Dissolved organic nutrient uptake by riverine phytoplankton varies along a gradient of nutrient enrichment

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HIGHLIGHTS

- Dissolved organic nitrogen and phosphorus are bioavailable to river phytoplankton.
- Nutrient limitation and dissolved organic nutrient use varies in time and space.
- Dissolved organic nutrient use is greatest when nutrient concentrations are low.
- For simple compounds, growth occurred even at low organic nutrient concentration.
- DOM in rivers has been overlooked as a potential nutrient source to phytoplankton.

GRAPHICAL ABSTRACT
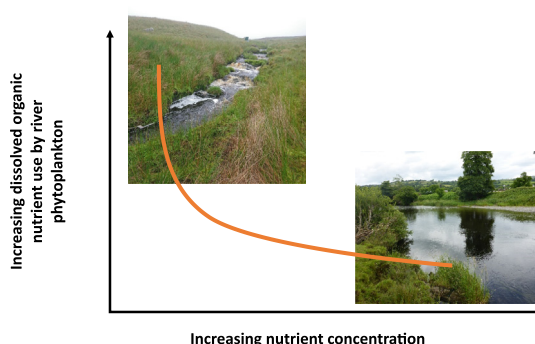
ABSTRACT

The concentration of dissolved organic matter (DOM) in freshwaters is increasing in large areas of the world. In addition to carbon, DOM contains nitrogen and phosphorus and there is growing concern that these organic nutrients may be bioavailable and contribute to eutrophication. However, relatively few studies have assessed the potential for dissolved organic nitrogen (DON) or dissolved organic phosphorus (DOP) compounds to be bioavailable to natural river phytoplankton communities at different locations or times. Temporal and spatial variations in uptake, relative to environmental characteristics were examined at six riverine sites in two contrasting catchments in the UK. This study also examined how the uptake by riverine phytoplankton of four DON and four DOP compounds commonly found in rivers, varied with concentration. Total nitrogen (TN) and phosphorus (TP) concentrations, the proportion of inorganic nutrient species, and nutrient limitation varied temporally and spatially, as did the potential for DON and DOP uptake. All eight of the DOM compounds tested were bioavailable, but to different extents. Organic nutrient use depended on the concentration of the organic compound supplied, with simple compounds (urea and glucose-6-phosphate) supporting algal growth even at very low concentrations. DON use was negatively correlated with the TN and ammonia concentration and DOP use was negatively correlated with soluble reactive phosphorus (SRP) and dissolved organic carbon (DOC) concentration. The evidence indicates that

DOM in rivers has been overlooked as a potential source of nutrients to phytoplankton and therefore as an agent of eutrophication.

1. Introduction

Nutrient enrichment of inland waters is a major global anthropogenic stressor, which can lead to excessive algal growth and algal blooms (Le Moal et al., 2019; Schindler, 1975; Smith et al., 2006). Nutrient limitation of algal growth, a key control on total biomass, is often viewed as a static condition in lakes, rivers and the ocean, while in reality both nitrogen and phosphorus can be limiting dynamically over space and time and frequently both resources can be depleted leading to widespread co-limitation (Elser et al., 2007). In lakes, seasonal patterns of nutrient limitation have been observed (Kolzau et al., 2014; Maberly et al., 2002), with phosphorus (P) limitation being common in spring, as supplies are rapidly drawn down during the spring plankton bloom, while nitrogen (N) limitation or co-limitation of N and P can occur in summer in response to internal P cycling or external P supply and the loss of available N through denitrification. Nutrient limitation of algal growth is therefore dependent on the balance of demand for nutrients to support growth during the growing season and the supply of nutrients from natural or anthropogenic sources.

While research on the agents of eutrophication has largely focussed on inorganic nutrients, in many systems concentrations of dissolved organic nitrogen (DON) can equal or exceed those of dissolved inorganic nitrogen (Campbell et al., 2000; Durand et al., 2011; Kortelainen et al., 2006; Lloyd et al., 2019; Perakis and Hedin, 2002; Yates et al., 2019). Similarly, although less well studied, dissolved organic phosphorus (DOP), sometimes referred to as soluble unreactive P, can be a major component of total phosphorus (TP) (Heathwaite and Johnes, 1996; Jordan et al., 2007, 2005; Yates et al., 2019). This organic material originates from natural and anthropogenic sources within catchments and autochthonous production in the water as products of excretion or decomposition (Wetzel, 2001). For example, areas of livestock farming are associated with DON and DOP sources from manures and slurries (Lloyd et al., 2019) such as urea, amino acids and phytic acid (Turner et al., 2002), while leachate from blanket peat is associated with DOM compounds with a higher aromaticity (Yates et al., 2019). In addition, sources such as septic tanks and sewage treatment works are likely to release DOM compounds of varying molecular weights, including proteinaceous and humic-like compounds (Liu et al., 2012). If these forms of nitrogen and phosphorus are bioavailable, they could have a major effect on algal growth, especially since concentrations of dissolved organic carbon (DOC) are increasing in many parts of the world probably as a result of numerous factors including climate change, land use and management change, and recovery from acidification (Lepistö et al., 2014; Monteith et al., 2007; Stackpoole et al., 2017).

DON exists in numerous forms in aquatic ecosystems including proteins, free amino acids, amino sugars, nucleic acids, excretion products such as urea and methylamines, and higher molecular weight substances typically deriving from the breakdown of polymers such as lignin and chitin (Berman, 2003; McCarthy et al., 1997; Osburn et al., 2016). Low molecular weight compounds such as urea, amino acids and sugar amines are often useable by phytoplankton (Berman and Chava, 1999; Flynn and Butler, 1986), while higher molecular weight compounds may also be partly bioavailable to phytoplankton (Bronk et al., 2007). DOP compounds include nucleic acids, phospho-sugars, phospholipids, polyphosphates and phosphonolipids (Diaz et al., 2018). The bioavailability of DOP to algae is less well studied but various DOP compounds have been shown to be bioavailable (Bai et al., 2014; Cotner and Wetzel, 1992; Diaz et al., 2018; Jansson et al., 2012; Michelou et al., 2011; Whitney and Lomas, 2019).

The bioavailability of many organic nutrient compounds and their increasing supply through the increased flux of DOM from terrestrial sources may therefore play a role in promoting algal growth in freshwaters (Feuchtmayr et al., 2019). The extent to which organic nutrients are likely to regulate primary production could vary depending on a variety of factors including the nutrient sources and the underlying nutrient and light regime of a system. For example, Seekell et al. (2015) found a threshold DOC concentration (4.8 mg C L^{-1}) below which DOC was positively related to lake primary production in boreal and arctic lakes, suggesting an enrichment effect where existing DOC concentrations are low. In highly enriched systems, where DON or DOP form only a small fraction of the total nutrient concentrations (Yates et al., 2019), it is generally assumed that most algal production is being driven by inorganic nutrients. Algae can access organic nutrients by direct uptake and indirectly after microbial remineralisation of DOM (Wetzel, 2001). This implies that nutrients derived from DOM are likely to be cycled between different nutrient pools and organic sources may potentially contribute to algal growth dynamically over time in sites at differing levels of nutrient enrichment.

To date, research has largely focussed on whether or not a specific DON or DOP compound is bioavailable rather than on how bioavailability varies with environmental conditions. Thus, the importance of nutrient concentration and nutrient limitation is largely unknown. Utilising the marked spatial and temporal gradients in nutrient concentrations identified in our previous work (Yates et al., 2019), this study addresses the following hypotheses:

1. Nutrient concentrations and nutrient limitation will reflect variations in catchment location and time of year.
2. DON and DOP compounds will differ in their overall bioavailability to algae and this will vary by site and season reflecting changes in nutrient concentration and limitation.
3. DON and DOP growth responses will depend on the total concentration of nutrients available instream.
4. Bioavailability to algae will depend on the concentration of the organic nutrient.

