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Nutrient enrichment induces a shift in dissolved organic carbon (DOC) metabolism in oligotrophic freshwater sediments

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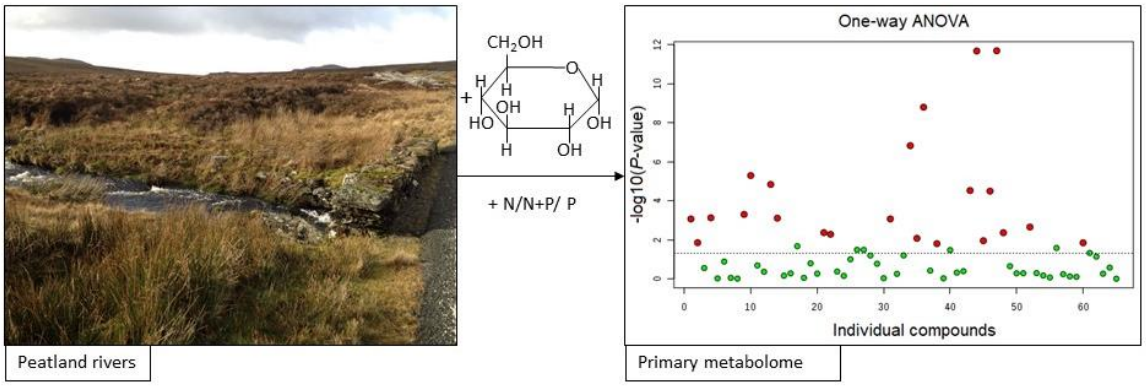
Abstract

Dissolved organic carbon (DOC) turnover in aquatic environments is modulated by the presence of other key macronutrients, including nitrogen (N) and phosphorus (P). The ratio of these nutrients directly affects the rates of microbial growth and nutrient processing in the natural environment. The aim of this study was to investigate how labile DOC metabolism responds to changes in nutrient stoichiometry using ^{14}C tracers in conjunction with untargeted analysis of the primary metabolome in upland peat river sediments. N addition led to an increase in ^{14}C-glucose uptake, indicating that the sediments were likely to be primarily N limited. The mineralization of glucose to $^{14}CO_2$ reduced following N addition, indicating that nutrient addition induced shifts in internal carbon (C) partitioning and microbial C use efficiency (CUE). This is directly supported by the metabolomic profile data which identified significant differences in 22 known metabolites (34 % of the total) and 30 unknown metabolites (16 % of the total) upon the addition of either N or P. ^{14}C-glucose addition increased the production of organic acids known to be involved in mineral P dissolution (e.g. gluconic acid, malic acid). Conversely, when N was not added, the addition of glucose led to the production of the sugar alcohols, mannitol and sorbitol, which are well known microbial C storage compounds. P addition resulted in increased levels of several amino acids (e.g. alanine, glycine) which may reflect greater rates of microbial growth or the P requirement for coenzymes required for amino acid synthesis. We conclude that inorganic nutrient enrichment in addition to labile C inputs has the potential to substantially alter in-stream biogeochemical cycling in oligotrophic freshwaters.

Graphical abstract



Keywords Metabolic profiling • Dissolved organic matter • DOM processing • Nutrient availability • Stoichiometry

1. Introduction

Carbon (C), nitrogen (N) and phosphorus (P) are the nutrients which most limit primary production and microbial growth in freshwater ecosystems (Hill et al. 2014). For dissolved organic nutrients in particular, the C, N and P cycles are inextricably linked as they can constitute parts of the same compound, however, there is still limited information on the composition of these molecules and how these cycles interact (Creamer et al. 2014; Swenson et al. 2015; Yates et al. 2019). Defined as the compounds that pass through a 0.45 µm filter, dissolved organic matter (DOM) can be a key transport mechanism for nutrients in terrestrial environments and a source of energy for aquatic communities in low-nutrient status waters (Thurman 1985; Minor et al. 2014; Worden et al. 2015; Yates et al. 2016). However, DOM has also been implicated in altering the bioavailability of pollutants (e.g. heavy metals), reducing the amount of aquatic oxygen via biological consumption, and forming carcinogens during the chlorination of drinking water (Matalinen et al. 2011; Smith et al. 2012; Kováčik et al. 2018).

Previous studies have suggested that the rates of N and P cycling are inter-related due to the potential of P limitation to develop under high N availability; both are also closely linked in terms of their impact on organic carbon (OC) processing under different nutrient statuses (Pilkington et al. 2005). Although aquatic P concentrations are decreasing in the EU following the implementation of the Urban Waste Water Treatment Directive, both C and N fluxes to coastal waters are increasing globally due to increasing C export from catchment headwaters and the inefficient use of fertilisers in agriculture, respectively (Evans et al. 2008; Vitousek et al. 2009). Although increasing inorganic nutrients have the potential to increase autochthonous DOC production in rivers, this may not necessarily lead to an increase in labile C due to the enhancement of microbial growth and rates of organic matter degradation (Stanley et al. 2011). The impact of inorganic inputs will therefore vary with changing nutrient status, as rivers move from being N/P limited to N/C limited from headwaters to the sea (Jarvie et al. 2018).

