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DOCTOR OF PHILOSOPHY

Dissolved organic matter (DOM) in freshwater ecosystems

Brailsford, Francesca

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Dissolved organic matter (DOM) in freshwater ecosystems

Francesca Louise Brailsford

August 2019

A thesis submitted to Bangor University

In candidature for the degree Philosophiae Doctor

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Thesis Summary

Dissolved organic matter (DOM) is broadly defined as the fraction of organic matter that passes through a 0.45 μm filter, encompassing compounds with a wide variation in size, solubility, charge and function. Although the composition of DOM in freshwaters is not currently well defined, ca. 20 % of DOM is present as labile, low molecular weight (LMW) DOM which is a key component of in-stream cycling of nutrients, including carbon (C), nitrogen (N), phosphorus (P) and sulphur (S). Presently, C and N export from freshwater to marine environments are increasing globally, due to climate change and agricultural intensification respectively, however, current water quality legislation rarely considers the monitoring, and management, of DOM. The overall aims of this thesis were therefore to: i) gain further insight into DOM processing in rivers across a range of spatial gradients (e.g. land-cover, inorganic/organic nutrient pool size); ii) compare DOM processing to inorganic nutrient processing; and iii) identify how DOM metabolism changes under different nutrient conditions. Radioisotope tracer techniques (^{14}C , ^{33}P , ^{35}S) were used to measure the uptake of DOM components (DOC, DON, DOP, DOS) in river waters and sediments. Due to the rapid cycling of LMW DOM compounds by the aquatic microbial biomass, sample preservation methods were investigated. Maintaining samples at a cool temperature, in the dark and commencing experiments within 24 h was the simplest and most efficient method to ensure that DOM within samples was not badly degraded. The use of freezing and acidification were also deemed to be viable options for long-term storage, however, the choice of method depends on their compatibility with subsequent analytical protocols. Landscape-scale analysis of DOM processing found that DOM uptake was faster in inorganic nutrient (N/P) enriched rivers, however the reverse was true for inorganic nutrient uptake. This suggests DOM uptake in nutrient-enriched rivers may not be driven by N/P demand but C limitation. Further work using dual-labelled isotopic methods may provide insight into DOM utilisation following uptake. Experimental work in oligotrophic (peat) and mesotrophic (improved grassland) rivers also found DOC uptake to be elevated in nutrient-enriched river waters and sediments. Microbial growth in sediments was indicated by a lag phase in DOC uptake. Sediments, particularly mesotrophic, had the capacity to process high DOC inputs (5-10 mM) which has implications for water quality management. Nutrient limitation removal by N and/or P addition to oligotrophic sediments led to changes in DOC uptake and metabolism. Metabolome analysis indicated that N addition led to increased DOC processing, while P addition increased amino acid synthesis, attributed to the P-containing enzymes required for the process. Additionally, DOS was found to be preferentially utilised by the microbial biomass, which goes against the tenet that inorganic S is the preferred source of S for most microorganisms. In conclusion, this thesis has provided a basis for exploring the mechanistic basis of DOM processing across physiochemical gradients in river catchments. Further research is now required to ground truth these findings across a wider range of global habitats. The capacity for LMW DOM to be processed by the microbial biomass of river waters and sediments, in addition to preferential uptake compared to inorganic nutrient sources in some contexts, highlight the importance for DOM to be at the forefront of water quality monitoring and management, alongside inorganic nutrients. This information will provide an evidence base from which effective legislation and management strategies can be designed to protect freshwater ecosystems.

Table of contents

Thesis Summary	iv
Acknowledgments	x
Abbreviations.....	xi
List of tables	xiii
List of figures.....	xv
Chapter 1: Introduction	1
1.1 Overview	2
1.2 Thesis background and rationale	3
1.3 Thesis aims and objectives.....	5
1.3.1 Thesis aims	5
1.3.2 Objective 1	5
1.3.3 Objective 2.....	6
1.3.4 Objective 3.....	6
1.3.4 Objective 4.....	7
1.4 Experimental Chapter information	7
1.5 References	9
Chapter 2: Literature review	11
2.1 Introduction	12
2.1.1 Classification of DOM	13
2.1.2 C:N:P Stoichiometry	15
2.2 Ecological importance of the topic.....	16
2.3 Methods of DOM quantification and characterisation	19
2.3.1 Quantification of DOM	19
2.3.2 Characterisation of DOM	21
2.4 Sources of DOM.....	23
2.4.1 Allochthonous sources	23
2.4.2 Autochthonous sources.....	27
2.5 Concentrations of DOM in freshwater ecosystems	29
2.5.1 Patterns in DOC concentrations.....	30
2.5.2 Patterns in DON concentrations.....	32
2.5.3 Patterns in DOP concentrations	34
2.6.4 Patterns in DOS concentrations	35
2.6 DOM flux in freshwater ecosystems	35
2.6.1 Patterns in DOC flux	36

2.6.2	Patterns in DON flux.....	38
2.6.3	Patterns in DOP flux	39
2.6.4	Patterns in DOS flux	40
2.7.	Microbial processing of DOM	40
2.7.1.	Intracellular processing	40
2.7.2	Extracellular processing of DOM	42
2.8	Concluding remarks.....	44
2.8.1	Summary.....	45
2.9	References	46
Chapter 3: Microbial use of low molecular weight DOM in filtered and unfiltered freshwater: Role of ultra-small microorganisms and implications for water quality monitoring		
3.1	Abstract.....	67
3.2	Introduction	68
3.3	Materials and methods	72
3.3.1	Field site and sampling.....	72
3.3.2	Nutrient depletion experiment	74
3.3.3	Statistical analysis	74
3.4	Results.....	75
3.4.1	Water quality characteristics.....	75
3.4.2	Microbial uptake of ¹⁴ C-labelled amino acids	77
3.4.3	Microbial uptake of ¹⁴ C-labelled glucose.....	80
3.4.4	Microbial uptake of ³³ P-labelled orthophosphate	81
3.5	Discussion	83
3.5.1	Role of ultra-small organisms in the processing of low MW DOM.....	83
3.5.2	Filtering as a method to preserve low MW DOM	85
3.5.3	Acidification as a preservative for low MW DOM.....	86
3.5.4	Recommendations for sampling low MW DOM.....	87
3.6	Acknowledgements	88
3.7	References	88
Chapter 4: Land cover and nutrient enrichment regulates dissolved organic matter (DOM) turnover in freshwater ecosystems		
4.1	Abstract.....	97
4.2.	Introduction.....	98
4.3	Materials and methods	100
4.3.1	Field site and sampling.....	100

4.3.2 Nutrient depletion experiment	102
4.3.3 Statistical analysis	103
4.4 Results.....	104
4.4.1 Physiochemical properties	104
4.4.2 Regression analysis of nutrient depletion versus physiochemical parameters	107
4.5 Discussion	114
4.5.1 Physiochemical parameters drive differences between rivers	114
4.5.2 Changes in DOM degradation with changing nutrient limitation.....	115
4.5.3 Nutrient stoichiometry and DOM depletion.....	116
4.5.4 Limitations	117
4.6 Conclusion	118
4.7 Acknowledgments	119
4.8 References	119
Chapter 5 Microbial uptake kinetics of dissolved organic carbon (DOC) compound groups from river water and sediments.....	127
5.1 Abstract.....	128
5.2 Introduction	129
5.3 Methods.....	132
5.3.1 Field sites and sampling	132
5.3.2 Background chemical analysis.....	133
5.3.3 Microbial community analysis.....	134
5.3.4 DOC depletion experiment	134
5.3.5 Statistical analysis	136
5.4 Results.....	137
5.4.1 Sediment and water characteristics	137
5.4.2 DOC uptake in sediment versus water	141
5.4.3 DOC uptake in two waters draining contrasting catchment types	142
5.4.4 Uptake of DOC compound groups.....	149
5.5 Discussion	150
5.6 Acknowledgments	156
5.7 References	156
Chapter 6: Nutrient enrichment induces a shift in dissolved organic carbon (DOC) metabolism in oligotrophic freshwater sediments	164
6.1 Abstract.....	165
6.2 Introduction	166

6.3 Materials and methods	168
6.3.1 Field site	168
6.3.2 ¹⁴ C-labelled nutrient metabolism assays	170
6.3.3 N and P sorption/desorption	171
6.3.4 Untargeted analysis of primary metabolism	172
6.4 Results.....	173
6.4.1 ¹⁴ C-labelled glucose depletion and metabolism.....	173
6.4.2 Non-targeted metabolite analysis by GC-MS	175
6.4.3 Compound-specific analysis	179
6.5 Discussion	181
6.5.1 Use of LMW carbon with nutrient limitation	181
6.5.2 Changes in primary metabolome with nutrient limitation.....	183
6.5.3 Compound-specific metabolome trends	184
6.5.4 Critical evaluation of the untargeted metabolomics approach	186
6.6 Conclusions	187
6.7 Acknowledgements	187
6.8 References	188
Chapter 7: Rapid microbial consumption of dissolved organic sulphur (DOS) in freshwaters.....	196
7.1 Abstract.....	197
7.2 Introduction	198
7.3 Materials and methods	200
7.3.1 Field site	200
7.3.2 Chemical characteristics	200
7.3.3 ³⁵ S-labelled nutrient depletion assays	201
7.3.4 Data analysis	202
7.4 Results and discussion	203
7.4.1 Sulphur composition in two contrasting land-cover types.....	203
7.4.2 ³⁵ S-labelled depletion	203
7.5 Conclusions	207
7.6 Acknowledgements	207
7.7 References	208
Chapter 8: Discussion.....	212
8.1 Introduction	213
8.2 Synthesis of findings.....	213

8.2.1 Determine how physiochemical changes across catchments influence DOM uptake in rivers	214
8.2.2 Explore the interaction between the pool size and uptake rate of DOM in rivers	216
8.2.3 Compare the relative importance of inorganic and organic nutrients to river systems	218
8.2.4 Investigate how the metabolism of LMW DOC changes with nutrient limitation	220
8.3 Strengths and limitations.....	221
8.4 Future work	224
8.5 General conclusions.....	228
8.6 References	229
Appendix 1: Supplementary material for Chapter 3	232
Appendix 2: Supplementary material for Chapter 4.....	238
Appendix 3: Supplementary material for Chapter 5	244
Appendix 4: Supplementary material for Chapter 6.....	260
Appendix 5: Supplementary material for Chapter 7	271

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Abbreviations

% - Percentage	GC-MS – Gas chromatography mass spectrometry
°C - Celsius	h - Hour(s)
ANOVA - Analysis of variance	h⁻¹ - Per hour
ATP - Adenosine triphosphate	H₂O - Water
AU – Absorbance units	H₃PO₄ – Phosphoric acid
AWI – Air : water interface	ha – hectare(s)
BGE - Bacterial growth efficiency	ha⁻¹ – per hectare
BOD – Biological oxygen demand	HAB – Harmful algal bloom
C - Carbon	HCl – Hydrochloric acid
Ca - Calcium	HDPE - high density polyethylene
cm - Centimetre(s)	HMW – High molecular weight
CO₂ - Carbon dioxide	HPLC – High performance liquid chromatography
COD – Chemical oxygen demand	IC – Ion chromatography
CUE - carbon use efficiency	K₂SO₄ - Potassium sulphate
d – Day(s)	kBq - Kilobecquerel
d⁻¹ – per day	KCl - Potassium chloride
Da – Dalton(s)	kDa – Kilodalton(s)
DBP – Disinfection by-products	kg - Kilogram(s)
dE_fOM – Dissolved effluent organic matter	K_m – Michaelis-Menten constant
dH₂O – Distilled water	L - Litre
DNA - Deoxyribonucleic acid	LCM - Land cover map
DOC - Dissolved organic carbon	LC-MS - Liquid chromatography mass spectroscopy
DON - Dissolved organic nitrogen	LMW – Low molecular weight
DOP – Dissolved organic phosphorus	m - Metre(s)
DW - Dry weight	mg - Milligram(s)
EC – Electroconductivity	min - Minute(s)
EU – European Union	min⁻¹ – per min
FTIR - Fourier transform infrared	mL - Millilitre

mM - Millimolar
mmol - Millimoles
MW - Molecular weight
MWC – Mid-water column
N - Nitrogen
n = sample size
Na - Sodium
Na₂³⁵SO₄ – Sodium sulphate
NaH₂PO₄ – Sodium phosphate
NaOH -Sodium Hydroxide
ng – Nanogram
NH₄⁺ - Ammonium
NH₄NO₃– Ammonium nitrate
nm – Nanometre(s)
nM – Nanomolar
nmol - Nanomoles
NO₃⁻ - Nitrate
NPOC - non-purgeable organic carbon
O₂ - Oxygen
OC – Organic carbon
P – Phosphorus
PCA – Principal component analysis
P_i – Inorganic phosphorus
PLFA - Phospholipid fatty acid
PLS-DA - PLS discriminant analysis
PO₄³⁻ - Phosphate
POCIS - Polar organic chemical integrative samplers
POM – Particulate organic matter
PP - Particulate P
rev min⁻¹ – Revolutions per minute
SEC – Size exclusion chromatography
SEM – Standard error of the mean
SMP - Soluble microbial products
SPW – Soil pore water
SRP – Soluble reactive phosphate
SUVA – Specific UV absorbance
TC- Total carbon
TDN- Total dissolved nitrogen
TDP – Total dissolved phosphorus
TFAA – Total free amino acid
TN – Total nitrogen
TP – Total phosphorus
UK - United Kingdom
UV – Ultraviolet
V_{max} – Maximal velocity
w/v – Weight per volume
WWTW - wastewater treatment works
y⁻¹ - Year
µg - Microgram
µL - Microlitre
µm - Micrometre
µM – Micromolar
µmol – Micromoles

List of tables

Table 2.1 Table listing the ranges of mean dissolved organic carbon (DOC) concentrations for rivers and streams in a number of locations, catchment types and seasons.

Table 2.2 Table listing the ranges of mean dissolved organic nitrogen (DON) concentrations for a range of locations. Adapted from Berman and Bronk (2003).

Table 2.3 Table listing the ranges of mean dissolved organic phosphorus (DOP) concentrations in $\mu\text{g L}^{-1}$ for a number of locations. Errors are ± 1 SEM. *Indirect calculation of DOP by subtracting inorganic P (P_i) from total P (TP).

Table 3.1 Chemical properties of water from the Hiraethlyn and Migneint sub-catchments used in the substrate mineralisation experiments. Values represent annual mean data \pm SEM ($n = 66$, except for low molecular weight fractionation parameters where $n = 3$).

Table 3.2 Results from a two-way mixed ANOVA for each isotopically-labelled nutrient, sub-catchment and temperature.

Table 4.1 Percentage depletion of ^{14}C -labelled DOC, DON and DOP and ^{33}P -labelled inorganic P (P_i) from river water across 5 major land use types after 24 h. Values represent percentage of tracer depleted within 24 h and are presented as means \pm SEM. The average value across all the sites is also presented at the foot of the table. Significant differences between land use types are shown with

lowercase superscript letters while differences between substrates are denoted by uppercase superscript letters ($P < 0.05$).

Table 4.2 Simple linear regression analysis of mean DOC, DON, DOP and P_i depletion after 24 hours (%) versus physicochemical parameters across 45 sites ($n = 3$).

Table 5.1 Chemical characteristics of the water and sediment samples used in the study. Values represent means \pm SEM, $n = 9$. *Denotes a significant P -value when comparing the two sites. The significance level was set at $P < 0.05$. All values for sediments are expressed on a dry weight basis unless otherwise stated.

Table 5.2 Analysis of total mass of Phospholipid-derived fatty acids (PLFA) and taxonomic groups of concentrated water samples and freeze-dried sediment samples used in the study. Values represent means \pm SEM, $n = 3$. *Denotes a significant P -value when comparing the two sites. The significance level was set at $P < 0.05$.

Table 6.1 Characteristics of the sediment samples used in the study. Values represent means \pm SEM, $n = 4$ (from Brailsford et al. 2019)

Table 7.1 Results from fitting a single first order curve to mean ^{35}S depletion curves.

List of figures

- Fig. 1.1** Schematic representation of the main allochthonous and autochthonous sources of dissolved organic matter (DOM) (adapted from Thomas 1997).
- Fig. 2.1** Schematic representation of the major pathways by which DOM can be transferred into and out of the microbial loop (adapted from Sinasbaugh and Foreman 2003).
- Fig. 2.2** Relationship between N/P ratio and nitrate concentration ($\mu\text{g l}^{-1}$) in a selection of large global rivers. The N/P Redfield ratio of 16:1 is indicated (Turner et al. 2001).
- Fig. 2.3** Diagram showing the biological and chemical pathways by which in-stream processing of DOM occurs.
- Fig. 3.1** Relative size of dissolved organic matter (DOM) and particulate organic matter (POM) components in comparison to bacteria, archaea and viruses. $\text{POM} > 0.45 \mu\text{m} > \text{DOM}$. $0.45/0.22 \mu\text{m}$ filter cut-offs indicated by a dashed line. * Some giant viruses $>1 \mu\text{m}$ exist.
- Fig. 3.2** Land use map of the Conwy catchment with upland peat bog (Migneint) and lowland improved grassland (Hiraethlyn) sub-catchments outlined in red.
- Fig. 3.3** Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{14}C -labelled amino acids for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means \pm SEM (n = 3). The legend is the same for all panels.
- Fig. 3.4** Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{14}C -labelled glucose for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25

°C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means \pm SEM ($n = 3$). The legend is the same for all panels.

Fig. 3.5 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{33}P -labelled orthophosphate for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means \pm SEM ($n = 3$). The legend is the same for all panels.

Fig. 4.1 Sampling locations across (a) Conwy and (b) Nadder catchments. Insert shows catchment locations in relation to the UK. Red boundaries represent catchments with $>50\%$ dominance of a single land cover. Land-cover maps were created with LCM2007 data provided by the Centre for Ecology and Hydrology (Emmett et al. 2016) using ArcGIS Hydrology toolbox (ESRI 2018. Version 10 Redlands, CA).

Fig. 4.2 Correlation bi-plot from the principal component analysis (PCA) river water chemical variables and rate data for radiolabelled DOC, DON, DOP and P_i compounds for each site across the three catchments ($n = 3$).

Fig. 4.3 Scatter plots of mean DOC depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Fig. 4.4 Scatter plots of mean DON depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Fig. 4.5 Scatter plots of mean DOP depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Fig. 4.6 Scatter plots of mean P_i depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Fig. 5.1 Effect of DOC concentration on the loss of ^{14}C -labelled amino acids for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

Fig. 5.2 Effect of DOC concentration on the loss of ^{14}C -labelled glucose for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

Fig. 5.3 Effect of DOC concentration on the loss of ^{14}C -labelled organic acids for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values

represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

Fig. 5.4 Effect of DOC concentration on the loss of ^{14}C -labelled phenolic compounds for:

a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

Figure 6.1. ^{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 \pm SEM, $n = 4$.

Figure 6.2. Microbial transformation of ^{14}C -glucose to $^{14}\text{CO}_2$ 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 transformation to $^{14}\text{CO}_2$ measured over a) 168 h and b) 48 h. Panel b) is derived from the data shown in panel a). Values represent means \pm SEM ($n = 4$). The legend is the same for both panels.

Figure 6.3 PLS-DA (PLS discriminant analysis) scores plot for the metabolome of control samples (+dH₂O 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.

Figure 6.4 Similarity dendrogram clustered by Euclidean distance (horizontal axis) for the metabolome of control samples (+dH₂O 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.

Figure 6.5. Hierarchical clustering heat map of the normalized metabolite log response in sediment primary metabolome for each treatment (0 h (control), 24 h (glucose, glucose + N, glucose + N + P, glucose + P). Metabolites which significantly decrease are displayed in blue, while metabolites which significantly increased are displayed in red. The brightness of each colour corresponds to the magnitude of the difference when compared with average value. Clustering of the roots nutrient treatments is depicted by the dendrogram at the top. Clustering of the metabolites is depicted by the dendrogram at the left. Metabolites are clustered by similarity according to Pearson correlation values. Boxplots of individual metabolites mean \pm 1 S.D.

Figure 7.1. Depletion of either ³⁵S-labeled DOS compounds (cysteine, methionine) or inorganic sulphur (Na₂³⁵SO₄) after addition to river water. DOS values are corrected for any cleaved sulphate groups remaining in solution. Values represents means \pm SEM (*n* = 3).

Chapter 1

Introduction

Dissolved organic matter (DOM) in freshwater ecosystems

Chapter 1

1.1 Overview

Dissolved organic matter (DOM) is a heterogeneous mixture of compounds, ranging from monomers (including sugars, amino acids and nucleic acids) through to large, macromolecular “humic” compounds (Dawson et al. 2001). DOM is defined operationally as carbon-containing compounds that can pass through a 0.45 µm filter (Thurman 1985). As it is defined by a size cut-off, the DOM fraction therefore contains both truly soluble components together with insoluble nano-particulate organic matter, such as viral particles and other genetic material (Mao et al. 2013). This diversity allows DOM to have a wide variety of biological, chemical and physical functions across aquatic and terrestrial environments. Global carbon (C), nitrogen (N) and phosphorus (P) cycles are intrinsically linked through DOM cycling, as DOM structures can take the forms of DOC-N, DOC-P or DOC-N-P (Karl and Björkman 2002). Whilst aquatic P concentrations are decreasing within the EU due to the implementation of the Urban Waste Water Treatment Directive and other management measures, the export of N and C to coastal waters is increasing globally, due to agricultural intensification and climate change respectively (Carcao and Cole 1999; Evans et al. 2005; Vitousek et al. 2009). In aquatic ecosystems, the total mass of DOM can exceed the mass of living organisms present, however, the source, fate and impact of DOM on the environment is not fully understood.

Chapter 1

1.2 Thesis background and rationale

The research presented in this thesis is set within the context of the biogeochemical C, N, P and S cycling within aquatic ecosystems. For a detailed analysis of how DOM is involved in these cycles, readers are referred to Chapter 2 (literature review). It is generally accepted that the three main nutrients required in nature, often termed macronutrients, are C, N and P. The stoichiometric ratio of C : N : P was originally established to be 106 : 16 : 1 in marine ecosystems, however, it has been suggested that the stoichiometric ratio for microbial communities is closer to 60 : 7 : 1 (Redfield 1934; Cleveland and Liptzin 2007). Although often overlooked, sulphur (S) requirements may be similar to those for P, with C : N : P : S stoichiometric ratios estimated to be 124 : 16 : 1 : 1.3 in aquatic ecosystems (Ksionzek et al. 2016).

Despite its broad composition, the majority of DOM is composed of high molecular weight (HMW) compounds > 1000 Dalton (Da) in size, containing high abundances of aromatic groups (Cui and Choo 2013). In general, HMW DOM is considered to be largely refractory, with its main function in aquatic ecosystems being light attenuation due to its chromophoric properties, however, HMW DOM may be microbially processed, albeit at a slower rate than more easily degradable DOM compounds (Farjalla et al. 2009). Only ca. 20 % of DOM is estimated to be in the labile, low MW (LMW) fraction, easily utilised by aquatic plants and microorganisms (Thomas 1997). Despite the small pool size, LMW DOM compounds can be rapidly cycled by aquatic organisms and have been demonstrated to be a key component of in-stream macronutrient processing (Lutz et al. 2011; Spencer et al. 2012).

Chapter 1

This thesis is focused on the degradation of labile LMW DOM components in freshwater ecosystems, particularly in river waters and at the water : sediment interface. There are a number of challenges to measuring riverine DOM in general, due to the complexity of the factors dictating the sources, transport and processing of DOM (Fig. 1.1). How the physical, chemical and biological gradients present across river catchments drive changes in labile DOM depletion is explored in Chapter 4. The interaction between the water column and sediments in the hyporheic zone is discussed in Chapter 5.

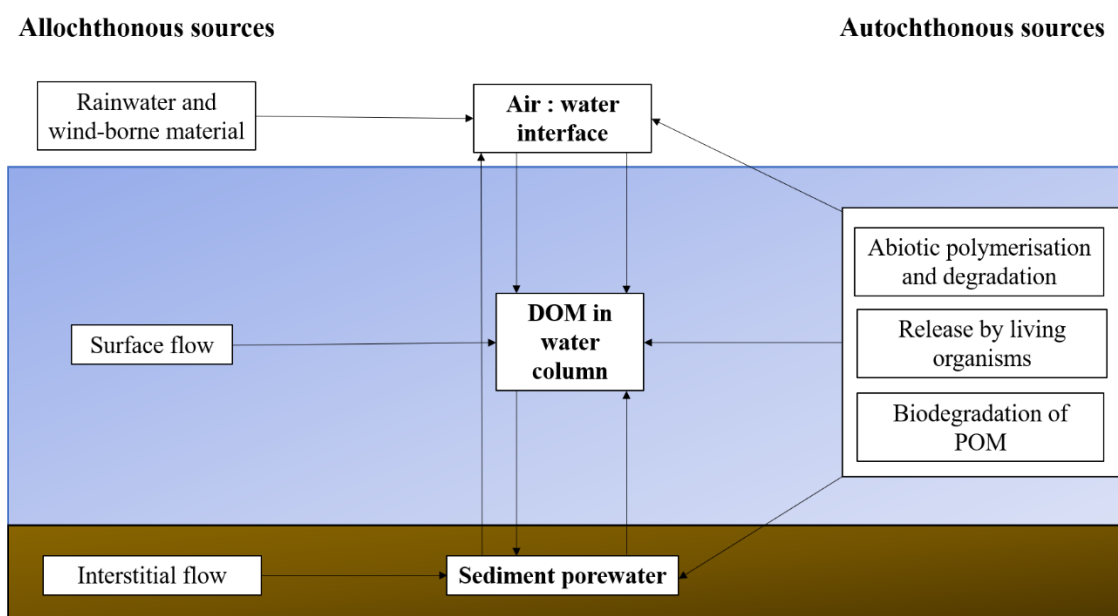


Fig. 1.1 Schematic representation of the main allochthonous and autochthonous sources of dissolved organic matter (DOM) (adapted from Thomas 1997).

A number of methods are currently available for DOM measurement, including the quantification of bulk chemical parameters and the use of optical parameters to provide semi-qualitative measures of DOM quality (Matilainen et al. 2011). This thesis focused on quantifying the turnover of specific labile DOM compounds. Radioisotope

Chapter 1

tracers (^{14}C , ^{33}P , ^{35}S) were therefore used to quantify depletion from river waters and sediments in the experimental chapters. In addition, this thesis also explores the metabolism of labile DOM compounds following uptake by the microbial biomass on a molecular level, using tools such as untargeted primary metabolomics. Whilst metabolomics and other molecular biological techniques have become more accessible over recent years, further research is required into their use in environmental applications. In chapter 6, the use of metabolomics is combined with ^{14}C -tracer experiments to better understand the response of the aquatic microbial biomass to labile DOM addition.

1.3 Thesis aims and objectives

1.3.1 Thesis aims

This PhD thesis broadly focuses on dissolved organic matter (DOM) processing across contrasting land cover types, in both river waters and sediments, with a specific focus on the influence of spatial gradients, intrinsic nutrient pool sizes, comparison to inorganic nutrient processing and changes in the metabolism of nutrients with changing nutrient limitation.

1.3.2 Objective 1

Determine how physiochemical changes across catchments influence DOM uptake in rivers

The uptake of DOC, DON and DOP compounds were measured across both spatial and temporal gradients at the catchment scale. The influence of intrinsic nutrient

Chapter 1

pool size and terrestrial land cover on the depletion of a range of DOM compounds in river waters was investigated in Chapters 3 and 4. In addition, the uptake kinetics of four different DOC compound groups (amino acids, sugars, organic acids, phenolics) by the river water and sediment microbial biomass was investigated in Chapter 5.

1.3.3 Objective 2

Explore the interaction between the pool size and uptake rate of DOM in rivers

The rates of LMW DOC compound group uptake were measured over a range of different pool sizes and concentrations (nM to mM) in Chapter 5. To examine the impact of nutrient limitation, the influence of inorganic nutrient (N/P) addition on labile DOC uptake and biodegradation was investigated in Chapter 6.

1.3.4 Objective 3

Compare the relative importance of inorganic and organic nutrients to river systems

Few studies have focused on the importance of DOM in comparison to inorganic nutrients as a source of major macronutrients (C, N, P, S). In Chapter 4, the uptake of inorganic and organic P are compared at the catchment scale. Chapter 6 investigates how the removal of inorganic N/P limitation affects labile DOC uptake and biodegradation in river sediments. In addition, Chapter 7 is a short communication characterising the uptake of inorganic S and organic S (S-containing amino acids) by the river water microbial biomass, in two contrasting sub-catchments.

Chapter 1

1.3.4 Objective 4

Investigate how the metabolism of LMW DOC changes with nutrient limitation

To complement the radioisotope tracer work conducted in this thesis, untargeted primary metabolome analysis was undertaken to determine how the metabolism of a simple DOC compound (glucose) responds to nutrient addition in sediments (Chapter 6).

1.4 Experimental Chapter information

The Experimental Chapters of this thesis are presented in manuscript format. For each Chapter, the title page lists all authors and how they have contributed to the manuscript. The current status of each Chapter is also listed (for example draft, submitted, accepted, published). There are four full experimental Chapters (Chapters 3-6) and one Chapter written as a short communication (Chapter 7). The titles of the experimental Chapters are as follows:

Chapter 3: Microbial use of low molecular weight DOM in filtered and unfiltered freshwater: Role of ultra-small microorganisms and implications for water quality monitoring

Chapter 4: Land cover and nutrient enrichment regulates dissolved organic matter (DOM) turnover in freshwater ecosystems

Chapter 5: Microbial uptake kinetics of dissolved organic carbon (DOC) compound groups from river water and sediments

Chapter 1

Chapter 6: Nutrient enrichment induces a shift in dissolved organic carbon (DOC) metabolism in oligotrophic freshwater sediments

Chapter 7: Rapid microbial consumption of dissolved organic sulphur (DOS) in freshwaters

Chapter 1

1.5 References

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Chapter 1

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Chapter 2

Literature review

Chapter 2

2.1 Introduction

The dissolved organic matter (DOM) fraction makes up the majority of organic matter in the freshwater ecosystems; it has been previously stated that rivers loadings can contain two orders of magnitude more DOM than particulate organic matter (POM) (He et al. 2016). DOM is widely defined as the matter that can pass through a 0.45 μm filter (Perdue and Ritchie 2003; He et al. 2016). This fraction contains both truly soluble DOC components together with insoluble nano-particulate organic matter, including viral particles and genetic material, which has previously been found at concentrations of $2.2 \pm 0.8 \mu\text{g L}^{-1}$ (Mao et al. 2013). Its soluble composition can vary considerably from large macromolecular humic substances ($> 1000 \text{ Da}$) down to low-molecular weight (LMW) monomeric units (e.g. sugars, amino acids). This diverse array of compounds enables DOM to have a range of physical and chemical functions within the aquatic ecosystem. The bioavailability of DOM in terms of transfer into the microbial food web varies with DOM composition, microbial community structure and the physical and chemical properties of the aquatic environment (Fig. 2.1; Sinasbaugh and Foreman 2003). In the freshwater environment DOM may originate from a number of different external or in-stream sources (autochthonous and allochthonous respectively), including inputs from precipitation, ground water flow, sediment pore water, terrestrial and aquatic plant production and the synthesis of compounds within the water column itself (Aitkinhead-Peterson et al. 2003). Although the total mass of DOM in the freshwater environment may be greater than that of the living organisms present, the processing of DOM and its influence on the ecology of an aquatic environment are not fully understood (Thomas 1997).

The aims of this literature review are to: 1) describe the composition and functions of DOM in freshwater ecosystems and the methods for its detection; 2) investigate the sources, concentrations and rates of flux of the key DOM compounds, specifically carbon (C), nitrogen

Chapter 2

(N), phosphorus (P) and sulphur (S) species, within freshwater ecosystems; 3) discuss the main mechanisms by which DOM compounds are processed.

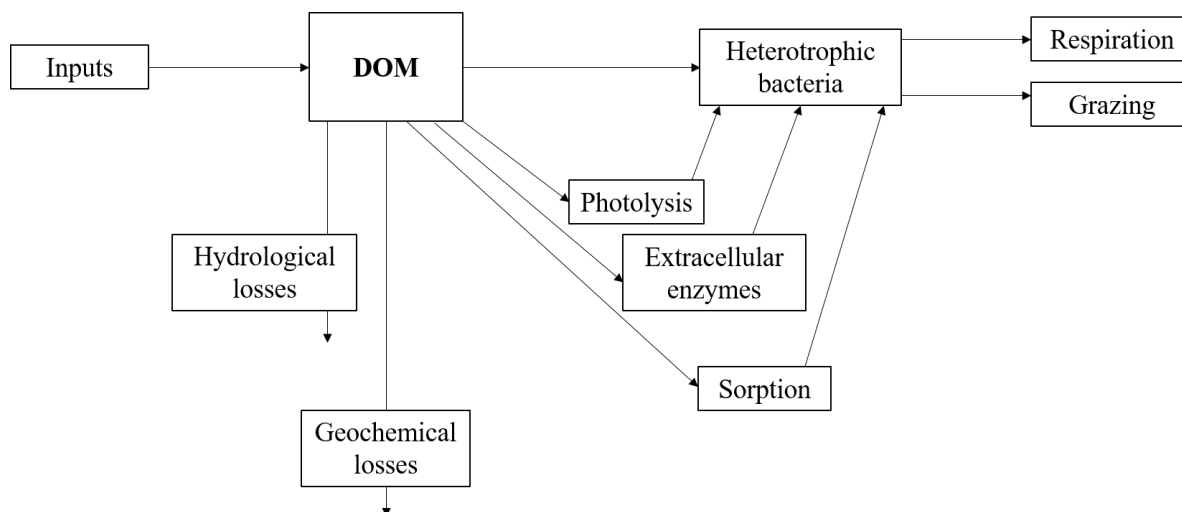


Fig. 2.1 Schematic representation of the major pathways by which DOM can be transferred into and out of the microbial loop (adapted from Sinasbaugh and Foreman 2003).

2.1.1 Classification of DOM

DOM can be broadly divided into high molecular weight and low molecular weight DOM, which are defined as being greater or less than 1000 daltons (Da) in size respectively (Cui and Choo 2013). The high molecular weight (HMW) fraction of DOM is dominated by aromatic humic substances and whilst low molecular weight (LMW) DOM contains simpler monomeric compounds. Humic substances have been defined as coloured polyelectric acids with high capacities to bind to other compounds such as metals, which can be isolated from water using ion exchange resins (Humbert et al. 2007; Boguta et al. 2019). These humic substances can be operationally split into humic acids, which are a mixture of acids with multiple carboxyl or phenol groups, and fulvic acids, which are smaller in molecular weight but contain a greater

Chapter 2

oxygen content compared to humic acids (Chen et al. 2002). These two compound groups constitute approximately 10 % and 40 % of DOM respectively (Thurman 1985). An additional 30% of DOM in freshwater consists of hydrophilic acids, which cannot be retained using an ion exchange resin. Compounds in the hydrophilic acid fraction tends to have many hydroxyl and carboxyl groups and can include volatile fatty acids and sugar acids (Leenheer and Croué 2003).

The remaining 20 % of DOM is made up of monomers and other simple organic compounds, often referred to as the labile fraction of DOM. The three classes of compound that make up the majority of monomeric DOM are carboxylic acids, amino acids and carbohydrates (Thomas 1997; Berggren et al. 2010). All carboxylic acids are monomers and therefore these are the largest DOM fraction. They play an important role in metal complexation, mineral dissolution as well as providing a direct energy source for microorganisms. Amino acids and oligopeptides are only present in small concentrations in freshwater, however, they can relay useful information to aquatic organisms as signalling compounds as well as providing a directly assimilatable form of organic N (Kaplan and Newbold 2003; Zhao et al. 2016).

DOM is a source of macronutrients, defined as elements required in large proportions for normal plant growth, including C, N, P and S, in freshwater ecosystems (Findlay and Sinasbaugh 2003). Carbon is the most abundant nutrient in the freshwater environment and rarely limits primary production. However, larger DOC compounds are coloured and therefore can act as a physical barrier to primary productivity through light attenuation, particularly in upland systems where DOC concentrations are high (Freeman et al. 2004; Moody and Worrall 2017).

Chapter 2

Dissolved organic N, P and S (DON, DOP and DOS respectively) are components of DOC compounds (Spitzzy and Lenheer 1991). Larger, humic and fulvic acids contain approximately 1 % and 2 % N respectively and only trace amounts of P (Thurman 1985). In terms of characterised DON compounds, the majority are amino acids, oligopeptides and nucleic acids, with a smaller fraction consisting of amino sugars (Benner 2002). DOP compounds include energy sources including adenosine triphosphate (ATP), in addition to nucleic acids, phosphorylated lipids and proteins however, DOP species as a whole have not yet been well characterised (Ged and Boyer 2013). Studies on DOS compounds (e.g. S-containing amino acids) are even more sparse, despite there being similar cellular requirements for P and S (Ksionzek et al. 2016). In the coastal zone, N is likely to be the nutrient limiting biological activity, whereas in lentic systems P is more likely to be limiting (Maberly et al. 2003). In-between these two systems, biological production may be co-limited by N and P, or else there might be an equilibrium between the two (Elser et al. 2007).

2.1.2 C:N:P Stoichiometry

The original stoichiometric ratio of C : N : P in the marine environment was identified by Redfield as 106 : 16 : 1 (Redfield 1934). Based on an analysis of previous literature, the C/N ratio of DOM in streams and rivers have been found to generally be in the range of 45-55 : 1 for fulvic acids and 18-30:1 for humic acids (Thurman 1985). The C : N ratio in humic substances has also been found to increase with increasing aromaticity (Steinberg 2003). These ratios are higher than both the Redfield ratio and those identified in soils, which indicates that aquatic DOM is likely to be N depleted in comparison (Thurman 1985). In addition, a global study found that the N/P Redfield ratio was exceeded in over 70 % of major world watersheds ($n = 82$) indicating that P concentration could also be a limiting nutrient in the freshwater

Chapter 2

ecosystem (Fig. 2.2; Turner et al. 2001; Dodds and Smith 2016). More recently, the C:N:P:S ratio has been estimated to be 124:16:1:1.3 (Hot et al. 2003).

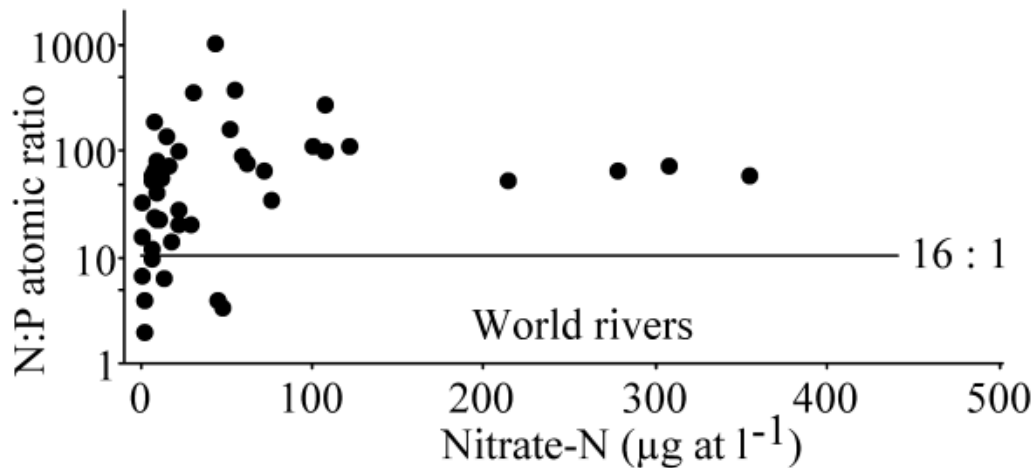


Fig. 2.2 Relationship between N/P ratio and nitrate concentration ($\mu\text{g l}^{-1}$) in a selection of large global rivers. The N/P Redfield ratio of 16:1 is indicated (Turner et al. 2001).

2.2 Ecological importance of the topic

Although aquatic P concentrations are decreasing within the EU following the implementation of Urban Waste Water Treatment Directive, C and N fluxes to coastal waters have continued to increase (Vitousek et al. 2009). Although the Directive has led to a reduced amount of surplus N being applied to agricultural land in Northern Europe, the long residence time of N in riverine systems means that fluxes into coastal waters have remained high, or in some cases have increased in recent years (Vitousek et al. 2009). In addition, on a global scale N fluxes are increasing due to intensified agricultural practices and population increases in urban coastal areas (Carcao and Cole 1999). In the UK and elsewhere, DOC input to coastal areas is also increasing due to anthropogenic inputs from wastewater treatment plants and polluted urban rivers (Butman et al. 2015).

Chapter 2

Excessive nutrient concentrations can cause issues with water quality in both rivers and coastal zones including eutrophication, which can lead to excessive plant growth and the development of harmful algal blooms (HABs) (Heisler et al. 2008). A previous study of historical HAB outbreaks in Chesapeake Bay found that DOC concentrations were increased during HAB events (Glibert et al. 2001). Eutrophication can lead to the loss of key species from ecosystems, changes in species composition and the loss of ecosystem services due to pollution (Smith and Schindler 2009; Glibert 2017). HABs can have many undesirable consequences, including risks to both human and animal health from toxic dinoflagellate species, the creation of anoxic bodies of water, elevated pH and economic losses through the damaging of fish and shellfish stocks in the coastal zone (Shumaway 1999; Landsberg 2002; Moore et al. 2008). These changes can lead to acute regime shifts, which have been previously seen to devastate freshwater ecosystems, particularly in shallow lakes (Scheffer et al. 2001; Scheffer and Carpenter 2003).

In terms of river management, eutrophication is one of the most significant issues in a number of countries. The management of eutrophication in freshwater ecosystems requires several issues to be addressed in order to make progress: defining the critical load of each nutrient, the amount by which these loadings are currently being exceeded and identifying site-specific seasonal variation in nutrient loadings (Smith et al. 1999). The mechanisms of nutrient loading and processing are complex, particularly for N, where a reduction in N input does not necessarily lead to a reduction in harmful blooms downstream (Smith and Schindler 2009). This may be related to the lack of consideration for organic nutrients (i.e. DON, DOP) within previous studies. There is therefore a need for further research in this area with a greater focus on organic forms of N, particularly in freshwater lakes where data are lacking (Smith and Schindler 2009).

Chapter 2

Climate change can also have variable effects on DOM: the rate of eutrophication may be accelerated by global warming, which has previously been reported for boreal lakes (Moser et al. 2002). It has been found that an increase in temperature increases primary productivity and DOM export from upland and boreal areas at higher latitudes, although research on DOC trends in lower latitudes is limited (Evans et al. 2005; Reiger et al. 2016). However, in areas with lower existing DOM concentrations, the warmer and drier weather attributed to climate change could cause a decrease in DOM concentrations (Häder et al. 2007). Reduced water saturation of the surrounding areas could lead to a reduced input of DOM into rivers and lakes, thus their ability to attenuate light would be reduced. This would allow for the increased breakdown of DOM as ultraviolet (UV) light would be able to penetrate to further depths, increasing the bioavailability of DOM compounds to aquatic organisms (Häder et al. 2007).

Increased loading of DOM in rivers can also lead to increased inputs of dissolved effluent organic matter (dE_fOM) from wastewater treatment plants, particularly as the reuse of wastewater increases in water-scarce areas (Michael-Kordatou et al. 2015). Wastewater is commonly reused for non-potable use, such as in agriculture, industry and recreation, or in some cases for potable use following treatment (Michael-Kordatou et al. 2015). One of the main concerns with recycling water for potable use is the formation of disinfection by-products (DBPs) from dE_fOM, a number of which have been linked to carcinogen formation and reproductive diseases (Sirivedhin and Gray 2005; Chaves et al. 2019). Humic substances in particular may be chlorinated or ozonated to produce carcinogens (Nikolaou et al. 2003). Relatively little is known about the effects that dE_fOM can have on the microbial community, although a large fraction of the DOC in wastewater effluents consists of soluble microbial products (SMPs) which can be transported across eukaryotic cell membranes (Michael-Kordatou et al. 2015). The majority of SMP compounds are known to be primarily composed

Chapter 2

of humic substances, carbohydrates and proteins, although their exact structures have not been identified (Liang et al. 2007). In some circumstances dE_fOM has been found to have toxic effects on invertebrate species, reducing biodiversity (Vasquez and Fatta-Kassinos 2013; Mor et al. 2019). More indirect effects of increased dE_fOM concentrations include the ability both to alter the bioavailability of key nutrients such as metals and to potentially increase the toxicity of microcontaminants to aquatic organisms (Shon et al. 2006). These microcontaminants may include pharmaceuticals and complexes such as plastics, detergents and pesticides (Pedersen et al. 2005).

2.3 Methods of DOM quantification and characterisation

Dissolved organic matter (DOM) is often quantified using a proxy such dissolved or total OC (DOC or TOC), therefore DOM is often represented as units of C (Kapalan and Newbold 2003). However, DOM may also be represented through other indirect measurements including chemical or biological oxygen demand (COD/BOD), DON or UV-light absorption (Zhao et al. 2012). Dissolved organic P and S quantification is also possible but is more complex and therefore not as frequently measured. In addition to these bulk quantification techniques, a number of more specific qualitative techniques have been developed over the last few decades which allow the characterisation of DOM, which takes into account the widely variable chemical composition of DOM in aquatic ecosystems (Her et al. 2003; Ohno and Bro 2006; Li et al. 2019). It is beyond the scope of this literature review to discuss the merits and limitations of individual techniques in great detail, however, the most common techniques used are outlined in the following sections.

2.3.1 Quantification of DOM

Chapter 2

There are a number of techniques for measuring TOC, all of which include some form of oxidation step following the removal of any inorganic C by acidification of the sample (Matilainen et al. 2011). The resulting CO₂ produced is then detected, generally using infrared spectroscopy (Sillanpää 2015). As some organic C may also be purged during the acidification process, the OC detected using this method is referred to as non-purgeable OC (NPOC). In order to measure DOC using this technique, the sample is pre-filtered through a 0.45 µm membrane.

DON and DOP can both be measured indirectly by measuring total dissolved N or P (TDN/TDP) and subtracting the inorganic fraction (Hansell and Carlson 2002). DON and DOP may be quantified using the persulphate digestion method, the safer alternative to the Kjeldahl method, combined with ion chromatography (IC) (De Borja et al. 2014).

Ultraviolet and visible (UV-vis) absorption spectroscopy can also be used as a semi-qualitative measurement of DOM (Matilainen et al. 2011; Roth et al. 2019). A number of single wavelength absorptions have previously been used as a proxy for DOC concentration, however, specific wavelengths and ratios can also be used to infer some characteristics of DOM (Peacock et al. 2014). Specific UV absorbance (SUVA), which is the absorbance at 254 nm divided by DOC concentration can be used to determine aromaticity, with a positive correlation between the SUVA value and both hydrophobicity and molecular weight of DOC (Edzwald and Tobiasson 1999; Weishaar et al. 2003). The E2:E3 ratio (absorbance at 250 nm and 365 nm) has also been found to correlate with aromaticity and molecular weight and the E4:E6 ratio (absorbance at approximately 400 and 600 nm) has previously been used as a measure of humification (Worrall et al. 2007; Peacock et al. 2014).

Chapter 2

2.3.2 Characterisation of DOM

In addition to analytical methods that quantify different classes of DOM, there are a range of techniques available with higher specificity that can combine the use of chromatographic techniques with high resolution detection methods, which can differentiate between compounds on a molecular scale (Jones 1997; Michael-Kordatou et al. 2015). Some of the main techniques utilised for DOM characterisation also include elements of spectroscopy and fractionation. In addition, pyrolysis may be combined with gas chromatography and mass spectrometry (GC/MS) for DOM characterization (Sulzberger and Durisch-Kaiser 2009).

Fluorescence spectroscopy can be used to detect fluorophores, which are an element of molecular structure which provide an indication of the composition of DOM chemistry (Zhang et al. 2008). Fluorescence techniques have a higher specificity than methods such as UV-vis and therefore have gained popularity in the last few decades (Matilainen et al. 2011). It is possible to use excitation emission fluorescence detectors *in situ* to collect real time data for characteristics such as the concentration of coloured DOM (CDOM) or chlorophyll- α , or else to use fluorescence excitation-emission matrix (EEM) spectrophotometry to detect fluorescence at a range a range of wavelengths, which can be used to identify wavelengths for more specific study (Yamashita et al. 2008; Her et al. 2003).

The physical fractionation of DOM components is a useful tool that allows the separation of DOM components in order to prepare samples for characterisation. Two of the most common methods are physical separation and adsorption chromatography. Physical separation of samples may be done manually using membrane filtration or through the use of size exclusion chromatographic (SEC) techniques. Membrane filtration has its limitations due to adsorption of compounds to the membrane gradually limiting pore size, however, SEC methods have been used in conjunction with high performance liquid chromatography (HPLC)

Chapter 2

in a technique known as HSPEC (Matilainen et al. 2011). This technique has many advantages including the ease of use and the volume of sample required (Tran et al. 2015). HSPEC has often been linked with either UV-vis, fluorescence or Fourier transform infrared (FTIR) detection to measure the molecular weights of the different DOM components. In recent years, FTIR detection has also been coupled with mass spectroscopy in order to yield further molecular information for DOM components (Sleighter and Hatcher 2007; Gonsior 2019).

Extraction and fraction may also be carried out using XAD resins, which fractionate DOM by adsorbing hydrophobic compounds but not hydrophilic compounds (Shapiro and Karavanova 2014). A technique based on these resins, known as the rapid fractionation technique, can separate compounds into general acidic, basic and neutral fractions (Chow et al. 2004; Al Juboori et al. 2016).

To detect trace elements of DOM, such as hormones, pharmaceuticals or other LMW compounds, passive samplers may be deployed which can collect samples in a time-integrated manner. An increasingly common method is using polar organic chemical integrative samplers (POCIS). POCIS discs are designed to collect small, hydrophobic compounds present in low concentrations and have been utilised for monitoring water quality at wastewater treatment works (WWTWs) (Jacquet et al. 2012). After being deployed for approximately 30 days, POCIS discs can be analysed using chromatographical or fractionation techniques in order to identify the compounds that have been concentrated. It is also possible to use solid phase extractions or liquid chromatography mass spectroscopy (LC-MS) to fractionate hormones and other hydrophilic low molecular weight compounds that may be present (Jacquet et al. 2012).