2. Methods

2.1. Site description

Two rivers with contrasting geologies were used in this study (Fig. 1), with sites selected to represent an enrichment gradient across different soils and land use. The lowland Hampshire Avon situated in the south of the UK is predominantly underlain by chalk, with thin organic mineral soils. The upland Conwy situated in north Wales is characterised by a mixture of Cambrian igneous and sedimentary rocks in the west of the catchment and Silurian mudstones in the east of the catchment, resulting in peat soils in the headwaters and brown earths and podzols towards the river estuary. Full details on the environmental characteristics and nutrient chemistry of these streams are available in Yates et al. (2019). Three sites previously monitored to determine their nutrient chemistry, were sampled within each river catchment (Table 1). These ranged from small headwater streams such as Priors Farm on the River Sem in the Hampshire Avon catchment and Nant-y-Brwyn in the Conwy catchment, to larger tributary streams including the River Nadder at Burcombe and River Wylye at South Newton in the Hampshire Avon catchment and Hiraethlyn at Bodnant in the Conwy catchment (Table 1).
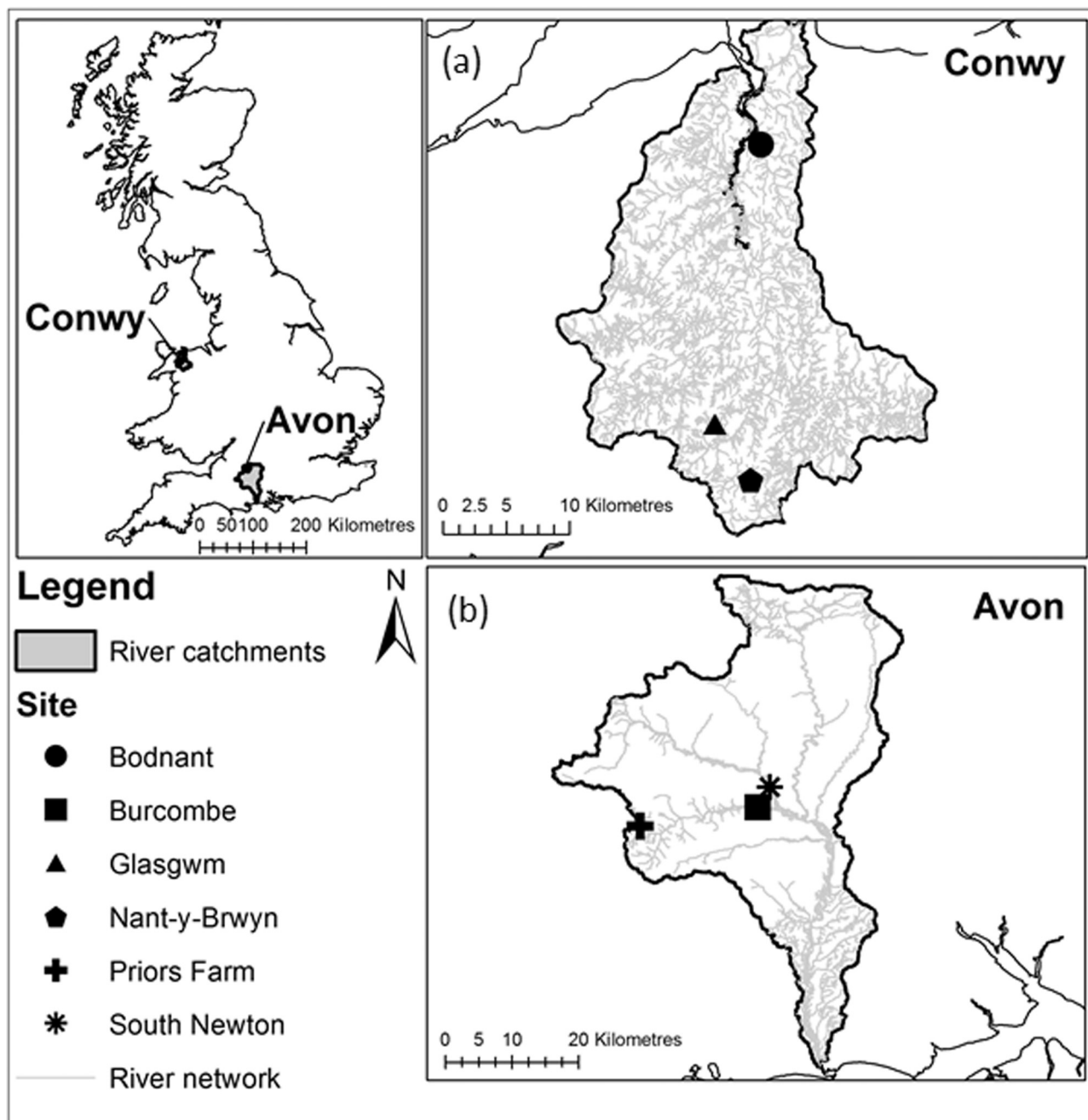
Fig. 1. Maps of studied catchments and sampling sites: Conwy, north Wales (a) and Hampshire Avon, southern England (b). Site latitude and longitude are given in Table 1.

2.2. Sampling and chemical analysis

All six sites were sampled on at least a fortnightly frequency using either spot sampling or an automatic water sampler (Yates et al., 2019) over the period February to November 2016. Inorganic nutrient analysis was conducted using a Skalar^{++} multi-channel continuous flow autoanalyser (Skalar Analytical B.V., The Netherlands), as detailed in Yates et al. (2019). DON and DOP concentrations were determined as the difference between total dissolved nitrogen (TDN) or total dissolved phosphorus (TDP) and dissolved inorganic nitrogen (DIN) or soluble reactive phosphorus (SRP) following persulphate oxidation of a 0.45 μm cellulose membrane filtered sample (Johnes and Heathwaite, 1992). Total N (TN) and total P (TP) were determined colourimetrically following the persulphate digestion of an unfiltered sample. Particulate

organic N and particulate P fractions were also determined as the difference between TN and TDN, and between TP and TDP, respectively.

At Bodnant, Burcombe and South Newton each monthly mean concentration was typically calculated from 23 to 26 samples. At Glasgwm, Nant-y-Brwyn and Priors Farm calculations were based upon 2 to 3 samples per month (Table 1). On seven occasions from February 2016 to January 2017, water samples for nutrient bioassays were transported to the laboratory in a cooled container within 48 h and acclimatised overnight in a 20 °C constant temperature room. In May and October 2017, further water samples were collected from the Nant-y-Brwyn and South Newton sites to examine the role of dissolved organic nutrient concentration in mediating the utilisation of the dissolved organic nutrient compounds at chemically-contrasting sites. In both cases, the initial phytoplankton chlorophyll *a* concentration was determined

Table 1
Characteristics of the six study sites ordered by increasing concentration of total nitrogen. Water chemistry data are means of monthly mean concentrations (mg of N, P or C L^{-1}) between February and November 2016, with monthly minima and maxima in parentheses. Land cover categories: A, Acid grassland; B, Bog; C, Calcareous grassland; H, Arable and horticultural; I, Improved grassland; R, Rough low productivity grassland; W, Coniferous woodland.

River catchment	Conwy	Conwy	Conwy	Hampshire Avon	Hampshire Avon	Hampshire Avon
Tributary	Glasgwm	Upper Conwy	Hiraethlyn	Sem	Wylye	Nadder
Site Name	Glasgwm	Nant-y-Brwyn	Bodnant	Priors Farm	South Newton	Burcombe
Latitude	53.027	52.988	53.226	51.055	51.107	51.080
Longitude	−3.845	−3.801	−3.799	−2.157	−1.878	−1.905
Elevation (m)	255	400	22	91	48	63
Catchment area (km^2)	2.6	1.3	20.5	2.3	447	208
Major land use (top two)	W (60%), A (18%)	B (80%), A (18%)	I (72%), R (12%)	I (59%), A (29%)	H (38%), C (32%)	H (48%), I (30%)
TN	0.49	0.61	3.30	3.93	7.01	7.51
	(0.34–0.65)	(0.38–1.18)	(3.02–3.66)	(1.97–6.13)	(6.56–7.28)	(6.18–8.85)
DIN	0.11	0.12	2.72	2.36	6.20	6.42
	(0.05–0.19)	(0.01–0.62)	(2.19–3.29)	(0.86–4.54)	(5.15–6.67)	(5.24–7.83)
DON	0.35	0.48	0.54	1.17	0.49	0.56
	(0.22–0.46)	(0.25–0.68)	(0.38–0.78)	(0.89–1.72)	(0.37–0.65)	(0.38–0.77)
TP	0.011	0.024	0.106	0.376	0.095	0.212
	(0.005–0.021)	(0.017–0.039)	(0.079–0.18)	(0.233–0.501)	(0.061–0.156)	(0.160–0.293)
SRP	0.000	0.005	0.071	0.151	0.041	0.087
	(0.000–0.002)	(0.000–0.016)	(0.035–0.150)	(0.048–0.275)	(0.025–0.055)	(0.033–0.143)
DOP	0.006	0.012	0.021	0.039	0.016	0.020
	(0.003–0.010)	(0.005–0.017)	(0.015–0.025)	(0.015–0.062)	(0.009–0.024)	(0.011–0.029)
DOC	4.31	11.06	2.72	12.97	2.10	2.84
	(1.98–6.62)	(3.74–18.73)	(1.75–3.71)	(10.44–19.87)	(1.89–2.31)	(2.27–3.39)
TN:TP (molar)	44.8	56.7	69.1	23.2	163.3	78.5
DON:TN	0.71	0.80	0.17	0.32	0.07	0.07
	(0.57 0.77)	(0.45–0.97)	(0.01–0.25)	(0.18–0.45)	(0.06–0.09)	(0.06–0.09)
DOP:TP	0.57	0.53	0.21	0.10	0.18	0.10
	(0.26–0.92)	(0.25–0.82)	(0.06–0.09)	(0.06–0.14)	(0.08–0.29)	(0.05–0.12)

prior to the experiments. For this, water samples were filtered onto glass fibre filters (Whatman GF/C; nominal pore size 1.2 μm), extracted in hot methanol and optical density was measured in a spectrophotometer (Talling, 1974). Concentrations of chlorophyll *a* were calculated using equations in Ritchie (2008).