Spatial and temporal shifts in nutrient inputs to aquatic systems will affect the in-stream stoichiometry of the DOM pool (Yates et al. 2019). This is likely to have a particular impact on river sediments, as the primary interface between the water column, hyporheic and groundwater flows, where the majority of nutrient and water exchange takes place (Boano et al. 2014). Based on the current literature, it is not clear how changes to nutrient stoichiometry in riverine sediments impact aquatic DOC metabolism; this paper aims to investigate the microbial response to changes in nutrient limitation. Previous studies investigating potential nutrient limitation have adopted a range of approaches including the modelling or direct measurement of nutrient chemistry in the water and the use of fluorescence properties or enzyme activity assays as a proxy for nutrient metabolism (Hill et al. 2012; Jarvie et al. 2018; Stutter et al. 2018; Luo and Gu 2018). However, direct measurement of C usage under different nutrient loading conditions has largely been limited to studies of soils and riparian areas (Creamer 2014; Heuck et al. 2015; de Sosa 2018). Here, we used the addition of a simple ^{14}C-

labelled organic compound (glucose) to measure the uptake and transformation of labile C under different nutrient-limited conditions. In addition, untargeted metabolomics using gas chromatography/mass spectrometry (GC/MS) was used to identify changes in C metabolism. In comparison to other methods, GC/MS has well-established spectral databases available for a range of metabolites and has previously been used for a range of environmental metabolomics applications including environmental stress, plant-animal interactions, ecotoxicology and ecophysiology (Bundy et al. 2008; Macel et al 2010; Viant and Somer 2013; Swenson et al. 2015).

The aims of this study were therefore to: 1) determine whether removing nutrient limitation increased microbial removal of low-molecular weight C from a high C, low inorganic N and P environment, and 2) identify any changes in C metabolism following the addition of inorganic N and P on intrinsic and newly formed extracellular compounds. The results were then used to assess the impact of inorganic nutrient enrichment on labile DOC processing in low-nutrient status river systems.

2. Materials and methods

2.1 Field site

Sediments were collected mid-stream from four independent sites within the Migneint sub-catchment of the Conwy catchment, North Wales in the summer of 2017. The Migneint is an area of upland blanket peat bog supporting acid heathland vegetation (e.g. *Calluna vulgaris*, *Vaccinium myrtillus*) and low intensity sheep production (<0.05 livestock units ha^{-1}). It has an approximate elevation of 400 m and a mean annual temperature of 6.42 ± 0.05 °C and annual rainfall of 2000-2500 mm (Emmett et al. 2016; Supplementary Fig. S1). It is an oligotrophic system with high mean annual DOC concentrations (>20 mg L^{-1}), low total N concentrations

116 (<0.4 mg N L^{-1}) and ultra-low total P concentrations (<10 μg P L^{-1}) (Yates et al. 2019) and can
117 be either N or P limited depending on seasonality (Emmett et al. 2016). Characteristics of the
118 sediments are presented in Table 1. After collection, sediment samples were kept on ice in the
119 dark during transportation to the laboratory and analysed within 24 h.

120

121 **Table 1** Characteristics of the sediment samples used in the study. Values represent
122 means ± SEM, $n = 4$ (from Brailsford et al. 2019).

	Mean sediment characteristic
$pH_{(H_2O)}$	4.75 ± 0.05
Electrical conductivity$_{(H_2O)}$ (μS cm^{-1})	15 ± 2
Moisture content (%)	80.3 ± 3.6
Silt content (%)	5.2 ± 1.3
Clay content (%)	0.7 ± 0.3
Sand content (%)	94.1 ± 1.6
Total C (mg C kg^{-1} sediment)	250 ± 42
Total free carbohydrates (mg C kg^{-1} wet sediment)	0.61 ± 0.08
Total phenols (mg C kg^{-1} wet sediment)	7.26 ± 2.58
Total N (mg N kg^{-1} sediment)	8.36 ± 1.28
NH_4^+ (mg N kg^{-1} wet sediment)	5.1 ± 1.8
NO_3^- (mg N kg^{-1} wet sediment)	0.91 ± 0.26
Total amino acids (mg N kg^{-1} wet sediment)	0.20 ± 0.01
Molybdate-reactive P (mg P kg^{-1} wet sediment)	0.21 ± 0.05

Phospholipid-derived fatty acid (PLFA) analysis	
Total PLFA biomass (nmol g^{-1} sediment)	621 ± 180
Gram– bacteria (%)	47.8 ± 0.7
Gram+ bacteria (%)	30.1 ± 1.9
Actinomycetes (%)	8.27 ± 2.09
Fungi (%)	4.51 ± 1.23
Eukaryote (%)	6.35 ± 2.64

Values represent means ± SEM, $n = 4$ independent sites. All values are expressed on a dry weight basis unless otherwise stated.

2.2 ^{14}C-labelled nutrient metabolism assays

Nutrient depletion was measured as follows: 2 g sediment was added to a sterile 15 mL polypropylene centrifuge tube (Corning, NY, USA). Subsequently, 200 µL of ^{14}C-[U]-glucose (Lot 3632475; PerkinElmer Inc., MA, USA) was added to the sediment surface to give a final C concentration of 1200 µM (500 µM glucose) (0.4 kBq ml^{-1}activity). This glucose was either added alone or in the presence of N, or P, or N + P at a C:N:P stoichiometric ratio of 60:7:1 ratio based on the C:N:P ratio of the microbial biomass (Cleveland and Liptzin 2007). The N was added as NH_4NO_3 and P was added as NaH_2PO_4. The pH of the solutions were similar to those of the background pH of the peat sediments (approximately pH 5) and were therefore not altered prior to addition. Glucose was chosen as it represents a major input of C into freshwater systems either in a monomeric or polymeric form and is thought to be used by almost all organisms within the microbial community (Rinnan and Bååth 2009). Although glucose may ferment in anaerobic systems, the samples in this experiment were contained in sterile centrifuge tubes with a large headspace and would have been subject to gaseous exchange at each sampling time point. The concentration of glucose was chosen based on the likely amount

that might be released into sediment porewater when microbial or plant cells die (Jones and Darrah 1996; Teusink et al. 1998).

To monitor the cumulative depletion of glucose in the sediment, samples were extracted at known times (0, 2, 4, 6, 24, 48 h) after glucose addition. The extraction was conducted by adding 10 mL ice-cold 1 M KCl to the sediment and shaking (200 rev min^{-1}) for 15 min, followed by centrifugation for 15 min at 20,817 *g*. A 1 mL aliquot of the supernatant was then recovered and mixed with HiSafe 3 scintillation fluid (PerkinElmer Inc.) and the amount of ^{14}C present determined with a Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK). Biological changes in sediment were accounted for by running the same experiments with sediments in which bacterial activity was inhibited by the addition of 100 µL 0.04 % formaldehyde (Tuominen et al. 1994). Respiration was also measured using a 1 M NaOH to capture any $^{14}CO_2$ released by the microbial biomass.

