Effects of phosphorus enrichment on the control of stream periphyton

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GENERAL INTRODUCTION AND AIM OF THE STUDY

The release of nutrients into the environment has been dramatically increasing over the past decades as a consequence of agricultural runoffs, industrialisation and other anthropogenic activities (Wurtsbaugh et al. 2019). While nutrients such as phosphorus (P) and nitrogen (N) are essential to maintain life, elevated inputs can have harmful consequences for ecosystem structures and food webs. In particular, nutrient loading into aquatic ecosystems often leads to an excessive growth of algae, a phenomenon known as eutrophication, which is closely associated with water quality degradation, biodiversity loss and an increased mortality of fish (Smith & Schindler 2009; Wurtsbaugh et al. 2019). Eutrophication research has largely focused on lentic, planktonic systems, where nutrient enrichment often leads to the formation of toxic cyanobacterial blooms (Schindler 2006; Paerl & Otten 2013; Ger et al. 2014; Ho & Michalak 2015; Paerl et al. 2018); less attention has been devoted to the consequences of nutrient loading on benthic ecosystems, particularly in lotic environments (Dodds & Smith 2016; Wurtsbaugh et al. 2019).

Streams and rivers are strongly dominated by benthic habitats (Dodds & Smith 2016). Benthic algae are dominant components of periphyton, i.e. the complex assemblage of surface-attached microorganisms and their extracellular substances, which commonly constitute the foundation of stream food webs (Vadeboncoeur & Power 2017; Weitere et al. 2018). The growth and structure of periphytic algae is controlled by both resource availability (bottom-up control) and grazing activity of herbivores (top-down control) (Hillebrand 2002; Beck et al. 2019). While nutrient enrichment is known to increase periphytic algal biomass in streams, grazing activity reduces it and has therefore the potential to limit the effects of eutrophication (Rosemond et al. 1993; Hillebrand 2002). However, nutrient enrichment may also alter the nutritional quality of periphyton, which can in turn affect grazer behaviour and hence the strength of top-down control.

Periphyton nutritional quality for herbivores is strongly related to the stoichiometry of mineral macrountrients (P and N) relative to carbon (C), namely periphyton C:P and C:N ratios (Sterner & Hessen 1994; Elser et al. 2000). High algal C:P and C:N ratios, i.e. low algal P and N content relative to C, have been shown to constrain growth and fecundity in a wide range of aquatic herbivores, including gastropods (Stelzer & Lamberti 2002; Fink & von Elert 2006), cladocerans (Elser et al. 2001; Hessen et al. 2002), and mayfly larvae (Frost & Elser 2002; Peckarsky et al. 2013). In addition to mineral nutrients, algal nutritional quality for herbivores is determined by the relative content of certain biochemical compounds, particularly amino acids (Koch et al. 2011; Wacker & Martin-Creuzburg 2012), sterols (Martin-Creuzburg et al. 2005; Gergs et al. 2014), and polyunsaturated fatty acids (PUFAs), i.e. fatty acids with more than one double bonds in their aliphatic chains (see Box 1; Brett & Müller-Navarra 1997; Guo et al. 2016a).

Box 1. Fatty acid morphology and classification

Fatty acids are aliphatic chains with a carboxyl group (-COOH) at one end and a methyl group (-CH_3) at the other end. Fatty acid morphology is designated as X:YωZ, where X is the number of carbon atoms in the aliphatic chain, Y is the number of double bonds, and Z is the position of the first double bond from the methyl end of the chain. Fatty acids are classified as:

Saturated fatty acids (SAFAs): fatty acids with no double bonds (e.g. palmitic acid, 16:0)

Monounsaturated fatty acids (MUFAs): fatty acids with one double bond (e.g. palmitoleic acid, 16:1ω7)

Polyunsaturated fatty acids (PUFAs): fatty acids with more than one double bond (e.g. linoleic acid, 18:2ω6)

(sources: Brett & Müller-Navarra 1997; Hill et al. 2011)

Periphyton relative content of mineral and biochemical compounds often depends on abiotic factors, particularly light and nutrient availability (Hill et al. 2011; Guo et al. 2016a). Low P and N concentrations in the water column respectively lead to high algal C:P and C:N ratios (Cross et al. 2005; Hill et al. 2011), as well as low PUFA content, as nutrients are needed for the synthesis of PUFA-rich phospholipids and galactolipids in algae (Guschina & Harwood 2006; Hill et al. 2011). To cope with nutrient deficiency in their food, herbivores may adopt behavioural strategies in order to increase their intake of limiting nutrients. In a heterogeneous environment, selective grazers may search for and preferentially feed on food patches with a higher nutrient content, which they can perceive through chemical signals (Moelzner & Fink 2014, 2015); alternatively, in a no-choice food scenario, grazers may increase consumption of nutrient-poor food in order to maximise their nutrient intake, a behaviour known as compensatory feeding (Fink & von Elert 2006).

So how can eutrophication affect the strength of top-down control of periphyton biomass? In **chapter I** of the present dissertation, I hypothesise that grazers will exhibit a higher consumption rate of nutrient-poor than nutrient-enriched periphyton (compensatory feeding), and as a result, the top-down control of periphyton biomass will be weaker in a eutrophied environment. The rheophilic pulmonate gastropod *Ancylus fluviatilis* was chosen as a model organism, as it is a widespread grazer in European streams, and it mainly feeds on periphyton (Cordellier & Pfenninger 2008; Schössow et al. 2016). Cohorts of *A. fluviatilis* were placed in flumes stocked with either phosphorus-poor or phosphorus-enriched natural periphyton, and allowed to graze *ad libitum* for one month, after which periphyton consumption rates were measured and compared between treatments.

Chapter I mainly focuses on short-term consequences of phosphorus enrichment on stream periphyton and grazer feeding behaviour. As I cultivated natural periphyton under phosphorus-poor conditions, and I only enriched part of it with phosphorus one day prior to the start of the experiment, I ensured that P-poor and P-rich periphyton only differed in their C:P ratios. Other parameters, such as periphyton biomass, fatty acids and taxonomic composition were kept the same between treatments, to avoid confounding effects. However, nutrient enrichment of stream periphyton can have additional consequences in the long term, rather than only altering periphyton stoichiometry. Such long-term effects may have more complex repercussions on periphyton nutritional quality for grazers, and thus on the strength of top-down control.

A common long-term consequence of nutrient enrichment is a change in the taxonomic composition of periphyton (Smith 2003). Oligotrophic streams are usually dominated by diatoms, while the proportion of periphytic chlorophytes and cyanobacteria often increases with phosphorus enrichment (Leland & Porter 2000; Pan et al. 2000; Whorley & Wehr 2016; McCall et al. 2017). However, different algal taxonomic groups considerably differ from one another in their nutritional quality for herbivores, most of all in their PUFA composition (Taipale et al. 2013). PUFAs are an essential component of animal diets because they regulate cell membrane fluidity, and long-chain PUFAs are precursors to eicosanoids, a class of signalling molecules that serve a wide range of physiological functions (Torres-Ruiz et al. 2007; Guo et al. 2016a). Several studies have demonstrated reduced growth or egg production in aquatic herbivores that fed on algae poor in PUFAs (Müller-Navarra et al. 2000; Wacker & von Elert 2004; Guo et al. 2016b; Windisch & Fink 2018). The most important PUFAs for herbivore nutrition are the long-chain eicosapentaenoic acid (EPA, 20:5ω3; see Box 1), docosahexaenoic acid (DHA, 22:6ω3) and arachidonic acid (ARA, 20:4ω6), which are mainly found in diatoms (Lang et al. 2011; Guo et al. 2016a). Chlorophytes lack long-chain PUFAs, but are rich in the short-chain PUFAs α-linolenic acid (ALA, 18:3ω3) and linoleic acid

(LIN, 18:2ω6), which are still nutritionally important for herbivores (Lang et al. 2011; Taipale et al. 2013; Guo et al. 2016b). Cyanobacteria, on the other hand, are considered a low-quality food source because they are generally poor in both PUFAs and sterols (von Elert et al. 2003; Brett et al. 2006), though some cyanobacteria contain moderate amounts of ALA and LIN (Lang et al. 2011). In addition, different algal groups may exhibit different C:P and C:N ratios, even when grown under the same conditions (Ho et al. 2003; Quigg et al. 2003), so periphyton C:N:P stoichiometry may not strongly correlate with nutrient loading if a taxonomic shift occurs in the algal community.

In **chapter II** I hypothesise that long-term phosphorus enrichment will lead to an increase in the proportion of chlorophytes and cyanobacteria over diatoms in stream periphyton, which will be associated to a decreased relative content of essential long-chain PUFAs in the periphytic community. For this experiment, I grew natural stream periphyton in laboratory flumes at five increasing concentrations of dissolved phosphorus, corresponding to a trophic gradient from highly oligotrophic to hypereutrophic. After one month, I collected periphyton samples from each flume and measured their taxonomic composition, biomass, C:P stoichiometry and fatty acid composition, to determine how these parameters would correlate with phosphorus availability.

In addition to algal nutritional quality for herbivores, another critical aspect of stream periphyton is its spatial variability, which can have profound effects on ecosystem dynamics (Stevenson 1997; Winemiller et al. 2010). Habitat heterogeneity, i.e. spatial and temporal variations in resources and abiotic factors within an ecosystem, is known to promote biodiversity and ecosystem stability (Stevenson 1997; Cardinale et al. 2000; Wellnitz & Poff 2001). In particular, periphytic communities in streams are highly heterogeneous due to variations in light, nutrient availability, current velocity and many other factors (Stevenson

1997; Winemiller et al. 2010; Warren et al. 2017). Periphyton heterogeneity increases nutrient flow and uptake (Riber & Wetzel 1987), favours coexistence of herbivore species (Chase et al. 2001) and generally mediates trophic interactions by influencing grazer behaviour and distribution (Vaughn 1986; Palmer 1995; Kawata & Agawa 1999). In turn, grazing activity may increase or decrease periphyton heterogeneity, depending on grazer density, mobility and feeding behaviour (Sommer 1999, 2000; Alvarez & Peckarsky 2005; Hillebrand 2008). However, despite its importance, very little is known about the effects of eutrophication on periphyton heterogeneity and its interaction with grazers. Under normal conditions, grazers may respond to periphyton heterogeneity by distributing unevenly in the environment, as they may be attracted to patches with a higher periphyton biomass and/or a higher nutritional quality, which they can perceive through chemical cues (Moelzner & Fink 2014, 2015). As nutrient availability increases, periphyton biomass and nutrient content may overall increase until algae become nutrient-saturated, which may further lead to a more uniform grazer distribution and therefore a homogeneous grazing pressure. This hypothesis is tested in **chapter I**, within the same study on the effects of nutrient enrichment on grazer feeding behaviour. After letting gastropods graze on stream periphyton for one month, I measured the heterogeneity of periphyton biomass in every experimental flume with a coefficient of variation, to determine any differences between phosphorus-poor and phosphorus-enriched periphyton.

In **chapter III**, on the other hand, I analyse the effects of phosphorus enrichment on the heterogeneity of both periphyton quantity and quality in an environment with uneven light conditions. Together with nutrient availability, light is a crucial factor determining both periphyton biomass and nutritional quality for herbivores (Hill et al. 2011), and is highly variable in streams (Warren et al. 2017). Periphyton biomass usually increases with light availability, whereas periphyton nutrient content relative to carbon decreases as the ratio of light to nutrient availability increases, in accordance with the light:nutrient hypothesis (Sterner et al. 1997; Fanta et al. 2010). Therefore, in a stream with low nutrient availability and heterogeneous light conditions, periphyton patches with high relative nutrient content are expected to occur in locations with low light availability, and vice versa. However, if nutrient inputs increase, periphyton relative nutrient content might increase until reaching a maximum saturating level even under high light availability. A similar outcome might be expected with periphyton relative PUFA content, which usually increases with phosphorus availability and decreases with increasing light (Hill et al. 2011). Therefore, in **chapter III**, I hypothesise that, in an environment with heterogeneous light conditions, periphyton C:P ratio and relative PUFA content will be more heterogeneous at low phosphorus than high phosphorus availability. Furthermore, I hypothesise that periphyton biomass will be higher under high light than low light irrespective of nutrient availability, so it will be equally heterogeneous between the two phosphorus treatments. To test these hypotheses, I grew natural stream periphyton in circular flumes, each of which was kept half in shade and half in light, at either low or high phosphorus availability. After three weeks, I used a coefficient of variation to determine the heterogeneity of periphyton biomass, C:P ratio and relative PUFA content for each flume.

Overall, the following questions are addressed in the upcoming chapters:

1. How does phosphorus enrichment affect the strength of periphyton top-down control? (**Chapter I**)
2. What are the long-term consequences of phosphorus enrichment for periphyton community structure and nutritional quality? (**Chapter II**)

3. What are the effects of phosphorus enrichment on periphyton heterogeneity? (**Chapter I, III**)

All chapters are based on individual research manuscripts. The first chapter is published in Freshwater Biology, while chapters II and III are under review at the Journal of Phycology and Limnology & Oceanography Letters, respectively. With the present dissertation, I aim to give new insights into the effects of phosphorus enrichment on the bottom-up and top-down control of stream periphyton.

CHAPTER I

High nutrient availability leads to weaker top-down control of stream periphyton: Compensatory feeding in *Ancylus fluviatilis*

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Summary

1. Benthic algal biomass and distribution in freshwater ecosystems are determined by both nutrient availability (bottom-up control) and grazing activity by herbivores (top-down control). Fluctuations in algal nutrient ratios may cause grazers to optimise their food intake through behavioural strategies in order to maintain a constant soft body stoichiometry. Such linkages between nutrient availability and grazing control of algal biomass are as yet poorly understood.
2. In this study, we tested whether the stream-dwelling freshwater gastropod *Ancylus fluviatilis* would increase its food consumption rate with decreasing periphyton nutrient content, a behaviour known as compensatory feeding. We performed a full factorial microcosm experiment in which two levels of periphyton phosphorus content (low versus high) were crossed with grazer presence/absence in 12 circular running water flumes. After one week of grazing, food consumption rates were measured by determining the periphyton difference between grazed and ungrazed flumes, and the periphyton biomass variability in every flume was described with a coefficient of variation (CV).
3. The food consumption rate of *A. fluviatilis* was significantly higher in the low phosphorus compared to the high phosphorus treatment, supporting the compensatory feeding hypothesis. As a result, in the presence of grazers, periphyton biomass was significantly lower under low phosphorus availability, while biomass was not affected by nutrient enrichment in the grazer-free flumes.
4. Despite the strong difference in periphyton phosphorus content, *A. fluviatilis* soft body stoichiometry did not differ between the two nutrient treatments, suggesting strong stoichiometric homeostasis. Furthermore, the distribution of algal biomass was

significantly more heterogeneous in the grazed phosphorus-poor than in the phosphorus-rich periphyton.

5. Our findings suggest that nutrient enrichment may lead to a weaker top-down control of algal biomass in stream ecosystems and to reduced spatial heterogeneity of periphyton abundance.

Introduction

Increasing nutrient inputs into freshwater ecosystems has become a major environmental issue over the past decades. Eutrophication, i.e. the excessive supply of nutrients into an ecosystem and the resulting overgrowth of primary producers, is a major cause of water quality degradation and biodiversity loss across all trophic levels (Evans-White et al. 2009; Smith & Schindler 2009). Consequences of nutrient enrichment have been widely studied in lake ecosystems (e.g. Downing et al. 2001; Hillebrand & Kahlert 2001; Schindler 2006; Ger et al. 2014), whereas less is known about eutrophication effects on plant-herbivore interactions in streams.

Compared to lakes, streams are more strongly dominated by benthic habitats, and benthic algal productivity in streams has been documented to increase in response to phosphorus (P) and nitrogen (N) enrichment (Smith et al. 1999; Dodds & Smith 2016). In addition to nutrient availability (bottom-up control), periphyton biomass is also controlled by the grazing activity of herbivores (top-down control) (Rosemond et al. 1993; Hillebrand 2002; Weitere et al. 2018). While it is widely acknowledged that both processes alter algal biomass and production, mechanisms determining the strength of the top-down pressure are still poorly understood.

Generally, nutrient availability may alter the nutritional quality of periphyton by changing its nutrient content relative to carbon (C). Together with fatty acid composition, C:N:P stoichiometry is a major indicator of food quality for herbivores. High primary producer C:P and C:N ratios are known to limit herbivore growth and reproduction (Elser et al. 2000; Stelzer & Lamberti 2002; Fink & von Elert 2006), which can in turn affect the intensity of top-down control. Hence bottom-up and top-down effects interact with each other, and ecological nutrient stoichiometry might provide a framework to mechanistically link nutrient availability and grazing activity.

As herbivores are generally more strictly homeostatic than their food resources (Persson et al. 2010), stoichiometric imbalances between autotrophs and herbivores are common in freshwater ecosystems (Elser et al. 2000; Cross et al. 2005; Fink et al. 2006). To cope with fluctuations in food stoichiometry, herbivores may adopt behavioural strategies to optimise their nutrient intake and thus maintain a balanced soft body elemental composition. Common strategies include active searching for food patches with a high nutrient content, which grazers can perceive with the help of chemical cues (Moelzner & Fink 2014, 2015); diet mixing of various food sources that are complementary in their nutrient composition (Groendahl & Fink 2016); increasing nutrient retention during digestion (Darchambeau et al. 2003); and last but not least, ingesting high amounts of low quality food to maximise the intake of limiting nutrients, a behaviour known as compensatory feeding (Cruz-Rivera & Hay 2000; Fink & von Elert 2006). Top-down control of algal biomass is inevitably influenced by such strategies, therefore nutrient loading might arguably impact grazer feeding behaviour and thus the strength of top-down control. Such an interaction between nutrient supply, food quality and grazing in the control of stream periphyton biomass was addressed in the present study.

In addition to controlling algal biomass, grazing activity may also alter the spatial variability of periphyton. Benthic algal assemblages in streams are highly heterogeneous due to spatial and temporal variations in resources and stressors, which normally lead to a patchy distribution of algal production (Palmer & Poff 1997). Grazers may respond to periphyton heterogeneity by distributing unevenly in space, and their activity may in turn enhance or reduce the heterogeneity of algal biomass, depending on several factors such as grazer mobility and/or density (Poff & Nelson-Baker 1997; Flecker & Taylor 2004; Alvarez & Peckarsky 2005). However, the effect of grazing on periphyton biomass heterogeneity in relation to nutrient enrichment and food quality is yet an unexplored topic.

To investigate how food quality affects the strength of grazer-periphyton interactions in streams, we performed a fully factorial microcosm experiment in which periphyton C:P ratio (low versus high) was crossed with grazer presence/absence in circular running water flumes. The following three hypotheses were tested:

1. Grazers feeding on P-depleted food would compensate for the low nutritional quality by increasing their consumption rate (compensatory feeding).
2. Grazers would maintain a constant soft body stoichiometry, thus soft body C:N:P ratios would not differ between grazers feeding on P-enriched and P-depleted periphyton.
3. As an implication of compensatory feeding, the strength of top-down control would increase at low P availability, resulting in a lower periphyton biomass.