Chapter 2

2.4 Sources of DOM

Organic matter ultimately enters the biosphere via autotrophic activity, particularly photosynthetic plants, however a range biochemical and physical processes are key to the transportation and processing of organic matter within the freshwater ecosystem, which will be discussed over the course of this section. Broadly speaking, terrestrial primary productivity is significantly higher than that of aquatic ecosystems, however there are a number of direct autochthonous sources of organic matter within freshwater ecosystems (Bertlissson and Jones 2003). In streams, the majority of DOM can originate from allochthonous sources, particularly in forested areas (Roelke et al. 2006; Heinz et al. 2015). This allochthonous DOM can be transported to the aquatic ecosystem through a number of mechanisms, including via rainwater, windborne material, surface flow and groundwater flow (Fig. 1.3; Thomas 1997). However, as a water body moves downstream and increases in size into the marine environment, the contribution of autochthonous sources gradually increases; ultimately only 2.5 % of oceanic DOM is thought to originate from terrestrial sources (Thomas 1997). This section will outline the main sources of allochthonous and autochthonous sources of DOM in river systems and the processes by which these nutrients reach the aquatic environment.

2.4.1 Allochthonous sources

Allochthonous sources of DOM are defined as those which originate outside of the aquatic ecosystem; this includes mainly terrestrial but also atmospheric origins (Hood et al. 2005). In some temperate areas, up to 99 % of the energetic input into a river system can be attributed to allochthonous sources (Fisher and Likens 1973). The bulk of allochthonous DOM can be divided into two categories: organic matter from plants and organic matter from soils. These

Chapter 2

two differ in that soil organic matter will have undergone decomposition to a greater extent compared to plant organic matter and therefore soil organic matter will be older, however both of these inputs are key for streams and smaller river systems (Thurman 1985; Hansen et al. 2016).

2.4.1.1 Plant-derived organic matter

Sources of plant organic matter can be divided into three components: leaf litter, roots and the organic horizon of the soil. The organic horizon of soil contains fresh and partially degraded plant matter and thus is a source of plant organic matter. In forested areas, it has been estimated that the majority of DOM comes from the organic horizon (~ 88 %), with approximately 15 % from both root exudate and decomposition and a further 7% from leaf litter leachate (Aitkenhead-Peterson 2000; Aitkinhead-Peterson et al. 2003). Plant organic matter can also dominate DOM composition in other habitats, including saltmarshes, bogs and wetlands. For the latter, a previous study found that over 90 % of DOC present in a wetland river was less than 30 years old and therefore too young to be considered soil organic matter (Thurman and Malcom 1983). Some of the main factors that affect plant organic matter production and its leaching into the aquatic environment include precipitation, the amount of leaf litter deposited on the ground and the characteristics of the soil (Qualls et al. 1992; Chow et al. 2008).

Plant organic matter is very labile: previous studies have shown that 25-40 % of plant litter DOC may be solubilised in distilled water within 24 hours (Thurman 1985; Hansen et al. 2016). In these studies, the majority of the DOC in the leachate was found to be simple carbohydrates rather than complex structures, with coloured organic acids (similar to aquatic fulvic acids) making up the remainder (Uselman et al. 2012). In contrast, fulvic acids from aquatic and soil interstitial water samples have been found to contain a higher carboxyl content

Chapter 2

and lower carbohydrate content than the compounds detected in the leaching experiments, indicating that the carbohydrate fraction of the leachate is rapidly degraded through oxidation (Uselman et al. 2012). In addition, between 30-100 % of DON in plant litter has previously been found to be soluble proteins and peptides (Yu et al. 2002). Different components of plant litter have been found to contain variable qualities of DOM, for example Uselman and colleagues (2012) found that there was 20-30 % soluble DOC in leaf litter in comparison to 6 % in root litter. Root litter samples were also found to leach less DOC in a simulated field experiment. In contrast, DOC:DON and DOC:DOP ratios in root litter were lower than in leaf litter, indicating that root litter has a larger amount of N and P available for degradation. This is thought to be because N and P are translocated during leaf senescence, in order to avoid excessive nutrient loss from the plant (Uselman et al. 2012).

The concentration of plant organic matter input to the aquatic ecosystem varies both spatially and seasonally, with the greatest accumulation of plant litter occurring in the autumn, when leaves are shed by deciduous shrubs and trees and other plants die back. The DOM from this plant organic matter is then flushed into the aquatic environment in the spring, through increased precipitation and snow melts. This nutrient input is generally great enough to increase DOM concentrations in aquatic ecosystems, despite the increased rate of discharge (Sebestyen et al. 2008). However, previous studies have found that some of the bulk of humic substances released as part of the spring flush differ from previous distilled water leaching studies, therefore it is likely that the flush also contains soil organic matter (Thurman 1985).

2.4.1.2 Soil organic matter

Soil organic matter is generally defined as organic matter that is greater than 100 years old that has been degraded and humified in the soil, although it has been proposed that some SOM

Chapter 2

could be newer, spanning continuum of progressively more decomposed OM (Coleman et al. 2004; Lehmann and Kleber 2015). It is mainly derived from the mineral soil, which is defined as having an organic matter content of less than 20 %. In general, soil organic matter is older than the autochthonous DOM found in freshwaters (Loh et al. 2006). The A horizon of soil contains organic matter that is actively being decomposed; it has the highest organic matter content. Some organic matter, often humic and fulvic acids, is transported down into the B horizon of soil, which are then retained through adsorption to clays and metal oxides (Thurman 1985). It follows that the higher the clay content of a soil, the lower the DOM output from those soils. The rate at which soil organic matter is exported to aquatic environments depends on a number of factors, including the gradient of the watershed, soil moisture and barriers affecting the ability of the interstitial water to move between the organic soil fraction and the mineral soil (Aitkinhead-Peterson et al. 2003). A higher rate of interstitial water infiltration to the mineral soil will reduce the concentration of soil organic matter percolating into adjacent aquatic environments, due to adsorption and other factors including microbial processing (Aitkinhead-Peterson et al. 2003). Areas such as peatland bogs have very high DOM outputs as there is almost no infiltration of the mineral soil, due to both physical barriers between the two horizons and the naturally high water table of these ecosystems (Eckhardt and Moore 1990).

The amount of organic matter stored in soils varies greatly between ecosystem types. For example, grasslands have a higher soil organic matter content in comparison to forest soils, however forests have a larger proportion of organic matter stored in plant matter; between 30-40 % of organic matter is stored in plants in forests, in comparison to 1-5 % in grasslands (Thurman 1985; Billings 2006). The greater organic matter content of grassland soils means that a higher concentration of DOM is dissolved by interstitial waters and transported into

Chapter 2

streams and rivers compared to forested areas, even during periods of nutrient flushing in the spring (Thurman 1985).

2.4.1.3 Transport methods

In addition to plant and soil organic matter, there are several transport mechanisms that can bring other allochthonous sources of DOM into the freshwater environment. Precipitation can act as a direct source of allochthonous DOM when it falls onto the aquatic environment. DOM from precipitation tends to contain dust and pollens being transported in the atmosphere, in addition to some gaseous N and C forms (Hejzlar et al. 2003). Previous analyses of DOM from precipitation have found low DOC/DON ratios, which could be attributed to marine nitrogenous gas emissions (Aitkinhead-Peterson et al. 2003). Some more indirect methods of transport of DOM into the aquatic atmosphere include windborne organic material, defecation and urination from livestock and throughfall through vegetation surrounding the aquatic ecosystem.

2.4.2 Autochthonous sources

The majority of autochthonous DOM originates from either macrophytes or algae, although they may also alter riverine DOM composition through the removal of nutrients from the water column (Schneider et al. 2016). Macrophyte production is the least well studied of the two, despite being the dominant organic matter producers in many aquatic ecosystems (Bertlison and Jones 2003). The literature has focused on algal production, which is dominated by phytoplankton in lentic systems and the periphyton community in lotic systems. Phytoplankton are seen as key contributors to aquatic DOM due to their high cellular concentrations of labile, low molecular weight compounds including carboxylic acids, amino acids and monomeric

Chapter 2

sugars (Bertlissson and Jones 2003). It follows that the DOM released by phytoplankton has a rapid turnover and that algal blooms are frequently found to coincide with increases in bacterial biomass (Gajewski and Chróst 1995).

The DOM produced by phytoplankton can enter the aquatic environment through a number of biotic and abiotic mechanisms. Although active algal cells release some nutrients directly, the processes involved are not very well understood (Cook et al. 2007). In general, the majority of the DOM released occurs via cell senescence and degradation. The rate of DOM release may also be increased by the “sloppy eating” and excretion by predator species who graze on phytoplankton and also the viral lysis of algal cells (Thomas 1997). Several abiotic factors may also affect the rate of algal DOM release, for example UV light, which can both increase the rate of primary production and cause an increase in DOM release, most likely through cell membrane damage (Farjalla et al. 2001). In addition, the background nutrient concentrations of the environment will also affect the rate of DOM release, for example large DOM losses from algal cells in nutrient-poor conditions have been attributed to a “culture shock” under laboratory conditions (Sharp 1977).

In shallow lentic and wetland areas, macrophyte production is a significant source of DOM for aquatic organisms. Similarly to algal DOM, macrophyte-originating DOM also contains quite a large labile fraction (Maranger et al. 2005). Although its production is focused in lentic ecosystems, the seasonal flushing of nutrients from these areas means that macrophyte production can also be a significant source of DOM for any surrounding aquatic ecosystems (Bertlissson and Jones 2003).

Chapter 2

2.5 Concentrations of DOM in freshwater ecosystems

Patterns in the concentration of DOM can be observed both spatially and temporally. In terms of the vertical distribution of DOM within the aquatic ecosystem, the concentrations of several DOM compounds, including amino acids and carbohydrates, have been found to be greatest at the air-water and soil-pore water interfaces in comparison to the mid water column (Thomas and Eaton 1996). It is generally agreed that the concentration of total DOM follows the same trend (Thomas 1997). However, more refractory compounds, such as humic substances, have been present at lower concentrations at the air-water interface; this can be attributed to the susceptibility of such compounds to photodegradation (Thomas 1997). In terms of spatial variation, it could be assumed that the increasing flow along the length of a water body could dilute the DOC concentration, the patterns observed on a global scale are inconsistent (Mulholland 2003). The majority of the temporal patterns observed in DOM concentrations can be linked to both seasonal changes and storm events (Mulholland 2003).

In addition to DOM concentration, the quality of DOM is also key in regulating the biological activity in the freshwater environment. DOM quality has previously been described using a number of indices relating to its chemical composition (Inamdar et al. 2012). Increasing quality of DOM implies the increased bioavailability of DOM to the aquatic community (Inamdar et al. 2012; Yates et al. 2019). The quality of DOM is not likely to correlate with the concentration of DOM observed, as greater concentrations of DOM can be attributed to larger, aromatic compounds that are not as easily utilised (Inamdar et al. 2012). The concentrations of smaller, monomeric compounds tend to play a more significant role in the structure and size of the microbial biomass (Jaffé et al. 2008; Kaplan et al. 2016).

Chapter 2

2.5.1 Patterns in DOC concentrations

The concentration of freshwater DOC is crucial due to its influence on both the physical properties and biological processes of an ecosystem. The average DOC concentration in freshwater river systems is between 0.5 and 50 mg L⁻¹ (Table 2.1; Mulholland 2003).

Precipitation is considered to be a key influential driver of DOC concentration, particularly on a global scale. It has been previously found that DOC concentration could be linked to the climate zone at different latitudes, with mean DOC increasing from 1 mg L⁻¹ in arid regions to 8 mg L⁻¹ in wet tropical regions (Meybeck 1988). However, several studies have highlighted that the influence of precipitation is not straightforward. A previous analysis of the discharge-weighted mean DOC concentrations of major world rivers by Spitzzy and Lenheer (1991) did not identify a significant correlation between DOC concentration and runoff. In a number of instances, rivers with the highest runoff had some of the lowest mean concentrations of DOC, whereas several rivers with low runoff had mean DOC concentrations that were much higher than global mean DOC (Spitzzy and Leenheer 1991). In addition, a study of alpine rivers by Rodríguez-Murillo et al. (2015) found that only 2.5 % of DOC variability could be attributed to precipitation.

Chapter 2

Table 2.1 Table listing the ranges of mean dissolved organic carbon (DOC) concentrations for rivers and streams in a number of locations, catchment types and seasons.

Mean range DOC (mg L ⁻¹)	Location	Catchment type	Season	Storm event	Reference
1.5-5.5	New York, USA	FW Tidal estuary	Annual	N	Findlay et al. 1991
3.3-7.8	N England	Limestone upland	Annual	N	Baker and Spencer 2004
8.2-15.3	N England	Peat-influenced	Annual	N	Baker and Spencer 2004
3.3-5.8	N England	Urbanised	Annual	N	Baker and Spencer 2004
4.8-15.6	Florida, USA	Estuarine (Mangrove)	Dry	N	Moyer et al. 2015
13.2-22.2	Florida, USA	Saltmarsh	Dry	N	Moyer et al. 2015
9.0-12.0	SW Scotland	Forest	Spring	Y	Grieve 1990
6.5-12.5	SW Scotland	Moorland	Spring	Y	Grieve 1990
7.8-8.6	Quebec, Canada	Clear mountain	Summer	N	Berggren and del Giorgio 2015
8.5-17.2	Quebec, Canada	Forest	Summer	N	Berggren and del Giorgio 2015
12.0-14.0	SW Scotland	Forest	Summer	Y	Grieve 1990
2.0-4.75	Ontario, Canada	Mixed catchment	Summer	N	Massicotte 2011
10.0-14.0	SW Scotland	Moorland	Summer	Y	Grieve 1990
1.2-6.1	NE Scotland	Moorland	Summer	N	Dawson et al. 2001
11.9-19.3	Quebec, Canada	Peat-influenced	Summer	N	Berggren and del Giorgio 2015
10.0-25.3	NE Scotland	Peat-influenced	Summer	N	Dawson et al. 2001
12.1-12.9	SE Australia	Semi-arid	Summer	Y	Westhorpe et al. 2011
18.9-28.5	SE Australia	Semi-arid	Summer	Y	Westhorpe et al. 2011
7.9-19.9	Florida, USA	Estuarine (Mangrove)	Wet	N	Moyer et al. 2015
20.7-25.9	Florida, USA	Saltmarsh	Wet	N	Moyer et al. 2015
14.0-17.0	SW Scotland	Forest	Winter	Y	Grieve 1990
5.5-7.0	SW Scotland	Moorland	Winter	Y	Grieve 1990

Chapter 2

In contrast, catchment land use, particularly percentage cover of wetlands or peatlands, has been found to consistently control the concentration of DOC in surface waters (Table 2.1; Mulholland 2003; Sachse et al. 2005). A previous study found that the percentage wetland cover accounted for 76 % of DOC variability in Canadian forested rivers (Dillon and Molot 1997). Within Scottish peatlands, soil organic content was found to account for > 85 % DOC variability in streams (Aitkinhead et al. 1999). In uplands, flowpath is also of importance: catchments with higher rates of surface flow, particularly in uplands, tend to have higher concentrations of DOC than well-drained areas due to the high organic content of surface soils (Mulholland 2003). In well-drained areas, precipitation is therefore more likely to affect aquatic DOC concentration as it would lead to the flushing of organic matter from surface soils, particularly as the gradient of the riparian area increases (Mulholland 2003). Finally, point of diffuse anthropogenic inputs will generally increase the concentration of DOC present in the water body (e.g. from septic tanks, wastewater treatment discharges).

2.5.2 Patterns in DON concentrations

DON is present at much lower concentrations in the freshwater environment than DOC (Table 2.2; Berman and Bronk 2003). These lower concentrations, in conjunction with the analytical challenges involved in DON measurement, have previously meant that research in this area was more limited than that of DOC, however over the last few decades the routine measurement of DON concentrations in studies has become much more commonplace (Bronk 2002).

Variation in DON concentration has a number of important consequences for the freshwater environment. For example, changes in the C : N ratio in DOM can cause changes in the bioavailability and rate of transport observed, due to changes in hydrophobicity (Inamdar et al. 2012; Miller et al. 2016; Shi et al. 2016). DON concentration can also be an indicator of

Chapter 2

stream and river health; previous studies have found that the percentage of DON in total dissolved N (TDN) can be as low as 2 % where anthropogenic influences were large, increasing to 60-90 % in pristine forested streams and semi-natural catchments (Perakis and Hedin 2002; Kortalainen et al. 2006). However, DON concentrations can increase due to N enrichment with increases from 0.15 mg L⁻¹ DON to > 5 mg L⁻¹ possible from oligotrophic uplands to eutrophic waters (Jones and Burt 1991).

Table 2.2 Table listing the ranges of mean dissolved organic nitrogen (DON) concentrations for a range of locations. Adapted from Berman and Bronk (2003).

Mean DON (mg L ⁻¹)	Location	N	Reference
0.38 ± 0.07	Russian Arctic Rivers	7	Gordeev et al. 1996; Wheeler et al. 1997
0.42 ± 0.2	Baltic Rivers	5	Stepanauskas et al. 2002
0.32	Maryland, USA	1	Hopkinson et al. 1998
0.83	Georgia, USA	1	Hopkinson et al. 1998
0.36	Maryland, USA	1	Hopkinson et al. 1998
0.42 ± 0.33	Delaware, USA	1	Seitzinger & Sanders 1997
0.47	New York, USA	1	Seitzinger & Sanders 1997
0.38	Maryland, USA	1	Bronk & Glibert 1993
0.5 ± 0.15	Georgia & South Carolina, USA	8	Alberts & Takács 1999
0.34 ± 0.1	Sweden Streams	2	Stepanauskas et al. 2000
1.26 ± 0.95	Sweden Wetlands	1	Stepanauskas et al. 1999
0.06 - 0.25	California, USA	1	Smith et al. 1991

Chapter 2

2.5.3 Patterns in DOP concentrations

In comparison to the characterisation of aquatic C and N pools, the P pool has been understudied, particularly the DOP pool which has often been considered an intermediate between the inorganic P forms (P_i) utilised by the aquatic microbial community (Karl and Björkman 2002). However, in nutrient depleted environments DOP may be utilised as a source of P by those species possessing phosphatase enzymes (Chróst and Overbeck 1987; Huang et al. 2005). The processing of DOP compounds is also directly linked to DOC/DON as all DOP compounds are either DOC-P or DOC-N-P in structure (Karl and Björkman 2002).

In oligotrophic aquatic ecosystems, DOP concentrations are generally in the magnitude of $1 \mu\text{M}$ ($30 \mu\text{g L}^{-1}$) (Table 2.3). DOP has been found to constitute up to 82 % of soluble P in some lentic systems, with a high proportion of this considered to be hydrolysable and thus part of the labile P pool (Chróst and Overbeck 1987).

Table 2.3 Table listing the ranges of mean dissolved organic phosphorus (DOP) concentrations in $\mu\text{g L}^{-1}$ for a number of locations. Errors are ± 1 SEM. *Indirect calculation of DOP by subtracting inorganic P (P_i) from total P (TP).

Mean DOP ($\mu\text{g L}^{-1}$)	Location	Season	N	Reference
9.3	Tamar Estuary, UK (LS)	Spring	4	Monbet et al. 2009
2.98	Tamar Estuary, UK (LS))	Summer	4	Monbet et al. 2009
13.44	Tamar Estuary, UK (LS)	Autumn	4	Monbet et al. 2009
10.78	Tamar Estuary, UK (LS)	Winter	4	Monbet et al. 2009
13.43	Tamar Estuary, UK (HS)	Spring	3	Monbet et al. 2009
11.37	Tamar Estuary, UK (HS)	Summer	3	Monbet et al. 2009
12.67	Tamar Estuary, UK (HS)	Autumn	3	Monbet et al. 2009

Chapter 2

14.77	Tamar Estuary, UK (HS)	Winter	3	Monbet et al. 2009
14.34 ± 3.45	Florida, USA	Annual	5	Ged and Boyer 2013
8.8 ± 1.39*	Québec, Canada	Summer	21	Kortelainen et al. 2006

2.6.4 Patterns in DOS concentrations

Concentrations of total DOS in riverine systems are underreported due to the difficulties in their measurement, however the concentrations of some DOS components such as thiols (10-160 nM) and free S-containing amino acids 0.2-5.0 nM have been reported (Marie et al. 2015; Horňák et al. 2016). The higher relative concentrations of thiols indicate their importance in S transport along the freshwater : marine continuum, whilst the low concentrations of S-containing amino acids in comparison to cellular concentrations 100-200 µM) suggests that they may be rapidly cycled in freshwaters (Marie et al. 2015; Horňák et al. 2016).

2.6 DOM flux in freshwater ecosystems

The flux of DOM through a freshwater catchment is generally measured as the mass of C, N or P exported from the catchment annually. As it passes along the water course, DOM is subject to both biological and chemical in-stream processing, which directly affects the rate of flux from the ecosystem (Fig. 2.3; Mulholland 2003). It has been estimated that just 50 % of total DOC input to rivers is exported to the world's oceans, with 25 % being processed in-stream and the remaining 25 % being adsorbed into the sediment (Hope et al. 1994).

The chemical processes affecting the flux of DOM include degradation by UV radiation, which can produce more bioavailable organic and inorganic compounds, which are more likely to be taken up by the microbial biomass (Moran and Covert 2003). The immobilisation of DOM through complexation with metal oxides, such as iron and aluminium,

Chapter 2

contained within the sediment of the stream is also possible (Hope et al. 1994). In a previous laboratory study, it was found that aluminium can remove 60 % of DOC from the water column, indicating that it can play a significant role in the flux of DOM (Hope et al. 1994; Gareis and Lesack 2018).

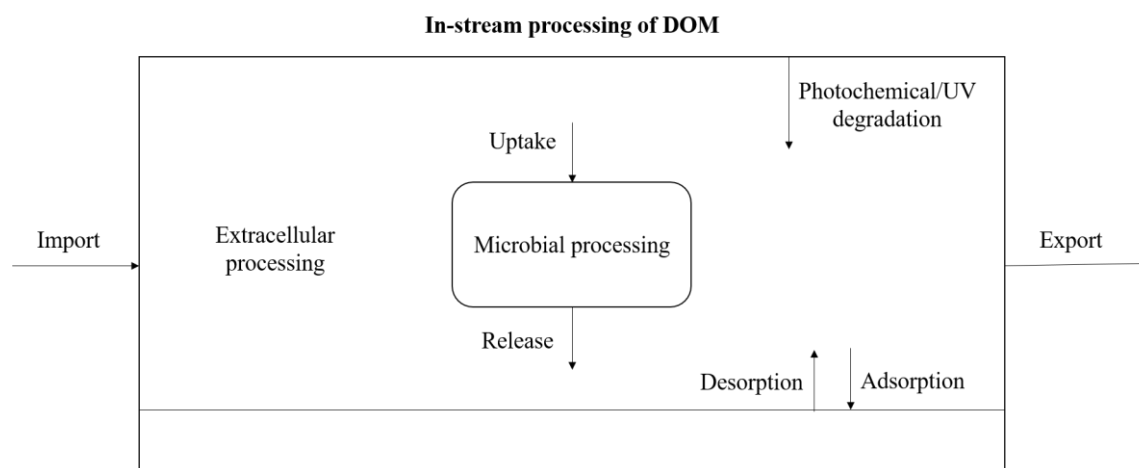


Fig. 2.3 Diagram showing the biological and chemical pathways by which in-stream processing of DOM occurs [Adapted from Mullholand 2003].

Some of the main biological processes influencing the rate of DOM flux include both the intracellular and extracellular biological breakdown of DOM, the release of carbon dioxide by respiration and the immobilisation of DOM by biofilms and epilithon present on the solid surfaces within any interfaces with the aquatic environment (Hope et al. 1994). These will be the subject of section 7.0.

2.6.1 Patterns in DOC flux

In a previous review estimates of global DOC export values, from 12 sources, between 0.3 and 10×10^{14} g C year⁻¹ (Hope et al. 1994). The main drivers of variability in DOC flux are similar

Chapter 2

to those previously identified for DOC concentrations; large-scale comparisons of DOC export from rivers have found that the antecedent conditions, particularly the rate of terrestrial runoff, was the primary influence of the rate of DOC flux observed (Mulholland 2003). This is not surprising as runoff encompasses a number of key factors, including the nature of the C source, precipitation and the hydrology of the catchment (Mulholland 2003). Biomes with low levels of precipitation were estimated to have an annual rate of DOC export of less than $1 \text{ g C m}^{-2} \text{ y}^{-1}$, whilst wet temperate and tropical regions were estimated to export between 2 and $10 \text{ g C m}^{-2} \text{ y}^{-1}$ (Shelsinger and Melak 1981). However, as with the effect of runoff on DOC concentrations, the impact of runoff DOC export has been disputed with more recent studies suggesting that runoff is of less importance in some catchments or that runoff can correlate negatively with DOC export (Laudon et al. 2004; Mattsson et al. 2009).

Other studies have highlighted additional potential influences on the variability in DOC flux. For example, a study of several Northern European catchments found that catchments with higher elevation correlated with the lower DOC flux rates (Mattsson et al. 2009). A review of DOC export for 164 rivers found that soil C:N ratio accounted for over 99 % of variability between biomes (Aitkenhead and McDowell 2000).

In individual catchments, a review of almost 100 rivers found that the DOC flux ranged between $1\text{-}50 \times 10^6 \text{ g C km}^{-2} \text{ y}^{-1}$, with wetlands and boreal forests exhibiting some of the highest DOC flux rates (Shelsinger and Melak 1981; Hope et al. 1994). In recent decades, several long-term studies of small catchments have found that DOC fluxes have been increasing annually, with the suggestion that this is related to temperature and pH changes linked to climate change (Evans 2005; Worrall and Burt 2007). On a global scale, it is difficult to discern such trends, as annual DOC flux rates reported are likely to be underestimated, due to

Chapter 2

the fact that many studies have sampled sub-monthly, which means that large increases in flux during storm events are likely to be missed (Mulholland 2003).

2.6.2 Patterns in DON flux

DON can constitute up to 90 % of total N in unpolluted rivers, therefore DON can be the main route by which N is lost from terrestrial and fluvial ecosystems (Perakis and Hedin 2002). Although it is often thought that N exists only as small percentage within high molecular weight (HMW) compounds such as humic and fulvic acids, it is also present in a number of smaller compounds which are more biologically labile that can be turned over in a matter of hours (Bronk 2002). The rate of DON flux is dependent on residence time, which in streams may be hours or days, increasing to months and years in high order rivers (Durand et al. 2011).

All DON compounds contain DOC, therefore the rate of DON flux is linked to the rate of DOC flux. In general, N-rich DOM compounds are more mobile than C-rich DOM compounds and therefore are more likely to be easier to become biologically degraded or immobilised (Inamdar et al. 2012). N-rich DOM will also have different rates of flux in comparison to C-rich DOM compounds (Inamdar et al. 2012). The rate of in-stream degradation will influence the rate of DON flux observed, however labelled-isotope data has also shown that DON can be synthesised by microbes using NO_3^- , therefore the flux of DON is quite complex (Benner et al. 1992).

A global review of DON fluxes to oceans found a mean range of 10-479 kg N km⁻² y⁻¹ (Alvarez-Cobelas et al. 2008). The mean DON flux at the tidal limit for the UK was been estimated to be 1.1×10^8 kg N y⁻¹ (Worrall et al. 2012). However, both of these studies indicated

Chapter 2

that there are shortcomings in their estimations, as it is difficult to account for all the possible sources and sinks of DON in the freshwater ecosystem.

2.6.3 Patterns in DOP flux

In contrast to DON, DOP makes up a relatively small fraction of the total P load in freshwater ecosystems; the majority of P is composed of particulate P (PP), followed by dissolved inorganic P (DIP) and DOP (Seitzinger et al. 2009). However, DOP is much more mobile than DON and can make its way from source to surface waters quickly (Aitkenhead-Pearson et al. 2003). DOP is also more hydrophobic than DON and therefore more bioavailable (Qin et al. 2015). In terms of eutrophication, this means that P is the more significant nutrient as it is transported to the aquatic ecosystem at the fastest rate (Aitkenhead-Pearson et al. 2003). Despite the importance of the P pool for both ecosystem health and nutrient cycling, the flux of P has been relatively understudied as the dynamics and patterns of P distribution, particularly for DOP, have not yet been defined (Meybeck 1982; Karl and Björkman 2002).

A recent study used the first spatially explicit model of DOM to estimate DOC, DON and DOP fluxes and estimated global DOP flux at approximately 6×10^{11} g P y^{-1} (Harrison et al. 2005). This is one magnitude lower than the only previous global estimate of total P flux of 2.3×10^{12} g P y^{-1} , however this value was calculated using the assumption that DIN : DON ratios are always constant (Meybeck 1982; Harrison et al. 2005). In terms of future outlook, several models predicting global P flux to 2030 have estimated that only small (1-6 %) increases in DOP flux will occur in the next few decades (Seitzinger et al. 2009). However, it was also predicted that the composition of the different P fractions would be altered, which would affect the rate of global P cycling (Seitzinger et al. 2009).

Chapter 2

2.6.4 Patterns in DOS flux

Presently, no studies could be identified that investigated the flux DOS compounds through aquatic environments, or how this relates to other S components such as inorganic sulphur (S_i) and particulate sulphur.

2.7. Microbial processing of DOM

Microbial processing includes the uptake, transformation and release of DOM by bacteria and algae. This section will discuss both intracellular and extracellular processing of DOM, in addition to the role of biofilms in DOM processing. The microbial processing of DOM is dependent on both its source and composition; these factors can influence both the rate of bacterial growth efficiency (BGE) (Berggren and del Giorgio 2015) and microbial community structure (Massicotte and Frenette 2011; Kujawinski et al. 2016). Significant spatial trends in the variability of BGE, reflecting trends in DOM composition, have previously been detected. For example, in a study of Canadian rivers by Berggren and del Giorgio (2015), microbial processing of DOC in streams in forested areas was found to occur with the highest BGE, whilst peatland areas utilised DOC with the lowest BGE, which corresponds to the rate of DOC export from peatlands. In addition, microbial activity has been previously found to correlate with both bacterial production and chlorophyll- α , which is thought to indicate the affinity of the microbes for the DOM substrates present (Massicotte and Frenette 2011).

2.7.1. Intracellular processing

The dynamics of LMW DOM compounds, particularly glucose and dissolved free amino acids (DFAA), are some of the best studied DOM components (Kirchman et al. 2003). Despite their

Chapter 2

low concentrations, LMW DOM can have very fast turnovers, meaning that these compounds can have very high rates of flux and can support a large amount of growth within the microbial biomass (Kirchman et al. 2003). It has been estimated that LMW DOM could support up to 100 % of bacterial C demand in some circumstances (Berggren et al. 2010). DFAAs are the largest contributor to bacterial production, with over 20 % of bacterial production being attributed to them. They are also one of the major sources of DON for the microbial community (Kirchman et al. 2003). In support of this, there is evidence that LMW DOM can be preferentially taken up at higher efficiencies than other DOM molecules, highlighting their significance (Berggren et al. 2010).

Unlike LMW DOM compounds, the high-molecular-weight (HMW) fraction of DOM is generally considered to be refractory, with its main functions in the aquatic ecosystem being physical and chemical (Bertilsson and Jones 2003). However, HMW DOM can be utilised by bacteria, albeit at a lower efficiency than LMW DOM (Farjalla et al. 2009). In contrast to LMW DOM, the microbial processing of HMW compounds is more likely to correlate with respiration, indicating that the processes involved are not very efficient (Farjalla et al. 2009; Massicotte and Frenette 2011). In humic-rich environments, this increase in respiration is likely to coincide with a shift in community structure from phytoplankton to bacteria, as autotrophic phytoplankton species would struggle with the limited light penetration and inter-species competition with better-adapted bacteria (Cotner and Biddanda 2002). A previous review of humic-rich environments found that any phytoplankton species present are likely to be mixotrophs that can feed directly on bacterial species, in order to avoid competition (Farjalla et al. 2009).

For the least labile HMW DOM, photodegradation can act as a "priming agent" for microbial processing of DOM (Moran and Covert 2003). HMW DOM can be broken down

Chapter 2

into more labile inorganic compounds and modified compounds by either natural or UV-light, which the microbial community is then able to process more efficiently (Moran and Covert 2003). Previous studies have estimated that these processes may provide between 4 % and 10 % of annual C demand for some ecosystems, although these estimates took into account a few known labile photo-products and therefore the true value may be higher (Moran and Covert 2003).

2.7.2 Extracellular processing of DOM

In addition to the photodegradation of HMW DOM products, less labile DOM compounds may be biologically modified extracellularly to allow membrane transport when competition for nutrients is high (Arnosti 2003). Approximately 25 % of DOC input to rivers is retained through sorption onto sediments, therefore the role of extracellular enzymes in biofilms must be key to the initial retention of DOC compounds in the aquatic ecosystem (Hope et al. 1994).

2.7.2.1 The role of biofilms in extracellular processing

A biofilm consists of a community of bacteria held within a matrix of extracellular polymeric substance (EPS) and can form at any solid:liquid interface within the aquatic system (Munn 2011). The EPS consists of bacterial-excreted chemicals, which can provide sites for the sorption of DOM compounds which bacteria can then utilise (Fischer 2003). The diffusion of DOM into the biofilm may also occur; previous studies have observed a positive correlation between the rate of DOM diffusion and biofilm thickness, which is likely to relate to changes in the biofilm's density as it expands (Fischer 2003). Once DOM has been transported into the biofilm, LMW compounds may diffuse into the bacterial cells through porins in the cell

Chapter 2

membrane, however, HMW compounds are likely to require enzymatic cleavage in order to be transported into the bacterial cell (Arnosti 2003).

2.7.2.2 Structure and function of extracellular enzymes

In general, the role of extracellular enzymes is to hydrolyse potentially labile DOM so that it may be transported into microbial cells. Although the production of extracellular enzymes is energetically costly for bacteria, it allows them a greater "foraging distance" from their static position within the biofilm (Vetter et al. 1998). Extracellular enzymes, for the cleavage of DOM compounds, generally fall into two categories: hydrolase and oxidative enzymes (Cunha et al. 2010). Hydrolase enzymes generally cleave both glycoside, peptide or ester bonds in order to break down larger molecules into their monomers, whilst oxidative enzymes will cleave C-C and C-O bonds in the presence of an electron acceptor (Cunha et al. 2010).

The production of extracellular enzymes is tightly regulated through a number of pathways, for example enzyme production will be repressed if more energetically favourable nutrients are present, or if a high concentration of the enzyme's product is present, in order to conserve energy (Cunha et al. 2010). Besides from a broad knowledge of the broad groupings of extracellular enzymes, only the extracellular enzymes of thermophiles have been focused on in detail due to their applications in biotechnology (Adams 1993). Generally, there are knowledge gaps concerning the rates at which these enzymes process DOM, as the activity of individual enzymes are hard to determine in laboratory settings due to the complexity of external factors that may affect the activity of these enzymes *in situ* (Arnosti 2003).

Chapter 2

2.8 Concluding remarks

DOM is composed of a diverse range of compounds in terms of molecular weight and chemical structure; the labile LMW fraction is preferentially utilised by the microbial community constitutes approximately 20 % of this chemical fraction. DOP and DOS are the least-studied classes of compound, primarily due to the difficulties in their measurement. DON compounds are relatively better characterised but are still far behind the study of DOC compounds. Generally, the measurement of all DOM compounds is carried out indirectly, by measuring total dissolved C, N or P and subtracting the inorganic fraction (Matalainen et al. 2011). No studies have been found quantifying total DOS, however there are some papers presenting the concentrations of specific DOS components (e.g. thiols, S-containing amino acids; Marie et al. 2015; Horňák et al. 2016). Semi-quantitative methods have improved the knowledge of certain DOM characteristic, for example using fluorescence at different wavelengths as an indication of aromaticity and molecular weight of DOC (Weishaar et al. 2003). New methods are also emerging that allow for specific compound identification, for example FTIR-MS (Sleighter and Hatcher 2007).

There are multiple sources of DOM in the aquatic ecosystem: although productivity is generally higher in terrestrial ecosystems, there are some key autochthonous sources of DOM in freshwaters, specifically algal and macrophyte productivity. However, the majority of DOM in freshwater ecosystems can generally be attributed to allochthonous plant and soil sources transported by water and air. Different DOM components can have a range of spatial distributions, from vertical distributions both within the column to nutrient gradients along the length of a water body (Thomas and Eaton 1996). The main factors that can influence the concentrations of DOM compounds are seasonal and rainfall events and the amount of sunlight-induced UV degradation (Mulholland 2003). Whilst DOC and DON compounds are often in

Chapter 2

the range of 0.5-50 mg L⁻¹ and 0.5-5 mg L⁻¹ respectively, DOP concentrations are usually much lower, generally less than 0.03 mg L⁻¹ (Johnes and Burt 1991; Mulholland 2003; Monbet et al. 2009). It has been estimated that up to half of riverine DOC is exported to the world's oceans, however less information is available about the flux of DON and DOP compounds. Whilst DON can be a major component of total N load in some circumstances, DOP is usually only a small part of the total P load in freshwater ecosystems which, in conjunction with the difficulties in measuring DOP concentrations, could be the reason why DOP is generally understudied (Perakis and Hedin 2002; Worsfold et al. 2008; Seitzinger et al. 2009). Despite the lack of studies, the DOS fraction may exceed the sulphur present as inorganic forms in the aquatic environment (Levine et al. 2016).

Previous studies of the intracellular processing of DOM have focused on LMW compounds such as amino acids and sugars, which are turned over rapidly in aquatic environments (Berggren et al. 2010). Larger HMW DOM molecules are generally considered to have a more refractory function, although it has been found that humic substances can be utilised by certain adapted bacteria, albeit at a slower rate (Farjalla et al. 2009). Extracellular degradation of DOM may also occur via the enzymes produced by some microbes or by photodegradation, which can break down HMW DOM into compounds that are more easily transported into the cell to be processed, however the mechanisms involved are not clear (Arnosti 2003).

2.8.1 Summary

In summary, this literature review demonstrates the importance of DOM for riverine nutrient processing. Further work is needed to address research gaps e.g. the differences in turnover and residence times of different DOM compounds (addressed in Chapters 3, 5 and 7), how riverine

Chapter 2

DOM depletion and metabolism rates vary in relation to changing pool sizes across physiochemical gradients (addressed in Chapter 4) and how DOM processing in sediments compares in pristine and enriched river systems (addressed in Chapters 5 and 6).

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Chapter 3

Microbial use of low molecular weight DOM in filtered and unfiltered freshwater: Role of ultra-small microorganisms and implications for water quality monitoring

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FLB, HCG, DLJ and PNG designed and conceived the experiment, FLB and HCG conducted the experimental work, CAY and ATO conducted the chemical analysis outlined in Table 3.1.

MRM produced the map presented in Fig. 3.2. FLB analysed the results and prepared the manuscript. All authors discussed results and contributed to the preparation of the manuscript.

Chapter 3

3.1 Abstract

Dissolved organic matter (DOM) plays a central role in regulating productivity and nutrient cycling in freshwaters. It is therefore vital that we can representatively sample and preserve DOM in freshwaters for subsequent analysis. Here we investigated the effect of filtration, temperature (5 and 25 °C) and acidification (HCl) on the persistence of low molecular weight (MW) dissolved organic carbon (DOC), nitrogen (DON) and orthophosphate in oligotrophic and eutrophic freshwater environments. Our results showed the rapid loss of isotopically-labelled glucose and amino acids from both filtered (0.22 and 0.45 µm) and unfiltered waters. We ascribe this substrate depletion in filtered samples to the activity of ultra-small (< 0.45 µm) microorganisms (bacteria and archaea) present in the water. As expected, the rate of C, N and P loss was much greater at higher temperatures and was repressed by the addition of HCl. Based on our results and an evaluation of the protocols used in recently published studies, we conclude that current techniques used to sample water for low MW DOM characterisation are frequently inadequate and lack proper validation. In contrast to the high degree of analytical precision and rigorous statistical analysis of most studies, we argue that insufficient consideration is still given to the presence of ultra-small microorganisms and potential changes that can occur in the low MW fraction of DOM prior to analysis.

Keywords Biodegradation • Metabolomics • Sampling method • Nutrients • Ultramicrobacteria • Uptake kinetics

Chapter 3

3.2 Introduction

Dissolved organic matter (DOM) represents a key source of nutrients and energy for plants and microorganisms living in pristine low nutrient status waters (Gardner et al. 1989; Lindell et al. 1996; Bernot et al. 2010; Durand et al. 2011; Stutter and Cains 2015). Conversely, DOM can also be seen as undesirable in freshwaters due to its potential to make pollutants more bioavailable, its ability to affect the hormone balance of freshwater organisms, its ability to generate significant reductions in dissolved oxygen concentrations owing to its uptake by microbial populations, and its potential to lead to the formation of carcinogens during chlorination of drinking water (Steinberg et al. 2008; Durand et al. 2011; McIntyre and Gueguen 2013). Understanding the origin, behaviour and fate of DOM in aquatic ecosystems is therefore important for predicting how it will influence primary productivity and overall water quality. It is clear from recent studies that DOM is composed of thousands of individual compounds which can be biologically processed within the river network leading to significant changes in the quality and quantity of DOM during passage from catchment to coast (Battin et al. 2003; Lusk and Toor 2016). While some high molecular weight (MW) compounds (> 1000 daltons (Da); Kujawinski 2011) may be relatively recalcitrant to microbial breakdown, some low MW compounds are highly labile, making representative sampling difficult due to potential losses during transport and storage prior to analysis.

DOM is operationally defined as C-containing compounds that can pass through a 0.45 µm filter (Thurman 1985; Nimptsch et al. 2014), this limit being historically linked to the microbiological standard for drinking water (Goetz and Tsuneishi 1951). This filtering process is designed to remove microorganisms and organic debris from the sample, although the passage of nano-particulate DOM is inevitable. It is now well established, however, that freshwaters contain a range of ultra-small organisms (e.g. viruses, bacteria, archaea) which can

Chapter 3

also readily pass through a 0.45 μm apertures (Fig. 3.1; Comolli et al. 2009; Maranger and Bird 1995). While viruses can be considered to be biologically inert from a DOM standpoint, the remaining ultra-small bacteria and archaea are thought to be physiologically active in a planktonic state (Baker et al. 2010; Luef et al. 2015). Currently, the ecological significance of these nano-organisms in nutrient cycling and DOM processing in natural freshwaters remains unknown. In addition, they also have the potential to compromise the quality of DOM in filtered samples destined for laboratory analysis.

One of the main approaches for assessing DOM concentrations in water is via manual grab sampling, during which samples are 0.45 μm filtered *in situ* or *ex situ* prior to storage in pre-washed bottles. Alternatively, automatic sampling systems may be employed to reduce the amount of time and resources required (Cassidy and Jordan 2011). However, automatically collected samples present challenges as they are not filtered after collection and are rarely recovered from site on a daily basis; therefore samples may be subject to significant periods of storage during which DOM biodegradation can occur. In addition, the samples may be exposed to higher temperatures than those of the river, potentially increasing the rate of microbial activity and loss or transformation of DOM (Ahad et al. 2006; Johnston et al. 2009). Although preservatives can be used to minimise nutrient transformations, these may interfere with subsequent metabolomics, biochemical and microbiological analysis and are frequently not used (Ferguson 1994; Kotlash and Chessman 1998).

Chapter 3

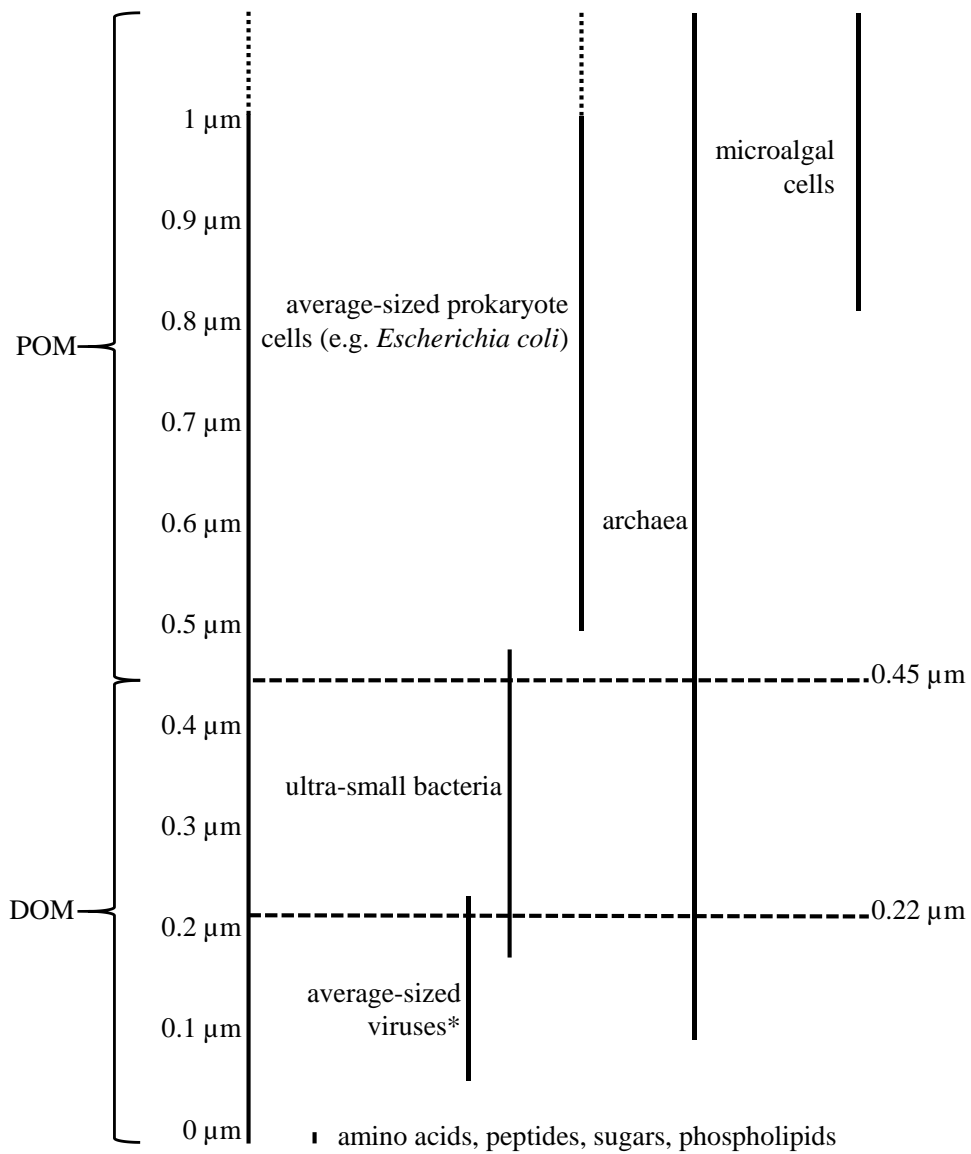


Fig. 3.1 Relative size of dissolved organic matter (DOM) and particulate organic matter (POM) components in comparison to bacteria, archaea and viruses. $POM > 0.45 \mu m > DOM$. 0.45/0.22 μm filter cut-offs indicated by a dashed line. * Some giant viruses $>1 \mu m$ exist.

Chapter 3

The three most commonly measured macronutrients that contribute to the molecular structure of DOM, and are key water quality parameters are C, N and P. Although the exact composition of all the dissolved organic C, N and P compounds in the aquatic environment is largely undefined, DOM can be divided into a high and low MW DOM fraction. The low MW DOM (< 1000 Da) fraction includes a wide range of common metabolites in either a monomer or oligomer form (e.g. amino acids, peptides, sugars, organic acids; Helms et al. 2008). As these compounds may be typically present at very low concentrations (< 500 nM), particularly in low nutrient-status waters, their significance is frequently overlooked relative to the more stable high MW humic DOM fraction (Kujawinski 2011). However, when their rapid rate of formation and turnover are considered, the overall flux of low MW DOM through the aquatic biota may be significant (Meon and Amon 2004). As these compounds are likely to have a quick rate of turnover in the aquatic environment, their detection can be challenging especially in non-sterile samples. The aim of this study was therefore to: (1) compare the rate of microbial uptake of three low MW DOM components over time in unfiltered (whole microbial community) and filtered (ultra-small microbial community) water samples; (2) determine the impact of temperature on the microbial utilization of low MW DOM; and (3) establish whether sample acidification provides an effective preservative for low MW DOM. The results of the study will be used to evaluate the significance of ultra-small microorganisms in low MW DOM turnover and also to devise potential strategies to representatively sample this DOM fraction.

Chapter 3

3.3 Materials and methods

3.3.1 Field site and sampling

Samples were collected from two contrasting sub-catchments within the Conwy catchment, North Wales (Fig. 3.2; Supplementary Fig. S3.2). The Hiraethlyn sub-catchment is an area of primarily lowland improved grassland used predominantly for agricultural livestock production (Cooper et al. 2014; Jones et al. 2016). It has an average elevation of 56 m a.s.l., an annual air temperature of 8.57 ± 0.04 °C and an annual rainfall of up to 1000 mm (Emmett et al. 2016). The Migneint sub-catchment is an area of upland blanket peat bog supporting acid heathland vegetation and low intensity sheep production. 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).

Samples were collected manually in high density polyethylene (HDPE) bottles in March, 2015. At each site, a sample of water was either, (1) left unfiltered, (2) filtered through a 0.45 µm cellulose nitrate filter (Whatman, Buckinghamshire, UK), (3) filtered through a 0.22 µm cellulose nitrate filter (Sartorius, Göttingen, Germany), or (4) unfiltered and acidified with 10 ml 0.1 M HCl. Filters were rinsed by passing 60 mL of sample water through before the sample was collected. During transportation back to the laboratory, samples (1 L) were kept cool and in the dark by placing them on ice (Supplementary Fig. S3.1).