2.3. Nutrient bioassay

Bioassays were performed in a laboratory-based study following Maberly et al. (2002). The aim was to balance environmental control, replication and system realism to allow us to assess whether dissolved organic nutrients were able to stimulate growth in the river phytoplankton community regardless of uptake mechanism. Water was filtered through a 100 μm mesh to remove any zooplankton or large particles and 35 mL transferred to 50 mL glass boiling tubes. Twelve different treatments were carried out in triplicate: a control with no chemical addition, additions of inorganic phosphorus (sodium phosphate),

Table 2
Treatments of bioassay incubations and organic chemicals. Nitrogen was added at 90 and phosphorus at 6 μmol L^{-1}. No addition is indicated by '-'.

Treatment name	Inorganic addition	Organic addition
Control		
Inorganic phosphorus	Sodium phosphate	–
Inorganic nitrogen	Ammonium nitrate	–
Inorganic N + P	Ammonium nitrate + Sodium phosphate	–
Urea + P	Sodium phosphate	Urea
Glycine + P	Sodium phosphate	Glycine
Glutamate + P	Sodium phosphate	L-Glutamic acid
Glucosamine + P	Sodium phosphate	N-Acetyl-D-glucosamine
Glucose 6-phosphate + N	Ammonium nitrate	D-Glucose 6-phosphate sodium salt
Phytic acid + N	Ammonium nitrate	Phytic acid sodium salt
Methylumbelliferyl phosphate + N	Ammonium nitrate	4-Methylumbelliferyl phosphate disodium salt
Methyl phosphonate + N	Ammonium nitrate	Methyl phosphonate

inorganic nitrogen (ammonium nitrate), inorganic P and N, four DON additions with inorganic P and four DOP additions with inorganic N (see Table 2). The selection of compounds was made to ensure that growth responses related to either organic nitrogen or organic phosphorus addition, so only compounds containing either N or P were used. Specific compound choices were made on the basis of our understanding of the catchment sites in terms of land use and activities such as manure and fertiliser applications, the likely presence of compounds in the freshwater environment, based on previous studies, and direct measurement of compounds, in the case of urea, which was present at all sites. Glycine and L-Glutamic acid are amino acids commonly found within freshwater biota and as free amino acids in water. *N*-acetyl glucosamine, an amino sugar, is a common compound in bacteria, algae and crustaceans where it is a constituent of chitin and is found across a range of freshwater habitats (Nedoma et al., 1994). Glucose-6-phosphate is a phosphorylated form of glucose which has been found to be widely used by freshwater bacterial communities (Brailsford et al., 2019; Rofner et al., 2016). Phytic acid, a key P storage compound in seeds, is common in agricultural soils receiving manures (Turner et al., 2002) and a constituent of lake organic P pools (Reitzel et al., 2007). Methyl phosphonate has been recently shown to be a potential P source to freshwater microbial communities, particularly in low nutrient systems, through the cleavage of the C—P bond (Whitney and Lomas, 2019; Yao et al., 2016). Methylumbelliferyl phosphate is commonly used in the assessment of extracellular phosphatase activities in freshwaters (e.g. Štrojsová et al., 2003), providing a substrate widely associated with the potential for use of organic phosphorus compounds. Dissolved organic nitrogen was added together with inorganic phosphorus and dissolved organic phosphorus was added with inorganic nitrogen in order to determine the potential for the use of the dissolved organic compound without limitation by the other nutrient (N or P), thereby avoiding a false negative response. Nutrients were added at approximate Redfield ratio proportions: N at 90 μmol L^{-1} (1.261 mg N L^{-1}) and P at 6 μmol L^{-1} (0.186 mg P L^{-1}) (Maberly et al., 2002).

The tubes were stoppered with foam bungs, to enable gas exchange, incubated in a 20 °C constant temperature room, and illuminated from below with cool white fluorescent lamps that generated a photon

irradiance of 80–120 μmol m^{-2} s^{-1} (photosynthetically available radiation, Macam Q102) with an 18 h light, 6 h dark cycle. The temperature and light cycle were chosen to reflect an optimal summer day in this climate zone and ensure that we could compare growth effects independent from other influences across different sites and seasons. After 14 days, the contents of each tube, including any biofilm on the inside walls, were resuspended by scraping and the use of a vortex mixer and filtered onto a Whatman GF/C glass fibre filter that was frozen at −20 °C until further analysis. Defrosted filters were analysed for chlorophyll *a* as described above.

The dissolved organic nutrient concentration experiments followed the same initial procedure described above. The same DON and DOP compounds (see Table 2) were added in triplicate at four concentrations per compound: 90 μmol N L^{-1}, 9 μmol N L^{-1} (0.126 mg N L^{-1}), 0.28 μmol N L^{-1} (0.039 mg N L^{-1}) and 0.09 μmol N L^{-1} (0.013 mg N L^{-1}) for the DON compounds and 6 μmol P L^{-1}, 0.6 μmol P L^{-1} (0.019 mg P L^{-1}), 0.186 μmol P L^{-1} (0.006 mg P L^{-1}) and 0.06 μmol P L^{-1} (0.002 mg P L^{-1}) for the DOP compounds. These concentrations were selected based on the ranges of DON and DOP concentration found at these sites (16–123 μmol N L^{-1} and 0.01–2 μmol P L^{-1}. Samples were then incubated and chlorophyll *a* analysed in the same way as described above.

2.4. Statistical analysis

All analyses were carried out in R version 3.5.1 (R Development Core Team, 2011). To assess the growth response of the bioassays to the organic nutrient additions, a natural log response ratio was calculated following Elser et al. (2007):

$$NR_X = \ln\left(\frac{T_x}{C_x}\right) \tag{1}$$

where NR_x is the nutrient response ratio for nutrient addition x, T_x is the chlorophyll *a* concentration of the nutrient treatment and C_x is the corresponding control treatment response. In the case of the inorganic nutrient additions, the control was the sample where no nutrients had been added. In the case of the organic nutrient additions, two different response ratios were calculated. Where the comparison was against the amount of chlorophyll produced compared to the inorganic N + P treatment, the control (for both) was the control sample without nutrient addition. Where the comparison was assessing if a particular organic compound was being used, the control for DON + inorganic phosphorus was + inorganic P alone and the control for DOP + inorganic nitrogen was + inorganic nitrogen alone. The response ratios were initially calculated for each of the three treatment replicates and then averaged.

2.4.1. Inorganic nutrient status

The inorganic nutrient limitation status of each site and date was determined using the critical effect size criteria of Harpole et al. (2011) from data resulting from bioassay incubations with inorganic nutrients (see Table 2). This approach uses a critical threshold value of 38.5% greater than the control values which corresponded to a *P* value <.05 derived from a meta-analysis of many nutrient limitation studies. This critical threshold is used to evaluate whether a response is greater (or less) than the control and is therefore indicative of a significant positive or negative growth response. It was adopted in this study to avoid the Type II errors associated with low replication or statistical power that can limit interpretation of traditional analyses. Where the inorganic nutrient addition, either singly or in combination, exceeded the control by the threshold value we assigned each site and date combination to either nitrogen, phosphorus or co-limitation by nitrogen and phosphorus, respectively. Where the threshold was not exceeded the site and date combination was assessed to have no overall limitation.

2.4.2. Causes of the organic nitrogen and phosphorus response

The first six experiments were used in this analysis as nutrient data were unavailable for the seventh experiment. Prior to statistical modelling, relationships between the water chemistry data and the organic nutrient response ratios were assessed visually to determine whether data transformation was necessary. Where non-linear relationships were found, the data were log-transformed and a small constant of 0.001 added to ensure there were no zeros in the data.