Compensatory feeding under nutrient deficiency has already been observed in the freshwater gastropods *Radix ovata* (Fink & von Elert 2006), *Fossaria* sp. and *Potamopyrgus antipodarum* (Hansen *et al*. 2016) in lentic systems; however, it has never been demonstrated in a running water system with natural periphyton. Our model organism was the rheophilic gastropod *Ancylus fluviatilis* (O.F. Müller, 1774; Pulmonata: Basommatophora), which is widespread in European rivers and streams and normally feeds on periphyton (Calow 1973; Cordellier & Pfenninger 2008).

Finally, we measured the spatial heterogeneity of periphyton biomass in our experimental flumes to investigate how it would be affected by the interaction of grazing and nutrient supply. To quantify periphyton heterogeneity, we calculated the coefficient of variation (CV) of periphyton biomass for every flume. CV measures heterogeneity by describing variation around the mean and is commonly used in periphyton studies (e.g. Biggs et al. 1999; Liess & Hillebrand 2004; Peters et al. 2007; Hillebrand 2008). Although CV is not informative about the spatial arrangement of biomass and distance effects on variability, its simplicity makes it suitable for small-scale experiments in which spatially explicit data are not needed. In our case, CV was a straightforward way to determine whether periphyton patches within one flume were similar to one another in terms of biomass.

Methods

Experimental set-up

The experiment consisted of four treatments, in which two levels of P content (P+ versus P-) were crossed with grazer presence/absence in a full factorial design. Each experimental unit consisted of a circular flume in a 22 × 27 cm (height × diameter) plastic bucket filled with 6 L aerated tap water. On the bottom surface of each flume were nine 5 × 5 cm slots for tiles, arranged in a circle around a small pump, which generated a circular water current. The average current velocity in the flumes was 14 cm s^{-1}. Each treatment was replicated three times, for a total of 12 flumes.

The flumes were placed outdoors, in the botanical garden of the University of Cologne, and covered with mesh to prevent animals and litter from entering. The average low and high temperatures in Cologne during the experimental period were 3.1°C and 9.3°C respectively. The water temperature in the flumes was ca. 2 degrees higher than the outside temperature, due to the heat generated by the pumps.

Study animal

The freshwater pulmonate limpet *Ancylus fluviatilis* was used as a model organism. A cohort of adult *A. fluviatilis* (shell length 5-8.5 mm) was collected on 25 January 2017 in the river Schlingenbach near Overath in western Germany (50°57'16.4"N, 7°20'16.1"E). For one week prior to the experiment, snails were kept in aerated aquaria in a climate chamber at 20°C under a 16:8 h light-dark cycle, and fed with natural periphyton.

Periphyton collection and manipulation

Natural periphyton was collected in the river Agger, near Overath (50°56'12.4"N, 7°17'37.1"E) and inoculated in a concrete outdoor pool in the University of Cologne botanical garden in November 2016. The pool measured 235 × 110 × 40 cm and was filled with 200 L diluted (1:15) WC medium (Guillard & Lorenzen 1972) with an adjusted phosphorus (P) concentration of 5 μg L^{-1}, typical of oligotrophic rivers (Dodds & Smith 2016). The pool was covered with a mesh to prevent animals and litter from entering, and the pool's bottom surface was covered with 4.7 × 4.7 cm unglazed ceramic tiles, as substrate for periphyton growth. Due to the cold weather, a thick ice layer formed on the water surface until mid-January 2017, slowing down algal growth. In late January 2017, all tiles were covered by a thin periphyton layer.

Periphyton-covered tiles for the P- treatment were directly taken from the outdoor pool, which was fertilised once a week with P-depleted WC medium (see above). To produce P+ periphyton, tiles were taken from the pool and transferred into a container filled with 10 L diluted (1:15) WC medium, with a final P concentration of 100 μg L^{-1} as commonly observed in eutrophic rivers (Dodds & Smith 2016), and transferred into the experimental flumes after one day of incubation. This procedure was repeated every time the tiles in the flumes had to be replaced, to ensure that all the experimental tiles were kept under the same conditions until one day prior to their usage.

Experimental procedure

Six flumes were stocked with two P+ periphyton-covered tiles each, and the remaining six with two P- periphyton tiles. Half of the flumes from both treatments were stocked with six *A. fluviatilis* individuals each, reflecting natural snail densities observed in the stream. Tiles were replaced once a week, and one third of the water in each flume was replaced three times a week with aerated tap water. The experiment was divided in two phases: acclimation phase (7 to 28 February 2017), to let the snails become accustomed to the new environment, and grazing phase (1 to 7 March 2017). In both phases, snails were allowed to graze *ad libitum* in the flumes, but grazing rates were quantified only in the grazing phase; therefore, the grazer-free treatment was excluded in the acclimation phase.

On 1 March 2017, the first day of the grazing phase, all tiles in the flumes were replaced. Additionally, three P- and three P+ periphyton tiles were randomly selected from the P- pool and the P+ container. Periphyton was scraped off each tile and homogenised with 40 ml tap water. Aliquots from each suspension were filtered onto pre-combusted glass fiber filters (Whatman GF/F, 25 mm Ø) for elemental and fatty acid analyses (see next section).

The experiment ended on 7 March 2017, when all tiles and snails were collected from the flumes. Periphyton was scraped off each tile and homogenised with 100 ml tap water, of which aliquots were filtered onto pre-combusted GF/F filters for C/N and P analyses. In addition, 1 ml of each suspension was mixed with 100 μl Lugol's iodine solution for taxonomic analysis. Snail soft bodies were removed from the shells, frozen at -80°C and freeze-dried to determine their dry mass, which was measured with a microbalance (Mettler UTM2, Giessen, Germany) to the nearest μg. Subsequently, fractions of each snail soft body were collected for C/N and P analyses.

Periphyton consumption rates were determined for every snail-containing flume, by calculating the difference between the average total periphyton particulate organic carbon (POC) in the grazer-free flumes and the total remaining periphyton POC in the grazed flumes, normalised to the total snail soft body dry mass per flume. Furthermore, the coefficient of variation (CV, ratio of standard deviation to mean) of periphyton POC between individual tiles was calculated for every flume as a measure of spatial heterogeneity of periphyton biomass.

Laboratory analyses

For C/N analysis, filters were dried for 24 h at 60°C, packed in tin capsules (HekaTech, Wegberg, Germany) and subsequently analysed with a Thermo Flash EA2000 Analyser (Schwerte, Germany). For P analysis, filters were transferred into a solution of potassium peroxodisulphate and 1.5% sodium hydroxide and autoclaved for 1 h at 120°C; soluble reactive phosphorus was subsequently analysed with the molybdate-ascorbic acid method (Greenberg et al. 1985) with a DR5000 UV-Vis spectrophotometer (Hach, Düsseldorf, Germany). Taxonomic analysis was performed by counting algal cells under an inverted microscope at 400-630x magnification; the taxonomic composition of each sample was described as chlorophyte:diatom ratio based on cell numbers.

For fatty acid extraction and analysis, each filter was placed in 5 ml extraction solvent (CH_2Cl_2/MeOH, 2:1 v/v) and stored at -20°C. Prior to the fatty acid extraction procedure, 10 μg heptadecanoic acid methyl ester (C17:0 ME) and 10 μg tricosanoic acid methyl ester (C23:0 ME) were added to each sample as internal standards. Samples were then sonicated in an ultrasound bath for 3 min and centrifuged at 5000 rpm for 5 min. The supernatants were dried at 40°C under a stream of nitrogen gas prior to fatty acid transesterification. This was achieved by adding 5 ml 3 N methanolic HCl to the samples and incubating them at 70°C for 20 min to quantitatively yield fatty acid methyl esters (FAMEs), which were extracted by adding 6 ml isohexane to each sample. The hexane phases were dried at 40°C under a nitrogen stream and redissolved in 100 μl isohexane. The fatty acid composition of the samples was subsequently determined via gas chromatography with a 6890 N GC System (Agilent Technologies, Waldbronn, Germany) according to Groendahl & Fink (2017).

Data analysis

Statistical analyses were performed in R (R Core Team, version 3.3.1, 2016. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria). All data were checked and approved for normal distribution with a Shapiro-Wilk's test and for homoscedasticity with a Levene's test. Next, two-sample t-tests were used to compare C:N, C:P, N:P ratios, chlorophyte:diatom ratio and POC/tile between P+ and P- periphyton at the beginning of the grazing phase, as well as to compare C:N, C:P, N:P ratios and food consumption rates between snails grazing on P+ and P- periphyton. In addition, interactive effects of P supply and grazing on total periphyton POC/flume, periphyton C:N, C:P and N:P ratios, and periphyton CV were determined using two-way analyses of variance (ANOVAs) followed by Tukey's HSD post-hoc tests.

Results

Periphyton initial conditions

Prior to the grazing phase, molar C:P and N:P ratios were significantly lower in the P+ than in the P- periphyton, with P- values being on average four times higher than P+ values (two-sample t-test; C:P $t = -16.161$, df = 4, $p < 0.001$; N:P $t = -14.698$, df = 4, $p < 0.001$; Table S1 in

Supporting Information). No significant differences between treatments were observed in periphyton C:N ratio (two-sample t-test, $t = 1.118$, df = 4, $p = 0.33$; Table S1 in Supporting Information) nor in periphyton biomass as POC per tile (two sample t-test, $t = 0.617$, df = 4, $p = 0.57$; Table S1 in Supporting Information). Therefore, any subsequent differences in grazer consumption rates could be interpreted to result from differences in periphyton P content, rather than N content or food quantity. Moreover, due to the short incubation with high P concentrations, no changes occurred in the periphyton taxonomic and fatty acid composition, with α-linolenic acid (C18:3ω3, ALA) being the most abundant polyunsaturated fatty acid in both treatments (Figure S1 in Supporting Information), and chlorophytes being the dominant taxonomic group (Table S1 in Supporting Information). No significant difference in periphyton chlorophyte:diatom ratio was detected between the two treatments (two-sample t-test, $t = 0.729$, df = 4, $p = 0.51$).

Table 1. Results of two-way ANOVAs on the effects of phosphorus (P) and grazing on periphyton C:P, C:N and N:P ratios, periphyton biomass expressed as particulate organic carbon (POC) per flume, and periphyton biomass heterogeneity expressed as coefficient of variation (CV) per flume at the end of the grazing phase. Significant effects ($p < 0.05$) are highlighted in bold.

Parameter	**Phosphorus**		**Grazing**		**P × Grazing**	
	$F_{1,8}$	p	$F_{1,8}$	p	$F_{1,8}$	p
C:P	29.44	**<0.001**	0.05	0.83	0.45	0.52
C:N	9.37	**0.02**	0.15	0.71	7.23	**0.03**
N:P	4.23	0.07	0.69	0.43	9.76	**0.02**
POC	28.40	**<0.001**	23.28	**0.001**	1.90	0.20
CV	6.89	**0.03**	4.93	0.06	3.70	0.09

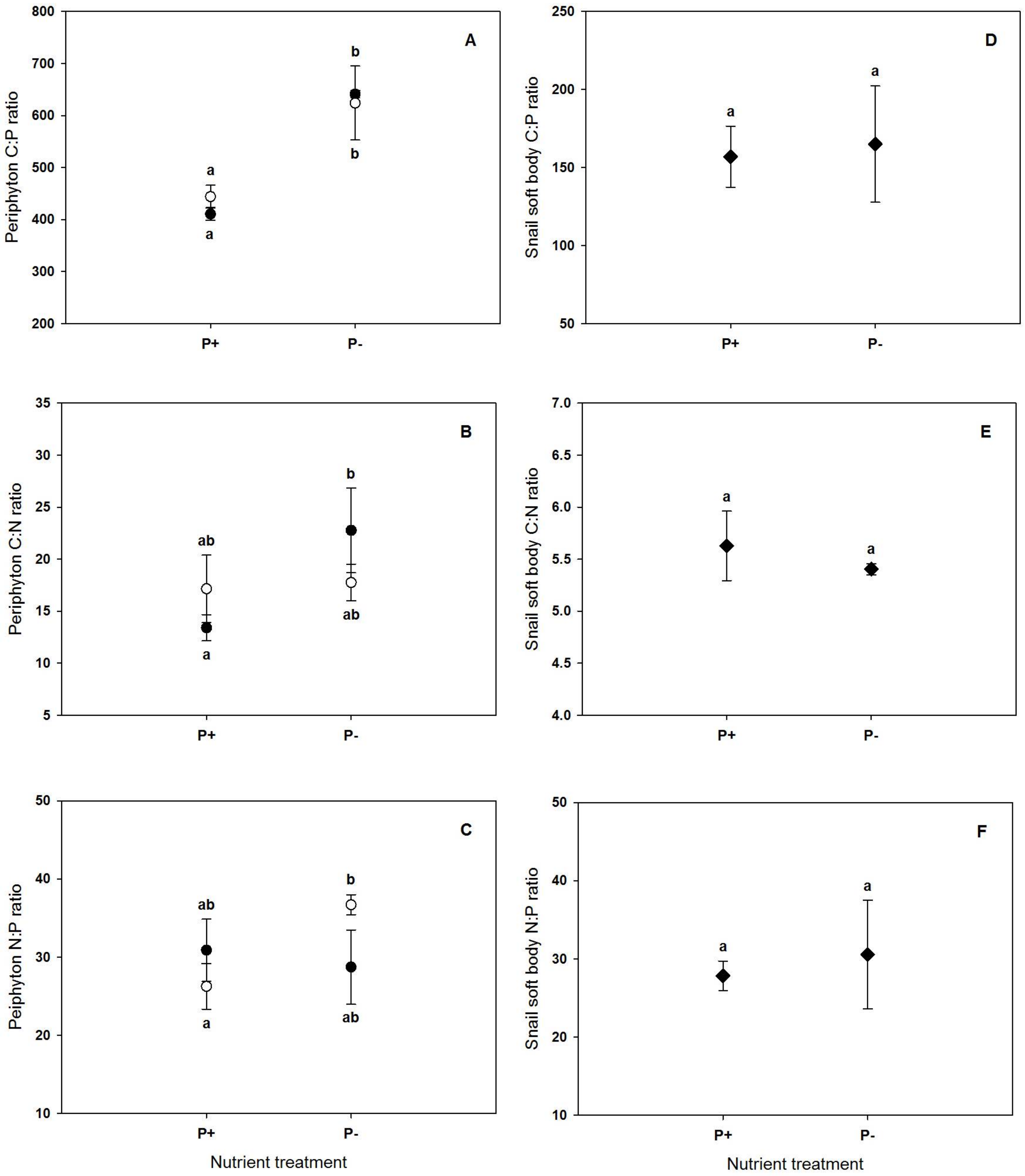

Figure 1. Molar C:P, C:N and N:P ratios of P-enriched (P+) and P-depleted (P-) periphyton (A-C) and of *Ancylus fluviatilis* soft bodies feeding on P+ and P- periphyton (D-F) at the end of the grazing phase. Filled circles, grazed periphyton; open circles, ungrazed periphyton. Values are mean ± SD of *n* = 3 replicate flumes. Different letters indicate significant differences between treatments.

Periphyton C:N:P ratios

At the end of the grazing phase, periphyton C:P ratio was still significantly lower in the P+ than in the P- treatment, and was unaffected by grazing (Fig. 1A; Table 1). However, periphyton C:N and N:P ratios were significantly affected by the interaction of P level and grazing (Table 1). C:N was significantly higher in the P- than in the P+ grazed periphyton, while it did not differ between P- and P+ ungrazed periphyton (Fig. 1B). On the other hand, N:P was significantly lower in the P+ than in the P- ungrazed periphyton, but it was not significantly different between P+ and P- grazed periphyton (Fig. 1C).

Snail soft body C:N:P ratios

In contrast to periphyton stoichiometry, C:N:P stoichiometry of *Ancylus fluviatilis* soft bodies was not affected by resource nutrient ratios (Fig. 1D-F). Neither the C:P, C:N nor N:P ratio of snail soft bodies did significantly differ between P+ and P- nutrient treatments (two-sample t-test; C:P $t = -0.331$, df = 4, $p = 0.76$; C:N $t = 1.145$, df = 4, $p = 0.32$; N:P $t = -0.653$, df = 4, $p = 0.55$).

Periphyton consumption and biomass

The food consumption rate of *A. fluviatilis*, adjusted to the total snail soft body dry mass per flume, was significantly higher in the P- than in the P+ nutrient treatment (two-sample t-test, $t = -4.2$, df = 4, $p < 0.05$; Fig. 2). Moreover, a two-way ANOVA revealed significant effects of both P level and grazing on periphyton POC, but no significant interaction between the two factors was detected (Table 1). Nevertheless, the lowest biomass expressed as POC per flume was observed in the grazed P- periphyton (Fig. 3A).

Periphyton heterogeneity

P level had a significant negative effect on the variability of periphyton biomass expressed as CV, whereas grazing had no effect, and the interaction between the two factors was not significant either (Table 1). Despite this, the highest periphyton CV was observed in the presence of grazers in the P- treatment, hence grazed P- periphyton was significantly more heterogeneous than both grazed and ungrazed P+ periphyton (Fig. 3B).

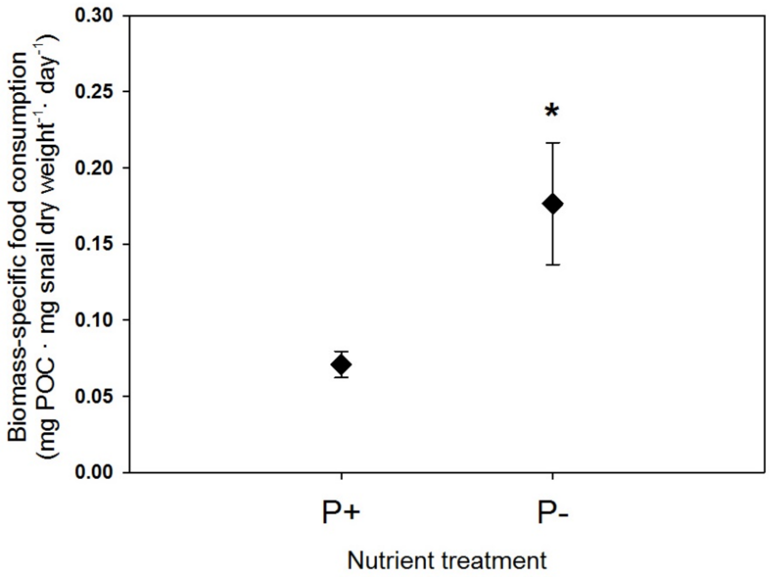

Figure 2. Biomass-specific food consumption rate of *Ancylus fluviatilis* feeding on P-enriched (P+) and P-depleted periphyton (P). Values are mean ± SD of $n = 3$ replicate flumes. The difference between the two treatments was significant ($p < 0.05$).

