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

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

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**24 Abstract**

25 Dissolved organic carbon (DOC) turnover in aquatic environments is modulated by the  
26 presence of other key macronutrients, including nitrogen (N) and phosphorus (P). The ratio of  
27 these nutrients directly affects the rates of microbial growth and nutrient processing in the  
28 natural environment. The aim of this study was to investigate how labile DOC metabolism  
29 responds to changes in nutrient stoichiometry using  $^{14}\text{C}$  tracers in conjunction with untargeted  
30 analysis of the primary metabolome in upland peat river sediments. N addition led to an  
31 increase in  $^{14}\text{C}$ -glucose uptake, indicating that the sediments were likely to be primarily N  
32 limited. The mineralization of glucose to  $^{14}\text{CO}_2$  reduced following N addition, indicating that  
33 nutrient addition induced shifts in internal carbon (C) partitioning and microbial C use  
34 efficiency (CUE). This is directly supported by the metabolomic profile data which identified  
35 significant differences in 22 known metabolites (34 % of the total) and 30 unknown metabolites  
36 (16 % of the total) upon the addition of either N or P.  $^{14}\text{C}$ -glucose addition increased the  
37 production of organic acids known to be involved in mineral P dissolution (e.g. gluconic acid,  
38 malic acid). Conversely, when N was not added, the addition of glucose led to the production  
39 of the sugar alcohols, mannitol and sorbitol, which are well known microbial C storage  
40 compounds. P addition resulted in increased levels of several amino acids (e.g. alanine, glycine)  
41 which may reflect greater rates of microbial growth or the P requirement for coenzymes  
42 required for amino acid synthesis. We conclude that inorganic nutrient enrichment in addition  
43 to labile C inputs has the potential to substantially alter in-stream biogeochemical cycling in  
44 oligotrophic freshwaters.

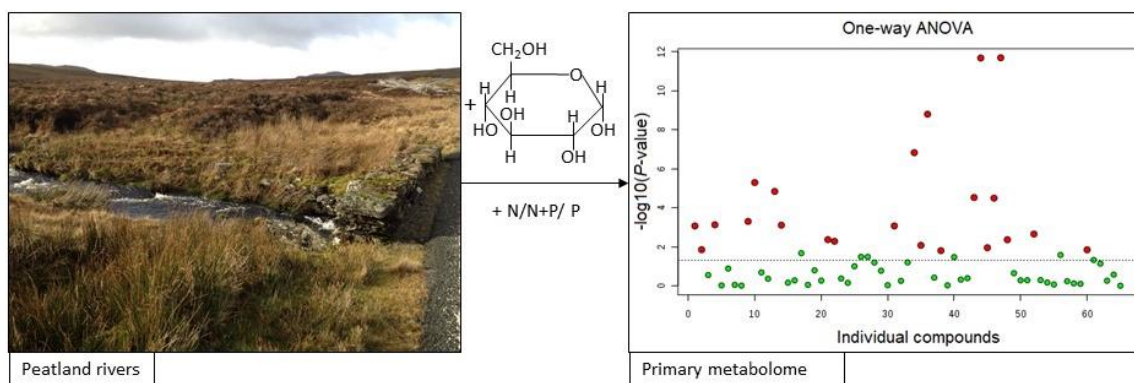
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48 **Graphical abstract**

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50

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

53

54 **1. Introduction**

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

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

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

92 labelled organic compound (glucose) to measure the uptake and transformation of labile C  
93 under different nutrient-limited conditions. In addition, untargeted metabolomics using gas  
94 chromatography/mass spectrometry (GC/MS) was used to identify changes in C metabolism.  
95 In comparison to other methods, GC/MS has well-established spectral databases available for  
96 a range of metabolites and has previously been used for a range of environmental metabolomics  
97 applications including environmental stress, plant-animal interactions, ecotoxicology and  
98 ecophysiology (Bundy et al. 2008; Macel et al 2010; Viant and Somer 2013; Swenson et al.  
99 2015).

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

106

## 107 **2. Materials and methods**

### 108 **2.1 Field site**

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

116 (<0.4 mg N L<sup>-1</sup>) and ultra-low total P concentrations (<10 µg P L<sup>-1</sup>) (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).

