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Biochemical quality of basal resources in a forested stream: effects of nutrient enrichment

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Abstract:	We studied biochemical changes in biofilm and suspended particulate and dissolved organic matter (OM) during the leaf emergence period (March-May 2008) in a forested headwater stream in response to a long-term (4 years, 2004-2008) experimental nutrient enrichment study. This study compared results from one reach upstream of the enrichment point and one reach downstream using moderate nutrient concentrations (nitrogen, N, from 388 to 765 µg L-1 and phosphorus, P, from 10 to 30 µg L-1, resulting in N:P ratios of 85 to 56). During the spring of 2008, we analysed the chlorophyll content, elemental composition (carbon, C, and N), bacterial density, and extracellular enzyme activities along with their biochemical composition (amino acids, fatty acids and sterols) on biofilm and OM. Nutrients caused changes in the biochemical composition of the biofilm, while changes in the OM were subtle. The C:N ratio of the biofilm decreased with nutrient enrichment likely due to the increase in protein (non-essential amino acids). The polysaccharide and total and essential fatty acid contents were higher when nutrient enrichment coincided with greater light availability. The peptidase extracellular activity was higher in the fertilised reach at early spring, while phosphatase activity decreased at late spring. The suspended and dissolved OM composition did not change due to the nutrient addition, likely due to the lower water residence time in the reach. Headwater systems are highly dynamic, and the biochemical composition of the biofilm changed in response to changes in nutrients but also to light in this study. These changes, although moderate, could influence

	higher trophic levels through modifications in their diet. This experiment exemplifies how small land use shifts may affect headwater streams.
Response to Reviewers:	Responses to the reviewer Reviewer #3: This is a comprehensive study that examined a breadth of biofilm and organic matter biochemical responses to a nutrient addition, and will be of interest to readers of Aquatic Sciences. The manuscript has greatly improved from the previous version, and most of the suggestions by the reviewers were incorporated. My comments are mainly minor in nature and seek to clarify various sections of the text. Thanks again for this new accurate revision. We think your suggestions have undoubtedly contributed to improve the manuscript. P2, L2: I suggest removing the words 'qualitative and quantitative' from this sentence (and from elsewhere in the text). Instead, consider using 'We studied compositional [or biochemical] changes in biofilm and…' Done P2, L4: Consider changing 'as part of' to 'in response to'. Done P2, L8-10: This sentence seems out of place. Consider making it the first sentence of the abstract as it provides context for the study. The sentence has been eliminated. As we have included at the end of the abstract a sentence related with the effects on consumers, we have considered this sentence unnecessary. P2, L13: Include 'on biofilm and OM' at the end of the sentence. Done P2, L15-16: Consider rewording this sentence: Add 'with nutrient enrichment' between 'decreased' and 'likely', and remove 'throughout the study period in the fertilized reach' on L16. Done P2, L18: Is the 'microbial use of peptides' the same as 'extracellular enzyme activity'? If so, use the latter for consistency with the rest of the manuscript. Done P2, L19: Replace 'initial phases' with 'early spring', and 'end of the study period' with 'late spring'. Done P2, L23: It would be nice to have a concluding sentence with potential implications as described in the Discussion section (i.e., potential effects of these biochemical changes on higher trophic levels). We have slightly modified the abstract and introduced these two last sentences: ‘These changes, although moderate, could influence higher trophic levels through modifications in their diet. This experiment exemplifies how small land use shifts may affect headwater streams’. P3, L5: Consider rephrasing to '…affects many freshwater and coastal systems' and remove 'other than large freshwater systems'. Done P3, L5-6: Consider removing this sentence, as there is quite a bit of research on the effects of nutrient increases on stream ecosystems (and your next few sentences also describe these effects). Considering this comments and the next one, we have rewritten these sentences. P3, L10-11: Consider changing 'in these systems' to 'in headwater streams' (appears in 2 places). See before P3, L19-20: Provide a citation for this sentence. Done. The new citation was included in the reference list P3, L20: Remove 'In'. Done P4, L8-10: Reword this sentence ('Previously' does not fit). Done P4, 22-23: But see Tant et al. 2013 (Freshwater Science) who examined changes in detrital C:N:P composition with nutrient enrichment.

Thank you for the reference. We have includes it in this part of the introduction and in the reference list. We have rewritten the sentence.

P5, L9: Do you mean 'as a result of an increase in the autotrophic component of the biofilm'…? Changed

P6, L24: Mention that 'daily' stream flow was calculated. Included

P7, L22: Mentioned that sterilized water = autoclaved. Changed

P8, L4: 'bulk water' is not very descriptive. Perhaps replace with 'unfiltered water'.
We have changed it here and along all the manuscript.

P8, L20-21: The authors mentioned in the response to the reviewers that the conductivity measurement was temperature corrected (i.e., specific conductivity), so 'conductivity' should be changed to 'specific conductivity' here. Specific conductivity is not always the default measurement for multi-probes, and many read both conductivity and specific conductivity, thus it needs to be specified which measurement was taken. Changed to specific conductivity.

P9, L10-11: Please describe how the biofilm was extracted from the cobbles. Was the entire cobble brushed/scraped, or just a small area?
The detailed description of the biofilm extraction from cobbles is described some lines above (page 8, line 12). There, we explain that an aliquot of the extraction was used to measure chlorophyll. In that section we have included entire cobbles to clarify.

P10, L18: My question on the previous review about 'sum of all amino acids' referred to whether this was expressed as 'total number of amino acids per cm 2 (i.e., 3 amino acids/cm2)' or 'the total mass of amino acids per cm2 (i.e., 1.6 ug/cm2)'. I assume it is the latter because the units on Figure 4b are in ug/cm2. Including the units that the sum of amino acids were reported in on L18 would be sufficient.
We have included “of the sum of all the amino acid concentrations …’ instead of the units, because we have not included units in any of the other chemical analyses.

P11, L25: It is still not clear to me why 'PAR' was not included as a continuous factor in the statistical analysis instead of the 'sampling date'. It is not as important that PAR was not measured separately in the fertilized and control reaches when including this factor as a continuous variable in the statistical analysis, because the factor you are currently using (date) also does not vary between the control and fertilized reaches. Date is mainly used as a proxy for PAR in this paper, as most of your results are interpreted in the context of PAR, given the strong responses observed when the canopy was open in early spring.
Date was preselected in the model as repeated observation, considering that samples were collected during different dates. This selection would not be possible with PAR because, as the reviewer comment, this variable would be continuous. We consider more appropriate that PAR is an environmental feature. Of course date would be a proxy for PAR but only because PAR change according the period selected for the study. At the same time, PAR is clearly different between the first and the second sampling dates but not during the rest of the dates sampled. Considering PAR as a variable did not improve the model and the results because there would not be differences during the April-May period (3 of the sampling dates).

P13, L5: Change 'studied' to 'study'. Changed

P13, L7: Change 'water conductivity' to 'specific conductivity'. Changed

P13, L7-8: Move the water temperature results to L4-5 as temperature is mentioned in this previous sentence and the temperature data do not appear in Table 1, which is referenced in the sentence on L8. However, be sure to clarify that both air and water temperature results are presented in this paragraph.
The paragraph has been rewritten and air and water temperatures have been separated.

P13, L11: Mention what the DOC concentration represents (average of 2 reaches over

	the study period?). We have included across both reaches. P13, L19: Add: 'Algal biomass (as chlorophyll a) was higher…' Added P14, L8: Add: '…first date, and lower in the fertilized reach on the last date' Added P14, L10-13: Rearrange the first two sentences so Figure 4a is discussed first, and Figure 4b is discussed second. The two sentences have been rearranged. P14, L14: Does 'joint' refer to 'total' here? If so, change to the latter. Changed P14, L15: Include the result for the control reach as well. The data in this line is referred to the results of the Pearson correlation. It is not the concentration value for the fertilized reach. P15, L3-4: Was the reported average fatty acid content the average across both reaches? Yes, we have included 'across both reaches' to clarify. P15, L7: Define SAFA (to remind the reader, since it was only defined once in the Methods). Done P15, L10: It would be useful to provide the ω ratios for diatom, chlorophytes, cyanobacteria, and bacteria again here to remind the reader that these indicators are determined from ω ratios. The ω3:ω6 ratio was only used to differentiate whether the resources are primarily of terrestrial origin (values < 1), or if the origin is aquatic (> 1). This ratio is not related with the indicators mentioned by the reviewer. We have included in the text the meaning of the ω3:ω6 ratio. P15, L14: Mention for the bacterial fatty acids that 'data not shown'. Done P15, L21 and 25: Provide the statistics to support these statements. We have included P >0.05 in line 21. The results in line 25 are referred to the average content of two different groups of sterols. Statistics cannot be applied here. P16, L5-6: It is not clear how the 'activities showed changes with time, but the variations were not significant' based on the results presented in Table 2 as most of the enzyme activities were significantly different between reaches and over time in the bulk water and DOM fraction (based on the stats in Table 2). We agree with this observation, the phrase did not give appropriate information. We have changed some parts of the paragraph and included a new text: "Although all activities showed changes with time, these variations did not follow the pattern of any of the measured abiotic parameters" P16, L9-10: Provide the statistics to support this statement. We have included: P > 0.05, in both lines. P17, L4: Change 'caused changes' to 'changed'. Changed P17, L9: Remove 'temporal' as the time period is mentioned at the end of the sentence ('on the first sampling date'). Removed P17, L12: What does 'autotrophic activity' refer to here? Activity (i.e., GPP, NPP) was not measured. Perhaps 'activity' should instead read 'changes in the autotrophic community composition'? We have changed activity by compartment. P18, L3-5: It is not clear what 'correlation between bacteria, chlorophyll, etc' is being referred to here. I don't believe a Pearson's correlation was conducted between bacterial density and chlorophyll a (or any measure of biofilm chemical composition), but I could have missed it. Or do you mean that because bacterial density was 2x higher in the fertilized reach, it was correlated with changes observed in the biofilm community? The link between bacterial density and biofilm composition needs to be clarified. Also, if the link is as I mentioned (2x higher density), please mention that it

was only significantly higher on one day, according to Figure 2B.
The results of the correlation in this paragraph were referred to the control site. However, as the reviewer has observed, the two phrases are not well linked and can cause a misunderstanding. We have eliminated the second sentence referred to the control site.
We have not included that it was higher on one day because at the beginning of the paragraph we already mention it:
“In the fertilized reach, the bacterial density in the biofilm was approximately twofold higher than that of the control reach during the first period. Hepinstall & Fuller (1994) observed a positive correlation between algae and bacteria under different light and nutrient availability conditions and attributed their findings to the relationships between the algal exudates (primarily composed of polysaccharides and proteins) used by bacteria as an energy source. In addition, algal exudates represent a major C source for bacteria (Romaní et al. 2004; Carr et al. 2005). This coupled dynamics between bacteria and algae in biofilm is observed only in the fertilized reach. “

P18, L7-10: What does 'initially' and 'later' mean in these sentences? It seems to refer to the progression of research on biofilm amino acids, but it's not clear.
We have changed both. The text in now: “The biofilm was characterised by the amino acids alanine and glycine at early spring, and the glycine content was likely related to the structural matrices of diatoms (Dauwe and Middelburg 1998; Dauwe et al. 1999). In the last sampling dates biofilms showed…”

P18, L10-11: Consider this edit to the sentence: '…a higher abundance of labile amino acids AS indicators of fresh OM'. “As” have been included in the text.
P18, L24: Due to 'GREATER algal abundance'? Included greater

P19, L1-2: The shift from terrestrial to aquatic fatty acids in the fertilized reach should be listed as a 'marginally significant shift' (based on the statistical results). Included 'marginally significant shift'

P19, L6: Change 'would' to 'could'. Done

P19, L8: Shifts in basal resource quality could also accelerate consumer growth. We have changed limit by affect

P19, L11: What was the observed effect on meiofaunal secondary production (increase? decrease?).
Included “higher meiofaunal secondary production”.