Three technical replicate samples were run for each treatment at each site. These technical replicates were subsequently averaged to provide a site mean upon which subsequent data analysis was performed. Statistical analysis was carried out in SPSS v22 (IBM UK Ltd., Portsmouth, UK). A two-way mixed analysis of variance (ANOVA) with Tukey's post-hoc testing was used to identify differences in treatments over time, with a significance level set at $P < 0.05$. One-way analysis of variance was used to detect differences between treatments at individual time-points. Graphs were produced using Sigmaplot v13.0 (Systat Software Inc., San Jose, CA USA).

2.3 N and P sorption/desorption

The amount of instant N and P sorption on the sediment's solid phase were determined using methods outlined by Marsden et al. (2016) (Supplementary Fig. S2). Briefly, a range of

concentrations of N as NH_4NO_3 (0, 2, 10, 50, 100, 200 mg L^{-1}) and P as Na_2HPO_4 (0, 2, 10, 50 mg L^{-1}) in 100 µL 0.01 M $CaCl_2$ were added to 0.5 g fresh sediment. Following this, 5 mL 0.01 M $CaCl_2$ was added to the sample and shaken (200 rev min^{-1}) for 15 min, followed by centrifugation (20,817 *g*; 15 min). Subsequently, the total N remaining in the supernatant were determined using a Multi N/C 2100S analyser (AnalytikJena, Jena, Germany) and molybdate-reactive P was measured according to Murphy and Riley (1962).

In addition, the natural and maximal sorption/desorption of P from the sediment's solid phase were measured using a ^{33}P tracer method (de Sosa et al. 2018; Supplementary Fig. S3). Briefly, a range of concentrations (0, 2, 10, 50 µM) P as Na_2HPO_4 in 100 µL deionised water spiked with ^{33}P (0.2 kBq ml^{-1} final activity; PerkinElmer, MA, USA) were added to 1 g fresh sediment and measuring the rates of instant sorption (<1 min) and subsequent desorption (30, 60 min). After the specified amount of time, either 5 mL of deionised water (to measure natural sorption/desorption) or 0.5 M citric acid (to measure maximal desorption capacity; De Luca et al. 2015) was added to the sample and shaken (200 rev min^{-1}) for 15 min, followed by centrifugation (20,817 *g*; 15 min). Subsequently 0.5 mL supernatant was mixed with Optiphase HiSafe scintillation cocktail (4 mL; PerkinElmer) and the remaining ^{33}P quantified on a Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK).

2.4 Untargeted analysis of primary metabolism

Nutrients in the same concentrations described above were added in 200 µL ultra-pure water (18 MΩ resistance) to 2 g of sediment in 1.5 mL microcentrifuge tubes (glucose, glucose + N, glucose + N + P, glucose + P). Control sediment samples had only ultra-pure water added to the sediment, while the blanks contained only ultra-pure H_2O (i.e. no sediment). Samples were snap frozen in liquid N_2 after 0 and 24 h and stored at -80 °C until shipping on dry ice to the

West Coast Metabolomics Center at UC Davis where samples were extracted using 3 : 3 : 2 (v/v/v) acetonitrile : isopropanol : water. Untargeted analysis of primary metabolism was carried out using an ALEX-CIS GC-TOF-MS (Gerstel Inc., Linthicum, MD; Supplementary Document 1).

Data analysis of identified and unknown compounds was carried out using MetaboAnalyst v3.5 and 4.0 (Xia and Wishart 2016; Chong et al. 2018). Prior to analysis, data were both $\log_{10}$ transformed and scaled using Pareto scaling (mean-centred and divided by the square root of the standard deviation of each variable). No missing value estimations of feature filtering were applied. Metabolic pathway maps were created using KEGG Mapper v3.1 (Kanehisa et al. 2012).

3. Results

3.1 ^{14}C-labelled glucose depletion and metabolism

The co-addition of N and P was found to have a significant effect on the uptake of ^{14}C-labelled glucose from the sediment over time (two-way mixed ANOVA, P = 0.002; Fig. 1; Supplementary Table S2). All treatments had a rapid response to the addition of labile C, however, overall uptake of C after 24 h was 13.7 ± 2.3 % higher for the glucose + N treatment compared to the glucose only and glucose + N + P treatments (one-way ANOVA, $F_{3,12}$ = 7.496, P = 0.004).