	<b>Mean sediment characteristic</b>
pH <sub>(H<sub>2</sub>O)</sub>	4.75 ± 0.05
Electrical conductivity <sub>(H<sub>2</sub>O)</sub> (µS cm <sup>-1</sup> )	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 <sup>-1</sup> sediment)	250 ± 42
Total free carbohydrates (mg C kg <sup>-1</sup> wet sediment)	0.61 ± 0.08
Total phenols (mg C kg <sup>-1</sup> wet sediment)	7.26 ± 2.58
Total N (mg N kg <sup>-1</sup> sediment)	8.36 ± 1.28
NH <sub>4</sub> <sup>+</sup> (mg N kg <sup>-1</sup> wet sediment)	5.1 ± 1.8
NO <sub>3</sub> <sup>-</sup> (mg N kg <sup>-1</sup> wet sediment)	0.91 ± 0.26
Total amino acids (mg N kg <sup>-1</sup> wet sediment)	0.20 ± 0.01
Molybdate-reactive P (mg P kg <sup>-1</sup> wet sediment)	0.21 ± 0.05

### Phospholipid-derived fatty acid (PLFA) analysis

Total PLFA biomass (nmol g <sup>-1</sup> 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

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

125

### 126 2.2 <sup>14</sup>C-labelled nutrient metabolism assays

127 Nutrient depletion was measured as follows: 2 g sediment was added to a sterile 15 mL  
 128 polypropylene centrifuge tube (Corning, NY, USA). Subsequently, 200 µL of <sup>14</sup>C-[U]-glucose  
 129 (Lot 3632475; PerkinElmer Inc., MA, USA) was added to the sediment surface to give a final  
 130 C concentration of 1200 µM (500 µM glucose) (0.4 kBq ml<sup>-1</sup> activity). This glucose was either  
 131 added alone or in the presence of N, or P, or N + P at a C:N:P stoichiometric ratio of 60:7:1  
 132 ratio based on the C:N:P ratio of the microbial biomass (Cleveland and Liptzin 2007). The N  
 133 was added as NH<sub>4</sub>NO<sub>3</sub> and P was added as NaH<sub>2</sub>PO<sub>4</sub>. The pH of the solutions were similar to  
 134 those of the background pH of the peat sediments (approximately pH 5) and were therefore not  
 135 altered prior to addition. Glucose was chosen as it represents a major input of C into freshwater  
 136 systems either in a monomeric or polymeric form and is thought to be used by almost all  
 137 organisms within the microbial community (Rinnan and Bååth 2009). Although glucose may  
 138 ferment in anaerobic systems, the samples in this experiment were contained in sterile  
 139 centrifuge tubes with a large headspace and would have been subject to gaseous exchange at  
 140 each sampling time point. The concentration of glucose was chosen based on the likely amount



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

143 To monitor the cumulative depletion of glucose in the sediment, samples were extracted  
144 at known times (0, 2, 4, 6, 24, 48 h) after glucose addition. The extraction was conducted by  
145 adding 10 mL ice-cold 1 M KCl to the sediment and shaking (200 rev min<sup>-1</sup>) for 15 min,  
146 followed by centrifugation for 15 min at 20,817 g. A 1 mL aliquot of the supernatant was then  
147 recovered and mixed with HiSafe 3 scintillation fluid (PerkinElmer Inc.) and the amount of <sup>14</sup>C  
148 present determined with a Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton  
149 Keynes, UK). Biological changes in sediment were accounted for by running the same  
150 experiments with sediments in which bacterial activity was inhibited by the addition of 100 µL  
151 0.04 % formaldehyde (Tuominen et al. 1994). Respiration was also measured using a 1 M  
152 NaOH to capture any <sup>14</sup>CO<sub>2</sub> released by the microbial biomass.

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

161

### 162 **2.3 N and P sorption/desorption**

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

165 concentrations of N as  $\text{NH}_4\text{NO}_3$  (0, 2, 10, 50, 100, 200  $\text{mg L}^{-1}$ ) and P as  $\text{Na}_2\text{HPO}_4$  (0, 2, 10, 50  
166  $\text{mg L}^{-1}$ ) in 100  $\mu\text{L}$  0.01 M  $\text{CaCl}_2$  were added to 0.5 g fresh sediment. Following this, 5 mL 0.01  
167 M  $\text{CaCl}_2$  was added to the sample and shaken (200  $\text{rev min}^{-1}$ ) for 15 min, followed by  
168 centrifugation (20,817 g; 15 min). Subsequently, the total N remaining in the supernatant were  
169 determined using a Multi N/C 2100S analyser (AnalytikJena, Jena, Germany) and molybdate-  
170 reactive P was measured according to Murphy and Riley (1962).

