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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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

1. 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 also readily pass through a 0.45 µm apertures (Fig. 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).

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.

2. Materials and methods

2.1 Field site and sampling

Samples were collected from two contrasting sub-catchments within the Conwy catchment, North Wales (Fig. 2; supplementary Fig. S2). 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 200-2500 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. S1).

2.2 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 $H_3{}^{33}PO_4$ (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 or 25 °C for the duration of the experiment.

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 CO_2 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).

2.3 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 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 ± the standard error of the mean (SEM) (n = 3).

3. Results

3.1 Water quality characteristics

The water samples collected from the two sub-catchments differed greatly in their chemical properties (Table 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 Mignient catchment and the N- and P-rich soils of the Hiraethlyn sub-catchment.

3.2 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 °C and 25 °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 2; Fig. 3).

In the samples from the agricultural catchment (Hiraethlyn) incubated at 5 °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. 3a). The latter two treatments however did not differ significantly from each other. In the acidified samples, the majority (91.4 ± 1.5 %) of the ^{14}C-amino acid 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 acid remaining in solution in the filtered and unfiltered samples after 7 d. When incubated at 25 °C, the rate of depletion was much faster than at 5 °C across all treatments, with 81.2 ± 0.4 % amino acids removed from the filtered and unfiltered water samples by 24 h (Fig. 3b). Increasing the incubation temperature to 25 °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 °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). Despite this, the trends in amino loss were broadly similar. Acidification largely prevented the loss of amino acids from solution, while filtering temporarily slowed, but did not prevent, amino acid depletion (Table 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.

3.3 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. 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 2; Fig. 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.

3.4 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 °C from the Migneint and 25 °C from the Hiraethlyn sub-catchments (two-way mixed ANOVA, $P < 0.001$; Table 2; Fig. 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. 5) and 25 °C Migneint (two-way mixed ANOVA, $P = 0.026$; Fig. 5).

At 5 °C, the amount of ^{33}P in the water from the Hiraethlyn sub-catchment did not drop below 91.5 ± 0.7 % for any treatment (Fig. 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. 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.

4. Discussion

4.1 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 (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 µm 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 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 1).

Across the different treatments and land-use types, the 20 ^{o}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).

4.2 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 dark (Johnes and Burt, 1991; Pearce, 1991). Although incubation at 5 ^{o}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 ^{14}C-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 supress 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.

4.3 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. HNO_3) may lead to the oxidation or depolymerisation of DOM during 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).

4.4 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 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.

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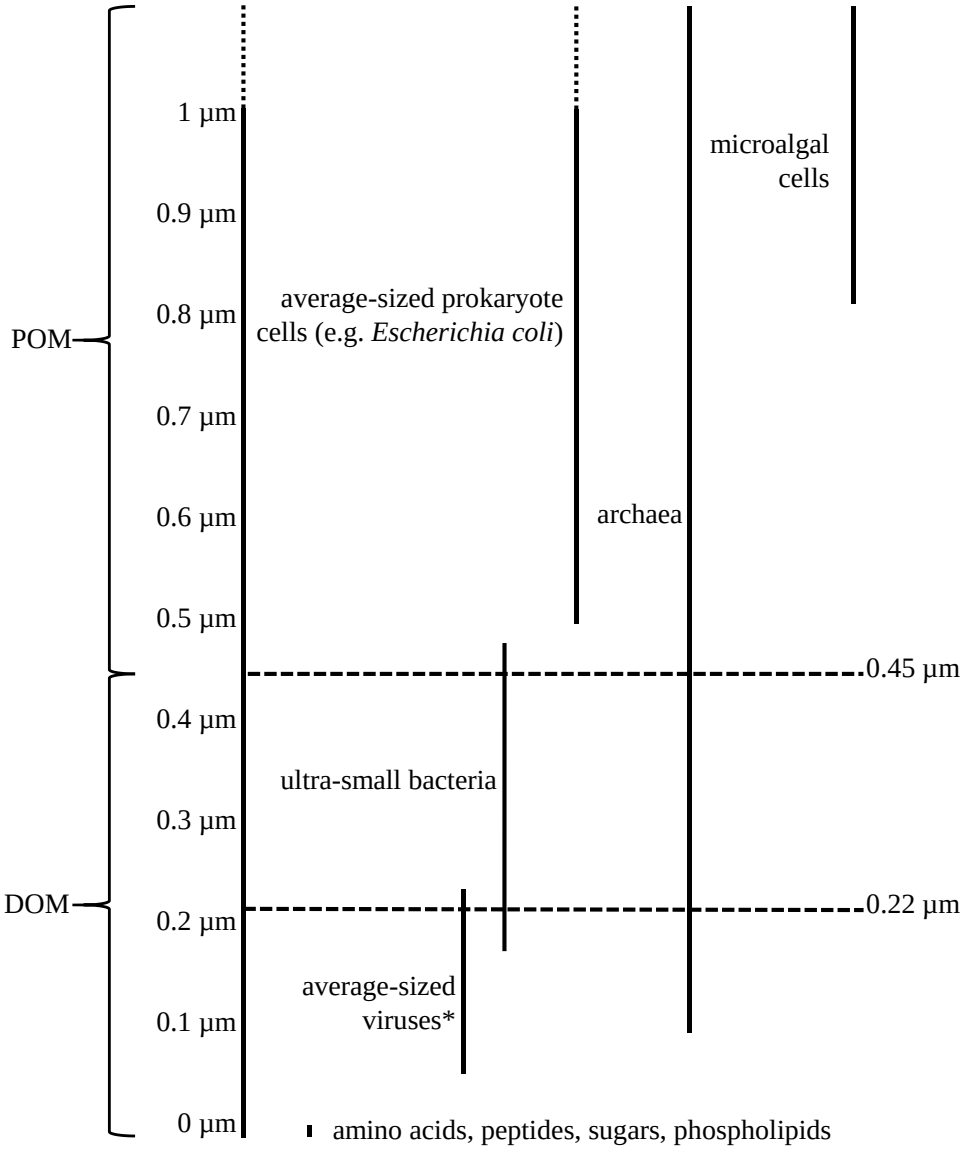