P19, L12-14: The meaning of the word 'prompt' is still not clear. In the responses to the previous review, the authors mention that the response was 'prompt' because it was observed only on the first sampling date. However, because samples were not collected earlier than the first date, it is not clear if this effect was truly 'prompt' (i.e., observed only in early spring), or if the same effects were observed for several months prior to the first sampling date (e.g., since the previous year's leaf fall opened the canopy). Also, it seems that this sentence should include the interaction with light: '…although the interaction between nutrients and light appears to have an effect….'

For the first sentence, I would suggest: 'These results suggest that the interaction between nutrients and light appears to affect the biochemical composition of the biofilm to the greatest degree in early spring; however, we do not know whether these effects observed in early spring also occurred in previous months.'

I would suggest rephrasing the second sentence on 'the effect could be repeated over time' to mention that this potential 'repeated effect' would only be important for stream consumers that lived longer than 1 year. However, I don't think this second sentence is needed, as the main argument that changes in basal food quality can affect stream consumers is already made above.
We have included the change proposed by the reviewer for the first sentence and eliminated the second one.

P20, L11: Change to '…related to changes in biofilm quality…' Done

	P20, L18: Provide units for these numbers. Done P21, L16: Include a 'Conclusions' subheading before the start of this paragraph. Done P21, L16: Remove 'permanent' - it's not clear what timescale is being referred to (time scale of the experiment? Geological time scales?). We have changed permanent to stable in order to emphasize that it was the only change maintained during all the experiment. P21, L19: Consider rephrasing this sentence for clarity: 'The progression in canopy closure in spring limited the effect of light on the biochemical…' Done P22, L4: Consider changing 'would' to 'could'. Done P22, L6: Consider changing 'global eutrophication scenario' to 'conditions of eutrophication'. Done Table 1: In the caption, mention that the sampling period was (17 Mar to 5 May 2008). Replace 'showed' with 'presented'. Done Table 2: In the caption, mention the interaction is with sampling date. Done

Biochemical quality of basal resources in a forested stream: effects of nutrient enrichment

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Short title: **Nutrient enrichment and resource quality**

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Abstract

We studied biochemical changes in biofilm and suspended particulate and dissolved organic matter (OM) during the leaf emergence period (March-May 2008) in a forested headwater stream in response to a long-term (4 years, 2004-2008) experimental nutrient enrichment study. This study compared results from one reach upstream of the enrichment point and one reach downstream using moderate nutrient concentrations (nitrogen, N, from 388 to 765 μg L^{-1} and phosphorus, P, from 10 to 30 μg L^{-1}, resulting in N:P ratios of 85 to 56). During the spring of 2008, we analysed the chlorophyll content, elemental composition (carbon, C, and N), bacterial density, and extracellular enzyme activities along with their biochemical composition (amino acids, fatty acids and sterols) on biofilm and OM. Nutrients caused changes in the biochemical composition of the biofilm, while changes in the OM were subtle. The C:N ratio of the biofilm decreased with nutrient enrichment likely due to the increase in protein (non-essential amino acids). The polysaccharide and total and essential fatty acid contents were higher when nutrient enrichment coincided with greater light availability. The peptidase extracellular activity was higher in the fertilised reach at early spring, while phosphatase activity decreased at late spring. The suspended and dissolved OM composition did not change due to the nutrient addition, likely due to the lower water residence time in the reach. Headwater systems are highly dynamic, and the biochemical composition of the biofilm changed in response to changes in nutrients but also to light in this study. These changes, although moderate, could influence higher trophic levels through modifications in their diet. This experiment exemplifies how small land use shifts may affect headwater streams.

Keywords *epilithic biofilms · amino acids · fatty acids · polysaccharides · headwater stream · riparian phenology*

Introduction

Eutrophication is a global phenomenon (Vitousek et al. 1997; Meybeck 2003) that affects many freshwater and coastal systems. In headwater streams, nutrients promote microbial activity and accelerate allochthonous organic matter (OM) processing (Greenwood et al. 2007; Benstead et al. 2009; Rosemond et al. 2015), increase the rate of biological production, and alter the biogeochemical cycles (Smith et al. 2006). Invertebrate secondary production, biomass and abundance increased in these systems because of nutrients, emphasizing that changes in food quality (*i.e.*, greater microbial OM conditioning) override changes in food quantity (*i.e.*, faster OM decomposition; Cross et al. 2006). However, increased nutrient supplies do not always propagate upwards in food webs, and some trophic decoupling between basal resources and consumers can occur over long periods (Davis et al. 2010). Elevated nutrient concentrations can also increase autochthonous biomass and primary production (Rosemond et al. 2000; Dodds et al. 2002), but these changes may be limited by light availability (Hill et al. 2001; Greenwood and Rosemond 2005; Ylla et al. 2007). In this sense, riparian canopies in forested streams modulate light reaching the streambed and consequently stream metabolism despite nutrient availability (Proia et al. 2012). Algal biomass could enhance heterotrophic OM utilization as a response of the algal-bacterial

coupling effect since algal exudates represent a major carbon source for bacteria (Romani et al, 2004).

Benthic algal communities can serve as a source of high-quality food and energy for consumers (1-60% of the total annual energy; Lamberti 1996). Because of the relatively low C:N and C:P ratios (Frost et al. 2002) and the high protein and lipid contents, algal-derived materials can serve as a more nutritious food source than terrestrial OM (Anderson and Cummins 1979; Lamberti 1996; Torres-Ruiz et al. 2007). The effects of nutrient enrichment on biofilm stoichiometry were described by Sabater et al. 2011 as an increase in the N and P contents and a decrease in the C:N ratio. These authors observed that these effects were reflected in the elemental composition of some key consumers (predators, grazers and detritivorous) as was also observed by other studies (Cross et al. 2003).

The OM present in the streams ~~(benthic, suspended particulate and dissolved) is~~ a complex mixture of polysaccharides, proteins, lipids, lignin, organic acids and other compounds, such as humic substances (Mannino and Harvey 2000). The primary energy-yielding compounds (biomolecules) in living organisms are polysaccharides, proteins and lipids. Many of these biomolecules play important structural and regulatory roles in organisms, and some are essential for consumers because they cannot be synthesised *de novo* (Brett and Müller-Navarra 1997; Dauwe and Middelburg 1998). Most studies focused on characterizing the composition of OM have mainly been limited to the dissolved fraction (Fellman et al. 2010; however, see, e.g., Gremare et al. 1997; Ledger and Hildrew 1998; Ylla et al. 2010; Kolmakova et al. 2013) but few (Tant et al. 2013) have examined the changes in OM composition in response to nutrient additions.

A previous study of forested Mediterranean streams has examined the stoichiometric changes in biofilms during a long-term nutrient enrichment experiment (Sabater et al. 2011) and has highlighted the time-dependent response depending on the ~~considered~~ biological compartment. Here, we extend this study by examining the changes in the biofilm and the suspended and dissolved OM ~~collected as part of the same experimental study.~~ OM quality was assessed based on its biochemical composition (*i.e.*, polysaccharides, proteins and lipids). In addition, functional (enzyme activity) changes in biofilm and suspended and dissolved OM were analysed. We hypothesised that nutrient enrichment in the biofilm would i) increase the polysaccharide, protein and lipid content; ii) change the amino acid and fatty acid composition of OM as a result of an increase in the autotrophic component in biofilm; and iii) increase the essential amino acid and fatty acid contents for consumers because most of these components are derived from primary producers. However, iv) the nutrient enrichment of the suspended and dissolved OM may exhibit minor changes in the biochemical composition due to the prevailing allochthonous origin and lower residence time in the reach. ~~This biochemical approach may provide clues for detecting mechanisms and key molecules that underlie the effects of abiotic environmental changes (nutrient addition) on OM composition. This paper contributes novel data and may represent a step forward in the knowledge of nutrient-rich headwater streams.~~

Methods

Experimental design

Our study was conducted in the Fuirosos stream, which is located in the northeastern Iberian Peninsula (41º 42’ N; 02º 34’ E) in the Montnegre-Corredor Natural Park. Fuirosos is a Mediterranean, oligotrophic, third-order stream with a basal

flow ranging from 0 to 25 L s^{-1} (Butturini et al. 2008). Nutrient addition was performed in a 50-m reach (termed the fertilised reach), and the reach upstream from the nutrient addition site was considered the control reach. The nutrient uptake length in this stream is approximately 50 m for N and P (Sabater et al. 2005). Both reaches were contiguous and similar in terms of slope, substrate type, water flow, light regime and riparian vegetation. The dominant riparian species were deciduous (*Platanus acerifolia* Willd., *Alnus glutinosa* (L.) Gaertn., *Populus nigra* L. and *Corylus avellana* L).

The riparian vegetation and the steep banks of the channel caused the light availability to be generally low, except during the early spring when the forest canopy was open (Acuña et al. 2004). As part of a long-term fertilisation experiment (June 2004 to June 2008), this study was performed in the spring of 2008 over 4 sampling dates (17/03, 7/04, 21/04, and 5/05) that corresponded with decreasing light availability in the streambed as the canopy closed. This experiment was performed during the fourth year of fertilization because previous results observed significant effects in biofilm stoichiometry only after long-term nutrient addition (Sabater et al. 2011).

Instantaneous underwater light was measured in the studied area using a portable meter (Li-192SB quantum sensor, LI-COR, Lincoln, NE, USA). These measurements were correlated with outdoor records from a nearby weather station to obtain the continuous light regime reaching the streambed. The air temperature during the sampling period was also obtained from a nearby weather station (Collsacreu and Pla de la Tanyada, Diputació de Barcelona). Both measures were for the whole studied section (both experimental reaches). The water level was continuously monitored during the sampling period with a pressure transducer (PDCR 1830, Druck limited, Leicester, UK) connected to a data logger. Stream flow was determined every 2 weeks by using the slug-injection method with NaCl as a conservative tracer (Gordon et al. 1992). These

measurements were used to determine a stage-flow relationship, and the daily stream flow during the study period was calculated. Nutrients were continuously added to the fertilised reach using a 200-L reservoir connected to a tap that dispensed dissolved nutrients at a constant flow rate. The tank was refilled with nutrient solution, and the concentrations were adjusted weekly to respond to variations in the nutrient concentrations and flow of the stream. Nutrients were added immediately downstream of a small waterfall to assure mixing and uniform dispersal. Inorganic N was added as ammonium nitrate, and P was added as ammonium phosphate. During the 4 years of fertilisation, the average inorganic N concentration increased twofold relative to the background concentration, the $N\text{-}NO_3$ concentration increased from 364 to 580 μg L^{-1}, and the $N\text{-}NH_4$ concentration increased from 24 to 185 μg L^{-1}. Inorganic P increased threefold, with $P\text{-}PO_4$ increasing from 10 to 30 μg L^{-1} (Sabater et al. 2011). This moderate increase in the dissolved nutrient concentrations, primarily of inorganic P, which is generally limiting in this stream (Sabater et al. 2005), decreased the N:P ratio from 85 in the control reach to 56 in the fertilised reach.