Chapter 3

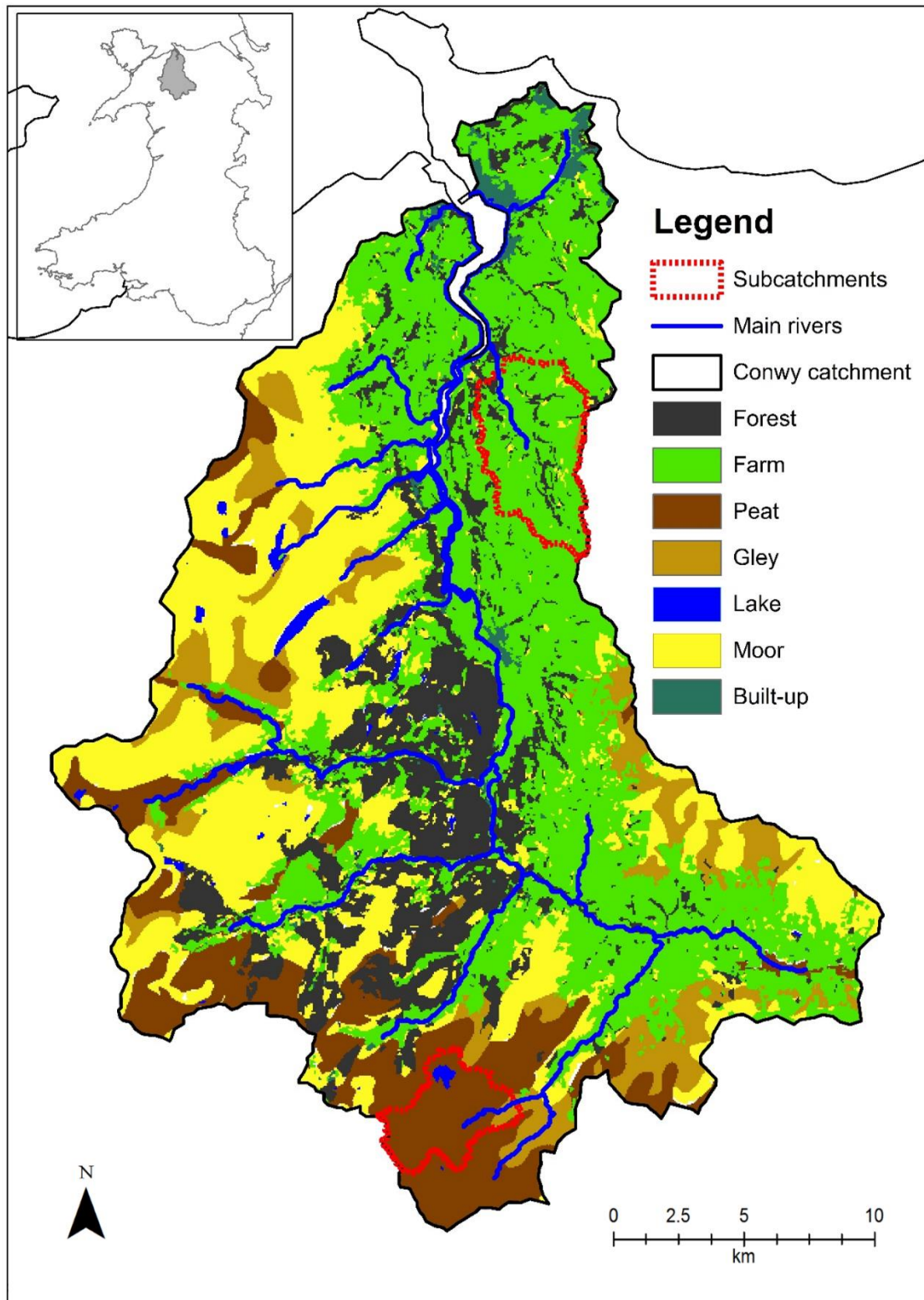


Fig. 3.2 Land use map of the Conwy catchment with upland peat bog (Migneint) and lowland improved grassland (Hiraethlyn) sub-catchments outlined in red.

Chapter 3

3.3.2 Nutrient depletion experiment

To evaluate C, N and P depletion in the different treatments, 3 different radioisotopes were used: ^{14}C -[U]-glucose (Lot 3632475; PerkinElmer, MA, USA), a mixture of 16 individual ^{14}C -[U]-amino acids (Lot 3590279; PerkinElmer) and $\text{H}_3^{33}\text{PO}_4$ (Lot 01305; PerkinElmer). For each isotope, three replicate 25 mL aliquots for each of the 4 treatments (acidified, unfiltered, 0.22 μm and 0.45 μm filtered) from the Hiraethlyn and Migneint sampling sites were added to sterile 50 mL polypropylene centrifuge tubes (Corning, NY, USA) and spiked with 0.2 kBq mL⁻¹ activity. The amount of isotope added was < 1 nM and therefore not expected to change the intrinsic concentration of the target compound within the samples. After sealing with sterile caps, the samples were subsequently incubated in the dark at either 5, 15 or 25 °C for the duration of the experiment. Data for 15 °C is presented in Supplementary Figs. S3.3-S3.5.

After incubation for 2, 5, 24, 48, 72, 144 or 168 h, 1 mL subsamples were taken, centrifuged to remove microbial cells (20,817 g, 5 min), and 0.5 mL of the supernatant placed in a scintillation vial. The subsamples were then acidified with 0.1 M HCl (50 μL), vortexed, left to stand for 3 h and then vortexed again to remove any dissolved CO₂ present. The subsample was then mixed with Optiphase HiSafe scintillation cocktail (4 mL; PerkinElmer) and the ^{14}C or ^{33}P quantified on a Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK).

3.3.3 Statistical analysis

All data analyses were carried out using SPSS 22.0 (IBM UK Ltd, Portsmouth, UK). Two-way mixed analysis of variance (ANOVA) was used to test for significant differences between treatments over time, with the significance level of the P-value being set at $p \leq 0.05$. If the data

Chapter 3

did not meet the criteria of Mauchly's test for sphericity, the Greenhouse-Geisser correction was applied to the P-value.

Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests respectively. If the data met the required assumptions a one-way ANOVA was subsequently used to test for differences between treatments at specific time points. Post-hoc multiple pairwise testing was carried out using Tukey's post-hoc multiple pairwise testing. Where data did not meet the assumptions for a one-way ANOVA, a Welch's test was used. Post-hoc multiple pairwise testing with the Games-Howell test was then carried out. All values are presented as means \pm the standard error of the mean (SEM) ($n = 3$).

3.4 Results

3.4.1 Water quality characteristics

The water samples collected from the two sub-catchments differed greatly in their chemical properties (Table 3.1). Values for pH, EC and temperature were found to be significantly lower in water collected from the acid heathland (Migneint) sub-catchment. Higher concentrations of both inorganic and organic N and P species were found in the agriculturally intensive (Hiraethlyn) sub-catchment. Higher concentrations of DOC were observed in samples from the Migneint sub-catchment, with a greater proportion of higher molecular weight DOC, than in the Hiraethlyn. These trends reflect the peaty soils of the Migneint catchment and the N- and P-rich soils of the Hiraethlyn sub-catchment.

Chapter 3

Table 3.1 Chemical properties of water from the Hiraethlyn and Migneint sub-catchments used in the substrate mineralisation experiments. Values represent annual mean data \pm SEM (n=66, except for low molecular weight fractionation parameters where n=3).

Determinand	Hiraethlyn	Migneint
pH	7.46 ± 0.09	5.36 ± 0.13
Electrical conductivity ($\mu\text{S cm}^{-1}$)	229 ± 25.3	35.9 ± 1.90
Temperature ($^{\circ}\text{C}$)	11.0 ± 0.35	11.3 ± 0.50
Dissolved organic carbon DOC (mg C L^{-1})	3.81 ± 0.24	11.7 ± 0.88
Absorbance at 254 nm (AU cm^{-1})	0.27 ± 0.02	0.51 ± 0.00
Nitrate NO_3^- (mg N L^{-1})	2.64 ± 0.11	0.07 ± 0.03
Ammonium NH_4^+ (mg N L^{-1})	0.05 ± 0.01	0.01 ± 0.00
Dissolved organic nitrogen DON (mg N L^{-1})	0.64 ± 0.09	0.44 ± 0.02
Particulate organic nitrogen PON (mg N L^{-1})	0.12 ± 0.06	0.03 ± 0.01
Orthophosphate (mg P L^{-1})	0.04 ± 0.00	0.01 ± 0.00
Dissolved organic phosphorus DOP (mg P L^{-1})	0.01 ± 0.00	0.01 ± 0.00
Particulate phosphorus (mg P L^{-1})	0.02 ± 0.01	0.01 ± 0.00
Percentage low molecular weight DOC ($\% <1 \text{ kDa}$)	99.7 ± 11.8	54.9 ± 4.06
Percentage low molecular weight aromatic compounds ($\% <1 \text{ kDa}$)	59.0 ± 7.81	31.2 ± 1.15

Chapter 3

3.4.2 Microbial uptake of ^{14}C -labelled amino acids

Significant interactions between treatment (acidified, unfiltered, 0.22 μm and 0.45 μm filtered) and time for samples incubated at 5 $^{\circ}\text{C}$ and 25 $^{\circ}\text{C}$ for both sample sites were observed for samples spiked with a mixture of ^{14}C -labelled amino acids, (two-way mixed ANOVA, $P < 0.001$; Table 3.2; Fig. 3.3).

In the samples from the agricultural catchment (Hiraethlyn) incubated at 5 $^{\circ}\text{C}$, the amount of amino acids remaining in the unfiltered treatment by 24 h was significantly lower than in the acidified, 0.22 μm or 0.45 μm filtered treatments (one-way ANOVA, $F_{3,8} = 207.32$, $P < 0.001$; Fig. 3.3a). The latter two treatments however did not differ significantly from each other. In the acidified samples, the majority ($91.4 \pm 1.5\%$) of the ^{14}C -amino acids still remained in solution at the end of the experiment (7 d). Although filtering did slow the rate of amino acid depletion, there was no difference in the amount of amino acids remaining in solution in the filtered and unfiltered samples after 7 d. When incubated at 25 $^{\circ}\text{C}$, the rate of depletion was much faster than at 5 $^{\circ}\text{C}$ across all treatments, with $81.2 \pm 0.4\%$ amino acids removed from the filtered and unfiltered water samples by 24 h (Fig. 3.3b). Increasing the incubation temperature to 25 $^{\circ}\text{C}$ decreased the half-lives of the unfiltered 0.45 μm and 0.22 μm filtered treatments from 17, 50 and 62 h to 4, 16, 17 h respectively. At 25 $^{\circ}\text{C}$ significant amounts of amino acid loss were also observed in the acidified samples after 3 d although the amount removed after 7 d was significantly less than observed in the other three treatments (one-way ANOVA, $F_{3,7} = 2847.27$, $P < 0.001$).

In contrast to the Hiraethlyn, the rate of amino acid depletion was much slower in water obtained from the Migneint sub-catchment (Fig. 3.3). Despite this, the trends in amino loss were broadly similar. Acidification largely prevented the loss of amino acids from solution,

Chapter 3

while filtering temporarily slowed, but did not prevent, amino acid depletion (Table 3.2). The rate of depletion was also much greater at 25 °C than in water incubated at 5 °C (one-way ANOVA, $F_{3,7} = 2847.27$, $P < 0.001$). The increase in incubation temperature to 25 °C decreased the half-life of the unfiltered treatment from 139 h to 56 h. Half-lives could not be calculated for the filtered treatments at 5 °C, but were 70 and 90 h for 0.22 µm and 0.45 µm filtered treatments respectively.

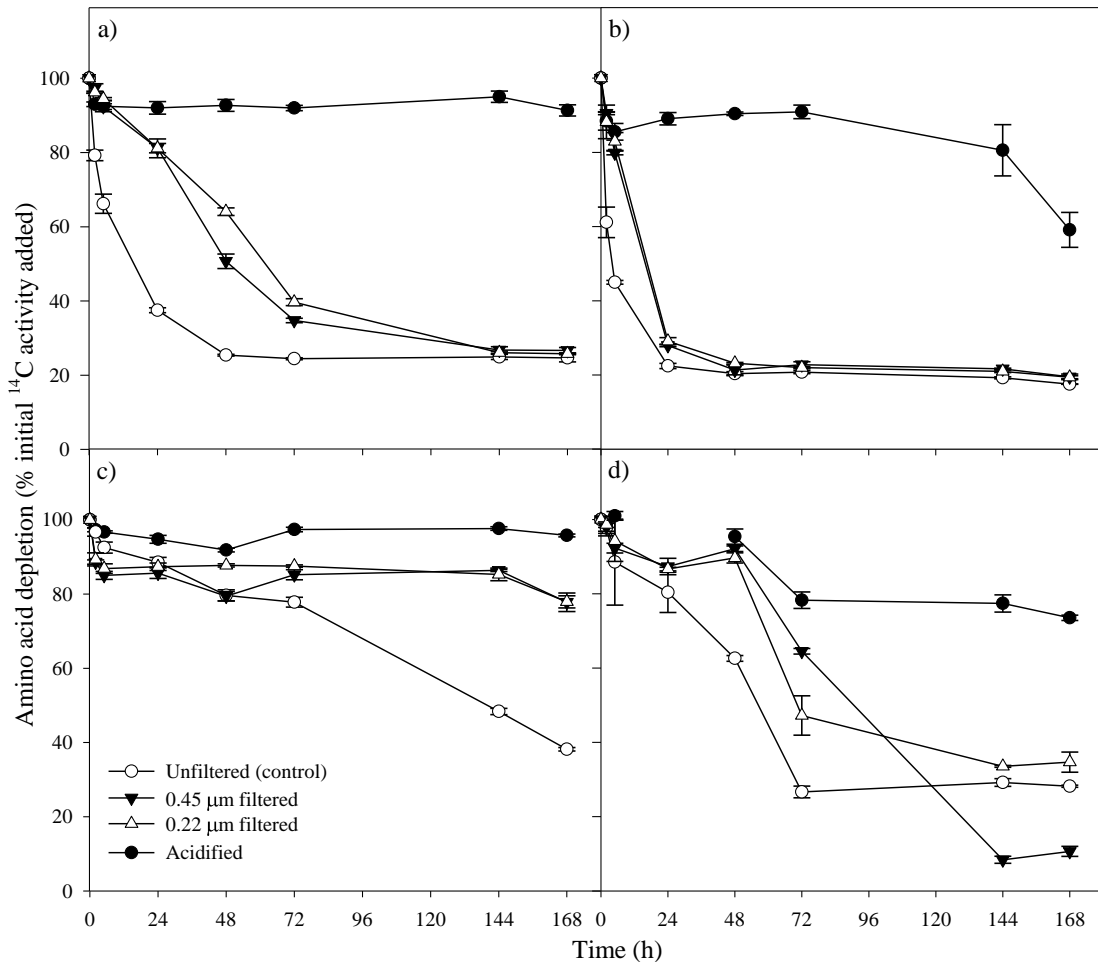


Fig. 3.3 Effect of filtering (0.45 or 0.2 µm) and acidification on the loss of ¹⁴C-labelled amino acids for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means ± SEM ($n = 3$). The legend is the same for all panels.

Chapter 3

Table 3.2 Results from a two-way mixed ANOVA for each isotopically-labelled nutrient, sub-catchment and temperature.

Sub-catchment	Nutrient	Temperature (°C)	Simple effect of time		Interaction time × treatment	
			F	<i>P</i> -value	F	<i>P</i> -value
Hiraethlyn	¹⁴ C amino acid mix	5	2156	<0.001*	276	<0.001*
Hiraethlyn	¹⁴ C amino acid mix	25	826	<0.001*	61	<0.001*
Migneint	¹⁴ C amino acid mix	5	332	<0.001*	114	<0.001*
Migneint	¹⁴ C amino acid mix	25	2103	<0.001*	164	<0.001*
Hiraethlyn	¹⁴ C glucose	5	4441	<0.001*	657	<0.001*
Hiraethlyn	¹⁴ C glucose	25	1730	<0.001*	140	<0.001*
Migneint	¹⁴ C glucose	5	139	<0.001*	52	<0.001*
Migneint	¹⁴ C glucose	25	481	<0.001*	57	<0.001*
Hiraethlyn	³³ P orthophosphate	5	15	<0.001*	4	0.001*
Hiraethlyn	³³ P orthophosphate	25	211	<0.001*	42	<0.001*
Migneint	³³ P orthophosphate	5	279	<0.001*	134	<0.001*
Migneint	³³ P orthophosphate	25	43	<0.001*	5	0.026*

* Denotes a significant *P*-value. The significance level was set at $P < 0.05$.

Chapter 3

3.4.3 Microbial uptake of ^{14}C -labelled glucose

The trends in ^{14}C -labelled glucose depletion from water were very similar to those observed for the ^{14}C -labelled amino acids (Fig. 3.4). Again, significant interactions between treatment and time for samples incubated at 5 °C, 25 °C and for both the agricultural (Hiraethlyn) and acid heathland (Migneint) sub-catchments were observed (two-way mixed ANOVA, $P < 0.001$; Table 3.2; Fig. 3.4).

Acidification with HCl largely prevented glucose uptake at 5 °C and greatly repressed its use at 25 °C, relative to the unfiltered control. Passing the water through a 0.22 or 0.45 μm filter also slowed the microbial immobilisation of ^{14}C -glucose. The half-life of glucose in the unfiltered Hiraethlyn water held at 5 °C was 18 h, while filtering to pass 0.45 or 0.22 μm extended this to 55 h and 65 h respectively. At 25 °C, the half-life for the unfiltered and 0.45 and 0.22 μm filtered samples was 5 h, 14 h and 15 h respectively. Although half-lives could not be calculated for the Migneint samples held at 5 °C, the half-life of glucose at 25 °C was 54 h for the unfiltered samples and 59 h and 77 h for the 0.45 μm and 0.22 μm filtered samples respectively.

Chapter 3

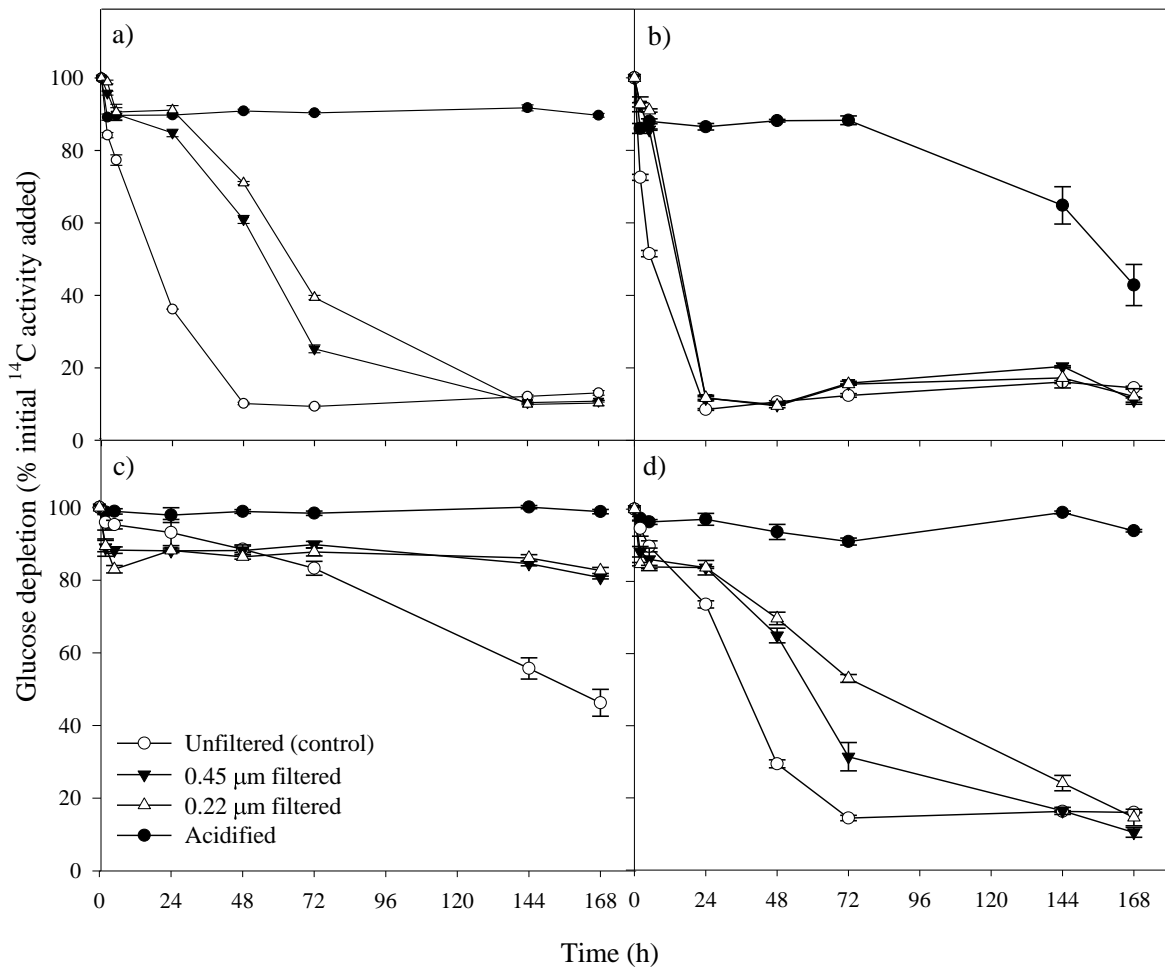


Fig. 3.4 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{14}C -labelled glucose for: a) Hiraethlyn sub-catchment 5 $^{\circ}\text{C}$, b) Hiraethlyn sub-catchment 25 $^{\circ}\text{C}$, c) Migneint sub-catchment 5 $^{\circ}\text{C}$, d) Migneint sub-catchment 25 $^{\circ}\text{C}$. Values represent means \pm SEM ($n = 3$). The legend is the same for all panels.

3.4.4 Microbial uptake of ^{33}P -labelled orthophosphate

Although there was notable similarity in trends observed between the two ^{14}C -labelled substrates, the results for ^{33}P -labelled orthophosphate followed a different pattern. A significant interaction between treatment and time was found for samples kept at 5 $^{\circ}\text{C}$

Chapter 3

from the Migneint and 25 °C from the Hiraethlyn sub-catchments (two-way mixed ANOVA, $P < 0.001$; Table 3.2; Fig. 3.5). This was observed to a lesser extent in samples incubated at 5 °C from the Hiraethlyn sub-catchment (two-way mixed ANOVA, $P = 0.001$; Fig. 3.5) and 25 °C Migneint (two-way mixed ANOVA, $P = 0.026$; Fig. 3.5).

At 5 °C, the amount of ^{33}P in the water from the Hiraethlyn sub-catchment did not drop below $91.5 \pm 0.7\%$ for any treatment (Fig. 3.5). At 25 °C, no significant differences were initially found between treatments (one-way ANOVA, $F_{3,8} = 4.39$, $P = 0.05$). However, after 24 h a progressive depletion was observed in the 0.45 and 0.22 μm filtered and unfiltered water relative to the acidified treatment (one-way ANOVA, $F_{3,8} = 10.69$, $P = 0.025$).

In contrast to the Hiraethlyn, a significant loss of ^{33}P was observed from the unfiltered water over 7 d in water from the Migneint (Fig. 3.5). This depletion was largely eliminated by passing the water through either a 0.22 or 0.45 μm filter prior to the addition of ^{33}P at 5 °C. At 25 °C the pattern of microbial ^{33}P immobilization were similar to those seen for the ^{14}C -labelled substrates. Overall, filtering slightly reduced the rate of ^{33}P loss during the first 24 h, however, few differences were observed between the filtered and unfiltered water beyond this time. A small amount of ^{33}P depletion was also observed in the acidified treatment, however, this only became apparent after 72 h and was much less than in the non-acidified treatments.

Chapter 3

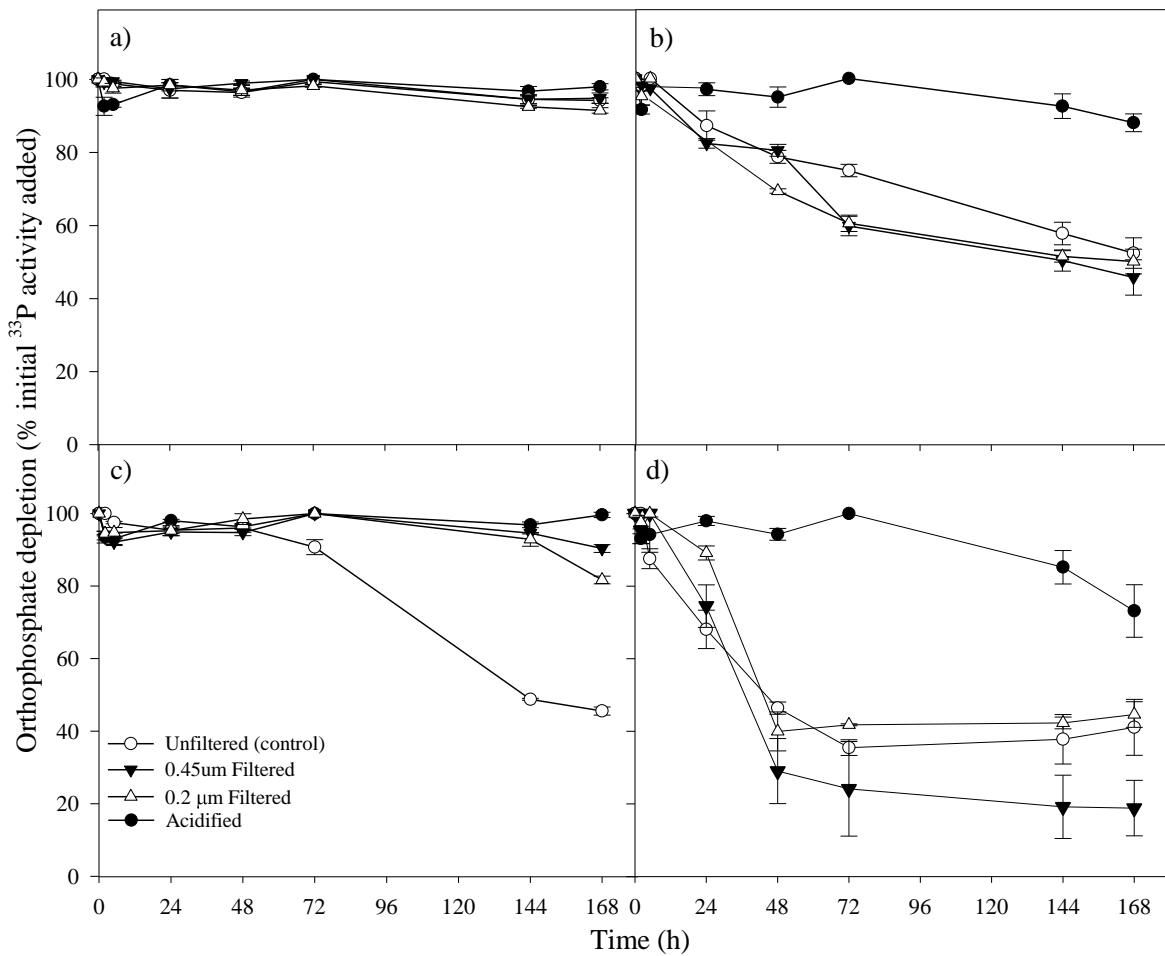


Fig. 3.5 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{33}P -labelled orthophosphate for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means \pm SEM ($n = 3$). The legend is the same for all panels.

3.5 Discussion

3.5.1 Role of ultra-small organisms in the processing of low MW DOM

Although 0.22 μm filters are often used and marketed as a method for water sterilisation, there have been studies indicating that microbes can even pass through 0.1 μm filters

Chapter 3

(Wang et al. 2007). Until recently, the identity of these organisms remained unknown, however, recent sequencing efforts have revealed them to contain a diverse range of taxa (Luef et al. 2015; Wu et al. 2016; Wurch et al. 2016). In addition, genome sequencing has indicated that these ultra-small organisms may contain genes which have the potential to facilitate a wide range of metabolic processes (Wu et al. 2016). This emerging area of research, however, remains highly controversial (Cisar et al. 2000; Martel et al. 2014; Abrol et al. 2015). Here, we present strong evidence to suggest that organisms $< 0.45 \mu\text{m}$ can take up sugars, amino acids and inorganic P from solution. In most cases, there was a lag-phase of ca. 24 h in substrate use in the filtered samples, indicative that the population may have become more active (e.g. broken from dormancy) or grown in size. Although we cannot discount the abiotic hydrolysis or precipitation of glucose and amino acids in solution, we expected these loss pathways to be minimal in our study. Firstly, the substrates are neutrally charged at the pH values used here and do not readily react with metals or particles that may sediment during the final centrifugation step. Secondly, abiotic cleavage would typically lead to the formation of by-products (e.g. keto acids) which would remain in solution rather than being completely mineralized. Thirdly, the patterns of inorganic ^{33}P depletion were similar to those observed for the organic substrates, and in prior studies on the bulk P chemistry (Johnes and Hodgkinson 1998).

Major differences in the rates of nutrient depletion were observed between the two sampling sites. Overall, DOC and DON depletion were much faster in water obtained from the intensive agricultural sub-catchment (Hiraethlyn). In contrast, much faster P depletion was observed in the acid heathland (Migneint) sub-catchment. As large amounts of inorganic N was present in the Hiraethlyn samples, we conclude that the amino acids were being used predominantly as a source of C rather than for the N they contain (Jones

Chapter 3

et al. 2004). The lower rate of glucose use in water from the Migneint probably reflects its lower intrinsic microbial population relative to the Hiraethlyn (Emmett et al. 2016), rather than a suppression of glucose uptake by the recalcitrant DOC already present in the sample. This intrinsic DOC requires photo-irradiation to promote its microbial use (Jones et al. 2016). The greater use of P in the water from the Migneint are consistent with very low levels of bioavailable P in these humic waters, in contrast to the inorganic P enriched waters at the Hiraethlyn site (Table 3.1).

Across the different treatments and land-use types, the 20 °C increase in temperature led to an increase in the rate of nutrient depletion by a factor of 3.6 ± 0.2 . This would approximately equate to a Q_{10} value of 1.81, which is similar to values found for freshwaters and sediments in previous studies (Bergström and Jansson 2000; Fischer et al. 2002).

3.5.2 Filtering as a method to preserve low MW DOM

While most studies typically measure bulk DOM in samples, advancements in analytical chemistry (e.g. FT-ICRMS) have seen an increasing trend towards the molecular separation and characterisation of individual low MW DOM compounds in freshwaters (Osborne et al. 2013; Hertkorn et al. 2016). Typically, the waters collected in these studies are transported back to the laboratory prior to filtering. Our results clearly show that even short periods of storage will result in a loss of low MW DOC and DON from the samples, potentially compromising any subsequent interpretation. This contrasts with some inorganic nutrient species such as nitrate (though not orthophosphate) which may be stable in solution for many days prior to analysis provided they are stored at 4 °C in the

Chapter 3

dark (Johnes and Burt 1991; Pearce 1991). Although incubation at 5 °C reduced the rate of sugar and amino acid loss by ca. 50 %, it did not prevent microbial activity and the loss of low MW DOM from the samples. Similarly, as discussed above, filtering failed to eliminate microbial transformation of low MW DOM, even in the short term. In addition, filtration may also increase microbial activity due to the removal of larger predator species (Gasol and Moran 1999). Our findings conflict to some extent with Kaplan (1994) who suggested that filtering was sufficient to preserve DOM for 24 h. This apparent contradiction can be explained by the typical dominance of high MW DOM in natural waters which is relatively recalcitrant to microbial attack, masking the loss of the low MW DOM fraction (Jones et al. 2016). In most cases, the depletion of ¹⁴C-labelled nutrients occurred at a similar rate in the 0.45 and 0.22 µm filtered treatments suggesting that either can be used to partially suppress microbial activity. This is in agreement with Fellman et al. (2008) and Nimptsch et al. (2014) who found little influence of filter pore size (0.2 to 0.7 µm) on DOM concentrations in a range of freshwaters.

3.5.3 Acidification as a preservative for low MW DOM

Acidification is routinely employed in the analysis of metal species in water samples to prevent complexation with DOM compounds (McCleskey et al. 2004). In our study, acidification was found to halt nutrient depletion for the majority of samples kept at 5 °C, however, at 25 °C some nutrient depletion still occurred after 72 h. These findings are in agreement with Tupas et al. (1994), where acidification was found to preserve DOC samples best when samples were stored at 4 °C. It should be noted, however, that the use of some acids (e.g. HNO₃) may lead to the oxidation or depolymerisation of DOM during

Chapter 3

long-term storage (Kaplan 1994) and preclude the subsequent analysis of these samples for DON owing to the resultant N contamination. The suitability of acidification therefore also depends on the parameter to be measured and the analytical procedure being used (McCleskey et al. 2004).

3.5.4 Recommendations for sampling low MW DOM

Maintaining sample integrity has been a recurring theme in aquatic science since the onset of water quality monitoring and formulation of legislation for environmental protection. Our study specifically focused on the persistence of common low MW metabolites produced and consumed by freshwater organisms. Based on our results, we recommend that if the rivers are located away from the laboratory then samples be directly filtered through pre-washed 0.45 μm filters in the field, refrigerated, and rapidly processed in the laboratory (< 3 h). Where possible, the samples should also be treated with an antimicrobial agent to limit subsequent transformation (e.g. HCl, H_3PO_4 ; Tupas et al. 1994), though phosphoric acid should clearly be avoided if subsequent determination of P species and fractions is planned. Alternatively, samples should be passed through pre-concentration cartridges in the field rather than waiting to get back to the laboratory. Freezing the samples *in situ* with liquid N_2 may also stabilise the samples, although freezing and thawing may induce unwanted and variable changes in the molecular structure of high MW DOM and in the N speciation and P fractionation if samples are unfiltered when frozen (Santos et al. 2010; Peacock et al. 2015). In the case of automated water samplers, our results strongly suggest that refrigeration and addition of a biocide to a filtered sample should be used during transport and storage. Whichever method is

Chapter 3

employed, we also recommend that low (10-100 nM) concentrations of internal standards (common metabolites) be added to the samples at the point of sampling to ensure that the loss of low MW compounds is minimal prior to their ultimate analysis. This validation process will be facilitated by the use of singly or dual labelled isotopically-labelled compounds (^{15}N , ^{13}C , ^{14}C , ^{33}P). It is clear from reviewing numerous studies in this area that great effort is made to obtaining analytical precision when quantifying DOM. In contrast, almost no attention is paid to ensuring that the sample is truly representative of the place from which it originated. While current approaches may be very satisfactory for relatively recalcitrant high MW DOM, our research strongly suggests that greater care is needed when sampling labile low MW DOM.

3.6 Acknowledgements

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Chapter 4

Land cover and nutrient enrichment regulates dissolved organic matter (DOM) turnover in freshwater ecosystems

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This is a draft manuscript

FLB, HCG and DLJ designed and conceived the experiment, FLB and HCG conducted the experimental work. FLB and HCG conducted the fieldwork in the Conwy catchment; CAY and ATO conducted the fieldwork in the Hampshire Avon. CAY produced the map presented in Fig. 4.1. FLB and HCG analysed the results and FLB prepared the manuscript. All authors discussed results and contributed to the preparation of the manuscript.

Keywords Radioisotope tracers • Biogeochemical cycling • Physiochemical gradients • Nutrients • Uptake kinetics

Chapter 4

4.1 Abstract

Dissolved organic matter (DOM) is a complex mixture of carbon-containing compounds, operationally defined as passing through a 0.45 μm filter. It represents a key component of macronutrient cycling for carbon (C), nitrogen (N), and phosphorus (P). Whilst the low-molecular weight (LMW) fraction constitutes ca. 20 % of DOM in aquatic ecosystems, the turnover of these pools can be extremely high. Due to the challenges of measuring this pool, comparatively little is known of the fate of LMW DOM compounds in lotic systems. This study aimed to investigate the processing of LMW DOM across 45 sites representing a range of physiochemical gradients and dominant land uses (arable, grassland, conifer forest, peatland and mixed) in the United Kingdom. $^{14}\text{C}/^{33}\text{P}$ -radioisotope tracers representing LMW DOC (sugars), DON (amino acids), DOP (sugar phosphates) and inorganic P (P_i) were used to measure the microbial uptake different DOM components in river waters. The amount of DOM biodegradation varied between different components ($\text{DON} \geq \text{DOC} > \text{DOP}$), however, the turnover of all three correlated positively with increasing organic N and P enrichment. Conversely, the uptake of P_i decreased with increasing intrinsic N and P concentrations. This was ascribed to increasing C limitation and decreasing P limitation with increasing inorganic nutrient enrichment respectively. DOC biodegradation strongly correlated with both increasing pH ($r^2 = 0.729$), in addition to increasing DON and DOP biodegradation ($r^2 = 0.906$ and $r^2 = 0.730$ respectively). We conclude that inorganic nutrient pollution events leading to eutrophication will lead to further DOC removal from the water column and increased microbial growth, further exacerbating the effects of eutrophication in river systems.

Chapter 4

4.2. Introduction

Dissolved organic matter (DOM) is a complex mixture of chemicals, traditionally defined as organic carbon (C)-containing compounds that can pass through a 0.45 μm filter (Thurman et al. 1985; Akkanen et al. 2012). DOM constitutes a key form in which terrestrially-derived C is transported from headwaters, through the catchment hydrological network and into the marine zone. It has been estimated that ~33 % of terrestrial C is exported to the ocean in this way (Stutter et al. 2013). In addition to C, DOM also contains nitrogen (N) and phosphorus (P), which are together considered to be the three major macronutrients required by freshwater organisms (Tipping et al. 2016). DOM therefore represents a key source of nutrients for microorganisms and plants along the aquatic gradient (Kirchman 2003; Cuss and Guéguen 2015). Further, recent research has shown that DOM quality and quantity can change from source to sea suggesting that components of the DOM pool are abiotically or biotically transformed during transit (Massicotte and Frenette 2011; Ejarque et al. 2017).

Over recent decades, DOC concentrations, the most commonly measured DOM component, have been reported to be increasing across the northern hemisphere (Clark et al. 2010; Shutova et al. 2014). This had led to concerns about the production of carcinogens known as disinfection by-products (DBPs) through the chlorination or bromination of DOM during the purification of water abstracted from rivers (Wu et al. 2010; Yan et al. 2014; Langsa et al. 2017). In addition, high concentrations of DOM are also considered to be undesirable due to its ability to mobilise heavy metals, pharmaceuticals and pesticides and its potential to act as an endocrine disrupting chemical (Maoz and Chefetz 2010; Mueller et al. 2012; Fenner et al. 2013; ElBishlawi and Jaffe

Chapter 4

2015). To protect human health and the wider environment it is therefore important to understand what regulates the fate of DOM in freshwaters.

DOM is a highly complex mixture composed of thousands of individual compounds differing in size, charge and solubility. Currently, there is no single analytical method which is able to fully characterise all the DOM components present in a sample (Greenwood et al. 2012). Fully quantitative studies, characterising lotic DOM on a molecular scale are therefore extremely limited. This contrasts with the abundance of studies using the optical properties of DOM to describe spatio-temporal changes in DOM quality within freshwater ecosystems (Stanley et al. 2011; Yates et al. 2016). Changes in DOM quality have also been assessed using molecular weight (MW) filtration. Broadly, DOM can be divided into two size categories, namely those compounds that are of high molecular weight (HMW; >1000 Da) and those that are of low molecular weight (LMW; <1000 Da) (Cui and Choo 2013). HMW DOM (e.g. humic substances) is considered to be relatively recalcitrant and not readily degraded by the microbial biomass. Therefore, it is often present at high concentrations, particularly in peat-rich headwater catchments. It can, however, have an abiotic function, reducing light attenuation in the water column due to its chromophoric properties, shielding the microbial biomass and extracellular compounds from UV degradation (Fellman et al. 2010). In general, LMW DOM (e.g. sugars, organic acids) is present in lower amounts (ca. 20 % of the total DOM pool) but is often more labile and rapidly metabolised by the microbial biomass (Dawson et al. 2001). This makes the measurement of this fraction particularly challenging, despite the labile DOM pool being an important component of in-stream CNP processing (Lutz et al. 2011; Spencer et al. 2012; Parr et al. 2014). In many cases, individual LMW DOM compounds (e.g. amino acids) are present at very low concentrations in freshwaters (1-10

Chapter 4

nM; Marie et al. 2015; Horňák et al. 2016). These concentrations are close to the influx-efflux equilibrium point for microbial transport systems (i.e. point of zero net flux). It is unclear, however, if this is a reflection of low rates of LMW DOM input or whether it is due to high rates of consumption, especially for a wide range of compound classes and freshwater types. The aim of this study was therefore to: (1) compare the rate of microbial uptake of LMW forms of DOC (sugars), DON (amino acids) and DOP (sugar phosphates) at different times of the year in 45 individual rivers spanning a range of physiochemical properties and land-uses; (2) determine which physiochemical parameters best correlated with DOC, DON and DOP uptake, and (3) compare the rates of DOP and inorganic (Pi) use by the microbial community. The results of the study will be used to evaluate how DOM and Pi are processed across catchment-scale gradients.

4.3 Materials and methods

4.3.1 Field site and sampling

Samples were collected from 45 independent rivers across 5 contrasting land uses (arable, grasslands, conifer forests, peatland and mixed) in the Conwy catchment, North Wales and the Nadder Catchment, Southern England (Yates et al. 2019; Fig. 4.1). This was undertaken alongside regular monitoring of all sites between February 2015 and December 2016. The sites selected encompass a wide range of chemical and physical gradients and contain a range of dominant land use classifications (Emmett et al. 2016; Yates et al. 2019).

The Conwy catchment (Fig. 4.1a) covers an area approximately 580 km², draining a wide range of land-cover types, from one of the largest areas of upland blanket peat bog

Chapter 4

in Wales in its headwaters to acid grasslands, coniferous plantations and broadleaf forests. The lower half of the catchment contains an area of lowland improved grassland used for agricultural livestock, primarily sheep, before entering the Irish Sea (Cooper et al. 2014; Emmett et al. 2016; Brailsford et al. 2019; Yates et al. 2019). The Nadder catchment covers a larger area (673 km²; Fig. 4.1b) but is dominated by two main underlying geologies: the headwaters of the Nadder are dominated by clay, while the Wylfe, its major tributary, is underlain by chalk and is heavily influenced by groundwater recharge (Yates et al. 2019). In contrast to the Conwy, large areas of the Nadder catchment are used for arable production, with some livestock production (dominated by intensive cattle production) taking place within the clay-dominated areas of the catchment (Yates et al. 2016; Yates et al. 2019).

At each site, 1 L mid-stream samples were manually collected in acid-washed, high density polyethylene (HDPE) bottles. The pH, electrical conductivity (EC) and surface water temperature were measured at the time of collection. For the laboratory studies, the samples were kept cool (ca. 4°C) in the dark during transportation to the laboratory. Chemical analyses were conducted and experiments commenced within 24 h of sample collection.

Chapter 4

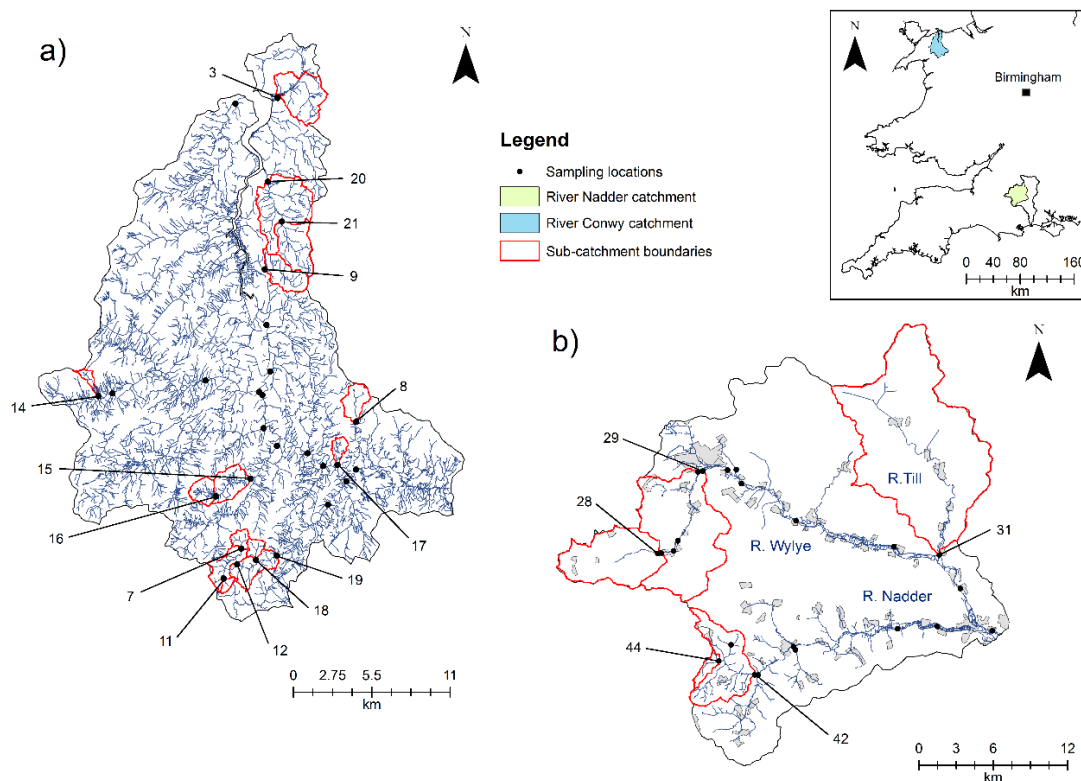


Fig. 4.1 Sampling locations across (a) Conwy and (b) Nadder catchments. Insert shows catchment locations in relation to the UK. Red boundaries represent catchments with >50 % dominance of a single land cover. Land-cover maps were created with LCM2007 data provided by the Centre for Ecology and Hydrology (Emmett et al. 2016) using ArcGIS Hydrology toolset (ESRI 2018. Version 10 Redlands, CA). [Reproduced with permission from Yates et al. 2019].

4.3.2 Nutrient depletion experiment

To evaluate DOC, DON, DOP and P_i depletion across the samples, individual samples were spiked with either ^{14}C -labelled glucose, a mixture of free amino acids, glucose-6-phosphate or $\text{H}_3^{33}\text{PO}_4$ respectively. For each isotope, three independent replicate 25 mL samples from each of the 45 sampling sites were added to sterile 50 mL polypropylene

Chapter 4

centrifuge tubes (Corning, NY, USA) and spiked with individual radioisotope (0.2 mL, 0.2 kBq mL⁻¹ final activity). The radioisotopes were added at small concentrations (< 1 nM) which would not greatly increase the intrinsic pool of the target compound or change its pH. After sealing with sterile caps, samples were incubated on an orbital shaker (200 rev min⁻¹) in the dark at 10 °C for the duration of the experiment.

After incubation for 2, 5, 24, 48, 72, 144 or 168 h, 1 mL subsamples were removed, centrifuged to remove microbial cells (20,817 g, 5 min), and 0.5 mL supernatant added to a plastic 7 mL scintillation vial (Meridian Biotechnologies, Tadworth., UK). The subsamples were then acidified with 0.1 M HCl (50 µL), vortexed, left to stand for 3 h and then vortexed again to remove any dissolved ¹⁴CO₂ present. The subsample was then mixed with Optiphase HiSafe 3 scintillation cocktail (4 mL; PerkinElmer, Waltham, MA) and the ¹⁴C or ³³P quantified on a Wallac 1404 liquid scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK).

4.3.3 Statistical analysis

Principal component analysis (PCA) of physiochemical parameters for the 45 sites and mean radioisotope tracer depletion data was conducted using SPSS 25.0 for Windows (IBM UK Ltd, Portsmouth, UK). Sites were grouped according to the dominant (> 50 %) land cover for the estimated catchment area. The catchment area for each sampling site was delineated using the Watershed tool (ArcGIS Hydrology Toolset, ESRI 2018, Version 10 Redlands, CA). The physicochemical parameters used in the analysis included DOC (mg C L⁻¹), TN (mg N L⁻¹), DON (mg N L⁻¹), NO₃⁻ (mg N L⁻¹), NH₄⁺ (mg N L⁻¹), TP (mg P L⁻¹), DOP (mg P L⁻¹), SRP (mg P L⁻¹), pH and electroconductivity (EC; µS cm⁻¹).

Chapter 4

¹). The percentage depletion at 24 h was used for comparison as this captured the linear phase of the degradation curve for the majority of sites and radioisotope tracers used.

The results from the four repeat experiments conducted over the different seasons were averaged together as season was not found to be a major driver of the differences between sites during initial analyses and many sites did not exhibit distinct variation between seasons (Supplementary Document 1). The resulting coordinates resulting from the PCA analysis were rotated using the Oblimin rotation method and plotted using Sigmaplot v13.0 (Systat Software Inc., San Jose, CA). Simple linear regressions were used to look for correlations between cumulative radioisotope depletion data over the first 24 h and individual physicochemical parameters across the 45 sites across the two catchments using Sigmaplot v13.0. One-way ANOVA with Tukey pairwise comparisons was undertaken using Minitab v18.0 using $P < 0.05$ as the cut-off for statistical significance (Minitab Inc., State College, PA). Where the data was not normally distributed (Shapiro-Wilk test) it was \log_{10} transformed prior to analysis.

4.4 Results

4.4.1 Physicochemical properties

As expected, significant variation was apparent between the chemistry of the riverwaters across the 45 sites (Supplementary Table S4.1; Yates et al. 2019). Comparison of the rate of substrate depletion over 24 h across all samples showed differences in substrate depletion rate following the series: glucose \geq amino acids \geq glucose-6-phosphate $>$ Pi ($P < 0.001$; Table 4.1; Supplementary Table S4.2). In addition, there were major differences in substrate depletion between land uses for all three organic substrates ($P < 0.001$),

Chapter 4

however, no difference in P_i use was observed across the land uses ($P = 0.613$). Overall, rates of substrate depletion were greatest in catchments draining agricultural land (arable and grassland) in comparison to sites draining peat moorland and coniferous forest. To identify the major factors associated with the depletion of isotope from solution, mean rates of DOC, DON, DOP and P_i were analysed alongside physiochemical parameters of the water (Table 4.1; Fig. 4.2). The PCA identified two principal components (PC; eigenvalue > 1) which explained 96.1 % of the overall variation between the 45 sites (Fig. 4.2).

Chapter 4

Table 4.1 Percentage depletion of ^{14}C -labelled DOC, DON and DOP and ^{33}P -labelled inorganic P (P_i) from river water across 5 major land use types after 24 h. Values represent percentage of tracer depleted within 24 h and are presented as means \pm SEM. The average value across all the sites is also presented at the foot of the table. Significant differences between land use types are shown with lowercase superscript letters while differences between substrates are denoted by uppercase superscript letters ($P < 0.05$).

Dominant land cover	Depletion 24 h (% initial activity added)			
	DOC	DON	DOP	P_i
Arable	92.7 \pm 0.9 ^a	75.6 \pm 0.4 ^a	74.6 \pm 0.5 ^a	32.9 \pm 4.7 ^a
Mixed	81.0 \pm 4.7 ^a	64.6 \pm 4.6 ^a	63.7 \pm 3.5 ^a	32.1 \pm 4.9 ^a
Grassland	78.3 \pm 2.2 ^a	60.6 \pm 1.9 ^a	54.4 \pm 3.1 ^a	21.1 \pm 5.1 ^a
Conifer forest	37.7 \pm 3.6 ^b	31.3 \pm 4.7 ^b	17.6 \pm 1.5 ^b	43.5 \pm 5.2 ^a
Peatland	37.0 \pm 3.9 ^b	38.8 \pm 3.4 ^b	20.2 \pm 2.1 ^b	36.2 \pm 13.2 ^a
Average	74.1 \pm 3.0 ^A	60.0 \pm 2.2 ^{AB}	55.2 \pm 3.1 ^B	30.7 \pm 3.5 ^C

When dominant land cover was plotted with coordinates resulting from the PCA analysis, rivers draining peatland (HMW DOC rich, N/P depleted) formed a distinct cluster in the bottom right quadrant (Fig. 4.2). There was also some separation between sites along the River Nadder and those of its main tributary, the Wylfe, on the right hand side of the plot, whilst the majority of sites located within the River Conwy catchment clustered on the left hand side. However, improved grassland-influenced rivers (DOC depleted, inorganic N/P enriched) sites from the Conwy catchment with similar levels of

Chapter 4

inorganic N and P enrichment to the Nadder Catchment were more closely associated with Nadder Catchment rivers, clustering together at the opposite end of x-axis (sites 2, 3, 20, 21; Fig. 4.2).

