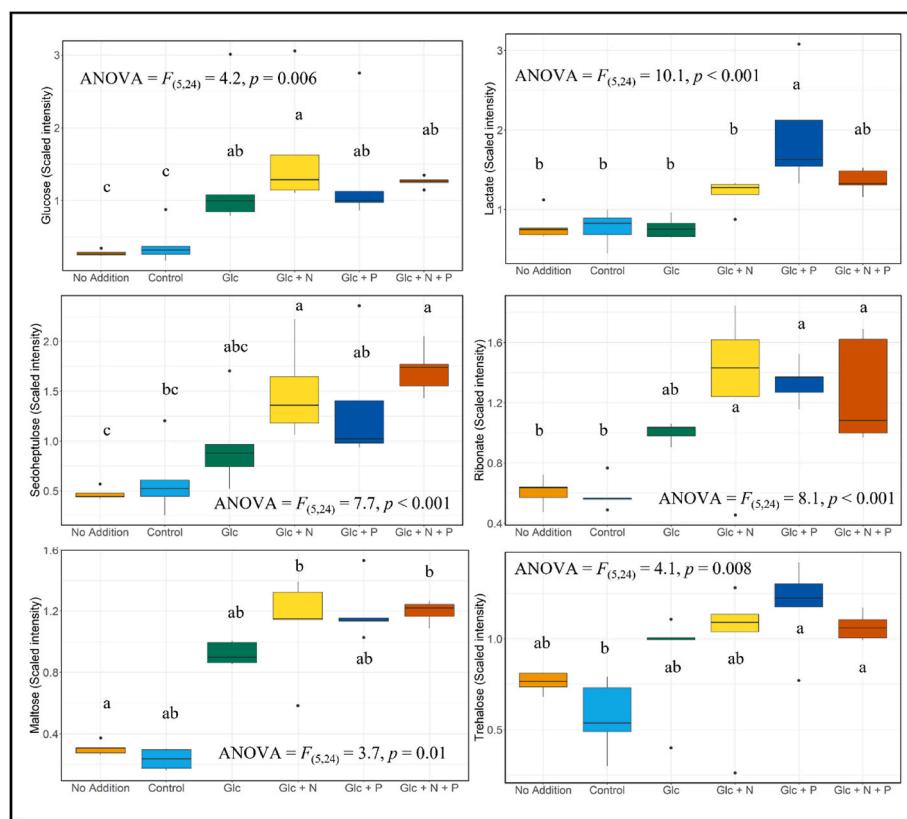
Magnitude of metabolic changes between treatments. Summary of the number of compounds significantly different between treatments, detected by pair-wise comparisons ( $p \leq 0.05$ ), and the direction of change.

Effect	Comparison	Compounds significant	Increase   Decrease
Effect of $\text{H}_2\text{O}$ (control)	Control / No addition	25	0   25
Effect of Glu	Glc / No addition	142	51   91
	Glc / Control	55	46   9
Effect of N	Glc + N / No addition	173	82   91
	Glc + N / Control	137	90   47
	Glc + N / Glu	175	95   80
Effect of P	Glc + P / No addition	233	202   31
	Glc + P / Control	223	204   19
	Glc + P / Glu	246	222   24
	Glc + P / Glc + N	215	170   45
Effect of N + P	Glc + N + P / No addition	237	131   106
	Glc + N + P / Control	142	117   25
	Glc + N + P / Glu	215	147   68
	Glc + N + P / Glc + N	85	67   18
	Glc + N + P / Glc + P	240	80   180

### 3.3. $^{14}\text{C}$ -glucose mineralisation

After incubating the soil with  $^{14}\text{C}$ -labelled glucose for 48 h, the microbial biomass had entered a phase of exponential growth, with a significant amount (greater than half of the final (7 d) total of respired  $^{14}\text{C}$ ) having been taken up by the microbial biomass and respired and emitted

as  $\text{CO}_2$  (Fig. 6). Initially, the Control treatment showed the most rapid mineralisation, with no lag phase, likely due to the ca. 1000-fold lower amounts of  $^{14}\text{C}$  being applied than in the other treatments (glucose was only added at a trace amount in this treatment), and high carbon use efficiency (CUE). Of the nutrient treatments, during this first 36 h period following  $^{14}\text{C}$ -Glc addition, Glc alone showed the most rapid



**Fig. 2.** Response of selected carbohydrate compounds (glucose, lactate, sedoheptulose, ribonate, maltose and trehalose) within the soil in response to nutrient (C, N and P) addition. Different letters indicate significant differences between treatment groups ( $p < 0.05$ ).

mineralisation rate, with Glc + N and Glc + P closely grouping and Glc + N + P being lower. Beyond 48 h, treatment differences became more pronounced with Glc having the lowest total  $^{14}\text{CO}_2$  production, while N containing treatments had higher respiration (Fig. 6).