Four cobbles were collected per reach and kept in a container with stream water to analyse chlorophyll and elemental and biochemical composition of biofilm. Glass tiles (1.44 cm^2) were submerged in the stream on February 7, 2008, before the sampling period began and were used as surrogate cobbles for biofilm development. Glass tiles were attached to a brick with silicon adhesive, and 4 bricks were installed per reach. Four glass tiles per reach were collected to measure bacterial density, and four more glass tiles were collected to measure extracellular enzyme activity. To analyse the bacterial density, each glass tile was placed in 10 mL of filtered (0.2-μm nylon membranes, Whatman), autoclaved water. To determine the enzyme activities, the glass tiles were placed in 4 mL of stream water. Water samples for nutrient analysis were

filtered through 0.2-µm nylon membranes (Whatman, Maidstone, UK) and additional water samples (four samples of 2 L) were collected for the analyses of the dissolved and particulate OM. All of the collected materials were immediately transported to the laboratory in a cooler.

Once in the laboratory, subsamples were obtained from each water sample to measure bacterial density and enzyme activities in the unfiltered water. Next, from each 2-L water sample 3 aliquots of 600 mL was filtered through three different 0.7-µm glass fibre precombusted filters (GF/F, Whatman) to separate particulate OM (POM) from dissolved OM (DOM). Each filter was used to measure the polysaccharide, protein and lipid contents of POM respectively. Subsamples were obtained from the filtered water to measure dissolved organic carbon (DOC), enzyme activities, polysaccharides, proteins and lipids associated with DOM. Biofilm material was obtained from entire cobbles immersed in distilled water (60 mL) that were brushed and sonicated (3 min, sonication bath at 40 W, 40 kHz). Aliquots from the 60-mL water samples containing the biofilm extract were used to analyse C, N, chlorophyll, polysaccharide, protein and lipid contents. The surface of each cobble was measured to allow for later standardisation of the measurements by area.

In general, the samples were kept frozen or refrigerated (for DOC) before analysis; however, the enzyme activity and bacterial density measurements were performed on the same day that the samples were collected.

Physicochemical parameters

Dissolved oxygen, water temperature, pH and specific conductivity were measured *in situ* using a portable multi-probe (Hach, Loveland, CO, USA) on each sampling date. Nitrate, ammonium and reactive P were analysed using standard methods (A.P.H.A. 1995).

Carbon and nitrogen content

The water samples used to measure DOC (20 mL) were acidified with 100 µL of 2 M HCl (2%), fixed with 100 µL of NaN_3 (2.7 mM), and maintained at 4ºC before analysis. DOC was measured using a TOC analyser (Multi NC/3100; Analytic Jena, Jena, Germany) with thermocatalytic oxidation (up to 950ºC).

The cobble samples used to measure the C and N contents in the biofilm were freeze-dried and weighed to the nearest 0.001 mg. Next, the elemental composition was determined using an elemental analyser (EA 1108, Thermo Fisher Scientific, Milano, Italy) with vanadium pentoxide as the oxidation catalyst.

Chlorophyll content

Chlorophyll *a* was used to estimate the algal biomass. Biofilm extracts from cobbles were collected on GF/F filters (Whatman) and extracted for 12 h with 90% acetone in the dark at 4ºC. Samples were sonicated (2 min, sonication bath at 40 W, 40 kHz), and filters were manually ground. The extract was filtered through 1.4-µm glass fibre filters (GF/C, Whatman), and the chlorophyll content was determined spectrophotometrically (Lambda 2 UV/VIS spectrophotometer, PerkinElmer, Waltham, MA, USA), as described by Jeffrey and Humphrey (1975).

Bacterial density and extracellular enzyme activities

The bacterial density (live and dead bacteria) was estimated for the colonised glass tiles and the unfiltered water samples (for a description of the methods, see the online resource, Appendix 1). The fraction of live bacteria was calculated as the abundance of live cells relative to the total cells.

The activities of four hydrolytic enzymes involved in OM degradation were measured: phosphatase (EC 3.1.3.1-2), β-D-1,4-glucosidase (EC 3.2.1.21), leucine-aminopeptidase (EC 3.4.11.1) and lipase (EC 3.1.1.3). Phosphatase is an enzyme that

degrades orthophosphoric monoesters to inorganic P (Klotz 1992), β-glucosidase is involved in ~~the use of simple autotrophic or heterotrophic~~ polysaccharides (Deshpande and Eriksson 1988), leucine-aminopeptidase activity is related to polypeptide hydrolysis, which is used to obtain leucine and other hydrophobic amino acids (Francoeur and Wetzel 2003), and lipase hydrolyses ester bonds in lipid substrates. These enzymes are derived from the microbial community, primarily heterotrophic bacteria (Romaní and Sabater 2001; Vrba et al. 2004). Colonised glass tiles and water samples (unfiltered water and DOM; 4 mL each) were analysed using spectrofluorometry to determine the activities of these four enzymes (Online resource, Appendix 2).

Biochemical composition

Biofilm samples from cobbles, POM (filters) and DOM (for polysaccharide, protein and lipid content analysis) were freeze-dried. The total polysaccharide content was measured using the 3-methyl-2-benzothiazolinone hydrochloride (MBTH) method (Pakulski and Benner 1992; Chanudet and Filella 2006) with some modifications (Online resource, Appendix 3; Ylla et al. 2010).

The amino acid composition was analysed using high-performance liquid chromatography (Online resource, Appendix 4; Ylla et al. 2011). All amino acids were quantified, except for cysteine, which was not quantified due to analytical problems. The total protein content was calculated as the sum of all amino acid concentrations in each sample. Amino acids were classified as essential (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, arginine and histidine) or non-essential (aspartic acid, serine, glutamic acid, glycine, alanine, proline and tyrosine) (Dauwe and Middelburg 1998).

Lipids from the biofilm and POM samples were extracted using a mixture of chloroform and methanol (2:1) (Bligh and Dyer 1959). The total lipid content was analysed using the colorimetric sulphophosphovanillin method (Zollner and Kirsch 1962). The dissolved fraction could not be calculated because the lipid content was below the detection limit of this method (< 0.01 mg L^{-1}). The fatty acid and sterol contents in the biofilm samples were determined using gas chromatography and mass spectrometry (Online resource, Appendix 5). Fatty acids were classified according to their number of double bonds, *i.e.,* saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA), and the position of the double bond, which is used to distinguish between ω3 and ω6 fatty acids (Parrish, 1999). A ω3:ω6 ratio of < 1 indicates that the resources are primarily of terrestrial origin, and a ratio > 1 indicates that the resources are primarily of aquatic origin. We also determined whether fatty acids were essential for consumers (essential fatty acids (EFA): 18:2ω6, 18:3ω3, 20:4ω6, 20:5ω3, and 22:6ω3) and if they could be considered indicative of an organisms presence; *i.e.,* fatty acids from diatoms (20:5ω3 and 16:1ω7), chlorophytes and cyanobacteria (18:2ω6 and 18:3ω3) and bacteria (15:0 and 15:1 and branched 13:0 and 15:0) (Desvilettes et al. 1997; Napolitano 1999; Olsen 1999). Sterols are essential for consumers, and they can be used as indicators of algal (fucosterol), fungi (ergosterol) and higher plants (campesterol, sitosterol and stigmasterol) origin (Martin-Creuzburg and Elert 2009).

Statistical analysis

The results are expressed as means ± standard errors. One-way ANOVA was used to compare the physicochemical parameters measured on each sampling date between the reaches. Linear Mixed-effect models were used with each response variable to examine the main effects of fertilisation during the experiment and fitted using the

restricted maximum likelihood (REML). The final model selected (using Akaike's Information Criterion, AIC) in the study was: reach as fixed effect factor with 2 levels: control and fertilised, and date as random effect factor with 4 levels: sampling occasions (date was preselected in the model as repeated observation). The main effects of fertilisation and the interaction with date were assessed. The models did not produce Type III results for random factors (Pinheiro and Bates 2000). When significant, pairwise comparisons were performed using the Bonferroni correction to determine differences among the data means. Correlations between the pairs of response variables in the biofilm were tested in order to explore possible drivers using Pearson's product-moment correlation coefficient (*r*) for the entire data set and for the separate reaches.

The normality of the residuals (Kolmogorov-Smirnov and Shapiro-Wilks tests) and homoscedasticity (Levene's test) were assessed and improved, if necessary, by using Box-Cox transformations. Results were considered significant at $\alpha < 0.050$ and marginally significant at $\alpha < 0.100$. These analyses were performed using IBM SPSS Statistics 20 software for Windows (SPSS Inc., Chicago, IL, USA).

The biomass of the biofilm per unit of surface area (g DM cm^{-2}) was tested using mixed linear models and a likelihood ratio test, and no differences were noted between the reaches or the reach x date interaction. This result indicated that the thickness of the biofilm was not significantly influenced by nutrient enrichment. Consequently, the variables measured in biofilms are given in units of surface area (cm^{-2}). The amino acid contents (given as relative abundance) of the biofilm and suspended and dissolved OM samples were analysed previously using detrended correspondence analysis (DCA). DCA showed that the longest gradient lengths (which estimate the heterogeneity in the biochemical composition) were shorter than 3.0 units, which indicated a linear response to the underlying environmental gradient. Therefore, linear ordination techniques were

appropriate (Lepš and Šmilauer 2003, pp. 43-59), and principal component analyses (PCA) were performed using CANOCO software (version 4.5, Biometris, Wageningen, The Netherlands).

Results

Physicochemical parameters

Light availability decreased from early to late spring, and this change was more evident from the first sampling date to the second one (Fig. 1a). However, the air temperature exhibited an inverse pattern (Fig. 1b). During the study period, water temperature increased from 8.6 to 13.6ºC, the stream flow was 23.1 (± 4.3) L s^{-1}, and no differences were observed between the 2 reaches regarding the specific conductivity, pH, oxygen or temperature (Table 1). The N and P concentrations remained higher in the fertilised reach, where the N:P ratio decreased (Table 1). The direction of these changes has remained unchanged since 2004 (Sabater et al. 2011). The DOC concentration did not change due to fertilisation, with an average value of 3.98 (± 0.13) mg L^{-1} across both reaches.

Biofilm characteristics

The average C content of the biofilm was 24.65 (± 0.86)%, without significant differences between the reaches, and the N content was higher in the fertilized reach (control reach: 2.98 ± 0.14%; fertilised reach: 3.53 ± 0.23%; $F_{1,24}$ = 4.74; P = 0.039). Thus, the C:N molar ratio of the biofilm was lower in the fertilised reach (control reach: 9.68 ± 0.26; fertilised reach: 8.29 ± 0.14; Reach: $F_{1,24}$ = 18.06; P = 0.004). None of these variables were affected by the sampling date.