Fig. 1 Relative size of dissolved organic matter (DOM) and particulate organic matter (POM) components in comparison to bacteria, archaea and viruses. POM > 0.45 µm > DOM. 0.45/0.22 µm filter cut-offs indicated by a dashed line. * Some giant viruses >1 µm exist.

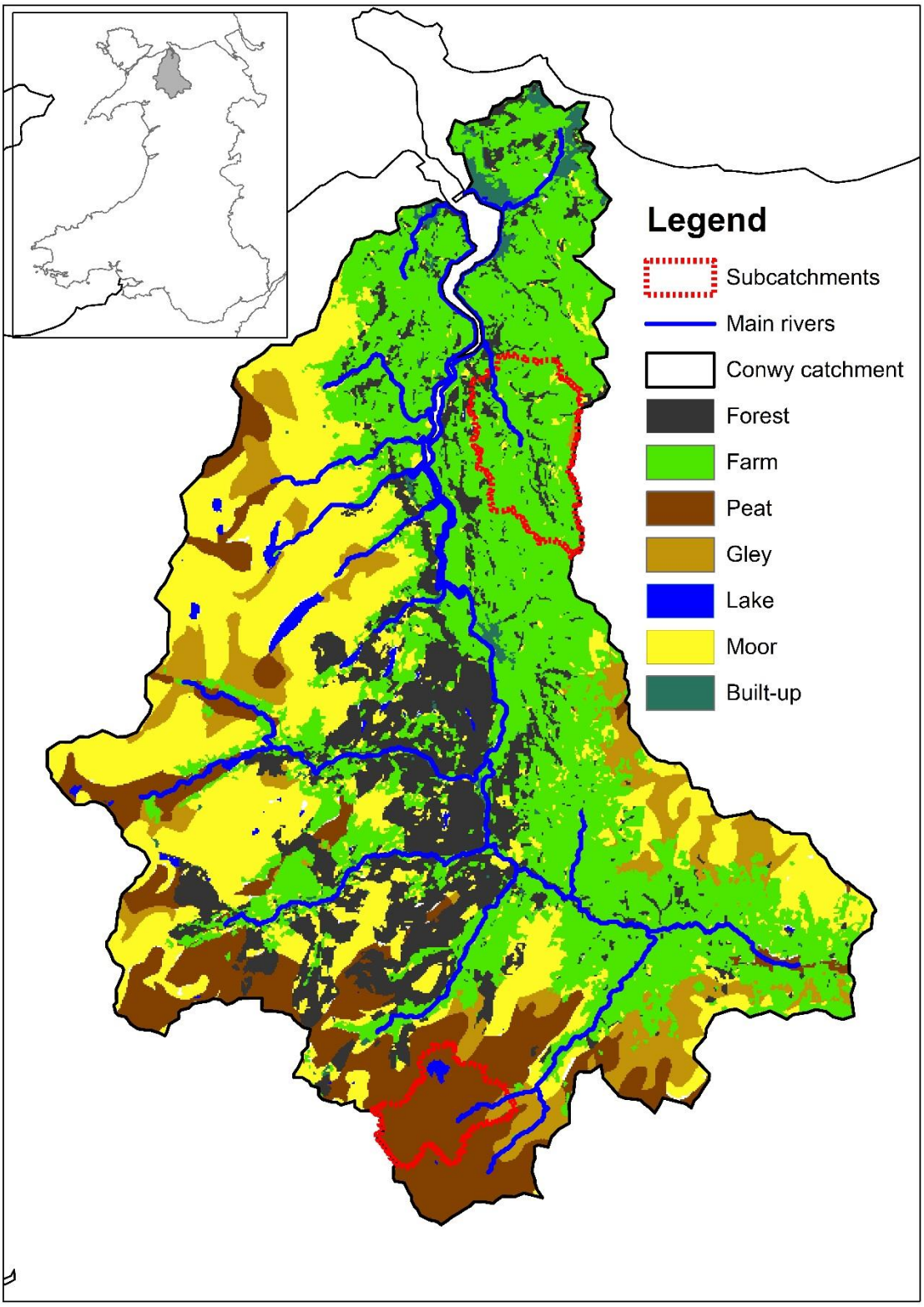

Fig. 2 Land use map of the Conwy catchment with upland peat bog (Migneint) and lowland improved grassland (Hiraethlyn) sub-catchments outlined in red.