By 168 h (7 d) total respiration (i.e., cumulative mineralisation) rates in all treatments had slowed and had appeared to enter a quasi-stationary phase (Fig. 6). Kruskall-Wallis followed by pairwise Wilcoxon tests showed there was a significant difference between treatments, specifically, between Control and all other treatments ( $p \leq 0.02$ ), Glc and Glc + N ( $p = 0.02$ ) and Glc + N + P ( $p = 0.016$ ), Glc + N and Glc + P ( $p = 0.02$ ), and Glc + P and Glc + N + P ( $p = 0.016$ ). There were also significant differences in the amount of  $^{14}\text{C}$  labelled compounds remaining in the soil ( $F_{(4,19)} p < 0.001$ ), with Glc and Glc + P treatments having a higher percentage of extractable  $^{14}\text{C}$  compounds compared to the other treatments (Fig. 7).

## 4. Discussion

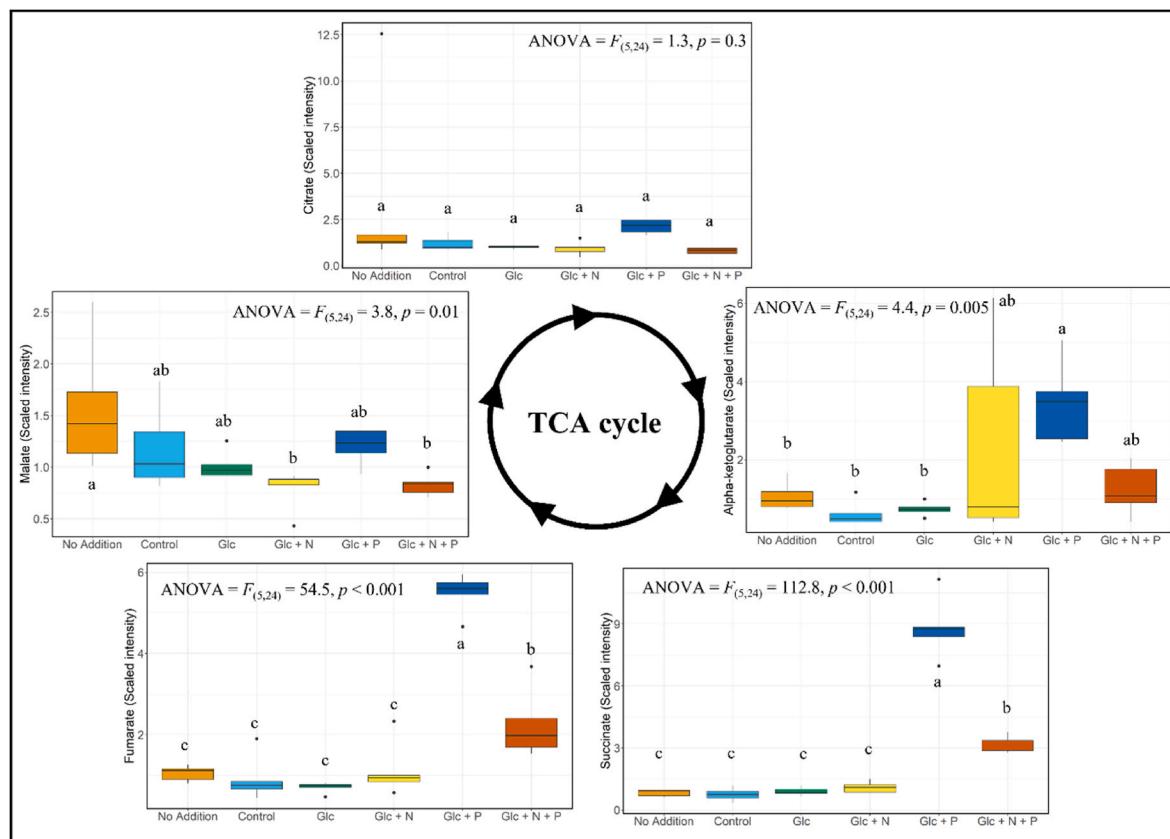
### 4.1. Primary metabolite changes induced by nutrient addition