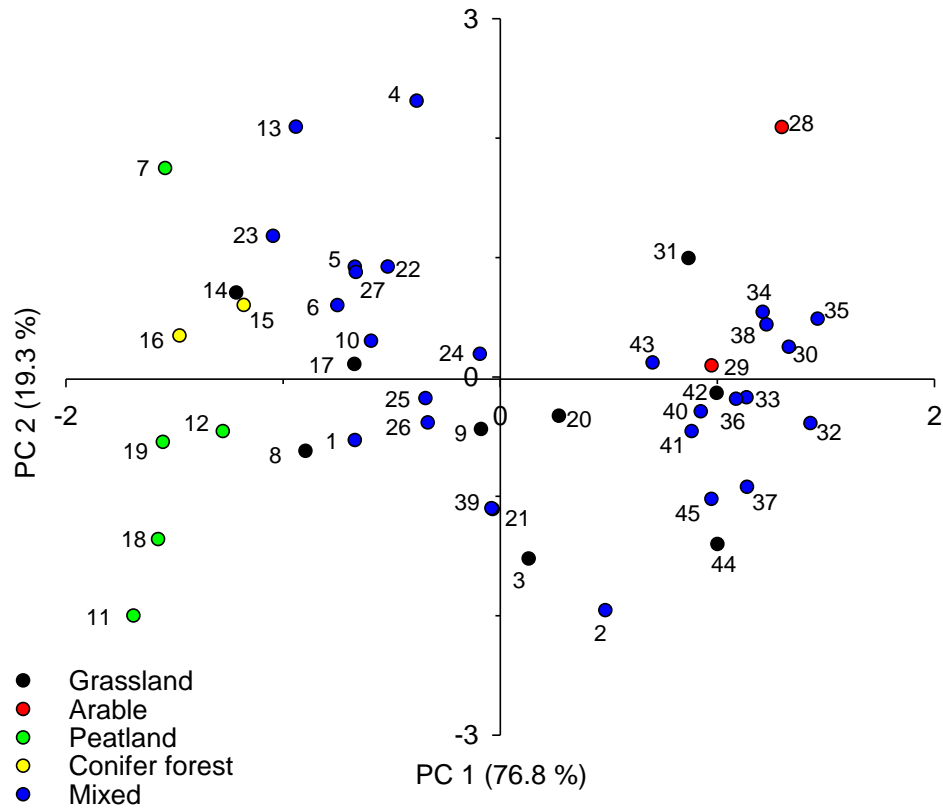


Fig. 4.2 Correlation bi-plot from the principal component analysis (PCA) for the main river water chemical variables and the DOC, DON, DOP and P_i depletion data for each of the 45 rivers sampled across 5 different land use types.

4.4.2 Regression analysis of nutrient depletion versus physiochemical parameters

The percentage depletion of labile DOC, DON and DOP after 24 h all had significant positive correlations with both pH and EC (linear regression, $P < 0.0001$ in all cases; Figs.

Chapter 4

4.3-4.5; Table 4.2). Conversely, P_i depletion correlated negatively with increasing EC values (linear regression, $P < 0.0001$; Fig. 4.6; Table 4.2). Generally, increasing ionic strength (EC) and inorganic nutrient enrichment of rivers (SRP, TP, TN) correlated with decreases in P_i uptake from solution by the microbial biomass, whereas the reverse was true for DON and DOP uptake, where both increasing inorganic and total N and P enrichment corresponded with higher rates of DON and DOP depletion from solution (linear regression, $P < 0.0001$ in all cases; Figs. 4.3-4.6; Table 4.2). No correlation was found between DOP depletion and P_i depletion across all the sites (linear regression, $P = 0.079$; Table 4.2).

Chapter 4

Table 4.2 Simple linear regression analysis of mean DOC, DON, DOP and *Pi* depletion after 24 hours (%) versus individual physicochemical parameters across 45 sites ($n = 3$).

Isotope	Variable	a	b	F	P-value	r²
DOC	DOC (mg C L ⁻¹)	-5.16	102.21	10	0.0030*	0.182
	pH	20.34	-71.08	118	<0.0001*	0.729
	Electroconductivity (EC; μ S)	0.05	59.53	46	<0.0001*	0.509
	<i>Pi</i> depletion (%)	-0.38	85.91	12	0.001*	0.211
	DON depletion (%)	1.27	-1.81	423	<0.0001*	0.906
	DOP depletion (%)	0.83	28.60	119	<0.0001*	0.730
DON	Nitrate (mg N L ⁻¹)	8.87	36.73	49	<0.0001*	0.526
	TN (mg N L ⁻¹)	8.62	30.8	54	<0.0001*	0.550
	SRP (mg P L ⁻¹)	407.59	41.03	46	<0.0001*	0.512
	TP (mg P L ⁻¹)	326.14	30.52	56	<0.0001*	0.558
	pH	14.63	-44.53	89	<0.0001*	0.669
	EC	0.04	48.25	59	<0.0001*	0.574
DOP	SRP (mg P L ⁻¹)	516.38	31.03	34	<0.0001*	0.437
	TP (mg P L ⁻¹)	384.9	20.27	31	<0.0001*	0.414
	pH	18.18	-74.81	54	<0.0001*	0.549
	EC	0.43	42.30	25	<0.0001*	0.360
	<i>Pi</i> depletion (%)	-0.31	47.03	3	0.0790	0.068
<i>Pi</i>	SRP	-561.13	54.51	27	<0.0001*	0.378
	TP	-417.19	68.02	24	<0.0001*	0.356
	TN	-12.15	71.46	33	<0.0001*	0.426
	EC	-0.05	46.37	32	<0.0001*	0.418

Chapter 4

The intrinsic DOC concentration had a weak negative correlation with the amount of labelled DOC uptake from solution, in a similar manner to the negative correlation observed between intrinsic P_i pool size and labelled P_i uptake (linear regression, $P = 0.003$ and $P < 0.0001$ respectively; Fig. 4.3; Table 4.2). However, overall the response to the addition DOC across all sites was more similar to those for DON and DOP addition, with strong positive correlations between DOC depletion and both DON and DOP depletion (linear regression, $P < 0.0001$; $r^2 = 0.906$ and $P < 0.0001$; $r^2 = 0.730$ respectively; Figs. 4.3-4.5; Table 4.2). DOC depletion had a weak negative correlation with P_i depletion overall (linear regression, $P < 0.0001$; $r^2 = 0.211$; Fig. 4.3; Table 4.2).

Chapter 4

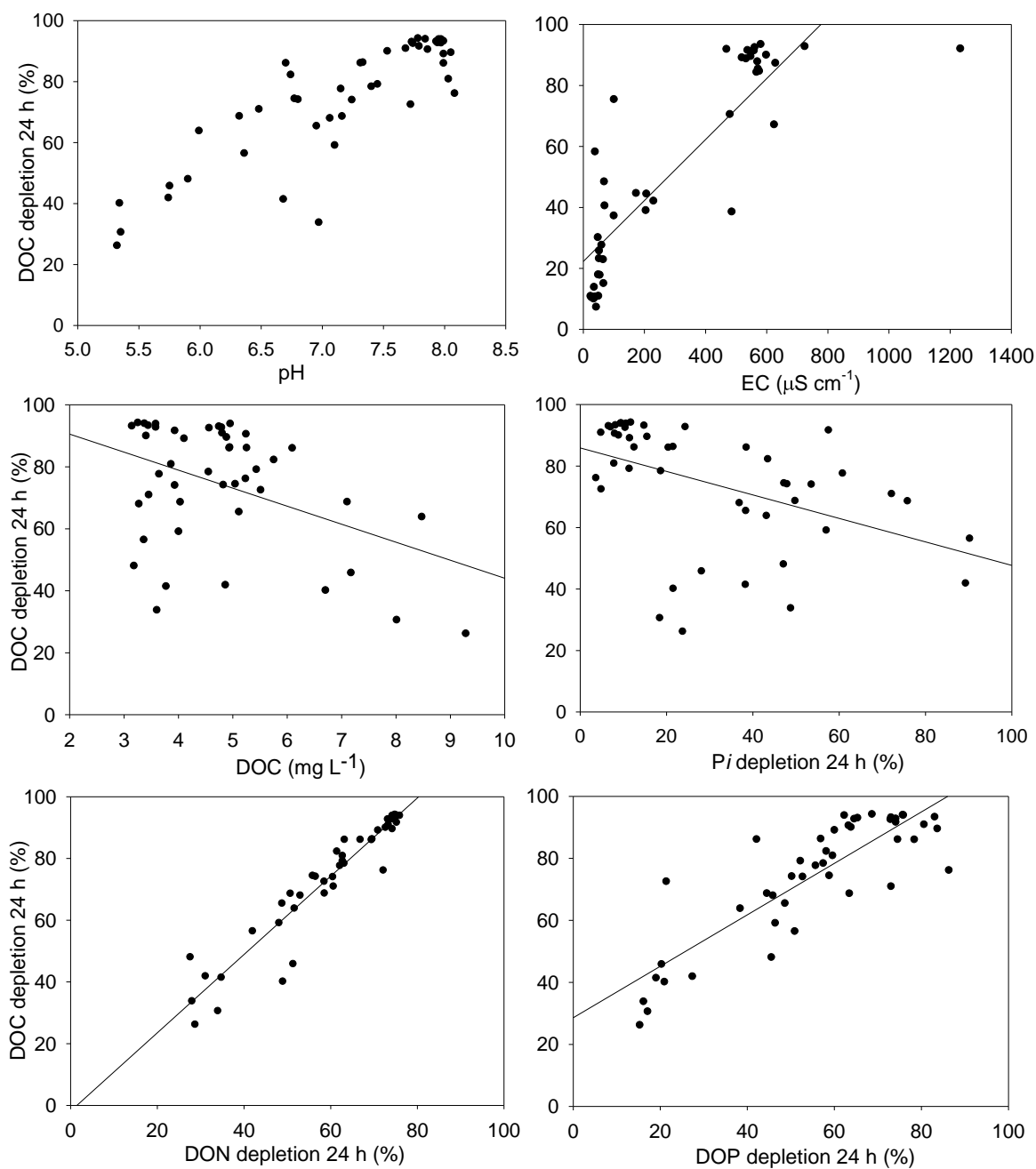


Fig. 4.3 Scatter plots of mean DOC depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Chapter 4

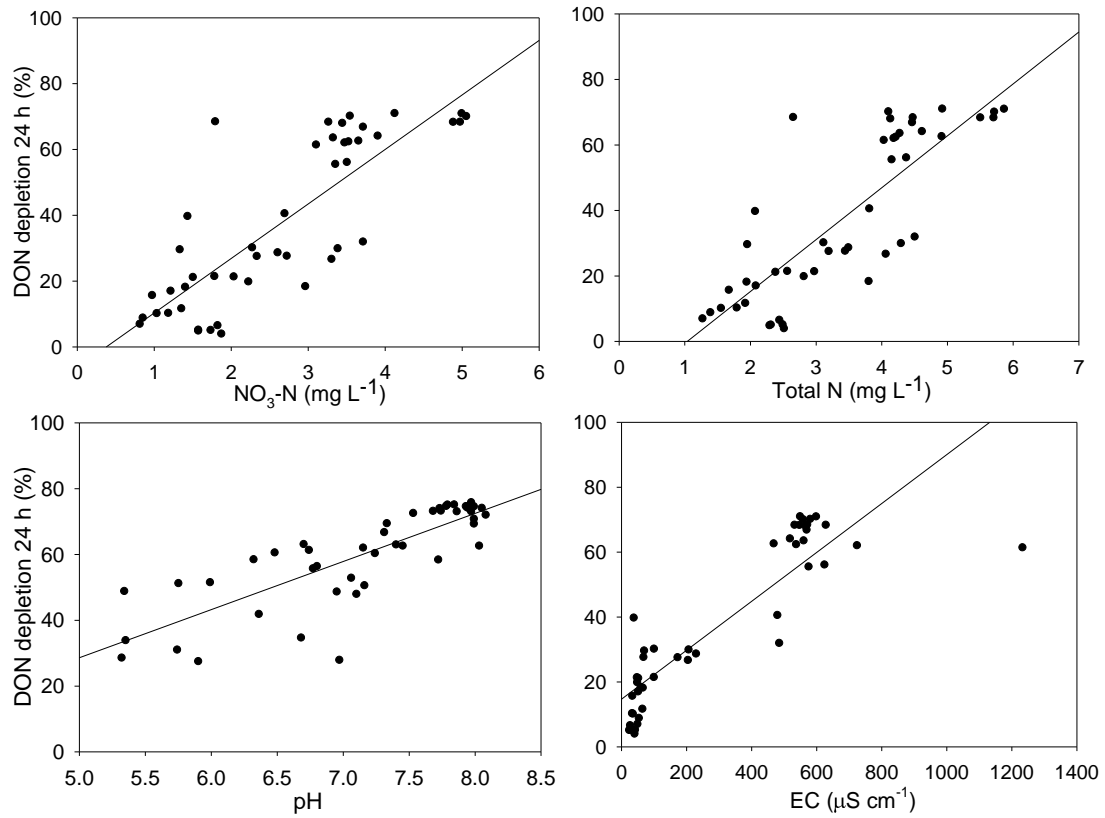


Fig. 4.4 Scatter plots of mean DON depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Chapter 4

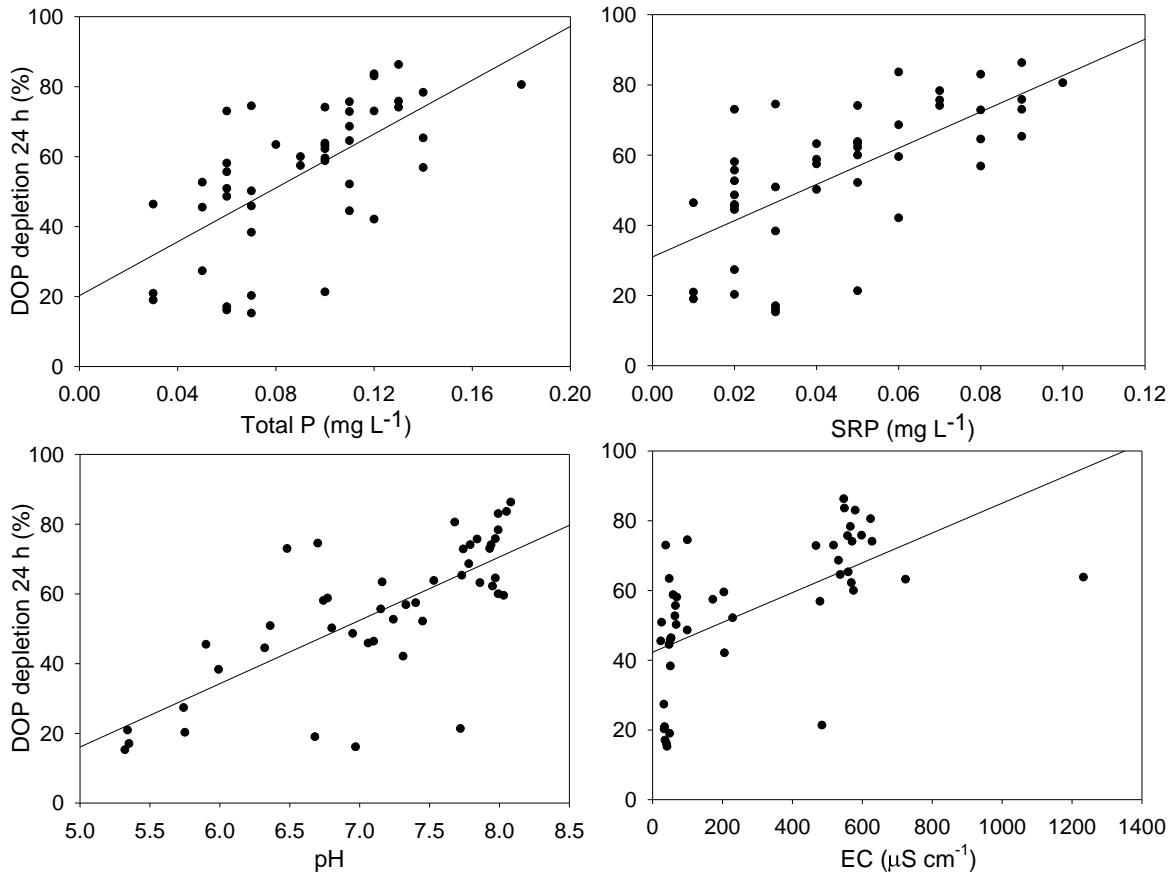


Fig. 4.5 Scatter plots of mean DOP depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

Chapter 4

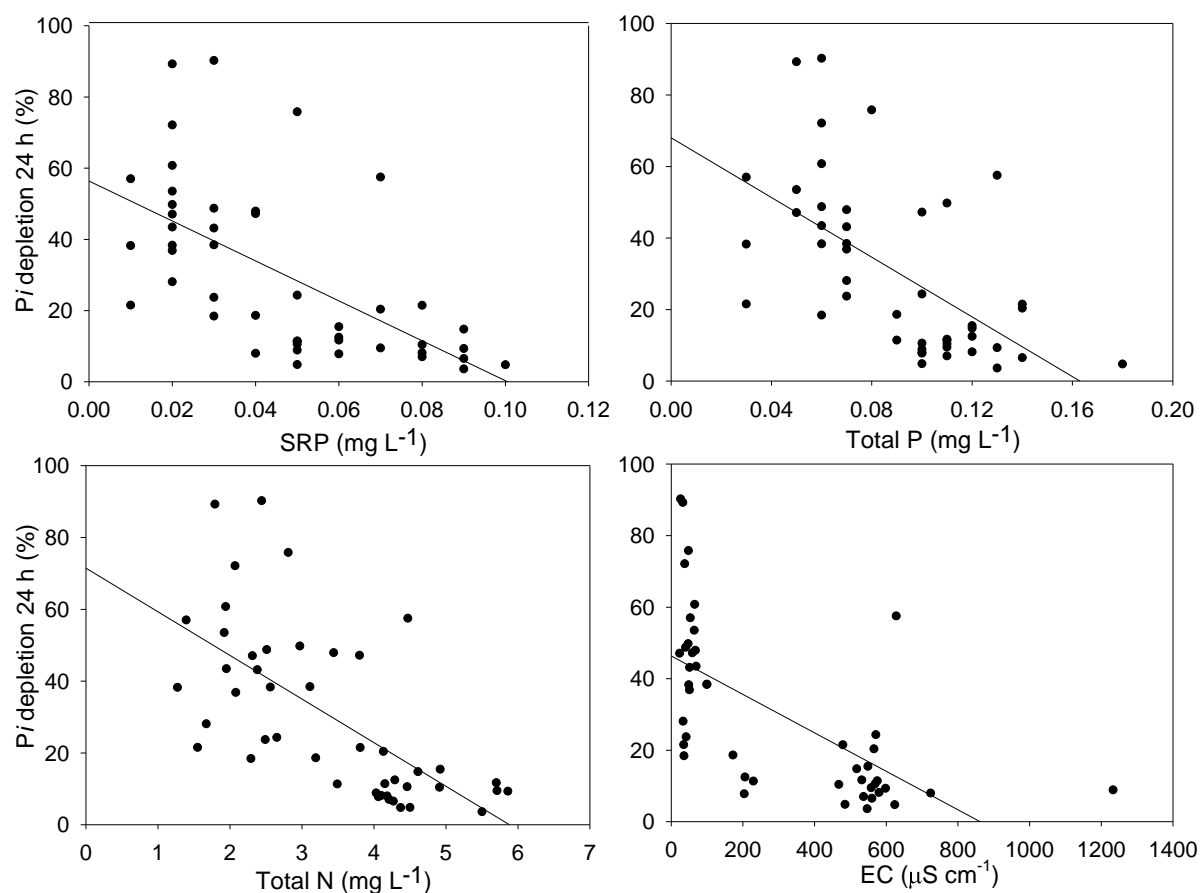


Fig. 4.6 Scatter plots of mean P_i depletion from solution after 24 h versus physiochemical parameters with simple linear regression lines displayed for 45 sites ($n=3$).

4.5 Discussion

4.5.1 Physiochemical parameters drive differences between rivers

The results presented in this study indicate that intrinsic physiochemical factors greatly influence how labile DOM components are biologically processed in freshwaters. Previous landscape-scale analysis of these sites has identified dominant land cover as an important determinant for DOM quality and stoichiometry, influencing C : N and DOC

Chapter 4

: DON/DOP ratios (Yates et al. 2019). Here, the intrinsic pool sizes of DOC and inorganic N/P appeared to directly influence the separation of sites (Fig. 2).

4.5.2 Changes in DOM degradation with changing nutrient limitation

DOC and inorganic N/P concentrations strongly correlated with the amount of microbial degradation of DOC, DON, DOP and P_i on a landscape scale (Table 4.2; Fig. 4.3). Across the two catchments, DOC depletion decreased with increasing intrinsic DOC concentration; the highest observed DOC concentrations were in peatland influenced rivers, where inorganic N and P concentrations were among the lowest observed (Table 4.2; Fig. 4.3; Emmett et al. 2016; Yates et al. 2019). In these headwater sites, N/P co-limitation is likely to be the main factor controlling the microbial processing of LMW DOM. For sites subject to inorganic N and P enrichment correlated with an increase in DOC uptake by the microbial biomass, which can be ascribed to the removal of metabolic constraints associated with N/P limitation on microbial growth and therefore labile LMW DOC uptake (Carlson and Ducklow 1996; Creamer et al. 2014). Generally, rivers transition from being N/P limited to N/C limited along the gradient from source to sea: although increasing inorganic N and P enrichment of rivers could potentially increase autochthonous DOC synthesis, the associated enhancement of microbial growth and OC degradation (as observed in the current study) can drive systems towards C limitation (Stanley et al. 2011; Emmett et al. 2016; Jarvie et al. 2018). This shift to C limitation with inorganic N/P enrichment has been observed in the current study, where labile DOC, DON and DOP depletion from river water increased with increasing intrinsic inorganic N and P across both catchments, confirming N/P enrichment (Table 4.2; Fig. 4.3; Yates

Chapter 4

et al. 2019). Conversely, P_i depletion from solution decreased with increasing inorganic N/P enrichment; this was in agreement with a previous study of an inorganic-enriched riparian zone where the addition of P_i , did not lead to an enhancement in microbial activity (represented by ^{14}C uptake) due to a lack of P limitation, and thus a lack of demand for P_i (de Sosa et al. 2018).

Based on the regression analysis parameters, overall, LMW DOM depletion followed the trend: $DON \geq DOC > DOP$, even in low nutrient status waters (Fig. 4.1; Table 4.2; Fig. 4.3; Yates et al. 2019). A previous catchment-scale study of DOM metabolism found that DON (in the form of amino acids) degraded quickest in peatland influenced rivers compared to those influenced by other land-covers, which is likely influenced by the N limitation of these ecosystems (Berggren and del Giorgio 2015). The microbial degradation of amino acids in oligotrophic peatland rivers was slower than in mesotrophic grassland rivers, however amino acids depletion was quicker than that of glucose, organic acids and phenolics compounds in both river waters and underlying sediments (Brailsford et al. 2019a). This supports the findings set out by Bronk and others (2007), who suggested that DON can be an extremely bioavailable source of N for both bacteria and phytoplankton.

4.5.3 Nutrient stoichiometry and DOM depletion

In recent years, the influence of stoichiometry of the three major macronutrients (CNP) has been brought to the forefront of catchment science, with several studies suggesting that modulating macronutrient stoichiometry could be the best approach for tackling

Chapter 4

eutrophication in freshwaters (Paerl et al. 2016; Stutter et al. 2018; Rankinen et al. 2019). In these studies and others, C : N ratios of soil have also been found to be a good predictor of both DOC: DON ratios and DOM bioavailability (Kroer 1993; Yates et al. 2019). Furthermore, N/P addition has been shown to alter the biodegradation of LMW DOC (Creamer et al. 2014; Brailsford et al. 2019b).

In terms of N/P quality, a number of studies have previously demonstrated that although increasing N/P is generally accompanied by increasing DON/DOP concentrations, a decrease in the proportion of DON/DOP components is usually also observed, as the influence of inorganic N/P increases (Berggren et al. 2015; Perakis and Hedin 2002; Durand et al. 2011; Yates et al. 2019). In the current study, both DON and DOP degradation correlated positively with increasing TN and TP (Table 4.2; Fig 4.3). We therefore consider that the increased DON/DOP demand at inorganically enriched sites could be due to: 1) a demand for easily biodegradable ON/OP (Bronk et al. 2007); 2) all DOM components (DOC, DON, DOP) being utilised for their carbon content due to C limitation (Jarvie et al. 2018); 3) changes in the way compounds are metabolised according to differences in nutrient limitation (Brailsford et al. 2019b).

4.5.4 Limitations

The current study utilised $^{14}\text{C}/^{33}\text{P}$ -labelled tracers to assess the fate of DOC, DON, DOP and P_i in river waters from 45 sites covering a range of physiochemical gradients. Apart from the DON component, a single tracer was used in each case. Although the LMW DOM fraction may represent a fraction of the total OM in aquatic ecosystems, it is a

Chapter 4

highly diverse mixture including sugars, amino acids, peptides, organic acids, carboxylic acids and nucleic acids (Dawson et al. 2001). Further studies are required using mixtures of radiolabelled compounds, in order to provide a more representative view of the uptake kinetics of labile LMW DOM in aquatic ecosystems (Brailsford et al. 2019a). In addition, for DOM compounds only the C was radiolabelled, therefore the ultimate fate of N in DON and P in DOP remains unknown. Finally, it is our opinion that downstream ‘omics’ approaches such as primary metabolome analysis could provide more insight into the fate of these compounds following uptake by the aquatic microbial biomass.

4.6 Conclusion

The comparison of the depletion of DOM components (DOC, DON, DOP) from solution demonstrated that DOC removal from solution proportionally increases with increases in DON and DOP depletion, with DON removal being slightly more rapid and DOP removal being slightly slower than DOC, respectively. The depletion of all three DOM fractions is predicted to increase along the freshwater : marine gradient. This study demonstrates that intrinsic water chemistry, which is in turn influenced by land-cover, can be a predictor of the capacity for DOM processing in the aquatic environment. While increasing ionic concentrations, inorganic N/P enrichment is likely to lead to increasing DOC, DON and DOP processing by the microbial biomass due to C limitation, P_i processing decreases with increasing inorganic enrichment. These results have implications for water quality management, as eutrophication is likely to result in further depletion of DOC from the water column, leading to increased microbial growth. Conversely, P_i inputs to eutrophic waters (e.g. due to intensive agriculture, sewage outfall

Chapter 4

events) are unlikely to be processed further, which could lead further water quality inputs downstream.

4.7 Acknowledgments

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Chapter 5

Microbial uptake kinetics of dissolved organic carbon (DOC) compound groups from river water and sediments

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FLB, HCG and DLJ designed and conceived the experiment, FLB and HCG conducted the experimental work. CAY produced the map presented in Fig. S5.1. FLB analysed the results and prepared the manuscript. All authors discussed results and contributed to the preparation of the manuscript.

Chapter 5

5.1 Abstract

Dissolved organic matter (DOM) represents a key component of carbon cycling in freshwater ecosystems. While the behaviour of bulk DOC in aquatic ecosystems is well studied, comparatively little is known about the turnover of specific dissolved organic carbon (DOC) compounds. The aim of this study was to investigate the persistence of ^{14}C -labelled low molecular weight (LMW) DOC at a wide range of concentrations (0.1 μM to 10 mM), in sediments and waters from oligotrophic and mesotrophic rivers within the same catchment. Overall, rates of DOC loss varied between compound groups (amino acids > sugars = organic acids > phenolics). Sediment-based microbial communities contributed to higher DOC loss from river waters, which was attributed, in part, to its greater microbial biomass. At higher DOC compound concentrations, DOC loss was greater in mesotrophic rivers in comparison to oligotrophic headwaters. A lag-phase in substrate use within sediments provided evidence of microbial growth and adaptation, ascribed here to the lack of inorganic nutrient limitation on microbial C processing in mesotrophic communities. We conclude that the higher microbial biomass and available inorganic nutrients in sediments enables the rapid processing of LMW DOC, particularly during high C enrichment events and in N and P-rich mesotrophic environments.

Keywords Metabolomics • Dissolved organic carbon • DOC processing • Nutrient availability • Stoichiometry

Chapter 5

5.2 Introduction

Dissolved organic carbon (DOC) is a complex mixture of compounds and represents a key component of carbon (C) transfer from terrestrial to freshwater environments and from headwaters to the marine zone¹. Further, allochthonous, terrestrially-derived DOC is frequently believed to be largely recalcitrant in freshwaters merely being transported rather than transformed in the aquatic environment. However, recently it has been shown to represent an important source of bioavailable carbon (C), fuelling aquatic heterotrophic ecosystem processes, particularly in streams and rivers influenced by peat-dominated headwaters where DOC concentrations are particularly high^{2,3}. DOC compounds can influence a wide range of processes occurring in the aquatic environment⁴. For example, high molecular weight (MW) DOC compounds have been found to bind to extracellular enzymes, modulating DOC breakdown along an aquatic continuum⁵. The fact that a DOC gradient exists along the majority of rivers, which abiotic degradation alone cannot account for, indicates that biological processing of DOC in-stream is occurring^{5,6,7}.

Sediments represent a crucial element of in-stream DOC processing due to the constant transfer of waters and nutrients occurring through the hyporheic and groundwater zone in catchments^{8,9,10}. These hyporheic-zone interactions are thought to have a major control on the residence time of organic matter compounds in freshwaters¹¹. Sediments can accumulate nutrients over time, particularly in lowland, low-gradient waters where sedimentation is more likely to occur⁶. Sediments can also be an autochthonous DOC source; it has been suggested that there is a net DOC efflux from sediments to overlying waters^{12,13}. Sediments also have the potential to become a primary

Chapter 5

source of pollutants, such as heavy metals, to overlying waters if there is a change to the aquatic chemical properties, leading to benthic nutrient export¹⁴.

Aquatic ecosystems are subject to increasing pressures; over the last few decades there have been increases in DOC fluxes from uplands across Europe and North America, particularly those dominated by peats, likely due to increasing global temperatures or a change in atmospheric N and S deposition¹⁵. In addition, anthropogenic inputs of excess inorganic nutrients to rivers promotes microbial activity, leading to reduced oxygen availability, eutrophication and disruption of entire food chains and loss of ecosystem services^{16,17,18}. In addition, it has been established that a small change in DOC concentrations can also lead to a shift in aquatic microbial community structure¹⁹. How microbial aquatic communities respond to changes in DOC inputs is not clear; consequently, this paper investigates the response of microbial communities to a range of DOC inputs.

While ultra-high resolution mass spectrometry has the potential to trace individual compounds through aquatic environments²⁰, few studies have quantified the pool sizes and fluxes of individual DOC compounds in freshwaters. A review of methods for measuring the microbial processing of DOC in lentic waters indicated that a ¹⁴C-labelled DOC tracer approach can be employed to measure DOC processing by the microbial community²¹. The two main approaches are to either (1) add ¹⁴C-tracers at intrinsic concentrations and measure uptake from solution and subsequent ¹⁴CO₂ respiration following metabolism, or (2) a kinetics approach measuring uptake at a wide range of concentrations, from concentrations below ambient conditions to high concentrations

Chapter 5

intended to fully saturate the system, in order to estimate rate parameters e.g. K_m and V_{max} for specific DOC compounds²¹.

To date, there have been a limited number of studies applying these methods to aquatic environments; such studies have focused on waters only, using simple ¹⁴C-labelled DOC compounds in isolation rather than compound groups, e.g. glucose or phenol^{22,23}. However, DOC is a heterogeneous mixture of compounds. Therefore we advocate that taking a specific compound group approach (using multiple compounds added together) will provide a more representative estimate of DOC loss rates in aquatic environments. This approach has been taken in some soil-based studies where more complex groups of DOC compounds have been investigated, such as amino acids²⁴ and organic acids²⁵. The kinetics-based approach using a large range of concentrations of the same compound or compounds has also been conducted successfully in some soil-based studies, primarily for glucose and other simple sugars^{26,27,28}. To our knowledge, there have been no previous studies using a kinetics-based approach for more complex phenolic compounds, which are a key component of the DOC pool in upland headwaters and sediments, accounting for up to 75% of the bioavailable DOC present^{29,30}.

The aims of this study were therefore to: (1) compare the rates of microbial uptake of four groups of low molecular weight (LMW) DOC compounds over time (sugars, amino acids, organic acids and phenolics); (2) determine the ability of microbial populations to process DOC under differing catchment conditions; and (3) establish the role of sediment and the hyporheic zone on DOC processing in rivers. The results of the study will be used to evaluate the relative importance of water-column versus hyporheic

Chapter 5

zone driven DOC processing and to establish trends in preferential uptake of any DOC fractions between mesotrophic and oligotrophic rivers.

5.3 Methods

5.3.1 Field sites and sampling

Sediment and water samples were collected from two contrasting land cover types within the Conwy catchment, North Wales, UK³¹ (Fig. S5.1). The first set were collected from three independent mesotrophic streams passing through lowland improved grasslands (mainly Cambisol soil type with some Gleysols present and *Lolium perenne* L. and *Trifolium repens* L. dominated swards). These livestock (sheep and beef) grazed grasslands have a long history (> 70 y) of receiving organic wastes in the form of cattle manures and slurries, inorganic NPK fertilisers and lime. The second set were collected from three independent oligotrophic headwater streams draining an upland blanket peat bog (mainly Histosol soil type) dominated by acid heathland vegetation (e.g. *Calluna vulgaris* (L.) Hull, *Vaccinium myrtillus* L., *Eriophorum vaginatum* L.), low intensity sheep grazing (< 0.1 ewe ha⁻¹) and no history of fertiliser application.

During the winter of 2016, three replicate 30 g samples of sediment (0-2 cm depth) were collected close to the riverbank at each site. In addition, three replicate unfiltered water samples were collected manually in acid-washed 1 L high density polyethylene (HDPE) bottles, 1 m upstream from the sediment sampling sites. Samples were placed in labelled bags and transported back to the laboratory at 10 °C in the dark within 4 hours of collection. pH and electroconductivity (EC) of river water and 1:2.5 (w/v) suspensions of sediment in e-pure water (18 MΩ resistance) were measured on the same day using

Chapter 5

standard electrode probes. Within 24 hours of collection, aliquots of river water, 1:5 sediment-to-1 M CH₃COOH extracts for P analysis and 1:5 sediment-to-0.5 M K₂SO₄ for all other analyses were frozen at -20 °C until subsequent laboratory analysis.

5.3.2 Background chemical analysis

Sediment moisture content was determined by oven drying < 2 mm sieved sediment at 105 °C for 24 h. Organic matter content was measured using loss-on-ignition in a muffle furnace (450 °C, 16h)³². Oven dried, root free sediment was analysed for C and N content using a TruSpec[®] analyzer (Leco Corp., St Joseph, MI, USA). Sediment samples were collected and shipped to Yara (Lincolnshire, UK) for texture analysis (Sand %, Silt %, Clay %) using a Mastersizer 3000 laser particle size analyzer (Malvern Panalytical). River water DOC and total dissolved N (TDN) content were determined using Multi N/C 2100S analyser (AnalytikJena, Jena, Germany). The following chemical parameters were determined using river water samples and 0.5 M K₂SO₄ sediment extracts: concentrations of NH₄⁺ and NO₃⁻ were measured according to the methods outlined by Mulvaney³³ and Miranda³⁴ respectively. Total free amino acids and total free carbohydrates were determined using the fluorometric OPAME procedure³⁵ and the Myklestad method³⁶ respectively. The concentration of phenolic compounds was measured using the Folin-Ciocalteu method³⁷. Finally, molybdate-reactive P was measured for river water samples and 1 M 1.0 M CH₃COOH sediment extracts³⁸.

Chapter 5

5.3.3 Microbial community analysis

To determine the size and structure of the microbial community, phospholipid-derived fatty acid (PLFA) analyses were carried out on both river water and sediment samples. From each site a 25 L water sample was collected and concentrated in the laboratory to 50 mL using a KrosFlo Research Iii Tangential Flow Filtration System (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Concentrated water samples and 25 g sediment samples were then freeze-dried and stored at -80 °C until shipping on dry ice to Microbial ID, DA, USA. The PLFA content of the samples was determined using the methods outlined by Buyer and Sasser³⁹.

5.3.4 DOC depletion experiment

Within 6 hours of collection, three independent replicate samples containing 9.9 mL unfiltered water and three replicates of 9.9 mL unfiltered water plus 1.00 g (\pm 0.01) sediment were added to sterile 15 mL polypropylene centrifuge tubes (Corning Inc., Corning, NY, USA). The river-water only treatment represented the response of the water column microbial biomass to different DOC inputs, whereas the sediment and river water (1:10 ratio) treatment represented the interaction of the water column and the sediment porewater microbial biomasses within the hyporheic zone. Each sample then had 100 μ L of solution containing unlabelled DOC compounds (at the final concentrations outlined below), spiked with the corresponding ¹⁴C-labelled compounds to act as a tracer, with a final activity 0.4 kBq mL⁻¹. The amount of ¹⁴C-tracer added to each DOC solution was < 1 nM and therefore not expected to change the overall concentration. The kinetic assays were divided into four compound groups: sugars, amino acids, organic acids and

Chapter 5

phenolic compounds. In total, 8 different radioisotopically labelled compounds were used (Table S5.1). Compounds were chosen to reflect compounds typically released during the breakdown of particulate organic matter entering soils and freshwaters. Sterile controls run with e-pure water (18 M Ω resistance) in place of river water resulted in no loss of ^{14}C -compounds from solution (Fig. S5.2). Abiotic loss of ^{14}C -compounds due to sediment sorption based on controls run with sediments sterilised with formaldehyde was used to correct for potential sorption at high and low ^{14}C -compound concentrations (Fig. S5.3).

The concentrations of amino acids (alanine, arginine, aspartate, glutamate, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, tyrosine, valine), glucose, organic acids (acetic acid, citric acid, malic acid) and phenolic compounds (*P*-coumaric acid, salicylic acid, vanillic acid) ranged from 0.1 μM to 0.5 mM for waters and 0.1 μM to 10 mM for sediments (Table S5.2). A higher concentration range for sediments was utilised to represent the higher background DOC concentrations found in sediments. The wide range of concentrations were selected to represent a broad range of DOC conditions, from low ambient concentrations through to an excess of DOC capable of fully saturating the system, which although unlikely to occur naturally for a prolonged period were used to assess the maximum concentration that could be processed in the two contrasting systems. After sealing with sterile caps, the samples were subsequently incubated on a shaker in the dark at 10 $^{\circ}\text{C}$, to ensure the samples remained well mixed for the duration of the experiment. This temperature represents the mean annual temperature within the catchment²³. The mean water temperature over the duration of the experiment was 8.28 ± 0.34 $^{\circ}\text{C}$.

After incubation for 1, 2, 5, 24, 40, 48, 72 and 168 h, by which point ^{14}C -compound depletion had plateaued, 0.5 mL subsamples were removed from the tubes,

Chapter 5

centrifuged to remove microbial cells (20,817 g, 3 min), and 0.25 mL of the supernatant placed into a scintillation vial. Destructive sampling was not possible due to the large number of samples, however samples were kept well mixed on a shaker throughout the experiment and a head space of at least 5 mL maintained in order to prevent samples from becoming anaerobic. The subsamples were then acidified with HCl (25 μ L, 0.1 M), left to stand overnight and then vortexed to remove any remaining dissolved $\text{H}^{14}\text{CO}_3/\text{}^{14}\text{CO}_2$ present. The subsample was then mixed with Optiphase HiSafe scintillation cocktail (4 mL; PerkinElmer, Waltham, MA, USA) and the ^{14}C quantified on a Wallac 1404 liquid scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK).

5.3.5 Statistical analysis

Initial rates of uptake of the DOC groups were calculated as the percentage of added ^{14}C depleted within 1 h. Lineweaver-Burke plots were used to estimate the Michaelis-Menten parameters V_{max} , the maximum rate of DOC from solution and K_m , the substrate concentration at which half the maximal uptake rate is achieved. Data analyses were conducted using SPSS 22.0 (IBM UK Ltd, Portsmouth, UK). Independent t-tests were used to determine any differences between sediment and water characteristics for each land cover type. Two-way mixed analysis of variance (ANOVA) was used to test for significant differences between sample type, land cover, DOC (sugars, amino acids organic acids, phenolic compounds) compound group and the concentration of the DOC compound group added. For comparisons of sediments and waters, only data from concentrations used in both water and sediment treatments were used in statistical

Chapter 5

analysis (Supplementary Table S5.2). The significance level of the P-value was set at $p \leq 0.05$. If the data did not meet the criteria of Mauchly's test for sphericity, the Greenhouse-Geisser correction was applied to the P-value.

5.4 Results

5.4.1 Sediment and water characteristics

The water samples from the two contrasting stream types used in the study were found to differ more widely in their chemical properties than the sediment samples (Table 5.1; Supplementary Tables S5.3). The pH, Electroconductivity (EC), total dissolved nitrogen, nitrate and orthophosphate values were significantly higher in samples from lowland mesotrophic sites, while DOC was found to be significantly higher in samples from the upland oligotrophic sites. These trends align with the peaty soils found in the upland oligotrophic soils and the manure and fertiliser nutrient-enriched soils in the lowland mesotrophic catchments (Emmett et al. 2016). For sediments, pH, EC and molybdate-reactive P values were higher in lowland mesotrophic sediments compared to upland oligotrophic sediments, whilst moisture content, organic matter, total carbon and total nitrogen were higher for upland oligotrophic sediments. This is likely due to the higher levels of variation observed within this dataset.

Chapter 5

Table 5.1 Chemical characteristics of the water and sediment samples used in the study.

Values represent means \pm SEM, $n = 9$. *Denotes a significant P -value when comparing the two sites. The significance level was set at $P < 0.05$. All values for sediments are expressed on a dry weight basis unless otherwise stated.

	Lowland mesotrophic	Upland oligotrophic	F	P-value
Water				
pH	7.09 ± 0.08	4.20 ± 0.16	14	$<0.001^*$
Electrical conductivity ($\mu\text{S cm}^{-1}$)	191 ± 8	49 ± 5	13	$<0.001^*$
Temperature	7.53 ± 0.37	9.00 ± 0.65	3	0.421
Dissolved organic C (mg C L^{-1})	2.86 ± 0.13	7.60 ± 0.62	6	$<0.001^*$
Total free carbohydrates (mg C L^{-1})	0.11 ± 0.02	0.09 ± 0.02	1	0.45
Total phenols (mg C L^{-1})	2.27 ± 0.66	1.78 ± 0.74	0	0.667
Total dissolved N (mg N L^{-1})	2.13 ± 0.24	0.38 ± 0.02	7	$<0.001^*$
NH_4^+ (mg N L^{-1})	0.05 ± 0.01	0.06 ± 0.01	0	0.818
NO_3^- (mg N L^{-1})	1.73 ± 0.24	0.02 ± 0.00	7	$<0.001^*$
Total free amino acids (mg N L^{-1})	0.10 ± 0.01	0.13 ± 0.01	2	0.142
Molybdate-reactive P (mg P L^{-1})	0.07 ± 0.01	0.03 ± 0.00	4	$<0.001^*$
Sediment				
$\text{pH}_{(\text{H}_2\text{O})}$	6.87 ± 0.06	4.75 ± 0.05	27	$<0.001^*$
Electrical conductivity ($\mu\text{S cm}^{-1}$)	37 ± 9	15 ± 2	4	0.002^*
Moisture content (%)	40.0 ± 3.6	80.3 ± 3.6	8	$<0.001^*$

Chapter 5

Silt content (%)	27.6 ± 14.6	5.2 ± 1.3	2	0.263
Clay content (%)	10.2 ± 5.8	0.7 ± 0.3	2	0.243
Sand content (%)	62.3 ± 20.3	94.1 ± 1.6	2	0.257
Total C (mg C kg sediment ⁻¹)	7.19 ± 1.22	250 ± 41.7	6	<0.001*
Total free carbohydrates (mg C kg wet sediment ⁻¹)	0.59 ± 0.03	0.61 ± 0.08	0	0.829
Total phenols (mg C kg wet sediment ⁻¹)	4.05 ± 1.37	7.26 ± 2.58	1	0.358
Total N (mg N kg sediment ⁻¹)	1.14 ± 0.08	8.36 ± 1.28	6	<0.001*
NH ₄ ⁺ (mg N kg wet sediment ⁻¹)	11.7 ± 5.5	5.1 ± 1.8	1	0.272
NO ₃ ⁻ (mg N kg wet sediment ⁻¹)	0.26 ± 0.10	0.91 ± 0.26	2	0.061
Total amino acids (mg N kg wet sediment ⁻¹)	0.20 ± 0.02	0.20 ± 0.01	0	0.834
Molybdate-reactive P (mg P kg wet sediment ⁻¹)	2.05 ± 0.21	0.21 ± 0.05	9	<0.001*

Higher abundances of PLFAs were recovered from sediment samples in comparison to water samples (Table 5.2; Supplementary Table S5.4). For waters, there were approximately half the amount of PLFAs of fungal origin in the upland oligotrophic sites in comparison to the lowland mesotrophic sites. No other taxa were found to differ. By contrast, approximately four times as many PLFAs were recovered from upland oligotrophic sediments in comparison to lowland mesotrophic sediments, which might reflect the higher abundance of submerged plants in the upland stream reaches. More

Chapter 5

PLFAs of gram positive bacterial origin were found in upland oligotrophic sediments than in their mesotrophic counterparts.

Table 5.2 Analysis of total mass of Phospholipid-derived fatty acids (PLFA) and taxonomic groups of concentrated water samples and freeze-dried sediment samples used in the study. Values represent means \pm SEM, $n = 3$. *Denotes a significant P -value when comparing the two sites. The significance level was set at $P < 0.05$.

	Lowland eutrophic	Upland oligotrophic	F	P-value
Water				
Total PLFA biomass (nmol ml water ⁻¹)	0.07 \pm 0.02	0.12 \pm 0.07	1	0.475
Gram - (%)	51.2 \pm 4.5	58.1 \pm 4.3	1	0.329
Gram + (%)	33.0 \pm 4.5	27.5 \pm 3.9	1	0.399
Actinomycetes (%)	3.25 \pm 1.37	2.23 \pm 2.09	1	0.428
AM Fungi (%)	4.10 \pm 4.71	5.50 \pm 3.59	1	0.680
Fungi (%)	3.92 \pm 0.26	1.86 \pm 0.22	6	0.004*
Eukaryote (%)	4.48 \pm 0.55	4.84 \pm 1.43	0	0.824
Sediment				
Total PLFA biomass (nmol g sediment ⁻¹)	152 \pm 34	621 \pm 180	3	0.048*
Gram – bacteria (%)	47.1 \pm 2.1	47.8 \pm 0.7	3	0.753
Gram + bacteria (%)	25.5 \pm 0.8	30.1 \pm 1.9	2	0.047*
Actinomycetes (%)	6.79 \pm 1.02	8.27 \pm 2.09	1	0.346
Fungi (%)	5.41 \pm 2.78	4.51 \pm 0.23	1	0.754
Eukaryote (%)	8.63 \pm 1.73	6.35 \pm 0.64	1	0.245

Chapter 5

5.4.2 DOC uptake in sediment versus water

For all DOC compound groups, the highest maximal reaction rates (V_{\max}) were observed for mesotrophic sediments, which were three orders of magnitude higher in comparison to mesotrophic river waters (Supplementary Fig. S5.3). No differences in V_{\max} were observed between sediment and water from oligotrophic rivers. The K_m values for the different DOC groups were also lowest in sediments from mesotrophic rivers, indicating that a lower concentration of DOC is required to reach the maximum uptake rates.

Higher rates of initial rate of ^{14}C -amino acid depletion ($\mu\text{mol cm}^{-3} \text{ hour}^{-1}$) were observed in sediments in comparison to waters (for comparable concentrations only) for both the oligotrophic and mesotrophic rivers (two-way ANOVA, $P < 0.001$). Whilst the mean initial rate of ^{14}C -glucose depletion was also higher in sediments than in waters for comparable concentrations ($<500 \mu\text{M}$) for both mesotrophic and oligotrophic rivers (two-way ANOVA, $P < 0.001$), the amount of glucose remaining in sediment and water samples at the end of the experiment was not found to differ in oligotrophic rivers (two-way ANOVA, $P = 0.873$; Supplementary Tables S5.5, S5.8).

For comparable concentrations of organic acids ($< 500 \mu\text{M}$), the initial rate of ^{14}C -labelled organic acid uptake was higher in both mesotrophic and oligotrophic sediments in comparison to waters from the same sites (two-way ANOVA, $P < 0.001$ for both). This corresponded with there being less organic acid remaining in solution for sediments in comparison to waters from mesotrophic sites (two-way ANOVA, $P = 0.019$; Tables S5.6-5.7).

In lowland mesotrophic sites there was no difference in the initial rate of phenolics depletion between sediments and waters (two-way ANOVA, $P = 0.579$), however, for

Chapter 5

upland oligotrophic sites the initial rate of phenolics depletion was higher in sediments in comparison to waters (two-way ANOVA, $P < 0.001$; Supplementary Table S5.7). In contrast, for both oligotrophic and mesotrophic sites there were more phenolics remaining in solution at the end of the experiment in water samples compared to sediment samples where the same concentration was used (two-way ANOVA, $P = 0.001$ and $P < 0.001$ respectively).

5.4.3 DOC uptake in two waters draining contrasting catchment types

5.4.3.1 ^{14}C -labelled amino acid uptake

For sediments, the mean initial amino acid depletion rate was double in oligotrophic rivers in comparison to mesotrophic rivers (two-way ANOVA, $P = 0.006$; Fig. 1; Tables S5.6-5.8). However, oligotrophic sediments had double the amount of amino acids remaining at the end of the experiment compared to mesotrophic sediments (two-way ANOVA, $P < 0.001$; Tables S5.6-5.8). This result was driven by the two highest amino acid concentrations, where there was high initial amino acid depletion followed by a period of saturation.

There was no difference in the mean ^{14}C -amino acid depletion rate detected for river waters, however, there was a significant interaction between the trophic state of the waters and amino acid concentration, driven by the difference in the amino acids remaining at the end of the assay at the highest concentration (500 μM) (two-way ANOVA, $P = 0.715$, $P < 0.001$ respectively). At the end of the experiment, there was double the amount of amino acids remaining in oligotrophic waters in comparison to mesotrophic waters (two-way ANOVA, $P < 0.001$; Supplementary Tables S5.6-5.8).