171 In addition, the natural and maximal sorption/desorption of P from the sediment's solid  
172 phase were measured using a  $^{33}\text{P}$  tracer method (de Sosa et al. 2018; Supplementary Fig. S3).  
173 Briefly, a range of concentrations (0, 2, 10, 50  $\mu\text{M}$ ) P as  $\text{Na}_2\text{HPO}_4$  in 100  $\mu\text{L}$  deionised water  
174 spiked with  $^{33}\text{P}$  (0.2  $\text{kBq ml}^{-1}$  final activity; PerkinElmer, MA, USA) were added to 1 g fresh  
175 sediment and measuring the rates of instant sorption (<1 min) and subsequent desorption (30,  
176 60 min). After the specified amount of time, either 5 mL of deionised water (to measure natural  
177 sorption/desorption) or 0.5 M citric acid (to measure maximal desorption capacity; De Luca et  
178 al. 2015) was added to the sample and shaken (200  $\text{rev min}^{-1}$ ) for 15 min, followed by  
179 centrifugation (20,817 g; 15 min). Subsequently 0.5 mL supernatant was mixed with Optiphase  
180 HiSafe scintillation cocktail (4 mL; PerkinElmer) and the remaining  $^{33}\text{P}$  quantified on a Wallac  
181 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK).

182

#### 183 **2.4 Untargeted analysis of primary metabolism**

184 Nutrients in the same concentrations described above were added in 200  $\mu\text{L}$  ultra-pure water  
185 (18  $\text{M}\Omega$  resistance) to 2 g of sediment in 1.5 mL microcentrifuge tubes (glucose, glucose + N,  
186 glucose + N + P, glucose + P). Control sediment samples had only ultra-pure water added to  
187 the sediment, while the blanks contained only ultra-pure  $\text{H}_2\text{O}$  (i.e. no sediment). Samples were  
188 snap frozen in liquid  $\text{N}_2$  after 0 and 24 h and stored at  $-80^\circ\text{C}$  until shipping on dry ice to the

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

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

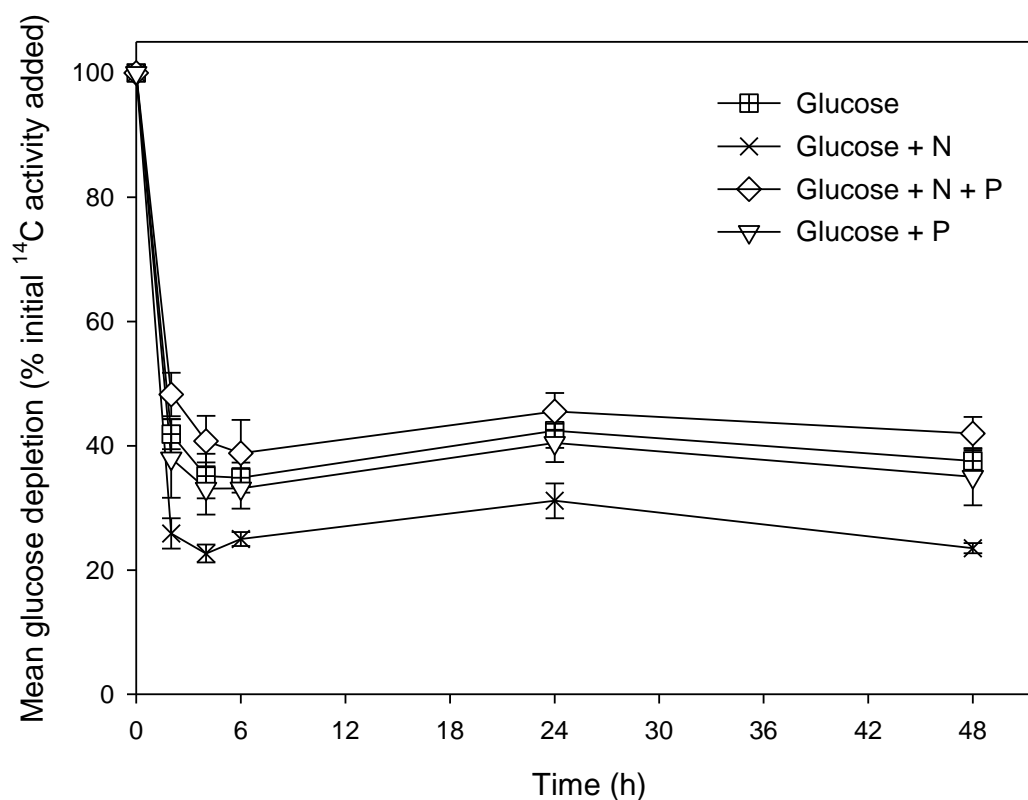
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### 200 **3. Results**