PCA ordination of the metabolite data (Fig. 1) illustrate the general clustering of the No addition, Control and Glc treatments, suggesting that these treatments caused little change in the overall metabolite profile of the soil. Generally, microbial growth in soils is limited by the stoichiometric ratio of C to macronutrients (i.e., N or P; Griffiths et al., 2012; Hobbie and Hobbie, 2013), therefore the addition of a labile C substrate (here glucose) is likely to have a significant impact on the growth of the microbial community and the metabolite profile. The Control treatment showed little difference to the No-addition treatment, likely representing the negligible biological effect of water addition (other than a potential dilution effect; Table 3) and a microbial biomass that is primarily limited by C. Equally, all treatments receiving glucose

had statistically significantly higher levels of glucose remaining in soil solution compared to the Control and No-addition treatments, suggesting that there was still a considerable amount of residual substrate that had not been metabolised within the 48 h incubation period. This confirms that we added sufficient glucose to ensure C was not limiting for the initial 48 h of the incubation. On average, after 1 week, 99% of the applied glucose had been consumed or respired from the soil (Fig. 7). The exact chemical nature of the  $^{14}\text{C}$  compounds remaining in solution after 1 week (ca. 1% of that added) remains uncertain, but may include compounds metabolised from glucose and subsequently excreted from the cell, compounds released from lysed cells (i.e. microbial biomass turnover) and some unutilised  $^{14}\text{C}$ -glucose. N and/or P addition led to more changes relative to the Glc group than were seen relative to the Control treatment (Table 3), consistent with the theory that limiting C may restrict pathways that provide substrates for N and P interactions (Griffiths et al., 2012). In the subsequent sections we will discuss the changes in metabolites within several major molecular groups, namely, carbohydrates, amino acids, fatty acids and compounds related to the TCA cycle.

#### 4.1.1. Carbohydrates

Carbohydrates (e.g. mono- and oligo-saccharides, starch and cellulose) contribute significantly to the makeup of SOM (Ratnayake et al., 2013; Reardon et al., 2018). Carbohydrates are also key metabolites in soil microorganisms, functioning as metabolic substrates, as well as structural and intra- and exo-cellular components (Lowe, 1978). Glucose was found in significantly higher concentrations in all amended treatments relative to the Control and No-addition. The conversion of glucose to lactate by fermentation is often used by cells that cannot produce enough energy (adenosine 5'-triphosphate (ATP)) through oxidative phosphorylation, due to oxygen limitation to their meet cellular demand (i.e., under anaerobic conditions) (Melkonian and Schury, 2021).



**Fig. 3.** Response of compounds related to the tricarboxylic acid (TCA) cycle (citrate, alpha-ketoglutarate, succinate, fumarate and malate) within the soil in response to nutrient (C, N and P) addition. Different letters indicate significant differences between treatment groups ( $p < 0.05$ ).

Unicellular organisms undergoing exponential growth have been shown to grow by glucose fermentation, producing a range of small organic molecules such as ethanol, lactate or other organic acids (Vander Heiden et al., 2009). Here, while the addition of glucose alone did not stimulate lactate production, lactate was increased in combination with the N + P additions (particular under P treatments; Fig. 2). Glucose also led to elevations of several other sugars, sugar acids, and sugar alcohols, as well as di- and tri-saccharides (Fig. 2). In most cases, the carbohydrate compounds were higher under the nutrient treatments relative to the glucose alone group, consistent with generally elevated metabolic activity (Fuhrer et al., 2005; Vénica et al., 2018). It is likely that through the N and P limited conditions induced under the Glc only treatment, some of the consumed glucose substrate was diverted to the synthesis of storage compounds. Equally, storage polysaccharide compounds (e.g., glycogen) are too large in size to be detected by the methods employed here, though these compounds are an important store of excess C (Mason-Jones et al., 2021).