The algal biomass (as chlorophyll *a*) was higher in the fertilised reach (Reach: $F_{1,24}$ = 7.12; P = 0.013), and the interactions between the reach and date (Reach x Date:

$F_{6,24}$ = 6.63; P < 0.001) indicated differences between the first and subsequent dates (pairwise comparison, Fig. 2a). The density of live bacteria (Fig. 2b) was higher in the fertilised reach during the first two sampling dates (Reach: $F_{1,24}$ =10.97; *P* = 0.003; Reach x Date: $F_{6,24}$ = 23.52; P < 0.001), and the percentage of live bacteria relative to the total bacteria (17.53 ± 2.56% on average over all dates and reaches) did not significantly change due to fertilisation.

The extracellular enzyme activities in the biofilms were differentially affected by fertilisation (Fig. 3). The phosphatase and β-glucosidase activities were lower in the fertilised reach from the 21st of April until the end of the experiment (Phosphatase, Reach x Date: $F_{6,24}$ = 17.92; *P* < 0.001; Glucosidase, Reach x Date: $F_{6,24}$ = 5.58; *P* = 0.001). The leucine-aminopeptidase activity was higher in the fertilised reach on the first date and lower on the last one (Reach x Date: $F_{6,24}$ = 8.91; *P* < 0.001), and the lipase activity did not show any significant changes due to any of the factors.

The polysaccharide content showed a significant interaction (Reach x Date: $F_{6,24}$ = 5.45; *P* < 0.001) but pairwise comparisons did not detect any difference between dates (*P* > 0.05) (Fig. 4a). However, the protein content in the biofilms was higher in the fertilised reach (Reach: $F_{1,22}$ = 6.56; *P* = 0.018) (Fig. 4b). The lipid content did not show any significant changes (Fig. 4c). The total polysaccharide and protein ~~content was~~ positively correlated with chlorophyll *a* in the fertilised reach (*r* = 0.763; *P* = 0.002; n = 14).

The higher protein content was primarily due to the non-essential amino acid increase (control reach: 12.51 ± 1.44; fertilized reach: 22.07 ± 2.82 µg cm^{-2}; Reach: $F_{1,22}$ = 9.56; *P* = 0.005). The essential amino acid content only marginally increased in the fertilised reach (control reach: 15.89 ± 1.93; fertilized reach: 22.52 ± 2.97 µg cm^{-2}; Reach: $F_{1,22}$ = 3.73; *P* = 0.066). The amino acid composition of the biofilm changed

with time in both the control and fertilised reaches. This change can be observed by the PCA ordination (Fig. 5), where the samples arranged on the first axis (explaining 34% of the variability) were separated between the first sampling dates on the positive side of the axis and were characterised by a high percentage of alanine and glycine, ~~and the~~ last sampling dates were situated on the negative side of this axis and were characterised by higher percentages of leucine, histidine and phenylalanine.

The average total identified fatty acid content in the biofilms across both reaches was 5.8 (± 0.8) µg cm^{-2}, which accounted for 34.1 (± 3.1)% of the total lipid content. The fatty acid content was greater on the first date in the fertilised reach (Reach x Date: $F_{6,23} = 9.52$; $P < 0.001$; Fig. 6a), and the essential fatty acids (EFA, Fig. 6b) and PUFA (polyunsaturated fatty acids) showed the same patterns (Reach x Date: $P < 0.029$). The SAFA:PUFA (saturated versus polyunsaturated FA) ratio did not show any differences (Fig. 6c). The ω3:ω6 ratio (values < 1 indicate that the resources are primarily of terrestrial origin, and > 1 of aquatic origin) was generally (marginally significantly) higher in the fertilised reach (control reach: 0.9 ± 0.2; fertilized reach: 1.2 ± 0.2; Reach: $F_{1,23} = 3.59$; $P = 0.070$; Fig. 6d). The fatty acid indicators of diatoms were more abundant on the first date in the fertilised reach (Reach x Date, $F_{6,23} = 22.48$; $P < 0.001$; Fig. 6e) and followed the same pattern as those of fatty acids that are characteristic of chlorophytes and cyanobacteria (Reach x Date: $F_{6,23} = 9.52$; $P < 0.001$; Fig. 6f), and bacterial fatty acids (Reach x Date: $F_{6,23} = 7.38$; $P < 0.001$, data not shown). The sum of the diatom and chlorophyte-cyanobacteria fatty acids was positively related to the chlorophyll *a* content ($r = 0.41$; $P = 0.021$; n = 31). However, the bacterial density and bacterial fatty acid contents were not correlated.

Six sterols were identified in the biofilms (campesterol, stigmasterol, sitosterol, fucosterol, cholesterol and cholestanol), representing 2.39 (± 0.26)% of the total lipids.

The total sterol content was 0.40 (± 0.05) µg cm^{-2}, with no differences among the reaches or dates ($P > 0.05$). The average fucosterol content was 0.03 (± 0.01) µg cm^{-2}, and the average content of sterols originating from higher plants was 0.29 (± 0.04) µg cm^{-2}.

Suspended and dissolved organic matter characteristics

The density of live bacteria in the unfiltered water did not change due to fertilisation (Table 2) and was higher at the end of the experiment in both reaches. The percentage of live bacteria was 21.79% (± 1.54%) and was not affected by any of the factors. The enzyme activities in the water were slightly higher in the fertilised reach and statistically significant for glucosidase, aminopeptidase and lipase in the unfiltered water and phosphatase, glucosidase and lipase in the dissolved fraction (Table 2). Although all activities showed changes with time, these variations did not follow the pattern of any of the measured abiotic parameters.

None of the biochemical components in the POM were affected by nutrient enrichment ($P > 0.05$, Table 2). No significant differences were observed between the tested factors regarding the amounts of essential (4.36 ± 0.62 µg L^{-1}) and non-essential amino acids (3.29 ± 0.43 µg L^{-1}) in the particulate material ($P > 0.05$).

In the DOM, the polysaccharide content increased in the control reach and decreased in the fertilised reach during the study period, and the protein content was generally higher in the control reach on the first date (Table 2). The essential and non-essential amino acid contents in the DOM were also higher in the control reach. Essential amino acids were higher in the control reach throughout the study period (control reach: 191.21 ± 14.50; fertilized reach: 125.55 ± 9.17 µg L^{-1}; Reach: $F_{1,20} = 17.88$; $P < 0.001$), and the non-essential amino acids were mainly higher in the control reach on the first 2 sampling dates (control reach: 167.29 ± 18.92; fertilized reach:

132.23 ± 12.80 µg L^{-1}; Reach: $F_{1,20} = 10.14$; $P = 0.005$; Reach x Date: $F_{6,20} = 7.57$; $P < 0.001$). The PCA performed with the amino acids in the DOM (Fig. 7) resulted in the arrangement of the samples along the first axis (explaining 62% of the variability) depending on the reach and date. Samples from the first dates of the fertilised reach and the last dates of the control reach were situated on the negative side of the axis and were characterised by the presence of histidine. Samples from the last dates of the fertilised reach and the first date of the control reach were found on the positive side of the axis and were characterised by the presence of glycine.

Discussion

Nutrient addition changed the biochemical composition of the biofilm in the forested headwater stream during leaf emergence, while changes in the suspended and dissolved OM were subtle. Nutrient enrichment resulted in a decrease in the C:N ratio of the biofilm and an increase in biofilm protein content. The increase in proteins was mainly explained by an increase in the non-essential amino acids, while the essential amino acid content only increased slightly. Moreover, an increase in polysaccharide content as well as total and essential fatty acid contents was observed on the first sampling date. On this date, the nutrient enrichment interacted positively with higher light availability stimulating especially the autotrophic compartment.

Biochemical quality of the biofilm and functional changes

Nutrient enrichment affected algae and bacteria, as previously shown (Sabater et al. 2011, Suberkropp et al. 2010). The chlorophyll concentration was positively correlated with the protein and polysaccharide content and with fatty acids from diatoms, chlorophytes and cyanobacteria, which indicated that the autotrophic

component of the biofilm was the main driver of the changes in the quality produced by the nutrient enrichment.

In the fertilized reach, the bacterial density in the biofilm was approximately twofold higher than that of the control reach during the first period. Hepinstall & Fuller (1994) observed a positive correlation between algae and bacteria under different light and nutrient availability conditions and attributed their findings to the relationships between the algal exudates (primarily composed of polysaccharides and proteins) used by bacteria as an energy source. In addition, algal exudates represent a major C source for bacteria (Romaní et al. 2004; Carr et al. 2005). This coupled dynamics between bacteria and algae in biofilm is observed only in the fertilized reach.

The relative individual amino acid contents were not different between the reaches, and only some temporary changes were identified. The biofilm was characterised by the amino acids alanine and glycine at early spring, and the glycine content was likely related to the structural matrices of diatoms (Dauwe and Middelburg 1998; Dauwe et al. 1999). In the last sampling dates biofilms showed a higher abundance of labile amino acid as indicators of fresh OM (leucine, histidine and phenylalanine; Ylla et al. 2011). Few studies have described the environmental factors that could determine amino acid production by algae, including as the most likely factors the nitrogen availability and the presence of light (Bates et al. 1991). In this experiment, light could be a determining factor for the amino acid composition changes in the biofilm. In contrast with our expectations, essential amino acids showed a very minimal increase in fertilized reach. It could be explained by a higher turnover of the essential components, an assumption that would be supported by the increase in leucine-aminopeptidase activity, especially at the first sampling day.

Although fatty acids and sterols are important compounds because they provide essential components to consumers, they only represented a small percentage of the total lipids in the biofilm (Brett and Müller-Navarra 1997; Martin-Creuzburg and Elert 2009). The fatty acid and EFA contents were higher in the fertilised reach during the early dates, most likely due to greater algal abundance (Hill et al. 2011). Furthermore, there was a marginally significant shift from fatty acids of predominantly terrestrial origin to fatty acids of predominantly aquatic origin (ω3:ω6 ratio < 1 to > 1) in the control versus the fertilised reach. This shift was related to the higher fatty acid contents from diatoms, chlorophytes-cyanobacteria, and bacteria in the fertilised reach (on the first date). The sterol concentration and composition were not affected by nutrients primarily because of their allochthonous (higher plant) origins.

These changes in the basal autochthonous resources, although moderate, could influence higher trophic levels because changes of essential compounds in the diet may affect consumer growth (Phillips 1984; Anderson et al. 2004; Brett and Müller-Navarra 1997). The effects of fertilization in this stream were transmitted to grazers (mainly the gastropod *Ancylus fluviatilis*) in the form of increased density and growth (Sabater et al. 2005; Sabater et al. 2011) and higher meiofaunal secondary production (Gaudes et al. 2004). These results suggest that the interaction between nutrients and light appears to affect the biochemical composition of biofilm to the greatest degree in early spring; however, we do not know whether these effects observed in early spring also occurred in previous months.