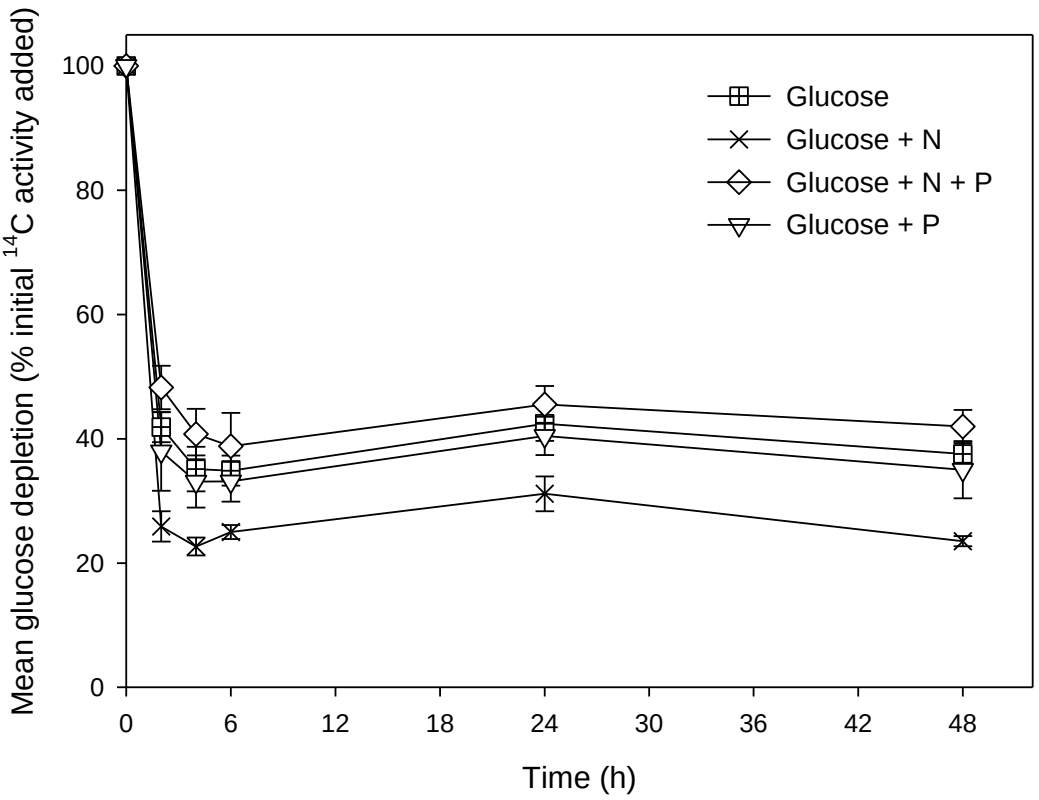
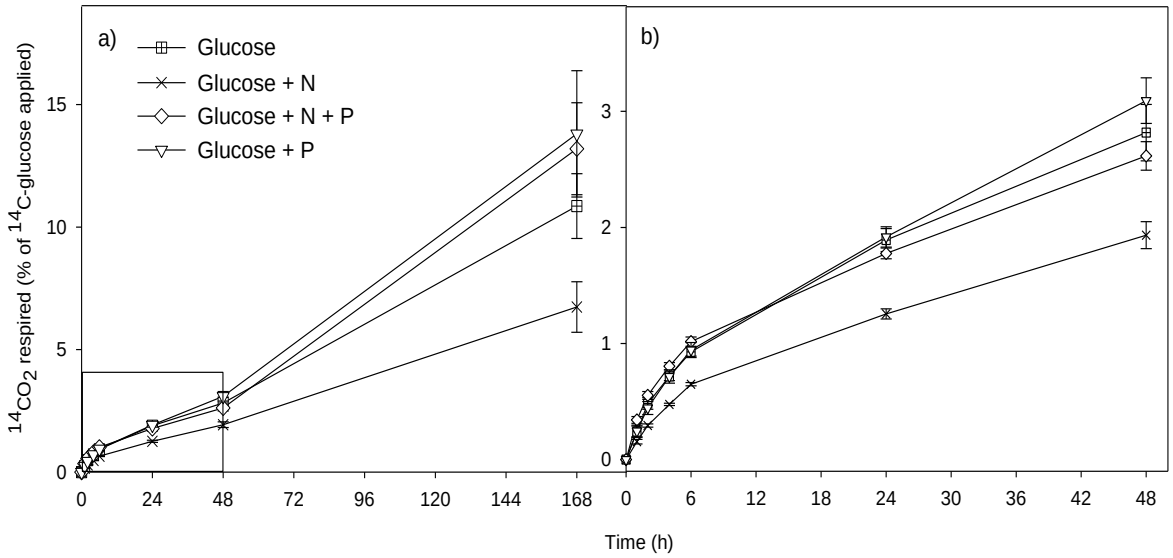


Figure 1. ^{14}C-labeled glucose depletion over time. The ^{14}C-glucose, in addition to N added as NH_4NO_3 and P added as NaH_2PO_4 was added to an oligotrophic river sediment and depletion measured over time. Values represent means ± SEM, n = 4.

A significant interaction between experimental treatment and time was observed for the percentage of $^{14}CO_2$ respiration by the sediment microbial communities (two-way mixed ANOVA, P = 0.018; Fig. 2; Supplementary Table S2). The initial rate of $^{14}CO_2$ respiration was lower for the glucose + N treatment in comparison to all other treatments from 4 to 24 h (one-way ANOVA, $P \leq 0.001$ in each case; Supplementary Table S1; Fig. 2). At 24 h, the rate of $^{14}CO_2$ respiration was still lower in the glucose + N treatment in comparison to the glucose and glucose + P treatments, with the glucose + N + P treatment falling in between (one-way ANOVA, $F_{3,12}$ = 5.804, P = 0.011; Fig. 2). By the final time-point, 168 h there were no

222 detectable differences between treatments, likely due to the increased variation observed at this
223 time-point (one-way ANOVA, $F_{3,12}$= 2.371, P = 0.122; Fig. 2).



224

225 **Figure 2.** Microbial transformation of ^{14}C-glucose to $^{14}CO_2$ over time. The ^{14}C-glucose, in
226 addition to N added as NH_4NO_3 and P added as NaH_2PO_4 was added to an oligotrophic river
227 sediment and transformation to $^{14}CO_2$ measured over a) 168 h and b) 48 h. Panel b) is derived
228 from the data shown in panel a). Values represent means ± SEM (n = 4). The legend is the
229 same for both panels.

230

326 **3.2 Non-targeted metabolite analysis by GC-MS**

327 Non-targeted metabolite analysis was conducted on four sediment samples of each nutrient
328 addition treatment after 24 h and the control from the beginning of the experiment. To identify
329 the main factors driving change in the metabolome, PLS discriminant analysis (PLS-DA) was
330 conducted with approximately 1040 peaks of identified non-targeted GC-MS metabolites
331 (Fig. 3). The first component of the PLS-DA results (63.8 % variance) likely reflects the
332 difference in nutrient addition. The treatments separated into three distinct clusters: the control

treatment consisting of the intrinsic metabolome of the river sediments, glucose + P addition and a final cluster containing the other three nutrient addition treatments (glucose, glucose + N and glucose + N + P). There was a complete overlap between the glucose + N and glucose + N + P treatments, indicating that the addition of N induces a similar response regardless of other nutrients added. The glucose only treatment appears to fall between the treatments with glucose + N addition and the glucose + P treatment.