To identify potential drivers of the organic nutrient ratio response, we undertook a three-stage statistical modelling approach based upon linear mixed effects models with a Gaussian error distribution. Firstly, a null model with only random intercept terms for site, date and organic nutrient treatment was fit to the response data to quantify how the variance in the organic nutrient response ratio was partitioned among these variables. Secondly, potential water chemistry predictors of each organic nutrient response ratio were added to this model as fixed effects, to determine how well these variables could account for the variability in response ratios across the different sites, dates and nutrient treatments. To consider combined effects of multiple water chemistry variables, a number of candidate models were constructed. Given the high likelihood of correlations among water chemistry predictors, a correlation matrix of the water chemistry variables was examined prior to modelling, and predictor variables were only included in the same candidate model when the Pearson correlation value between those variables was <0.65. The null models and models with the fixed effects were compared using Akaike Information Criterion (AIC) values, which reflect the balance between model goodness of fit and model simplicity. Following the identification of the best candidate water chemistry model, we simplified this model further by sequentially removing variables with the lowest F statistic value and comparing the resulting model AIC and R^2 value to the previous models. The most optimal model was then determined as the most simple model with the lowest AIC and highest R^2. These models all contained a nested random intercept term for each site and date and a separate random intercept term for organic nutrient treatment to account for the structure of the data. Thirdly, individual models of responses to specific organic chemical treatments were constructed, with a random intercept term for site, using the same model selection procedure detailed above. These models allowed assessment of whether the drivers of the chlorophyll *a* growth response differed according to the organic chemical used. Model residuals were assessed visually for conformity to underlying model assumptions of normality, homogeneity of variance, and independence of observations. Analyses were conducted using the lme4 package in R (Bates et al., 2014)

2.4.3. Dissolved organic nutrient concentration effects

To examine the effect of the concentration of dissolved organic nutrients on growth, chlorophyll *a* concentrations from the different organic nutrient treatments from the May and October 2017 experiments were modelled using a linear model for each site and chemical, followed by post-hoc testing among treatment pairs, with adjusted *p* values to account for multiple testing. We compared whether growth responses at different dissolved organic nutrient concentrations were significantly different from the respective inorganic nutrient control. Prior to the statistical analysis, chlorophyll *a* concentrations were natural logged to ensure a more even distribution of observations across the explanatory variable concentration gradient. Finally, model residuals were again visually assessed to ensure conformity to model assumptions.

3. Results

3.1. Nutrient concentration and limitation

The total nitrogen (TN) concentration across the six sites ranged 26-fold from 0.34 to 8.85 mg L^{-1}, and the site average varied 15-fold, while

the fraction of TN that was DON, on average, varied from 7% at South Newton and Burcombe to 80% at Nant-y-Brwyn (Table 1). The total phosphorus (TP) concentrations varied 100-fold from 0.005 to 0.50 mg L^{-1} across sites and the site average varied 34-fold, with the contribution of the DOP fraction to TP varying from 10% at Priors Farm and Burcombe to 57% at Glasgwm (Table 1). Inorganic nutrient concentrations were consistently low at Nant-y-Brwyn and Glasgwm, where soluble reactive phosphorus (SRP) concentrations were, on average, <0.005 mg P L^{-1} and dissolved inorganic nitrogen (DIN) concentrations ~0.1 mg N L^{-1}. DOC concentrations varied 6-fold across sites and between the catchments, being highest at Nant-y-Brwyn and Priors Farm, intermediate at Glasgwm and lowest at Bodnant, Burcombe and South Newton.

The bioassays assessing nutrient limitation by addition of inorganic nutrients revealed a wide range of variability in response across sites and over time (Fig. 2). Phosphorus was the most common limiting nutrient, with co-limitation by both nitrogen and phosphorus also occurring frequently and at all sites on at least one occasion. Nitrogen limitation occurred at three sites, in particular at Nant-y-Brwyn where the phytoplankton were strongly co-limited or nitrogen limited throughout the year. Priors Farm was the most variable in nutrient limitation status over time, with no-limitation, co-limitation, nitrogen limitation or phosphorus limitation occurring on different dates throughout one year. In contrast, the site at Burcombe was mostly phosphorus limited.

3.2. Response to DON and DOP addition

There was a significant positive effect of all four DON compounds, urea, glycine, L-glutamic acid and N-acetyl glucosamine, on phytoplankton growth for two to five sites for one or more dates (Fig. 3a). In particular, Glasgwm and Nant-y-Brwyn had a significant response to all compounds throughout the year. The other sites showed a more variable response, with a greater number of positive responses between

June and September. The frequency of utilisation of the DON compounds was lowest in South Newton and Burcombe; there were five occasions when a significant negative response to their addition occurred at South Newton. However, throughout much of the year the addition of the DON compounds had no significant effect across these sites and those at Bodnant and Priors Farm.

Significant positive effects of DOP compounds on phytoplankton growth were more common than similar effects of DON compounds (Fig. 3b). Significant growth responses to both Phytic acid and Methyl phosphonate addition were less frequent than responses to the addition of Glucose-6-phosphate and Methylumbelliferyl-phosphorus. The Glasgwm and Nant-y-Brwyn sites had the highest frequency of significant positive responses throughout the year for all compounds, while Priors Farm had the lowest.

Dissolved organic nutrient response ratios for both DON and DOP compounds were compared to the response ratios where equivalent concentrations of inorganic nitrogen and phosphorus were added. For the majority of samples, the combined addition of inorganic nitrogen and inorganic phosphorus produced the largest growth response in the phytoplankton. However, on a number of occasions, at different sites and for different compounds, growth resulting from organic nutrient addition exceeded that resulting from inorganic nutrient addition (Fig. 4a and b). For the DON compounds, this occurred on 67 occasions out of a total of 168, while for the DOP compounds it occurred on 39 occasions out of 168.

3.3. Effect of water chemistry on dissolved organic nutrient response

The null (random effects only) model indicated that chlorophyll *a* response ratios for the DON bioassays (DON + inorganic phosphorus vs + inorganic phosphorus alone) were more variable among sites (82.5% of total variance) than by date or compound (14.4% and 0.5% of total variance, respectively). The same pattern occurred for the DOP chlorophyll *a* response ratio (DOP + inorganic nitrogen vs + inorganic
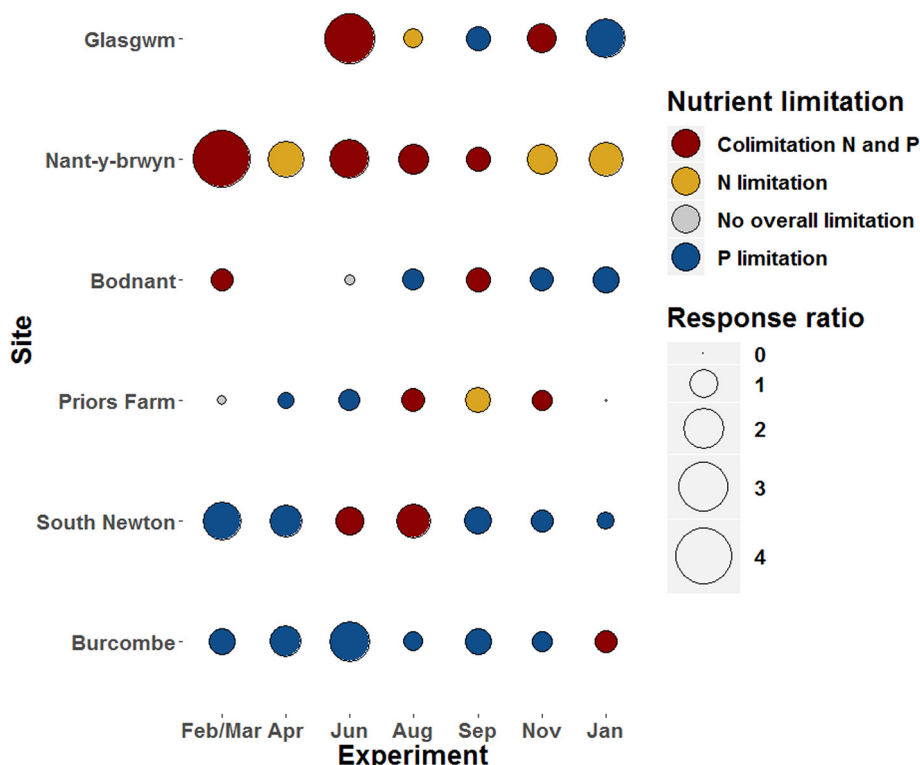
Fig. 2. Seasonal and site-based variation in nutrient limitation assessed by the phytoplankton biomass response to bioassays of inorganic N, P and NP.
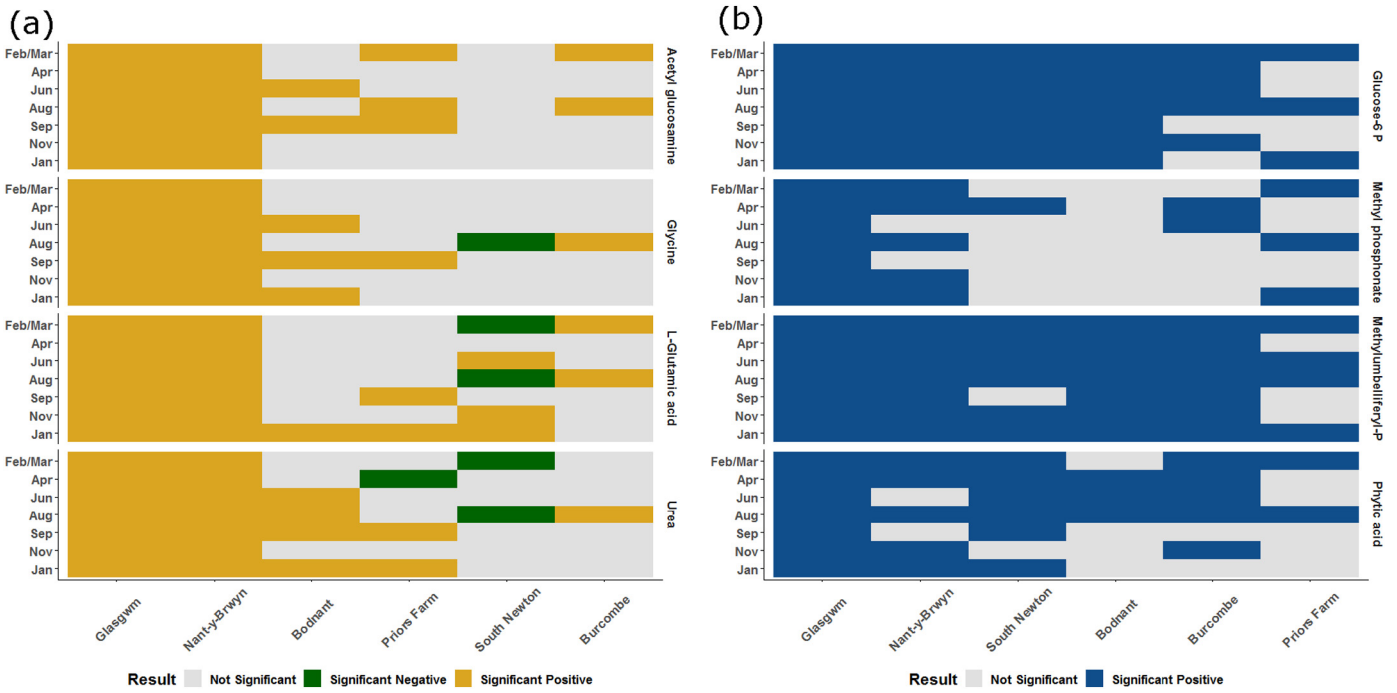
Fig. 3. Seasonal and site-based variation in the use of the fixed concentration of a) DON and b) DOP compounds assessed by the phytoplankton biomass response to bioassays in 2016/17.