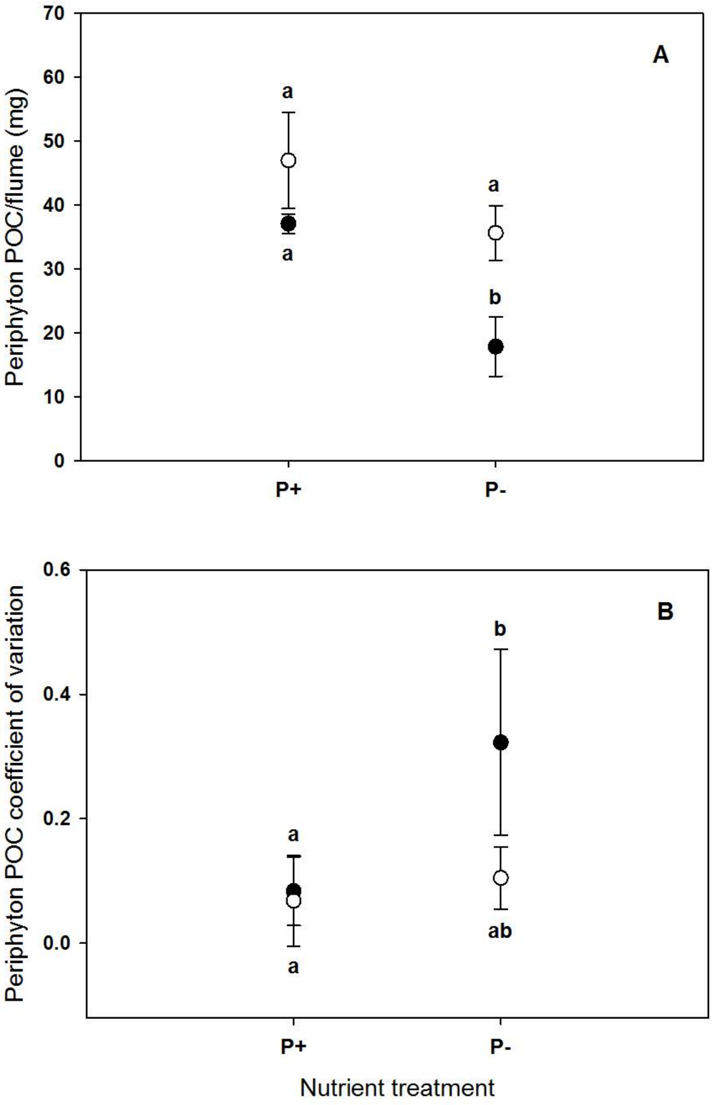

Figure 3. Biomass expressed as particulate organic carbon (POC) (A) and coefficient of variation (CV) (B) of P-enriched (P+) and P-depleted (P-) periphyton at the end of the grazing phase. Filled symbols, grazed periphyton; open symbols, ungrazed periphyton. Values are mean ± SD of *n* = 3 replicate flumes. Different letters indicate significant differences between treatments.

Discussion

The food consumption rate of *Ancylus fluviatilis* was significantly higher on P-depleted than on P-enriched periphyton, supporting our compensatory feeding hypothesis (hypothesis 1). Such an increase in consumption rate when feeding on nutrient-poor periphyton may have allowed the snails to maximise their nutrient intake. As a consequence, snail soft body C:N:P stoichiometry did not vary between the two nutrient treatments despite the marked differences in periphyton C:P and N:P ratios, as predicted by hypothesis 2.

Compensatory feeding may help herbivores to maintain an optimal body stoichiometry when the nutrient content of their food is low. However, the ingestion of high amounts of nutrient-poor food requires mechanisms for the disposal of excess assimilated carbon, which might involve further energetic costs and thus decrease the benefits of compensatory feeding (Darchambeau et al. 2003; Fink & von Elert 2006; Shimizu & Urabe 2008). Suggested mechanisms to cope with excess carbon include lowered assimilation, storage in lipids, or release through excretion or respiration (Hessen & Anderson 2008). Due to the water current, our experimental design did not allow us to sample and analyse *A. fluviatilis* fecal pellets to test whether their C:P ratios would be increased by compensatory feeding, as was observed by Fink and von Elert (2006) with *Radix ovata*. Therefore, potential adaptive mechanisms to get rid of excess carbon in *A. fluviatilis* require further investigation.

Increased consumption rates of phosphorus-depleted periphyton led to a stronger top-down control under low phosphorus availability, as the lowest periphyton standing stock was observed in the grazed low phosphorus treatment, supporting hypothesis 3. No significant difference in total biomass was detected between P+ and P- ungrazed periphyton, although nutrient enrichment is often associated with an increase in algal biomass, particularly when algae are nutrient-limited (Hillebrand 2002). However, in our experimental design, all flumes initially contained similar periphyton biomass, and the six-day long grazing phase was probably too short to result in a growth increment under phosphorus enrichment. The positive correlation between nutrient availability and benthic algal biomass in streams has been widely recognised (Smith et al. 1999; Dodds & Smith 2016); our findings further suggest that elevated nutrient inputs may also indirectly increase algal biomass by leading to a more relaxed top-down control, as grazers may lower their consumption rate on nutrient-rich food. The mechanism of compensatory feeding is a short-term behavioural response to fluctuations in resource nutrient stoichiometry. In the long-term, such effects might be supplemented by other mechanisms acting on longer time scales. Low nutrient availability may constrain consumer growth and reproduction and thus eventually reduce grazing intensity, but nutrient enrichment may favour dominance of grazer species with high nutrient demands and thus reduce consumer diversity, as was observed by Evans-White et al. (2009). Furthermore, nutrient enrichment is likely to alter not only periphyton nutrient content, but also its taxonomic (Stelzer & Lamberti 2001) and fatty acid composition (Hill et al. 2011), which are further components of algal nutritional quality (Brett et al. 2000; Wacker & von Elert 2004; Windisch & Fink 2018). As a consequence, more studies are needed to investigate the long-term effects of eutrophication on periphyton food quality and grazer behaviour in stream communities.

Phosphorus has traditionally been considered the primary limiting nutrient for freshwater systems, but recent studies have pointed out that both phosphorus and nitrogen inputs are a major cause of eutrophication in streams and lakes (Elser et al. 2007; Dodds & Smith 2016). Moreover, the accessibility of both nutrients to primary producers can be enhanced by grazers, which recycle nutrients via excretion (Mulholland et al. 1991; Hillebrand et al. 2004; Liess & Haglund 2007). However, the degree of nutrient regeneration strongly depends on differences in stoichiometry between primary producers and grazers (Liess & Hillebrand 2004). In our study, periphyton N:P ratio was significantly higher in the P- depleted than in the P-enriched treatment at the start of the grazing phase; as grazers consumed significantly more P-depleted than P-enriched periphyton, they would have been expected to recycle excess nitrogen while retaining phosphorus, thus leading to a more pronounced nitrogen regeneration in the P-depleted treatment. After grazing, periphyton C:N and N:P ratios were significantly affected by the interaction of phosphorus enrichment and grazing, whereas periphyton C:P ratio was affected by phosphorus enrichment only. Therefore, the presence of grazers did not increase the relative phosphorus content of periphyton, suggesting strong phosphorus assimilation by grazers. On the other hand, grazing appeared to increase periphyton relative nitrogen content in the phosphorus-rich treatment, most likely through nitrogen recycling, while surprisingly decreasing it in the phosphorus-poor treatment. However, this reduction in relative nitrogen content might be simply due to a sampling bias, as a possibly heterogeneous nitrogen distribution might have led snails to preferentially graze on, and thus eliminate, nitrogen-rich periphyton.

Variability of periphyton nutrient content, and hence of grazing activity, might have further affected the observed distribution of periphyton biomass, which was more heterogeneous in the phosphorus-poor than in the phosphorus-rich treatment after grazing. Grazing has been reported to increase periphyton heterogeneity under certain conditions, including high consumer mobility (Alvarez & Peckarsky 2005) and presence of predators (McIntosh et al. 2004). In our case, the increased grazing pressure in the low phosphorus treatment resulting from compensatory feeding might have had a positive effect on periphyton biomass heterogeneity, consistent with the results of a meta-analysis by Liess & Hillebrand (2004). Alternatively, the observed difference in periphyton biomass heterogeneity after grazing might have been an indirect consequence of phosphorus enrichment. Periphyton nutrient content might have been homogenised by phosphorus enrichment, leading to a uniform grazing distribution in the high phosphorus treatment; in contrast, snails in the low phosphorus treatment may have aggregated on food patches with a higher local nutrient content. Enhanced phosphorus availability might thus not only lead to a reduced grazing pressure on periphyton but also to a decrease in spatial heterogeneity of benthic algal assemblages.

Overall, nutrient limitation might be costly for grazers, but beneficial for the ecosystem as a whole. Here we provide evidence that compensatory feeding is an important mechanism for the control of benthic algal biomass, resulting from moderate nutrient inputs to primary producers. Eutrophication might lead to excessive algal production not only directly, but also indirectly by reducing the strength of grazing, thus disrupting the balance between bottom-up and top-down control at the base of the trophic pyramid (see also Vadeboncoeur & Power 2017).

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Supporting Information

Table S1. Periphyton biomass expressed as particulate organic carbon (POC) per tile, molar C:P, C:N and N:P ratios, and taxonomic composition expressed as chlorophyte:diatom ratio in the P-enriched (P+) and P-depleted treatment (P-) at the beginning of the grazing phase. Values are mean ± SD of $n = 3$ replicate tiles. Significant results ($p < 0.05$) are highlighted in bold.

	P+	**P-**	**t-test significance**		
			t	df	*p*
POC (mg)	9.07 ± 1.76	8.40 ± 0.65	0.617	4	0.57
C:P	171.49 ± 16.10	680.48 ± 39.04	-16.161	4	**<0.001**
C:N	17.32 ± 5.14	13.98 ± 0.59	1.118	4	0.33
N:P	10.55 ± 3.44	48.70 ± 2.90	-14.698	4	**<0.001**
Chlorophyte:diatom	16.81 ± 4.21	14.37 ± 3.99	0.729	4	0.51

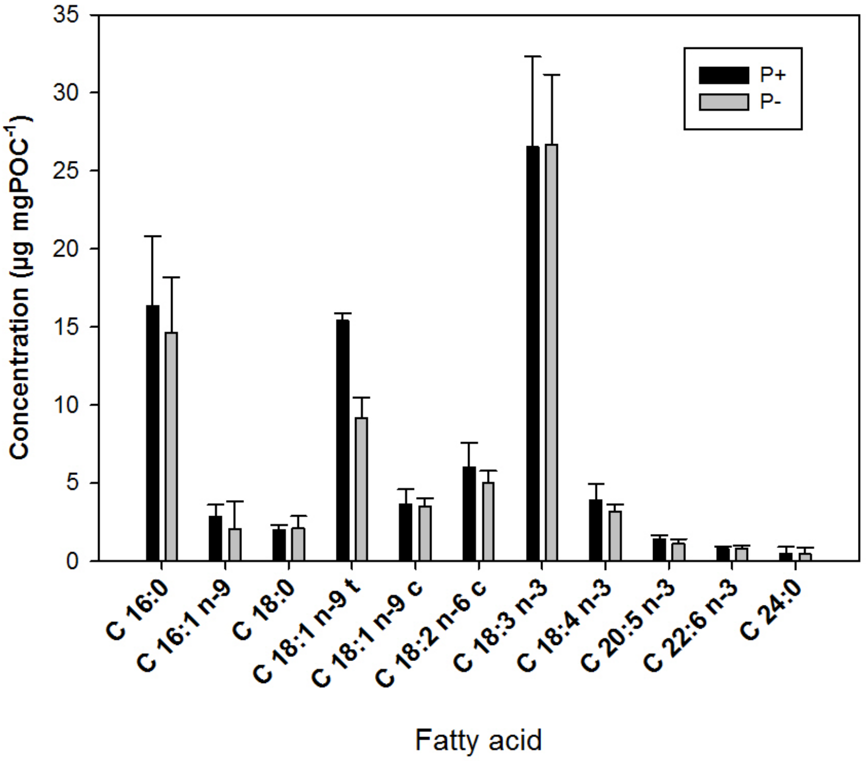

Figure S1. Periphyton fatty acid composition in the P-enriched (P+, black bars) and P-depleted (P-, grey bars) treatment at the beginning of the grazing phase. Values are mean ± SD of $n = 3$ replicate tiles.

CHAPTER II

Taxonomic shift over a phosphorus gradient affects the stoichiometry and fatty acid composition of stream periphyton

Abstract

Phosphorus enrichment of stream ecosystems generally increases primary production in the benthos, but the consequences of eutrophication for the nutritional quality of periphyton for grazers are less clear. On short time scales, high phosphorus inputs may lead to reduced C:P ratios and high essential fatty acid contents of periphyton, which are both considered important determinants of food quality for grazers. However, nutrient enrichment may alter the taxonomic composition of periphyton and favour the growth of less palatable algal taxa. In this study, periphyton was grown under a gradient of dissolved phosphorus availability from 5 to 100 μg P L^{-1}, to investigate eutrophication effects on periphyton taxonomy, C:N:P stoichiometry and fatty acid composition. After one month, periphyton grown under oligotrophic conditions was mainly composed of diatoms (~86%). With increasing phosphorus availability, diatoms were gradually outcompeted by chlorophytes and cyanobacteria, which were the predominant taxon under eutrophic conditions. Unexpectedly, periphyton C:P was on average 277 under oligotrophic conditions and increased with phosphorus availability, reflecting a tendency of chlorophytes and cyanobacteria to produce more biomass per unit of assimilated phosphorus compared to diatoms. Periphyton content of essential polyunsaturated fatty acids relative to biomass followed a unimodal relationship with phosphorus availability and peaked at intermediate phosphorus levels, likely as a result of both taxonomic and nutrient effects. Our results demonstrate that phosphorus-driven eutrophication of freshwater ecosystems may worsen periphyton nutritional quality due to taxonomic sorting, which may further lead to lower growth and reproduction of herbivores.

Introduction

Benthic algae are the most nutritious food source for herbivores in stream ecosystems (Brett et al. 2017). Compared to allochthonous organic matter, algae provide higher relative amounts of essential nutrients (i.e. phosphorus and nitrogen) and polyunsaturated fatty acids (PUFAs), which are major determinants of food quality for grazers (Torres-Ruiz et al. 2007; Lau et al. 2009). However, periphyton nutritional quality is strongly influenced by environmental factors such as light intensity and nutrient availability (Hill et al. 2011), whose fluctuations may significantly alter the efficiency of energy transfer to consumers. In particular, low relative contents of nutrients and/or PUFAs in periphyton are known to constrain grazer growth and reproduction (Stelzer & Lamberti 2002; Guo et al. 2016a). Therefore, there has been growing interest in determining how abiotic resources affect benthic algal food quality in streams (Guo et al. 2016b).

Nutrient concentrations in streams have been increasing in the past few decades due to agricultural runoffs, wastewater discharge and other anthropogenic activities, often leading to an increment in algal production known as eutrophication (Dodds & Smith 2016; Wurtsbaugh et al. 2019). Elevated inputs of phosphorus (P) or nitrogen (N) may result in a high algal nutrient content relative to carbon (C), and thus low C:P or C:N stoichiometric ratios, as demonstrated in several laboratory studies with natural periphyton (Stelzer & Lamberti 2001; Cross et al. 2005; Fanta et al. 2010; Hill et al. 2011). A few studies have also shown that high phosphorus availability may lead to an increased proportion of PUFAs relative to total fatty acids in algal cells, because high phosphorus concentrations aid the formation of PUFA-rich galactolipids and phospholipids, while at low nutrient availability, carbon is stored and accumulated in saturated fatty acids (SAFAs) (Reitan et al. 1994; Guschina & Harwood 2006; Hill et al. 2011). Therefore, phosphorus enrichment might improve the nutritional quality of periphyton for grazers by increasing its relative content of nutrients and PUFAs. However, eutrophication is often associated with an increase in the proportion of periphytic cyanobacteria and/or chlorophytes, and a subsequent decrease in diatom abundance (Carrick & Lowe 1988; Leland & Porter 2000; Pan et al. 2000; Marcarelli & Wurtsbaugh 2006; McCall et al. 2017). Diatoms are considered a high-quality food source for aquatic herbivores because they are rich in long-chained PUFAs, particularly eicosapentaenoic acid (EPA, 20:5ω3), which serve a wide range of essential physiological functions for cell membranes and hormone regulation in animals (Brett & Müller-Navarra 1997; Lang et al. 2011; Twining et al. 2016). Chlorophytes are poor in EPA and other long-chained PUFAs, but are considered of intermediate dietary quality because they contain high relative amounts of C_{18} PUFAs, particularly α-linolenic acid (ALA, 18:3ω3) and linoleic acid (LIN, 18:2ω6), which also have important physiological roles for invertebrate growth (Torres-Ruiz et al. 2007; Taipale et al. 2013; Guo et al. 2016b). Cyanobacteria, on the other hand, are a low-quality food source because they are generally poor in PUFAs and sterols (von Elert et al. 2003; Brett et al. 2006; Burns et al. 2011). As a consequence, a shift in taxonomic composition caused by nutrient enrichment is likely to impact the fatty acid profile of periphyton, resulting in lower amounts of available essential PUFAs for herbivores. However, only a few field studies have investigated how nutrient enrichment affects periphyton fatty acid composition in streams, with complex and differing results, as nutrients were often interacting with other variables, such as light (Guo et al. 2015, 2016a; Cashman et al. 2013; Whorley & Wehr 2016).

While it is acknowledged that nutrient enrichment may increase algal nutrient content, little is known about the C:P stoichiometry of communities experiencing taxonomic shifts in response to eutrophication. Laboratory studies on algal monocultures have shown that diatoms tend to maintain lower C:P ratios than chlorophytes when grown under the same conditions (Ho et al. 2003; Quigg et al. 2003). As a consequence, positive effects of increasing P availability on periphyton relative P content, as expected from observations on stable communities, may be counteracted by negative effects, when chlorophytes and cyanobacteria are favoured over diatoms along the nutrient gradient. A number of studies observed high variation in the relationship between P availability and periphyton C:P stoichiometry in natural streams, though no mechanistic explanations were provided (Fanta et al. 2010; O'Brien & Wehr 2010; Liess et al. 2012; Hiatt et al., 2019). Therefore, controlled experiments are needed to investigate how eutrophication affects periphyton C:P stoichiometry in relation to its taxonomic composition.

In this study, stream periphyton was grown under a gradient of dissolved P availability in laboratory flumes, to test nutrient enrichment effects on periphyton food quality in terms of fatty acid composition and nutrient stoichiometry. We manipulated water P concentrations to mimic conditions in natural streams from highly oligotrophic to highly eutrophic. We tested the following hypotheses: (1) Diatoms would dominate the periphytic community at low P availability, while the fraction of chlorophytes and cyanobacteria would increase with P availability; (2) As a consequence of the change in taxonomic composition, periphyton content of essential PUFAs (particularly EPA) would decrease with increasing P availability; (3) Periphyton C:P ratio would not strongly correlate with P availability as two contrasting effects (i.e. direct P effect on stoichiometry of single populations versus indirect P effect on stoichiometry of communities mediated by taxonomic sorting) would negatively interact.