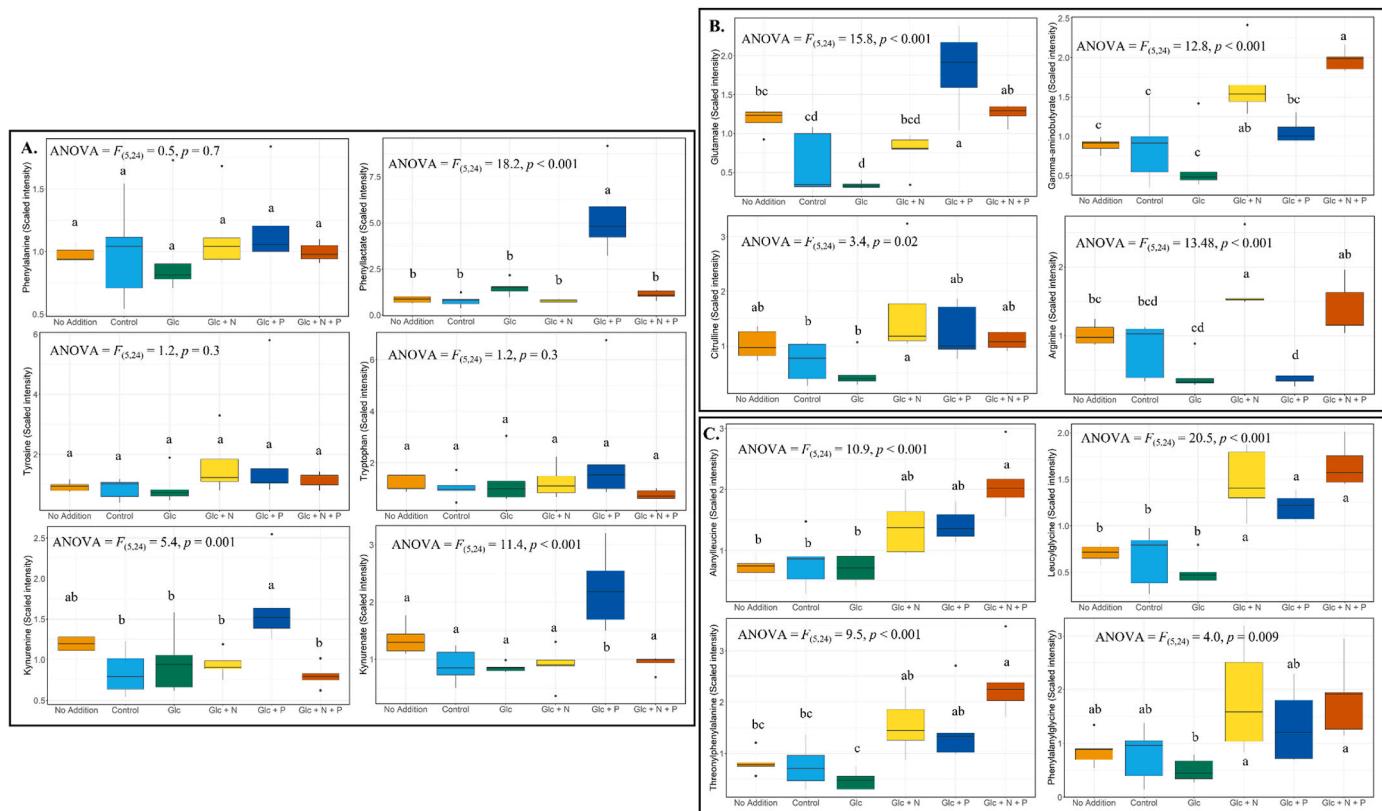
#### 4.1.2. TCA cycle related compounds

The tricarboxylic acid cycle (TCA cycle) is the series of chemical reactions that release stored energy through the oxidation of organic molecules. Inorganic phosphate is the key compound in the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP), providing energy to drive cellular processes (Berg et al., 2002a; Phillips et al., 2009). In this case it is possible that P was limiting for maximum oxidative phosphorylation so, P addition led to significant increases in key compounds related to TCA, namely succinate and fumarate. However, there was little increase in TCA associated compounds in the treatments without P, suggesting that the microbial community was not putting additional C and energy resources into P solubilization using organic acids (Alori et al., 2017).