nitrogen alone) null model, although variability between compounds was higher than for the DON compounds (55.4%, 11.8% and 15.5% of total variance, respectively). In both cases, the addition of fixed effects for water chemistry explanatory variables improved the model fit based on a comparison of the AIC values and reduced the variability associated with the random effect of site. Following model selection for the DON response ratio full model, the most optimal model contained TN and ammonia. Both variables had significant negative effects on the DON response ratio (Table 3, Fig. 5a and b), indicating that the phytoplankton showed a lower growth response to the addition of DON compounds as the ambient concentrations of TN and ammonia in the

water increased. Models of the individual DON compounds generally reflected the same result as that of the full model, with a significant negative effect of TN, except for L-glutamic acid where DON:TN had a significant positive effect on the DON response ratio, while ammonia and DOP concentrations had a significant negative effect on the response ratio (Appendix Table A.1).

Following model selection of the DOP chlorophyll *a* response ratio full model, the most optimal model contained DOC and SRP, where both variables had a significant negative effect on the chlorophyll *a* response to DOP additions (Fig. 5c and d). This pattern also occurred across the individual DOP compound models, except for methyl
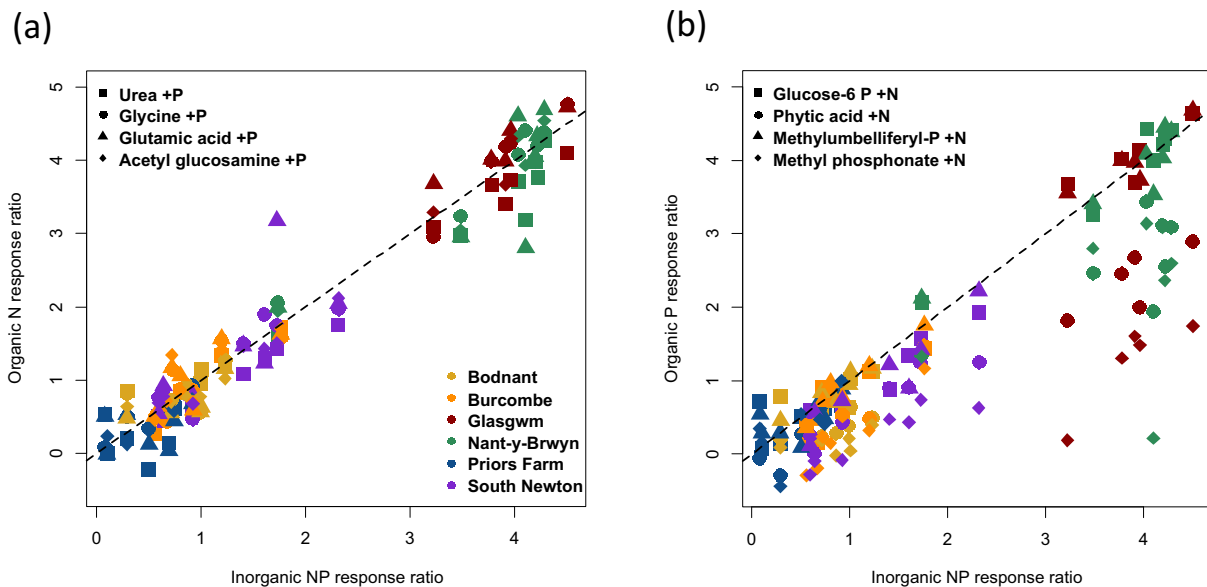
Fig. 4. Comparison of the response ratios for the addition of the DON +P (a) and DOP+N (b) with the inorganic nitrogen and phosphorus addition. The dotted line denotes the 1:1 line. Values above the 1:1 line denote samples where the response of phytoplankton biomass to the organic compound was higher than the response to inorganic N and P addition.

Table 3
Model coefficients, standard errors and F statistic for the dissolved organic nitrogen and phosphorus treatments. Significant coefficients terms are shown in bold with *** denoting $P < .001$, ** $P < .01$ and * $P < .05$.

Model	Explanatory variable	Coefficient	Standard error	F statistic
Organic nitrogen response ratio for all compounds	Log TN	**−1.17*****	0.12	88.12
	Log Ammonia	**−0.34****	0.12	7.79
Organic phosphorus response ratio for all compounds	Log SRP	**−0.48*****	0.05	79.89
	DOC	**−0.05***	0.02	8.63

phosphonate where SRP alone was the most optimal model (Appendix Tables A.2).

3.4. Effect of dissolved organic nutrient concentration on bioavailability

The use of dissolved organic nutrient compounds by river phytoplankton varied according to concentration across the two sites and seasons tested (Tables 4 and 5 and Figs. 6 and 7). Significant positive growth effects at Nant-y-Brwyn occurred for most of the DON compounds at the highest concentrations (90 and 9 μmol L^{-1}) in both seasons. In addition, three compounds, urea, glycine and glucosamine,

Fig. 5. Model fit of the key water chemistry drivers for DON use (a and b) and DOP use (c and d). Solid black line denotes model fitted slope and dashed lines represent at 95% confidence interval.

Table 4
Significant chlorophyll *a* growth responses to varying dissolved organic nitrogen compound additions in spring and autumn 2017. + denotes a positive growth response, − a negative growth response, and ns no significant response.