Methods

Experimental set-up and procedure

Each experimental unit consisted of a circular flume in a 22 × 27 cm (height × diameter) plastic bucket with a circular water current generated by a pump in the centre. On the bottom surface of each flume were nine 5 × 5 cm slots for tiles. The experiment consisted of five treatments of increasing dipotassium phosphate (K_2HPO_4) concentrations in the water:

oligotrophic (5 μg P L^{-1}), oligo-mesotrophic (25 μg P L^{-1}), mesotrophic (50 μg P L^{-1}), meso-eutrophic (75 μg P L^{-1}) and eutrophic (100 μg P L^{-1}), according to Dodds & Smith (2016). Each flume was filled with 6 L modified WC medium (Guillard & Lorenzen 1972; Table S1 in Supplementary Information) diluted in aerated tap water (1:15) with an adjusted P concentration according to the treatment. The average current velocity in the flumes was 14 cm s^{-1}. Each treatment was replicated twice, resulting in a total of ten flumes. Nine 4.7 × 4.7 cm unglazed ceramic tiles were placed horizontally in the tile slots on the bottom surface of each flume, arranged in a circle, as substrate for periphyton growth.

Natural periphyton was collected in the river Rhine in Cologne (50°54'25.5"N, 6°58'41.7"E) in August 2018 and homogenised in 500 ml tap water, of which 40 ml were inoculated into each flume on the same day. Additionally, aliquots of the collected periphyton were filtered onto pre-combusted glass fibre filters (Whatman GF/F, 25 mm Ø) for elemental, fatty acid and pigment analyses (see following sections). The flumes were placed in a row on a shelf in a climate chamber kept at 18 ± 1°C in a 16:8 hours light:dark cycle. The water temperature in the flumes was approximately 2°C greater than the room temperature, due to the heat generated by the pumps, but did not differ between treatments. The average PAR intensity above the flumes, produced by LED lamps, was 38.6 μmol m^{-2} s^{-1}, measured at the water surface.

Periphyton was allowed to grow in the flumes for 32 days. The water in the flumes was replaced every seven days with fresh medium of the respective P level. Each flume was shifted by one position to the right every two days, to exclude position effects and ensure equal average lighting in all flumes. The experiment ended on 3rd September 2018, three days after the last medium exchange. Periphyton was scraped off each tile and homogenised in 100 ml tap water, of which aliquots were filtered onto pre-combusted glass fibre filters for elemental, fatty acid and pigment analyses. In addition, 1 ml of each suspension was mixed with 100 μl

Lugol's iodine solution for microscopic analysis under an inverted microscope, to identify the algal classes present in the flumes and include them in the pigment ratio matrix for CHEMTAX (see *Pigment analysis*).

Nutrient analyses

For C/N analysis, filters were dried for 48 h at 60°C, packed in tin capsules (HekaTech, Wegberg, Germany) and analysed with a Thermo Flash EA2000 Analyser (Schwerte, Germany). For the analysis of periphyton particulate P, filters were placed in a solution of potassium peroxodisulphate and 1.5% sodium hydroxide, and autoclaved for 1 h at 120°C to convert particulate P into soluble reactive P, which was subsequently analysed with the molybdate-ascorbic acid method (Greenberg et al. 1985) with a DR5000 UV-Vis spectrophotometer (Hach, Düsseldorf, Germany).

Fatty acid analysis

Fatty acids extraction and analysis was performed according to Windisch & Fink (2018). Each filter was transferred into 5 ml extraction solvent (CH_2Cl_2/MeOH, 2:1 v/v) and stored at -20°C. Internal standards (10 μg heptadecanoic acid methyl ester, C17:0, and 10 μg tricosanoic acid methyl ester, C23:0) were added to each sample, which were subsequently sonicated for 3 min and centrifuged at 5000 rpm for 5 min, to remove filter residues. The supernatants were evaporated to dryness at 40°C under a stream of nitrogen gas, and 5 ml 3 N methanolic HCl were added to each sample. The samples were transesterified at 70°C for 20 min. Fatty acid methyl esters were extracted by adding 6 ml isohexane to each sample. The hexane phases were evaporated to dryness at 40°C under a nitrogen stream and redissolved in 100 μl isohexane.

The fatty acid composition of the samples was subsequently determined via gas chromatography with a 6890 N GC System (Agilent Technologies, Waldbronn, Germany) equipped with a DB-225 capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) and a flame ionisation detector. Instrument settings were as follows: injector and flame ionisation detector temperatures were 200°C; the initial oven temperature was 60°C for 1 min, followed by a 120°C min^{-1} temperature ramp to 180°C and a 50°C min^{-1} ramp to 200°C; after 10.5 min at 200°C, the temperature was increased to 220°C at 120°C min^{-1} and kept for 10 min. Helium with a flow rate of 1.5 ml min^{-1} was used as carrier gas. Fatty acid methyl esters were quantified by referring to the known amounts of internal standards and previously established calibration functions for each fatty acid.

Pigment analysis

Pigment extraction and analysis was performed according to Ilic (2019) and Trench-Fiol & Fink (2020) to determine the taxonomic composition of periphyton based on pigment ratios. Filters were wrapped in aluminium foil and stored at -20°C. For pigment extraction, each filter was transferred into 5 ml 100% acetone, and 0.5 *µ*g trans-*ß*-apo-8'-carotenal (Sigma Aldrich) was added to each sample as internal standard. The samples were sonicated for 2 min and placed on ice for 1 min; this procedure was repeated five times, for a total of 10 min sonication time. The samples were stored at 4°C for 24 h to allow further extraction and subsequently centrifuged for 15 min at 4500 rpm after the filters were removed. The supernatants were transferred into vials for pigment analysis via high-pressure liquid chromatography (HPLC).

The samples were analysed with a Shimadzu Prominence HPLC System equipped with a binary pump (LC-20AB), an autosampler (SIL-A20C), a column oven (CTO-10AC) set at 40°C, a photodiode array detector (SPD-M20A) and a degasser. Pigments were separated following a method modified after Garrido and Zapata (1993), with a reverse phase Spherisorb ODS2 column (dimensions: 25 cm × 4.6 mm, particle size: 5 μm). The solvents used were 50:20:30 methanol: 1 M ammonium acetate: acetonitrile (solvent A) and 50:50 acetonitrile:ethyl acetate (solvent B). The time program was as follows: 0 min: 90% A, 10% B; 26 min: 40% A, 60% B; 28 min: 10% A, 90% B. The flow rate was 1 ml min^{-1}.

Pigment measurements from the HPLC analysis were used to determine the taxonomic composition of periphyton with CHEMTAX (version 1.95, Wright & Mackey, Hobart, Australia) according to Mackey et al. (1996). Microscopic counts allowed us to identify the algal classes present in the samples (namely diatoms, chlorophytes and cyanobacteria), thus the pigment ratio table for CHEMTAX was adjusted accordingly. The pigment/chlorophyll *a* ratio matrix from Schlüter et al. (2016) was used as initial ratio matrix, from which 60 different ratio matrices were generated for each treatment. Of these, the six matrices (10%) with the lowest residual root mean square were averaged to create a new input ratio matrix, which was run repeatedly until the ratios and root mean squares were stable. The final results from CHEMTAX gave an estimation of the relative abundance of the identified algal groups (diatoms, chlorophytes and cyanobacteria) from each sample, in terms of contribution to the total chlorophyll *a* (Mackey et al. 1996; Schlüter et al. 2016).

Data analysis

All data were checked for normal distribution with a Shapiro-Wilk’s test and for homogeneity of variances with a Levene’s test. Regression analyses were performed to test for relationships between P availability and periphyton taxonomic composition, stoichiometry and fatty acid profile; for each analysis, the regression model that best explained the observed variance (i.e. with the highest R^2 value) was selected. Statistical analyses were conducted in SigmaPlot (version 11, SysStat). Type I error was set at 0.05 for significance level.

Results

Periphyton initial conditions

Periphyton collected from the river Rhine was mainly composed of diatoms (87 ± 1.5% contribution to total chlorophyll *a*, mean ± SD), together with a small proportion of chlorophytes (11 ± 1%) and cyanobacteria (2 ± 0.5%, see Fig. S1 in Supplementary Information). No other algal classes were observed. Periphyton average molar C:N:P ratio was 160:13:1 (Table S2 in Supplementary Information). Total fatty acid (FA) content was 40.62 ± 4.3 μg mg C^{-1} and total polyunsaturated fatty acid (PUFA) content was 9.25 ± 0.7 μg mg C^{-1} (Table S2 in Supplementary Information). Eicosapentaenoic acid (EPA) was the most abundant PUFA at 3.75 ± 0.3 μg mg C^{-1}, followed by linoleic acid (LIN, 1.60 ± 0.1 μg mg C^{-1}) and α-linolenic acid (ALA, 1.55 ± 0.1 μg mg C^{-1}, Fig. S2 in Supplementary Information).

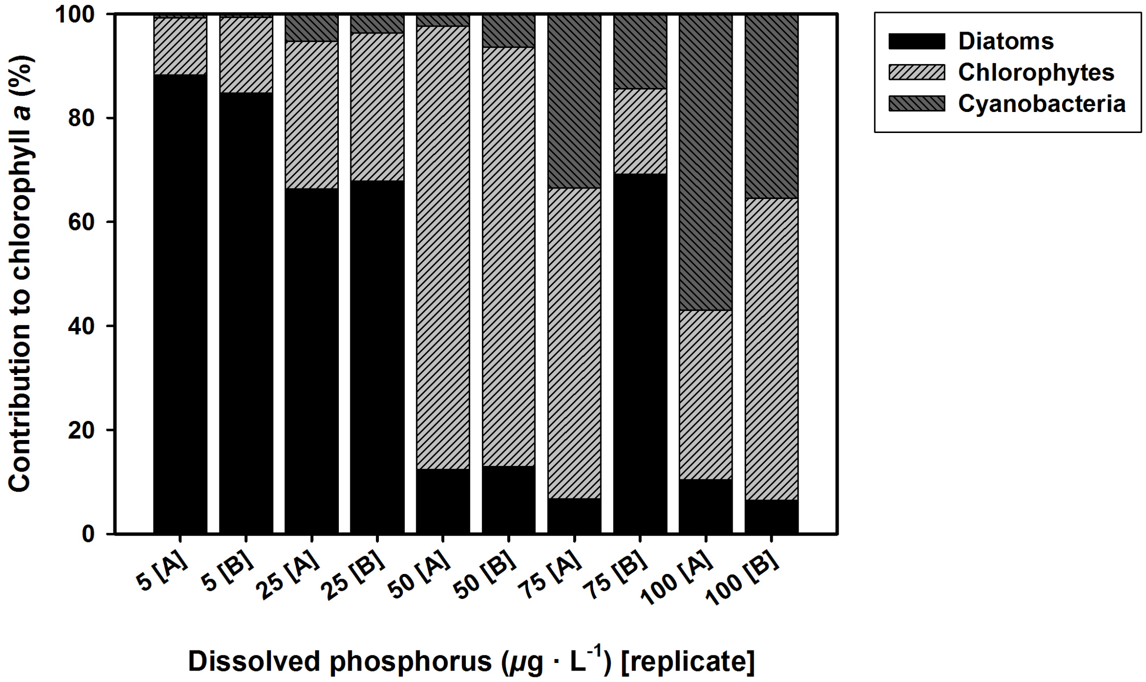

Figure 1. Periphyton taxonomic composition over the phosphorus gradient, expressed as percentage contribution to total chlorophyll *a*. Both replicates (A and B, in square brackets) are shown individually for every treatment.

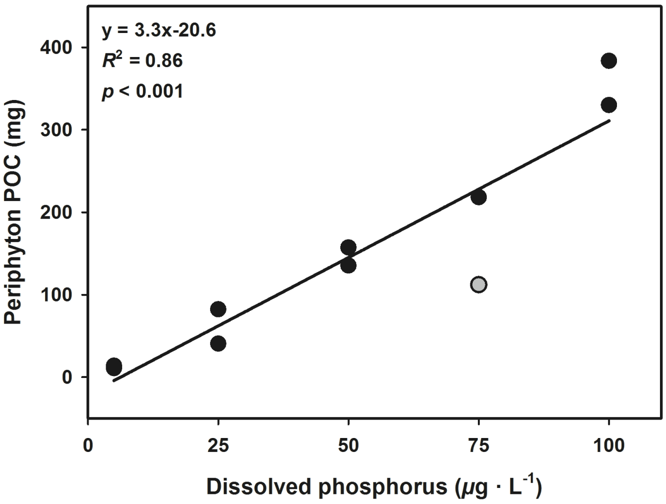

Figure 2. Linear regression (R^2 = 0.86, p < 0.001) between P availability and total periphyton biomass per flume, expressed as mg particulate organic carbon (POC). Replicate B of the meso-eutrophic treatment, the only replicate in the higher end of the P gradient with a >50% diatom relative abundance (see Figure 1), is marked in grey.

Periphyton taxonomic composition

One month after inoculation, periphyton taxonomic composition was strongly affected by P availability (Fig. 1). Diatoms were the predominant algal group in the assemblages at 5 and 25 μg P L^{-1}, on average constituting 86% and 67% of the total, respectively. At 50 μg P L^{-1} and above, diatom relative abundance was markedly lower (<13%), except in replicate B of the meso-eutrophic treatment (75 μg P L^{-1}), where diatoms accounted for 69% of the periphyton community. For this reason, the same replicate is marked in grey in the remaining figures (Fig. 2-5, Fig. S3 in Supplementary Information) though it was not excluded from statistical analyses. Diatom abundance decreased exponentially with increasing P availability (nonlinear regression, 2 parameter exponential decay equation, R^2 = 0.67, p = 0.004, Fig. S3a in Supplementary Information), whereas chlorophyte abundance peaked at 50 μg P L^{-1} (83% on average, Figure S3b in Supplementary Information). The proportion of cyanobacteria increased exponentially from <1% at the lowest P availability to an average of 46% at 100 μg

P L^{-1} (nonlinear regression, 1 parameter exponential equation, $R^2 = 0.85$, $p < 0.001$, Fig. S3c in Supplementary Information).

Periphyton biomass and C:N:P stoichiometry

Periphyton biomass expressed as particulate organic carbon (POC) followed a linear relationship with P availability (linear regression, $R^2 = 0.86$, $p < 0.001$, Fig.2). Periphyton C:P ratio was the lowest at 5 μg P L^{-1} with an average value of 277, and it increased logarithmically with P availability, reaching an average value of 794 at 100 μg P L^{-1} (nonlinear regression, dynamic fitting, 2 parameter logarithmic equation; $R^2 = 0.72$, $p = 0.002$, Fig. 3a). An analogous trend was observed with the N:P ratio (nonlinear regression, dynamic fitting, 2 parameter logarithmic equation; $R^2 = 0.76$, $p = 0.001$, Fig. 3b). In contrast, periphyton C:N ratio was approximately 10 in all treatments, thus it was not affected by P availability (linear regression; $R^2 = 0.0034$, $p = 0.9$, Fig. 3c).

Periphyton fatty acid composition

Periphyton total FA and PUFA content relative to C followed a unimodal relationship with P availability, both peaking at 50 μg P L^{-1} (nonlinear regression, dynamic fitting, 3 parameter Gaussian equation; FA: $R^2 = 0.74$, $p = 0.008$, Fig. 4a; PUFA: $R^2 = 0.72$, $p = 0.01$, Fig. 4b). SAFA:PUFA ratio was highest at 5 μg P L^{-1} and declined logarithmically with P availability (nonlinear regression, dynamic fitting, 2 parameter logarithmic equation; $R^2 = 0.91$, $p < 0.001$, Fig. 4c). The nutritionally important fatty acids EPA, ALA, and LIN also showed a unimodal relationship with P availability, with EPA peaking at 25 μg P L^{-1} (nonlinear regression, dynamic fitting, 3 parameter Gaussian equation; $R^2 = 0.61$, $p = 0.037$, Fig. 5a) and ALA and LIN at 50 μg P L^{-1} (nonlinear regression, dynamic fitting, 3 parameter Gaussian equation; ALA: $R^2 = 0.63$, $p = 0.031$, Fig. 5b; LIN: $R^2 = 0.67$, $p = 0.02$, Fig. 5c).

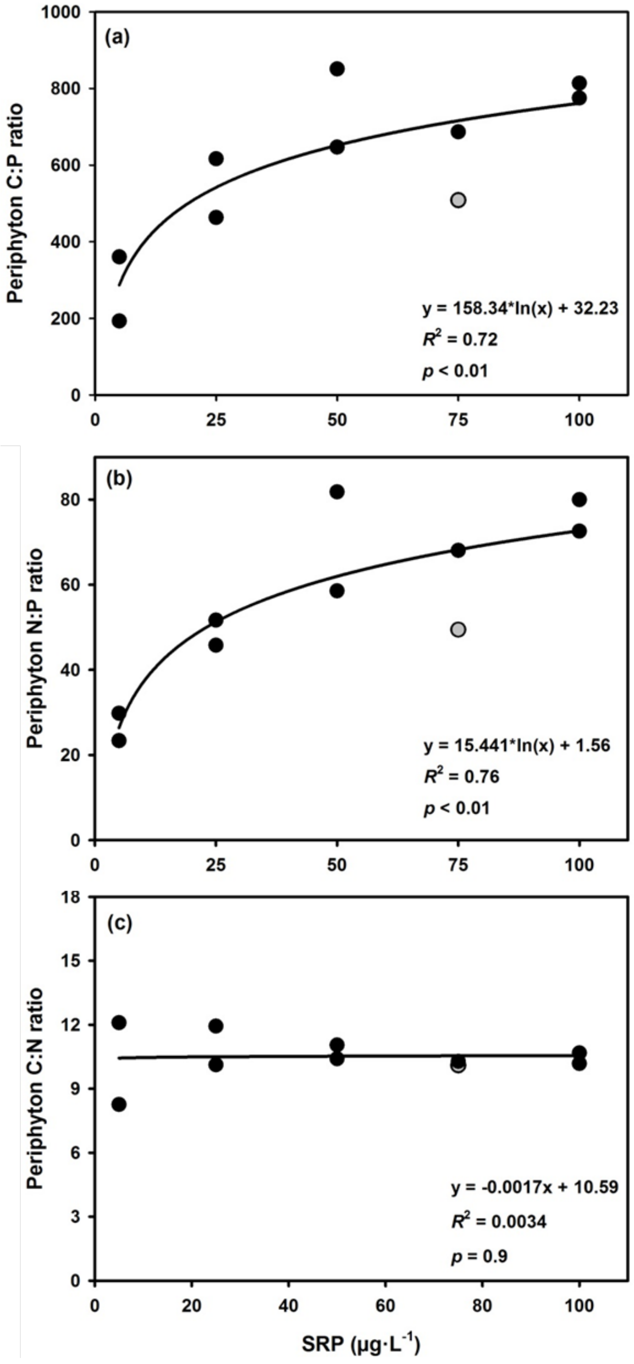

Figure 3. Periphyton molar C:P (a), N:P (b) and C:N (c) ratios against P availability. Periphyton C:P and N:P ratio were lowest in the oligotrophic treatment (5 μg P L^{-1}) and followed a logarithmic curve along the P gradient (nonlinear regression, dynamic fitting, 2 parameter logarithmic equation; C:P ratio: R^2 = 0.72, p = 0.002; N:P ratio: R^2 = 0.76, p = 0.001), while C:N ratio was not affected (linear regression; R^2 = 0.0034, p = 0.9). Replicate B of the meso-eutrophic treatment, the only replicate in the higher end of the P gradient with a >50% diatom relative abundance (see Figure 1), is marked in grey.