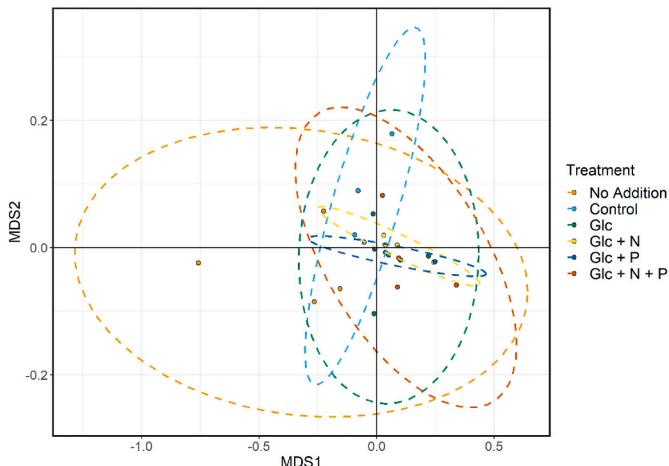
#### 4.1.3. Amino acids

Amino acids are the structural units required for protein (and enzyme) synthesis, as well as providing substrates for other biochemical compounds such as nucleic acids (Moe, 2013). The Glc treatment contained the lowest levels of these amino acids, even below the native levels in the control soil, while Glc + N and Glc + N + P in most cases had significantly higher levels. Typically, relative N deficit (and/or C excess) leads to the presence of lower levels of amino acids and other nitrogenous compounds, as the microbial community scavenges nitrogenous compounds from the soil (Geisseler and Horwath, 2014; Hicks et al., 2021). Thus, when (inorganic) N availability increases, organic N compounds also increase as there is less requirement for high N use efficiency (Mooshamer et al., 2014). We observed this scenario for many amino acids and their derivatives, especially in the class of amino acids derived from the glutamate pathways (arginine, glutamine, proline, histamine) (Fig. 4B). Glutamate is key to a number of metabolic processes in cells, including protein synthesis and glycolysis, and the TCA cycle (Helling, 1998; Feehily and Karatzas, 2013), and is one of the most ubiquitous amino acids in soil, as a component of root exudates, and the dissolved organic nitrogen (DON) pool (Paynel et al., 2001; Forde and Lea, 2007).

An exception to this pattern in amino acid concentration was the pathway for aromatic amino acids (Fig. 4A). The proteinogenic aromatic amino acids were found at similar levels in all groups, regardless of treatment. Amino acids possessing aromatic ring structures are generally less attractive to soil microbes as a source of organic N, due to their higher C:N ratio and complexity (Sauheitl et al., 2009). However, the Glc + P treatment did cause increases in several aromatic amino acid derivatives, for example phenylacetate, kynurenone, and kynurename, presumably through stimulation of catabolic pathways. Aromatic amino acids are often the metabolic starting point for the production of a variety of secondary metabolites (Parthasarathy et al., 2018). While



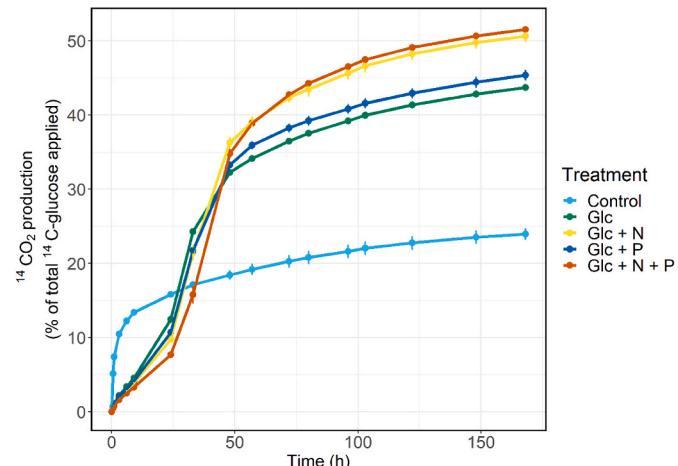
**Fig. 4.** Response of proteinogenic amino acids and oligopeptide compounds within the soil in response to nutrient (C, N, and P) addition. Panel A shows selected amino acid compounds related to the aromatic family (phenylalanine, phenyllactate, tyrosine, tryptophan, kynurenone and kynurenamine), Panel B summarises selected amino acid compounds related to the glutamate pathway (glutamate, gamma-aminobutyrate, citrulline and arginine) and Panel C summarises selected oligopeptides (alanlyleucine, leucylglycine, threonylphenylalanine and phenylalanylglycine). Different letters indicate significant differences between treatment groups ( $p < 0.05$ ).



**Fig. 5.** Influence of nutrient (C, N or P) addition on the PLFA derived microbial community structure of soil. NMDS plot of PLFA profiles of each soil treatment. Ellipses represent 95% confidence intervals for each treatment.

secondary metabolism was not specifically examined in this study, future research is recommended on secondary metabolites due to their importance in organismal interaction and sensing (Karlovsky, 2008).