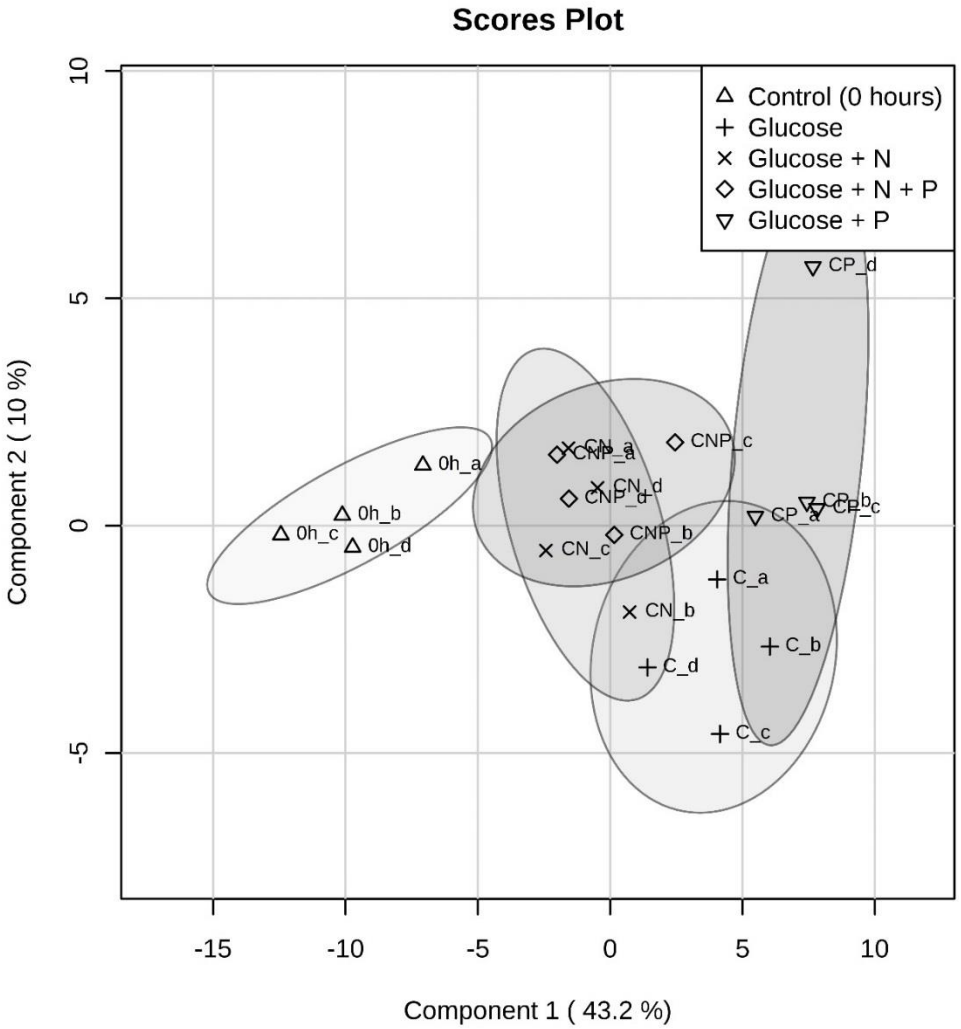


Figure 3 PLS-DA (PLS discriminant analysis) scores plot for the metabolome of control samples ($+_dH_2O$ only) at 0 h and all treatments at 24 h after the addition of treatments (+ glucose (C); + glucose and N (CN); + glucose, N and P (CNP) and + glucose and P (CP). Lower case letters represent individual sampling sites.

In general, the glucose and glucose + P treatment were found to cluster closely together in terms of Euclidean distance, whilst the glucose + N and glucose + N + P treatments formed their own separate cluster (Fig. 4). The control samples clustered separately to all other treatments. The two N-containing treatments were found to overlap with the other treatments for samples from site B.