Chapter 5

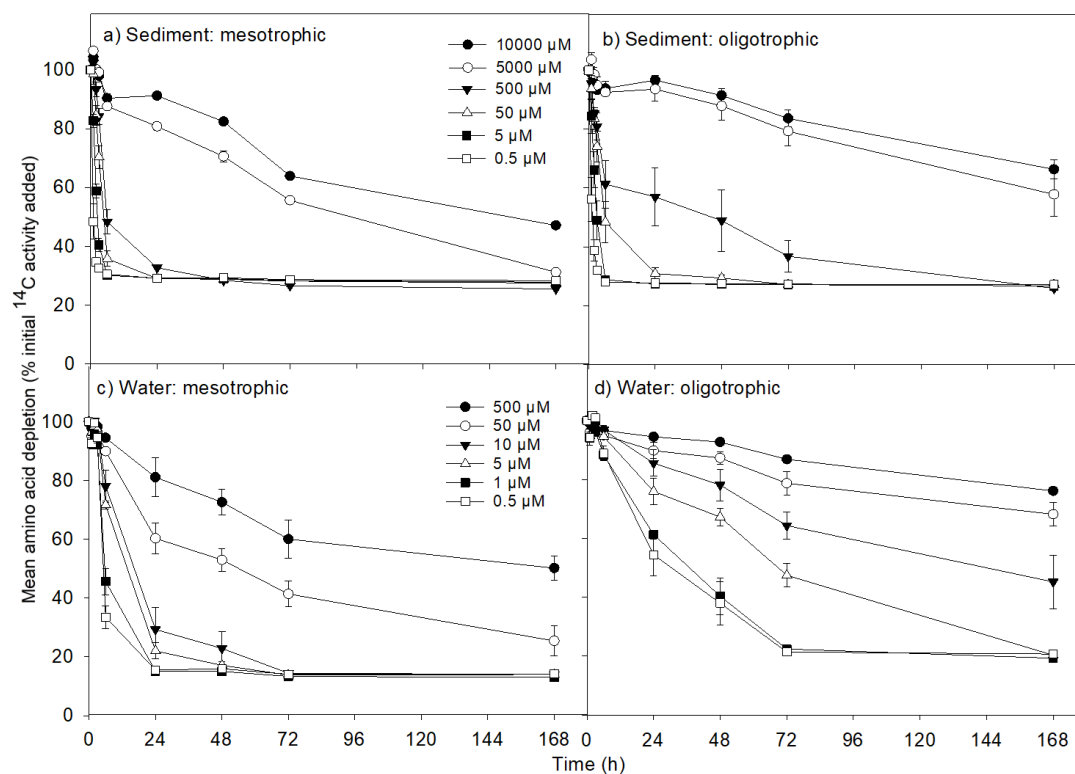


Fig. 5.1 Effect of DOC concentration on the loss of ^{14}C -labelled amino acids for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

5.4.3.2 ^{14}C -labelled glucose uptake

Chapter 5

Overall, there was two thirds of the initial ^{14}C -glucose remaining at the end of the experiment in samples from mesotrophic river sediments in comparison to oligotrophic river sediments (two-way ANOVA, $P = 0.037$; Fig. 5.2). The difference between the percentage of ^{14}C -glucose remaining in waters from the two contrasting land cover types was greater, with $\sim 23\%$ more glucose remaining in solution for oligotrophic waters (; two-way ANOVA, $P = 0.020$; Supplementary Tables S5.6-5.8), despite the higher initial ^{14}C -glucose depletion rate in sediments and waters from oligotrophic rivers (two-way ANOVA, $P < 0.001$).

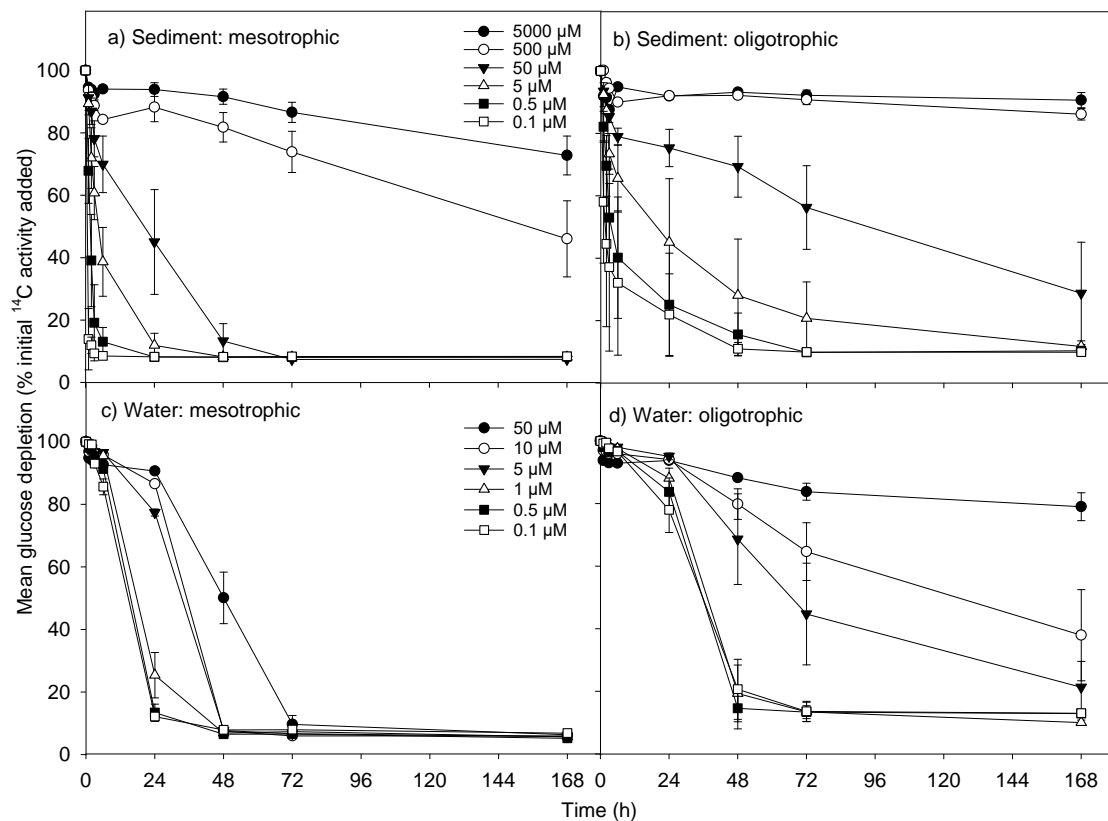


Fig. 5.2 Effect of DOC concentration on the loss of ^{14}C -labelled glucose for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland

Chapter 5

improved grassland river waters (oligotrophic). Values represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

5.4.3.3 ^{14}C -labelled organic acids uptake

When the results for the two contrasting land cover types were compared, the initial organic acid depletion rate was $\sim 60\%$ higher in oligotrophic sediments than mesotrophic sediments, however, no difference was found between mesotrophic and oligotrophic waters (two-way ANOVA, $P < 0.001$ and $P = 0.947$ respectively; Fig. 5.3; Supplementary Tables S5.6-5.8). There was also no difference in the amount of organic acids remaining in the mesotrophic and oligotrophic sediments by the end of the assay, whilst overall more organic acid compounds remained in the oligotrophic waters at the end of the experiment, in comparison to the mesotrophic waters (two-way ANOVA, $P = 0.202$ and $P < 0.001$ respectively; Fig. 5.3).

Chapter 5

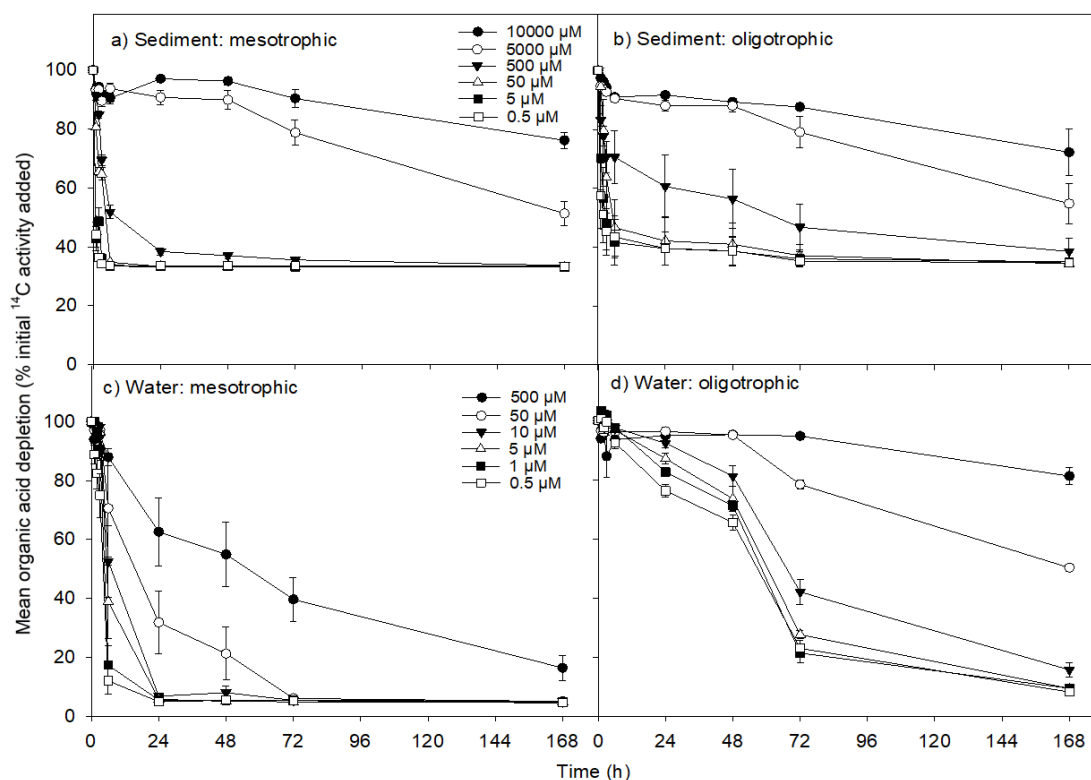


Fig. 5.3 Effect of DOC concentration on the loss of ¹⁴C-labelled organic acids for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values represent means \pm SEM ($n = 3$). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

Chapter 5

5.4.3.4 ¹⁴C-labelled phenolic compounds uptake

For upland oligotrophic sediments, despite an initial spike in mean phenolics uptake ~ 4 times higher than the initial rate observed in lowland mesotrophic sediments (two-way ANOVA, $P < 0.001$; Fig. 5.4), there was no effect of land cover at the end of the experiment (two-way ANOVA, $P = 0.715$). This can be attributed to the higher levels of variance observed in this dataset. In contrast, although there was no initial difference in phenolic compounds uptake rates between waters from the two land cover types (two-way ANOVA, $P = 0.249$), by the end of the experiment a greater uptake of phenolics had occurred in the lowland mesotrophic water in comparison to the upland oligotrophic waters (two-way ANOVA, $P < 0.001$).

Chapter 5

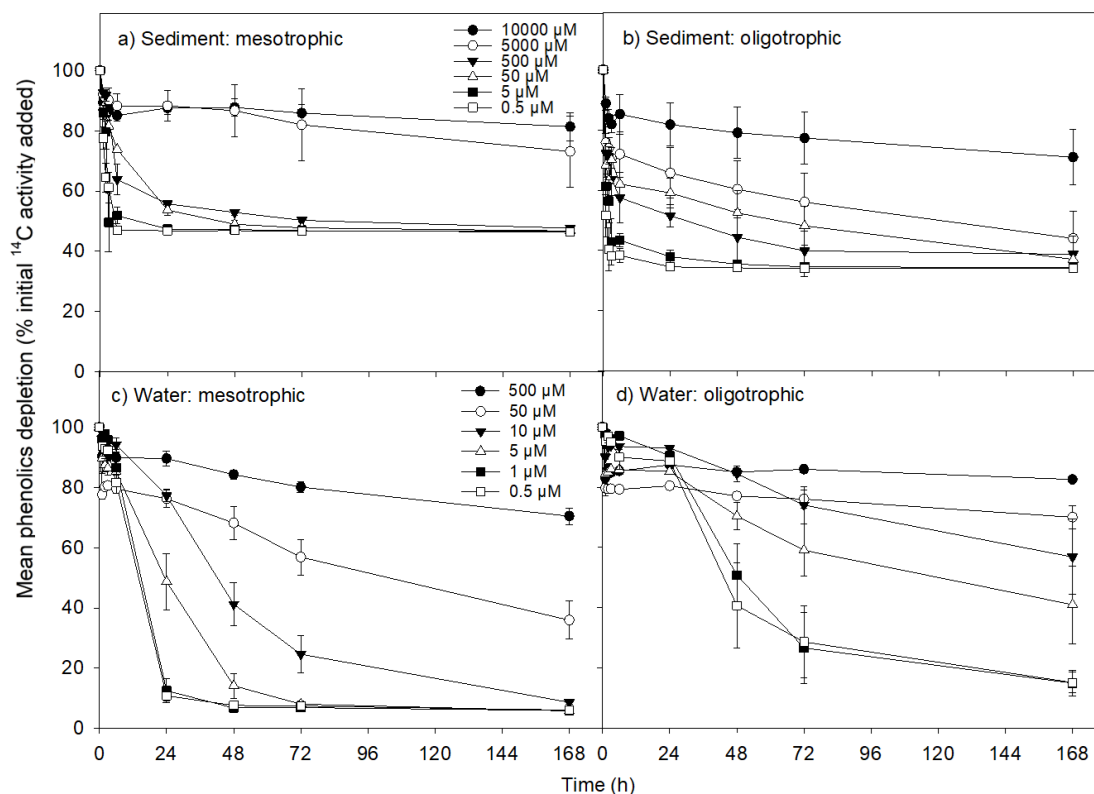


Fig. 5.4 Effect of DOC concentration on the loss of ¹⁴C-labelled phenolic compounds for: a) lowland improved grassland river sediments (mesotrophic), b) upland peat bog sediments (oligotrophic), c) lowland improved grassland river waters (mesotrophic), d) upland improved grassland river waters (oligotrophic). Values represent means ± SEM (*n* = 3). Please note the legends are different for the top two panels (a and b) and bottom two panels (c and d) to represent the different concentration ranges found in each substrate type; sediment and water respectively. The legend is the same for the top two (a and b) and bottom two (c and d) panels respectively.

Chapter 5

5.4.4 Uptake of DOC compound groups

The highest initial DOC uptake rate was observed for the phenolic compounds, for both mesotrophic sediments and oligotrophic waters (two-way ANOVA, $P < 0.001$). This was reflected in phenolics having the highest maximum velocity for the reaction for mesotrophic sediments (Supplementary Fig. S5.4; Supplementary Table S5.8). The mean initial ^{14}C -labelled organic acid depletion rate in mesotrophic sediments was approximately one third of ^{14}C -labelled glucose depletion rate, however no difference was detected between organic acids and amino acids (two-way ANOVA, $P < 0.001$; Supplementary Table S5.5). For oligotrophic sediments, the initial ^{14}C -organic acid depletion rate was not different from that observed for amino acids and phenolic compounds. However, it was lower than the initial glucose depletion rate (two-way ANOVA, $P < 0.001$). The initial phenolic compounds depletion rate was higher than that of both amino acids and glucose (two-way ANOVA, $P < 0.001$). For both land cover types, the initial glucose uptake rate in sediments was half that of the amino acid uptake rate (two-way ANOVA, $P < 0.001$; Supplementary Tables S5.5-5.6).

Although there was an overall significant effect of DOC compound group on the percentage DOC remaining at the end of the experiment, there was no difference between organic acids and glucose remaining by the end point (two-way ANOVA, $P < 0.001$ and $P > 0.05$ respectively; Supplementary Table S5.6). However, there were less organic acids remaining at the end of the experiment compared to the phenolic compounds in the mesotrophic sediments. Despite the high initial depletion rates, glucose had the highest amount remaining by the end of the assay (two-way ANOVA, $P < 0.001$). The elevated phenolics uptake rates were also not sustained over the duration of the study; in both

Chapter 5

mesotrophic and oligotrophic sediments there were more phenolic compounds remaining in solution at the end point compared to glucose (two-way ANOVA, $P < 0.001$).

In contrast to the results for the sediment samples, the initial ^{14}C -organic acid depletion rate in both mesotrophic and oligotrophic waters was not found to be different to the initial depletion rates of amino acids and glucose (two-way ANOVA, $P < 0.001$ for both). However, the initial ^{14}C -glucose depletion rates in mesotrophic and oligotrophic waters were higher than the rates observed for ^{14}C -labelled amino acids (two-way ANOVA, $P < 0.001$ in both cases; Fig. 5.2; Supplementary Table S5.6). Although the initial phenolic compounds depletion rate was higher in upland oligotrophic waters, there was no difference in the initial phenolics depletion in lowland mesotrophic waters compared to the other compound groups (two-way ANOVA, $P = 0.199$). The combined mean percentage of both organic acids and glucose remaining at the end of the experiment was approximately half of the combined mean percentage of amino acids and phenolic compounds remaining in solution (two-way ANOVA, $P < 0.001$ for both).

5.5 Discussion

The overall amino and organic acid processing rates were approximately double the rates for sediments in comparison to waters, for both mesotrophic and oligotrophic rivers, over the duration of the experiment. The elevated DOC processing rates in sediments highlight the importance of the hyporheic zone for in-stream carbon cycling; this includes sediments of the active channel and riparian zones, both of which provide a stable environment for higher microbial processing rates and comprise the main interface where surface and groundwaters mix⁴⁰. As expected, there was a significant effect of ^{14}C -

Chapter 5

compound concentration on DOC processing rates for the majority of treatments. The higher intrinsic nutrient loadings observed for sediments in comparison to waters are likely to be a contributing factor to the elevated processing rates; intrinsic DOC concentrations have previously been found to have a positive correlation with the in-stream organic matter processing rate^{11,41,42}. Sediments were also found to have a higher microbial biomass compared to waters at both oligotrophic and mesotrophic sites in the current study, which may also increase their uptake capacity for DOC compounds. Alternatively, the greater depletion observed in these treatments could be ascribed to abiotic sorption of the compounds to the sediment's solid phase, however this is known to be low, particularly for weakly or neutrally charged solutes and sediments with low cation exchange capacity such as those used here^{43,44}. This was confirmed by control experiments performed as part of this study (Supplementary Fig. S5.4).

In upland oligotrophic waters, glucose was initially processed more quickly in the water column than in the sediment at comparable concentrations, although a higher proportion of glucose added was processed in sediments over the whole experimental period. Phenolic compounds were also initially processed at higher rates in upland oligotrophic waters in comparison to upland oligotrophic sediments, although more phenolic compounds were processed in sediments overall, as observed for glucose. These results are in agreement with the earlier findings of Dawson and colleagues⁴⁵ who reported in-stream processing of DOC in carbon-rich upland waters as a major factor governing DOC gradients on a spatial scale along the length of a lotic water body. ¹⁴C-glucose uptake has previously been used as a proxy for microbial activity^{11,46}; therefore the higher initial rate of glucose processing in the oligotrophic water column could

Chapter 5

indicate that initially there is more microbial activity in oligotrophic waters in comparison to mesotrophic waters. It has previously been found that bacterial growth efficiency in terms of DOC utilisation can be higher in carbon-rich waters, compared to carbon-poor waters, which is in agreement with the above results²². However, the lag phase observed in sediments previously described could indicate that faster microbial growth is occurring in the sediments, allowing more rapid glucose processing.

For both sediment and water, our study showed that microbial communities from lowland mesotrophic rivers were able to process higher DOC concentrations than those from upland oligotrophic rivers, with the exception of organic acids and phenolic compounds in sediments only. We hypothesise that this could be attributed to a range of factors including: (i) the higher background inorganic nutrient concentrations in lowland mesotrophic waters, thereby removing metabolic constraints on substrate uptake and microbial growth^{28,47}; (ii) the elevated high MW humic substances concentrations in the oligotrophic waters which may limit biological activity via binding and inhibiting free enzymes responsible for substrate catalysis^{5,30,48}; (iii) the binding of the added substrates to DOC in the water, removing these from solution early in the experiment and thus preventing microbial uptake, (iv) the binding of intrinsic DOC to the organisms present, thereby suppressing membrane bound transport systems^{49,50}; or (v) the higher concentration of organisms in the mesotrophic waters and sediments in comparison to those from the oligotrophic environment, as shown by the PLFA data.

Initially, there was a slightly higher rate of DOC uptake in oligotrophic waters, however, this was not sustained over the duration of the experiment. This, alongside the higher inorganic nutrient concentrations typically present in the mesotrophic waters,

Chapter 5

lends support to hypothesis (i), but does not support hypothesis (v) based on our PLFA data. In contrast, based on the abundant microbial transport systems for LMW substrates discovered by metagenomic sequencing (which suggests largely intracellular LMW DOC breakdown) we do not favour hypothesis (ii). Similarly, we discount (iii) as most of our C substrates have neutral charge at the pH values used here and are therefore unlikely to interact strongly with intrinsic DOC present in the samples or with mineral surfaces. Hypothesis (iv) cannot be critically evaluated in our study and further work would need to be undertaken to evaluate its significance.

A lag phase in substrate uptake was observed for mesotrophic rivers, such that DOC processing was initially quicker in oligotrophic rivers; this was particularly evident in sediments. This lag phase in mesotrophic rivers could be attributed to microbial growth or the microbial community becoming more active over time (e.g. resuscitation from a starvation/viable-but-non-culturable state)⁵¹. Previous studies have found that mixing sediment with water containing a lower concentration of DOC than in the natural overlying waters can halt growth or reduce the biomass by approximately 50% over the short term, with the community reaching a new steady state after 4 days⁵². In the current study, this lag phase generally did not exceed 48 hours and was followed by an increase in the DOC uptake rate.

The fastest initial DOC processing rate observed was for amino acids in oligotrophic sediments at $0.23 \pm 0.09 \mu\text{mol cm}^{-3} \text{ h}^{-1}$. A higher proportion of amino acids were processed in sediments than in waters from both land cover types. Previously we have ascribed the rapid uptake of amino acids as relating to its labile DOC (i.e. readily used by a C-limited microbial community)²³, but it is also a source of DON. ¹⁴C-labelled

Chapter 5

amino acid studies have found that the C skeleton produced during intracellular amino acid processing (e.g. pyruvate) can be excreted with intracellular N increasing following amino acid uptake⁵³. This is supported by the percentage of amino acids remaining in solution never falling below 20% for waters in particular; a trend which has also been observed in previous studies^{23,54}. Catchment-scale studies of DOM processing have found that amino acid uptake capacity was the highest in peat-influenced streams, which is likely linked to the N limitation characteristic of these ecosystems⁵⁵. However, the greatest overall DOC loss from solution was observed for organic acids and glucose in mesotrophic sediments. All of the initial DOC uptake rates measured were within the same order of magnitude as those found for ¹⁴C-labelled glucose uptake in soils and for glucose²⁶ and acetate⁵⁶ by bacteria and algae in aquatic environments.

A study of ¹⁴C-glucose rates versus ¹⁴C-phenol uptake in humic and clear waters, which also measured bacterial abundance alongside the assays, found that glucose uptake peaked during the exponential phase of bacterial growth, with the biggest peak seen in clear waters²². Although approximately the same amount of glucose was processed over the duration of the experiment, higher bacterial growth efficiency was observed in humic waters, with the bacterial biomass reaching a higher abundance relative to clear water and mixed clear water/humic water samples. Microbes from humic waters began to process phenol once the glucose had been almost completely utilised, indicating that they will preferentially use more labile DOC, but can also adapt to use more aromatic compounds. The usage of phenol by microbes from clear waters did not exceed the limit of detection for the duration of the experiment²². Our results appear to mirror this earlier study, with higher sustained glucose uptake rates in comparison to phenolic compounds over the course of the experiment, and an initial lag-phase evident before glucose uptake begins,

Chapter 5

indicating that this too could coincide with a period of microbial growth. However, more evidence of phenolic compound processing was observed in this study, which may relate to the fact that our samples were collected at slightly warmer temperatures than in the work of Tranvik and Höfle²², or that the phenolic compounds used in this study contained more aliphatic bonds than phenol, which were used in the earlier study.

The phenolic compound processing in the current study may be limited by abiotic factors such as photodegradation, as samples were incubated in the dark. Previous studies have shown that photodegradation alone, in the absence of microbial processing, can result in the production of labile DOC from larger, humic-like compounds, which are more bioavailable to the microbial community⁵⁷. Photochemical degradation of DOC compounds is particularly important in upland mountain, heath and bog habitats where the amount of shading by riparian vegetation is lowest; this can also act to influence water temperature, moderating biotic processes⁵⁸.

We conclude that the higher inorganic nutrient concentrations and greater microbial biomass of sediments allows more rapid processing of LMW DOC compounds, particularly at higher background DOC enrichment. For mesotrophic sediments in particular, the greater availability of N and P to the stream biota may remove the inorganic nutrient limitation barrier on DOC uptake, providing them with a greater capacity for in-stream DOC processing. In comparison, oligotrophic rivers processed less DOC than the mesotrophic rivers; initial DOC processing primarily took place in the water column in oligotrophic rivers, although the sediments processed more DOC overall, with a preference for the simplest compounds (amino acids, glucose). If DOC is not processed fully in the uplands and DOC export from peatlands continues to increase over the coming

Chapter 5

years, this may exacerbate problems in downstream lowland areas, which has implications for future water quality management.

5.6 Acknowledgments

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Chapter 6

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

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FLB, HCG and DLJ designed and conceived the experiment, FLB and HCG conducted the experimental work. MRM produced the map presented in Fig. S6.1. FLB analysed the results and prepared the manuscript. All authors discussed results and contributed to the preparation of the manuscript.

Keywords Metabolomics • Dissolved organic carbon • DOC processing • Nutrient availability • Stoichiometry

Chapter 6

6.1 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 $^{14}\text{CO}_2$ reduced following N addition, indicating that nutrient addition induced shifts in internal C partitioning and microbial C use efficiency. 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.

Chapter 6

6.2 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

Chapter 6

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

Chapter 6

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.

6.3 Materials and methods

6.3.1 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. S6.1). It is an oligotrophic system with high mean annual DOC concentrations (>20 mg L⁻¹), low total N concentrations (<0.4 mg N L⁻¹) and ultra-low total P concentrations (<10 µg P L⁻¹) (Yates et al. 2019) and can be either N or P limited

Chapter 6

depending on seasonality (Emmett et al. 2016). Characteristics of the sediments are presented in Table 6.1. After collection, sediment samples were kept on ice in the dark during transportation to the laboratory and analysed within 24 h.

Table 6.1 Characteristics of the sediment samples used in the study. Values represent means \pm SEM, $n = 4$ (from Brailsford et al. 2019).

	Mean sediment characteristic
pH _(H₂O)	4.75 \pm 0.05
Electrical conductivity _(H₂O) (μ S cm ⁻¹)	15 \pm 2
Moisture content (%)	80.3 \pm 3.6
Silt content (%)	5.2 \pm 1.3
Clay content (%)	0.7 \pm 0.3
Sand content (%)	94.1 \pm 1.6
Total C (mg C kg ⁻¹ sediment)	250 \pm 42
Total free carbohydrates (mg C kg ⁻¹ wet sediment)	0.61 \pm 0.08
Total phenols (mg C kg ⁻¹ wet sediment)	7.26 \pm 2.58
Total N (mg N kg ⁻¹ sediment)	8.36 \pm 1.28
NH ₄ ⁺ (mg N kg ⁻¹ wet sediment)	5.1 \pm 1.8
NO ₃ ⁻ (mg N kg ⁻¹ wet sediment)	0.91 \pm 0.26
Total amino acids (mg N kg ⁻¹ wet sediment)	0.20 \pm 0.01
Molybdate-reactive P (mg P kg ⁻¹ wet sediment)	0.21 \pm 0.05
Phospholipid-derived fatty acid (PLFA) analysis	
Total PLFA biomass (nmol g ⁻¹ sediment)	621 \pm 180
Gram- bacteria (%)	47.8 \pm 0.7
Gram+ bacteria (%)	30.1 \pm 1.9
Actinomycetes (%)	8.27 \pm 2.09
Fungi (%)	4.51 \pm 1.23
Eukaryote (%)	6.35 \pm 2.64

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

Chapter 6

6.3.2 ^{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 200 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

Chapter 6

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 $^{14}\text{CO}_2$ 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).

6.3.3 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. S6.2). 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

Chapter 6

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. S6.3). 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).

6.3.4 Untargeted analysis of primary metabolism

Nutrients in the same concentrations described above were added in 200 μL ultra-pure water (18 $\text{M}\Omega$ 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 $-80\text{ }^\circ\text{C}$

Chapter 6

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; Appendix 6).

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 or feature filtering were applied. Metabolic pathway maps were created using KEGG Mapper v3.1 (Kanehisa et al. 2012).

6.4 Results

6.4.1 ^{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. 6.1; Supplementary Table S6.2). 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, $F_{3,12} = 7.496$, $P = 0.004$).

Chapter 6

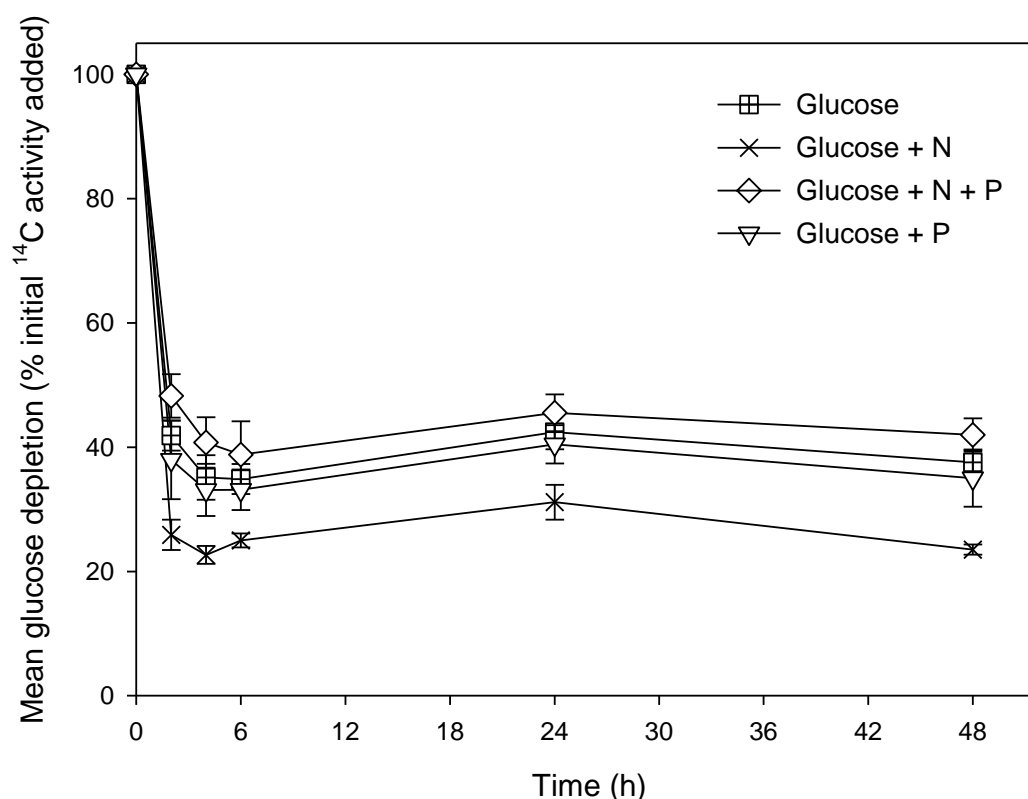


Figure 6.1. ¹⁴C-labeled glucose depletion over time. The ¹⁴C-glucose, in addition to N added as NH₄NO₃ and P added as NaH₂PO₄ was added to an oligotrophic river sediment and depletion measured over time. Values represent means ± SEM, *n* = 4.

A significant interaction between experimental treatment and time was observed for the percentage of ¹⁴CO₂ respiration by the sediment microbial communities (two-way mixed ANOVA, *P* = 0.018; Fig. 6.2; Supplementary Table S6.2). The initial rate of ¹⁴CO₂ respiration was lower for the glucose + N treatment in comparison to all other treatments from 4 to 24 h (one-way ANOVA, *P* ≤ 0.001 in each case; Supplementary Table S6.1; Fig. 6.2). At 24 h, the rate of ¹⁴CO₂ respiration was still lower in the glucose + N treatment in comparison to the glucose and glucose + P treatments, with the glucose + N + P

Chapter 6

treatment falling in between (one-way ANOVA, $F_{3,12} = 5.804$, $P = 0.011$; Fig. 6.2). By the final time-point, 168 h there were no detectable differences between treatments, likely due to the increased variation observed at this time-point (one-way ANOVA, $F_{3,12} = 2.371$, $P = 0.122$; Fig. 6.2).

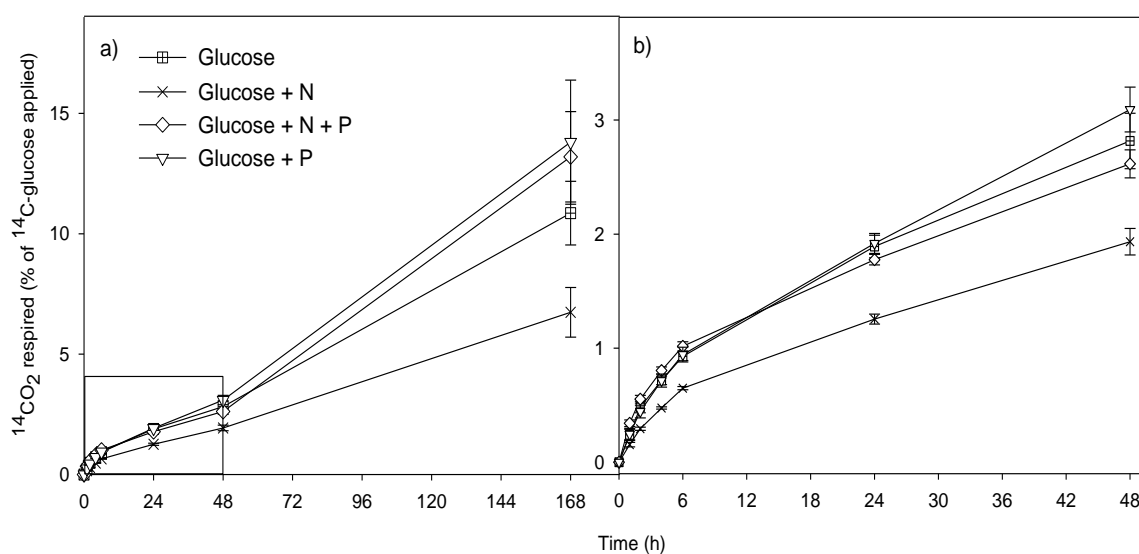


Figure 6.2. Microbial transformation of ^{14}C -glucose to $^{14}\text{CO}_2$ over time. The ^{14}C -glucose, in addition to N added as NH_4NO_3 and P added as NaH_2PO_4 was added to an oligotrophic river sediment and transformation to $^{14}\text{CO}_2$ measured over a) 168 h and b) 48 h. Panel b) is derived from the data shown in panel a). Values represent means \pm SEM ($n = 4$). The legend is the same for both panels.

6.4.2 Non-targeted metabolite analysis by GC-MS

Non-targeted metabolite analysis was conducted on four sediment samples of each nutrient addition treatment after 24 h and the control from the beginning of the

Chapter 6

experiment. To identify the main factors driving change in the metabolome, PLS discriminant analysis (PLS-DA) was conducted with approximately 1040 peaks of identified non-targeted GC-MS metabolites (Fig. 6.3). The first component of the PLS-DA results (63.8 % variance) likely reflects the 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.

Chapter 6

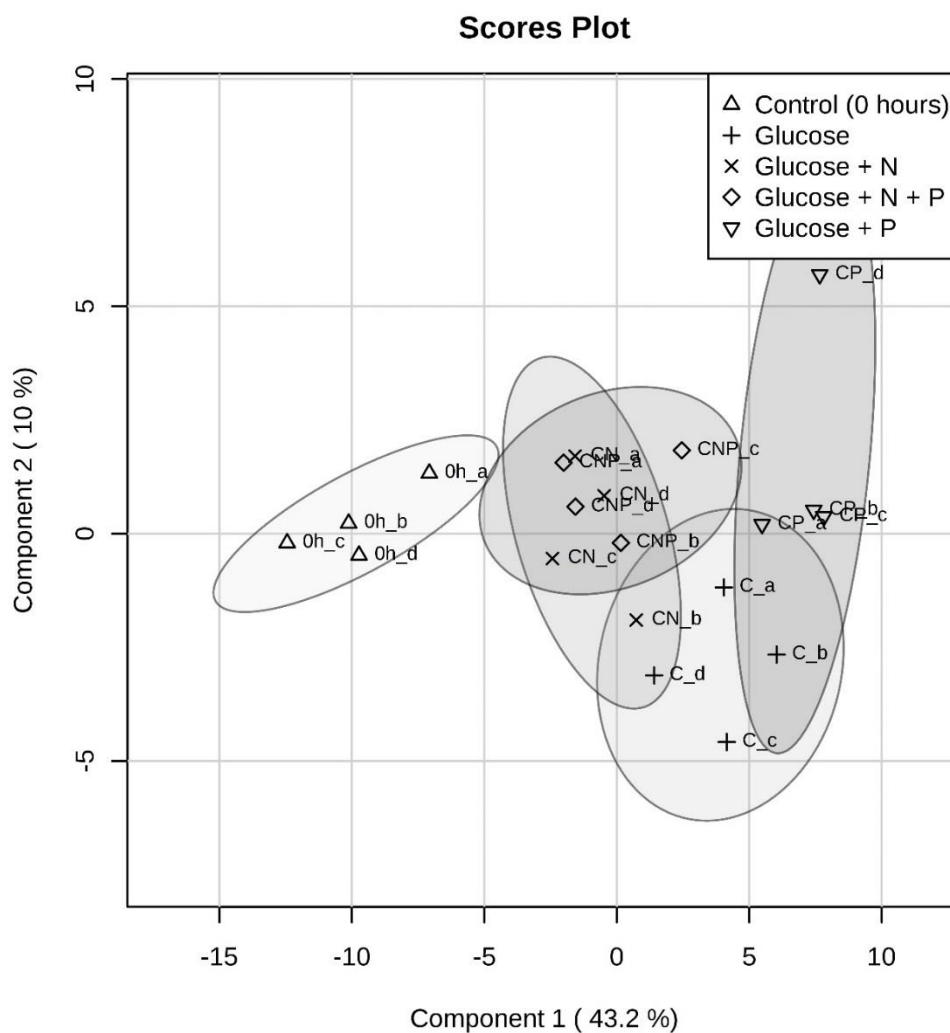


Figure 6.3 PLS-DA (PLS discriminant analysis) scores plot for the metabolome of control samples (+_dH₂O only) at 0 h and all treatments at 24 h after the addition of treatments (+ glucose (C); + glucose and N (CN); + glucose, N and P (CNP) and + glucose and P (CP). Lower case letters represent individual 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. 6.4). The control samples clustered

Chapter 6

separately to all other treatments. The two N-containing treatments were found to overlap with the other treatments for samples from site B.

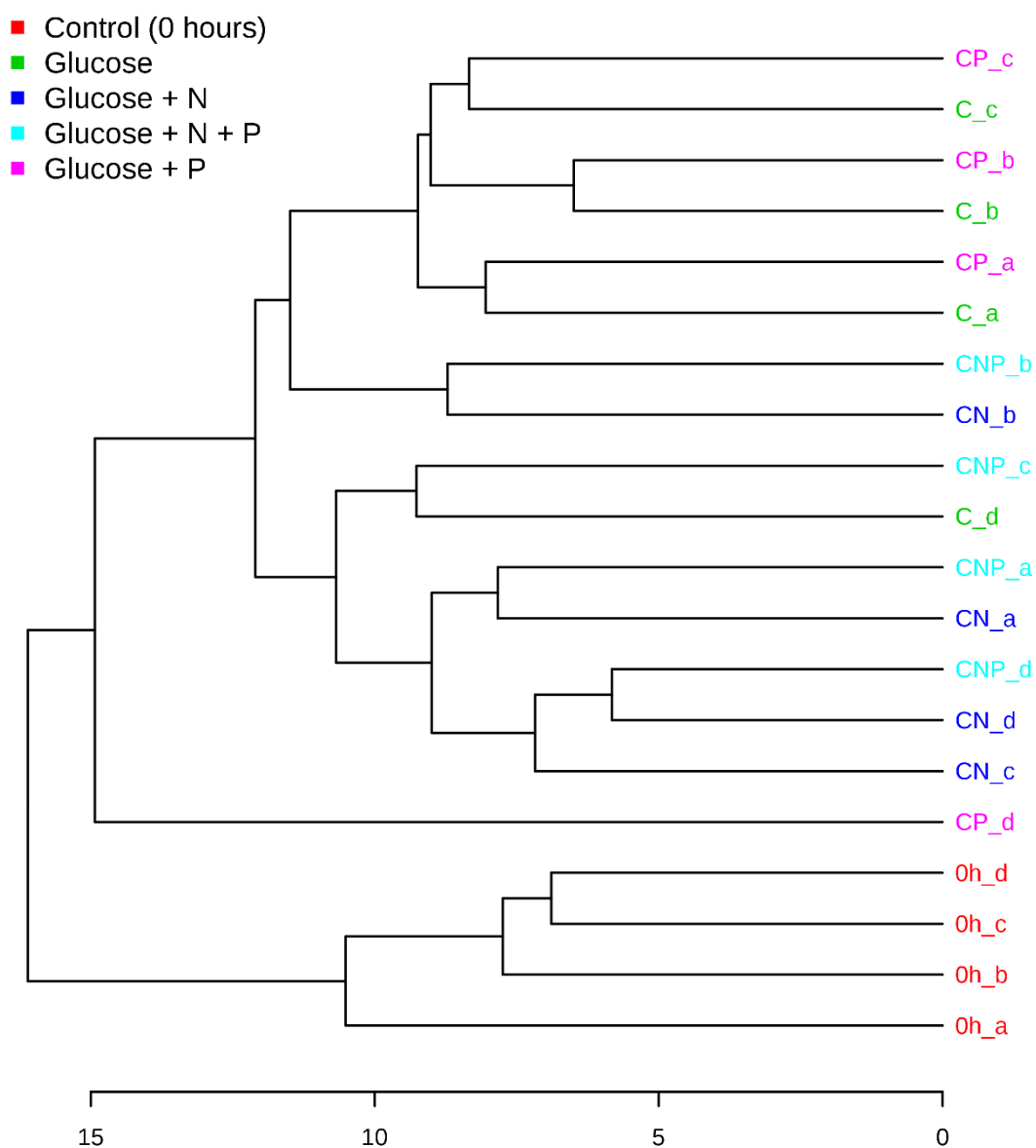


Figure 6.4 Similarity dendrogram clustered by Euclidean distance (horizontal axis) for the metabolome of control samples (+_dH₂O only) at 0 h and all treatments at 24 h after

Chapter 6

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.

6.4.3 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. S6.4). 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. 6.5; Supplementary Table S6.3). 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. 6.5).

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

Chapter 6

amino acid metabolism, could indicate amino acid synthesis (one-way ANOVA, $P < 0.05$;

Fig. 6.5).

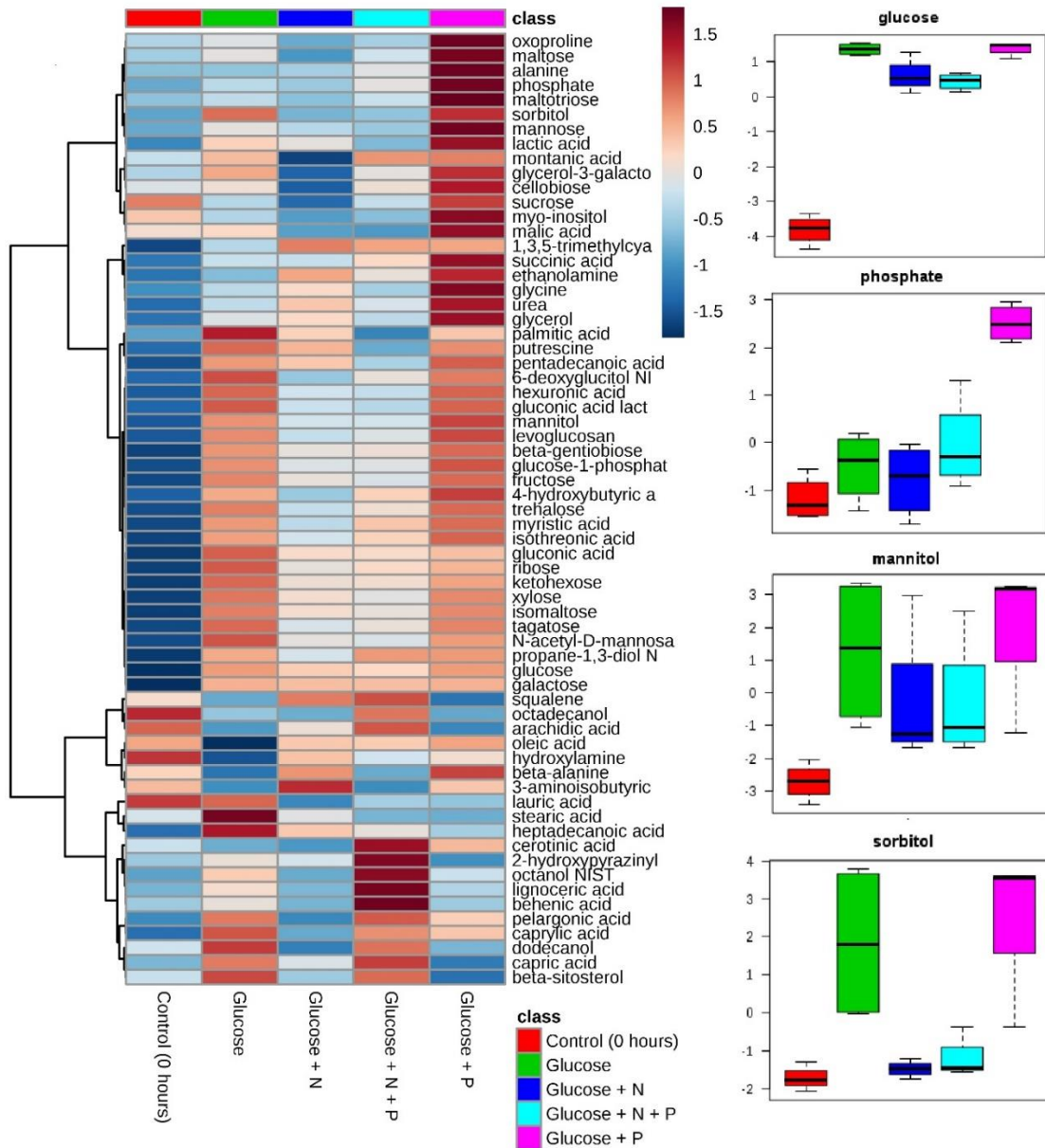


Figure 6.5. Hierarchical clustering heat map of the normalized metabolite log response in sediment primary metabolome for each treatment (0 h (control), 24 h (glucose, glucose + N, glucose + N + P, glucose + P)). Metabolites which significantly decrease are displayed in blue, while metabolites which significantly increased are displayed in red.

Chapter 6

The brightness of each colour corresponds to the magnitude of the difference when compared with average value. Clustering of the metabolites is depicted by the dendrogram at the left. Metabolites are clustered by similarity according to Pearson correlation values. Boxplots of individual metabolites mean \pm 1 S.D.

6.5 Discussion

6.5.1 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. 6.2). 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. 6.5; Wetzl 1992; Findlay and Sinsabaugh 1999). 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 $^{14}\text{CO}_2$ respiration over the duration of the experiment for the upland river sediments was an order of magnitude lower than rates previously observed for

Chapter 6

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 $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ respiration after 168 h, although the addition of glucose + N resulted in the lowest initial rate of $^{14}\text{CO}_2$ 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 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 Dijkstra 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

Chapter 6

glucose in the N addition treatment, overall C mineralisation to CO₂ 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 respire 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.

6.5.2 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. 6.3). 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 ¹⁴C 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

Chapter 6

treatments largely clustered together, as did the glucose + N and glucose + N + P treatments (Fig. 6.4).

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

Phosphate utilisation by the sediment microbiome appeared to be higher in the glucose + N + P treatment in comparison to the glucose + P treatment, with a greater concentration of phosphate remaining in the former treatment (Fig. 6.5). Nitrogen addition to peat bogs has also been observed to enhance P uptake in other studies

Chapter 6

(Williams and Silcock 2001). This increase in P uptake following co-addition of P and N addition also indicates that the system was initially N limited at the time of the study. The glucose-only treatment produced significantly higher concentrations of gluconic acid, in addition to other weak organic acids such as malic acid, compared to the control and other N addition treatments after 48 h (Fig. 6.3). Such compounds have previously been demonstrated to be produced directly from glucose by microbes, in order to encourage P dissolution from mineral surfaces (Stella and Halimi 2015; Chen et al. 2016).

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

Chapter 6

6.5.4 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. S6.5-S6.7; 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).

Chapter 6

6.6 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 CO₂ 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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Chapter 6

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Chapter 7

Rapid microbial consumption of dissolved organic sulphur (DOS) in freshwaters

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A draft of this short communication has been submitted to Biogeochemistry.

FLB and DLJ designed and conceived the experiment, FLB conducted the experimental work, analysed the results and prepared the manuscript. CAY produced the map presented in Fig. S7.1. All authors discussed results and contributed to the preparation of the manuscript.

Chapter 7

7.1 Abstract

Sulphur (S) is a key macronutrient for all organisms, with similar cellular requirements to that of phosphorus (P). As for nitrogen and phosphorus, studies of S cycling have often focused on the inorganic fraction, however, there is strong evidence to suggest that freshwater microbial communities may also access dissolved organic S (DOS) compounds (e.g. S-containing amino acids). The aim of this study was to compare the relative concentration and uptake rates of organic ^{35}S -labelled amino acids (cysteine, methionine) with inorganic S ($\text{Na}_2^{35}\text{SO}_4$) in oligotrophic versus mesotrophic river waters draining from low nutrient input and moderate nutrient input land uses respectively. Our results showed that inorganic SO_4^{2-} was present in the water column at much higher concentrations than free amino acids. In contrast to SO_4^{2-} , however, cysteine and methionine were both rapidly depleted from the mesotrophic and oligotrophic waters with a halving time < 1 hour. Only a small proportion of the DOS taken up by the microbial community was mineralized and excreted as SO_4^{2-} ($< 16\%$ of the total taken up) suggesting that the DOS satisfies a microbial demand for carbon (C) and S. In conclusion, even though inorganic S was abundant in freshwater, the microbial communities retained the capacity to take up and assimilate DOS.