The phosphatase activity in the biofilm was lower in the fertilised reach at the end of the studied period, and the leucine-aminopeptidase activity was only higher on the first sampling date. Extracellular enzymes play an important role in OM and nutrient flow in streams, and their synthesis is activated by the presence of specific substrates (Arnosti

2003). In biofilms, the responses of extracellular enzymes are further modulated by the quality of the available OM and the microbial interactions within the biofilm (Romaní et al. 2012). In the fertilised reach and during the first period, the use of peptides is higher, potentially due to the higher availability of substrates for these enzymes (an increase in protein content in the biofilm). Moreover, higher leucine-aminopeptidase activities have been related to nutrient enrichment (Romaní et al. 2004), incident light and photosynthetic activity (Espeland et al. 2001; Francoeur and Wetzel 2003; Ylla et al. 2009). At the end of the study period, the biofilm from the fertilised reach showed a decrease in phosphatase activity that was potentially related to the reduction of algal biomass and the availability of inorganic P, making phosphatase enzyme production unnecessary in the biofilm of the fertilised reach (Romaní et al. 2004; Allison and Vitousek 2005). In the control reach, high polysaccharide and peptide use (indicated by high β-glucosidase and leucine-aminopeptidase activities) was measured at the end of the experiment, which indicated greater requirements for C and N sources compared with the biofilm from the fertilised reach and the use of larger amounts of the accumulated polysaccharides within the biofilm.

Changes in light availability during the study period could be related to changes in biofilm quality (in polysaccharides, fatty acids, EFAs) observed during the study period. Light availability in the streambed showed an initial maximum value of approximately 60 µmol photons m^{-2} s^{-1}; however, it rapidly decreased to values of approximately 10 µmol photons m^{-2} s^{-1} (83% reduction). These values were similar to those observed previously by Sabater et al. (2011) and lower than those observed by Veraart et al. (2008) in the same stream in preceding years. In addition, the rate of change due to leaf growth was similar to that of other forested streams at similar latitudes and during similar seasons (changing from > 750 to < 100 µmol photons m^{-2} s^{-}

[1] (Ledger and Hildrew, 1998) or from > 1000 to 20 µmol photons m^{-2} s^{-1} (Hill et al. 2001). Fuirosos has moderate light availability during the leafless stage of riparian vegetation, while primary producers are limited by light after leaves have developed (Acuña et al. 2004). This change in light availability results in transient effects on periphyton and, consequently, on biochemical composition.

Biochemical quality of the suspended and dissolved OM

Minor changes were observed in the suspended and dissolved OM compartments. These changes were not surprising because most of this material would have been derived from upstream areas and quickly exported downstream. The effects observed regarding the density of bacteria in the biofilm due to enrichment were not reflected in the bacterial density of water. Instead, an increase was only observed at the end of the experiment and was not related to the nutrient enrichment. In addition, we did not observe any indirect effects of increases in the algal biomass and the release of algal exudates on the quality of DOM in the fertilised reach. The amino acid composition of DOM changed with time, shifting from fresh material (histidine) to structural amino acids (glycine). However, this shift was not directly related to fertilisation or changes in the biofilm. In general, forested streams (specifically Fuirosos) are primarily heterotrophic, with the DOM in the stream water primarily resulting from allochthonous sources (Thurman 1985; Butturini et al. 2008). Higher enzyme activities in water with nutrient enrichment have also been observed (Williams et al. 2012), but the temporal differences observed in our study are not related to light, as observed for biofilms.

Conclusions

~~In conclusion,~~ after long-term moderate fertilisation, the only stable change in the quality of OM was the lower C:N ratio of the biofilms, which was related to a higher protein content, mainly in non-essential amino acids. The progression in canopy closure

in spring limited the effect of light on the biochemical composition of the biofilm. Thus, most of the potential changes caused by moderate nutrient enrichment in forested headwater streams are transient due to light limitations. The suspended and dissolved OM composition did not reflect the nutrient effects due to their mainly allochthonous origin (upstream influence) and lower residence time in the reach. Changes in land-use in many parts of the world (FAO 2012) highlight increases in the nutrient diffuse inputs in rivers and their associated risks. Our results showed that the effects of low-moderate nutrient enrichment in OM composition are minor but evident over short periods, especially in autotrophic compartments and during times at which other favourable environmental factors (e.g., light) co-occur. This experiment exemplifies how small land use changes could affect near-pristine headwater streams. Although limited to one case-study, our results offer information for headwater resource management under conditions of eutrophication.

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Figure captions

Fig. 1 Abiotic parameters during the sampling period: photosynthetically active radiation (PAR) reaching the streambed; the *right inset* figure shows the PAR dynamics during 2007-08, and the grey area indicates the study period, which is shown in detail in the main figure (a) along with air temperature (b). The data indicate daily mean values

Fig. 2 Changes in the algal and bacterial contents in the biofilm: chlorophyll *a* content (a) was used to estimate algal biomass, and the density of live bacteria (b) is shown in the control (open bars) and fertilised (black bars) reaches. Different letters indicate significant differences among sampling dates based on pairwise comparisons adjusted with the Bonferroni correction. Error bars represent +1 SE. N = 4. C: control, F: fertilised

Fig. 3 Extracellular enzyme activities in biofilm: phosphatase (a), β-glucosidase (b), leucine-aminopeptidase (c) and lipase (d) in the control (open bars) and fertilised (black bars) reaches. Different letters indicate significant differences among sampling dates based on pairwise comparisons adjusted with the Bonferroni correction. Error bars represent +1 SE. N = 4. C: control, F: fertilised

Fig. 4 Biochemical composition of the biofilm: polysaccharide (a), protein (b) and lipid (c) contents in the control (open bars) and the fertilised (black bars) reaches. Error bars represent +1 SE. Note the different scale of the Y-axes. C: control, F: fertilised

Fig. 5 Principal component analysis (PCA) of the relative abundance of amino acids (in % pg) in the biofilm showing the samples from the control (open symbols) and the fertilised (black symbols) reaches over the sampling dates. The first and second axes are represented, and the percentage of variability explained by each is indicated. Arrows represent amino acid loading, and the axes are indicated in grey. Abbreviations: ASP, aspartic acid; SER, serine; GLU, glutamic acid; GLY, glycine; HIS, histidine; ARG, arginine; THR, threonine; ALA, alanine; PRO, proline; CYS, cysteine; TYR, tyrosine; VAL, valine; MET, methionine; LYS, lysine; ILE, isoleucine; LEU, leucine; and PHE, phenylalanine. C: control, F: fertilised

Fig. 6 Fatty acid composition of the biofilm in terms of the total fatty acid content (a), essential fatty acid content (EFA) (b), ratio of saturated fatty acids (SAFA) to polyunsaturated fatty acids (PUFA) (c), ratio of ω3:ω6 fatty acids (d) and concentrations of fatty acids indicating the presence of diatoms (e) and chlorophytes and cyanobacteria (f) in the control (open bars) and the fertilised (black bars) reaches. The dashed line in (d) indicates a shift from organic matter (OM) of terrestrial origin (ω3:ω6 < 1) to OM of aquatic origin (ω3:ω6 > 1). Different letters indicate significant differences based on

pairwise comparisons adjusted with the Bonferroni correction. Error bars represent +1 SE. N = 4. C: control, F: fertilised

Fig. 7 Principal component analysis (PCA) of the relative abundance of amino acids (in % mol) in the dissolved organic matter (DOM) showing the samples from the control (open symbols) and the fertilised (black symbols) reaches over the sampling dates. The first and second axes are represented, and the percentage of variability explained by each component is indicated. Arrows represent the amino acid loading, and the axes are indicated in grey. Amino acid abbreviations are given in figure 5. C: control, F: fertilised

Tables

Table 1 Physico-chemical parameters in the stream water across sampling period (March to May 2008). Results of the comparison between reaches (1-way ANOVA) are presented.

	C reach	**F reach**	**1-way ANOVA**
	Mean ± SE	Mean ± SE	*P*-value
Conductivity (µS cm^{-1})	202 ± 17	206 ± 18	0.886
pH	7.6 ± 0.1	7.6 ± 0.2	0.831
Dissolved oxygen (mg L^{-1})	11.15 ± 0.89	11.13 ± 0.83	0.987
Oxygen (%)	108.2 ± 8.6	107.9 ± 8.4	0.981
Temperature (ºC)	10.7 ± 1.1	10.8 ± 1.0	0.935
SRP (µg P-PO_4 L^{-1})	5.78 ± 0.70	107.73 ± 43.40	**<0.001**
Nitrate (µg N-NO_3 L^{-1})	365.66 ± 60.58	1387.02 ± 401.72	**0.032**
Ammonium (µg N-NH_4 L^{-1})	19.12 ± 5.35	201.47 ± 47.80	**<0.001**
N : P (molar ratio)	173.85 ± 21.02	103.40 ± 21.79	**0.028**

C: control, F: fertilised.
P-values < 0.05 are indicated in bold.
N = 4

Table 2 Effect of fertilisation and the interaction with sampling date in the density of live bacteria, the extracellular enzyme activities and in the biochemical composition in water. Note that Date is considered as a random effect factor (no Type III results).

		C reach	F reach	Reach		Reach x Date	
		Mean ± SE	Mean ± SE	$F_{1,24}$	*P*	$F_{6,24}$	*P*
Enzyme activities	**Unfiltered water**						
	Live bacteria	30021 ± 4107	25380 ± 2919	1.03	0.320	7.16	**<0.001**
	Phosphatase	0.36 ± 0.04	0.37 ± 0.02	1.66	0.210	1.02	***0.070***
	β-glucosidase	0.08 ± 0.004	0.12 ± 0.01	55.02	**< 0.001**	10.24	**< 0.001**
	Leucine-aminopeptidase	0.89 ± 0.04	1.04 ± 0.04	34.38	**< 0.001**	12.80	**< 0.001**
	Lipase	0.06 ± 0.01	0.08 ± 0.01	23.64	**< 0.001**	18.09	**< 0.001**
	DOM						
	Phosphatase	0.11 ± 0.004	0.14 ± 0.01	20.58	**< 0.001**	2.52	0.114
	β-glucosidase	0.06 ± 0.003	0.09 ± 0.003	162.22	**< 0.001**	10.29	**< 0.001**
	Leucine-aminopeptidase	0.80 ± 0.07	0.81 ± 0.03	0.55	0.820	24.18	**< 0.001**
	Lipase	0.05 ± 0.01	0.07 ± 0.01	14.04	**0.001**	13.10	**< 0.001**
Biochemical composition	**POM**						
	Polysaccharides	203.11 ± 25.22	276.28 ± 40.52	2.63	0.120	2.15	*0.088*
	Proteins	6.81 ± 0.90	8.40 ± 2.52	0.51	0.484	1.01	0.443
	Lipids	346.71 ± 50.94	392.40 ± 60.78	2.80	0.110	2.75	*0.066*
	DOM						
	Polysaccharides	894.33 ± 107.70	619.43 ± 41.44	14.30	**0.001**	7.40	**<0.001**
	Proteins	358.50 ± 32.09	257.77 ± 18.30	15.57	**0.001**	4.67	**0.004**

C: control and F: fertilised reaches.
Bacterial density is expressed in cells mL^{-1}, phosphatase, β-glucosidase and lipase activities in nmol MUF mL^{-1} h^{-1} and leucine-aminopeptidase activity in nmol AMC mL^{-1} h^{-1}. Biochemical composition is expressed in µg L^{-1}.
P-values < 0.050 are indicated in bold and *P*-values < 0.100 in italics.
N = 32 (4 per reach and date)