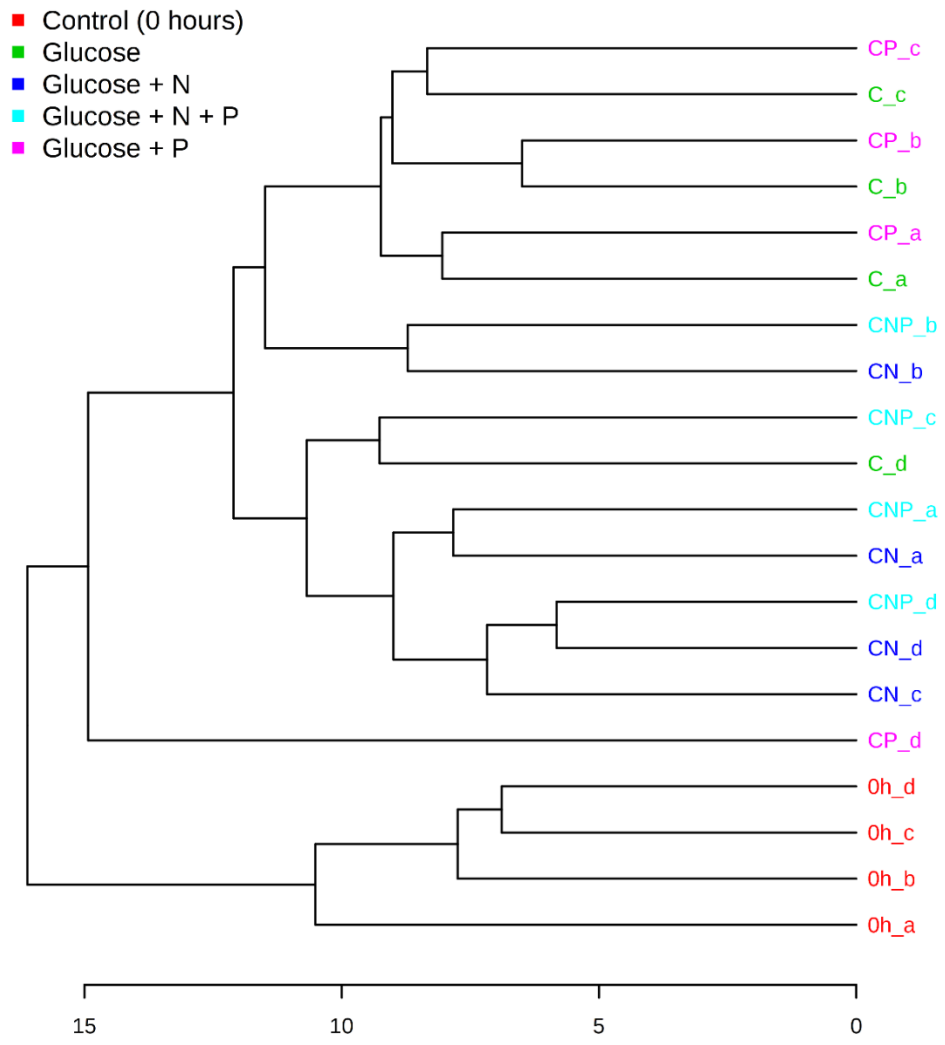
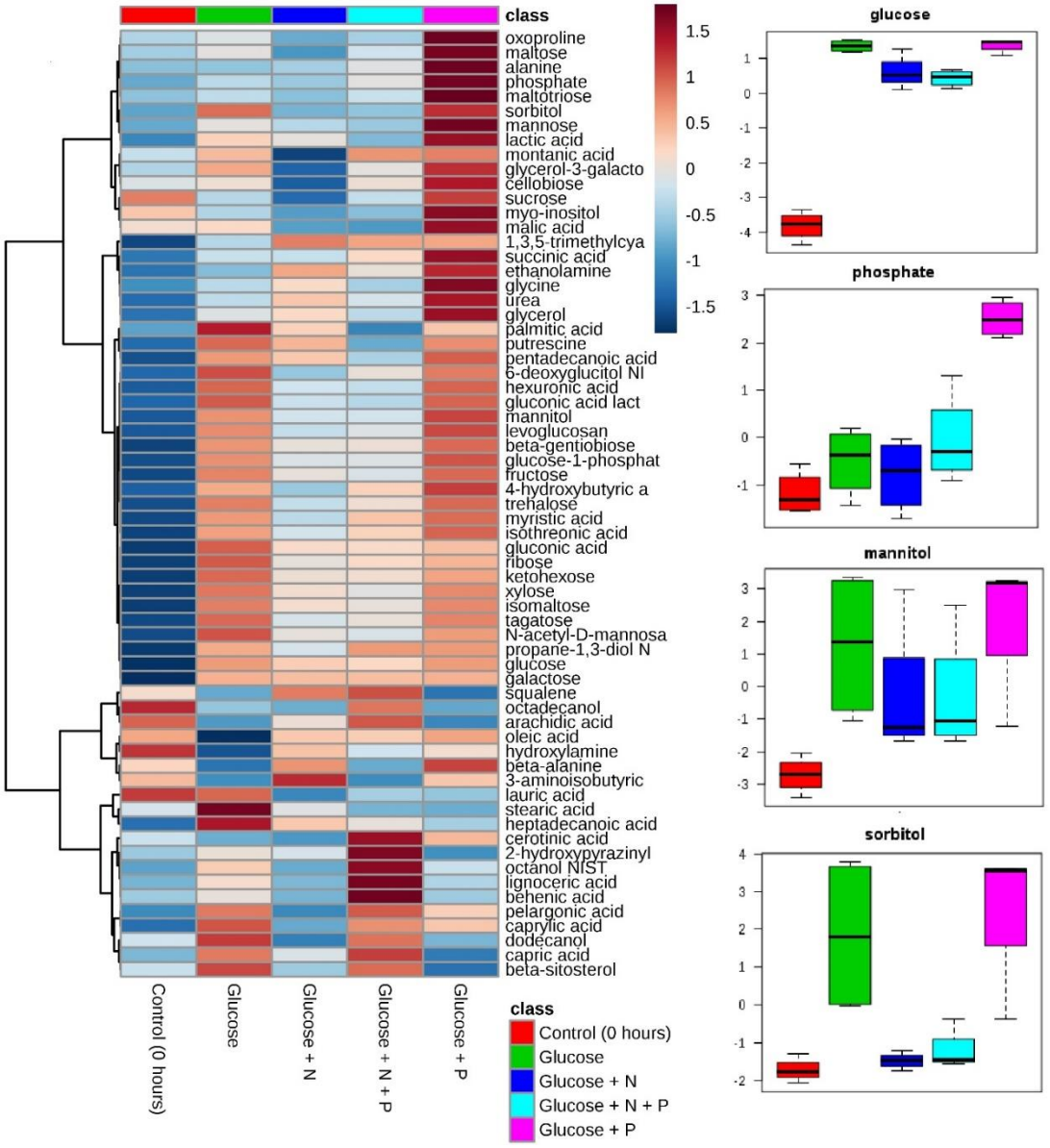


Figure 4 Similarity dendrogram clustered by Euclidean distance (horizontal axis) for the metabolome of control samples ($+_dH_2O$ only) at 0 h and all treatments at 24 h after the addition of either glucose alone (C), glucose + N (CN), glucose + N + P (CNP), and glucose + P (CP). Lower case letters represent individual sampling sites.