Though whole proteins are too large to be considered in a metabolomic analysis, oligopeptides are considered markers for protein turnover (Doherty and Beynon, 2006). In this analysis the addition of glucose alone (Glu) did not lead to changes in oligopeptide levels relative to the residual amounts in untreated soil, but both N and P (and their combination) did, however, increase contents of oligomers

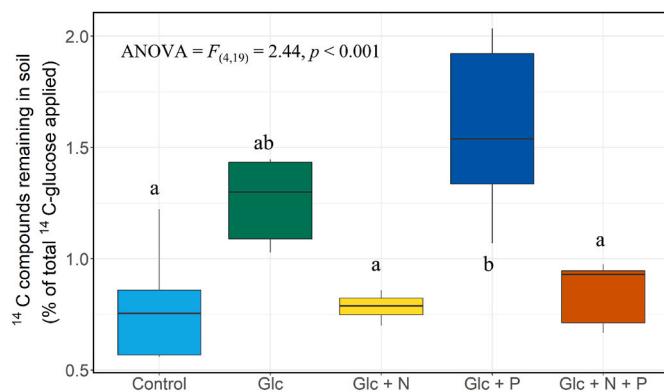


**Fig. 6.** Microbial mineralisation of  $^{14}\text{C}$ -labelled glucose to  $^{14}\text{CO}_2$  in soil over 1 week (168 h), in the presence or absence of C, N or P amendments. Treatments were replicated in quintuplicate ( $n = 5$ ), and error bars indicate the SEM.

(Fig. 4C). These results are consistent with increased metabolic activity (protein production and growth), resulting from the relief of nutrient limitations (Hartman and Richardson, 2013).

#### 4.1.4. Fatty acids

FAs are key to cellular function, contributing to a number of roles, including membrane lipids (i.e., PLFAs) as well as storage and cell signalling (Carvalho and Caramujo, 2018). FA biosynthesis pathways are highly conserved across the kingdoms of life (Berg et al., 2002b). Here



**Fig. 7.** Percentage of <sup>14</sup>C-labelled compounds remaining in the soil after 1 week following the addition of <sup>14</sup>C-labelled glucose either in the presence or absence of additional C, N or P amendments. Different letters indicate significant differences between treatment groups ( $p < 0.05$ ).

we illustrate that soil microbial lipid metabolism was dramatically and consistently altered by the addition of Glc + P to the soil, but not by the combined treatment of Glc + N + P (Table 2, Fig. S1). P is an essential component of lipid metabolism, particularly in the synthesis of PLs, which under unstressed conditions are the dominant polar membrane lipid class. P is liberated and solubilised from organic P by phosphatase enzymes (Jones and Oburger, 2011; Alori et al., 2017), and it has previously been suggested that P mineralisation and P solubilization are constrained by soil stoichiometry, because N and organic C are required for the synthesis of phosphatases (Widdig et al., 2019).

Equally, an absence of P has also been shown to induce lipid accumulation (Warren, 2020; Yang et al., 2018). Here we also showed that C and P surplus (Glc + P) caused lipid accumulation, particularly long chain FAs, which are generally involved in cell structure (i.e., PLs) and storage (e.g., triacylglycerols (TAGs)) (Salati and Goodridge, 1996; Brown et al., 2021).

The amount of short chain fatty acids (SCFAs) decreased significantly under the Glc + P and Glc + N + P treatments (Table 2). It is likely that this is a result of the increase in the ratio of labile C and P substrates, which were used preferentially relative to the soil's inherent more recalcitrant organic matter derived substrates. SCFAs are the metabolic end products of the anaerobic fermentation of recalcitrant polymeric carbohydrates (e.g., cellulose, starch, chitin) (Silva et al., 2020). SCFAs are functionally important metabolites, serving as electron donors for other microorganisms (e.g. fermentative Fe(III)-reducing microorganisms) and also act as a substrate for the SCFA-utilising bacterial population (He and Qu, 2008; Awasthi et al., 2018). It should be noted, however, that our soils were kept in an aerobic state and therefore anaerobic processes were not likely to be prevalent even in microsites.

N deprivation has also been shown to induce storage lipid accumulation (Weng et al., 2014). While N deprivation was not a direct treatment here, it may have been induced as a result of the unbalanced soil stoichiometry (i.e., C:N:P ratio), particularly under Glc + P treatments. However, here, N provision without P (Glc + N) led to decreased levels of longer chain fatty acids, but not for SCFAs. This potentially illustrates the partitioning of microbial resources, with N addition leading to the biosynthesis of a greater number of nitrogenous compounds (i.e., amino acids and proteins), while P addition led to the metabolism of a greater number of P-reliant compounds (i.e., FAs and compounds dependent on oxidative phosphorylation).