Online resource captions

Appendix 1. Analysis method of bacterial density

Appendix 2. Analysis method of extracellular enzyme activities

Appendix 3. Analysis method of polysaccharide content

Appendix 4. Analysis method of amino acid composition

Appendix 5. Analysis method of fatty acid and sterol composition

Figure

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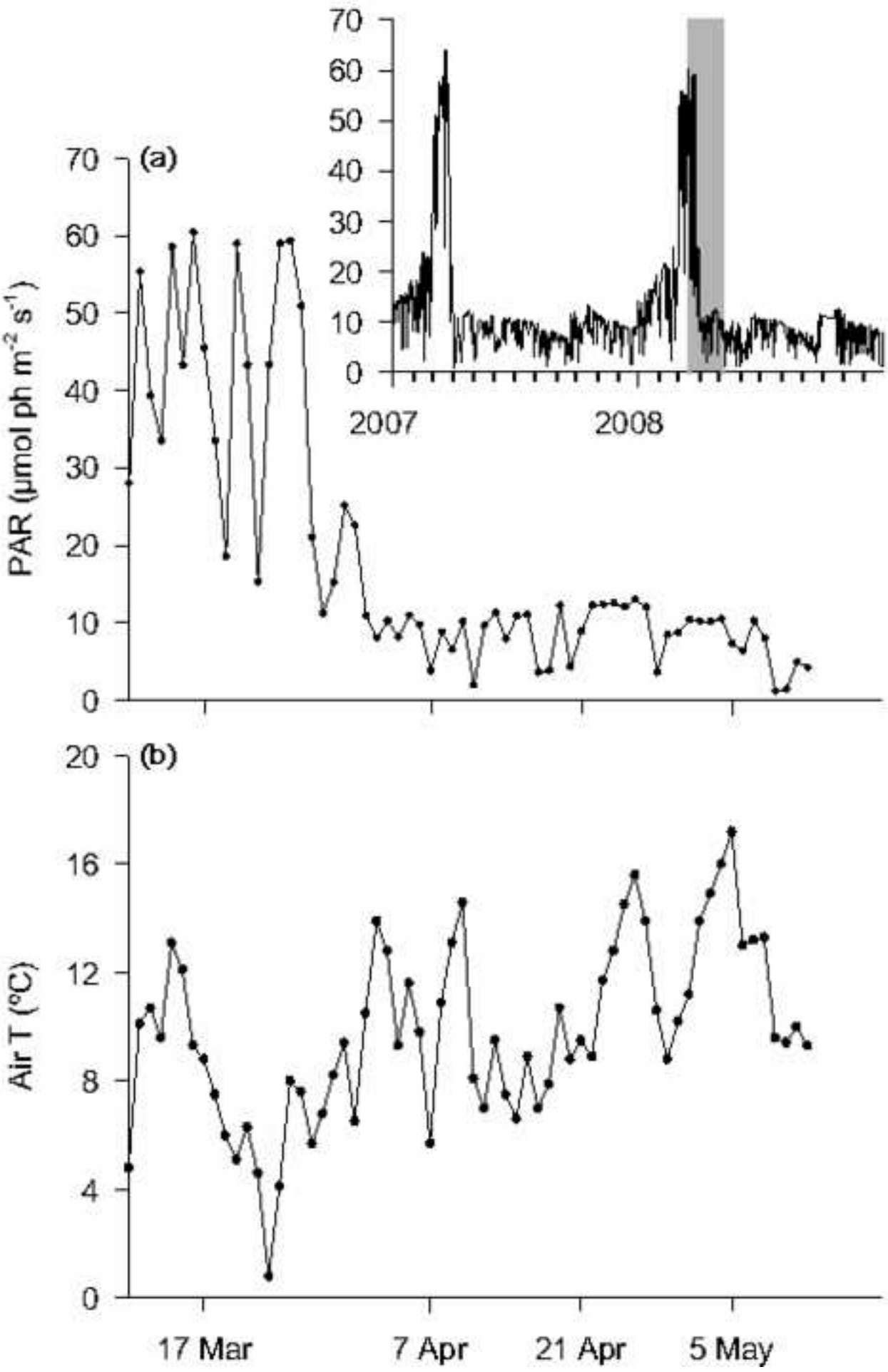

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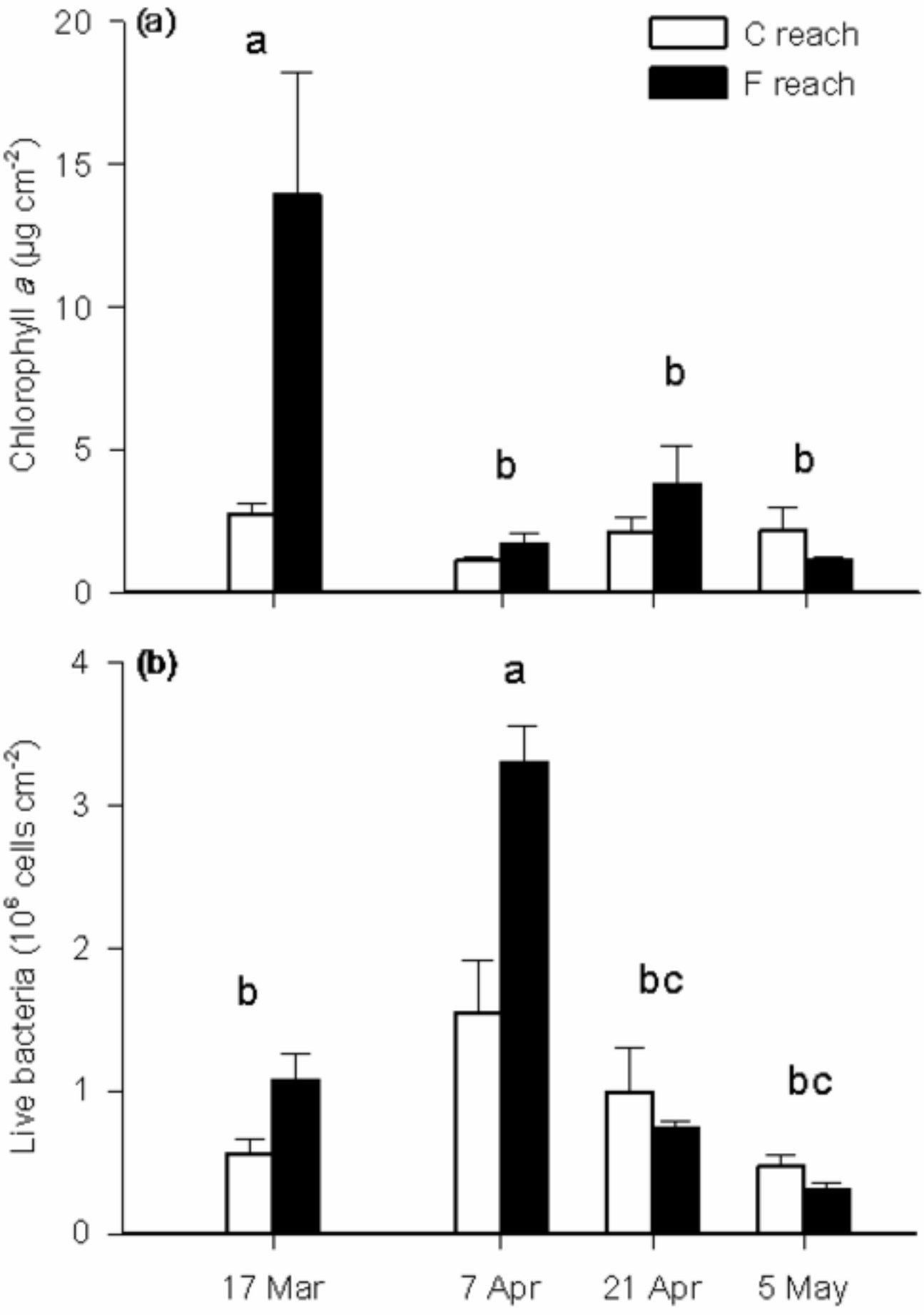

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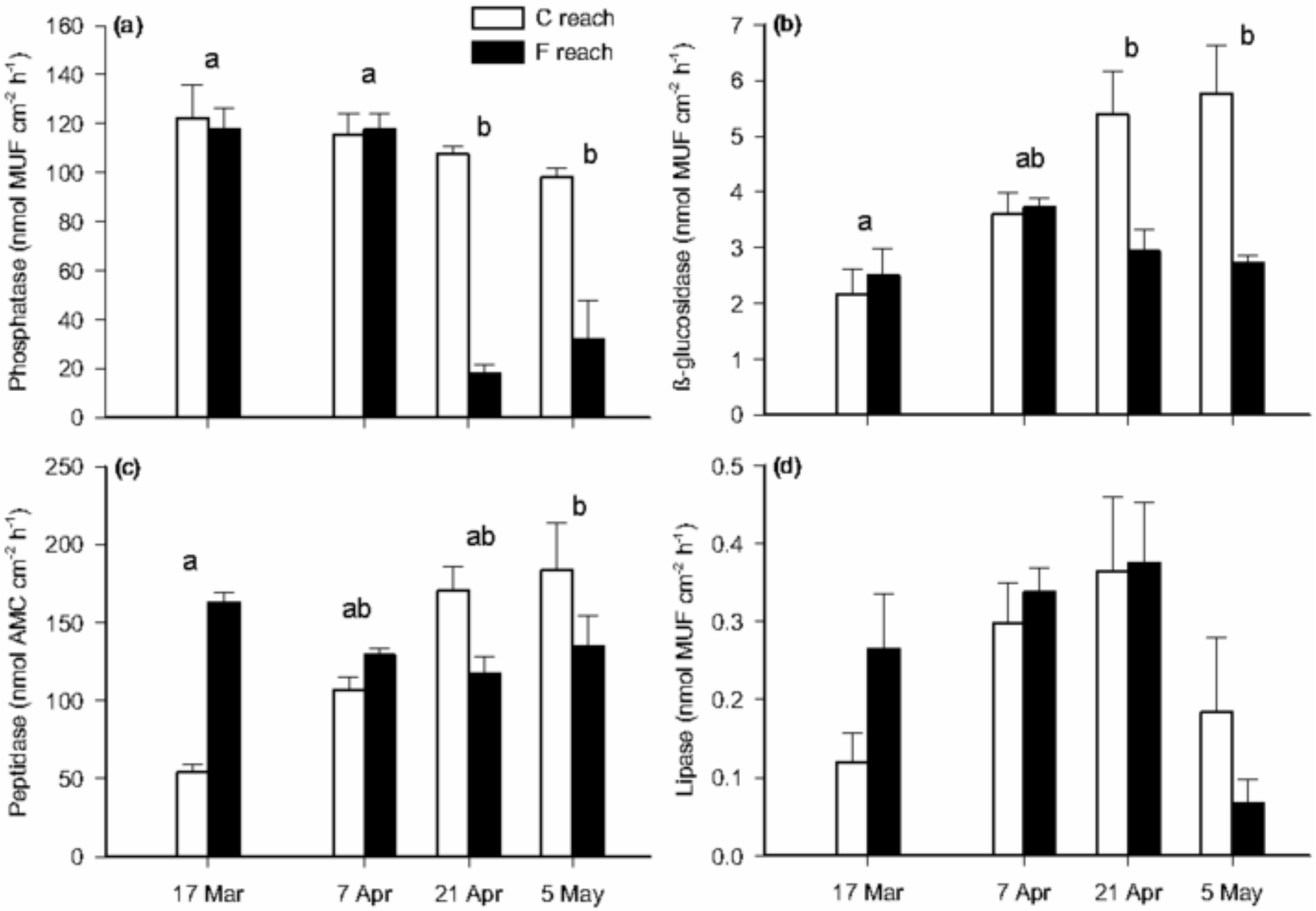