Keywords Dissolved organic matter • DOS processing • Nutrient cycling • Radioisotopes
• Sulphate

Chapter 7

7.2 Introduction

Carbon (C), nitrogen (N) and phosphorus (P) have long been considered to be the key macronutrients regulating primary productivity in freshwater environments. Although S is rarely limiting in freshwaters (Dodds and Whiles 2010), recent stoichiometric studies have found that average cellular sulphur (S) requirements are close to those of P (C:N:P:S 124:16:1:1.3; Ho et al. 2003; Ksionzek et al. 2016). As the C, N, P and S cycles are intrinsically linked through dissolved organic matter (DOM), it is therefore important to consider the cycling of dissolved organic sulphur (DOS). Further, through S-containing peptides, DOS may also play a role in the transport and bioavailability of trace metals (Marie et al. 2015; Ksionzek et al. 2016). Despite the lack of studies on DOS, the concentration of dissolved and particulate forms of organic S has been shown to exceed that present in an inorganic form (i.e. SO_4^{2-}) in some ecosystems (Levine 2016; Ksionzek et al. 2016). While comprehensive studies of the composition of the DOS pool are lacking, some compounds have been frequently identified and are considered to be of importance in overall S cycling (e.g. S-containing amino acids, dimethylsulfoniopropionate, DMSP) (Ginzburg et al. 1998; Sela-Adler et al. 2016).

Of the S-containing amino acids, only methionine and cysteine are biosynthetically incorporated into proteins and these are expected to represent the main forms of DOS entering unpolluted freshwaters (Brosnan and Brosnan 2006). Relative to other amino acids, they are generally present at low concentrations in the cell (ca. 1.5-3.5% of the total amino acid pool; Okayasu et al. 1997). However, they are also precursors for many other cellular metabolites, which can be present at high concentrations. For example, S-adenosyl methionine (SAM) is a co-factor involved in the majority of methyl

Chapter 7

transfer reactions across all organisms, *N*-formyl methionine acts as the translation initiator in prokaryotic protein synthesis and *S*-methylmethionine is a precursor for DMSP (Brosnan and Brosnan 2006; Ferla and Patrick 2014; Sela-Adler et al. 2016). The main role of cysteine is the creation of complex protein structures through the formation of disulphide bonds with other cysteine residues while methionine plays a key role in the initiation of protein translation and often forms part of the hydrophobic core of proteins (Brosnan and Brosnan 2006; Ferla and Patrick 2014). Cysteine may also be utilised to synthesize methionine in prokaryotes, a process that is reversed in eukaryotic cells (Cooper 1983; Ferla and Patrick 2014; Qiao et al. 2018).

Total DOS and particulate organic S (POS) concentrations in freshwater are rarely reported due to challenges in their measurement, however, measurements of total thiol concentrations (R-SH group; 10-160 nM) indicate their potential importance in long-distance S transport (Marie et al. 2015). In contrast, measurements of individual free S-containing amino acids in the water column indicate that they are only present at extremely low concentrations (0.2-5.0 nM; Horňák et al. 2016), despite their presence in cells at much higher concentrations (Li et al. 2017). This suggests that they may be rapidly cycled within freshwaters. We have not identified any previous work investigating the direct uptake of DOS compounds by aquatic microbial communities or how this compares to inorganic sulphate uptake. The aims of this study were therefore to use ³⁵S-labelled isotopic tracers to: 1) quantify rates of DOS uptake by the aquatic microbial biomass; 2) compare the rates of uptake of DOS compounds (S-containing amino acids) versus inorganic S (SO₄²⁻) in river waters, and 3) identify differences in S uptake in two contrasting land cover types.

Chapter 7

7.3 Materials and methods

7.3.1 Field site

Samples were collected in the spring of 2018, from three independent streams draining each of two contrasting land cover types within the Conwy catchment, Wales, UK (Supplementary Fig. S7.1; Emmett et al. 2016). Three replicate 1 L mid-stream samples were collected manually from each site in high-density polyethylene (HDPE) bottles. Samples were kept on ice in the dark during transportation to the laboratory and experiments commenced within 6 hours. The first set of samples were collected from mesotrophic streams passing through lowland improved grasslands, subject to moderate livestock grazing and fertiliser applications. The second set were collected from three independent oligotrophic headwater streams draining an upland blanket peat bog dominated by acid heathland vegetation, with low intensity sheep grazing and no history of fertiliser application. General water and sediment characteristics of these sites have been previously described (Supplementary Table S7.1; Brailsford et al. 2019; Yates et al. 2019).

7.3.2 Chemical characteristics

Thiol concentrations in river water samples were measured using a thiols fluorescent detection kit (Lot: 18S037A; Invitrogen Inc., Carlsbad, CA). Total Reflection X-ray Fluorescence (TXRF) elemental analysis of underlying river sediments was measured on dried (40 °C), sieved (< 125 µm) sediment using a Bruker S2 Picofox TXRF spectrometer (Bruker Inc., MA, USA). The quantification of sulphate was conducted on river water

Chapter 7

samples by ion chromatography using a 930 Compact IC Flex (Metrohm, Herisau, Switzerland).

7.3.3 ³⁵S-labelled nutrient depletion assays

Within 6 hours of collection, aliquots of unfiltered river water (25 mL) were placed in sterile 50 mL polypropylene centrifuge tubes (Corning Inc., Corning, NY). Each sample was spiked with 200 µL of ultrapure water (18 MΩ resistance) containing ³⁵S labelled methionine, cysteine or sodium sulphate (Na₂³⁵SO₄) to give a final activity 0.2 kBq mL⁻¹. Compounds were chosen to reflect possible organic (methionine, cysteine) or inorganic (Na₂³⁵SO₄) S compounds typically released during the breakdown of particulate organic matter entering soils and freshwaters. We assumed that the amount of ³⁵S-isotope added to the water (<0.1 nM) did not change the intrinsic concentration of the pool being measured. Sterile controls run with ultrapure water in place of river water resulted in no loss of ³⁵S-compounds from solution.

After sealing with sterile caps, the samples were subsequently incubated on a shaker (200 rev min⁻¹) in the dark at 10 °C for the duration of the experiment. This temperature represents the mean annual temperature within the Conwy catchment (Brailsford et al. 2019). After incubation for 1, 2, 4, 6, 24, 48, 72, 144 h, two 1.0 mL aliquots were removed from the tubes and centrifuged to remove microbial cells (20,817 g, 3 min). 0.5 mL of the supernatant was either: 1) placed directly into a scintillation vial for ³⁵S quantification, or 2) added to 0.5 mL BaCl₂ (0.5 M) and centrifuged again (20,817 g, 3 min) to precipitate and remove any inorganic sulphate present in the methionine and cysteine treatments (i.e. S mineralized from DOS and present in the external media). The

Chapter 7

subsamples were then mixed with Optiphase HiSafe 3 scintillation cocktail (4 mL; PerkinElmer Inc., Waltham, MA) and the ^{35}S quantified on a Wallac 1404 liquid scintillation counter with automated quench correction (Wallac EG&G, Milton Keynes, UK).

7.3.4 Data analysis

Data analysis was carried out in SPSS v22 (IBM UK Ltd., Portsmouth, UK). DOS values were corrected for any cleaved sulphate groups remaining in solution and two-way mixed (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 ANOVA was used to detect differences between treatments at individual time-points with a significance level set at $P < 0.05$. Graphs and curve-fitting data were produced using Sigmaplot v13.0 (Systat Software Inc., San Jose, CA). To determine the halving time ($t_{1/2}$) of each S compound in river water, single first order exponential decay curves were fitted to the data according to:

$$s = y_0 + (a \times \exp^{-kt}) \quad (1)$$

Where s is the ^{35}S remaining in solution, y_0 represents an asymptote, k is the exponential coefficient describing ^{35}S depletion by the aquatic microbial biomass, a is the sulphur pool size and t is time (h) (Hill et al. 2008). The halving time ($t_{1/2}$) of each S form can then be calculated as:

$$t_{1/2} = \ln(2)/k \quad (2)$$

Chapter 7

7.4 Results and discussion

7.4.1 Sulphur composition in two contrasting land-cover types

The characterisation of DOS in riverine ecosystems is historically underreported (Marie et al. 2015). Here, thiol concentrations were much lower than reported in previous studies, being 0.4 nM for mesotrophic rivers and below the limit of detection (<0.05 nM) for the upland oligotrophic rivers. The former is two orders of magnitude lower than values presented in other studies of freshwaters (Superville et al. 2013; Marie et al. 2015; Supplementary Tables S7.1-7.2). Sulphate concentrations were similar across the mesotrophic and oligotrophic rivers (3438 nM; one-way ANOVA, $F_{1,4} = 0.161$, $P = 0.709$). Although we did not identify individual amino acids, the total concentration of free amino acids was similar between the two river types (7 μM).

7.4.2 ^{35}S -labelled depletion

To our knowledge, no other study has compared the rates of microbial uptake of DOS compounds (cysteine, methionine) versus inorganic S ($\text{Na}_2^{35}\text{SO}_4$) in aquatic systems. Here, the addition of trace amounts of S-containing amino acids resulted in a rapid depletion for both mesotrophic and oligotrophic waters, whereas $\text{Na}_2^{35}\text{SO}_4$ was not readily depleted from solution in either treatment (Fig. 7.1). However, the higher background inorganic S concentrations present in both mesotrophic and oligotrophic waters will have diluted the isotope pool, reducing the uptake of $\text{Na}_2^{35}\text{SO}_4$ from solution. For both mesotrophic and oligotrophic waters, the compound added had a significant effect on the rate of ^{35}S uptake over time (two-way mixed ANOVA, $F_{1,7} = 917$, $P < 0.0001$ and $F_{1,7} = 543$, $P < 0.0001$ respectively). Overall, very little of the ^{35}S -label present in

Chapter 7

the amino acid was released into solution as $^{35}\text{SO}_4^{2-}$ (15.2 ± 3.7 % of the total added), indicating that the majority of the ^{35}S -labelled amino acids were taken up intact by the microbial community. Similarly, a study of S availability to plants in paddy field soils demonstrated higher rates of uptake from ^{35}S incorporated into the organic components of rice straw compared to S addition as $\text{Na}_2^{35}\text{SO}_4$ (Zhou et al. 2006).

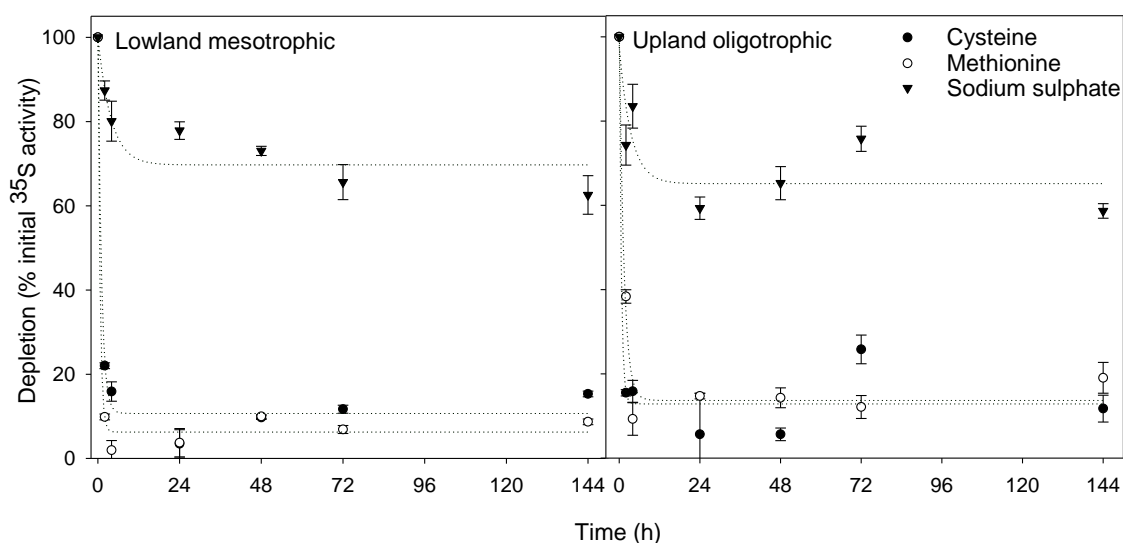


Figure 7.1. Depletion of either ^{35}S -labeled DOS compounds (cysteine, methionine) or inorganic sulphur ($\text{Na}_2^{35}\text{SO}_4$) after addition to river water. DOS values are corrected for any cleaved sulphate groups remaining in solution. Values represents means \pm SEM ($n = 3$).

In lowland mesotrophic waters, the calculated $t_{1/2}$ of methionine was quicker than for cysteine, whereas the reverse was true for upland oligotrophic waters (Table 7.1). This was reflected in an increase in $t_{1/2}$ for methionine and a decrease in $t_{1/2}$ for cysteine from lowland to upland waters. However, whilst land-cover was found to significantly affect ^{35}S -methionine depletion overall, no significant effect of land-cover was identified for cysteine (two-way mixed ANOVA, $F_{1,7} = 121$, $P < 0.0001$ and $F_{1,7} = 2$, $P = 0.193$

Chapter 7

respectively). In addition, the increase in instantaneous methionine uptake (after 2 h) between lowland and upland waters was statistically significant whilst the increase in cysteine uptake was not (one-way ANOVA, $F_{1,4} = 73$, $P = 0.001$; one-way ANOVA, $F_{1,4} = 0.2$, $P = 0.905$ respectively). In all treatments the $t_{1/2}$ for the DOS compounds was < 1 h. A rapid initial loss of inorganic S ($\text{Na}_2^{35}\text{SO}_4$) from solution was observed, with more depletion observed in upland mesotrophic rivers compared to lowland mesotrophic rivers (28.0 ± 3.1 % versus 12.6 ± 3.9 %; one-way ANOVA, $F_{1,4} = 16$, $P = 0.016$). However, depletion did not fall below 50 % in mesotrophic or oligotrophic waters, therefore the $t_{1/2}$ value could not be calculated (Table 7.1). We attribute this to the limitation of the microbial biomass by another nutrient, such as carbon.

Table 7.1 Model parameters describing the size and turnover of ^{35}S over time for lowland mesotrophic and upland oligotrophic systems. The models are described by a single first order exponential decay equation fitted with an asymptote. Values represents means SEM ($n = 3$)

	Compound	y_0	a	k	$t_{1/2}$ (h)	r^2
Lowland mesotrophic	Cysteine	10.7	89.3	0.99	0.70	0.99
	Methionine	6.3	93.7	1.68	0.41	0.99
	Inorganic S	69.7	30.2	0.3	-	0.85
Upland oligotrophic	Cysteine	12.9	87.1	1.72	0.40	0.96
	Methionine	13.7	86.8	0.73	0.96	0.98
	Inorganic S	65.2	32.3	0.29	-	0.74

Equation of model: $s = y_0 + a \exp(-kt)$

Chapter 7

It has generally been considered that inorganic S forms, in addition to cysteine, are the preferred source of S for the microbial biomass, although fungal species may also use methionine as a preferred source (Kertesz 2000). Previous community analysis of the rivers in the current study found that < 5 % of phospholipid-derived fatty acids (PLFAs) originated from a fungal source, suggesting that the rapid cysteine uptake cannot be ascribed to high proportions of fungal cells within the microbial community (Brailsford et al. 2019). There is literature to support the presence of sulphate starvation induced (SSI) proteins, which are synthesised by a range of cultures of microorganisms when preferred sources of S are not available (Stipanuk 1986; Kertez 1993; Scott et al. 2007). These enzymes allow other sources of S to be utilised, altering S cycling in sulphate-limited conditions, however, the SO_4^{2-} trigger concentration at which their expression occurs in aquatic environments remains unknown.

The current study measured the biological uptake of methionine, cysteine and $\text{Na}_2^{35}\text{SO}_4$ by the aquatic microbial biomass in isolation. Further studies measuring the uptake kinetics of ^{35}S -labelled amino acids in river waters may advance our understanding of the capacity for uptake of S-containing amino acids by the aquatic microbial biomass. A previous study of amino acid metabolism of *Streptococcus thermophilus* in batch fermentations, combining metabolomic and transcriptomic approaches, observed increases in enzymes associated with both cysteine and methionine uptake, despite both amino acids being added to the nutrient broth. This suggests that the rate of uptake from solution may not meet microbial demand for S-containing amino acids (Lahtvee et al. 2011; Qiao et al. 2018). The combination of radiolabelled tracers in conjunction with

Chapter 7

omics approaches could provide additional insights of the pathways involved in S uptake and metabolism.

7.5 Conclusions

In summary, we found that the S-containing amino acids cysteine and methionine were rapidly removed from solution by the aquatic microbial biomass, whereas inorganic sulphur ($\text{Na}_2^{35}\text{SO}_4$) uptake was limited. Halving times for the compounds in solution were < 1 h in both mesotrophic and oligotrophic rivers. This finding goes against the consensus that sulphate is the preferred source of S for most microorganisms (Kertesz 2000). We also hypothesise that it is more energetically efficient to take up free amino acids and directly incorporate them in proteins in comparison to the uptake of SO_4^{2-} and *de novo* amino acid biosynthesis. This is especially the case for S-containing amino acids which are energetically costly to produce in comparison to other amino acids (Akashi and Gojobori 2002). As the DOS compounds studied here represent a tri-nutrient source (C, N and S) these low molecular weight forms of S also negate the need to take up as much inorganic N. Further work is required to investigate the microbial uptake of a wider range of DOS compounds and also to quantify their rates of production. This will enable a quantitative assessment of the overall role of DOS in freshwater S cycling.

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Chapter 7

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Chapter 7

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Chapter 8

Discussion

Chapter 8

8.1 Introduction

In this section, the results of the experimental work (Chapters 3-7) are summarised and then discussed in relation to the overall initial objectives of the thesis alongside the broader implications of the findings. More detailed discussions of the results are presented in the experimental chapters. An outline of the main strengths, and potential limitations, of this thesis are presented, followed by a summary of research questions identified for future studies.

8.2 Synthesis of findings

The broad aims of this thesis were to i) gain further insight into DOM processing in rivers across a range of spatial gradients (e.g. land-cover, inorganic/organic nutrient pool size); ii) to compare DOM processing to inorganic nutrient processing; and iii) to identify how DOM metabolism changes under different nutrient conditions. The results of the experimental chapters, in relation to thesis objectives derived from these aims, are laid out in the following sections.

The literature review highlighted that DOM is a broad, heterogenous mixture, therefore the differences in turnover and residence times between DOM components needed to be addressed (Akkanen et al. 2012; Jones et al. 2016). All of the experimental chapters within this thesis focused on tracing the processing of compounds present in the labile LMW DOM fraction using radiolabelled tracers (^{14}C , ^{33}P , ^{35}S). Chapters 3, 4, 5 and 7 directly compared the processing of different DOM components, investigating the persistence of DOM compounds in river waters and sediments, uptake kinetics and

Chapter 8

changes in response to DOM addition in the presence of different intrinsic pool sizes. A different approach was used in Chapter 6, which focused on changes in metabolism for a single DOM compound under different levels of inorganic nutrient enrichment, using untargeted primary metabolomics.

The main focus of this thesis was DOM processing in the water column, however in terms of the biogeochemical processes affecting DOM processing, the hyporheic zone, including river sediments, is the most understudied component. This is due to difficulties in measuring these processes in-stream and being able to design a realistic laboratory study. In addition to the study of DOM processing in river waters in Chapters 3, 4, 5 and 7, Chapters 5 and 6 addressed how DOM processing in sediments compared in pristine and enriched river systems. Chapter 5 compared the uptake kinetics of LMW DOM in both river waters and sediments from oligotrophic and mesotrophic rivers with contrasting background nutrient concentrations, whilst Chapter 6 focused on DOM metabolism in sediments from oligotrophic rivers only, with artificially altered levels of inorganic nutrient enrichment.

8.2.1 Determine how physiochemical changes across catchments influence DOM uptake in rivers

Two approaches were taken in order to study the influence of physicochemical parameters on labile DOM processing in river systems. Firstly, Chapters, 3, 5 and 7 compared DOM uptake using river waters and/or sediments from two contrasting sub-catchments: blanket peat bog-influenced oligotrophic waters (the Migneint) and improved grassland-

Chapter 8

influenced mesotrophic waters (the Hiraethlyn). A greater overall DOM depletion from solution was observed in waters and sediments from mesotrophic rivers in comparison to oligotrophic rivers, which was in accordance with previous studies, where higher rates of DOM uptake were detected in the presence of higher background nutrient concentrations (Peterson et al. 1993; Tank et al. 2010; Mutschlechner et al. 2018). In general, initial DOM depletion rates were also higher for mesotrophic rivers, although in Chapter 5 a lag phase was observed in some mesotrophic sediments following addition of DOC compounds. This was attributed to either: i) a period of microbial growth facilitated by a lack of nutrient limitation (Carlson et al. 1996; Lleo et al. 2005; Creamer et al. 2014); or ii) a temporary halting of growth or reduction in microbial biomass due sediment mixing with waters containing a lower DOC concentration than overlying waters (Bott et al. 1984).

The second approach taken was to cover a broader scale of physiochemical gradients by conducting experiments with samples across 45 sites, spanning 5 dominant cover use classifications (Chapter 4). Initially, this experiment was conducted as a quarterly seasonal campaign, following the observed increase in DOM uptake by the aquatic microbial biomass with increasing incubation temperatures in Chapter 3. However, preliminary data analysis appeared to show that the underlying physicochemical differences between sites from each sub-catchment were the key drivers of differences observed in DOC, DON and DOP depletion, as observed in previous studies of the sites used in this experiment (Chapter 3; Yates et al. 2019). Subsequent analysis was undertaken using the four quarterly experiments as replicates. Cluster analysis revealed distinct clustering of low inorganic enrichment, high DOC sites, whilst

Chapter 8

the clustering of agricultural and mixed land cover sites was more disparate, demonstrating their higher variability in physiochemical parameters. Across all the sites, there were overall differences in processing rates for the different DOM components ($\text{DON} \geq \text{DOC} > \text{DOP}$). However, a common trend was observed whereby the turnover of all three positively correlated with increasing inorganic N and P concentrations. This was reflected in the higher depletion rates observed for mesotrophic rivers in experiments focusing on the two contrasting catchments. DOC biodegradation also correlated positively with increasing pH, which tends to increase down the freshwater : marine continuum. This was supported by the comparison of contrasting sub-catchments in Chapters 3 and 5, in addition to other studies, where DOC biodegradation was greater in nutrient-enriched rivers (Peterson et al. 1993; Tank et al. 2010; Mutschlechner et al. 2018). Broadly, these experiments demonstrated that the presence higher background organic/inorganic nutrient pools usually correlates with an increased uptake of DOM compounds.

8.2.2 Explore the interaction between the pool size and uptake rate of DOM in rivers

The influence of intrinsic nutrient pool size and dominant terrestrial land cover on the depletion of a range of DOM compounds in river waters was investigated in Chapters 3, 4 and 7. These experiments were conducted using low concentrations ($< 1 \text{ nM}$) of radioisotope, in order to avoid producing a priming effect and therefore allowing the natural differences in pool size to be compared. However, in order to fully explore the effect of pool size on DOM uptake, in Chapter 5 a number of LMW DOC compound groups (sugars, amino acids, organic acids and phenolics) at a wide range of

Chapter 8

concentrations (nM to mM final concentration) were added to river waters and sediments from two contrasting sub-catchments. This allowed the determination of: i) nutrient loading capacities and ii) uptake kinetics parameters (K_m , V_{max}).

The differences in processing rates (amino acids > sugars = organic acids > phenolics versus) reflected the results of Chapter 4, where DON compounds were processed at a quicker rate than DOC compounds. This was observed in the kinetics parameters calculated, where the V_{max} for amino acids was an order of magnitude higher than that of glucose. Overall, river sediments were observed to have a higher capacity for labile DOC uptake compared to the overlying river waters. For example, the microbial biomass was capable of removing $\geq 20\%$ of high amounts of amino acids and organic acids (10 mM) after 7 days, whilst for river waters a saturation in uptake appeared at much lower concentrations (0.5 mM), particularly for oligotrophic waters. This was in part attributed to the higher levels of PLFA biomarkers in sediments compared to waters, which indicates a larger microbial biomass. This was supported by a proportional increase in glucose depletion, commonly used as a proxy for microbial activity (Peters et al. 1989; Tank et al. 2010) and the presence of a lag time between DOC addition and subsequent depletion, particularly for mesotrophic rivers, which was attributed to microbial growth and therefore increased microbial activity.

The effect of inorganic nutrient pool sizes on LMW DOC processing was investigated in Chapter 6. In this case, only one DOC compound (glucose) was used to test whether changes to N and/or P concentrations influenced DOC uptake and metabolism in river sediments from an oligotrophic sub-catchment. Glucose depletion was rapid in all treatments with ca. 60-80 % loss from sediment porewater within 48 h,

Chapter 8

however the greatest uptake was observed when sediments were enriched with N. This treatment saw the least amount of initial mineralisation of C to $^{14}\text{CO}_2$, although there were no statistically significant differences between nutrient treatments by the end of the experiment. This indicated that N enrichment altered the partitioning of C within the microbial biomass, with more C being preferentially used for storage or growth rather than catabolism.

When the results of these two studies are considered together, they indicate that the higher microbial biomass and inorganic nutrient enrichment in river sediments enables the rapid processing of high quantities of LMW DOC and increased microbial activity, which particularly in N and P-rich environments could have consequences for water quality during high C enrichment events.

8.2.3 Compare the relative importance of inorganic and organic nutrients to river systems

The use of radioisotope tracers in the experimental chapters of this thesis have demonstrated that the aquatic microbial biomass can readily remove DOC, DON, DOP and DOS from solution at a range of concentrations. For aquatic systems in particular, we have not found any studies which have directly compared the microbial uptake of inorganic and organic compounds. In order to address this, ^{33}P and ^{35}S -labelled radioisotope tracers were used to compare the uptake of inorganic versus organic nutrients in river waters, in Chapters 4 and 7 respectively.

Chapter 8

In addition to the analysis of catchment-scale trends in DOM component processing, Chapter 4 also compared DOP and inorganic P (P_i) processing across the same sites. Whilst trends in DOP uptake generally followed those observed for other DOM fractions (e.g. increased depletion with increasing nutrient enrichment), P_i uptake tended to correlate negatively with N/P enrichment. This indicates that there is no additional P_i demand under nutrient-enriched conditions, when systems become C limited, which has previously been observed in soils (de Sosa et al. 2018). This can be attributed to due to the lower cellular requirements for P compared to C and N (60:7:1; Cleveland and Liptzin 2007). Conversely, when P is not a limiting nutrient, DOP may be taken up by the microbial biomass for its C content (Jarvie et al. 2018).

Similar findings were observed for DOS uptake versus inorganic sulphur uptake in Chapter 7. Rapid uptake of DOS (S-containing amino acids) was observed in river waters from both mesotrophic and oligotrophic sites, whereas little inorganic S was depleted from solution. The intrinsic pool of inorganic S was an order of magnitude higher than the total free amino acids present, which could indicate that the demand for inorganic S was lower, in a manner similar to that observed for P_i in Chapter 4. In this case, the S-containing amino acids may have been taken up by the microbial biomass in order to meet other cellular nutrient demands (e.g. C, N), as suggested for DOP uptake in inorganic-enriched systems. However, the utilisation of a radioisotope tracer labelled with ^{35}S rather than ^{14}C demonstrated that the S entered the microbial biomass and therefore was not being cleaved from the C-N skeleton prior to the molecule being taken up or being excreted from the biomass following uptake. In addition, it is more energetically costly for microorganisms to synthesise S-containing amino acids than to take them up directly

Chapter 8

from solution (Akashi and Gojobori 2002). In summary, despite being relatively understudied, both DOP and DOS can be taken up by the microbial biomass, however further work is required to determine how the compounds are utilised following uptake. Whilst the S component of DOS was demonstrated to be retained, further work is required to demonstrate this for the P component of DOP.

8.2.4 Investigate how the metabolism of LMW DOC changes with nutrient limitation

Radiolabelled tracer experiments have been used in this thesis to measure the processing of different DOM compounds under natural conditions, across wide physiochemical gradients (Chapter 4) and in contrasting river sub-catchments (Chapters 3, 5 and 7). In Chapter 6, a single DOC compound (glucose) was used to investigate how the metabolism of DOC changes under altered states of nutrient limitation. Sediments from nutrient-poor rivers, primed with inorganic N and/or P at stoichiometric ratios for the microbial biomass, were spiked with either: i) radiolabelled glucose, to monitor its uptake and metabolism; or ii) non-labelled glucose, to allow the primary metabolome of the sediments to be analysed.

Whilst the glucose + N treatment demonstrated the quickest depletion from solution it also led to the lowest initial rate of $^{14}\text{CO}_2$ respiration, indicating that there had been a shift in glucose utilisation between treatments. When a cluster analysis of the metabolomes was performed, overlaps were identified between the glucose + N and glucose + N + P treatments and the glucose and glucose + P treatments respectively. Although all treatments showed elevated glucose in their primary metabolome, more

Chapter 8

glucose had been depleted from the sediment porewater in treatments with N addition, indicating that the river system was N limited at the time of the experiment. This was confirmed by the elevated P uptake when P was added alongside N and the synthesis of sugar alcohols in treatments with no N addition, which have previously been suggested to act as cell storage compounds (Yu et al. 2016). Conversely, amino acid synthesis appeared to increase in treatments with P addition, which could indicate that this process is P-limited, potentially due to the number of P-containing enzymes required for this process. These findings provide an insight into the molecular mechanisms of nutrient limitation in low-nutrient status waters and highlight the need to further explore -omics approaches for understanding biogeochemical processes. Future work should explore changes in metabolic response depending on which nutrient is limiting to the system at the time of sampling.

8.3 Strengths and limitations

The experimental chapters in this thesis combined two sampling regimes: i) sampling many sites across multiple catchments, encompassing gradients in physicochemical properties (Chapter 4); or ii) sampling rivers within two contrasting sub-catchments that are part of the same larger catchment (Chapters 3, 5, 6 and 7). In Chapter 3, where samples were being manipulated through both incubation at a range of temperatures and the use of preservation techniques such as filtration and acidification, three independent replicate samples were taken from one river within each sub-catchment. However, in the experimental chapters where the aim was to capture the natural variation and keep conditions closer to natural conditions (Chapters 5, 6 and 7, samples unfiltered, kept at

Chapter 8

10 °C, shaken to keep aerated), three replicate samples were taken from each of three independent rivers within the sub-catchments. In future studies, the collection of samples from independent rivers, in addition to replicates from each river, is recommended to capture natural environmental variation.

The methodological findings of Chapter 3 of this thesis demonstrate that maintaining the integrity of river water samples is challenging, particularly if samples need to be transported long distances. While filtration, acidification and freezing may all act to preserve samples, the use of these methods is dependent on the type of analysis to be performed. Filtration is not feasible if the aim is to investigate the activity of the microbial biomass, while both acidification and freezing may depolymerise larger DOM compounds in long-term storage (Kaplan 1994; Peacock et al. 2015). The simplest method of maintaining sample integrity in the short term (< 24 h) is to keep samples in the dark at ≤ 5 °C, through the use of refrigerated coolers or keeping samples on ice. All experiments conducted in this thesis were commenced within ≤ 24 h of sample collection, in order to minimise sample degradation.

The experiments in this thesis combined existing mesocosm techniques to further the understanding of LMW DOM processing in aquatic environments. In Chapter 5, radiolabelled compounds were added as compound groups (e.g. amino acids, organic acids, sugars, phenolics), in order to ensure that the DOC added was more representative of potential inputs to aquatic systems, to provide a broad result for that DOC group. However, as all the radioisotope tracers utilised in this study were ^{14}C -labelled, it was not possible to distinguish between individual compounds. Evidence from Chapter 7 demonstrated that differences could be identified between the uptake rates of two S-

Chapter 8

containing amino acids, therefore future studies could combine the grouped and individual tracer approaches, in order to assess the individual variation between compounds in specific groups.

A similar limitation was found in the measurement of DON and DOP uptake in Chapters 3 and 4, where ^{14}C -labelled tracers were used to measure DON and DOP depletion. In both cases, this allowed for the monitoring of the carbon skeleton of the compound but did not indicate whether the N/P itself is being taken up into the microbial biomass or cleaved prior to uptake of the molecule or excreted as a waste product following uptake. Radioisotope tracers are available for DOP compounds, however only stable isotopes are available for DON compounds, which do not allow for similar volumes of samples to be run due to the comparative cost and specialist equipment required. For this reason, it was also not possible to compare the rates of DON versus inorganic N uptake in this study. However, the use of dual-isotopic labels could allow for further study of DOS and DOP processing in aquatic systems. In chapter 7, ^{35}S -labelled compounds were used to directly trace S uptake, but it would be possible to study multiple compounds in the same samples if ^{14}C -labelled S-containing amino acids were also utilised.

In Chapter 6, primary metabolomics was used in conjunction with radioisotope tracers to further the understanding of LMW DOC processing on a molecular level. While this work has provided new insights into how changes in nutrient limitation affect DOC metabolism, currently such techniques are cost prohibitive, therefore sample numbers are limited. To counteract this, the replicates used in this study were taken from four independent rivers within the same sub-catchment, in order to capture more of the variation observed. Environmental metabolomics is currently an emerging field; it is

Chapter 8

anticipated that these types of analysis will continue to decrease in cost as the instrumentation becomes more accessible. Future work should therefore build on the work done here, to increase our understanding of the molecular mechanisms behind DOM processing in aquatic environments.

8.4 Future work

The experimental work presented in this thesis has provided new insights into LMW DOM processing in aquatic environments, under a broad range of conditions. However, several research gaps have been identified in the synthesis of this work, which could be addressed in more detail. Several research questions are outlined below, which have been divided into those which are answerable short-term and those which address more long-term research needs.

8.4.1 Short-term research questions

The following research questions could follow on directly from the research conducted as part of this thesis, using similar techniques to address some of the limitations identified:

1. Broadly, the experimental work presented in this thesis has demonstrated that the presence of higher background N/P pools usually correlates with an increased uptake of DOM compounds. These experiments, however, were primarily conducted in peat bog-influenced oligotrophic rivers and improved grassland-influenced mesotrophic rivers from a single catchment (although the work in Chapter 4 captured the response of a wider range of nutrient status rivers). In order demonstrate that the results of this thesis apply

Chapter 8

to rivers with a wider range of physicochemical gradients, it will be necessary to apply these techniques to river waters from multiple river catchments, preferably over a range of latitudes.

2. Tracers encompassing both individual compounds and compound groups were used to explore DOM uptake in the experimental chapters of this thesis, however another approach for future research would be to combine these two approaches. Chapter 7 found that individual S-containing amino acids were utilised differently by the microbial biomass, therefore exploring the response to addition of the different amino acids, organic acids and phenolic compounds used in Chapter 5 individually may provide further insight into the mechanisms of DOM cycling in the aquatic environment.

3. Further to the previous point, the exploration of DOS cycling in Chapter 7 considered DOS compounds individually. Although there were differences in the uptake kinetics of the compounds used, dual labelling of the system using ^{14}C - ^{35}S would allow multiple substrate to be added concurrently, so that preferences for individual compounds could be confirmed. A kinetics approach, similar to that used in Chapter 5, could also be adopted to study the capacity of river systems to utilise DOS compounds, as the current study used trace additions ($< 1 \text{ nM}$) only.

4. One aim of this thesis was to demonstrate that organic forms of N, P and S were taken up by the microbial biomass. Whilst the uptake of DOS by the aquatic microbial biomass was confirmed using ^{35}S radioisotope tracers, the uptake of N and P as DOC-N DOC-P could not be fully confirmed due to the limitations of using ^{14}C -labelled tracers. Future work should utilise ^{33}P -labelled DOP compounds in order to follow the fate of P in the system. In addition, the use of multi-labelled stable isotope compounds (i.e. a combination of ^{13}C , ^{15}N , ^{18}O , ^{34}S , ^{41}K) could be used to study the fate of N and other

Chapter 8

elemental components of DOM compounds in river waters and sediments. This could also be expanded to include micronutrients held in DOM (e.g. $^{56}\text{Fe}/^{57}\text{Fe}$, $^{66}\text{Zn}/^{68}\text{Zn}$, $^{63}\text{Cu}/^{65}\text{Cu}$).

5. The experimental chapters of this thesis focused on the use of radiolabelled LMW DOM compounds. The compounds used are expected to be present in the labile LMW fraction of DOM and have been demonstrated to be taken up rapidly by the aquatic microbial biomass in this thesis. However, 80 % of DOM is comprised of HMW DOM compounds, which despite being considered to be largely refractory may be biodegraded, albeit at a slower rate (Farjalla et al. 2009). Whilst LMW DOM compounds are often available as radioisotope tracers off the shelf, HMW compounds require either custom synthesis or the labelling of plant species with a LMW compound (e.g. $^{14}\text{CO}_2$) in order to produce higher MW compounds. Future work should explore the relative importance of HMW DOM using similar techniques.

8.4.2 Long-term research needs

The synthesis of this thesis has highlighted new research areas that could be explored in order to further the understanding of DOM processing in aquatic environments. Some of the key future research needs include:

1. The uptake rates determined for individual DOM compounds and compounds groups in this thesis provide an insight as to how uptake changes across broad nutrient gradients, although currently the compound-specific rates determined cannot be aligned with existing chemistry data, which is usually available for bulk chemical parameters (e.g.

Chapter 8

DOC, DON, DOP, inorganic N/P/S). When the means to quantify individual compounds become available, it will be possible to combine rate data and intrinsic pool sizes to determine flux rates for individual compounds, thus allowing targeted monitoring and management of river systems. In this thesis, uptake rates varied between compounds and with changing background chemical conditions indicate that influxes of different DOM components would need to be managed differently.

2. This thesis mainly considered the direct uptake of LWM DOM solutes by the microbial community, however, it did not evaluate the role of exoenzymes in this process. As the depletion of the LMW solutes from solution was relatively fast, we assume that exoenzyme cleavage of high MW polymers to LWM products in the rate limiting step in organic matter processing in freshwaters, however, further work is needed to confirm this.

3. Only the rate of LMW DOM consumption was measured in this thesis, however, it would also be good to measure the rates of *in situ* production. Although DOM enters freshwaters from the surrounding area (e.g. runoff, soil baseflow), the *in situ* production of DOM should also be considered (e.g. from primary productivity or biomass predation and turnover). This would allow the calculation of net and gross rates of DOM production and consumption. This would be useful for mathematical modelling DOM fluxes in freshwater ecosystems.

4. Finally, the use of metabolomics in this thesis gave an insight into the mechanisms involved in DOC metabolism under different nutrient-limited conditions. This thesis was limited to two time points and sites within one sub-catchment; future work should explore how the metabolism of DOM compounds changes across a broader range of physiochemical gradients. Metabolomics provides a snapshot of one time point; whilst combining with ^{14}C -radiolabelled tracers gave more of an insight in this thesis, future

Chapter 8

work could combine metabolomics with more sensitive tracer techniques such as stable isotope probing. It would also be useful to combine this with microbial metagenomic and metatranscriptomic approaches to see which organism and metabolic pathways are triggered in response to substrate addition. If done by stable isotope probing (^{13}C , ^{18}O , ^{15}N) it would allow an evaluation of which organisms were involved in DOM processing. This would provide higher resolution data, such as which groups of organisms the tracer is entering within the aquatic microbial biomass (Kaplan et al. 2008; Hotchkiss and Hall 2015).

8.5 General conclusions

Through the experimental work of this thesis, I have explored how LMW DOM compounds are processed in river waters and sediments. Detailed work on the uptake rates and kinetics of a range of compounds has been conducted in two contrasting sub-catchments, peat-influenced oligotrophic rivers and improved grassland-influenced mesotrophic rivers, alongside broader work on DOC, DON and DOP uptake across wider physicochemical gradients. The results of this thesis have demonstrated that DOM is readily taken up by the microbial biomass of river waters and sediments and in the case of DOS can be taken up preferentially compared to inorganic S. Whilst the results of radiolabelling studies cannot provide information on the molecular mechanisms underlying DOM uptake and processing, metabolomics and other “omics” tools have the potential to fill some knowledge gaps in the future. This thesis has highlighted that DOM processing can have an impact on riverine water quality; careful monitoring and management of DOM inputs to aquatic ecosystems should be considered a priority alongside the inorganic nutrients currently legislated for.

Chapter 8

8.6 References

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Chapter 8

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Chapter 8

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Appendix 1

Appendix 1: Supplementary material for Chapter 3

**Microbial use of low molecular weight DOM in filtered and unfiltered freshwater:
Role of ultra-small microorganisms and implications for water quality monitoring**

Francesca L. Brailsford, Helen C. Glanville, Miles M. Marshall, Peter N. Golyshin,
Penny J. Johnes, Christopher A. Yates, Alun T. Owen, Davey L. Jones.

Appendix 1

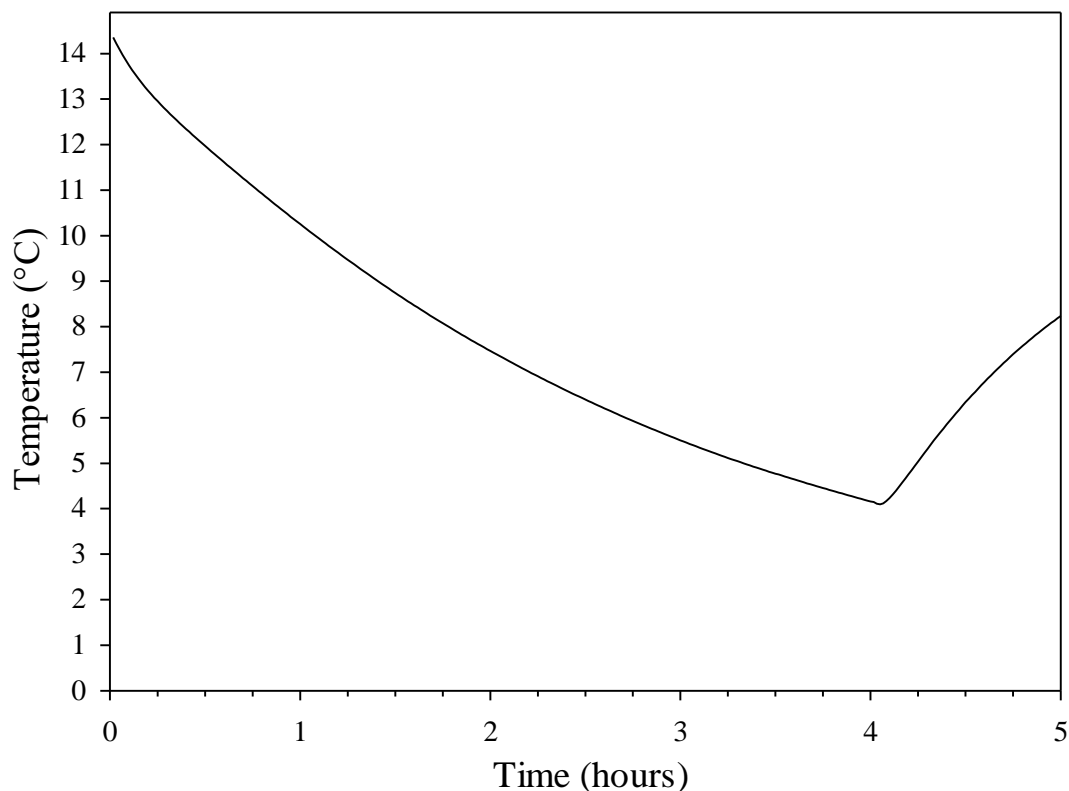


Fig. S3.1 Temperature of river water samples collected in 1 L HDPE bottles. Samples were collected in the field and immediately stored on ice for 4 h (representing the transportation time from the field to the laboratory). The samples were then removed from the ice and held at room temperature for 1 h (to represent dispensing time prior to spiking with either ^{14}C or ^{33}P -labelled nutrients). The 5 hour time point therefore equates to the start of the labelling experiment. Samples were then stored at 10 °C immediately after being spiked with the labelled isotopes. Temperature was recorded every minute using a Tinytag Talk 2 datalogger (Gemini, UK).

Appendix 1



Fig. S3.2 Images of a) the Hiraethlyn (lowland improved grassland) and b) Migneint (upland blanket peat bog) sub-catchments.

Appendix 1

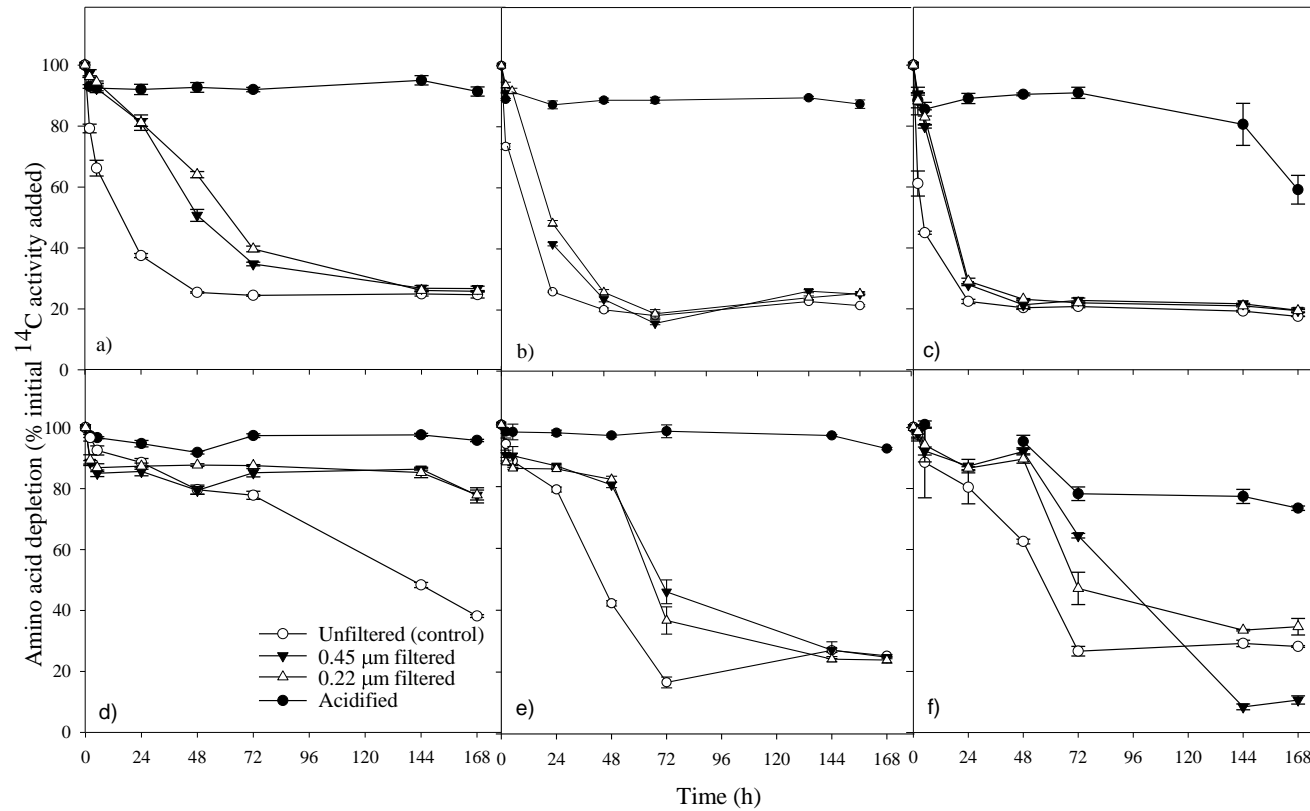


Fig. S3.3 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ¹⁴C-labelled amino acids for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 15 °C, c) Hiraethlyn sub-catchment 25 °C d) Migneint sub-catchment 5 °C, e) Migneint sub-catchment 25 °C, f) Migneint sub-catchment 25 °C. Values represent means ± SEM (*n* = 3). The legend is the same for all panels.

Appendix 1

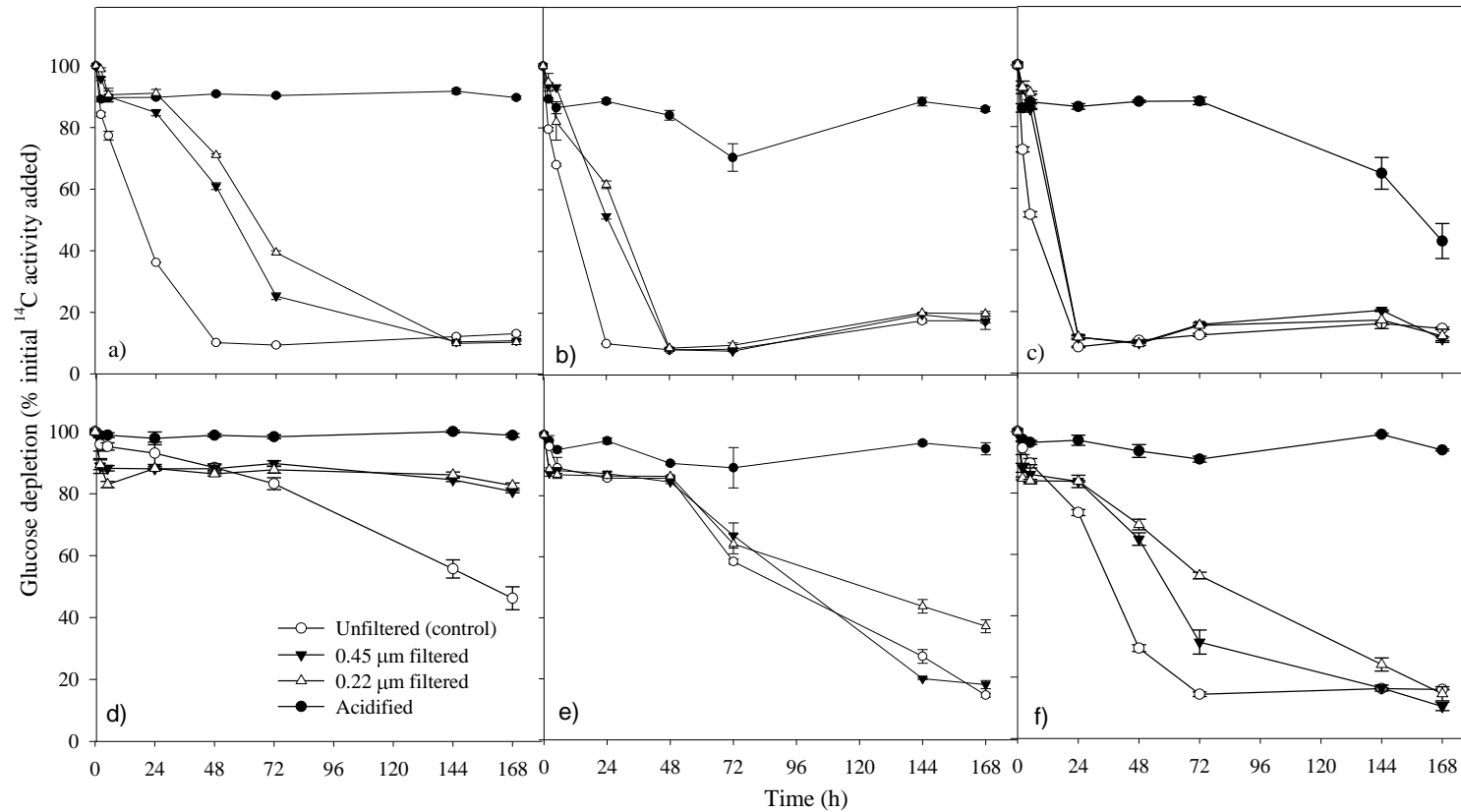


Fig. S3.4 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ¹⁴C-labelled glucose for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 15 °C, c) Hiraethlyn sub-catchment 25 °C d) Migneint sub-catchment 5 °C, e) Migneint sub-catchment 25 °C, f) Migneint sub-catchment 25 °C. Values represent means ± SEM (*n* = 3). The legend is the same for all panels.