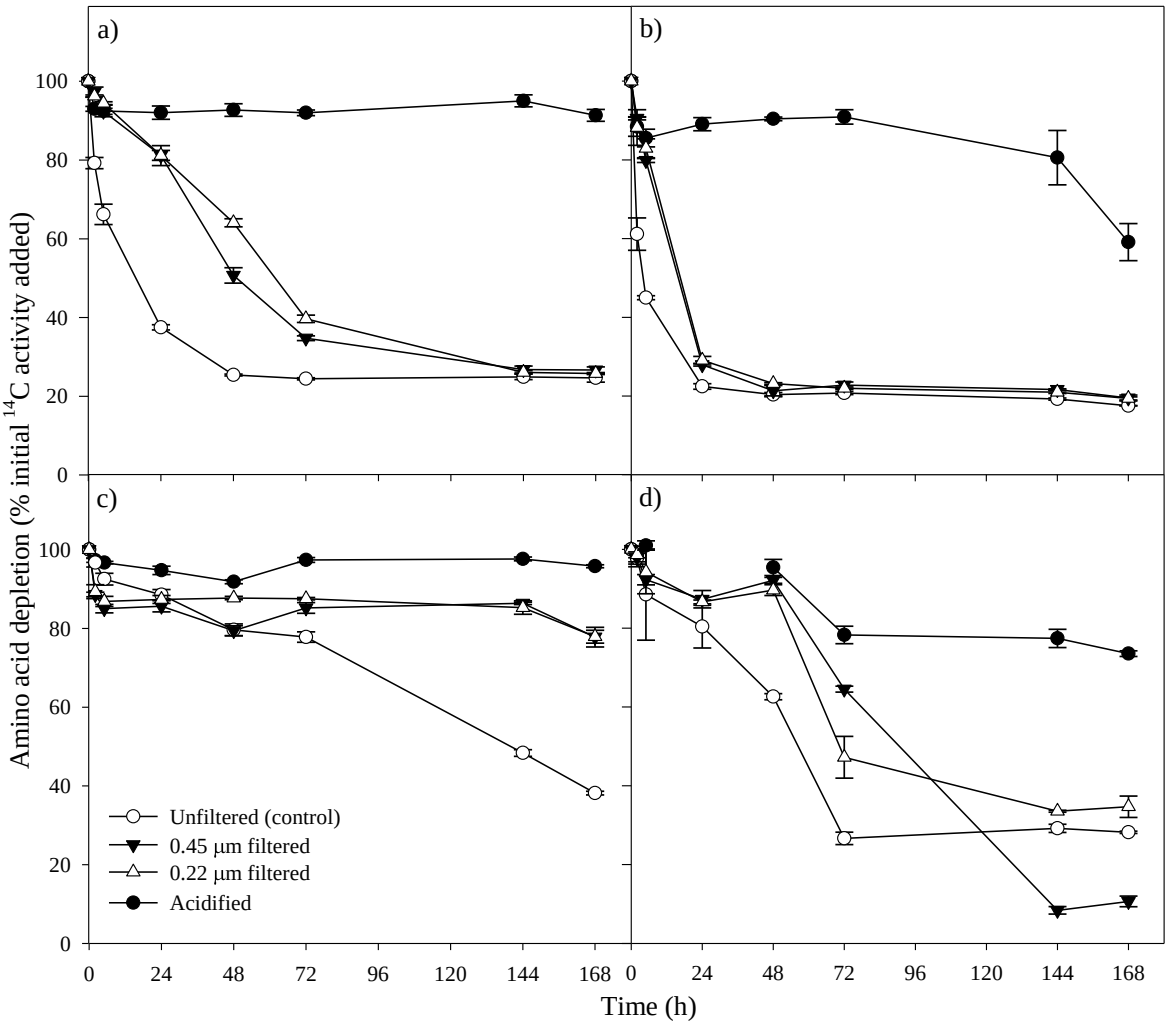

Fig. 3 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{14}C-labelled amino acids for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means ± SEM (*n* = 3). The legend is the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