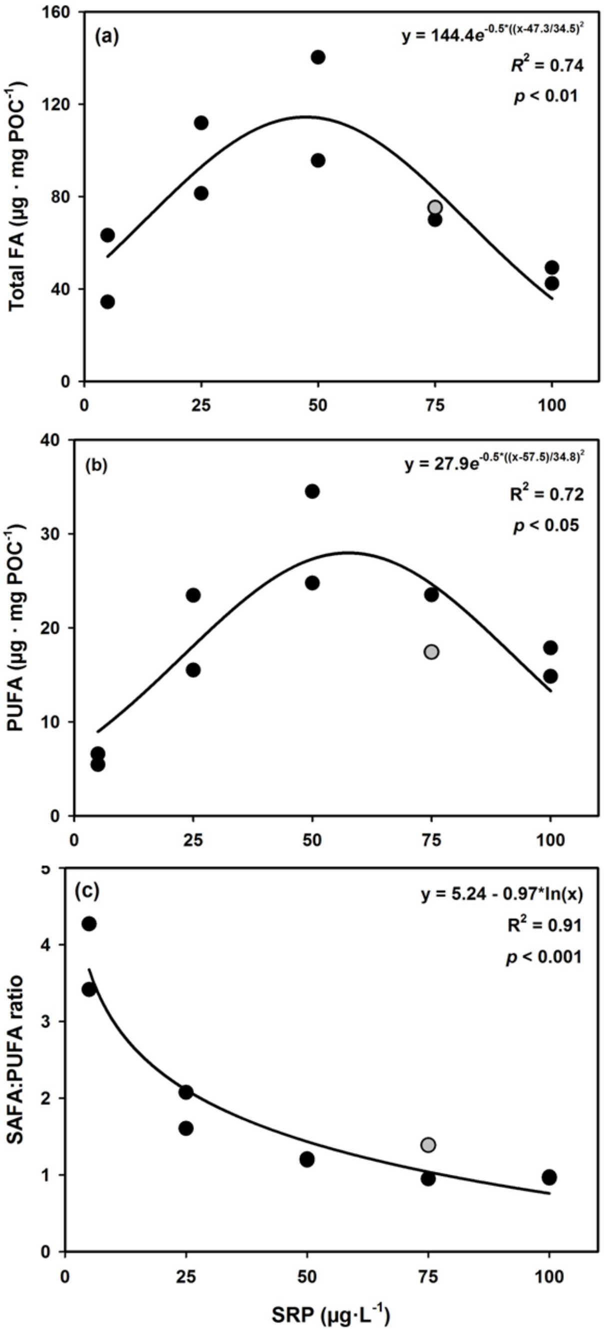

Figure 4. Periphyton content of total fatty acids (FA, a), polyunsaturated fatty acids (PUFA, b) and SAFA:PUFA ratio (c) along the P gradient. Total FA and PUFA content followed a bell-shaped relationship with P availability (nonlinear regression, dynamic fitting, 3 parameter Gaussian equation; FA: $R^2 = 0.74$, $p = 0.008$; PUFA: $R^2 = 0.72$, $p = 0.01$), while SAFA:PUFA ratio was highest in the oligotrophic treatment and declined in a logarithmic fashion (nonlinear regression, dynamic fitting, 2 parameter logarithmic equation; $R^2 = 0.91$, $p < 0.001$). Replicate B of the meso-eutrophic treatment, the only replicate in the higher end of the P gradient with a >50% diatom relative abundance (see Figure 1), is marked in grey.

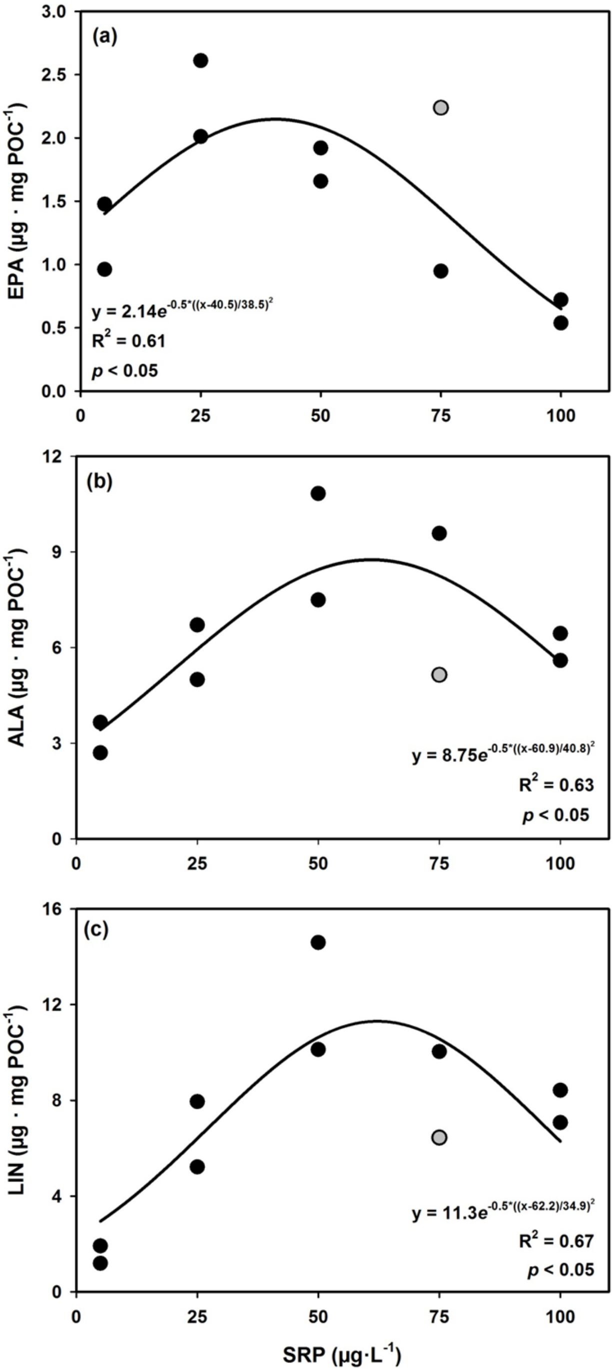

Figure 5. Periphyton content of EPA (a), ALA (b) and LIN (c) along the P gradient. All three fatty acids followed a unimodal relationship with P availability, with EPA peaking in the oligo-mesotrophic treatment, and ALA and LIN in the mesotrophic treatment (nonlinear regression, dynamic fitting, 3 parameter Gaussian equation; EPA: R^2 = 0.61, p = 0.037; ALA: R^2 = 0.63, p = 0.031; LIN: R^2 = 0.67, p = 0.02). Replicate B of the meso-eutrophic treatment, the only replicate in the higher end of the P gradient with a >50% diatom relative abundance (see Figure 1), is marked in grey.

Discussion

In contrast to our expectations based on current knowledge, we observed negative relationships between increasing P availability and some essential nutritional quality variables of stream periphyton, at least in the higher ranges of nutrient concentrations. While the patterns in fatty acid content along the P gradient were unimodal, with decreases in the transition from mesotrophic to eutrophic conditions, periphyton molar C:P and N:P ratios were the lowest in the oligotrophic treatment and increased continuously over the range from oligotrophic to eutrophic conditions. This is the first time that a positive relationship between P availability and periphyton C:N:P stoichiometry is observed, with periphyton relative P content decreasing rather than increasing under eutrophication. Previous studies have observed the opposite trend (Stelzer & Lamberti 2001; Fanta et al. 2010; Hill et al. 2011). We believe our results were mainly due to the observed taxonomic shift, which did not occur in the mentioned studies. At low P, the periphyton community was mainly composed of diatoms, whose relative abundance significantly declined with increasing P concentration. Chlorophytes were the predominant algal group in the mesotrophic treatment, while cyanobacteria abundance increased exponentially with P availability, as it is often observed in eutrophic systems (Dodds & Smith 2016). Diatoms may have tolerated P deficiency by minimising biomass production, whereas chlorophytes and cyanobacteria may have built more biomass per unit of assimilated P, resulting in algal blooms under high P availability and relatively high C:P ratios in the equilibrium phase of periphyton succession. This reflects findings by Quigg et al. (2003) and Ho et al. (2003), in which chlorophytes had significantly higher C:P ratios than diatoms when grown under identical conditions. Therefore, eutrophication may result in a nutrient dilution effect under high algal productivity, and the high variance often observed between periphyton C:P stoichiometry and stream nutrient status (Fanta et al. 2010; O'Brien & Wehr 2010; Liess et al. 2012; Hiatt et al. 2019) may be explained by differences in taxonomic composition among sampling sites. Furthermore, diatoms are known to maintain lower N:P ratios than chlorophytes and cyanobacteria (Quigg et al. 2003; Weber & Deutsch 2010,2012; Hillebrand et al. 2013), which may also explain the positive relationship we observed between periphyton N:P ratio and P availability, even though N availability was kept constant in all treatments.

The fatty acid profile of periphyton was influenced by both taxonomic composition and P availability. Algal total FA and PUFA contents peaked in the mesotrophic treatment, where periphyton was dominated by chlorophytes. Although chlorophytes contain lower amounts of long-chained PUFAs compared to diatoms, they are rich in C_{18} PUFAs, particularly ALA and LIN (Torres-Ruiz et al. 2007; Taipale et al. 2013). The total FA and PUFA contents in the chlorophyte-dominated periphyton were higher than in the diatom-dominated periphyton likely as a result of the higher P availability, as P is needed for the synthesis of PUFA-rich galactolipids and phospholipids in algae (Reitan et al. 1994; Guschina & Harwood 2006; Hill et al. 2011). At the higher end of the P gradient, instead, the greater proportion of cyanobacteria resulted in a decrease in periphyton FA and PUFA contents, as cyanobacteria are generally poorer in PUFAs (Lang et al. 2011). On the other hand, as predicted, periphyton relative EPA content was the highest at relatively low P availability, coinciding with a high diatom proportion in the community, and declined as diatoms were replaced by chlorophytes and cyanobacteria along the P gradient. EPA is one of the most important PUFAs for herbivore nutrition and occurs primarily in diatoms (Torres-Ruiz et al. 2007; Lang et al. 2011; Taipale et al. 2016). However, at the lowest P availability, periphyton EPA content was relatively low despite the high proportion of diatoms, likely as a result of nutrient stress. In this treatment, periphyton SAFA:PUFA ratio was the highest, as under P deficiency, diatoms tend to store carbon as SAFAs and limit the synthesis of PUFAs (Hill et al. 2011).

While we only manipulated P concentrations under constant background conditions, other factors should be taken into accounts when natural ecosystems are considered. Nutrient availability inevitably interacts with light intensity, which was kept moderate in our flumes but is typically variable in natural environments. High light intensity increases periphyton C:P and C:N ratios, especially at low nutrient concentrations, and dilutes algal PUFA content (Fanta et al. 2010; Hill et al. 2011; Guo et al. 2015). Together, both effects result in a lower nutritional quality of periphyton. Furthermore, while it is not uncommon that diatoms are replaced by chlorophytes and cyanobacteria when nutrient inputs increase, the taxonomic composition of stream periphyton is also influenced by several factors in concomitance with P availability. In particular, high water temperatures are known to favour the growth of chlorophytes and cyanobacteria over diatoms (Tilman et al. 1986; Marcarelli & Wurtsbaugh 2006). As a consequence, such a taxonomic shift from diatoms to chlorophytes and/or cyanobacteria following P enrichment does not occur ubiquitously. Instead, a change in diatom species composition may be observed under eutrophication, particularly in cold waters (Winter & Duthie 2000; Sonneman et al. 2001; Lavoie et al. 2008; Korhonen et al. 2013).

Our results show that the maximum nutritional quality of stream periphyton, in terms of low C:P ratios and high PUFA content, is achieved at low to intermediate P availability. In highly oligotrophic conditions, nutrient stress will prevent the synthesis of PUFAs, as well as limit biomass production and thus food quantity for grazers; at high P availability, PUFA-poor taxa such as cyanobacteria may become dominant, and increased algal productivity will result in a lower nutrient content relative to biomass. Stream eutrophication will thus be detrimental to periphyton nutritional quality by reducing the relative amounts of essential nutrients and PUFAs available to herbivores, which will compromise the efficiency of energy transfer to the upper trophic levels. PUFA- and nutrient-poor periphyton will lead to lower growth and reproduction rates of herbivores (Stelzer & Lamberti 2002; Fink & von Elert 2006; Guo et al. 2016a), which will in turn affect the survival and fitness of secondary consumers, such as fish. The fatty acid content of consumers has been widely observed to match the fatty acid composition of their food (Brett et al. 2006; Guo et al. 2016a), and essential PUFA content of phytoplankton and fish in lakes is often negatively correlated with lake trophic status (Ahlgren et al. 1996; Müller-Navarra et al. 2004; Taipale et al. 2016). Furthermore, the nutritional quality of periphyton may indirectly influence the strength of top-down control. Grazer behaviour is known to affect periphyton community structure through selective feeding and nutrient regeneration (Mulholland et al. 1991; Rosemond et al. 2000; Beck et al. 2019); fluctuations in nutrient availability are likely to interact with top-down control by affecting grazer behaviour, as in e.g. compensatory feeding (Iannino et al. 2019). Future studies should focus on how long-term bottom-up effects would alter grazer growth and behaviour, and how this would in turn influence periphyton biomass and nutritional quality.

Overall, we predict that the best periphyton nutritional quality for grazers is likely to be achieved in oligo- to mesotrophic environments, especially under moderate light intensity. With this study, we demonstrate for the first time that periphyton C:P ratio may increase under P enrichment due to a taxonomic shift towards dominance of chlorophytes and cyanobacteria, which exhibit a higher biomass productivity than diatoms. While nutrient enrichment may improve algal nutritional quality in the short term, our results highlight the importance of taxonomic sorting in a complex community, which can, in the long run, overlay physiological effects of single species.

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Supplementary Information

Table S1. Chemical composition of the WC growth medium (from Guillard and Lorenzen 1972). All concentrations were subsequently diluted (1:15) for use in the experimental flumes. The dipotassium phosphate concentration was adjusted according to each treatment (see Materials and Methods).

Ingredient	**Molecular formula**	**Concentration (mg L^{-1})**
Sodium nitrate	$NaNO_3$	85
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	37
Calcium chloride	$CaCl_2 \cdot 2H_2O$	36.8
Sodium metasilicate	$NaSiO_3 \cdot 5H_2O$	21.2
Sodium bicarbonate	$NaHCO_3$	12.6
TES (buffer)[1]	$C_6H_{15}NO_6S$	115
Dipotassium phosphate	$K_2HPO_4 \cdot 3H_2O$	See Materials and Methods
Trace elements		
Ingredient	**Molecular formula**	**Concentration (μg L^{-1})**
Disodium EDTA	Na_2EDTA	4.36
Iron(III) chloride	$FeCl_3 \cdot 6H_2O$	3.15
Boric acid	H_3BO_3	1
Manganese chloride	$MnCl_2 \cdot 4H_2O$	0.18
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	0.022
Copper(II) sulphate	$CuSO_4 \cdot 5H_2O$	0.01
Cobalt(II) chloride	$CoCl_2 \cdot 6H_2O$	0.01
Sodium molybdate	$Na2MoO_4 \cdot 2H_2O$	0.006

[1] N-tris[Hydroxymethyl]-methyl-2-aminoethan-sulphonic acid

Table S2. Molar C:P, C:N and N:P ratios, total fatty acid (FA) and polyunsaturated fatty acid (PUFA) content of periphyton inoculated in the flumes at the start of the growing phase. Values are mean ± SD of *n* = 3 replicates.

C:P	160.37 ± 13.63
C:N	12.37 ± 0.08
N:P	12.97 ± 1.11
FA (μg/mg C)	40.62 ± 4.33
PUFA (μg/mg C)	9.25 ± 0.71

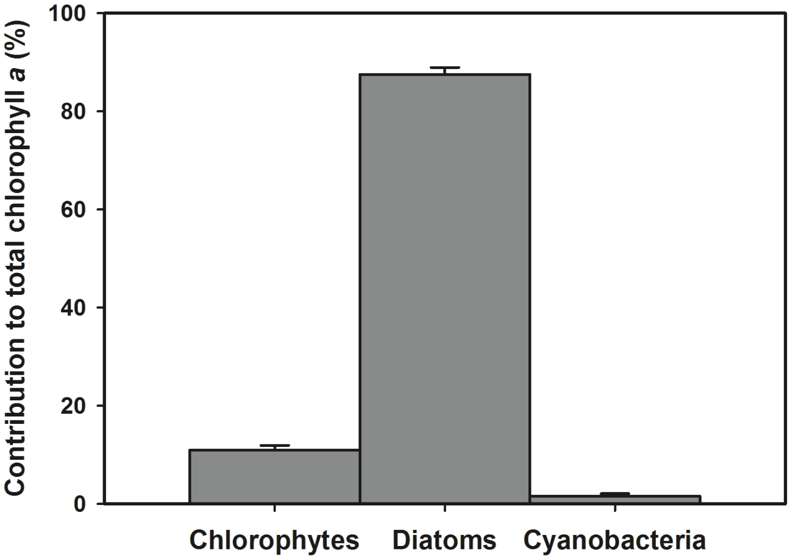

Figure S1. Taxonomic composition of periphyton inoculated into the flumes at the beginning of the experiment, expressed as percentage contribution to chlorophyll *a*. Values are mean ± SD of $n = 3$ replicates.

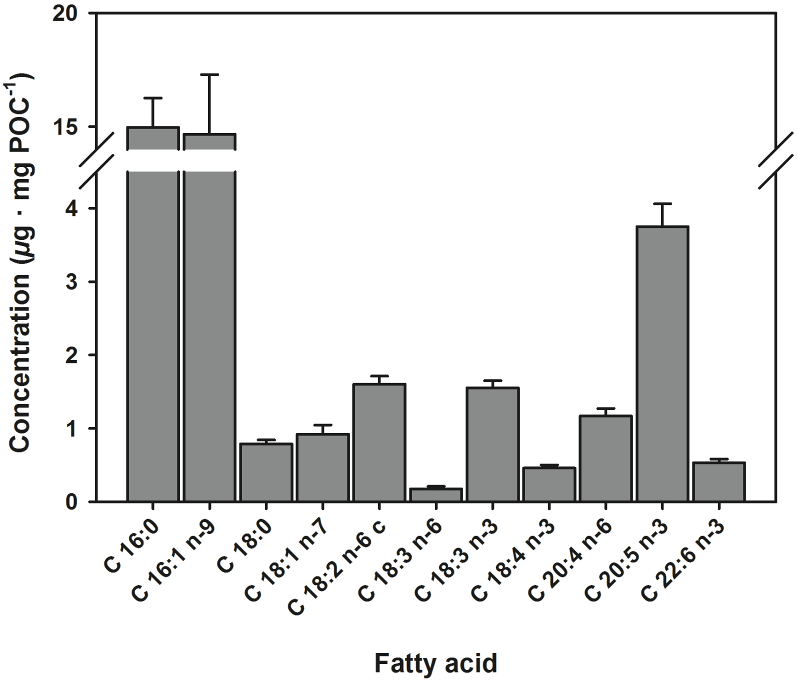

Figure S2. Fatty acid composition of periphyton inoculated into the flumes at the beginning of the experiment. Values are mean ± SD of $n = 3$ replicates.