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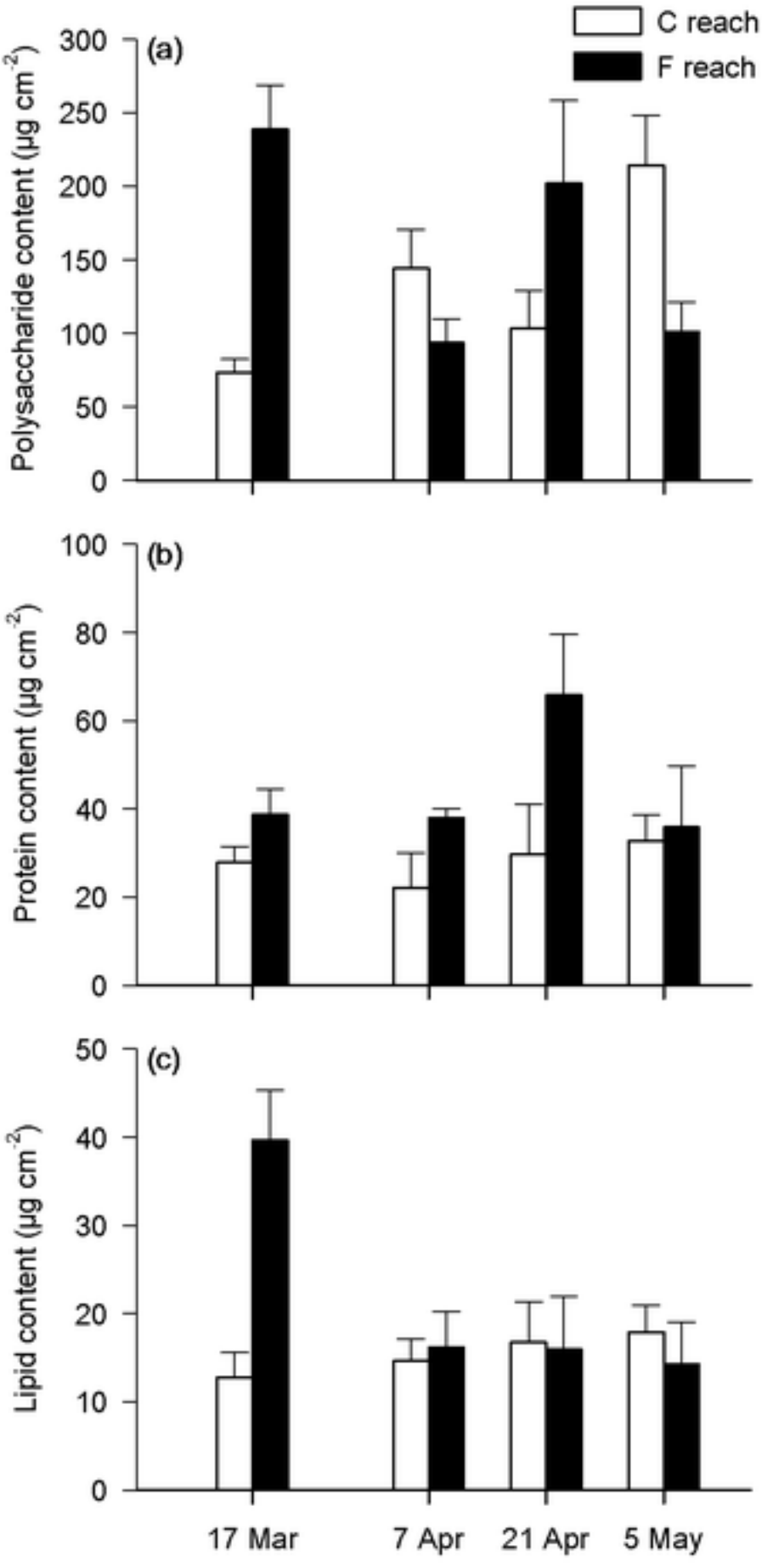

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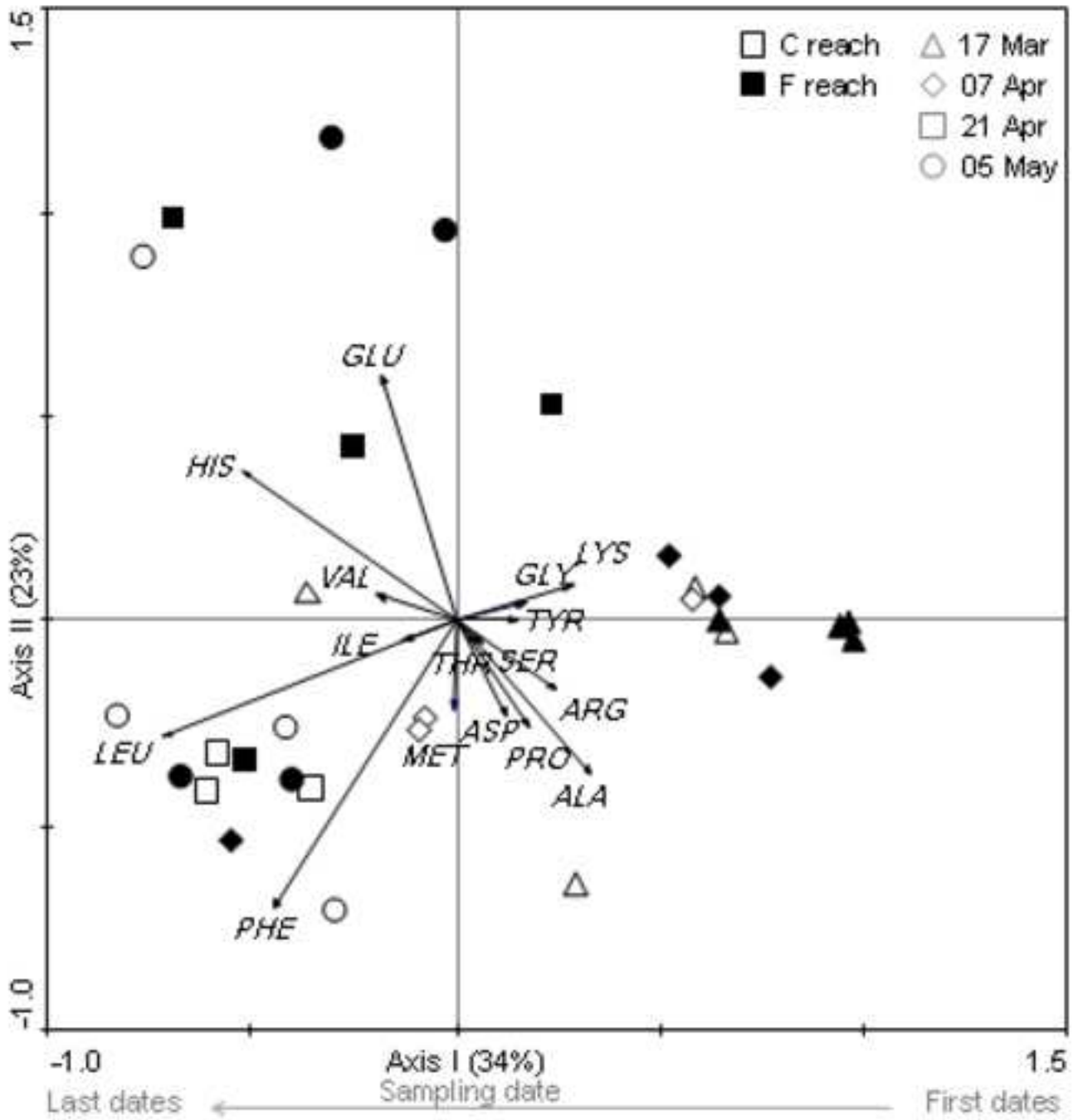

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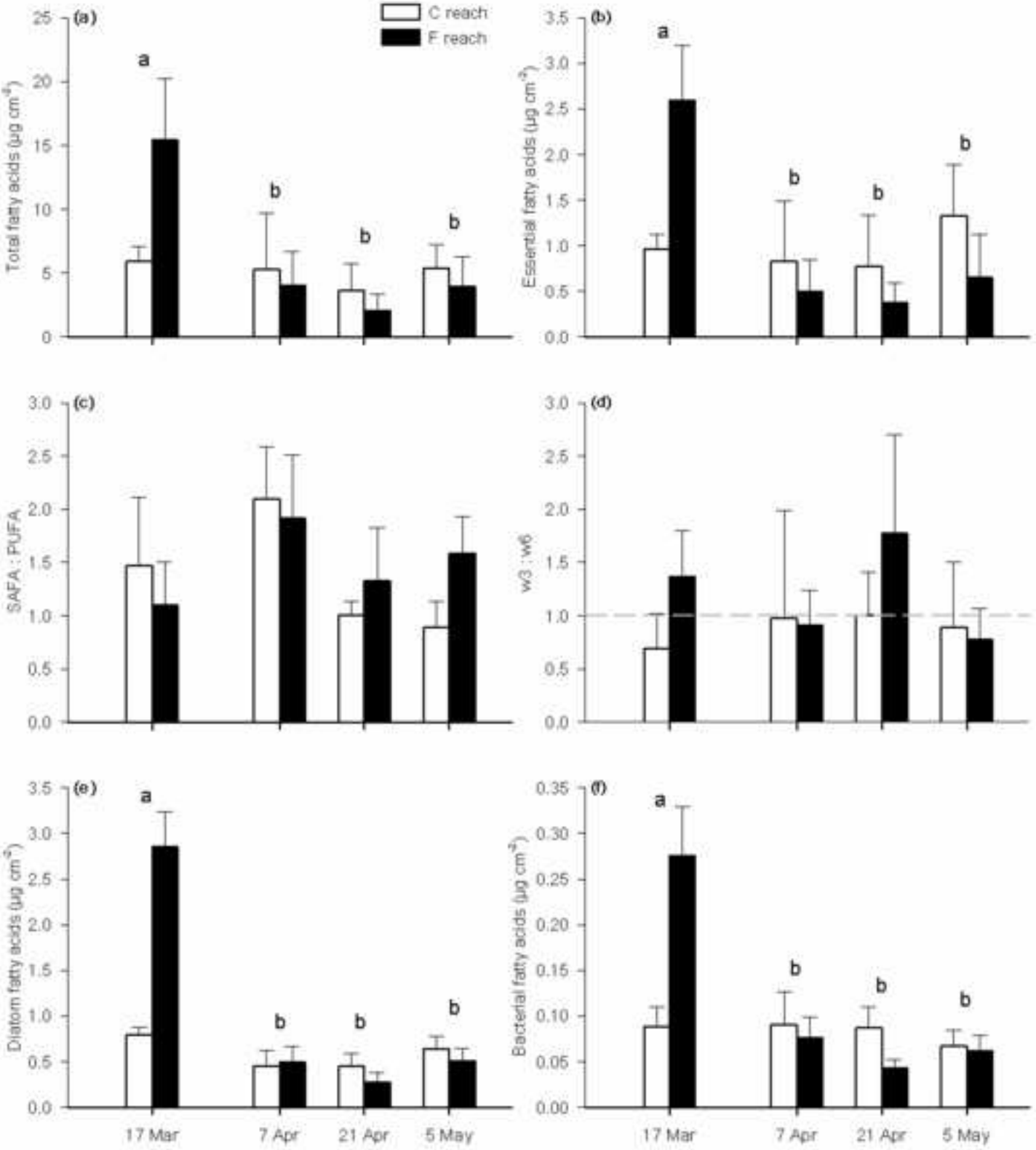