In terms of lipid metabolism, we can speculate that plentiful P (but a deficit of N) led to increased oxidative phosphorylation, which provided sufficient ATP for robust fatty acid synthesis, but the enzymes and regulatory loops for lipid metabolism and were not triggered under N deficit. The combination of P and N may have either alleviated this bottleneck to allow normal metabolism to utilize the fatty acids or may

have allowed for alternative pathways that preclude the generation of the fatty acids in the first place. We hereby accept hypothesis i as there was a significant shift in the metabolic profile of the soil (as illustrated in Figs. 1–4 and Fig. S1).

#### 4.2. Soil biological community response

The size and structure of the soil biological community underpins soil function (Wagg et al., 2014), driving SOM turnover and biogeochemical cycling (Rousk and Bengtson, 2014). Thus, C-induced shifts in soil microbial community structure may result in changes in soil function, notwithstanding the functional redundancy, which exists within the community. Surprisingly, we observed no significant change in the ratio of key microbial taxa in response to labile C and nutrient addition when assayed using the conventional PLFA biomarker approach (Fig. 5; Frostegård et al., 2011). This is somewhat surprising and suggests that PLFAs are not sensitive unless used in an isotope-specific context (i.e., tracing the incorporation of <sup>13</sup>C-glucose into taxonomically-relevant PLFAs) (Joergensen, 2022; Yao et al., 2015). In accordance with other studies, we did show an initial lag phase in <sup>14</sup>C-glucose use (ca. 10–16 h), followed by a short-lived exponential mineralisation phase, a pattern consistent with microbial growth (Hill et al.; Rousk and Bååth, 2007). From the mineralisation response and the total PLFA data, we conclude that glucose addition did stimulate *de novo* biomass production. The results also suggest that all components of the biomass grew equally, or that the conventional PLFA-biomarker approach failed to capture rapid changes in the active microbial community. However, in recent years several limitations have been identified with the PLFA method including the incorrect assignment of FAs to biological groups (Joergensen, 2022; Willers et al., 2015). We hypothesized that glucose addition would lead to an increase in the Gram-negative-to-Gram-positive bacterial ratio and a decrease in the fungal-to-bacterial ratio due to preferential bacterial growth (Fanin et al., 2019). However, this was not observed at the 48-h point of sampling potentially due to the relatively moderate amount of added substrate not being sufficient to induce significant shifts in the microbial community in the short term, we therefore reject hypothesis ii. Overall, our results suggest the soil microbial community demonstrated plasticity (Morrissey et al., 2017) and that metabolite extraction and analysis by metabolomic methods may have much greater sensitivity than conventional GC-MS based analysis of PLFAs.

#### 4.3. Microbial use of LMW carbon

The soil microbial community is expected to experience large pulse inputs of C, N and P in response to rhizodeposition, fertilisers and abiotic stress events (e.g., dry-rewet, freeze-thaw) (Göransson et al., 2013; Jones et al., 2009; Warren, 2014). The treatments used here were chosen to reflect these. Although the addition and subsequent depletion of <sup>14</sup>C-labelled glucose (50 mM) occurred rapidly in all treatments (implied by the <sup>14</sup>CO<sub>2</sub> emission), an initial lag phase was apparent (Fig. 6). We ascribe this slow initial response to a low microbial biomass and an initial saturation of internal metabolic pathways, rather than an overloading of the membrane transport systems (Hill et al.). Note that no lag phase was observed when only a natural trace amount of <sup>14</sup>C-labelled glucose (ca. 50  $\mu$ M) was added, and that it was also catabolised more rapidly. At the time our metabolomic measurements were made (48 h), the amount of glucose-C recovered as <sup>14</sup>CO<sub>2</sub> in the 50 mM glucose treatments was much larger (ca. 50%) than that in the trace glucose addition (50  $\mu$ M, ca 18%; Fig. 6). This is consistent with a major shift in microbial C partitioning and thus C use efficiency, with more glucose-derived C being channelled into energy intensive growth rather than maintenance metabolism. The addition of extra nutrients also induced changes in internal C partitioning, with the presence of N leading to a reduction in C use efficiency, while P addition generally had no observable effect. We ascribe this response to the removal of N limitation, allowing slightly more glucose-C (ca. 5%) to be channelled into