3.3 Compound-specific analysis

All treatments saw an increase in metabolite production after 24 h. However, the CNP treatment saw the greatest increase in the number of metabolites present. Of the metabolites identified, the key pathways they were attributed to included sugar metabolism, amino acid synthesis and lipid metabolism (Supplementary Fig. S4). Treatments with no N addition saw a significant increase in the production of sugar alcohols such as sorbitol (one-way ANOVA, $P < 0.05$; Fig. 5; Supplementary Table S3). There were also higher concentrations of glucose and other sugars such as fructose, ketohexose, tagatose and glucose-1-phosphate in the sediment for treatments with no N addition, suggesting that glucose had been utilised internally at a slower rate in the absence of N (one-way ANOVA for glucose, $P < 0.05$; Fig. 5). In addition, there was a higher amount of products from anaerobic respiration (e.g. lactic acid) suggesting some fermentative metabolism within the sediment.

In comparison to the glucose + N + P treatment, the glucose + P treatment had a higher proportion of added phosphate present after 24 h, indicating that less of the added phosphate had been utilised in the absence of N (one-way ANOVA, $P < 0.05$; Fig. 5). The glucose + P treatment also showed a significant elevation in the amount of alanine present, and a similar, non-significant elevation in the amount of glycine present in comparison to the other treatments, including the control. This, in conjunction with an increased concentration of urea in comparison to other treatments, a known product of amino acid metabolism, could indicate amino acid synthesis (one-way ANOVA, $P < 0.05$; Fig. 5).



374

375 **Figure 5.** Hierarchical clustering heat map of the normalized metabolite log response in
376 sediment primary metabolome for each treatment (0 h (control), 24 h (glucose, glucose + N,
377 glucose + N + P, glucose + P). Metabolites which significantly decrease are displayed in blue,
378 while metabolites which significantly increased are displayed in red. The brightness of each
379 colour corresponds to the magnitude of the difference when compared with average value.
380 Clustering of the roots nutrient treatments is depicted by the dendrogram at the top. Clustering
381 of the metabolites is depicted by the dendrogram at the left. Metabolites are clustered by
382 similarity according to Pearson correlation values. Boxplots of individual metabolites mean ±
383 1 S.D.

4. Discussion

4.1 Use of LMW carbon with nutrient limitation

The depletion of ^{14}C-glucose from solution was rapid in all treatments; after 48 h between 20-40 % of ^{14}C-glucose remained in the sediment, depending on the treatment (Fig. 2). Although the results of the metabolomic analyses demonstrated that a proportion of the glucose added remained unchanged in solution, it is likely that some of the ^{14}C-glucose remaining had been transformed following uptake by microbes or through the action of extracellular enzymes (Fig. 5; Wetzl 1992; Findlay and Sinsabaugh 1999). However the treatments without N addition had lactic acid present after 48 h, so it is possible that some glucose fermentation could have taken place in these treatments (Fig. 5). The concentration of glucose added was such that glucose would be available in excess to the microbial population of the sediment without fully saturating the system, based on previously observed glucose uptake in sediments from the same upland peat sites (Brailsford et al. 2019). The amount of C added was approximately 4 orders of magnitude higher than the baseline concentrations of C present as total free carbohydrates and 5 orders of magnitude higher than concentration in overlying river waters (0.61 ± 0.08 mg C kg wet sediment^{-1} and 0.09 ± 0.02 mg C L^{-1} respectively; Brailsford et al. 2019).

Cumulative $^{14}CO_2$ respiration over the duration of the experiment for the upland river sediments was an order of magnitude lower than rates previously observed for lowland agricultural soils (Hill et al. 2008; Rousk et al. 2014). This could be indicative of a higher C use efficiency (CUE), which is typical of areas of upland blanket peat bog and of aquatic systems in comparison to terrestrial systems (Kayranli et al. 2010; Sinsabaugh et al. 2013). This apparent high CUE may reflect the partitioning of glucose-C into storage metabolites which may be mineralised later. This is supported by the near-linear rate of $^{14}CO_2$ accumulation over 7 d despite most of the ^{14}C being depleted from the sediment pore water very quickly

(within 6 h). There were no detectable differences in cumulative $^{14}CO_2$ respiration after 168 h, although the addition of glucose + N resulted in the lowest initial rate of $^{14}CO_2$ respiration (first 24 h) in comparison to the other treatments. This was in contrast to the rate of ^{14}C-glucose depletion from the sediment after 24 h, where the glucose + N treatment had the highest rate of glucose depletion from the sediment in comparison to the glucose, and the glucose + P treatments, with the glucose + N +P treatment falling in between. The addition of N alongside P has previously been found to increase N loss from low-P systems after 48 h due to enhanced nitrification and denitrification processes, which could explain why the glucose + N + P treatment did not produce the same response as the glucose + N treatment (He and Dijksta 2015).

Oligotrophic peat systems can be either N or P limited depending on seasonality. In our study, the increased rate of C mineralisation in the N-enriched treatment, in conjunction with the timing of the current study (conducted in summer when N inputs from atmospheric deposition are at their lowest), indicate that the system was N limited at the time of the study (Elser et al. 2009; McGovern et al 2014; Emmett et al. 2016). After a rapid initial uptake in the glucose + N treatment, it is possible that P then became the growth-limiting nutrient, which could explain why despite the initial rapid uptake of glucose in the N addition treatment, overall C mineralisation to CO_2 was lower than for the other treatments. The addition of P alongside a C source has previously been observed to have no effect on or to even suppress C uptake in lowland agricultural soils, which has been attributed to a lack of P limitation and changes in soil chemistry, making conditions unfavourable to soil biota respectively (de Sosa et al. 2018). Alternatively, labile C could have entered an alternative C pool within the microbial biomass, which respires C at a slower rate (Glanville et al. 2016). As neither P addition nor the combination of N + P appeared to have an effect on the uptake of C into the biomass, it strongly suggests that the different nutrient treatments induced shifts in internal C partitioning.