Appendix 1

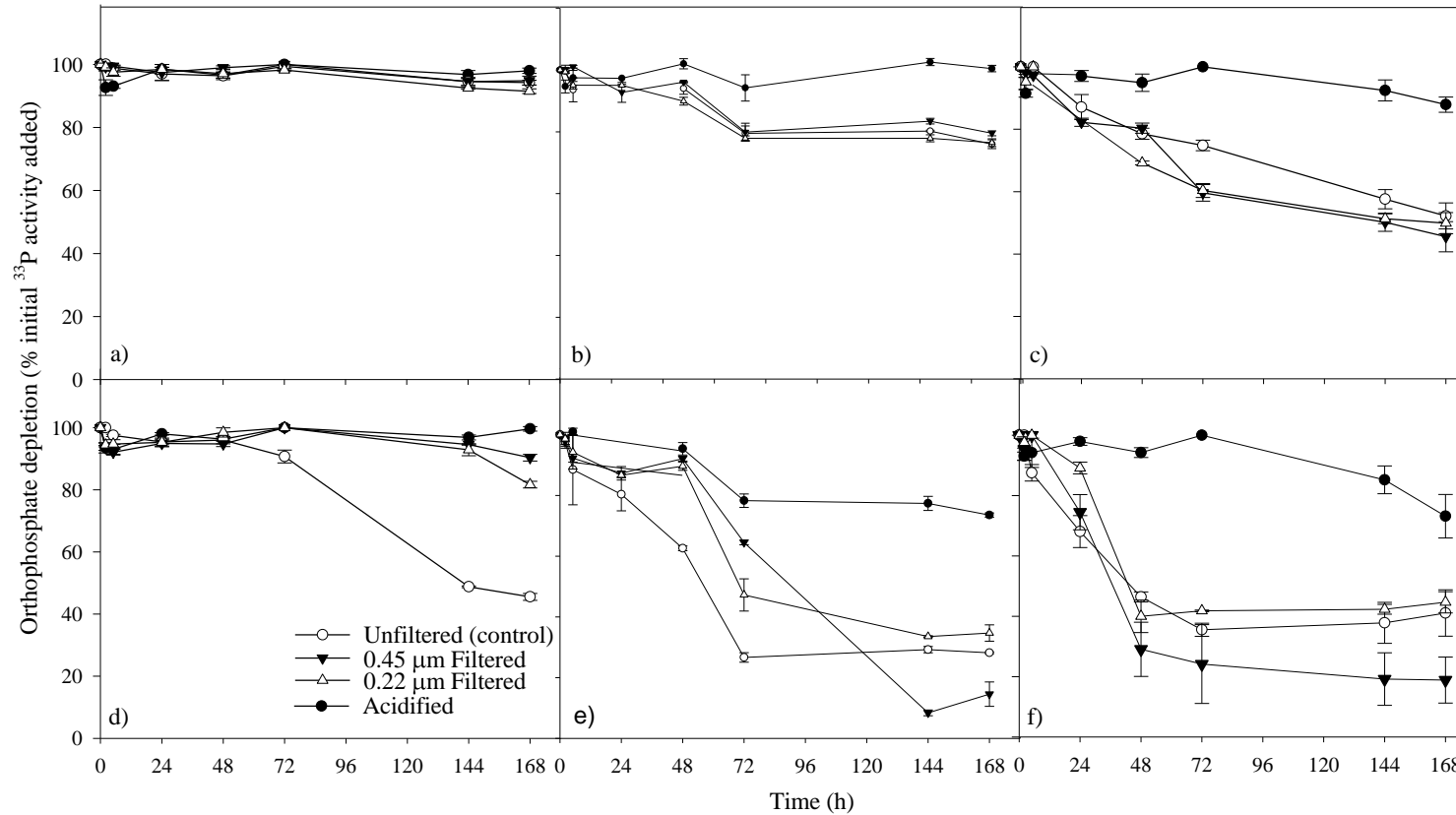


Fig. S3.5 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ¹⁴C-labelled glucose for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 15 °C, c) Hiraethlyn sub-catchment 25 °C d) Migneint sub-catchment 5 °C, e) Migneint sub-catchment 25 °C, f) Migneint sub-catchment 25 °C. Values represent means ± SEM (*n* = 3). The legend is the same for all panels.

Appendix 2

Appendix 2: Supplementary material for Chapter 4

Land cover and nutrient enrichment regulates dissolved organic matter (DOM) turnover in freshwater ecosystems

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Appendix 2

Supplementary Table S4.1 Mean river water chemical characteristics and dominant land cover for all sites used in this study.

Site code	Land cover	pH		EC ($\mu\text{S cm}^{-1}$)		DOC (mg C l^{-1})		DON (mg N l^{-1})		DOP (mg P l^{-1})	
1	Mixed	7.31	± 0.09	206	± 6	5.25	± 0.68	0.54	± 0.04	0.02	± 0.00
2	Mixed	7.33	± 0.10	479	± 12	4.94	± 0.36	0.57	± 0.03	0.02	± 0.00
3	Grasslands	8.03	± 0.93	204	± 3	3.86	± 0.15	0.47	± 0.01	0.01	± 0.00
4	Mixed	7.45	± 0.09	229	± 24	5.43	± 0.70	0.58	± 0.05	0.02	± 0.00
5	Mixed	7.40	± 0.10	172	± 3	4.55	± 0.29	0.55	± 0.04	0.02	± 0.00
6	Mixed	7.15	± 0.28	66	± 4	3.64	± 0.29	0.37	± 0.03	0.02	± 0.00
7	Mixed	6.77	± 0.13	59	± 2	5.04	± 0.74	0.57	± 0.05	0.02	± 0.00
8	Grasslands	7.16	± 0.42	48	± 3	4.03	± 0.81	0.48	± 0.07	0.02	± 0.00
9	Grasslands	7.24	± 0.39	64	± 4	3.93	± 0.34	0.40	± 0.03	0.02	± 0.00
10	Mixed	7.10	± 0.41	53	± 3	4.00	± 0.78	0.38	± 0.06	0.01	± 0.00
11	Mixed	7.06	± 0.40	51	± 3	3.27	± 0.36	0.38	± 0.04	0.01	± 0.00
12	Mixed	6.68	± 0.33	49	± 2	3.77	± 0.32	0.34	± 0.03	0.02	± 0.00
13	Mixed	6.97	± 1.03	40	± 1	3.60	± 0.22	0.36	± 0.02	0.01	± 0.00
14	Grasslands	5.32	± 0.14	42	± 2	9.28	± 0.88	0.55	± 0.04	0.02	± 0.00
15	Conifer	5.75	± 0.41	33	± 1	7.17	± 0.74	0.41	± 0.04	0.01	± 0.00
16	Conifer	5.74	± 0.44	33	± 1	4.86	± 0.84	0.37	± 0.03	0.02	± 0.00
17	Grasslands	5.35	± 0.12	36	± 2	8.01	± 0.86	0.46	± 0.03	0.02	± 0.00
18	Mixed	5.34	± 0.17	35	± 2	6.70	± 0.81	0.42	± 0.04	0.02	± 0.00
19	Mixed	6.32	± 0.08	47	± 1	7.10	± 0.31	0.52	± 0.01	0.02	± 0.00
20	Grasslands	5.99	± 0.12	51	± 2	8.47	± 0.85	0.58	± 0.04	0.01	± 0.00
21	Grasslands	6.70	± 0.05	100	± 2	4.93	± 0.16	0.51	± 0.01	0.02	± 0.00
22	Mixed	6.95	± 0.05	99	± 2	5.11	± 0.45	0.50	± 0.03	0.02	± 0.00
23	Mixed	6.80	± 0.06	68	± 4	4.82	± 1.33	0.61	± 0.19	0.02	± 0.00
24	Mixed	6.74	± 0.07	70	± 4	5.75	± 0.64	0.45	± 0.02	0.02	± 0.00
25	Mixed	6.48	± 0.08	38	± 2	3.45	± 0.57	0.38	± 0.03	0.02	± 0.01
26	Mixed	6.36	± 0.08	26	± 1	3.36	± 0.25	0.38	± 0.02	0.01	± 0.00
27	Mixed	5.90	± 0.08	24	± 1	3.18	± 0.30	0.36	± 0.02	0.02	± 0.01
28	Arable	7.68	± 0.09	624	± 28	4.80	± 0.92	0.48	± 0.05	0.02	± 0.00
29	Arable	7.78	± 0.07	532	± 26	3.25	± 0.31	0.46	± 0.01	0.02	± 0.00
30	Mixed	7.79	± 0.11	628	± 15	3.93	± 0.13	0.56	± 0.05	0.01	± 0.00
31	Grasslands	7.84	± 0.09	558	± 6	3.37	± 0.13	0.42	± 0.01	0.01	± 0.00
32	Mixed	7.95	± 0.08	569	± 12	3.58	± 0.93	0.50	± 0.06	0.01	± 0.00
33	Mixed	7.97	± 0.07	598	± 6	4.95	± 0.65	0.53	± 0.10	0.01	± 0.00
34	Mixed	7.94	± 0.03	571	± 3	3.58	± 0.35	0.53	± 0.05	0.01	± 0.00
35	Mixed	7.99	± 0.03	580	± 7	3.44	± 1.13	0.38	± 0.05	0.01	± 0.00
36	Mixed	7.99	± 0.04	566	± 2	6.09	± 0.69	0.47	± 0.05	0.02	± 0.00
37	Mixed	8.05	± 0.03	549	± 3	4.88	± 0.62	0.44	± 0.07	0.01	± 0.00
38	Mixed	8.08	± 0.05	547	± 2	5.23	± 0.76	0.38	± 0.02	0.01	± 0.00
39	Mixed	7.97	± 0.04	537	± 28	4.79	± 0.30	0.39	± 0.04	0.02	± 0.00
40	Mixed	7.93	± 0.05	518	± 19	3.14	± 0.80	0.50	± 0.06	0.02	± 0.01

Appendix 2

41	Mixed	7.74 ± 0.04	468 ± 32	4.56 ± 1.28	0.80 ± 0.13	0.02 ± 0.00
42	Grasslands	7.72 ± 0.03	485 ± 48	5.51 ± 0.63	0.39 ± 0.04	0.02 ± 0.00
43	Mixed	7.73 ± 0.06	560 ± 85	4.74 ± 0.69	0.61 ± 0.09	0.02 ± 0.00
44	Grasslands	7.99 ± 0.08	575 ± 19	4.10 ± 0.12	0.45 ± 0.03	0.01 ± 0.00
45	Mixed	7.53 ± 0.10	1233 ± 39	3.40 ± 0.99	0.60 ± 0.06	0.01 ± 0.00
Site code	Land cover	Nitrate (mg N l ⁻¹)	Ammonia (mg N l ⁻¹)	TN (mg N l ⁻¹)	SRP (mg P l ⁻¹)	TP (mg P l ⁻¹)
1	Mixed	3.38 ± 0.46	0.10 ± 0.03	4.29 ± 0.46	0.06 ± 0.01	0.12 ± 0.02
2	Mixed	2.69 ± 0.22	0.27 ± 0.04	3.81 ± 0.24	0.08 ± 0.01	0.14 ± 0.01
3	Grasslands	3.30 ± 0.11	0.06 ± 0.00	4.06 ± 0.12	0.06 ± 0.00	0.10 ± 0.00
4	Mixed	2.60 ± 0.43	0.05 ± 0.01	3.49 ± 0.45	0.05 ± 0.01	0.11 ± 0.02
5	Mixed	2.33 ± 0.22	0.05 ± 0.01	3.19 ± 0.25	0.04 ± 0.00	0.09 ± 0.01
6	Mixed	1.40 ± 0.38	0.03 ± 0.01	1.94 ± 0.40	0.02 ± 0.01	0.06 ± 0.01
7	Mixed	2.96 ± 0.43	0.04 ± 0.01	3.80 ± 0.44	0.04 ± 0.01	0.10 ± 0.01
8	Grasslands	2.22 ± 0.63	0.02 ± 0.01	2.81 ± 0.68	0.05 ± 0.02	0.08 ± 0.03
9	Grasslands	1.35 ± 0.39	0.04 ± 0.01	1.92 ± 0.41	0.02 ± 0.01	0.05 ± 0.01
10	Mixed	0.85 ± 0.33	0.03 ± 0.01	1.39 ± 0.39	0.01 ± 0.00	0.03 ± 0.01
11	Mixed	1.21 ± 0.41	0.02 ± 0.00	2.08 ± 0.55	0.02 ± 0.00	0.07 ± 0.03
12	Mixed	0.81 ± 0.29	0.04 ± 0.01	1.27 ± 0.32	0.01 ± 0.00	0.03 ± 0.01
13	Mixed	1.87 ± 0.34	0.04 ± 0.01	2.51 ± 0.37	0.03 ± 0.01	0.06 ± 0.01
14	Grasslands	1.57 ± 0.29	0.05 ± 0.01	2.49 ± 0.33	0.03 ± 0.01	0.07 ± 0.01
15	Conifer	0.97 ± 0.34	0.06 ± 0.03	1.67 ± 0.39	0.02 ± 0.01	0.07 ± 0.02
16	Conifer	1.18 ± 0.38	0.06 ± 0.04	1.79 ± 0.41	0.02 ± 0.01	0.05 ± 0.01
17	Grasslands	1.57 ± 0.29	0.03 ± 0.01	2.29 ± 0.32	0.03 ± 0.00	0.06 ± 0.01
18	Mixed	1.03 ± 0.42	0.02 ± 0.01	1.55 ± 0.44	0.01 ± 0.01	0.03 ± 0.01
19	Mixed	2.03 ± 0.15	0.04 ± 0.00	2.97 ± 0.16	0.02 ± 0.00	0.11 ± 0.01
20	Grasslands	1.50 ± 0.27	0.05 ± 0.02	2.38 ± 0.30	0.03 ± 0.01	0.07 ± 0.01
21	Grasslands	2.27 ± 0.12	0.04 ± 0.00	3.11 ± 0.13	0.03 ± 0.00	0.07 ± 0.00
22	Mixed	1.78 ± 0.27	0.02 ± 0.00	2.56 ± 0.29	0.02 ± 0.00	0.06 ± 0.01
23	Mixed	2.72 ± 0.90	0.03 ± 0.01	3.44 ± 1.04	0.04 ± 0.02	0.07 ± 0.02
24	Mixed	1.33 ± 0.34	0.02 ± 0.00	1.95 ± 0.36	0.02 ± 0.01	0.06 ± 0.01
25	Mixed	1.43 ± 0.45	0.03 ± 0.01	2.07 ± 0.48	0.02 ± 0.01	0.06 ± 0.01
26	Mixed	1.82 ± 0.34	0.03 ± 0.00	2.44 ± 0.37	0.03 ± 0.01	0.06 ± 0.01
27	Mixed	1.73 ± 0.32	0.02 ± 0.00	2.31 ± 0.35	0.02 ± 0.00	0.05 ± 0.01
28	Arable	3.50 ± 0.47	0.07 ± 0.03	4.37 ± 1.18	0.10 ± 0.03	0.18 ± 0.03
29	Arable	4.97 ± 0.11	0.04 ± 0.00	5.70 ± 0.57	0.06 ± 0.01	0.11 ± 0.02
30	Mixed	3.26 ± 0.72	0.06 ± 0.02	4.47 ± 0.11	0.07 ± 0.00	0.13 ± 0.00
31	Grasslands	5.05 ± 0.11	0.05 ± 0.00	5.71 ± 0.51	0.07 ± 0.01	0.11 ± 0.01
32	Mixed	3.71 ± 0.57	0.06 ± 0.01	4.46 ± 0.11	0.05 ± 0.00	0.10 ± 0.00
33	Mixed	4.99 ± 0.83	0.04 ± 0.01	5.86 ± 0.47	0.09 ± 0.02	0.13 ± 0.02
34	Mixed	1.79 ± 0.49	0.04 ± 0.01	2.65 ± 0.51	0.05 ± 0.01	0.10 ± 0.02
35	Mixed	3.54 ± 0.49	0.03 ± 0.01	4.10 ± 0.49	0.08 ± 0.02	0.12 ± 0.02
36	Mixed	3.44 ± 0.46	0.04 ± 0.01	4.13 ± 0.57	0.07 ± 0.01	0.14 ± 0.03
37	Mixed	4.12 ± 0.51	0.04 ± 0.01	4.92 ± 0.50	0.06 ± 0.01	0.12 ± 0.02
38	Mixed	4.88 ± 0.11	0.05 ± 0.00	5.50 ± 0.66	0.09 ± 0.02	0.13 ± 0.02
39	Mixed	3.52 ± 0.48	0.07 ± 0.03	4.21 ± 0.54	0.08 ± 0.01	0.11 ± 0.02
40	Mixed	3.90 ± 0.56	0.04 ± 0.01	4.61 ± 0.89	0.09 ± 0.02	0.12 ± 0.03

Appendix 2

41	Mixed	3.65	±	0.55	0.09	±	0.03	4.91	±	0.57	0.08	±	0.01	0.11	±	0.02
42	Grasslands	3.71	±	0.50	0.04	±	0.01	4.50	±	0.91	0.05	±	0.01	0.10	±	0.02
43	Mixed	3.32	±	0.51	0.04	±	0.01	4.27	±	0.47	0.09	±	0.01	0.14	±	0.02
44	Grasslands	3.35	±	0.55	0.05	±	0.01	4.15	±	0.49	0.05	±	0.01	0.09	±	0.01
45	Mixed	3.10	±	0.65	0.04	±	0.00	4.03	±	0.12	0.05	±	0.00	0.10	±	0.00

Appendix 2

Supplementary Table S4.2 Mean depletion of $^{14}\text{C}/^{33}\text{P}$ -labelled compounds from solution. Values represent mean cumulative depletion (% of total added) \pm SEM ($n = 3$). AA, ^{14}C -labelled amino acids; G, ^{14}C -labelled glucose; G-6-P, ^{14}C -labelled glucose-6-phosphate.

Site code	Depletion															
	^{33}P 5 h		^{33}P 24 h		AA 5 h		AA 24 h		G 5 h		G 24 h		G-6-P 5 h		G-6-P 24 h	
1	14.7	\pm 3.3	49.8	\pm 8.9	21.4	\pm 5.8	58.5	\pm 6.7	30.2	\pm 12.8	68.7	\pm 8.1	15.9	\pm 5.1	44.5	\pm 14.7
2	12.4	\pm 4.3	21.5	\pm 10.4	40.6	\pm 11.6	69.5	\pm 4.6	70.6	\pm 22.2	86.3	\pm 5.7	8.00	\pm 2.8	56.9	\pm 5.9
3	3.4	\pm 0.6	12.5	\pm 4.9	30.0	\pm 7.8	66.8	\pm 2.8	44.5	\pm 12.9	86.2	\pm 3.3	4.40	\pm 2.7	42.1	\pm 10.2
4	45.3	\pm 18.7	72.1	\pm 15.3	39.8	\pm 17.6	60.6	\pm 8.2	58.4	\pm 9.9	71.0	\pm 9.6	35.5	\pm 18.3	73.0	\pm 12.3
5	13.4	\pm 4.0	60.8	\pm 10.8	18.3	\pm 4.3	62.1	\pm 3.4	15.2	\pm 2.7	77.7	\pm 6.4	6.90	\pm 3.0	55.7	\pm 10.7
6	11.3	\pm 4.5	53.5	\pm 12.0	11.7	\pm 3.3	60.4	\pm 5.2	23.0	\pm 8.5	74.1	\pm 10.1	5.50	\pm 2.0	52.7	\pm 13.7
7	71.1	\pm 13.0	89.3	\pm 1.7	10.4	\pm 2.1	31.1	\pm 3.9	10.6	\pm 3.1	41.9	\pm 6.3	11.6	\pm 2.2	27.4	\pm 2.6
8	13.3	\pm 2.1	43.1	\pm 4.3	21.2	\pm 8.4	51.6	\pm 7.0	25.8	\pm 8.6	63.9	\pm 13.6	17.9	\pm 8.0	38.3	\pm 11.0
9	8.8	\pm 4.1	18.6	\pm 7.4	27.6	\pm 13.3	63.0	\pm 7.3	44.7	\pm 21.2	78.4	\pm 13.2	14.7	\pm 7.6	57.4	\pm 14.8
10	12.7	\pm 3.8	43.5	\pm 7.7	29.7	\pm 8.5	61.4	\pm 1.8	40.6	\pm 22.3	82.3	\pm 3.0	14.0	\pm 5.0	58.1	\pm 11.6
11	7.7	\pm 2.1	23.7	\pm 6.9	5.2	\pm 1.7	28.7	\pm 5.9	7.40	\pm 3.10	26.3	\pm 5.0	4.7	\pm 2.2	15.3	\pm 3.2
12	13.0	\pm 5.9	28.1	\pm 10.1	15.7	\pm 3.6	51.3	\pm 5.5	10.1	\pm 3.0	45.9	\pm 4.8	8.7	\pm 3.1	20.3	\pm 2.2
13	42.7	\pm 12.1	90.2	\pm 1.8	6.6	\pm 2.1	41.9	\pm 8.5	10.6	\pm 4.0	56.5	\pm 11.1	12.0	\pm 3.7	50.9	\pm 13.7
14	11.2	\pm 3.8	47.1	\pm 18.4	5.1	\pm 2.8	27.6	\pm 14.5	11.0	\pm 3.2	48.1	\pm 15.6	12.7	\pm 4.2	45.5	\pm 12.0
15	11.4	\pm 0.5	48.7	\pm 12.4	4.0	\pm 2.1	27.9	\pm 9.9	10.8	\pm 4.0	33.8	\pm 13.9	8.7	\pm 3.6	16.1	\pm 3.4
16	10.0	\pm 3.7	38.3	\pm 11.3	7.0	\pm 3.7	34.7	\pm 11.9	11.0	\pm 2.0	41.5	\pm 14.6	5.4	\pm 2.2	19.0	\pm 2.0
17	9.8	\pm 4.3	38.3	\pm 18.1	21.5	\pm 13.7	48.7	\pm 8.6	37.3	\pm 24.9	65.5	\pm 11.6	19.0	\pm 7.8	48.6	\pm 14.9
18	7.0	\pm 1.1	18.4	\pm 7.6	5.0	\pm 1.3	33.9	\pm 3.6	10.5	\pm 1.6	30.7	\pm 7.0	9.50	\pm 3.6	17.1	\pm 3.6
19	9.4	\pm 3.7	21.5	\pm 7.2	10.2	\pm 1.2	48.9	\pm 1.4	13.9	\pm 2.0	40.2	\pm 3.1	11.1	\pm 3.1	20.9	\pm 3.2
20	2.8	\pm 0.3	7.8	\pm 3.3	26.8	\pm 8.2	62.7	\pm 6.9	39.1	\pm 2.7	80.9	\pm 11.0	4.80	\pm 1.8	59.6	\pm 9.7

Appendix 2

21	5.2 ± 1.1	11.3 ± 4.0	28.7 ± 10.9	62.7 ± 7.0	42.2 ± 15.9	79.2 ± 12.3	8.70 ± 4.0	52.2 ± 14.7
22	27.9 ± 8.5	75.8 ± 8.5	19.9 ± 8.2	50.7 ± 12.8	18.0 ± 2.7	68.7 ± 13.6	8.90 ± 2.4	63.4 ± 8.5
23	12.4 ± 3.1	57.0 ± 15.9	8.9 ± 4.9	48.0 ± 12.9	17.9 ± 7.5	59.2 ± 18.2	6.30 ± 3.1	46.4 ± 7.6
24	8.8 ± 1.2	38.5 ± 7.7	30.3 ± 13.1	63.1 ± 2.4	75.5 ± 1.4	86.2 ± 5.6	21.4 ± 8.4	74.5 ± 6.3
25	13.3 ± 4.6	47.9 ± 15.2	27.7 ± 13.5	56.5 ± 6.8	48.5 ± 19.8	74.2 ± 14.8	14.9 ± 5.7	50.2 ± 13.4
26	12.4 ± 3.7	47.2 ± 8.0	18.4 ± 6.5	55.8 ± 10.9	27.7 ± 7.3	74.5 ± 10.4	10.2 ± 3.6	58.8 ± 8.4
27	7.4 ± 2.3	36.9 ± 9.1	17.1 ± 9.8	52.9 ± 11.6	23.3 ± 10.6	68.1 ± 19.8	7.4 ± 2.8	45.9 ± 7.8
29	51.0 ± 24.1	57.5 ± 20.9	68.4 ± 3.6	75.2 ± 1.1	87.4 ± 6.3	91.7 ± 0.7	67.2 ± 19.2	74.1 ± 16.1
30	4.0 ± 0.6	8.2 ± 0.2	70.1 ± 1.7	76.0 ± 0.3	90.5 ± 3.4	93.6 ± 0.5	18.4 ± 2.8	75.0 ± 10.6
31	5.1 ± 1.3	9.5 ± 0.7	70.1 ± 1.6	75.2 ± 0.6	91.5 ± 2.6	94.0 ± 0.6	18.8 ± 2.8	75.7 ± 10.5
31	6.0 ± 3.8	10.6 ± 2.9	66.9 ± 2.9	74.2 ± 1.1	87.9 ± 6.0	93.9 ± 0.2	16.4 ± 4.1	62.3 ± 13.3
33	4.7 ± 1.2	9.3 ± 0.3	71.0 ± 0.8	75.9 ± 0.4	90.1 ± 4.4	94.0 ± 0.2	20.2 ± 5.0	75.8 ± 11.3
34	9.5 ± 3.3	24.3 ± 8.1	68.5 ± 3.6	74.3 ± 1.4	85.5 ± 7.4	92.8 ± 0.5	39.0 ± 10.3	74.1 ± 18.0
35	11.7 ± 3.8	20.4 ± 4.8	68.1 ± 0.8	69.4 ± 4.9	84.5 ± 8.3	86.1 ± 5.0	26.1 ± 7.2	78.3 ± 10.3
36	8.5 ± 1.7	15.4 ± 2.6	71.1 ± 1.0	74.1 ± 1.9	91.3 ± 1.8	89.6 ± 0.5	30.6 ± 9.7	83.6 ± 2.1
37	2.6 ± 1.1	4.8 ± 0.7	56.2 ± 5.5	73.2 ± 1.4	67.2 ± 11.5	91.0 ± 0.2	21.6 ± 9.7	80.6 ± 5.0
37	4.6 ± 0.8	8.1 ± 0.6	70.2 ± 0.2	74.6 ± 0.7	93.6 ± 0.5	93.4 ± 0.2	17.2 ± 2.5	83.0 ± 7.2
38	2.5 ± 0.9	3.6 ± 0.9	68.4 ± 0.2	72.1 ± 0.3	89.6 ± 0.2	76.2 ± 2.9	62.8 ± 6.5	86.3 ± 2.1
39	4.2 ± 1.6	7.0 ± 1.5	62.5 ± 6.5	73.1 ± 1.9	91.6 ± 0.9	92.8 ± 0.9	18.9 ± 5.7	64.6 ± 16.2
40	3.7 ± 1.2	4.8 ± 1.9	32.0 ± 9.2	58.4 ± 7.4	38.6 ± 9.9	72.6 ± 12.8	5.10 ± 0.4	21.3 ± 5.3
41	5.9 ± 1.6	10.4 ± 1.5	62.7 ± 5.3	73.3 ± 1.9	92.0 ± 0.3	92.6 ± 0.9	18.6 ± 3.8	72.9 ± 18.9
42	7.0 ± 2.7	14.8 ± 7.8	64.2 ± 4.2	74.7 ± 1.3	89.2 ± 4.5	93.3 ± 1.0	15.6 ± 2.9	73.0 ± 18.7
44	5.0 ± 1.7	11.7 ± 1.3	68.4 ± 0.5	74.7 ± 0.2	88.8 ± 4.8	94.3 ± 0.4	17.1 ± 1.9	68.6 ± 10.6
44	4.9 ± 0.9	6.50 ± 1.6	63.6 ± 4.5	74.1 ± 1.1	92.5 ± 1.0	93.1 ± 0.6	14.0 ± 3.4	65.3 ± 15.7
45	6.2 ± 2.5	11.4 ± 3.8	55.6 ± 10.5	70.9 ± 3.3	84.8 ± 4.1	89.2 ± 3.9	13.1 ± 3.5	60.0 ± 16.9

Appendix 3

Appendix 3: Supplementary material for Chapter 5

Microbial uptake kinetics of dissolved organic carbon (DOC) compound groups from river water and sediments

Francesca L. Brailsford, Helen C. Glanville, Peter N. Golyshin, Penny J. Johnes, Christopher
A. Yates and Davey L. Jones

Appendix 3

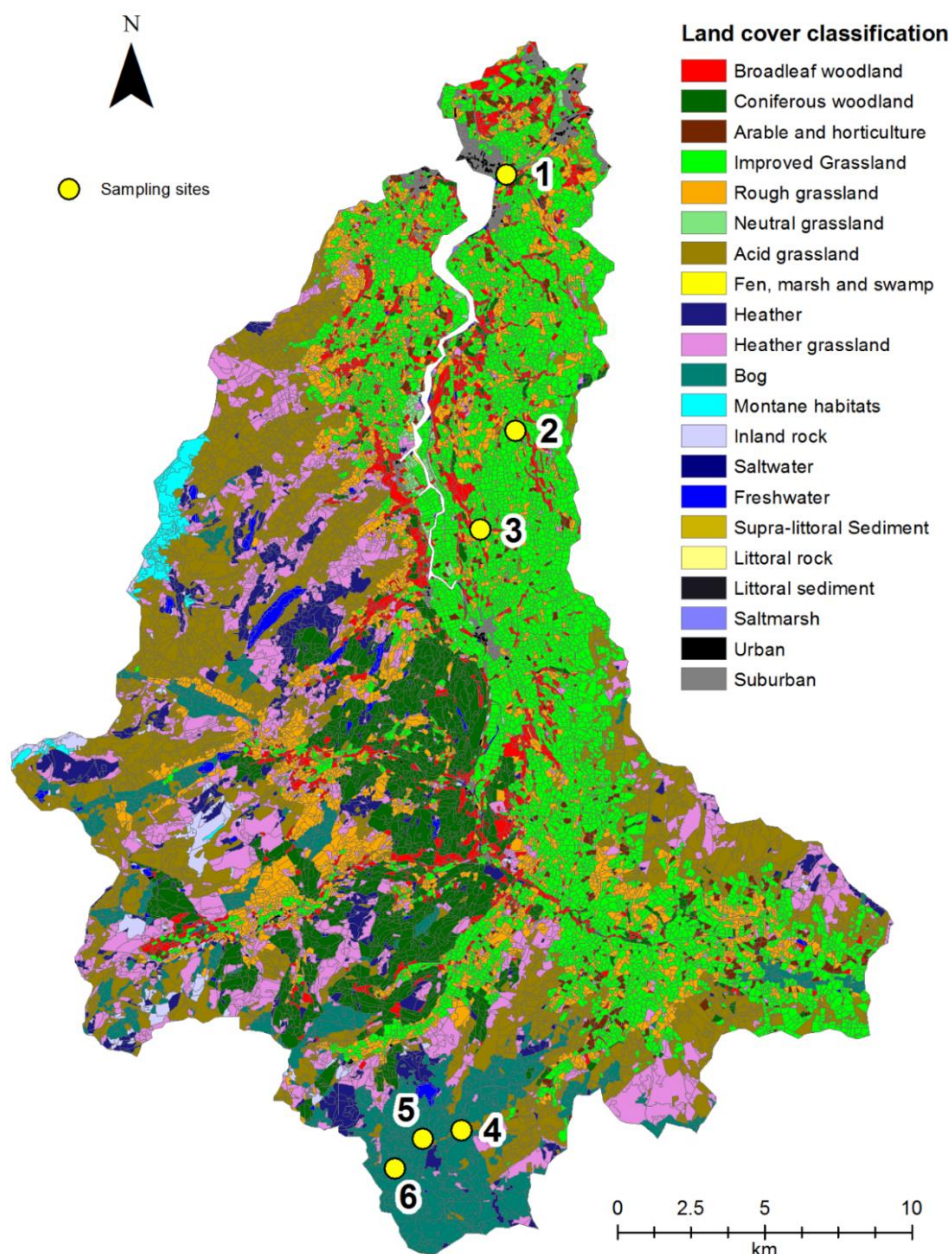


Fig. S5.1 Land cover map of the Conwy catchment with lowland improved grassland sites (1-3) and upland peat bog sites (4-6) indicated. Created with ArcGIS Hydrology toolbox (ESRI 2018. Version 10 Redlands, CA) using LCM2007 data provided by the Centre for Ecology and Hydrology (Emmett et al. 2016).

Appendix 3

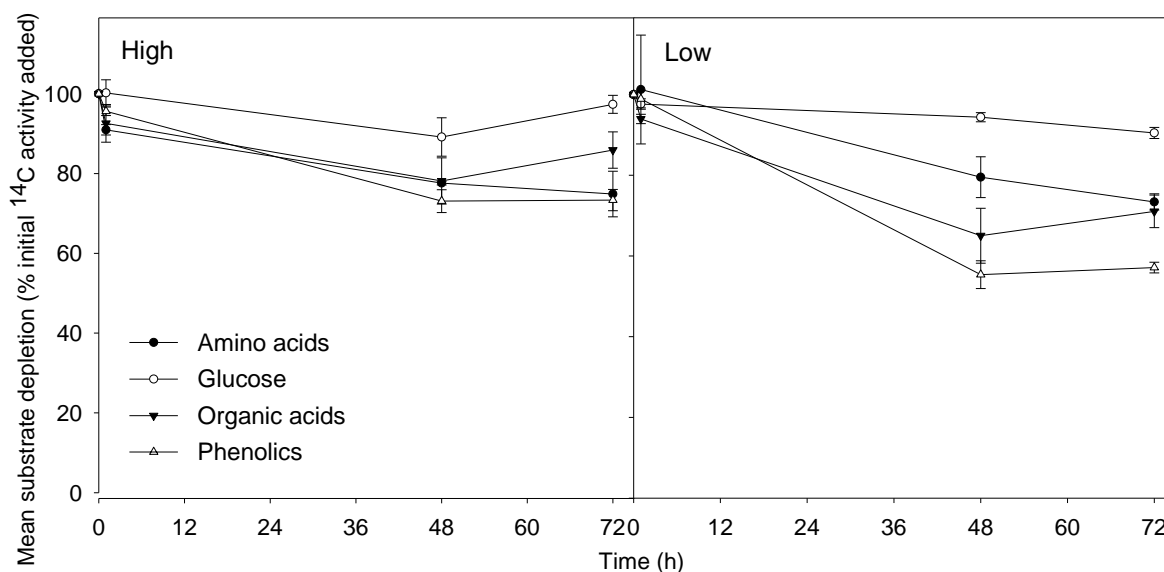


Fig. S5.2 Abiotic loss of ¹⁴C-labelled amino acids, glucose, organic acids and phenolics compounds from river sediments sterilised with formaldehyde. Values represent means \pm SEM ($n = 3$).

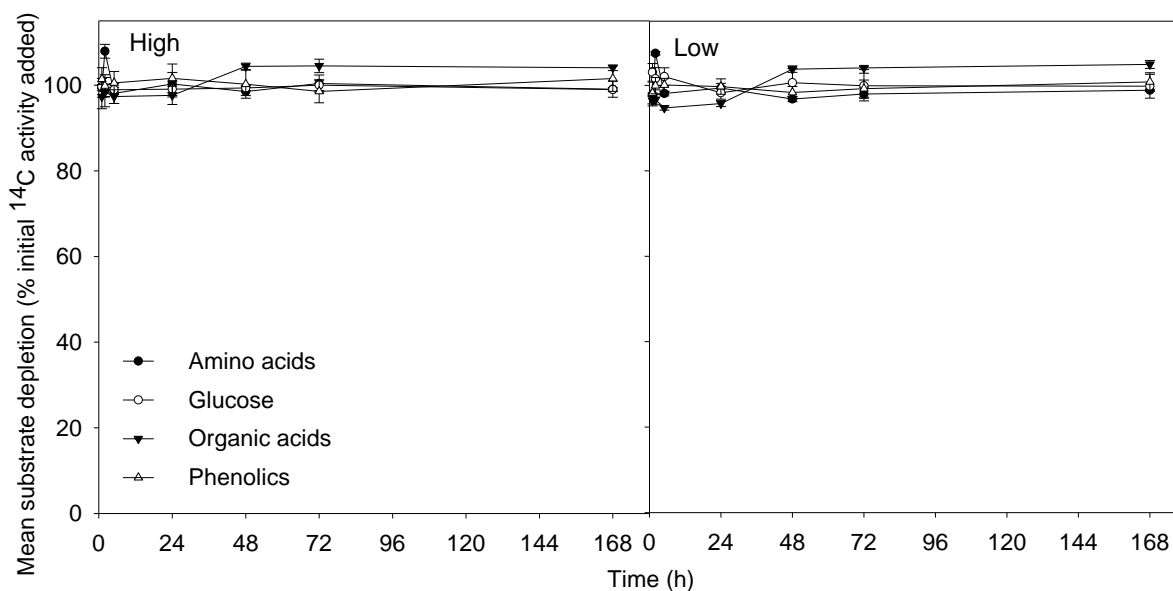


Fig. S5.3 Abiotic loss of ¹⁴C-labelled amino acids, glucose, organic acids and phenolics compounds from water. Values represent means \pm SEM ($n = 3$).

Appendix 3

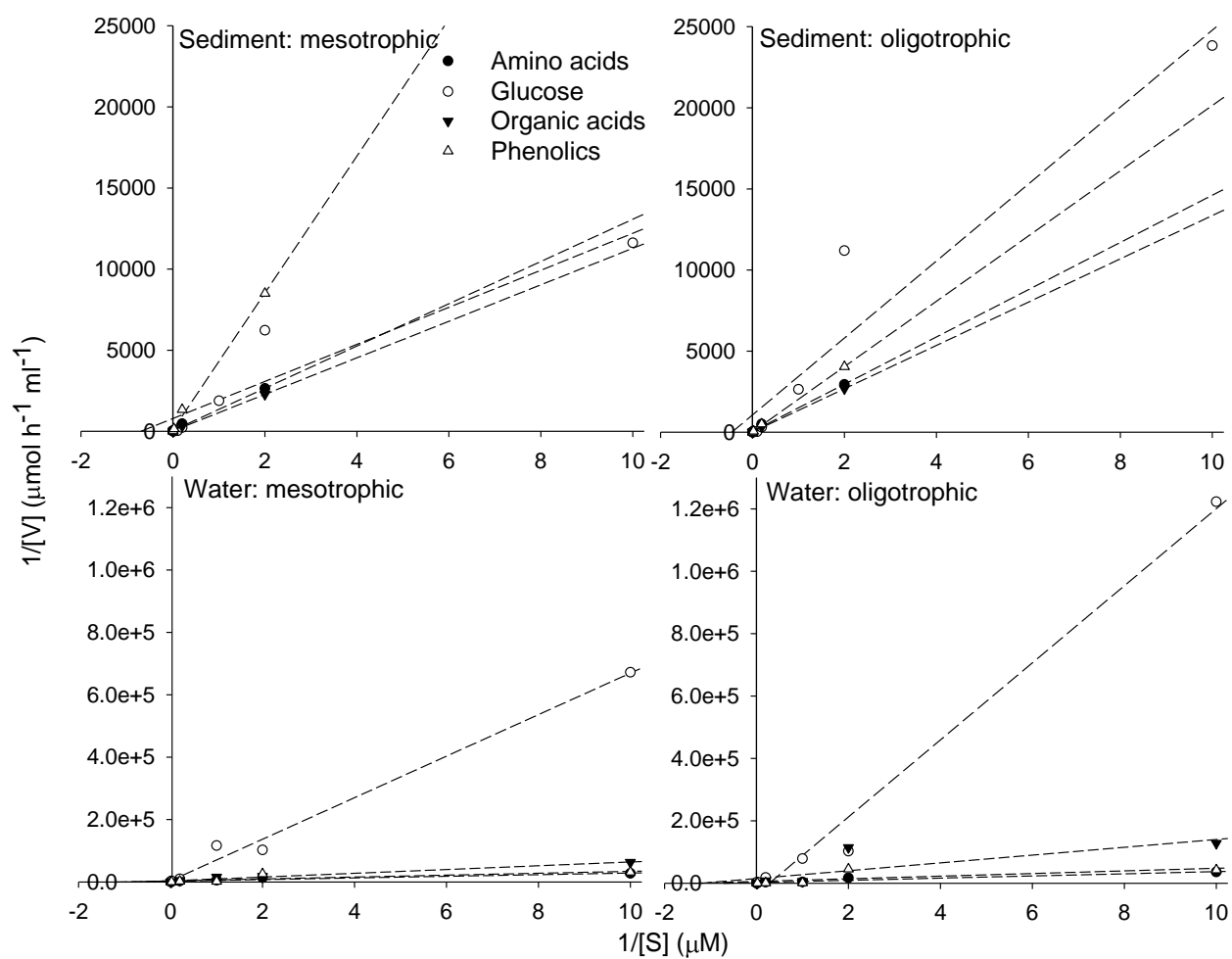


Fig. S5.4 Lineweaver-Burke plots for amino acids, glucose, organic acids and phenolics compounds for: lowland improved grassland river sediments (mesotrophic), upland peat bog sediments (oligotrophic), lowland improved grassland river waters (mesotrophic) and upland improved grassland river waters (oligotrophic).

Appendix 3

Table S5.1 Compounds used in the kinetics experiments.

Functional group	Compound	Isotope	Supplier	Lot Number
Sugars	Glucose	¹⁴ C-[U]-glucose	Perkin Elmer	3632475
	Alanine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
Amino acids	Arginine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Aspartate	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Glutamate	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Glycine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Isoleucine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Lysine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Methionine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Phenylalanine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Proline	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Serine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Tyrosine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Valine	¹⁴ C-[U]-amino acids	Perkin Elmer	2132273
	Organic acids	Acetic acid	¹⁴ C-[U]-acetic acid	Perkin Elmer
Citric acid		¹⁴ C-[U]-citric acid	Perkin Elmer	3604237
Malic acid		¹⁴ C-[U]-malic acid	American Radiolabeled Chemicals (ARC)	150508
Phenolics	<i>P</i> -coumaric acid	¹⁴ C-[U]- <i>P</i> -coumaric acid	American Radiolabeled Chemicals (ARC)	161117
	Salicylic acid	¹⁴ C-[U]-salicylic acid	American Radiolabeled Chemicals (ARC)	070502
	Vanillic acid	¹⁴ C-[U]-vanillic acid	American Radiolabeled Chemicals (ARC)	160311

Appendix 3

Table S5.2 Concentrations of functional groups used in the kinetic experiments.

	Sediments				Waters			
	Amino acids	Sugars	Organic acids	Phenolics	Amino acids	Sugars	Organic acids	Phenolics
Concentration (μM)	10000	-	10000	10000	500	-	500	500
	5000	5000	5000	5000	50	50	50	50
	500	500	500	500	-	10	-	-
	50	50	50	50	5	5	5	5
	5	5	5	5	1	1	1	1
	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	-	0.1	-	-	0.1	0.1	0.1	0.1

Appendix 3

Table S5.3 Mean chemical characteristics of the sediment and water samples used in the study. Values represent mean \pm SEM ($n = 3$) except for soil texture analysis. BLD, below limit of detection.

	Lowland mesotrophic sites			Upland oligotrophic sites		
	1	2	3	4	5	6
Water						
pH _(H2O)	7.27 \pm 0.20	6.87 \pm 0.05	7.15 \pm 0.10	4.34 \pm 0.35	4.14 \pm 0.37	4.11 \pm 0.33
Electrical conductivity (μ S cm ⁻¹)	201 \pm 6	207 \pm 12	163 \pm 15	46 \pm 6	51 \pm 15	49 \pm 9
Dissolved organic C (mg C L ⁻¹)	2.88 \pm 0.56	2.58 \pm 0.35	3.11 \pm 0.46	7.48 \pm 1.31	6.43 \pm 1.33	8.89 \pm 1.53
Total free carbohydrates (mg C L ⁻¹)	0.08 \pm 0.03	0.10 \pm 0.03	0.16 \pm 0.04	0.08 \pm 0.04	0.12 \pm 0.05	0.06 \pm 0.01
Total phenols (mg C L ⁻¹)	0.49 \pm 0.09	1.65 \pm 0.00	3.67 \pm 0.26	3.24 \pm 0.00	1.06 \pm 0.18	BLD
Total dissolved N (mg N L ⁻¹)	1.33 \pm 0.08	2.78 \pm 0.18	2.29 \pm 0.25	0.36 \pm 0.01	0.38 \pm 0.05	0.41 \pm 0.02
NH ₄ ⁺ (mg N L ⁻¹)	0.05 \pm 0.02	0.05 \pm 0.02	0.06 \pm 0.02	0.06 \pm 0.02	0.06 \pm 0.02	0.05 \pm 0.02
NO ₃ ⁻ (mg N L ⁻¹)	0.96 \pm 0.16	2.31 \pm 0.23	1.91 \pm 0.37	0.01 \pm 0.01	0.02 \pm 0.01	0.02 \pm 0.02
Total free amino acids (mg N L ⁻¹)	0.10 \pm 0.04	0.11 \pm 0.02	0.10 \pm 0.01	0.10 \pm 0.01	0.14 \pm 0.03	0.14 \pm 0.01
Molybdate-reactive P (mg P L ⁻¹)	0.07 \pm 0.01	0.09 \pm 0.01	0.05 \pm 0.01	0.02 \pm 0.01	0.03 \pm 0.00	0.04 \pm 0.00
Sediment						
pH _(H2O)	6.90 \pm 0.06	6.85 \pm 1.10	6.84 \pm 0.17	4.69 \pm 10.0	4.87 \pm 0.14	4.70 \pm 2.04
Electrical conductivity (μ S cm ⁻¹)	55 \pm 4	29 \pm 2	27 \pm 4	15 \pm 3	18 \pm 5	10 \pm 0
Moisture content (%)	44.4 \pm 9.2	40.3 \pm 6.4	35.2 \pm 2.3	80.9 \pm 6.2	76.1 \pm 9.4	83.8 \pm 2.1
Silt content (%)	56.53	15.85	10.33	3.32	4.51	7.69
Clay content (%)	21.7	4.31	4.44	0.2	0.55	1.34
Sand content (%)	21.77	79.84	85.23	96.48	94.94	90.97
Total C (mg C kg dry sediment ⁻¹)	8.09 \pm 0.70	3.11 \pm 0.33	10.4 \pm 1.88	414 \pm 12.5	144 \pm 7.2	194 \pm 2.9
Total free carbohydrates (mg C wet sediment ⁻¹)	0.56 \pm 0.05	0.72 \pm 0.03	0.53 \pm 0.04	0.65 \pm 0.03	0.42 \pm 0.04	0.76 \pm 0.23
Total phenols (mg C kg wet sediment ⁻¹)	3.08 \pm 2.88	11.4 \pm 5.7	4.97 \pm 2.96	5.91 \pm 2.28	2.86 \pm 0.00	3.30 \pm 0.85
Total N (mg N kg dry sediment ⁻¹)	1.12 \pm 0.15	0.95 \pm 0.08	1.36 \pm 0.06	13.4 \pm 0.17	5.15 \pm 0.32	6.53 \pm 0.15
NH ₄ ⁺ (mg N kg wet sediment ⁻¹)	2.55 \pm 0.88	1.19 \pm 0.01	1.38 \pm 0.06	0.99 \pm 0.10	1.60 \pm 0.37	1.84 \pm 0.89
NO ₃ ⁻ (mg N kg wet sediment ⁻¹)	0.41 \pm 0.28	0.11 \pm 0.04	0.20 \pm 0.04	1.70 \pm 0.00	0.81 \pm 0.00	0.19 \pm 0.00
Total free amino acids (mg N kg wet sediment ⁻¹)	0.27 \pm 0.03	0.13 \pm 0.02	0.19 \pm 0.01	0.18 \pm 0.01	0.21 \pm 0.03	0.22 \pm 0.02
Molybdate-reactive P (mg P kg wet sediment ⁻¹)	2.59 \pm 0.26	1.64 \pm 0.43	1.92 \pm 0.19	0.20 \pm 0.04	0.12 \pm 0.04	0.30 \pm 0.13

Appendix 3

Table S5.4 Analysis of total mass of phospholipid-derived fatty acids (PLFA) and taxonomic groups of concentrated water samples and freeze-dried sediment samples used in the study. Sediment values represent mean \pm SEM ($n = 3$).

	Lowland mesotrophic sites			Upland oligotrophic sites		
	1	2	3	4	5	6
Water						
Total PLFA biomass (nmol ml water ⁻¹)	0.04	0.08	0.08	0.04	0.25	0.07
Gram – bacteria (%)	50	44.1	59.5	55	66.7	52.7
Gram + bacteria (%)	30.7	41.7	26.7	28.8	20.1	33.4
Actinomycetes (%)	3.97	3.08	2.71	4.42	1.13	1.16
Fungi (%)	4.22	4.14	3.4	1.43	2.1	2.05
Eukaryote (%)	5.54	3.7	4.19	3.09	7.68	3.75
Sediment						
Total PLFA biomass (nmol g sediment ⁻¹)	117 \pm 54	239 \pm 55	100 \pm 1	1134 \pm 186	531 \pm 38	199 \pm 47
Gram - bacteria (%)	43.8 \pm 6.1	46.7 \pm 1.6	50.7 \pm 0.3	47.2 \pm 1.8	48.4 \pm 0.5	47.8 \pm 1.6
Gram + bacteria (%)	24.5 \pm 1.6	24.8 \pm 1.7	27.2 \pm 0.4	28.3 \pm 2.7	32.9 \pm 5.3	29.0 \pm 1.9
Actinomycetes (%)	5.25 \pm 1.64	5.59 \pm 1.14	9.53 \pm 0.54	8.79 \pm 0.85	6.45 \pm 3.18	9.57 \pm 1.32
Fungi (%)	11.0 \pm 8.1	3.6 \pm 1.2	1.7 \pm 0.0	4.5 \pm 0.3	4.2 \pm 0.5	4.9 \pm 0.4
Eukaryote (%)	10.2 \pm 1.0	11.3 \pm 3.9	4.4 \pm 1.1	7.6 \pm 0.5	5.6 \pm 1.8	5.8 \pm 0.6

Appendix 3

Table S5.5 Results from a two-way ANOVA for each isotopically-labelled nutrient, land-cover and sample type for assay end points (% of initial activity remaining). * Denotes a significant *P*-value. The significance level was set at $P < 0.05$.