#### 201 **3.1 $^{14}\text{C}$ -labelled glucose depletion and metabolism**

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

208



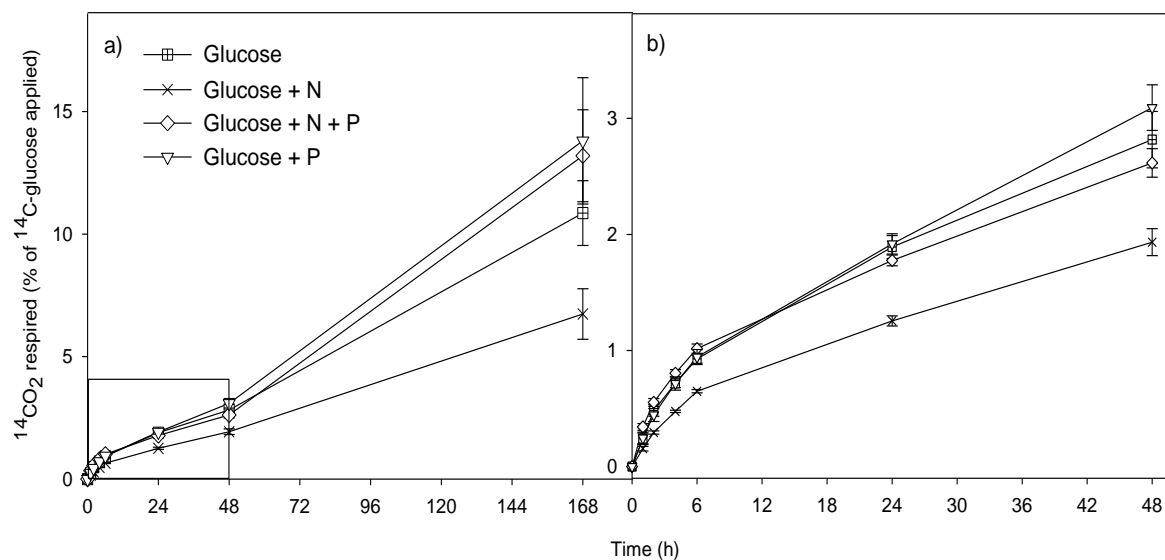
209

210 **Figure 1.**  $^{14}\text{C}$ -labeled glucose depletion over time. The  $^{14}\text{C}$ -glucose, in addition to N added as  
 211  $\text{NH}_4\text{NO}_3$  and P added as  $\text{NaH}_2\text{PO}_4$  was added to an oligotrophic river sediment and depletion  
 212 measured over time. Values represent means  $\pm$  SEM,  $n = 4$ .

213

214 A significant interaction between experimental treatment and time was observed for the  
 215 percentage of  $^{14}\text{CO}_2$  respiration by the sediment microbial communities (two-way mixed  
 216 ANOVA,  $P = 0.018$ ; Fig. 2; Supplementary Table S2). The initial rate of  $^{14}\text{CO}_2$  respiration was  
 217 lower for the glucose + N treatment in comparison to all other treatments from 4 to 24 h (one-  
 218 way ANOVA,  $P \leq 0.001$  in each case; Supplementary Table S1; Fig. 2). At 24 h, the rate of  
 219  $^{14}\text{CO}_2$  respiration was still lower in the glucose + N treatment in comparison to the glucose and  
 220 glucose + P treatments, with the glucose + N + P treatment falling in between (one-way  
 221 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