catabolic rather than anabolic processes (Mooshammer et al., 2014; S. Y. Zhang et al., 2019). The higher concentrations of  $^{14}\text{C}$  labelled compounds remaining in the soil in the Glc and Glc + P treatments after 7 d (Fig. 7) may also have been a result of this N limitation, reflecting the *de novo* production of exoenzymes (proteases) required to mine extra N from SOM to sustain further growth (Wild et al., 2019). It is also possible that some of the glucose-derived C in the N-free treatments was allocated to storage C pools, a phenomenon that is induced by nutrient imbalance (Manzoni et al., 2021).

#### 4.4. Implications for SOC cycling

This study examined the basic biochemical responses of soil to labile organic and inorganic substrate inputs. While the general processes of mineralisation and metabolism are well defined in biology particularly for simple, ubiquitous molecules (i.e., glucose), nutrient stoichiometry is also a key factor in determining ability of the community to mineralise available compounds (Karhu et al., 2022; Lehmann et al., 2020). The biochemical response (carbohydrates, amino acids and fatty acids) of the whole soil microbiome in this study was similar to the biochemical response of a community in simpler environments (Jenkins et al., 2017; Swenson et al., 2018). Together, these demonstrate the ability of metabolomic fingerprinting as a tool to provide fine scale insight into shifts in soil biogeochemical cycling. The value of this data is extremely high as small organic molecule cycling underpins many biogeochemical processes in soil yet remains relatively unexplored, and greater understanding of small organic molecule dynamics is critical in understanding SOM cycling and C storage.

As plants develop, they have been shown to influence and select their soil microbial community through rhizosphere engineering (Sasse et al., 2018). Root exudates provide the substrate for soil microbial growth and are hotspots of interspecies interactions and biochemical cycling (Nadarajah, 2016; Canarini et al., 2019). While this experiment examined the fundamental response of the soil microbial community to relatively low concentrations of nutrient inputs, the field environment is vastly more complex than the microcosms examined here. Metabolomics has already been identified as a nascent field with potential for the study of the complex relationships within the rhizosphere (Mhlongo et al., 2018; Oburger and Jones, 2018). However, further work must establish the effect of nutrient addition on the competition and interactions between plants and the soil microbial community. These experiments must also consider the extraction method used, e.g., water, where the water-soluble fraction is most likely to yield the most representative of compounds readily accessible to the biological community (exo-metabolites), or a solvent extraction, which will yield more extensive intra- and exo-metabolites (Swenson et al., 2015).

Finally, here we used glucose as a simple, labile substrate, however, in the natural environment glucose would typically be accompanied by the release of other monomers (e.g., organic acids, amino acids) and oligomers (e.g., oligopeptides, oligosaccharides). For example, cellobiose, as a product of cellulose degradation, is one of the most ubiquitous and abundant disaccharides in soil, which can then be further broken down to glucose (Schellenberger et al., 2011; Chmadowska et al., 2016). Further studies of the soil microbial community's metabolic response to complex mixtures of organic compounds are therefore recommended.

#### 5. Conclusions

Addition of labile nutrients in stoichiometrically balanced (and unbalanced) ratios led to significant, rapid (<48 h) changes within the soil metabolome, as well as a difference in cumulative soil respiration rates over 7 days. Treatments with a combination of glucose and mineral elements tended to have a greater effect on the soil metabolism than glucose alone and in most cases, this was attributed to an elevated microbial activity as nutrient limitations were alleviated. The most

profound of these changes was the significant increase in FAs under Glc + P treatment likely attributed to increased oxidative phosphorylation, while a relative N deficit prevented lipid metabolism and utilisation. Treatments without N addition had significantly lower cumulative soil respiration rates over 7 days, while P substrate addition had no significant impact on soil respiration rates, suggesting N was the main nutrient limiting microbial growth in this soil (after C). Inorganic nutrient enrichment of soils is likely to have substantial implications for labile and recalcitrant C cycling and microbial resource partitioning within the soil system. Understanding the fundamental changes in small molecule cycling is therefore likely to improve our knowledge of both, chemical ecology and soil and microbial function. Further research is suggested to better understand metabolic and biochemical changes in soil microbial communities with regard to C inputs from plants (particularly in the rhizosphere) and under more complex substrate mixtures.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2022.108779>.

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