4.2 Changes in primary metabolome with nutrient limitation

In terms of the primary metabolome, cluster analysis of known metabolites separated treatments into two distinct groups: control samples from the beginning of the experiment and a cluster consisting of the glucose, glucose + N, glucose + N + P treatments and glucose + P (Fig. 3). There was an almost complete overlap between the glucose + N and glucose + N + P treatments, indicating that N addition has elicited a similar response regardless of what other nutrients are added. This supports the evidence that the peat sediments were N limited at the time of sampling. There was also a partial overlap between the glucose and glucose + P treatments, which was also evident in the ^{14}C depletion and respiration measurements, where the response to the nutrients added could not be distinguished. Similar trends were detected when samples were clustered using Euclidean distance for known metabolites; the control (0 h no addition) treatment was a distinct cluster to the treatments with nutrient addition, whereby glucose and glucose + P treatments largely clustered together, as did the glucose + N and glucose + N + P treatments (Fig. 4).

4.3 Compound-specific metabolome trends

All treatments saw an increase in the relative concentration of glucose in their metabolome compared to the control (0 h), indicating that not all the glucose had been metabolised within the 48 h period. This corresponds to the ^{14}C-glucose depletion data where a proportion of the glucose added remained in the sediment after the same time period. However, a lower relative concentration of glucose remained in the sediment for the glucose + N and glucose + N + P treatments in comparison to the glucose only and glucose + P treatments, indicating that glucose may have been utilised at a slower rate in treatments that did not receive additional N. The glucose + N treatment also saw the highest rate of ^{14}C-glucose removal from the sediment

457 over the course of the experiment. In previous studies the addition of labile DOC compounds
458 has increased inorganic N uptake in similar upland headwater streams (Robbins et al. 2017)
459 and agricultural rivers (Johnson et al. 2012; Oveido-Vargas et al. 2013), therefore meeting this
460 demand through the provision of an inorganic N source is likely to have led to the increased
461 uptake of labile C observed here.

462 Phosphate utilisation by the sediment microbiome appeared to be higher in the glucose
463 + N + P treatment in comparison to the glucose + P treatment, with a greater concentration of
464 phosphate remaining in the former treatment (Fig. 5). Nitrogen addition to peat bogs has also
465 been observed to enhance P uptake in other studies (Williams and Silcock 2001). This increase
466 in P uptake following co-addition of P and N addition also indicates that the system was initially
467 N limited at the time of the study. The glucose-only treatment produced significantly higher
468 concentrations of gluconic acid, in addition to other weak organic acids such as malic acid,
469 compared to the control and other N addition treatments after 48 h (Fig. 3). Such compounds
470 have previously been demonstrated to be produced directly from glucose by microbes, in order
471 to encourage P dissolution from mineral surfaces (Stella and Halimi 2015; Chen et al. 2016).

472 The addition of inorganic nutrients (N and/or P) appeared to alter the metabolism of
473 glucose for use in other pathways. For example glucose + P addition increased the synthesis of
474 amino acids, including alanine and glycine, in addition to waste products from amino acid
475 synthesis such as urea. The process of amino acid synthesis requires several P-containing co-
476 enzymes, for example pyridoxal phosphate (PLP) which is required for transamination
477 reactions, indicating that the production of amino acids could be P-limited. In this experiment,
478 after 48 h higher concentrations of glucose, other sugars (fructose, ketohexose and tagatose)
479 and their derivatives were present in the treatments that did not receive N addition, suggesting
480 that glucose had been utilised at a slower rate in the absence of N. Treatments with no N
481 addition also saw a significant increase in the production of sugar alcohols such as mannitol

and sorbitol compared to control and N addition treatments; these compounds can act as storage compounds for microbial cells and may provide protection from cellular stress (Yu et al. 2016).

4.4 Critical evaluation of the untargeted metabolomics approach

Untargeted metabolomics using GC-MS has been the primary choice for environmental samples due to its relative affordability, the possibility of identifying specific compounds and the potential to produce quantitative results (Viant and Sommer 2013). Fragmentation spectra resulting from GC-MS can be screened against large databases which currently contain over 1000 metabolites (Kind et al. 2009). However, library building has been centred around medical and cell biology samples and the derivitisation required for GC-MS may bias the metabolite profile towards specific functional groups (Lin et al. 2006; Viant and Sommer 2013). In this study only ~35 % of fragmentation spectra detected could be matched to a metabolite. The inclusion of unknown metabolites in statistical analyses separated treatments in a similar manner compared to when unknown metabolites were excluded. However, when unknown metabolites were included, 59 % of the top 75 metabolites with the greatest differences between treatments were unidentified compounds (Supplementary Fig. S5-S7; Supplementary dataset 1). The primary metabolome presented in this study represents a single point in time, while C assimilation is a dynamic process and the metabolic profile may change over time following the initial uptake. Future work could combine study of the primary metabolome with more dynamic techniques such as the use of stable isotopes to trace C into different organism groups (Kaplan et al. 2008; Hotchkiss and Hall 2015).

5. Conclusions

The addition of N led to an increase in labile DOC uptake, which was evident in the reduction of sugars present in the metabolome of N addition treatments. In contrast, N addition corresponded with a decrease in CO_2 respiration over the duration of the experiment, indicating that N is required to allocate more C to storage and cell protection as opposed to respiration. When N and P were added simultaneously P uptake was enhanced compared to the addition of P only. A lack of N addition led to an increased production of storage compounds such as alcohol sugars, in addition to the synthesis of amino acids (glycine, alanine) and associated waste products. Due to the P-containing co-enzymes required for amino acid synthesis, this may be a P-limited process. The addition of labile C only led to specific increases in the production of organic acid-like compounds, which can aid P release from both organic and inorganic P held on the sediment's solid phase. These results provide an insight into the molecular mechanisms of nutrient enrichment in low-nutrient status rivers. We found that whilst nutrient stoichiometry is important for nutrient cycling N addition appears to be a key driver of changes to DOC metabolism in oligotrophic stream sediments.

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