| | | Nant-y-Brwyn | | | | South Newton | | | |
		Concentration (mg N L^{-1})				Concentration (mg N L^{-1})			
	Chemical	1.261	0.126	0.039	0.013	1.261	0.126	0.039	0.013
Spring	Urea + P	+	+	+	+	−	ns	ns	ns
	Glycine + P	+	+	+	ns	ns	ns	ns	ns
	Glutamate + P	ns	+	ns	ns	ns	ns	ns	ns
	Glucosamine + P	+	+	+	ns	ns	ns	ns	ns
Autumn	Urea + P	+	+	ns	+	ns	ns	ns	ns
	Glycine + P	+	+	ns	ns	ns	ns	ns	ns
	Glutamate + P	+	+	ns	+	ns	ns	ns	ns
	Glucosamine + P	+	+	+	ns	ns	ns	ns	ns

caused significant growth at 2.8 μmol N L^{-1} in spring. Urea and L-glutamic acid produced significant positive growth at the lowest concentration in the autumn, while urea produced a significant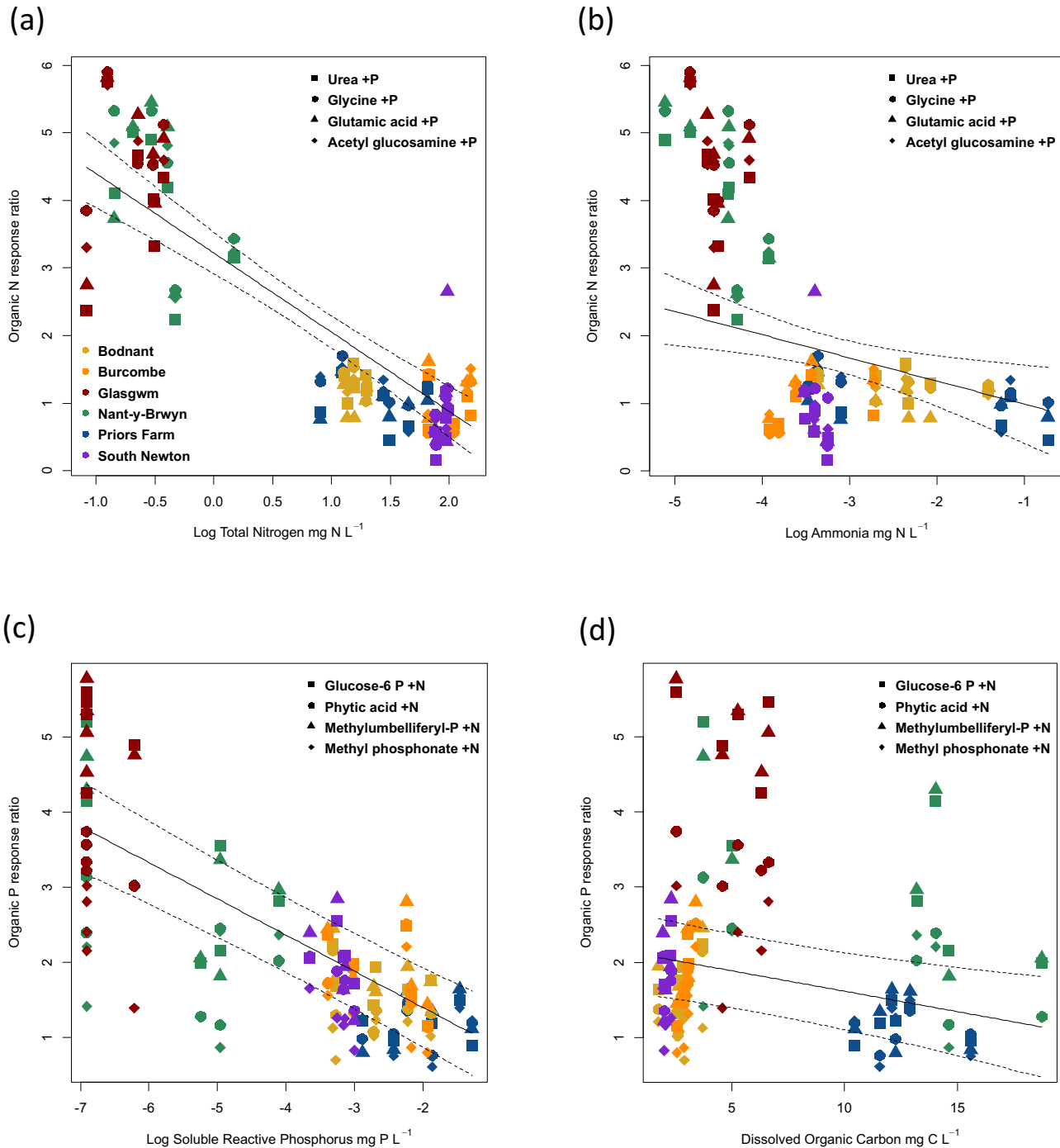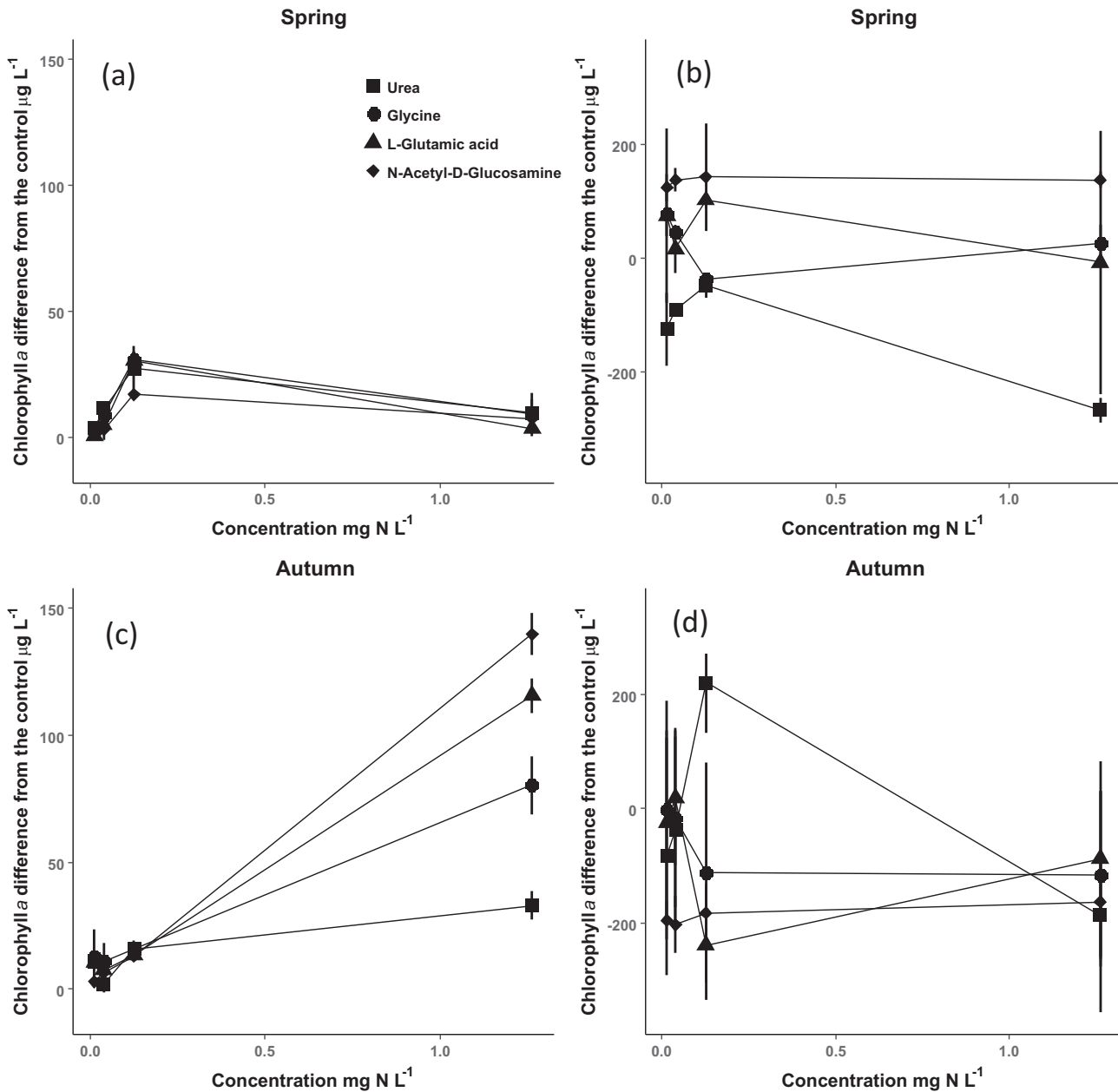 positive response at this concentration in spring. In contrast, none of the concentrations of the DON compounds generated a significant positive growth response at the South Newton site, while in spring, urea produced a significant negative growth response relative to the control (Fig. 6). DOP responses were similar, in that the significant positive growth responses occurred at the highest concentrations at Nant-y-Brwyn in both seasons and at South Newton in spring. Glucose-6-phosphate and Methylumbelliferyl phosphorus only produced significant positive growth at South Newton in spring at the highest concentration. Glucose-6-phosphate produced significant positive growth at all concentrations at Nant-y-Brwyn in spring (Fig. 7). These results for the growth response to the dissolved organic nutrient additions reflected the results of the inorganic nutrient bioassays. In 2017, nutrient limitation was similar to the results from 2016, with the nitrogen limitation in spring and co-limitation of nitrogen and phosphorus in autumn at Nant-y-Brwyn and phosphorus limitation in both spring and autumn at South Newton.