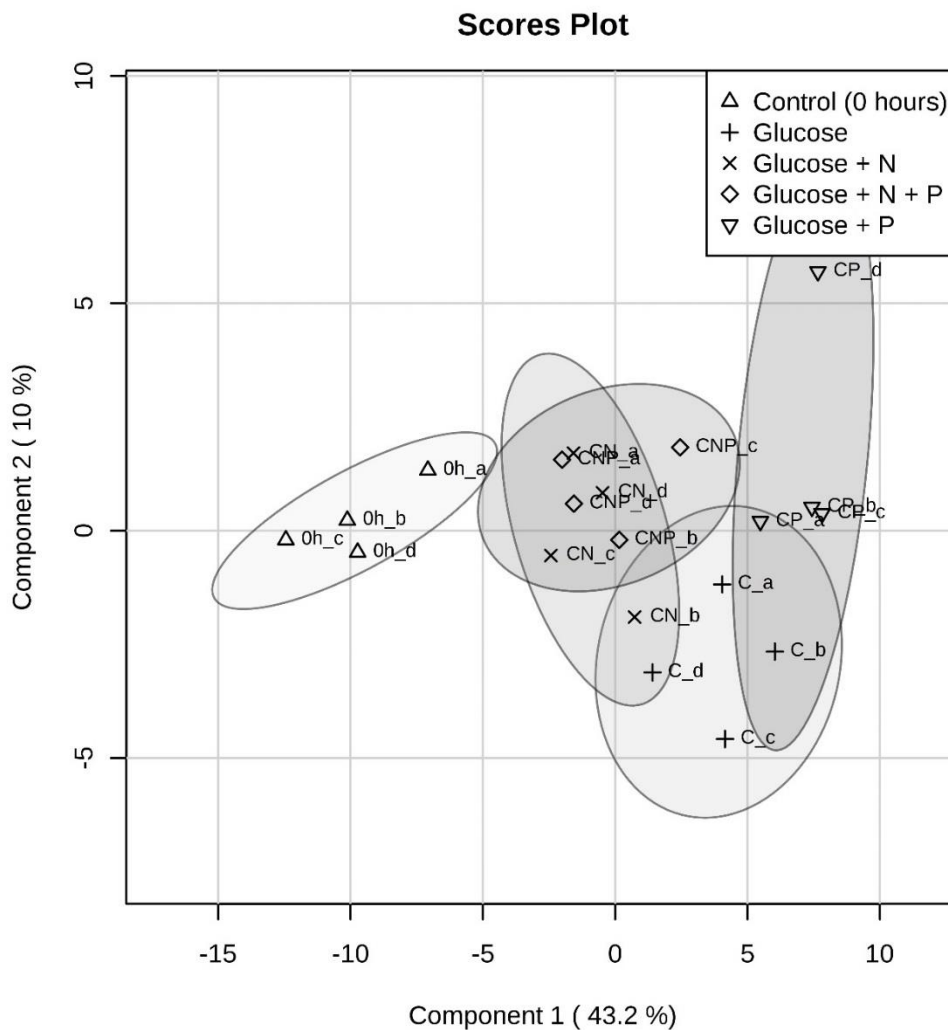
225 **Figure 2.** Microbial transformation of  $^{14}\text{C}$ -glucose to  $^{14}\text{CO}_2$  over time. The  $^{14}\text{C}$ -glucose, in  
 226 addition to N added as  $\text{NH}_4\text{NO}_3$  and P added as  $\text{NaH}_2\text{PO}_4$  was added to an oligotrophic river  
 227 sediment and transformation to  $^{14}\text{CO}_2$  measured over a) 168 h and b) 48 h. Panel b) is derived  
 228 from the data shown in panel a). Values represent means  $\pm$  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

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



339

340 **Figure 3** PLS-DA (PLS discriminant analysis) scores plot for the metabolome of control  
 341 samples (+<sub>d</sub>H<sub>2</sub>O only) at 0 h and all treatments at 24 h after the addition of treatments (+ glucose  
 342 (C); + glucose and N (CN); + glucose, N and P (CNP) and + glucose and P (CP). Lower case  
 343 letters represent individual sampling sites.