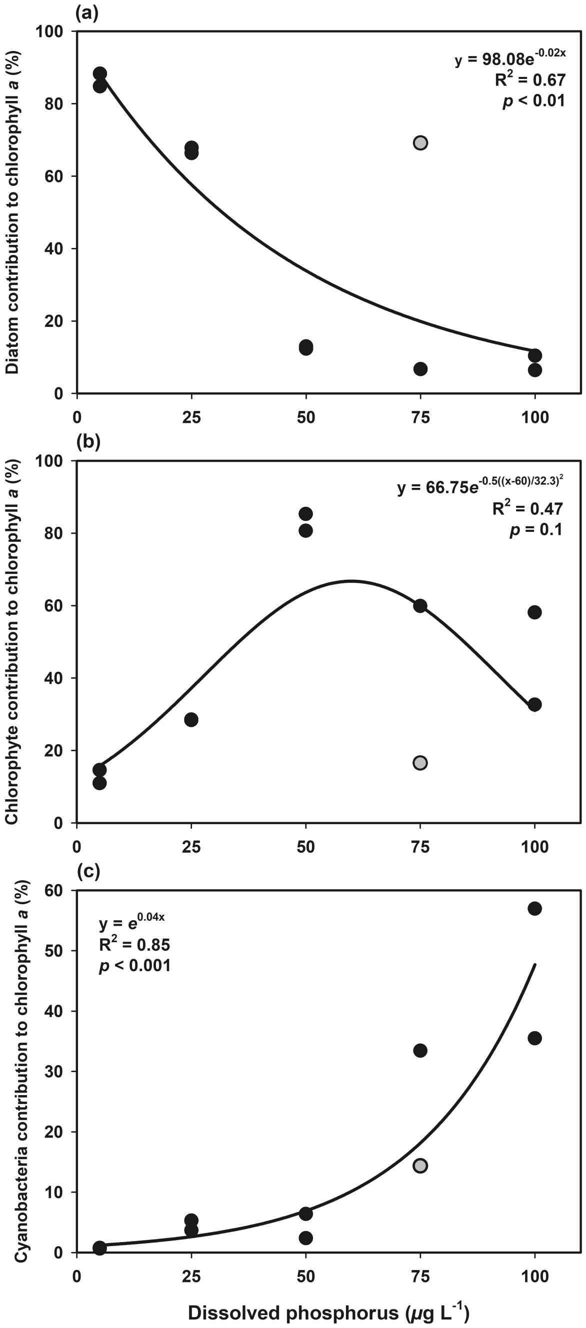

Figure S3. Relative abundance of diatoms (a), chlorophytes (b) and cyanobacteria (c) along the P gradient expressed as percentage contribution to total chlorophyll *a*. Diatom abundance declined exponentially with increasing P availability (nonlinear regression, 2 parameter exponential decay equation, $R^2 = 0.67$, $p = 0.004$), whereas chlorophyte abundance peaked in the mesotrophic treatment (nonlinear regression, 3 parameter Gaussian equation; $R^2 = 0.47$, $p = 0.1$). Cyanobacteria abundance increased exponentially with P availability (nonlinear regression, 1 parameter exponential equation, $R^2 = 0.85$, $p < 0.001$). Replicate B of the meso-eutrophic treatment, the only replicate in the higher end of the P gradient with a >50% diatom relative abundance (see Figure 1), is marked in grey.

CHAPTER III

The light:nutrient hypothesis in a heterogeneous environment: Effects of nutrient enrichment on the spatial variability of stream periphyton

Abstract

Quantity and quality of stream periphyton are strongly limited by light and nutrients. According to the light:nutrient hypothesis, periphyton C:P and C:N stoichiometric ratios increase as the ratio of light to nutrient availability increases. Similar results have also been observed with periphyton relative content of polyunsaturated fatty acids (PUFA), as P is needed for the production of phospholipids and galactolipids. Spatial variations in light availability are expected to result in a heterogeneous distribution of periphyton biomass within the same stream. However, the effects of abiotic factors on the heterogeneity of periphyton quality are still poorly understood. In this study, we tested nutrient enrichment effects on periphyton heterogeneity in an environment with uneven light conditions. Natural periphyton was grown at either high or low P availability in circular running water flumes, each of which was kept half in shade and half in light. After three weeks, periphyton biomass was significantly more heterogeneous in the high P than in the low P treatment, as algal production increased in the illuminated half of the flume only in the P-enriched treatment. However, periphyton quality parameters (i.e. C:P ratio and relative PUFA content) were equally homogeneous in both treatments, despite the light differences within the flumes. Although this outcome seems to contrast with the light:nutrient hypothesis, it is likely that high-quantity periphyton patches acquired more P and depleted the available P for the low-quantity patches, leading to an even C:P ratio and PUFA content within each flume. Therefore, in an environment with heterogeneous light availability, nutrient enrichment has the potential to increase the heterogeneity of periphyton quantity without affecting the heterogeneity of periphyton quality, due to periphyton patches not being independent of one another.

Introduction

Light and nutrients are the main abiotic factors that limit the growth of autotrophic periphyton in stream ecosystems (Rosemond 1993; Warren et al. 2017). While periphyton quantity increases with both light and nutrient availability, periphyton nutritional quality for herbivores is usually enhanced by a balance between light and nutrients. Periphyton quality is often quantified as algal content of phosphorus (P) and nitrogen (N) relative to carbon (C), i.e. C:P and C:N stoichiometric ratios (Stelzer & Lamberti 2002; Fink & von Elert 2006). As a general rule, periphyton relative content of nutrients decreases as the ratio of light to nutrient availability increases, consistent with the light:nutrient hypothesis (Sterner et al. 1997; Fanta et al. 2010; Hill et al. 2011). A similar outcome has also been observed with periphyton relative content of polyunsaturated fatty acids (PUFA; Hill et al. 2011), another major determinant of algal food quality for grazers (Guo et al. 2016; Twining et al. 2016). Algal PUFA content increases with P availability, as P is needed for the biosynthesis of PUFA-rich phospholipids and galactolipids, but high light availability dilutes algal PUFA content while C is accumulated (Hill et al. 2011).

As natural streams are highly heterogeneous systems (Palmer & Poff 1997), light and nutrient availability can vary considerably within the same stream (Warren et al. 2017). This variability can lead to a high degree of heterogeneity of both periphyton biomass and nutritional quality. The spatial heterogeneity of periphyton is a critical factor in regulating biotic interactions, nutrient cycling and foodweb processes (Stevenson 1997; Hillebrand 2008; Winemiller et al. 2010). However, while most research has focused on the heterogeneity of periphyton quantity (Winemiller et al. 2010), very few studies so far have investigated how the heterogeneity of periphyton quality is affected by variations in abiotic factors. Yet, the heterogeneity of periphyton nutritional quality may play an important role in shaping community dynamics, as grazers are likely to be attracted to, and therefore aggregate on high-quality food patches in a heterogeneous environment (Moelzner & Fink 2015), and their uneven distribution may further enhance the heterogeneity of the whole system.

In a previous experiment (Iannino et al. 2019), we observed that, after a grazing period, periphyton biomass was more heterogeneously distributed in phosphorus-poor than in phosphorus-rich flumes. We speculated that nutrient enrichment might have homogenised the relative P content of periphyton, leading to a more uniform grazer distribution in the P-enriched flumes. Excessive nutrient inputs into streams are a severe and widespread environmental issue that commonly leads to eutrophication, with harmful consequences for water quality and biodiversity (Dodds et al. 2016; Wurtsbaugh et al. 2019). Although the effects of nutrient enrichment on periphyton quantity and quality have been widely studied (e.g. Fanta et al. 2010; Hill et al. 2011; Cashman et al. 2013; Guo et al. 2016), consequences for the spatial heterogeneity of periphyton are still poorly understood. At low to moderate nutrient concentrations, variations in light availability within a stream are likely to result in a highly heterogeneous periphyton quality; as nutrient concentrations increase, algae might become nutrient-saturated, and thus exhibit a uniformly high nutrient content relative to carbon regardless of light availability (see Hill et al. 2011).

In this study, we investigated how phosphorus enrichment would affect the heterogeneity of both periphyton quantity and quality in a system with heterogeneous light conditions. The following hypotheses were tested: 1) heterogeneous light conditions would lead to a heterogeneous distribution of periphyton biomass, with higher biomass in areas of higher light intensity, at both high and low P availability; but 2) periphyton nutritional quality, both in terms of C:P stoichiometry and PUFA content relative to carbon, would be more heterogeneous in P-poor than P-rich systems under heterogeneous light conditions, due to nutrient saturation at elevated P availability.

Materials and methods

Experimental set-up and procedure

Each experimental unit consisted of a circular flume in a 22 × 27 cm (height × diameter) plastic bucket with a circular water current generated by a pump in the centre. On the bottom surface of each flume were nine 5 × 5 cm slots for tiles. Half of the total surface of each flume (including the top opening) was covered with a shading net sunscreen fabric, which reduced incoming irradiance (measured as photosynthetically active radiation, PAR) by 60%. The average PAR intensity above the flumes, produced by LED lamps, was 38.59 μmol m^{-2} s^{-1} in the non-shaded half and 15.5 μmol m^{-2} s^{-1} in the shaded half of the flumes, measured at the water surface.

The experiment consisted of two treatments: low P availability (15 μg P L^{-1}, P-) and high P availability (100 μg P L^{-1}, P+). Each flume was filled with 6 L modified WC medium (Guillard & Lorenzen 1972) diluted in tap water (1:15) with an adjusted P concentration according to the treatment. The average current velocity in the flumes was 14 cm s^{-1}. Each treatment was replicated five times, resulting in a total of ten half-shaded flumes. The flumes were placed in a row on a shelf in a climate chamber at 18 ± 1°C with a 16:8 hours light:dark cycle. The water temperature in the flumes was approximately 2°C higher than the room temperature, due to the heat generated by the pumps, but did not differ between treatments.

Natural periphyton was collected in the river Agger, near Overath in western Germany (50°56'12.4"N, 7°17'37.1"E) and inoculated in a concrete outdoor pool in the University of Cologne botanical garden in June 2019. The pool measured 235 × 110 × 40 cm and was filled with 200 L diluted (1:15) WC medium (Guillard & Lorenzen 1972) with an adjusted P concentration of 15 μg L^{-1}. The pool was covered with mesh to prevent leaf litter and animals from entering, and the pool's bottom surface was covered with 4.7 × 4.7 cm unglazed ceramic tiles, as substrate for periphyton growth. After three days, a thin periphyton layer covered the tiles. Two periphyton-covered tiles were placed in each experimental flume, i.e. one in the shaded half and one in the non-shaded half. Additionally, three periphyton-covered tiles were randomly selected to analyse initial stoichiometry, fatty acid and taxonomic composition of periphyton. For this, periphyton was scraped off each tile and homogenised with 40 ml tap water. Aliquots from each suspension were filtered onto pre-combusted glass fiber filters (Whatman GF/F, 25 mm Ø) for elemental and fatty acid analyses (see following sections), and 1 ml of each suspension was mixed with 100 μl Lugol's iodine solution for microscopic counts under an inverted microscope.

Periphyton was allowed to grow in the flumes for 21 days. The water in the flumes was replaced every six days with fresh medium of the respective P level. Each flume was shifted by one position to the right every two days, to exclude position effects and ensure equal average lighting in all flumes. The experiment ended on 15 July 2019, three days after the last medium exchange. Periphyton was scraped off each tile and homogenised in 100 ml tap water, of which aliquots were filtered onto pre-combusted GF/F filters for elemental and fatty acid analyses. In addition, 1 ml of each suspension was mixed with 100 μl Lugol's iodine solution for microscopic counts under an inverted microscope, to determine periphyton taxonomic composition.

Nutrient analyses

For periphyton P analysis, filters were placed in a solution of potassium peroxodisulphate and 1.5% sodium hydroxide, and autoclaved for 1 h at 120°C. Soluble reactive P was subsequently analysed with the molybdate-ascorbic acid method (Greenberg et al. 1985) with a DR5000 UV-Vis spectrophotometer (Hach, Düsseldorf, Germany) against a freshly prepared calibration curve of orthophosphate standards. For periphyton C analysis, filters were dried at 60°C for

48 h, packed in tin capsules (HekaTech, Wegberg, Germany) and analysed with a Thermo Flash EA2000 Analyser (Schwerte, Germany).

Fatty acid analysis

Each filter was transferred into 5 ml extraction solvent (CH_2Cl_2/MeOH, 2:1 v/v) and stored at -20°C. Internal standards (10 μg heptadecanoic acid methyl ester, C17:0 ME, and 10 μg tricosanoic acid methyl ester, C23:0 ME) were added to each sample, which were subsequently sonicated for 3 min and centrifuged at 4500 × *g* for 5 min. The supernatants were dried at 40°C under a stream of nitrogen gas. Subsequently, 5 ml 3 N methanolic HCl were added to each sample, which were then incubated at 70°C for 20 min to achieve transesterification. Fatty acid methyl esters were extracted by adding 6 ml isohexane to each sample. The hexane phases were dried at 40°C under a nitrogen stream and redissolved in 100 μl isohexane.

The fatty acid composition of the samples was determined via gas chromatography with a 6890 N GC System (Agilent Technologies, Waldbronn, Germany) equipped with a DB-225 capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) and a flame ionisation detector. Instrument settings were as follows: injector and flame ionisation detector temperatures were 200°C; the initial oven temperature was 60°C for 1 min, followed by a 120°C min^{-1} temperature ramp to 180°C and a 50°C min^{-1} ramp to 200°C; after 10.5 min at 200°C, the temperature was increased to 220°C at 120°C min^{-1} and kept for 10 min. Helium with a flow rate of 1.5 ml min^{-1} was used as carrier gas. Fatty acid methyl esters were quantified by referring to the internal standards and previously established calibration functions for each fatty acid.

Heterogeneity analysis

The heterogeneity of periphyton biomass, C:P stoichiometry and PUFA relative content for each flume was measured with a coefficient of variation (CV), i.e. the ratio of standard deviation to the mean. CV is a basic, yet straightforward method to describe variability within a system, and therefore has been widely used in periphyton studies (e.g. Liess & Hillebrand 2004; Peters et al. 2007; Hillebrand 2008; Iannino et al. 2019). Although CV only gives a simple measure of overall variation and ignores dependence on spatial distance, we believe it was suitable for our small-scale experiment, in which spatially explicit data were not relevant.

Data analysis

All data were checked and approved for normal distribution with a Shapiro-Wilk's test and for homoscedasticity with a Levene's test. Two-sample t-tests were used to compare coefficients of variation (CV) of periphyton biomass, C:P and PUFA relative content as a measure of heterogeneity. In addition, interactive effects of P availability and light on periphyton biomass, C:P ratio, PUFA relative content and diatom:chlorophyte ratio were determined using two-way analyses of variance (ANOVAs) followed by Tukey's HSD post-hoc tests. Statistical analyses were performed in R (R Core Team, version 3.3.1, 2016).

Results

Periphyton initial conditions

At the beginning of the experiment, periphyton was mainly composed of chlorophytes (98 ± 1.1%, mean ± SD), with a small proportion of diatoms (2 ± 1.13%, Table S1 in Supplementary Information). No other algal classes were observed. Periphyton average molar C:N:P ratio was 293:22:1, and the average PUFA relative content was 17.93 ± 1.6 μg mg C^{-1} (Table S1 in Supplementary Information).

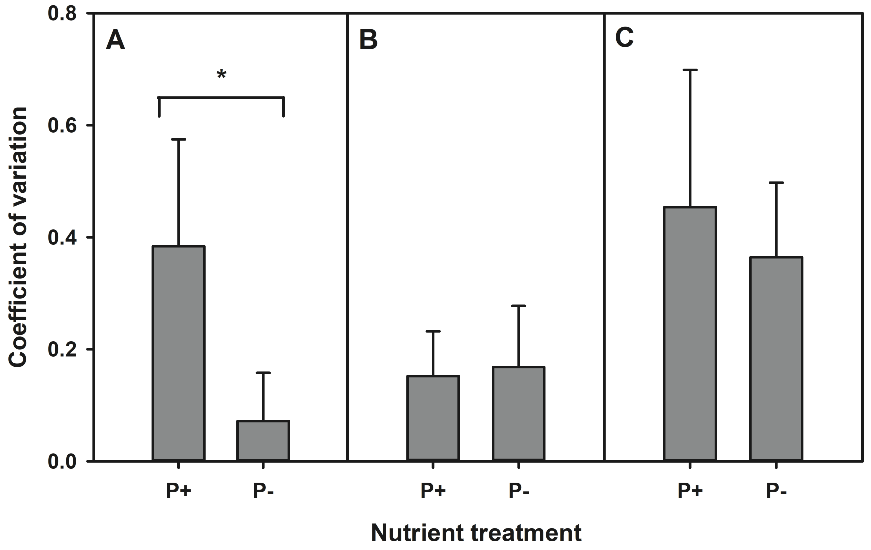

Figure 1. Coefficient of variation of (A) biomass as particulate organic carbon (POC), (B) molar C:P ratio and (C) relative content of polyunsaturated fatty acids (PUFA) of P-enriched (P+) and P-depleted (P-) periphyton. Values are mean ± *SD* of $n = 5$ replicate tiles. The difference between the two treatments was significant for POC ($p < 0.05$), but not for C:P ($p = 0.8$) nor for PUFA ($p = 0.5$).

Periphyton heterogeneity

At the end of the experiment, periphyton biomass was significantly more heterogeneous in the P+ than in the P- treatment (two-sample t-test; $t = 3.28$, df = 8, $p < 0.05$; Fig. 1A), whereas the CV of both periphyton C:P ratio and PUFA relative content did not differ between the two treatments (two-sample t-test; C:P ratio: $t = -0.26$, df = 8, $p = 0.8$, Fig. 1B; PUFA content: $t = 0.72$, df = 8, $p = 0.5$, Fig. 1C).

Light intensity had a significant effect on periphyton biomass and significantly interacted with P level in determining the distribution of periphyton biomass (Table 1), which was significantly higher under high light intensity in the P+ treatment (Fig. 2). P level had a significant effect on periphyton C:P stoichiometry and periphyton PUFA relative content, but it did not significantly interact with light, which had no effects on either parameter (Table 1).

Periphyton C:P ratio was significantly higher in the P- than in the P+ treatment, but it did not differ between high light and low light conditions (Fig. 3). Periphyton PUFA relative content was significantly higher under high light in the P+ treatment than in the P- treatment under any light conditions, while no significant differences in PUFA relative content were observed between high light and low light conditions within any of the P treatments (Fig. 4).

Periphyton was mainly composed of chlorophytes (>95%) in all treatments, with a small percentage of diatoms (<4%; Table S2 in Supplementary Information). P level and light had no significant effects on the taxonomic composition of periphyton, expressed as diatom:chlorophyte ratio (Table 1).