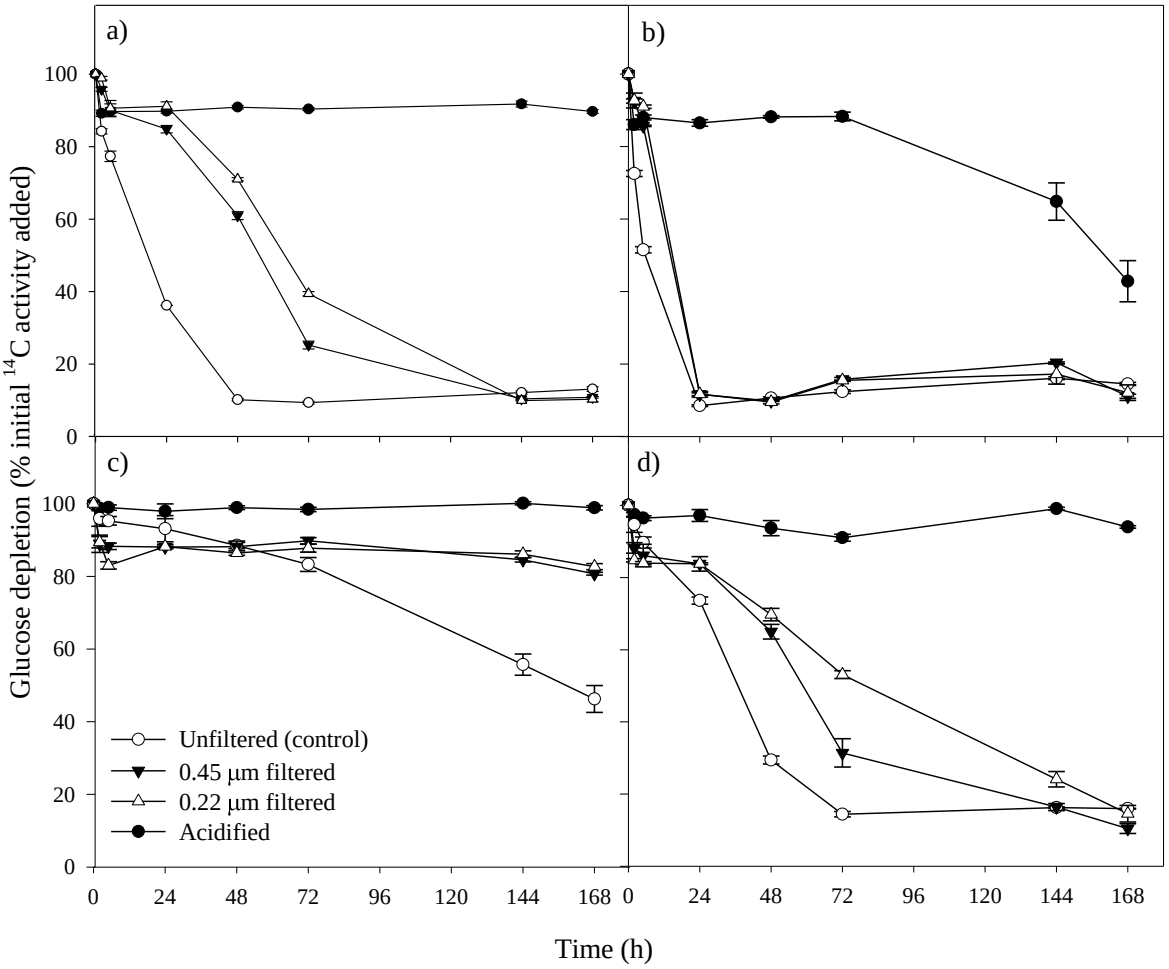

Fig. 4 Effect of filtering (0.45 or 0.2 μm) and acidification on the loss of ^{14}C-labelled glucose for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means ± SEM (n = 3). The legend is the same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

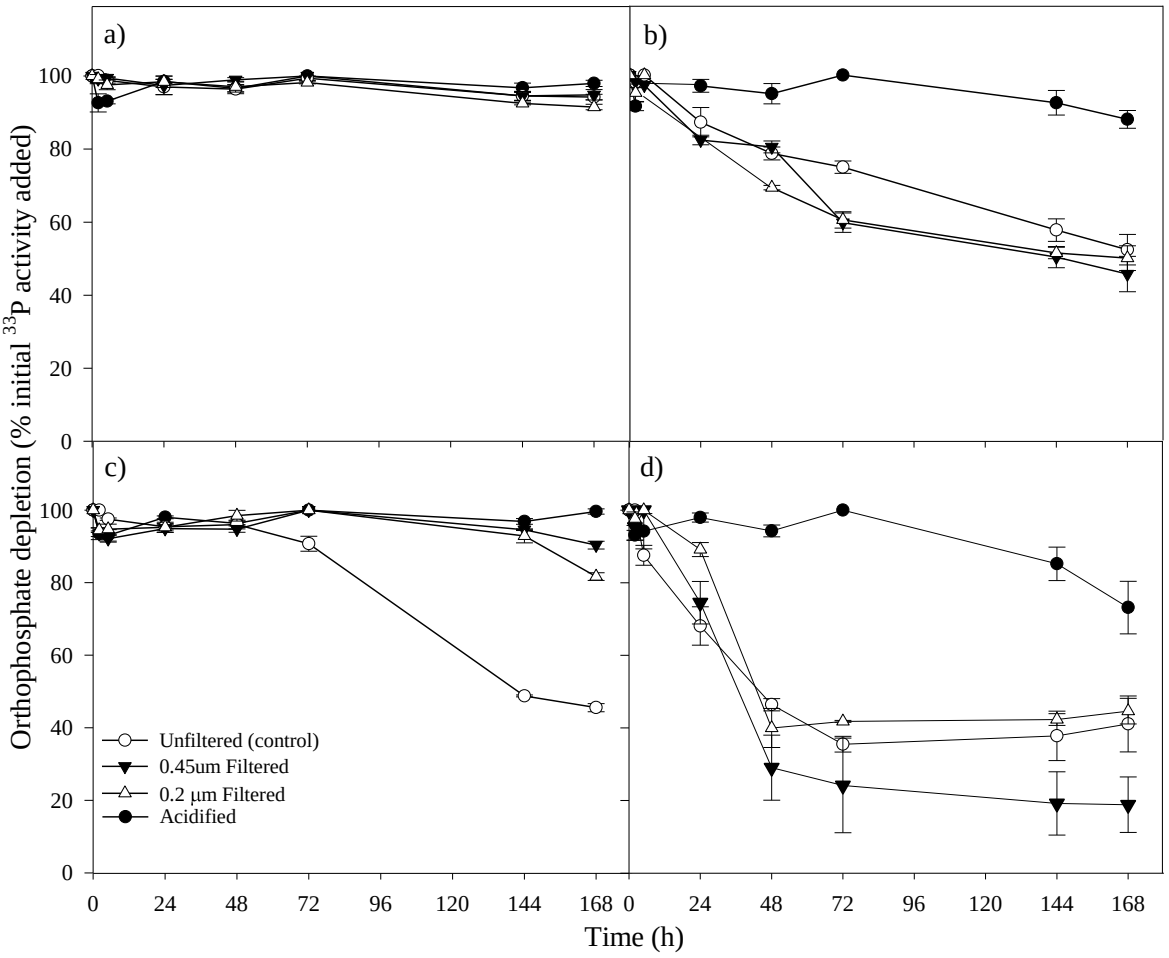

Fig. 5 Effect of filtering (0.45 or 0.2 µm) and acidification on the loss of ^{33}P-labelled orthophosphate for: a) Hiraethlyn sub-catchment 5 °C, b) Hiraethlyn sub-catchment 25 °C, c) Migneint sub-catchment 5 °C, d) Migneint sub-catchment 25 °C. Values represent means ± SEM (n = 3). The legend is the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