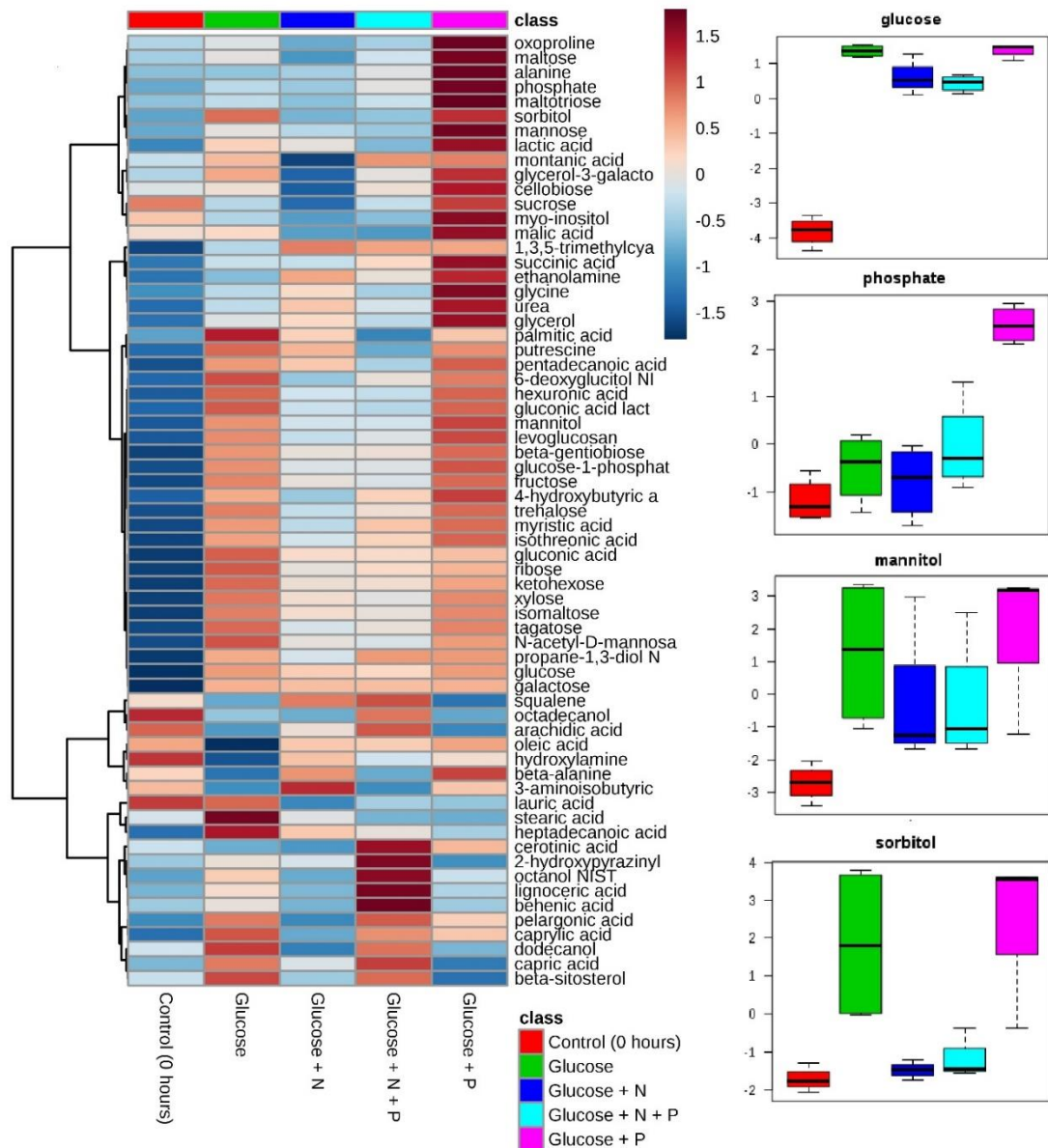


### 354 3.3 Compound-specific analysis

355 All treatments saw an increase in metabolite production after 24 h. However, the CNP  
356 treatment saw the greatest increase in the number of metabolites present. Of the metabolites  
357 identified, the key pathways they were attributed to included sugar metabolism, amino acid  
358 synthesis and lipid metabolism (Supplementary Fig. S4). Treatments with no N addition saw a  
359 significant increase in the production of sugar alcohols such as sorbitol (one-way ANOVA,  $P$   
360  $< 0.05$ ; Fig. 5; Supplementary Table S3). There were also higher concentrations of glucose and  
361 other sugars such as fructose, ketohexose, tagatose and glucose-1-phosphate in the sediment  
362 for treatments with no N addition, suggesting that glucose had been utilised internally at a  
363 slower rate in the absence of N (one-way ANOVA for glucose,  $P < 0.05$ ; Fig. 5). In addition,  
364 there was a higher amount of products from anaerobic respiration (e.g. lactic acid) suggesting  
365 some fermentative metabolism within the sediment.