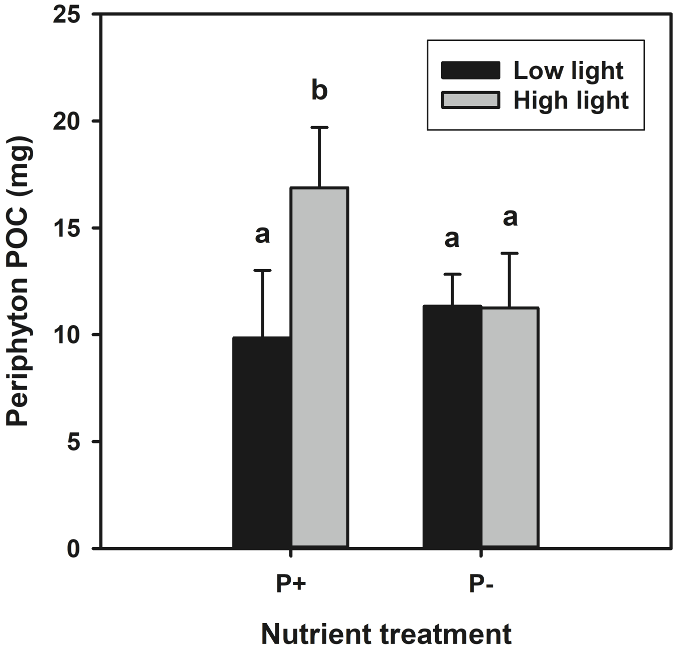

Figure 2. Biomass expressed as particulate organic carbon (POC) of P-enriched (P+) and P-depleted (P-) periphyton, in low light (black) and high light availability (grey). Values are mean ± *SD* of *n* = 5 replicate tiles. Different letters indicate significant differences between treatments, determined via Tukey's post-hoc test.

Table 1. Results of two-way ANOVAs on the effects of phosphorus and light on periphyton biomass as particulate organic carbon (POC), periphyton C:P ratio, periphyton relative content of polyunsaturated fatty acids (PUFA), and periphyton diatom:chlorophyte ratio. Significant effects ($p < 0.05$) are highlighted in bold.

	Phosphorus		**Light**		**Phosphorus × Light**	
	$F_{1,16}$	p	$F_{1,16}$	p	$F_{1,16}$	p
POC	3.18	0.09	8.97	**0.008**	9.39	**0.007**
C:P	50.3	**<0.001**	0.03	0.87	0.04	0.84
PUFA	9.23	**0.007**	2.93	0.11	1.93	0.18
Diatom:chlorophyte	0.56	0.46	3.64	0.07	0.56	0.46

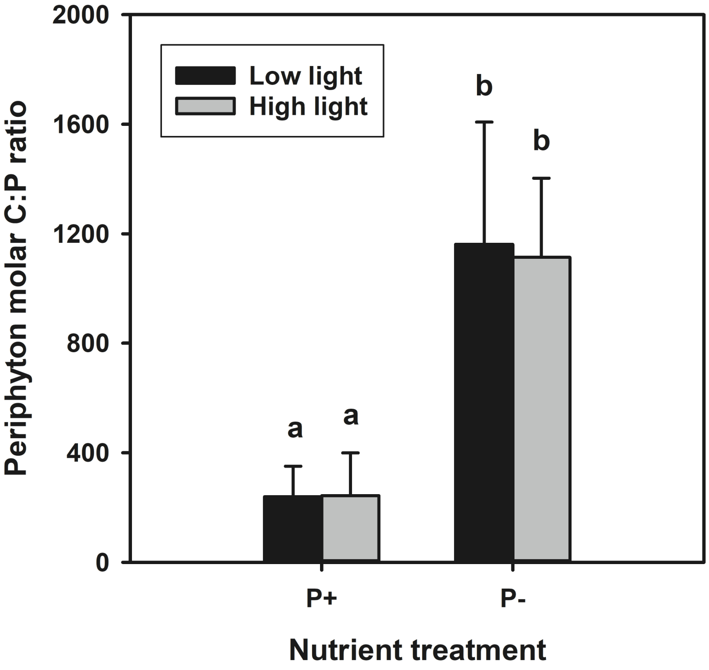

Figure 3. Molar C:P ratio of P-enriched (P+) and P-depleted (P-) periphyton, in low light (black) and high light availability (grey). Values are mean ± *SD* of $n = 5$ replicate tiles. Different letters indicate significant differences between treatments, determined via Tukey's post-hoc test.

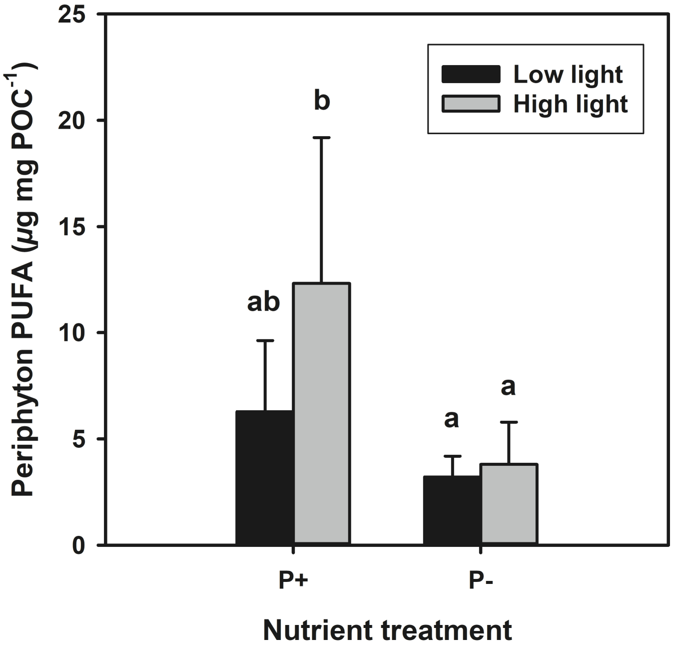

Figure 4. Relative content of polyunsaturated fatty acids (PUFA) of P-enriched (P+) and P-depleted (P-) periphyton, in low light (black) and high light availability (grey). Values are mean ± *SD* of *n* = 5 replicate tiles. Different letters indicate significant differences between treatments, determined via Tukey’s post-hoc test.

Discussion

Contrary to our expectations, in a closed system with heterogeneous light conditions, periphyton biomass was more heterogeneously distributed under P enrichment than P deficiency. In the P-enriched flumes, the heterogeneous light availability interacted with P addition and resulted in a significantly higher periphyton biomass in the non-shaded half of the flume compared to the shaded half, in which periphyton growth was light-limited. In the P-depleted flumes, however, periphyton biomass was homogeneously distributed, i.e. equally low between the differently illuminated areas, which suggests a primary P-limitation of periphyton growth. However, even though high light availability increased biomass production in the P-enriched treatment, this increase did not translate into a higher C:P ratio or lower PUFA content. Unexpectedly, algal C:P ratio and PUFA relative content were homogeneous between periphyton patches under different light conditions, at both high and low P availability. While P enrichment significantly improved periphyton quality parameters, light had no effects on periphyton C:P ratio or PUFA relative content, which only differed between the two nutrient treatments and not between the differentially illuminated areas. These results may seem to contrast with the light:nutrient hypothesis, which states that periphyton C:P ratio should increase together with the ratio of light to nutrient availability (Sterner et al. 1997). However, most studies that found patterns consistent with the light:nutrient hypothesis were full-factorial experiments, in which every experimental unit was independent and assigned to a given set of homogeneous conditions, i.e. either completely shaded or completely in the light (Elser et al. 2003; Dickman et al. 2006; Hill et al. 2011). In our experimental design, the illuminated and shaded areas were not independent of one another, but were part of the same heterogeneous environment. Therefore, in the P-enriched flumes, in which periphyton biomass was more abundant under high light than under low light, the available dissolved P was absorbed by the periphyton patches in proportion to their biomass, resulting in a homogeneous C:P ratio even if periphyton biomass was heterogeneously distributed. This explanation may also be valid for the lack of light effects on periphyton C:P stoichiometry in previous tests of the light:nutrient hypothesis, in which different light treatments were close to one another within the same flumes (Hill and Fanta 2008; Hill et al. 2009).

As PUFA production is closely linked to P availability (Hill et al. 2011), periphyton PUFA relative content followed a similar pattern as periphyton C:P ratio. P availability was the only factor that had a significant effect on periphyton PUFA content, which was homogeneous among differentially illuminated areas in the flumes. Yet, only the PUFA content of periphyton in the non-shaded half of P-enriched flumes was significantly higher than the periphyton

PUFA content in P-poor flumes, suggesting a weak interaction between P availability and light. Hence PUFA production was P-limited in the P-poor flumes, whereas it approached light limitation in the shaded half of the P-enriched flumes.

Our results shed more light on the effects of nutrient enrichment and light on periphyton heterogeneity in streams. As streams are highly heterogeneous environments (Palmer & Poff 1997), periphyton patches under different light conditions may occur in close proximity within the same stream, without being independent of one another like in a controlled experiment. Therefore, variations in light and nutrient availability will influence the spatial distribution of stream periphyton biomass and nutrient content in a more complex way than what is predicted by the light:nutrient hypothesis. This study shows for the first time that, in an environment with heterogeneous light conditions, periphyton biomass may be more heterogeneously distributed at high P than at low P availability, but despite this, periphyton nutritional quality will be homogeneous, because periphyton patches with a higher biomass will acquire more nutrients than periphyton with low biomass. In such a scenario, periphyton quantity more than quality has the potential to lead to an uneven distribution of grazers, which may, nevertheless, increase the heterogeneity of periphyton quality through an uneven nutrient regeneration mediated by excretion (Liess & Hillebrand 2004).

Overall, in an ecosystem with a homogeneously low background nutrient availability, nutrient enrichment will not reduce the spatial heterogeneity of periphyton. However, further studies should investigate how eutrophication may affect periphyton heterogeneity in streams with a heterogeneous nutrient availability, owing to e.g. biogeochemical hotspots (McClain et al. 2003) and/or local hydrogeological variations (Valett et al. 1997). In such cases, periphyton quality will be heterogeneous under normal conditions, and external nutrient enrichment is more likely to homogenise the nutrient content of periphyton. Furthermore, variations in light and nutrient availability in streams are likely to interact with variations in water current velocity, which will add a further level of complexity to the heterogeneity of periphyton quantity and quality. High water current alters periphyton biomass and composition, and increases periphyton nutrient uptake (Hiatt et al. 2019; Risse-Buhl et al. 2020). Nevertheless, it is important to remember that different environments in nature are often not independent of one another, but may instead interact with one another in unexpected ways. In heterogeneous ecosystems, interacting factors may have complex, counter-intuitive outcomes, not always in accordance with theoretical models such as the light:nutrient hypothesis.

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Supplementary information

Table S1. Molar C:P, C:N and N:P ratios, polyunsaturated fatty acid (PUFA) content, and percentage of diatoms and chlorophytes of periphyton placed in the flumes at the beginning of the experiment. Values are mean ± SD of *n* = 3 replicates.

C:P	293.38 ± 130.57
C:N	13.24 ± 0.32
N:P	22 ± 9.46
PUFA (μg/mg C)	17.93 ± 1.6
Chlorophyte %	97.96 ± 1.13
Diatom %	2.04 ± 1.13

Table S2. Percentage of diatoms and chlorophytes in P-enriched (P+) and P-depleted (P-) periphyton at the end of the experiment. Values are mean ± SD of *n* = 5 replicate tiles.

	P+		**P-**	
	High light	**Low light**	**High light**	**Low light**
Diatom %	3.3 ± 1.6	1.5 ± 0.9	2.3 ± 2.2	3.6 ± 4.9
Chlorophyte %	96.7 ± 1.6	98.5 ± 0.9	97.7 ± 2.2	96.4 ± 4.9

GENERAL DISCUSSION

Nutrient enrichment of stream ecosystems is a widespread ecological issue that can dramatically alter the interaction of bottom-up and top-down control of periphyton. Increasing nutrient inputs in streams are likely to influence the nutritional quality and spatial variability of periphyton, which can in turn significantly affect the growth and behaviour of grazers. With the present dissertation, I demonstrate that nutrient enrichment can lead to a weaker top-down control of periphyton (**chapter I**), lower periphyton nutritional quality for herbivores (**chapter II**), and increase or decrease the heterogeneity of periphyton biomass, depending on other factors (**chapter I**, **III**). An overview of the results from each chapter is summarised in Box 2.

Box 2. Overview of main results

Chapter I: Phosphorus enrichment resulted in a weaker top-down control of stream periphyton, as grazer consumption rates were lower with phosphorus-rich than phosphorus-poor periphyton. In addition, phosphorus enrichment was associated with a lower heterogeneity of periphyton biomass in the presence of grazers.

Chapter II: Phosphorus enrichment led to higher periphyton C:P ratios and lower relative PUFA content, by favouring the growth of cyanobacteria and chlorophytes over diatoms.

Chapter III: In a system with heterogeneous light availability, phosphorus enrichment increased periphyton biomass heterogeneity without affecting the heterogeneity of periphyton C:P ratio and relative PUFA content.

Nutrient enrichment in streams is known to increase periphyton biomass, an effect that is often counteracted by the grazing activity of herbivores (Hillebrand 2002). However, grazer growth and behaviour may be strongly influenced by the relative nutrient content of periphyton. When periphyton nutrient content is too low to meet grazer demands, grazers may increase their food consumption rates in order to maximise nutrient intake. Such a 'compensatory feeding' response has been observed in laboratory experiments with marine amphipods (Cruz-Rivera & Hay 2000) and freshwater gastropods (Fink & von Elert 2006; Hansen et al. 2016). In **chapter I**, compensatory feeding is observed for the first time in a running-water microcosm experiment with natural periphyton. In this study, compensatory feeding allowed the gastropod *Ancylus fluviatilis* to maintain an optimal soft body stoichiometry, which did not differ between the phosphorus-enriched and phosphorus-depleted treatment. Furthermore, as I tested the effects of nutrient enrichment on grazer consumption rates, I provide the first study in which the implications of compensatory feeding are elucidated as a link between bottom-up and top-down control of stream periphyton. **Chapter I** shows that nutrient enrichment may result in a higher periphyton biomass not only directly, but also indirectly, by reducing grazer consumption rates and hence the strength of top-down control.

However, although compensatory feeding may help grazers maintain a constant soft body stoichiometry, previous studies have shown that such a behavioural response may come at an energetic cost, and therefore it may still lead to reduced growth rates and survivorship for some grazers (Cruz-Rivera & Hay 2000; Fink & von Elert 2006). The ingestion of excessive amounts of carbon requires costly mechanisms for its disposal, such as increased carbon release through excretion or respiration (Hessen & Anderson 2008). As a consequence, while compensatory feeding may result in a strong top-down pressure on periphyton in the short term, nutrient limitation may nevertheless decrease the growth and survival of herbivores, eventually leading to a weaker top-down control in the long term. On the other hand, nutrient enrichment may itself have long-term effects on periphyton community structure and nutritional quality, which will further affect grazer growth and behaviour and hence the strength of top-down control. Such long-term bottom-up effects on stream periphyton were studied in **chapter II**.

Long-term nutrient enrichment of periphytic communities often leads to an increase in the proportion of cyanobacteria (Dodds et al. 2016; Wurtsbaugh et al. 2019), which are considered a low-quality food source for herbivores because they lack essential polyunsaturated fatty acids (PUFAs) and sterols (von Elert et al. 2003; Brett et al. 2006). In **chapter II**, I grew natural stream periphyton for six weeks under a gradient of dissolved phosphorus, and observed that phosphorus availability significantly affected the taxonomic composition of periphyton. Diatoms dominated the algal community at low phosphorus levels, and were gradually replaced along the phosphorus gradient first by chlorophytes, whose abundance peaked at intermediate phosphorus availability, and further by cyanobacteria under eutrophic conditions. Such a shift in taxonomic composition resulted in strong and sometimes surprising changes in nutritional quality parameters. The relative content of PUFAs in algae, a major determinant of food quality for many herbivores (Twining et al. 2016), is known to increase with phosphorus enrichment (Reitan et al 1994; Hill et al 2011). Therefore, periphyton relative PUFA content initially increased with phosphorus availability, peaked at intermediate phosphorus levels, and decreased again as the proportion of cyanobacteria increased. On the other hand, the relative content of EPA, one of the most important PUFAs for herbivore nutrition (Twining et al. 2016), peaked at relatively low phosphorus availability, coinciding with a high diatom proportion and supporting the evidence that EPA is mainly found in diatoms (Lang et al. 2011). These results suggest that the best periphyton nutritional quality for herbivores, in term of fatty acid composition, may be found at low to intermediate phosphorus availability, due to an interaction between taxonomic and nutrient effects. Similarly, a phytoplankton study by Müller-Navarra et al.

(2004) has observed that algal relative content of long-chained PUFAs is negatively correlated with lake trophic status, and other lake studies have found that, likely due to phytoplankton taxonomic differences, PUFA relative content in fish is significantly higher in oligo-mesotrophic than eutrophic lakes (Ahlgren et al. 1996; Taipale et al 2016). With **chapter II**, I provide complementary evidence that such a negative relationship between algal PUFA content and trophic status may also be observed in stream periphytic communities, though algal PUFA production might still be phosphorus-limited in highly oligotrophic streams.

The most unexpected result from **chapter II**, however, was the observed relationship between dissolved phosphorus and periphyton C:P ratio. Periphyton stoichiometry is an important indicator of food quality for herbivores (Sterner & Hessen 1994; Elser et al. 2000) and algal C:P ratios usually decrease as phosphorus availability increases (Cross et al. 2005; Hill et al. 2011). In **chapter II**, the opposite trend was observed, as periphyton C:P ratio was the lowest in the highly oligotrophic treatment and increased with phosphorus availability in a logarithmic fashion. The most likely explanation for this outcome is that diatoms may build less biomass per unit of assimilated phosphorus than chlorophytes and cyanobacteria, resulting in moderately low C:P ratios even in an oligotrophic environment. Laboratory studies have indeed shown that, when grown under the same conditions, diatoms exhibit lower C:P ratios than chlorophytes (Ho et al. 2003; Quigg et al. 2003). Moreover, even though a negative relationship between phosphorus availability and periphyton phosphorus content has never been observed before, several field studies in streams have failed to find a strong positive relationship between the two variables (Fanta et al. 2010; O'Brien & Wehr 2010; Liess et al. 2012; Hiatt et al. 2019). Therefore, the results from **chapter II** suggest that, if a taxonomic shift from diatoms to chlorophytes and/or cyanobacteria occurs, increasing phosphorus inputs may decrease the relative phosphorus content of an algal community instead of increasing it. As low algal nutrient content significantly constrains the fitness of herbivores (Sterner & Hessen 1994; Elser et al. 2000), stream eutrophication may lower periphyton food quality not only in terms of fatty acid composition, but also of nutrient stoichiometry.

If eutrophication is associated with a lower relative content of nutrients in the periphytic community, in theory this effect might lead to a stronger top-down control of periphyton biomass due to compensatory feeding. However, a strong top-down pressure in a eutrophic environment may still not be enough to counteract the excessive growth of algal biomass caused by high nutrient availability. Furthermore, given the energetic costs of compensatory feeding and the low nutritional quality of cyanobacteria, top-down control in a eutrophied environment may be eventually weakened due to reduced grazer growth and survival rates in the long term. As a result, stream eutrophication may be deleterious for the food web structure to the same extent as nutrient deficiency.