Table 1 Chemical properties of water from the Hiraethlyn and Migneint sub-catchments used in the substrate mineralisation experiments. Values represent annual mean data ± 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 (μS cm^{-1})	229 ± 25.3	35.9 ± 1.90
Temperature (°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 kDa)	99.7 ± 11.8	54.9 ± 4.06
Percentage low molecular weight aromatic compounds (% <1 kDa)	59.0 ± 7.81	31.2 ± 1.15

Table 2 Results from a two-way mixed ANOVA for each isotopically-labelled nutrient, sub-catchment and temperature.

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

Sub-catchment	Nutrient	Temperature (°C)	Simple effect of time		Interaction time × treatment	
			F	*P* -value	F	*P* -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*

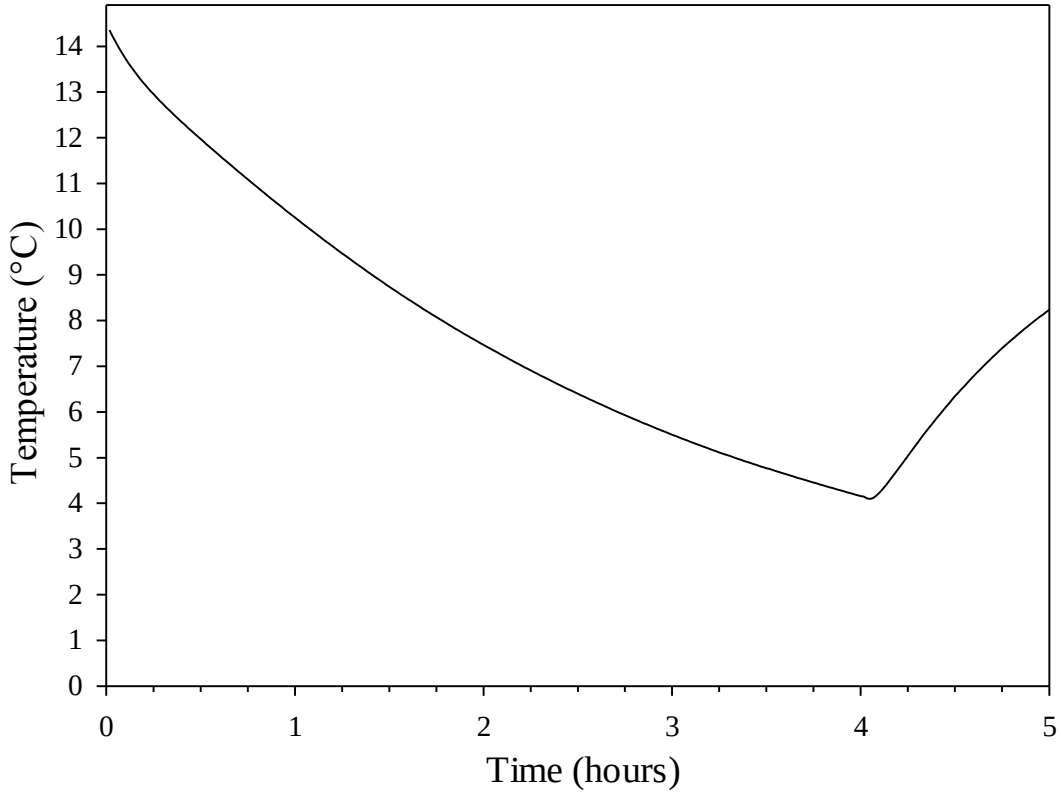

Fig. S1 Temperature of river water samples collected in 1 L HDPE bottles. Samples were collected in the field and immediately stored on ice for 4 h (representing the transportation time from the field to the laboratory). The samples were then removed from the ice and held at room temperature for 1 h (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).

Fig. S2 Images of a) the Hiraethlyn (lowland improved grassland) and b) Migneint (upland blanket peat bog) sub-catchments.