366 In comparison to the glucose + N + P treatment, the glucose + P treatment had a higher  
367 proportion of added phosphate present after 24 h, indicating that less of the added phosphate  
368 had been utilised in the absence of N (one-way ANOVA,  $P < 0.05$ ; Fig. 5). The glucose + P  
369 treatment also showed a significant elevation in the amount of alanine present, and a similar,  
370 non-significant elevation in the amount of glycine present in comparison to the other  
371 treatments, including the control. This, in conjunction with an increased concentration of urea  
372 in comparison to other treatments, a known product of amino acid metabolism, could indicate  
373 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  $\pm$   
 383 1 S.D.

## 384 4. Discussion

### 385 4.1 Use of LMW carbon with nutrient limitation

386 The depletion of  $^{14}\text{C}$ -glucose from solution was rapid in all treatments; after 48 h between 20-  
387 40 % of  $^{14}\text{C}$ -glucose remained in the sediment, depending on the treatment (Fig. 2). Although  
388 the results of the metabolomic analyses demonstrated that a proportion of the glucose added  
389 remained unchanged in solution, it is likely that some of the  $^{14}\text{C}$ -glucose remaining had been  
390 transformed following uptake by microbes or through the action of extracellular enzymes (Fig.  
391 5; Wetzl 1992; Findlay and Sinsabaugh 1999). However the treatments without N addition had  
392 lactic acid present after 48 h, so it is possible that some glucose fermentation could have taken  
393 place in these treatments (Fig. 5). The concentration of glucose added was such that glucose  
394 would be available in excess to the microbial population of the sediment without fully  
395 saturating the system, based on previously observed glucose uptake in sediments from the same  
396 upland peat sites (Brailsford et al. 2019). The amount of C added was approximately 4 orders  
397 of magnitude higher than the baseline concentrations of C present as total free carbohydrates  
398 and 5 orders of magnitude higher than concentration in overlying river waters ( $0.61 \pm 0.08$  mg  
399 C kg wet sediment<sup>-1</sup> and  $0.09 \pm 0.02$  mg C L<sup>-1</sup> respectively; Brailsford et al. 2019).

400 Cumulative  $^{14}\text{CO}_2$  respiration over the duration of the experiment for the upland river  
401 sediments was an order of magnitude lower than rates previously observed for lowland  
402 agricultural soils (Hill et al. 2008; Rousk et al. 2014). This could be indicative of a higher C  
403 use efficiency (CUE), which is typical of areas of upland blanket peat bog and of aquatic  
404 systems in comparison to terrestrial systems (Kayranli et al. 2010; Sinsabaugh et al. 2013).  
405 This apparent high CUE may reflect the partitioning of glucose-C into storage metabolites  
406 which may be mineralised later. This is supported by the near-linear rate of  $^{14}\text{CO}_2$  accumulation  
407 over 7 d despite most of the  $^{14}\text{C}$  being depleted from the sediment pore water very quickly

408 (within 6 h). There were no detectable differences in cumulative  $^{14}\text{CO}_2$  respiration after 168 h,  
409 although the addition of glucose + N resulted in the lowest initial rate of  $^{14}\text{CO}_2$  respiration (first  
410 24 h) in comparison to the other treatments. This was in contrast to the rate of  $^{14}\text{C}$ -glucose  
411 depletion from the sediment after 24 h, where the glucose + N treatment had the highest rate  
412 of glucose depletion from the sediment in comparison to the glucose, and the glucose + P  
413 treatments, with the glucose + N + P treatment falling in between. The addition of N alongside  
414 P has previously been found to increase N loss from low-P systems after 48 h due to enhanced  
415 nitrification and denitrification processes, which could explain why the glucose + N + P  
416 treatment did not produce the same response as the glucose + N treatment (He and Dijkstra  
417 2015).