In addition to periphyton nutritional quality for herbivores, nutrient enrichment may also affect periphyton spatial heterogeneity, another key driver of trophic interactions and community dynamics in streams (Stevenson 1997; Winemiller et al. 2010). When faced with a spatially heterogeneous food source, grazers may in turn distribute unevenly among food patches and further contribute to the overall heterogeneity of the system. In **chapter I**, at the end of the experiment with *A. fluviatilis*, periphyton biomass was significantly more heterogeneous in the grazed phosphorus-depleted flumes than in the phosphorus-enriched flumes, suggesting that grazers were more heterogeneously distributed in the phosphorus-poor than in the phosphorus-rich treatment. Under nutrient-poor conditions, grazers may have aggregated on a periphyton patch with a slightly higher nutrient content, whereas nutrient enrichment may have homogenised periphyton nutrient content by leading to algal nutrient saturation, resulting in a more homogeneous grazer distribution among food patches. In other words, nutrient enrichment may have lowered the heterogeneity of periphyton quality, and thus indirectly lowered the heterogeneity of periphyton quantity by affecting the distribution of grazing pressure. However, this is only an assumption that requires further testing.

In **chapter III**, on the other hand, nutrient enrichment did not decrease the heterogeneity of either periphyton quantity or quality in an environment with uneven light conditions. Light intensity is a major limiting factor for periphyton growth, and is highly variable in streams (Warren et al. 2017). High irradiance is known to increase algal biomass (Hill et al. 2011) and to generally decrease algal nutritional quality for herbivores, according to the light:nutrient hypothesis (Sterner et al. 1997; Fanta et al. 2010). In **chapter III**, I grew periphyton biomass in half-shaded flumes at different phosphorus concentrations, to investigate how phosphorus enrichment would interact with heterogeneous light conditions to determine periphyton heterogeneity. After three weeks, the different light conditions within the same flume resulted in a heterogeneous distribution of algal biomass only at high phosphorus availability, suggesting that algal growth was strongly phosphorus-limited in the low phosphorus treatment and thus unaffected by light. Nutrient enrichment therefore increased the heterogeneity of periphyton biomass under uneven light conditions. However, periphyton C:P ratio and PUFA relative content did not differ between the differentially illuminated areas of the flumes, at either low or high phosphorus availability. On the contrary, both C:P ratio and PUFA content were homogeneous within every flume, even when different light conditions caused a heterogeneous distribution of algal biomass. At first sight, this outcome might be met with scepticism, as numerous tests of the light:nutrient hypothesis have previously demonstrated that algal C:P ratios increase as the ratio of light to nutrient availability increases (Elser et al. 2003; Dickman et al. 2006; Fanta et al. 2010; Hill et al. 2011); nevertheless, no study so far has purposely investigated how light and nutrients interact in a heterogeneous light environment. In my experimental design, different light patches were not independent of each other as in previous tests of the light:nutrient hypothesis, but were part of the same heterogeneous system and therefore influenced each other, which is common in nature (Winemiller et al. 2010; Warren et al. 2017). It is likely that different periphyton patches within a flume absorbed phosphorus from the water in proportion to their biomass, resulting in a homogeneous C:P ratio even when algal biomass was not homogeneously distributed. The same conclusion can be drawn with periphyton PUFA content, which increases with phosphorus availability (Reitan et al. 1994; Hill et al. 2011). As a consequence, **chapter III** shows for the first time that, while nutrient enrichment can increase the heterogeneity of periphyton biomass in a stream with uneven light conditions, the relative nutrient content of periphyton may remain homogeneous, because periphyton patches with a high biomass will deplete the available nutrients for periphyton patches with a low biomass. This conclusion is at least valid for environments with heterogeneous light and homogeneous nutrient availability; further tests should investigate how nutrient enrichment would affect periphyton heterogeneity in a stream with heterogeneous nutrient availability, which can occur due to e.g. biogeochemical hotspots (McClain et al. 2003).

Further research should also test how the bottom-up effects on periphyton heterogeneity elucidated in **chapter III** would influence grazer behaviour and distribution, and thus interact with top-down effects. According to the ideal free distribution model (Fretwell & Lucas 1970; Haugen et al. 2006), grazers may tend to aggregate on food patches in proportion to the amount of resources available in each patch. However, the assumptions of the ideal free distribution model are often violated in nature (Matsumura et al. 2010), so it is unlikely that the distribution of grazers will exactly match the distribution of resources. Still, in an environment with a heterogeneous periphyton quantity and a homogeneous nutritional quality, as caused by nutrient enrichment and uneven light in **chapter III**, differences in periphyton quantity rather than quality might lead to a heterogeneous distribution of grazers, which will be more numerous on food patches with a higher biomass. In turn, the uneven grazing pressure may enhance the heterogeneity of both periphyton quantity and quality, depending on rates of grazer consumption, nutrient recycling and algal growth.

In both **chapter II** and **chapter III**, periphyton was grown at increasing phosphorus concentrations for a relatively long time (four weeks in **chapter II**, three weeks in **chapter III**). However, phosphorus enrichment led to very different results in periphyton community structure and nutritional quality between the two experiments. In **chapter II**, phosphorus enrichment caused a taxonomic shift from diatoms to chlorophytes and cyanobacteria, resulting in higher periphyton C:P ratios and lower PUFA relative content; in **chapter III**, instead, periphyton taxonomic composition did not change with increasing phosphorus availability, therefore phosphorus enrichment led to lower C:P ratios and higher PUFA content in periphyton. This discrepancy is likely due to differences in periphyton collection and cultivation methods before the start of the two experiments, and is a reminder of the several factors at play in an ecosystem that inevitably interact with each other. In **chapter II**, natural periphyton was collected in the Rhine, a large river where diatoms are the predominant algal group (Ackermann et al. 2011), and inoculated into the experimental flumes on the same day of collection. In **chapter III**, instead, periphyton was collected in the much smaller river Agger, and pre-grown for three days on ceramic tiles in an outdoor pool during summer; although nutrient concentrations in the pool were moderate, the warm weather favoured the growth of chlorophytes, which constituted almost 100% of the periphytic community on the tiles when these were placed in the experimental flumes at the beginning of the experiment. This cultivation method was designed to establish a homogeneous algal community that would not change throughout the experiment, to avoid confounding taxonomic effects; nevertheless, the lack of a taxonomic shift in **chapter III**, as opposed to **chapter II**, is a reminder that nutrient availability is not the only factor that determines the taxonomic composition of algal communities. In **chapter II**, where the initial algal community in the flumes was strongly dominated by diatoms, the relatively high water temperature likely interacted with phosphorus enrichment to favour the growth of chlorophytes and cyanobacteria, consistent with results by Tilman et al. (1986) and Marcarelli & Wurtsbaugh (2006); if the water temperature in the flumes had been lower (e.g. below 10°C), chlorophytes and cyanobacteria may not have been able to outcompete diatoms, even at high phosphorus concentrations. In fact, nutrient enrichment of aquatic ecosystems does not always result in a taxonomic shift from diatoms to chlorophytes and/or cyanobacteria. On the contrary, a change in diatom species composition is often observed in eutrophied lakes and streams, especially in cold environments (Winter & Duthie 2000; Soininen 2002; Korhonen et al. 2013). Current velocity, turbidity, pH and substrate type are further factors that may affect the taxonomic composition of periphyton in streams (Leland & Porter 2000).

Overall, with the present thesis I demonstrate that phosphorus enrichment can have profound effects on the control of stream periphyton, beyond simply increasing algal biomass. Eutrophication of aquatic ecosystems is a widespread ecological issue that has been linked to water quality degradation and biodiversity loss across all trophic levels (Evans-White et al. 2009; Wurtsbaugh et al. 2019); my results further show that nutrient enrichment can disrupt the balance between bottom-up and top-down control, by altering periphyton nutritional quality for grazers and thus reducing the strength of top-down pressure in the short term (**chapter I**). In the long term, instead, nutrient enrichment can favour the growth of algal groups with low essential fatty acid and nutrient content relative to carbon (**chapter II**), which can eventually reduce grazer growth and survival rates. Moreover, nutrient enrichment may not only affect periphyton quantity and quality, but also their spatial variability, through both direct and indirect effects. In **chapter I**, phosphorus enrichment was associated to a more homogeneous periphyton biomass in the presence of grazers, probably caused by a more uniform grazer distribution; however, when interacting with uneven light conditions, nutrient enrichment increased the heterogeneity of periphyton biomass (**chapter III**), which can have positive consequences for ecosystem dynamics. All in all, the effects of nutrient enrichment on aquatic ecosystems will be complex and will interact with several other factors; nevertheless, the study of periphyton nutritional quality for grazers, and its variability in space and time, can provide useful insights on the link between nutrient enrichment and food web structure, and thus the interaction of bottom-up and top-down control of algal biomass.

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ABSTRACT

The release of nutrients into aquatic ecosystems has been dramatically increasing over the past decades as a consequence of human activities, often leading to an excessive growth of primary producers. While the effects of harmful algal blooms in lakes have been widely studied, less is known about the consequences of nutrient enrichment for stream food webs, which are strongly dominated by benthic habitats. The growth of benthic algal communities (periphyton) in streams is controlled by nutrient availability (bottom-up control) and by the grazing activity of herbivores (top-down control), which is known to counteract the effects of nutrient enrichment on algal biomass. However, nutrient availability may also affect the nutritional quality of periphyton, which can in turn influence the growth and behaviour of grazers and hence the strength of top-down control. Furthermore, nutrient enrichment may alter periphyton spatial heterogeneity, an important ecosystem aspect that mediates trophic interactions and favours biodiversity.

In the present thesis I investigate the effects of phosphorus enrichment on the interaction between bottom-up and top-down control of stream periphyton, with a focus on periphyton nutritional quality for herbivores and periphyton spatial heterogeneity. In a microcosm experiment with the rheophilic gastropod *Ancylus fluviatilis*, I demonstrate that phosphorus enrichment may lead to a weaker top-down control of stream periphyton, because grazers need lower amounts of nutrient-rich food to maintain an optimal soft body stoichiometry, compared to nutrient-poor food. In addition, I demonstrate that, in the long term, phosphorus enrichment may lead to an overall lower periphyton nutritional quality for grazers by favouring the growth of cyanobacteria and chlorophytes over diatoms. I demonstrate for the first time that, when such a taxonomic shift occurs, phosphorus enrichment decreases the relative phosphorus content of periphyton, because cyanobacteria and chlorophytes generally have higher C:P ratios than diatoms.

I observed both positive and negative effects of phosphorus enrichment on periphyton heterogeneity, depending on other factors. In the presence of grazers, phosphorus enrichment resulted in a more homogeneous periphyton biomass, possibly because it led to a homogeneous nutrient content of periphyton and therefore a more even grazer distribution. However, in an environment with heterogeneous light conditions, phosphorus enrichment resulted in a more heterogeneous periphyton biomass, while the heterogeneity of periphyton relative phosphorus content was not affected by phosphorus availability, because periphyton patches of different sizes acquired nutrients in proportion to their biomass.

The present thesis shows that nutrient enrichment can have a significant impact on the control of stream periphyton. In addition to directly increasing algal production, it can alter periphyton nutritional quality and spatial heterogeneity, and therefore affect the growth and behaviour of grazers. Nutrient enrichment can thus interfere with the balance between bottom-up and top-down control of stream periphyton, with possibly harmful consequences for stream food webs.

ZUSAMMENFASSUNG

Die Freisetzung von Nährstoffen in aquatische Ökosysteme als Konsequenz menschlicher Aktivitäten hat in den letzten Jahrzehnten dramatisch zugenommen und häufig zu einem übermäßigen Wachstum der Primärproduzenten geführt. Während die Folgen schädlicher Algenblüten in Seen weitgehend untersucht sind, ist über die Auswirkungen von Nährstoffanreicherungen auf Nahrungsnetze in Fließgewässern, die stark von benthischen Lebensräumen dominiert werden, weniger bekannt. Das Wachstum benthischer Algengemeinschaften (Periphyton) in Fließgewässern wird nicht nur durch die Nährstoffverfügbarkeit (Bottom-up-Kontrolle), sondern auch durch die Weideaktivität von Pflanzenfressern (Top-down-Kontrolle) gesteuert, was den Auswirkungen der Nährstoffanreicherung auf die Algenbiomasse entgegenwirkt. Die Nährstoffverfügbarkeit kann jedoch auch die Nahrungsqualität des Periphytons beeinflussen, was wiederum das Wachstum und Verhalten von Weidegängern und damit die Stärke der Top-Down-Kontrolle beeinflussen kann. Darüber hinaus kann eine Nährstoffanreicherung die räumliche Heterogenität des Periphytons verändern. Dies ist ein wichtiger Aspekt des Ökosystems, der trophische Interaktionen vermittelt und die Biodiversität begünstigt.

In der vorliegenden Arbeit untersuche ich die Auswirkungen von Phosphoranreicherungen auf die Interaktion zwischen Bottom-Up-Kontrolle und Top-Down-Kontrolle des Fließgewässerperiphytons, mit einem Schwerpunkt auf der Ernährungsqualität für Pflanzenfresser und der räumlichen Heterogenität des Periphytons. In einem Mikrokosmos-Experiment mit dem rheophilen Gastropoden *Ancylus fluviatilis,* zeige ich, dass eine Phosphoranreicherung zu einer schwächeren Top-Down-Kontrolle des Fließgewässerperiphytons führen kann, weil Weidetiere im Vergleich zu nährstoffarmer Nahrung geringere Mengen an nährstoffreicher Nahrung benötigen, um eine optimale

Weichkörperstöchiometrie aufrechtzuerhalten. Darüber hinaus zeige ich auf, dass eine Phosphoranreicherung langfristig zu einer insgesamt niedrigeren Nahrungsqualität des Periphytons für Weidegänger führen kann, indem sie das Wachstum von Cyanobakterien und Chlorophyten gegenüber Kieselalgen begünstigt. Ich zeige zum ersten Mal, dass bei einer solchen taxonomischen Verschiebung eine Phosphoranreicherung den relativen Phosphorgehalt des Periphytons verringert, da Cyanobakterien und Chlorophyten generell höhere C:P-Verhältnisse aufweisen als Kieselalgen.

Ich konnte sowohl positive als auch negative Auswirkungen von Phosphoranreicherungen auf die Periphyton-Heterogenität beobachten, abhängig von anderen Faktoren. In Gegenwart von Weidegängern führte eine Phosphoranreicherung zu einer homogeneren Periphyton-Biomasse, möglicherweise weil sie zu einem homogenen Nährstoffgehalt des Periphytons und damit zu einer gleichmäßigeren Weidegängerverteilung führte. In einer Umgebung mit heterogenen Lichtverhältnissen führte eine Phosphoranreicherung jedoch zu einer heterogeneren Periphyton-Biomasse, während die Heterogenität des relativen Phosphorgehalts des Periphytons nicht durch die Phosphorverfügbarkeit beeinflusst wurde, da Periphyton-Felder unterschiedlicher Größe Nährstoffe im Verhältnis zu ihrer Biomasse erwarben.

Die vorliegende Arbeit zeigt, dass eine Nährstoffanreicherung einen signifikanten Einfluss auf die Kontrolle des Fließgewässerperiphytons haben kann. Zusätzlich zur direkten Steigerung der Algenproduktion kann sie die Nahrungsqualität und die räumliche Heterogenität des Periphytons verändern und somit das Wachstum und Verhalten von Weidegängern beeinflussen. Eine Nährstoffanreicherung kann daher das Gleichgewicht zwischen der Bottom-up- und Top-down-Kontrolle des Fließgewässerperiphytons stören, mit möglicherweise schädlichen Folgen für die Nahrungsnetze der Fließgewässer.

RECORD OF ACHIEVEMENT

CHAPTER I: High nutrient availability leads to weaker top-down control of stream periphyton: Compensatory feeding in *Ancylus fluviatilis*

The experiment described in this chapter was exclusively performed by me or under my direct supervision. Patrick Fink and Markus Weitere were involved in the design of the experiment. Patrick Fink, Markus Weitere and Alexander T.L. Vosshage have critically read and commented on the manuscript.

CHAPTER II: Taxonomic shift over a phosphorus gradient affects the stoichiometry and fatty acid composition of stream periphyton

The experiment described in this chapter was exclusively performed by me or under my direct supervision. Patrick Fink was involved in the design of the experiment. Patrick Fink, Markus Weitere and Alexander T.L. Vosshage have critically read and commented on the manuscript.

CHAPTER III: The light:nutrient hypothesis in a heterogeneous environment: effects of nutrient enrichment on the spatial variability of stream periphyton

The experiment described in this chapter was exclusively performed by me or under my direct supervision. Patrick Fink was involved in the design of the experiment. Patrick Fink, Markus Weitere and Alexander T.L. Vosshage have critically read and commented on the manuscript.

LIST OF PUBLICATIONS

[1]Iannino, A., Vosshage, A.T.L., Weitere, M. & Fink, P. (2019) High nutrient availability leads to weaker top-down control of stream periphyton: Compensatory feeding in *Ancylus fluviatilis*. *Freshwater Biology,* 64: 37-45.

[2]Iannino, A., Vosshage, A.T.L., Weitere, M. & Fink, P. Taxonomic shift over a phosphorus gradient affects the stoichiometry and fatty acid composition of stream periphyton. *Journal of Phycology*, under review.

[3]Iannino, A., Vosshage, A.T.L., Weitere, M. & Fink, P. The light:nutrient hypothesis in a heterogeneous environment: Effects of nutrient enrichment on the spatial variability of stream periphyton. *Limnology & Oceanography Letters*, under review.

[1]Chapter I

[2]Chapter II

[3]Chapter III

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ERKLÄRUNG

Bonn, 8.06.2020

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

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CURRICULUM VITAE

PERSÖNLICHES

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STUDIUM

2016- **PhD Freshwater Ecology**, Universität zu Köln

Titel der Dissertation: *Effects of phosphorus enrichment on the control of stream periphyton*

2013-2015 **M.Sc. Biological Sciences**, Universität zu Köln

Titel der Dissertation: *Interactive effects of phosphorus availability and grazer species richness on the biodiversity of benthic freshwater algae*

2009-2013 **B.Sc. Natural Sciences**, University of East Anglia (UK)

Titel der Dissertation: *The effect of inbreeding on dispersal behaviour in the red flour beetle* Tribolium castaneum

PRÄSENTATIONEN

Juli 2019 *Taxonomic shift over a phosphorus gradient affects the stoichiometry and fatty acid composition of stream periphyton*

11th Symposium for European Freshwater Sciences

Zagreb, Kroatien

Februar 2019	*High nutrient availability leads to weaker top-down control of stream periphyton: Compensatory Feeding in* Ancylus fluviatilis **Aquatic Sciences Meeting of the Association for the Sciences of Limnology and Oceanography (ASLO)** San Juan, Puerto Rico
September 2018	*High nutrient availability leads to weaker top-down control of stream periphyton: Compensatory Feeding in* Ancylus fluviatilis **111. Jahrestagung der Deutsche Zoologische Gesellschaft (DZG)** Greifswald, Deutschland

BERUFLICHE ERFAHRUNG

2013-2014	**Hilfswissenschaftlerin** AG von Elert (Aquatic Chemical Ecology), Universität zu Köln