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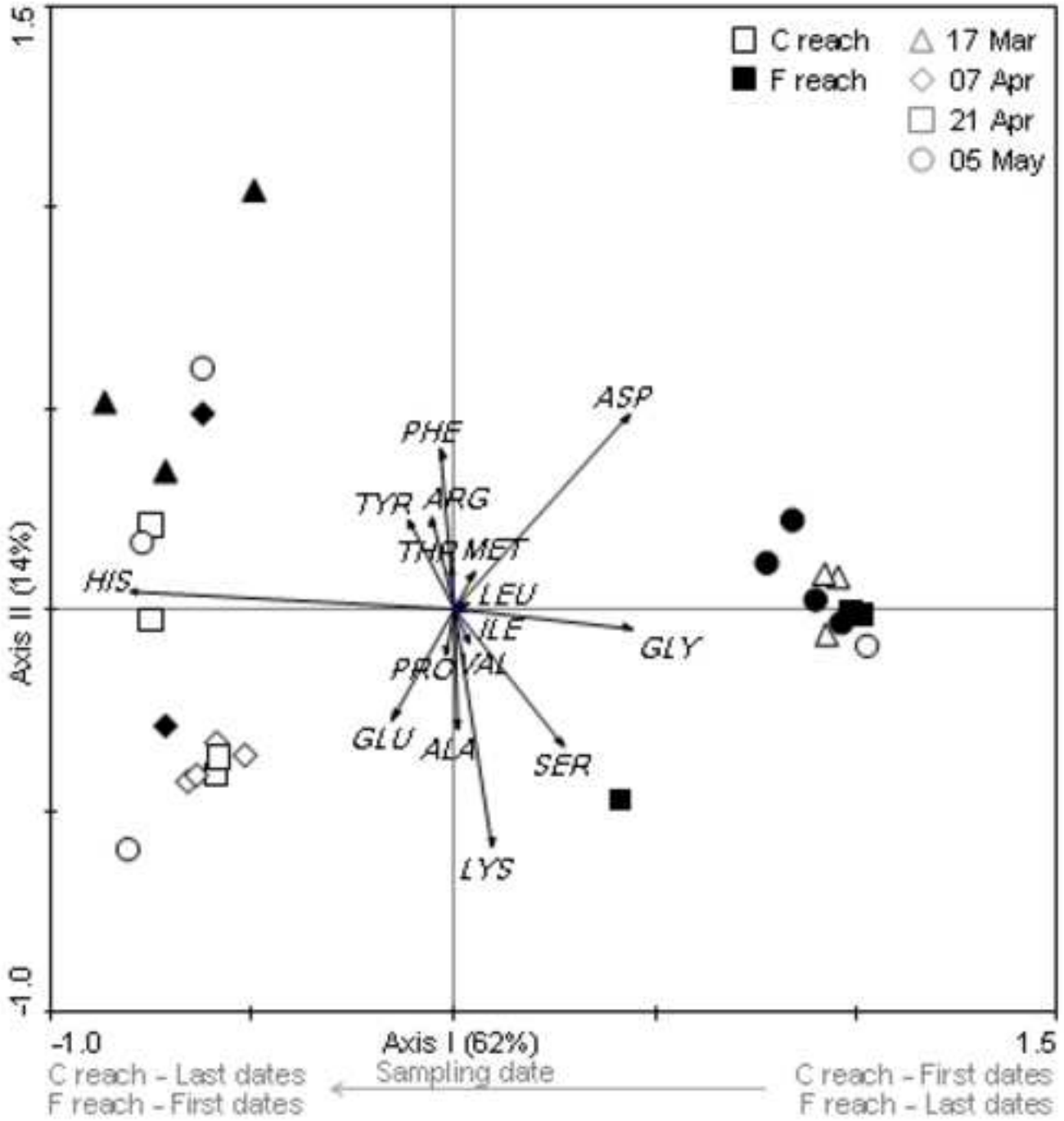

Biochemical quality of basal resources in a forested stream: effects of nutrient enrichment

Journal: **Aquatic Sciences**

Online resource

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Short title: **Nutrient enrichment and resource quality**

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Appendix 1. Analysis method of bacterial density

Live and dead bacteria were counted using the Live/Dead Baclight bacterial viability kit, which contains a mixture of SYTO® 9 and propidium iodide. Colonised glass tiles were placed into glass vials with 10 mL of autoclaved stream water. Once in the lab, samples were sonicated (< 1 min). Aliquots of 200 µL of the glass tile extracts were diluted with 2 mL of sterilised stream water. For the water samples, 4 mL was taken directly (no dilution). A 1 : 1 mixture of SYTO® 9 and propidium iodide was added (3 µL), and samples were incubated for 20 min. Samples were then filtered through 0.2 µm black polycarbonate filters (Nucleopore, Whatman, Maidstone, UK). The filters were dried, placed on a slide with mounting oil and examined by epifluorescence microscopy (E600, Nikon Instruments, Melville, NY, USA). At least 20 random fields were examined on each slide for a minimum of 100 bacteria cells.

Appendix 2. Analysis method of extracellular enzyme activities

Extracellular enzyme activities were determined using the artificial substrates 4-methylumbelliferyl-phosphate for phosphatase, 4-methylumbelliferyl-β-D-glucopyranoside for β-glucosidase, L-leucine-7-amido-4-methylcoumarin hydrochloride for leucine-aminopeptidase and 4-methylumbelliferyl palmitate for lipase (Sigma-Aldrich, St. Louis, MO, USA), as the respective substrate analogues. Samples were incubated with 120 µL of artificial substrate to a final concentration of 0.3 mM (saturated conditions; Romaní and Sabater 2001) in the dark under continuous shaking for 1 h at an ambient temperature. Blanks and standards of methylumbelliferone (MUF) and aminomethyl-coumarin (AMC) were also incubated. At the end of the incubation, glycine buffer (pH 10.4) was added (1 : 1, V : V), and the fluorescence was measured at 365/455 nm excitation/emission for MUF and 364/445 nm excitation/emission for AMC.

Appendix 3. Analysis method of polysaccharide content

Samples for polysaccharide content were acidified with 1 mL of 12 M H_2SO_4 for 2 h at an ambient temperature. Then, the samples were diluted with 4 mL of Milli-Q water, sonicated (2 min) and hydrolysed at 100ºC for 3 h. After cooling, the pH of the hydrolysis solution was neutralised with NaOH. Next, monosaccharides were reduced to alditols by the addition of potassium borohydride. The reduction reaction was terminated by the addition of 2 M HCl. The samples were left overnight at 4ºC. The

following day, triplicate aliquots of the hydrolysis products (and duplicate blanks) were placed in test tubes and oxidised to formaldehyde by the addition of 0.025 M periodic acid. The oxidation reaction was terminated by the addition of 0.25 M sodium metaarsenite. After the addition of 2 M HCl, the aldehyde was reacted with 3-methyl-2-benzothiazolinone hydrochloride (MBTH) reagent, ferric chloride solution and acetone. Absorbance was measured at 635 nm with a spectrophotometer (Spectronic® 20 Genesys, Thermo Spectronic, Cambridge, UK). Glucose standard curves were generated concurrently.

Appendix 4. Analysis method of amino acid composition

Samples for amino acid composition and concentration were hydrolysed with 6 M HCl at 110ºC for 20 h. The HCl remaining in the sample after hydrolysis was removed by N_2 flush and the residue derivatised with a fluorescent reagent (AccQ Fluor reagent, Waters, Milford, MA, USA). Samples were filtered and amino acids analysed using high performance liquid chromatography (HPLC, Waters). The injection volumes were 5 μL for biofilm samples and 10 μL for water samples. The validity of the method was verified by the addition of an internal standard (50 pmol of α-aminobutyric acid), which was recovered at nearly 100% (50 pmol ± 5 for POM and DOM and 50 pmol ± 10 for cobble samples) during the treatment and analysis of the standards and samples. Amino acids were identified on the basis of the retention times and quantified by a comparison between the standard (mixture of 17 primary amino acids) and sample peaks.

Appendix 5. Analysis method of fatty acid and sterol composition

Samples for fatty acid and sterol composition and concentration were frozen with liquid N_2 and freeze-dried for 48 h. Then, samples were extracted with a dichloromethane-methanol (MeOH) 2:1 solution, and were sonicated for 20 min. Samples were centrifuged for 5 min, and the organic extract was concentrated up to 0.5 mL, (*) saponified with KOH (6% in MeOH) and left overnight. On the second day, water and hexane were added. From the hexanic phase, the sterols extract was obtained and concentrated under N_2. The aqueous phase was acidified with HCl and extracted with hexane. The hexanic phase was concentrated up to 0.5 mL and methylated using BF_3 (20% W:V in MeOH) overnight. The next day, water and hexane were added. From

the hexanic phase, the methylated acids extract was obtained and concentrated under N2.. All extractions during the protocol were repeated 3 times for each replicate. For DOM, the beginning of the protocol was slightly different, an aliquot of 100 mL. was taken and NaCl and dichloromethane were added. Samples were shaken and the dichloromethane phase (lower phase) was collected and concentrated up to 0.5 mL. From here on the protocol was the same as described above (*). Samples were kept frozen until analysis. Procedural blanks were processed simultaneously with samples. Internal standards (heptadecanoic acid and 5-α-cholestane) were added to the samples and blanks to calculate the yield of the extraction (71% for FA and 53% for sterols) and to correct for the final concentrations. Prior to analysis, sterols were derivatised with bis(trimetilsylil)trifluoroacetamide (BSTFA) for 30 min at 150ºC, and samples were resuspended in hexane. Samples were analysed with a gas chromatograph (GC 8000 series) equipped with a mass spectrometer detector (MD 800; Thermo Fisher Scientific, San Jose, CA, USA). The gas chromatograph was fitted with a SGE BPX70 capillary column (30 m x 0.25 mm, 0.25μm) for FA methyl esters (FAME) detection and an Agilent J&W DB5 MS (30 m x 0.25 mm, 0.25 μm) for sterols. Samples ran in splitless (48 s or 1 min) or split mode depending on the concentration of the sample, with helium as the carrier gas at a flow of 1 mL min^{-1} and the injector temperature at 250ºC / 270ºC. The mass spectrometer was in electronic ionisation mode. External standards (Supelco 37 component FAME Mix, Sigma-Aldrich and single sterol standards) were used to identify (by retention time and mass spectra) and quantify (by calibration curves) FAME and sterols, although extra components were also identified. Results were analysed with Xcalibur 2.0.7 software (Thermo Fisher Scientific Inc., 1998-2007).

Reference

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