Sample type	Nutrient	Effect of land-cover		Effect of concentration		Interaction concentration × land-cover	
		F	<i>P</i> -value	F	<i>P</i> -value	F	<i>P</i> -value
Sediment	¹⁴ C amino acids	28	<0.001*	58	<0.001*	14	<0.001*
Sediment	¹⁴ C glucose	16	0.001*	55	<0.001*	3	0.032*
Sediment	¹⁴ C organic acids	2	0.202	58	<0.001*	11	<0.001*
Sediment	¹⁴ C phenolics	18	<0.001*	14	<0.001*	1	0.477
Water	¹⁴ C amino acids	95	<0.001*	61	<0.001*	10	<0.001*
Water	¹⁴ C glucose	14	<0.001*	14	<0.001*	14	<0.001*
Water	¹⁴ C organic acids	482	<0.001*	195	<0.001*	114	<0.001*
Water	¹⁴ C phenolics	52	<0.001*	39	<0.001*	4	0.009*
Sample type	Land-cover	Effect of functional group		Effect of concentration		Interaction concentration × functional group	
		F	<i>P</i> -value	F	<i>P</i> -value	F	<i>P</i> -value
Sediment	Lowland mesotrophic	92	<0.001*	49	<0.001*	3	<0.001

Appendix 3

Sediment	Upland oligotrophic	4	0.018*	42	<0.001*	5	<0.001*
Water	Lowland mesotrophic	70	<0.001*	135	<0.001*	20	<0.001*
Water	Upland oligotrophic	10	<0.001*	81	<0.001*	2	0.028*
Land-cover	Nutrient	Effect of sample type		Effect of concentration		Interaction concentration × sample type	
		F	<i>P</i> -value	F	<i>P</i> -value	F	<i>P</i> -value
Lowland mesotrophic	¹⁴ C amino acids	1	0.424	23	<0.001*	30	<0.001*
Lowland mesotrophic	¹⁴ C glucose	7	0.019*	9	0.001*	13	<0.001*
Lowland mesotrophic	¹⁴ C organic acids	580	<0.001*	8	0.002*	7	0.003*
Lowland mesotrophic	¹⁴ C phenolics	99	<0.001*	80	<0.001*	75	<0.001*
Upland oligotrophic	¹⁴ C amino acids	314	<0.001*	180	<0.001*	188	<0.001*
Upland oligotrophic	¹⁴ C glucose	0	0.873	48	<0.001*	1	0.524
Upland oligotrophic	¹⁴ C organic acids	1	0.348	150	<0.001*	127	<0.001*
Upland oligotrophic	¹⁴ C phenolics	17	0.001*	18	<0.001*	13	<0.001*

Appendix 3

Table S5.6 Results from a two-way ANOVA for each isotopically-labelled nutrient, land-cover and sample type for initial rate of activity ($\mu\text{mol h}^{-1}$). * Denotes a significant P -value. The significance level was set at $P < 0.05$.

Sample type	Nutrient	Effect of land-cover		Effect of concentration		Interaction concentration \times land-cover	
		F	P -value	F	-value	F	P -value
Sediment	^{14}C amino acids	9	0.006*	33	<0.001*	5	0.003*
Sediment	^{14}C glucose	5	0.037*	123	<0.001*	5	0.003*
Sediment	^{14}C organic acids	18	<0.001*	105	<0.001*	6	0.010*
Sediment	^{14}C phenolics	31	<0.001*	89	<0.001*	12	<0.001*
Water	^{14}C amino acids	0.136	0.715	5	0.002*	0	0.991
Water	^{14}C glucose	6	0.020*	1011	<0.001*	9	<0.001*
Water	^{14}C organic acids	0	0.947	77	<0.001*	0	1.000
Water	^{14}C phenolics	2	0.189	23	<0.001*	2	0.139

Sample type	Land-cover	Effect of functional group		Effect of concentration		Interaction concentration \times functional group	
		F	P -value	F	P -value	F	P -value
Sediment	Lowland mesotrophic	9	<0.001*	113	<0.001*	5	<0.001*
Sediment	Upland oligotrophic	20	<0.001*	142	<0.001*	9	<0.001*
Water	Lowland mesotrophic	2	0.199	13	<0.001*	1	0.429

Appendix 3

Water		60	<0.001*	238	<0.001*	48	<0.001*
Land-cover	Nutrient	Effect of sample type		Effect of concentration		Interaction concentration × sample type	
		F	<i>P</i> -value	F	<i>P</i> -value	F	<i>P</i> -value
Lowland mesotrophic	¹⁴ C amino acids	5	0.036*	9	<0.001*	2	0.226
Lowland mesotrophic	¹⁴ C glucose	43	<0.001*	40	<0.001*	29	<0.001*
Lowland mesotrophic	¹⁴ C organic acids	61	<0.001*	174	<0.001*	29	<0.001*
Lowland mesotrophic	¹⁴ C phenolics	0	0.579	8	0.001*	0	0.923
Upland oligotrophic	¹⁴ C amino acids	21	<0.001	22	<0.001*	12	<0.001*
Upland oligotrophic	¹⁴ C glucose	136	<0.001*	148	<0.001*	101	<0.001*
Upland oligotrophic	¹⁴ C organic acids	67	<0.001*	117	<0.001*	38	<0.001*
Upland oligotrophic	¹⁴ C phenolics	26	<0.001*	209	<0.001*	20	<0.001*

Appendix 3

Table S5.7 Results from a two-way ANOVA for each isotopically-labelled nutrient, land-cover and sample type for initial rate of activity ($\mu\text{mol cm}^{-3} \text{h}^{-1}$). * Denotes a significant *P*-value. The significance level was set at $P < 0.05$.

Land-cover	Sample type	Concentration (μM)	End point (%)			
			Amino acids	Glucose	Organic acids	Phenolics
Lowland mesotrophic	Sediment	10000	47.1 \pm 0.1	-	76.1 \pm 2.7	81.4 \pm 4.7
		5000	31.2 \pm 1.3	72.8 \pm 6.2	51.3 \pm 4.1	73.1 \pm 11.8
		500	25.5 \pm 0.3	46.1 \pm 12.2	33.7 \pm 0.2	47.6 \pm 0.3
		50	27.5 \pm 0.3	7.4 \pm 0.1	33.0 \pm 0.1	46.7 \pm 0.4
		5	27.8 \pm 0.3	8.1 \pm 0.0	33.1 \pm 0.1	46.8 \pm 0.3
		0.5	28.3 \pm 0.3	8.3 \pm 0.0	33.3 \pm 0.0	46.5 \pm 0.1
		0.1	-	8.4 \pm 0.1	-	-
Upland oligotrophic	Sediment	10000	66.3 \pm 3.3	-	72.2 \pm 7.8	71.0 \pm 9.2
		5000	57.8 \pm 7.4	90.7 \pm 2.4	54.8 \pm 6.9	44.0 \pm 9.1
		500	25.8 \pm 0.4	86.2 \pm 1.8	38.5 \pm 4.6	38.7 \pm 6.0
		50	27.0 \pm 0.4	28.8 \pm 16.4	34.3 \pm 0.9	37.1 \pm 2.6
		5	26.7 \pm 0.2	11.7 \pm 1.8	35.0 \pm 1.3	34.3 \pm 0.1
		0.5	27.1 \pm 0.2	10.4 \pm 0.6	34.7 \pm 1.0	34.0 \pm 0.2
		0.1	-	9.9 \pm 0.1	-	-
Lowland mesotrophic	Water	500	50.0 \pm 4.2	-	16.4 \pm 4.2	70.5 \pm 2.8
		50	25.5 \pm 5.1	6.3 \pm 0.7	5.0 \pm 0.4	35.9 \pm 6.4
		10	13.8 \pm 0.5	6.0 \pm 0.6	4.9 \pm 0.5	8.6 \pm 0.7

Appendix 3

		5	14.0 ± 0.5	5.7 ± 0.7	4.7 ± 0.3	5.8 ± 0.3
		1	13.1 ± 0.5	5.7 ± 0.6	4.8 ± 0.4	5.9 ± 0.2
		0.5	14.2 ± 0.2	5.2 ± 0.4	4.4 ± 0.2	6.1 ± 0.2
		0.1	-	6.8 ± 0.6	-	-
		500	75.2 ± 1.0	-	81.2 ± 2.9	82.6 ± 0.9
		50	68.2 ± 4.0	79.0 ± 4.5	50.2 ± 1.3	70.0 ± 4.0
		10	44.5 ± 9.0	38.0 ± 14.6	15.6 ± 2.4	56.8 ± 12.5
Upland oligotrophic	Water	5	20.3 ± 1.2	21.3 ± 8.3	9.0 ± 1.2	41.0 ± 13.0
		1	19.3 ± 1.2	9.9 ± 0.8	9.0 ± 0.5	14.9 ± 4.4
		0.5	20.7 ± 0.8	12.9 ± 0.6	7.9 ± 0.7	15.1 ± 3.4
		0.1	-	13.0 ± 0.2	-	-
Land-cover	Sample type	Concentration (µM)	Initial rate (µmol cm ⁻³ h ⁻¹)			
			Amino acids	Glucose	Organic acids	Phenolics
		10000	0.45815 ± 0.13368	-	0.63589 ± 0.06816	1.05436 ± 0.11036
		5000	0.12552 ± 0.04502	0.27064 ± 0.03011	0.33935 ± 0.09734	0.39688 ± 0.07032
		500	0.03335 ± 0.01149	0.03152 ± 0.00472	0.07488 ± 0.00685	0.03657 ± 0.00786
Lowland mesotrophic	Sediment	50	0.01277 ± 0.00165	0.00438 ± 0.00201	0.01275 ± 0.00035	0.00664 ± 0.00025
		5	0.00214 ± 0.00014	0.00053 ± 0.00018	0.00316 ± 0.00020	0.00075 ± 0.00012
		0.5	0.00038 ± 0.00003	0.00016 ± 0.00005	0.00044 ± 0.00002	0.00012 ± 0.00002
		0.1	-	0.00009 ± 0.00001	-	-
		10000	1.02168 ± 0.16661	-	0.98712 ± 0.09294	1.55920 ± 0.21682
Upland oligotrophic	Sediment	5000	0.26823 ± 0.11391	0.40233 ± 0.05102	0.63706 ± 0.05637	1.43057 ± 0.15824
		500	0.06761 ± 0.01459	0.03273 ± 0.00283	0.11430 ± 0.01074	0.16049 ± 0.01572
		50	0.01513 ± 0.00182	0.00324 ± 0.00026	0.01873 ± 0.00325	0.01634 ± 0.00210

Appendix 3

		5	0.00198	±	0.00030	0.00038	±	0.00019	0.00308	±	0.00054	0.00199	±	0.00020
		0.5	0.00034	±	0.00004	0.00009	±	0.00002	0.00037	±	0.00006	0.00025	±	0.00003
		0.1	-		0.00004		±	0.00002	-			-		
		500	0.01340	±	0.00816		-		0.03042	±	0.00323	0.04803	±	0.02653
		50	0.00243	±	0.00039	0.00260	±	0.00014	0.00138	±	0.00044	0.01118	±	0.00076
		10	0.00034	±	0.00008	0.00032	±	0.00003	0.00021	±	0.00008	0.00039	±	0.00024
		5	0.00019	±	0.00005	0.00010	±	0.00002	0.00006	±	0.00006	0.00051	±	0.00026
		1	0.00008	±	0.00001	0.00001	±	0.00001	0.00002	±	0.00001	0.00004	±	0.00001
		0.5	0.00004	±	0.00001	0.00001	±	0.00000	0.00006	±	0.00002	0.00003	±	0.00002
		0.1	-		0.00000		±	0.00000	-			-		
		500	0.01022	±	0.00541		-		0.03057	±	0.00603	0.08454	±	0.00370
		50	0.00198	±	0.00025	0.00310	±	0.00009	0.00171	±	0.00018	0.01014	±	0.00132
		10	0.00031	±	0.00009	0.00030	±	0.00002	0.00013	±	0.00004	0.00099	±	0.00008
		5	0.00026	±	0.00007	0.00006	±	0.00001	0.00017	±	0.00011	0.00078	±	0.00005
		1	0.00006	±	0.00000	0.00001	±	0.00001	0.00001	±	0.00001	0.00002	±	0.00002
		0.5	0.00003	±	0.00001	0.00001	±	0.00000	0.00001	±	0.00001	0.00002	±	0.00000
		0.1	-		0.00000		±	0.00000	-			-		

Appendix 3

Table S5.8 Parameters derived from Lineweaver-Burke plots based on kinetics data for ^{14}C amino acids, glucose, organic acids and phenolics.

V_{\max} is the maximum reaction velocity ($\mu\text{mol h}^{-1} \text{mL}^{-1}$) and K_m (Michaelis-Menten constant) is the substrate concentration at which half V_{\max} can be achieved (μM).

	Sediment mesotrophic			Sediment oligotrophic			Water mesotrophic			Water oligotrophic		
	V_{\max}	K_m	r^2	V_{\max}	K_m	r^2	V_{\max}	K_m	r^2	V_{\max}	K_m	r^2
Amino acids	23.2	0.02	0.99	0.0004	1.03	0.92	0.0199	28.99	1.00	0.0004	1.41	0.93
Glucose	1.43	0.00	0.89	0.0002	16.6	0.99	0.0009	2.216	0.92	0.0000	3.59	0.99
Organic acids	36.1	0.03	1.00	0.0003	1.62	0.98	0.0564	75.34	1.00	0.0001	0.83	0.64
Phenolics	39.0	0.01	1.00	0.0003	0.85	0.69	0.0429	86.37	1.00	0.0002	0.72	0.53

Appendix 4

Appendix 4: Supplementary material for Chapter 6

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

Francesca L. Brailsford, Helen C. Glanville, Peter N. Golyshin, Miles R. Marshall, Charlotte
E. Lloyd, Penny J. Johnes, Davey L. Jones.

Appendix 4

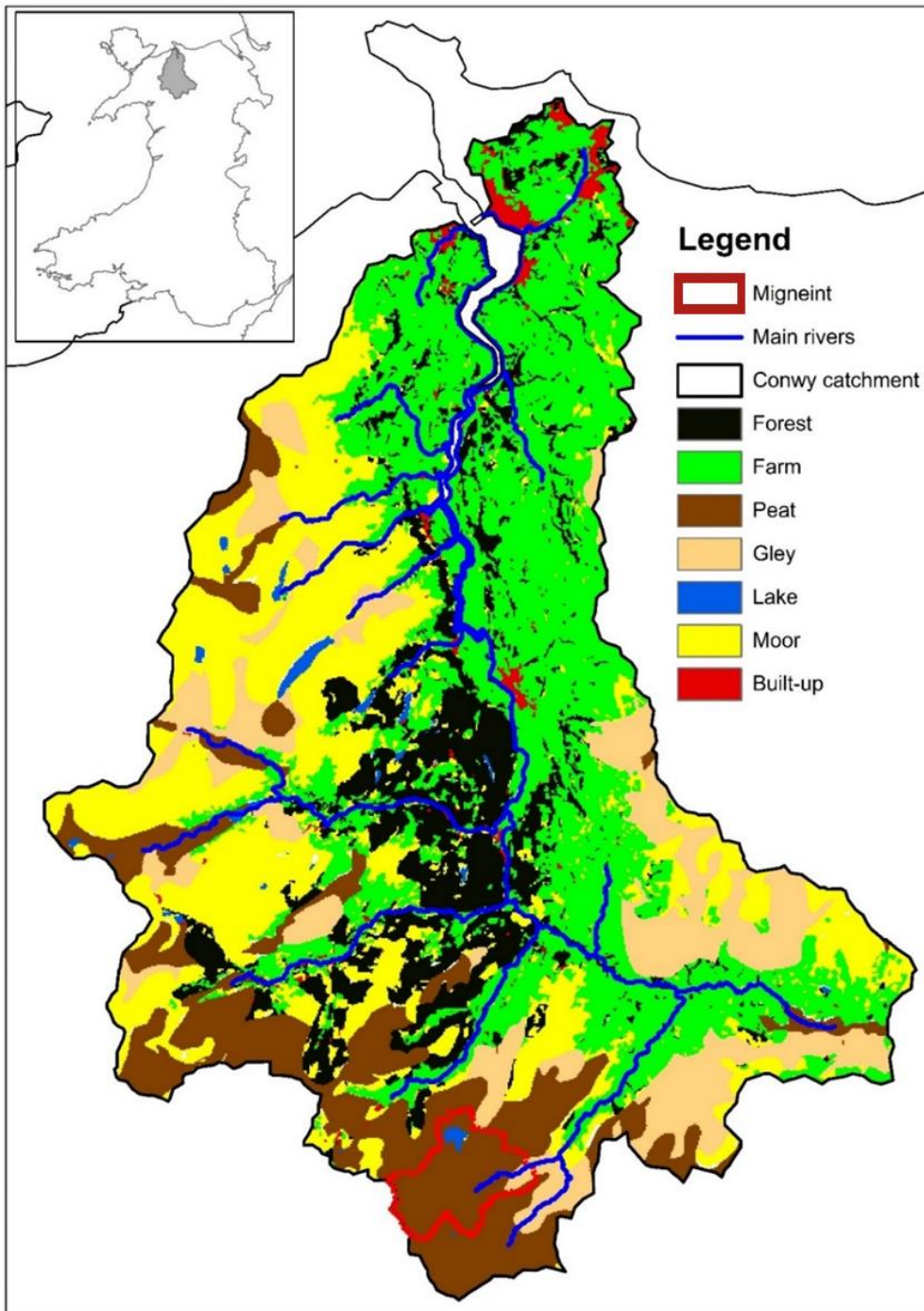


Figure S6.1 Land use map of the Conwy catchment with upland peat bog (Migneint) sub-catchment in the headwaters of the catchment (outlined in red).

Appendix 4

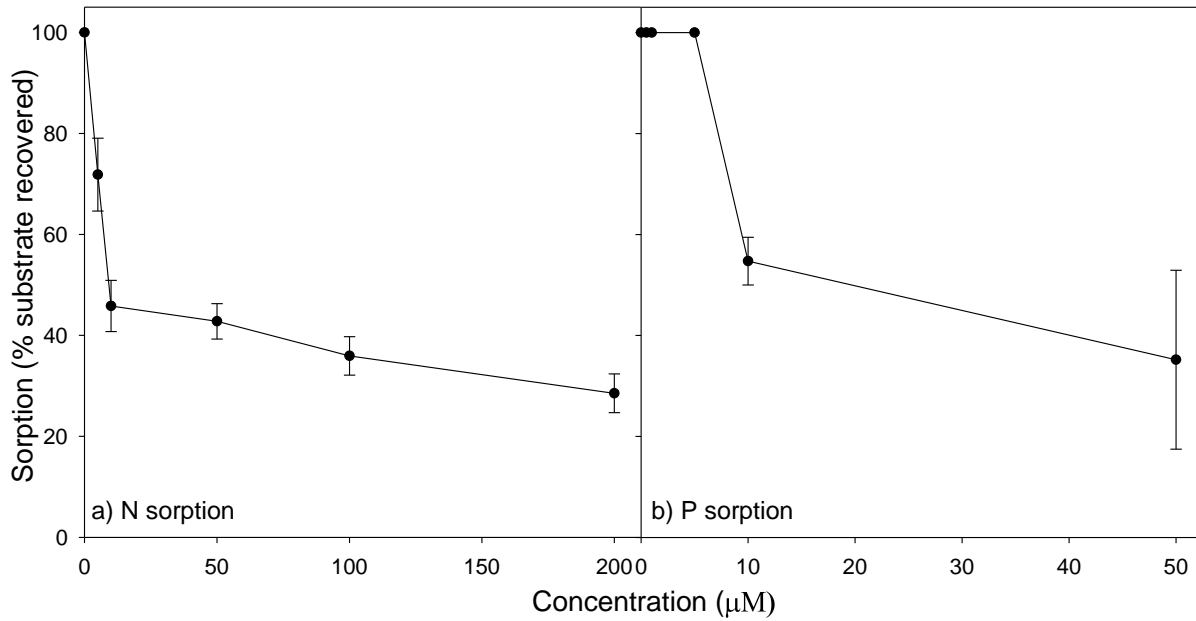


Figure S6.2 Sorption of N added as NH_4NO_3 and P added as NH_2PO_4 to the solid phase of river sediment measured at different concentrations of added N and P. Values represent means \pm SEM, $n = 3$.

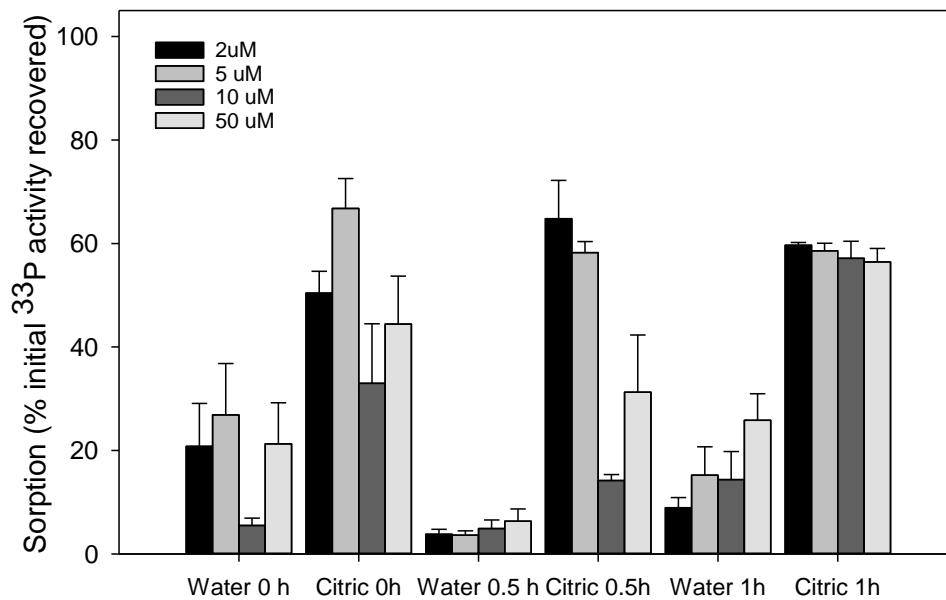


Figure S6.3 Natural (water extract) and maximal (citric acid extract) desorption of ^{33}P -orthophosphate from the solid phase of river sediment. Different coloured bars represent concentrations of P added as NH_2PO_4 . Values represent means \pm SEM, $n = 3$.

Appendix 4

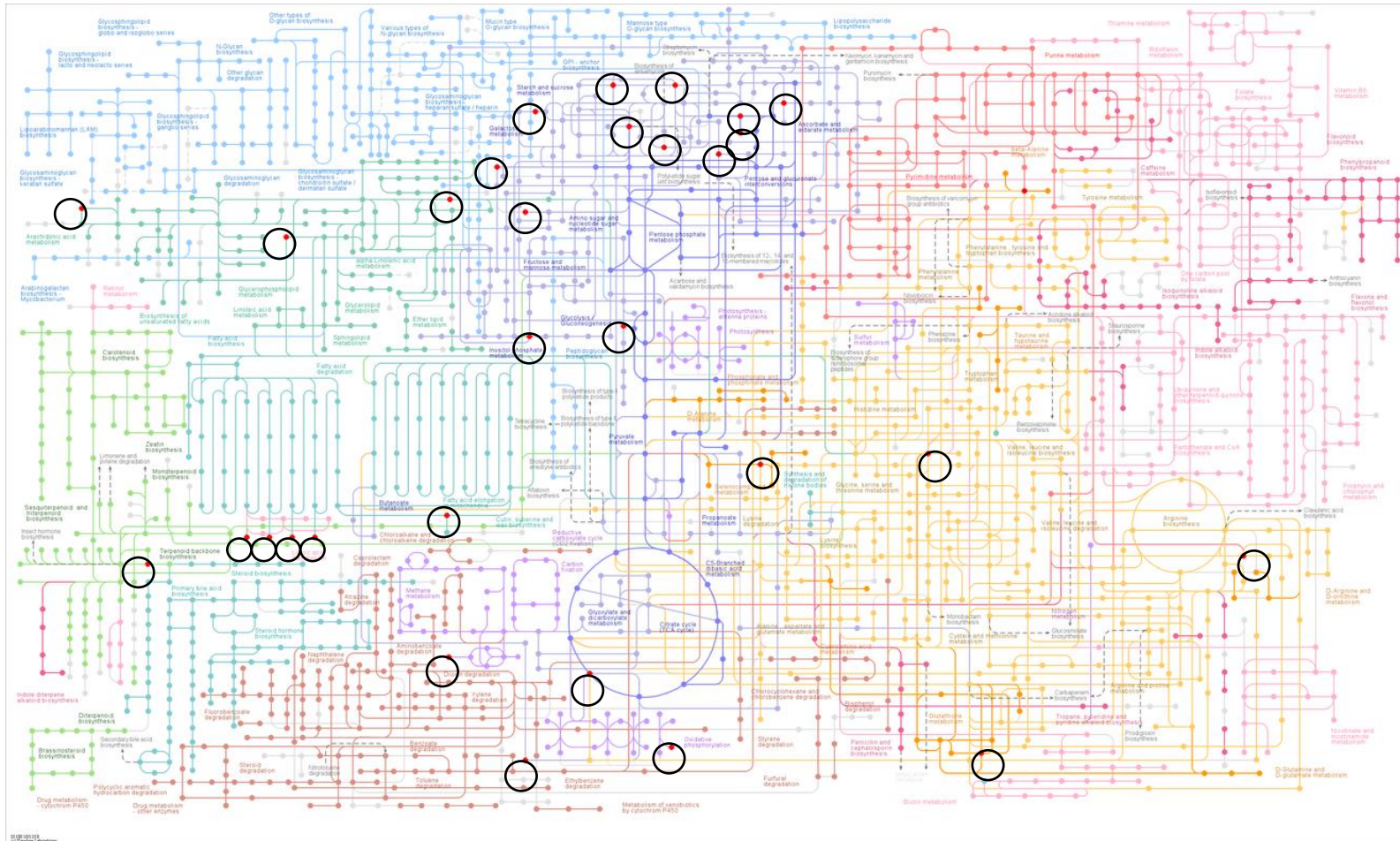


Figure S6.4. Map showing metabolic pathways detected in samples using untargeted metabolomics. Red dots (circled) indicate the main compounds identified within the samples collected for this experiment (Created using KEGG Mapper: <https://www.genome.jp/kegg/mapper.html>).

Appendix 4

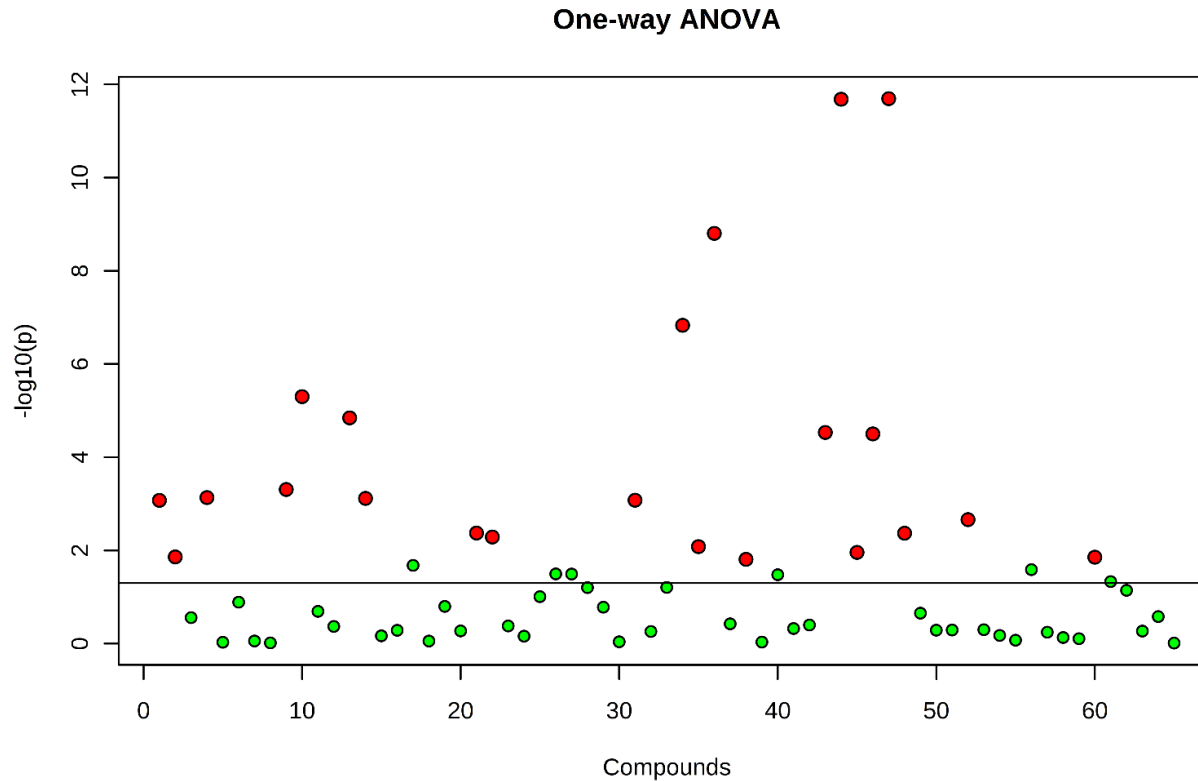


Figure S6.5 Results of a one-way ANOVA between concentrations of assigned metabolites in upland peat river sediments. The P -values shown on the y-axis for each metabolite are transformed by $-\log_{10}$. The black line denotes the threshold P -value: $P = 0.05$. Individual red dots correspond to a metabolite with significant differences between treatments, whilst green dots indicate a metabolite with no significant differences between treatments. The higher up the y-axis, the lower the P -value for that metabolite.

Appendix 4

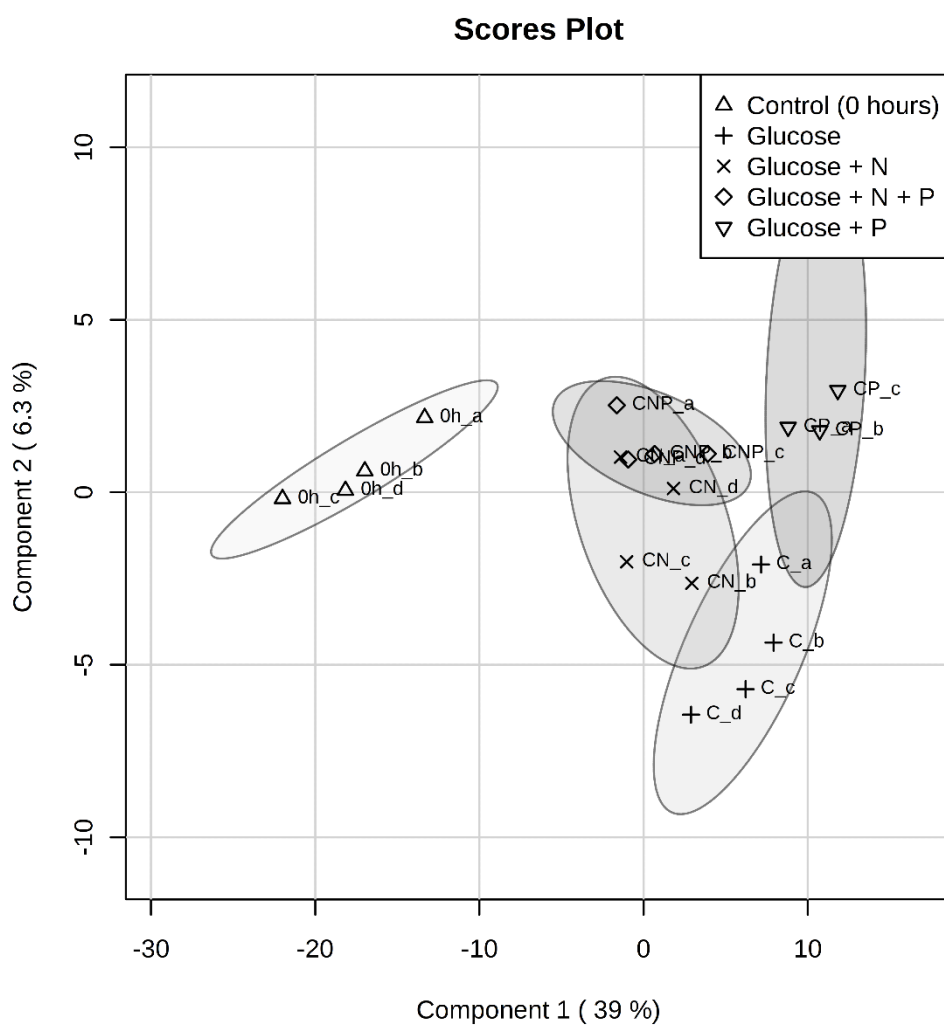


Figure S6.6 PLS-DA (PLS discriminant analysis) scores plot for the sediment primary metabolome for each treatment (0 h (control), 24 h (glucose, glucose + N, glucose + N + P, glucose + P)), including unidentified metabolites. Lowercase letters indicate sample site.

Appendix 4

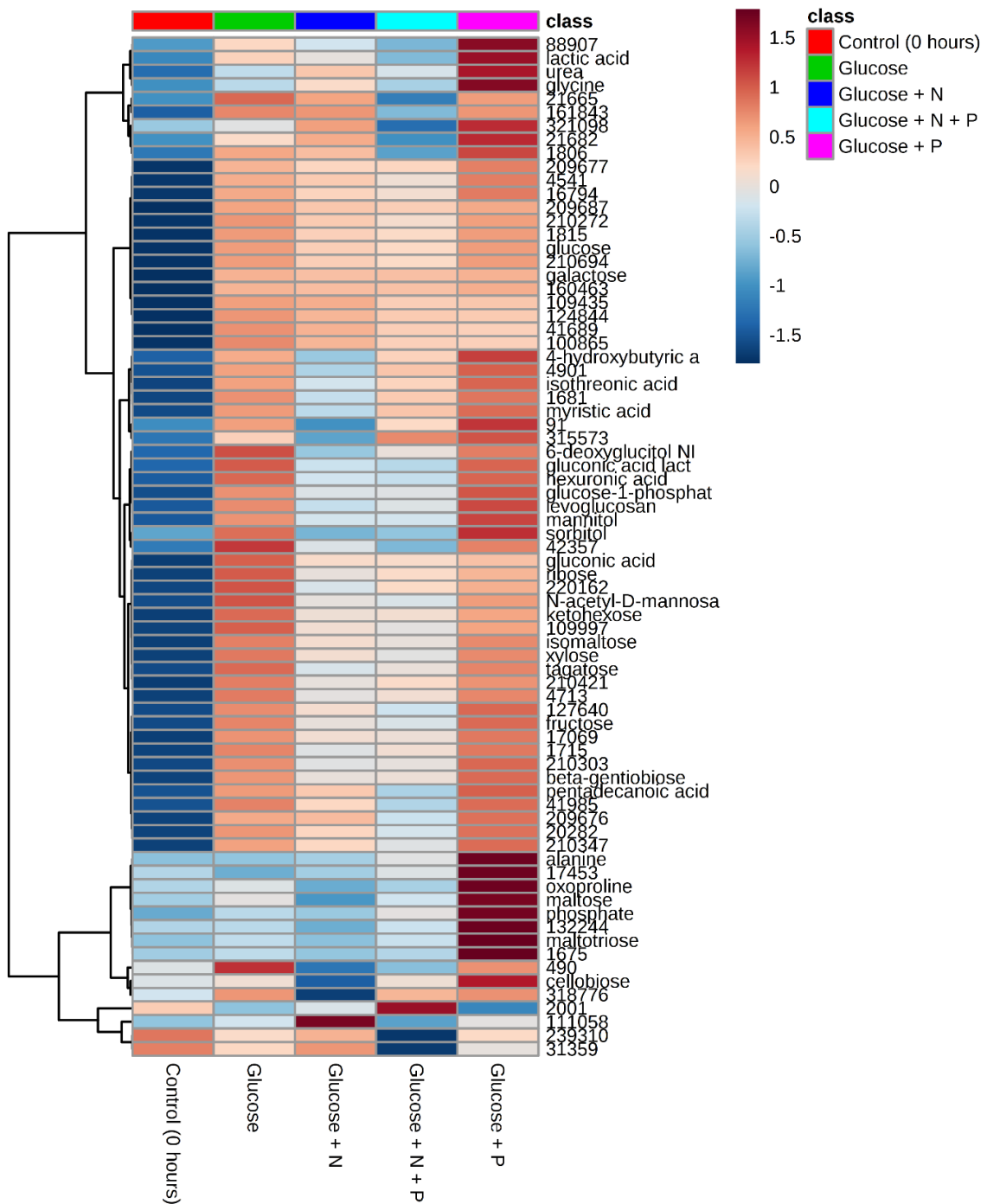


Figure S6.7 Heat map of mean changes in sediment primary metabolome for each treatment (0 h (control), 24 h (glucose, glucose + N, glucose + N + P, glucose + P)) including unidentified metabolites. Metabolites are clustered by similarity according to Pearson correlation values. The top 75 metabolites according to two-way analysis of variance (ANOVA) are displayed.

Appendix 4

Table S6.1. Results from one-way ANOVA for $^{14}\text{CO}_2$ respiration and ^{14}C -glucose depletion for each time-point. * Denotes a significant P -value. The significance level was set at $P < 0.05$. Superscript letters represent the results of post-hoc testing with different letters indicating significant differences between treatments at the $P < 0.05$ level.

Variable	Time (h)	Glucose	Glucose + N	Glucose + N + P	Glucose + P	F -value	P -value
$^{14}\text{CO}_2$ respiration	1	0.24 ± 0.05	0.15 ± 0.02	0.34 ± 0.03	0.25 ± 0.05	3	0.08
$^{14}\text{CO}_2$ respiration	2	0.48 ± 0.05 ^{a,b}	0.29 ± 0.01 ^a	0.55 ± 0.03 ^b	0.44 ± 0.06 ^{a,b}	5	0.016*
$^{14}\text{CO}_2$ respiration	4	0.72 ± 0.04 ^a	0.48 ± 0.01 ^b	0.80 ± 0.03 ^a	0.72 ± 0.06 ^a	10	0.001*
$^{14}\text{CO}_2$ respiration	6	0.93 ± 0.04 ^a	0.65 ± 0.01 ^b	1.02 ± 0.04 ^a	0.94 ± 0.06 ^a	11	0.001*
$^{14}\text{CO}_2$ respiration	24	1.89 ± 0.10 ^a	1.26 ± 0.04 ^b	1.78 ± 0.05 ^a	1.92 ± 0.09 ^b	13	<0.001*
$^{14}\text{CO}_2$ respiration	48	2.82 ± 0.24 ^a	1.93 ± 0.12 ^b	2.62 ± 0.12 ^{a,b}	3.09 ± 0.20 ^b	6	0.011*
$^{14}\text{CO}_2$ respiration	168	10.9 ± 1.32	6.74 ± 1.03	13.2 ± 1.88	13.8 ± 2.58	2	0.122
^{14}C -glucose depletion	2	41.9 ± 2.42 ^{a,b}	25.90 ± 2.44 ^a	48.3 ± 3.51 ^b	38.0 ± 6.34 ^{a,b}	6	0.013*
^{14}C -glucose depletion	4	35.1 ± 3.58 ^{a,b}	22.70 ± 1.44 ^a	40.8 ± 4.07 ^b	33.1 ± 4.19 ^{a,b}	5	0.022*
^{14}C -glucose depletion	6	34.9 ± 2.42	25.00 ± 1.12	38.8 ± 5.38	33.1 ± 3.26	3	0.079
^{14}C -glucose depletion	24	42.4 ± 2.76 ^{a,b}	31.20 ± 2.80 ^a	45.5 ± 2.95 ^b	40.5 ± 3.09 ^{a,b}	5	0.024*
^{14}C -glucose depletion	48	37.6 ± 1.98 ^a	23.50 ± 0.81 ^b	42.0 ± 2.69 ^a	35.0 ± 4.61 ^{a,b}	7	0.004*

Appendix 4

Table S6.2. Results from a two-way mixed ANOVA for ^{14}C -labelled glucose depletion and $^{14}\text{CO}_2$ respiration.* Denotes a significant P -value. The significance level was set at $P < 0.05$.

Variable	Simple effect of time		Interaction time \times treatment	
	F	P -value	F	P -value
^{14}C -glucose depletion	75	<0.001*	3	0.002*
$^{14}\text{CO}_2$ respiration	6	0.019*	3	0.018*

Appendix 4

Table S6.3. Summary of significant results from one-way ANOVA for each metabolite. The significance level was set at $P < 0.05$ for the adjusted P -value (false discovery rate, FDR). Post-hoc multiple pairwise testing was used to identify differences between treatments using Tukey's HSD testing. Significant pairwise comparisons are listed.

Metabolite	<i>F</i> -value	<i>P</i> -value	$-\log_{10}(P)$	FDR	Tukey's HSD
Galactose	177	<0.001	11.7	6.78E-11	Control ^a , Glucose ^b , Glucose + N ^b , Glucose + N +P ^b , Glucose + P ^b
Glucose	176	<0.001	11.7	6.78E-11	Control ^a , Glucose ^b , Glucose + N ^c , Glucose + N +P ^c , Glucose + P ^b
Isomaltose	70	<0.001	8.80	3.42E-08	Control ^a , Glucose ^b , Glucose + N ^b , Glucose + N +P ^b , Glucose + P ^b
Ketohexose	36	<0.001	6.83	2.40E-06	Control ^a , Glucose ^b , Glucose + N ^c , Glucose + N +P ^c , Glucose + P ^b
Ribose	21	<0.001	5.30	6.53E-05	Control ^a , Glucose ^b , Glucose + N ^c , Glucose + N +P ^{b,c} , Glucose + P ^{b,c}
Phosphate	18	<0.001	4.84	1.55E-04	Control ^a , Glucose ^a , Glucose + N ^a , Glucose + N +P ^a , Glucose + P ^b
Glucose-1-phosphate	16	<0.001	4.53	2.57E-04	Control ^a , Glucose ^{b,c} , Glucose + N ^b , Glucose + N +P ^b , Glucose + P ^c
Gluconic acid	16	<0.001	4.50	2.57E-04	Control ^a , Glucose ^b , Glucose + N ^b , Glucose + N +P ^b , Glucose + P ^b
Sorbitol	10	<0.001	3.31	3.56E-03	Control ^a , Glucose ^b , Glucose + N ^c , Glucose + N +P ^c , Glucose + P ^d
Tagatose	9	<0.001	3.13	4.22E-03	Control ^a , Glucose ^b , Glucose + N ^a , Glucose + N +P ^b , Glucose + P ^b

Appendix 4

Pentadecanoic acid	9	<0.001	3.12	4.22E-03	Control ^a , Glucose ^b , Glucose + N ^b , Glucose + N +P ^{a,b} , Glucose + P ^b
Levoglucozan	9	<0.001	3.08	4.22E-03	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b
Xylose	9	<0.001	3.07	4.22E-03	Control ^a , Glucose ^b , Glucose + N ^b , Glucose + N +P ^{a,b} , Glucose + P ^b
Cellobiose	7	<0.001	2.66	1.02E-02	Control ^{a,b} , Glucose ^{a,b} , Glucose + N ^a , Glucose + N +P ^{a,b} , Glucose + P ^b
N-acetyl-D-mannosamine	6	<0.001	2.37	1.74E-02	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b
Fructose	6	<0.001	2.37	1.74E-02	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b
Myristic acid	6	0.010	2.29	1.97E-02	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^b , Glucose + P ^b
Isothreonic acid	5	0.010	2.08	3.00E-02	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b
Gluconic acid lactone	5	0.010	1.96	3.78E-02	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b
Urea	4	0.010	1.86	4.33E-02	Control ^a , Glucose ^{a,b} , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b
Alanine	4	0.010	1.85	4.33E-02	Control ^a , Glucose ^a , Glucose + N ^a , Glucose + N +P ^{a,b} , Glucose + P ^b
Hexuronic acid	4	0.020	1.81	4.59E-02	Control ^a , Glucose ^b , Glucose + N ^{a,b} , Glucose + N +P ^{a,b} , Glucose + P ^b

Appendix 6

Appendix 5: Supplementary material for Chapter 7

Rapid microbial consumption of dissolved organic sulphur (DOS) in freshwaters

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Appendix 6

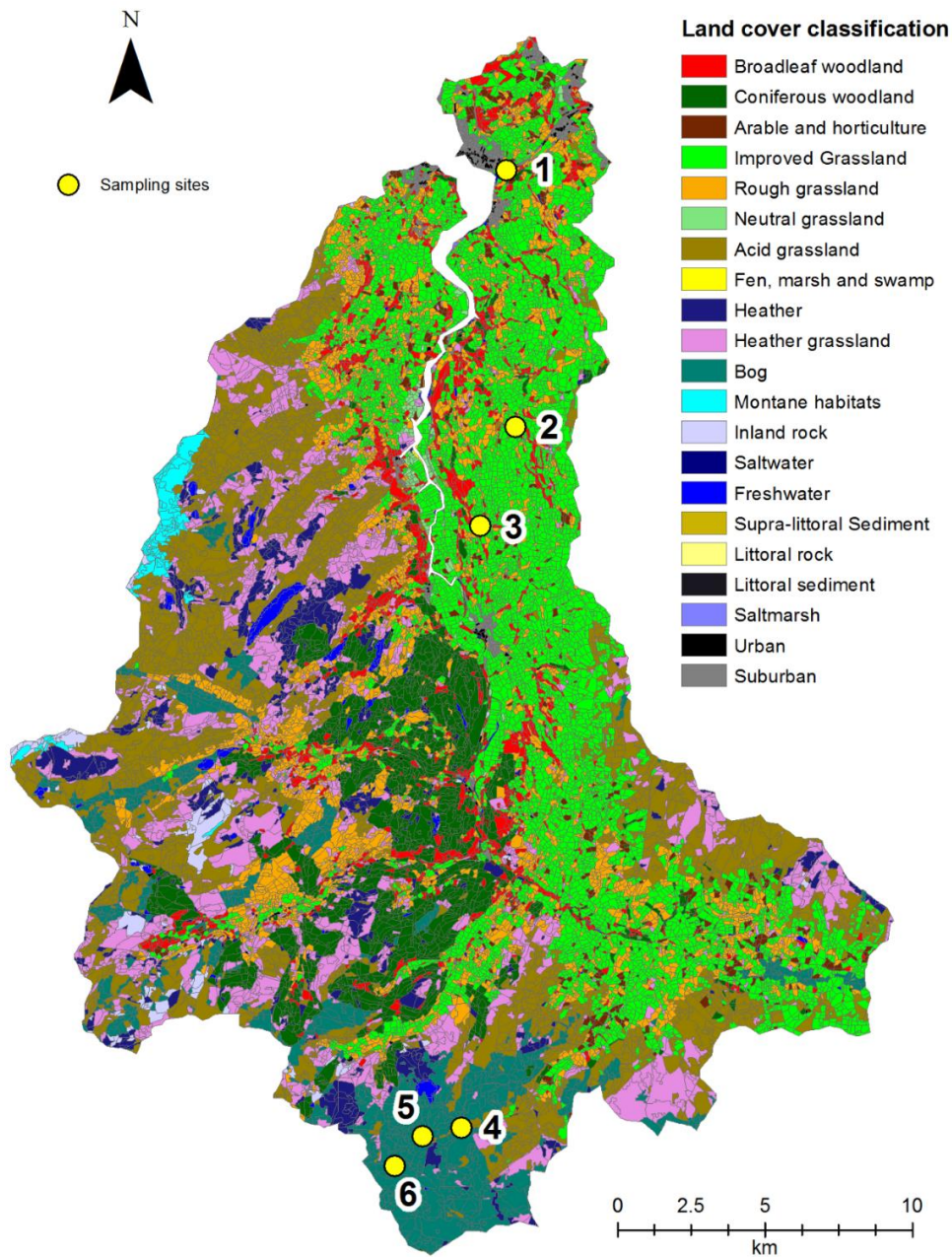


Fig. S7.1 Land cover map of the Conwy catchment with lowland improved grassland sites (1-3) and upland peat bog sites (4-6) indicated. Created with ArcGIS Hydrology toolbox (ESRI 2018, Version 10 Redlands, CA) using LCM2007 data provided by the Centre for Ecology and Hydrology (Emmett et al. 2016). Reproduced from Brailsford et al. 2019.

Appendix 6

Table S7.1 Chemical characteristics of the water samples used in the study. Values represent means \pm SEM, rows 1-3 are $n = 3$, rows 4-11 are $n = 9$. *Denotes a significant P -value when comparing the two sites. The significance level was set at $P < 0.05$. Rows 4-11 reproduced from Brailsford et al. (2019). BLD indicates below the limit of detection ($<1 \text{ ng S L}^{-1}$).

	Lowland	Upland		
	mesotrophic	oligotrophic	F	P-value
Sulphate (mg S L^{-1})	0.11 ± 0.01	0.11 ± 0.02	0	0.877
Total free thiols (ng S L^{-1})	12.7 ± 3.9	BLD	-	-
pH	7.09 ± 0.08	4.20 ± 0.16	14	$<0.001^*$
Electrical conductivity ($\mu\text{S cm}^{-1}$)	191 ± 8	49 ± 5	13	$<0.001^*$
Temperature	7.53 ± 0.37	9.00 ± 0.65	3	0.421
Dissolved organic C (mg C L^{-1})	2.86 ± 0.13	7.60 ± 0.62	6	$<0.001^*$
Total free carbohydrates (mg C L^{-1})	0.11 ± 0.02	0.09 ± 0.02	1	0.450
Total phenols (mg C L^{-1})	2.27 ± 0.66	1.78 ± 0.74	0	0.667
Total dissolved N (mg N L^{-1})	2.13 ± 0.24	0.38 ± 0.02	7	$<0.001^*$
NH_4^+ (mg N L^{-1})	0.05 ± 0.01	0.06 ± 0.01	0	0.818
NO_3^- (mg N L^{-1})	1.73 ± 0.24	0.02 ± 0.00	7	$<0.001^*$
Total free amino acids (mg N L^{-1})	0.10 ± 0.01	0.13 ± 0.01	2	0.142
Molybdate-reactive P (mg P L^{-1})	0.07 ± 0.01	0.03 ± 0.00	4	$<0.001^*$