418 Oligotrophic peat systems can be either N or P limited depending on seasonality. In our  
419 study, the increased rate of C mineralisation in the N-enriched treatment, in conjunction with  
420 the timing of the current study (conducted in summer when N inputs from atmospheric  
421 deposition are at their lowest), indicate that the system was N limited at the time of the study  
422 (Elser et al. 2009; McGovern et al 2014; Emmett et al. 2016). After a rapid initial uptake in the  
423 glucose + N treatment, it is possible that P then became the growth-limiting nutrient, which  
424 could explain why despite the initial rapid uptake of glucose in the N addition treatment, overall  
425 C mineralisation to  $\text{CO}_2$  was lower than for the other treatments. The addition of P alongside a  
426 C source has previously been observed to have no effect on or to even suppress C uptake in  
427 lowland agricultural soils, which has been attributed to a lack of P limitation and changes in  
428 soil chemistry, making conditions unfavourable to soil biota respectively (de Sosa et al. 2018).  
429 Alternatively, labile C could have entered an alternative C pool within the microbial biomass,  
430 which respire C at a slower rate (Glanville et al. 2016). As neither P addition nor the  
431 combination of N + P appeared to have an effect on the uptake of C into the biomass, it strongly  
432 suggests that the different nutrient treatments induced shifts in internal C partitioning.

## 433 **4.2 Changes in primary metabolome with nutrient limitation**

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

447

## 448 **4.3 Compound-specific metabolome trends**

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

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

484

#### 485 **4.4 Critical evaluation of the untargeted metabolomics approach**

486 Untargeted metabolomics using GC-MS has been the primary choice for environmental  
487 samples due to its relative affordability, the possibility of identifying specific compounds and  
488 the potential to produce quantitative results (Viant and Sommer 2013). Fragmentation spectra  
489 resulting from GC-MS can be screened against large databases which currently contain over  
490 1000 metabolites (Kind et al. 2009). However, library building has been centred around  
491 medical and cell biology samples and the derivitisation required for GC-MS may bias the  
492 metabolite profile towards specific functional groups (Lin et al. 2006; Viant and Sommer  
493 2013). In this study only ~35 % of fragmentation spectra detected could be matched to a  
494 metabolite. The inclusion of unknown metabolites in statistical analyses separated treatments  
495 in a similar manner compared to when unknown metabolites were excluded. However, when  
496 unknown metabolites were included, 59 % of the top 75 metabolites with the greatest  
497 differences between treatments were unidentified compounds (Supplementary Fig. S5-S7;  
498 Supplementary dataset 1). The primary metabolome presented in this study represents a single  
499 point in time, while C assimilation is a dynamic process and the metabolic profile may change  
500 over time following the initial uptake. Future work could combine study of the primary  
501 metabolome with more dynamic techniques such as the use of stable isotopes to trace C into  
502 different organism groups (Kaplan et al. 2008; Hotchkiss and Hall 2015).

503

504

505

## 506 **5. Conclusions**

507 The addition of N led to an increase in labile DOC uptake, which was evident in the reduction  
508 of sugars present in the metabolome of N addition treatments. In contrast, N addition  
509 corresponded with a decrease in CO<sub>2</sub> respiration over the duration of the experiment, indicating  
510 that N is required to allocate more C to storage and cell protection as opposed to respiration.  
511 When N and P were added simultaneously P uptake was enhanced compared to the addition of  
512 P only. A lack of N addition led to an increased production of storage compounds such as  
513 alcohol sugars, in addition to the synthesis of amino acids (glycine, alanine) and associated  
514 waste products. Due to the P-containing co-enzymes required for amino acid synthesis, this  
515 may be a P-limited process. The addition of labile C only led to specific increases in the  
516 production of organic acid-like compounds, which can aid P release from both organic and  
517 inorganic P held on the sediment's solid phase. These results provide an insight into the  
518 molecular mechanisms of nutrient enrichment in low-nutrient status rivers. We found that  
519 whilst nutrient stoichiometry is important for nutrient cycling N addition appears to be a key  
520 driver of changes to DOC metabolism in oligotrophic stream sediments.

521

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