4. Discussion

4.1. Seasonal and spatial changes in nutrient limitation

We show that there are large seasonal changes in nutrient limitation and the potential for river phytoplankton growth utilising dissolved organic nutrient compounds in the simplified system of a microcosm experiment, supporting hypothesis 1. Although the determination of nutrient limitation at sites is frequently restricted to single samples or seasons, similar seasonal changes in nutrient limitation have been shown previously. For example, in lakes, Maberly et al. (2002) showed a transition from single nutrient limitation, particularly of P, in spring to co-limitation in summer for phytoplankton and periphyton in 30 UK upland lakes. Similarly, in detailed studies on four lowland lakes,

Kolzau et al. (2014) showed large seasonal variation in nutrient limitation in three lakes with P-limitation most prevalent in spring and N-limitation or co-limitation most prevalent later in the year. A similar pattern is seen at the South Newton site, but in our study there also appears to be greater variability in the nutrient limitation pattern across sites, with the headwater streams (Glasgwm, Nant-y-Brwyn and Priors Farm) tending to show more temporal variation than the sites further down the river network. This greater variability in headwater nutrient limitation was also found by Jarvie et al. (2018) when assessing limitation using nutrient ratios and concentrations across the UK. These seasonal changes in nutrient limitation largely result from input to and removal from different nutrient pools throughout the growing season and more variation may be expected at headwater river sites caused by the high surface area to flow volumes and lower dilution capacity for anthropogenic inputs (Bernal et al., 2015; Triska et al., 2007).

Within each site, the concentration of DON and DOP was generally less variable than that of the inorganic fractions DIN and SRP. This could imply that these dissolved organic nutrient forms potentially provide a more consistent supply for algal growth throughout the year, or it may also represent a residual nutrient pool that is not being utilised either through lack of bioavailability or limitation by other factors that could restrict their uptake (Mahaffey et al., 2014). This lower variability in DOP concentration compared to inorganic P has also been reported in the marine environment occurring both temporally and spatially over a number of years in the north Pacific (Björkman et al., 2018). It should however be noted that we are using a pore-sized operational definition of TDN and TDP and therefore colloids and small particles, possibly with lower bioavailability, may also contribute to the dissolved pool of nutrients from which we derived DON and DOP concentrations.

4.2. Relationship between dissolved organic nutrient use and inorganic nutrient concentration and limitation

Evidence of the potential for dissolved organic nutrient use by river phytoplankton was highest, both for DON and DOP, in the

Table 5
Significant chlorophyll *a* growth responses to varying dissolved organic phosphorus compound additions in spring and autumn 2017. + denotes a positive growth response, − a negative growth response and ns no significant response.

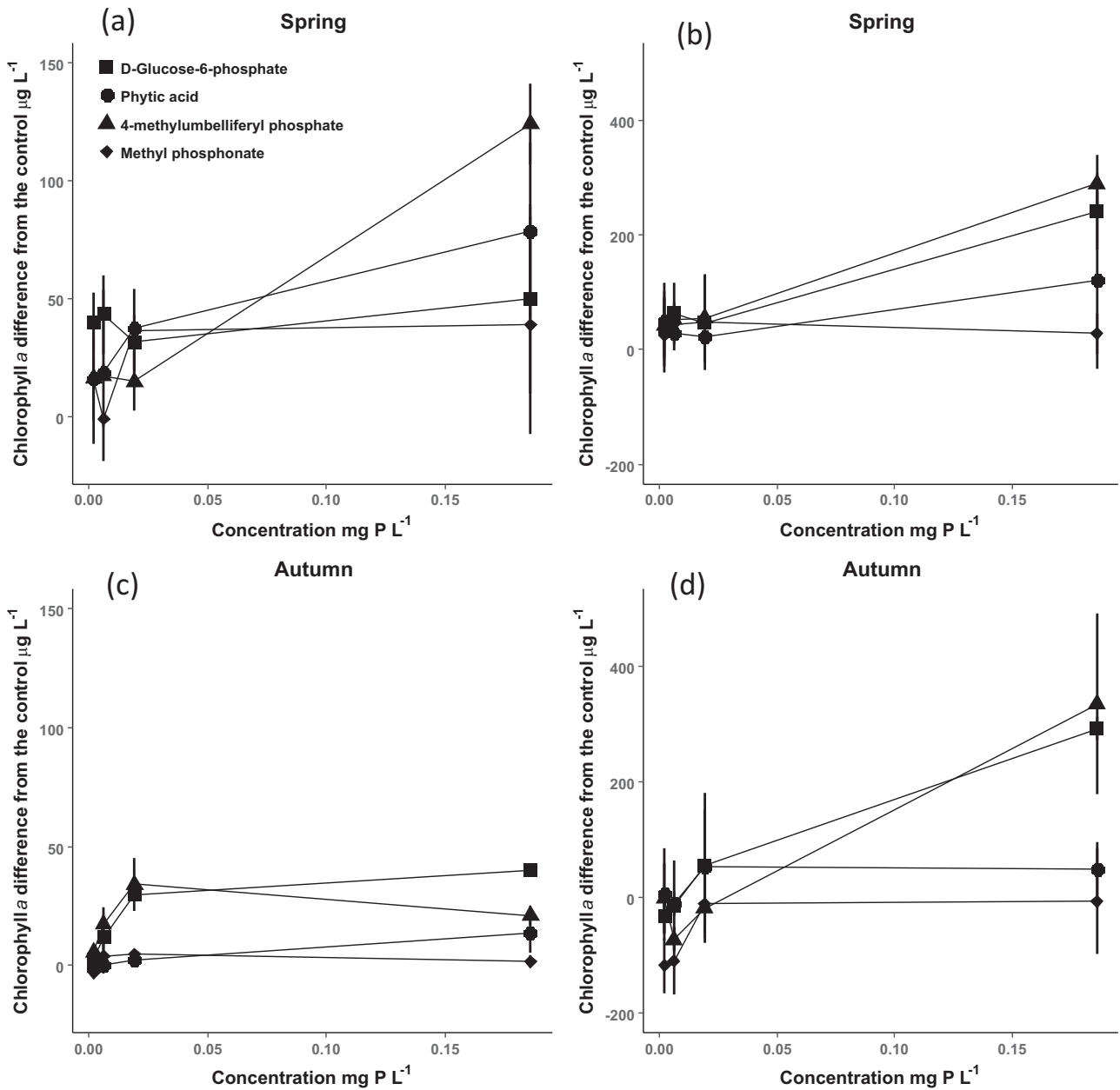| | | Nant-y-Brwyn | | | | South Newton | | | |
		Concentration (mg P L^{-1})				Concentration (mg P L^{-1})			
	Chemical	0.186	0.019	0.006	0.002	0.186	0.019	0.006	0.002
Spring	Glucose 6-phosphate + N	+	+	+	+	+	ns	ns	ns
	Phytic acid + N	+	+	ns	ns	ns	ns	ns	ns
	Methylumbelliferyl phosphate + N	+	ns	ns	ns	+	ns	ns	ns
	Methyl phosphonate + N	ns	ns	ns	ns	ns	ns	ns	ns
Autumn	Glucose 6-phosphate + N	+	+	ns	ns	ns	ns	ns	ns
	Phytic acid + N	+	ns	ns	ns	ns	ns	ns	ns
	Methylumbelliferyl phosphate + N	+	+	ns	ns	ns	ns	ns	ns
	Methyl phosphonate + N	ns	ns	ns	ns	ns	ns	ns	ns

Fig. 6. The mean and standard deviation of the bioassay growth response of river phytoplankton to varying concentrations of DON compounds in spring and autumn 2017 at Nant-y-Brwyn (a and c) and South Newton (b and d).

sites where the underlying total nutrient concentrations were lowest, in particular at the Nant-y-Brwyn and Glasgwm sites where inorganic nutrient concentrations were typically below the values considered limiting in river systems (Jarvie et al., 2018; Maberly et al., 2002; Mainstone and Parr, 2002). At the other sites, DON use reflected the patterns seen in nutrient co-limitation, with a tendency for utilisation of more compounds during the summer months. These results, showing DON and DOP use reflect patterns in ambient nutrient concentration and limitation, support hypothesis two. DOP use was more widespread than that of DON use, which again probably reflects the higher frequency of P limitation across the sites. The differing composition of the phytoplankton communities and their interaction with heterotrophic bacteria across these sites is also likely to have influenced the utilisation of the DON and DOP compounds and in turn be influenced by the prevailing nutrient environment. The interpretation of these results should, however, be made

in the context of the experimental design of the study and the limitation that microcosms cannot fully replicate real world conditions.

Despite the potential for dissolved organic nutrient use being most commonly observed in the nutrient-poor sites, we also found significant positive growth effects in the phytoplankton community in response to dissolved organic nutrient additions at highly nutrient-enriched sites. For example, at Priors Farm, where nutrient concentrations were above limiting concentrations throughout 2016, significant positive growth was found for all compounds on at least one occasion. The additional energetic costs of upregulating organic molecule transporters or extracellular enzymes has generally been assumed to inhibit dissolved organic nutrient use under conditions of sufficient inorganic nutrient supply. However, there is also some evidence that organic compounds may be utilised irrespective of the prevailing background nutrient concentration by

Fig. 7. The mean and standard deviation of the bioassay growth response of river phytoplankton to varying concentrations of DOP compounds in spring and autumn 2017 at Nant-y-Brwyn (a and c) and South Newton (b and d).

either bacterial or algal communities in other environments (Björkman et al., 2018; Diaz et al., 2018). This suggests that the growth response at different sites is likely to be closely linked to the specific compound a community is exposed to and the composition, in terms of algae and microbial community. We have not attempted to infer the mechanisms of dissolved organic nutrient uptake, as we are only observing the final growth stimulation of the algal community in this study. However, it is highly likely that there are both direct and indirect pathways for nutrient uptake in these systems, depending on the compound, with the microbial community potentially playing an important role in remineralising organic nutrient compounds. For example, the urease gene that breaks down urea to ammonia and CO_2 internally is present in cyanobacteria and diatoms, but absent in green algae (Kanamori et al., 2005; Veaudor et al., 2019). It also implies that dissolved organic nutrients may be supplementing or in some instances

replacing inorganic nutrient use to support algal growth, contributing to eutrophication in these water bodies.

4.3. Dissolved organic nutrient compounds as growth inhibitors or enhancers

The DON compounds glycine, L-glutamic acid and urea produced a significant negative growth effect on two occasions at South Newton and on one occasion for urea at Priors Farm. This may be due to an inhibitory effect on uptake of these compounds caused by the high background concentrations of inorganic N forms, which has been previously found in the case of urea (Solomon et al., 2010). High concentrations of organic compounds such as glutamate and other amino acids have also been reported to be inhibitory to growth in some algal species (Baldia et al., 1991; Chapman and Meeks, 1983). The initial algal community composition of the sites may also have played a role

in the growth response, where the affinity of different algal groups to different N sources has been found to alter the dominant species contributing to the algal biomass (Donald et al., 2011; Glibert et al., 2004). If the dominant species at a site were not able to assimilate the DON addition, and community reorganisation, including competition for nutrients from heterotrophic bacteria, occurred in response to the addition, it may be possible that the phytoplankton growth in the DON addition treatments was lower than that of the controls, which had ambient nutrient concentrations and an addition of inorganic P. It should be noted though, that community reorganisation in response to dissolved organic nutrient addition is also likely to have been influenced by the experimental design and therefore may not fully reflect community changes in the natural environment.

Enhanced growth of river phytoplankton on DON and DOP compounds over and above the expected maximum yield of the combined inorganic N and P treatment was found on 67 site-date combinations for DON and 39 site-date combinations for DOP. This additional growth could be a result of the addition of carbon within the system, which can be used as an additional energy substrate to drive bacterial remineralisation of organic molecules, freeing inorganic or lower molecular weight dissolved organic N and P for further algal growth. Carbon supplementation has previously been found to increase alkaline phosphatase activity that was, in turn, highly correlated with bacterial density and suggestive of increased microbial remineralisation of P in other experimental studies (Anderson, 2018). It has also been observed that high rates of microbial hydrolysis of adenosine-5′-triphosphate resulted in net release of inorganic P into seawater when it exceeded the requirements of the microbial community (Björkman et al., 2018).

4.4. Effect of water chemistry on organic nutrient bioavailability

The statistical models confirmed that nutrient limitation and potential for use of dissolved organic nutrients are closely linked to ambient nutrient concentrations (hypothesis three). The significant driver of the DON usage was the concentration of TN and ammonia in the water. For DOP, the most significant driver was found to be the ambient SRP concentration in the water. These findings imply that, as may be expected, phytoplankton biomass in nutrient-rich sites, responded less to the DON and DOP compounds than in nutrient-poor sites. However, residual variation in the models is likely to reflect our finding that significant growth on DON and DOP compounds also occurred at more enriched sites. The underlying reasons for this are likely to reflect the adaptation of the phytoplankton and microbial communities, as agents of remineralisation, to their prevailing nutrient environment. This is both in terms of species composition and an upregulation of enzyme activities and other organic molecular transporter mechanisms where more readily assimilated inorganic molecules are scarce. The additional variance explained by adding the nutrient concentration data into the null model was nearly all attributable to the site variable, implying that these conditions are likely to reflect the site specific adaptation or composition of the phytoplankton and bacterial communities to the nutrients they are exposed to (Diaz et al., 2018; Donald et al., 2011). The consistency in these drivers was also present in the response ratios for individual chemicals, which suggests that, within this dataset, the driver of use is generally not specific to the organic molecule being used. However, we did find that for L-glutamic acid the positive relationship with DON:TN was the most important explanatory variable, along with ammonia and DOP concentration. This may indicate that usage of this compound has a more complicated relationship to nutrient enrichment than solely the ambient TN concentration. The significant negative response to the underlying DOC concentration of the DOP response ratio appears potentially counterintuitive, given the general pattern of lower inorganic nutrient concentrations with higher DOC concentration. However, this result may be evidence of enhanced competition of P for heterotrophic growth in environments where carbon is in abundant supply (Currie and Kalff, 1984) or be related to the chemical composition of

DOC at different sites and times reflecting differences in the proportion of labile vs refractory compounds. Further research is required to disentangle these possibilities.

4.5. Effect of dissolved organic nutrient concentration on bioavailability

The growth response to differing concentrations of DON and DOP compounds followed the expected pattern of significant use at the highest concentrations and in the location where background nutrient concentrations tended to be lowest, confirming hypothesis four. This growth response also reflected the inorganic bioassay results, with N and co-limitation at Nant-y-Brwyn and P limitation at South Newton. However, we also found some evidence that the simplest DON and DOP forms, urea and glucose-6-phosphate, were producing a significant algal growth response at all concentration levels at the Nant-y-Brwyn site in spring. This indicates that these compounds are capable of being utilised for algal growth, whether directly or indirectly via bacteria, even at concentrations which are likely to be more similar to the low natural background concentrations of these specific compounds, since they are likely to have high turnover rates (Bronk et al., 2007). Local sources of these compounds in this type of catchment include sheep urine from the extensive sheep grazing on moorland areas, suggesting that stream biota may be acclimated to these compounds as a nutrient resource. The natural communities tested here, particularly at the low nutrient Nant-y-Brwyn site, are likely to be well adapted to scavenging these particular forms of dissolved organic nutrient at very low concentrations (Moschonas et al., 2017).

5. Conclusions

An experimental ex-situ microcosm approach has been used to study nutrient limitation and the potential for dissolved organic nutrient use in river phytoplankton, providing new insights under controlled laboratory conditions into the role of dissolved organic nutrients in rivers along a nutrient gradient. Nutrient limitation of riverine phytoplankton in this study varied spatially and seasonally, with all sites showing some variability in whether they were P, N or co-limited during the year, supporting hypothesis one. In accordance with hypothesis two, DON and DOP compounds differed in their algal bioavailability. The DON compounds generally showed similar levels of bioavailability among compounds, although there was limited evidence for occasional significant negative growth response. DOP compounds tended to stimulate growth more frequently than the DON compounds, likely reflecting the greater occurrence of P limitation at the sites in this study. However, we also found some evidence for growth enhancement on organic nutrient substrates above that on inorganic nutrient alone. Ambient nutrient concentrations were significant predictors of the growth response to dissolved organic nutrient additions. TN and ammonia concentration significantly explained the variation in DON growth response and SRP concentration was the most significant predictor of the DOP growth response, along with DOC concentration, supporting Hypothesis three. The concentration of the organic nutrient influenced bioavailability (Hypothesis four), however for the simplest compounds tested, urea and glucose-6-phosphate, there was evidence of growth even at the lowest concentration of nutrient addition. We conclude, therefore, that DOM is a potential nutrient resource used to support primary production in freshwaters, and that the specific algal growth response is likely to be controlled by site-specific conditions and adaptations. We argue that DOM is an overlooked nutrient resource for primary producers, and could be a key contributor to the process of eutrophication in freshwaters, particularly in relatively unenriched sites, where site-specific adaptations supporting the utilisation of dissolved organic nutrients may facilitate enhanced algal growth should fluxes of DOM increase.

CRediT authorship contribution statement

E.B. Mackay: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **H. Feuchtmayr:** Investigation, Visualization, Writing - original draft, Writing - review & editing. **M.M. De Ville:** Investigation. **S.J. Thackeray:** Methodology, Formal analysis, Writing - review & editing. **N. Callaghan:** Investigation. **M. Marshall:** Investigation, Writing - review & editing. **G. Rhodes:** Investigation, Writing - review & editing. **C.A. Yates:** Investigation, Writing - review & editing. **P.J. Johnes:** Funding acquisition, Methodology, Writing - review & editing. **S.C. Maberly:**Funding acquisition, Methodology, Investigation, Writing - original draft, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.137837.

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