

Periphyton-grazer interactions in headwater streams

by

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Preliminary note

Although in large parts of this thesis the first person singular is used, it should be noted that each study contributing to this thesis is the work of more than one person. The co-authors of the respective publication or manuscript are given at the beginning of the specific chapters. In accordance with the applicable doctoral regulations, the author contributions are given in a separate document.

Figures and tables are numbered continuously throughout the thesis. Therefore, the displayed figure and table numbers may deviate from the published or submitted version of the respective manuscript.

Summary

Small headwater streams comprise most of the total channel length and catchment area in fluvial networks. They are tightly connected to their catchments and, thus, are highly vulnerable to changes in catchment hydrologic budgets and land use. Although these small, often fishless streams are of little economic interest, they are vital for the ecological and chemical state of larger water bodies. Although numerous studies investigate the impact of various anthropogenic stressors or altered catchment conditions, we lack an in-depth understanding of the natural conditions and processes in headwater streams. This natural state, however, largely affects how a headwater stream responds to anthropogenic or climatic changes. One of the major threats to aquatic ecosystems is the excessive anthropogenic input of nutrients leading to eutrophication. Nutrients exert a bottom-up effect in the food web, foremost affecting primary producers and their consumers, i.e. periphyton and benthic grazers in headwater streams. The periphyton-grazer link is the main path of autochthonous (in-stream) production into the stream food web and the strength of this link largely determines the effectiveness of this pathway. Therefore, this thesis aims at elucidating important biological processes with the explicit focus on periphyton-grazer interactions. I assessed different aspects of periphyton-grazer interactions using laboratory experiments to solve methodological problems, and using a field study to compare the benthic communities of three morphologically similar, phosphorus-limited, near-natural headwater streams. With the results of the laboratory experiments, I was able to show that periphyton RNA/DNA ratios can be used as proxy for periphyton growth rates in controlled experiments and that the fatty acid composition of grazing mayfly nymphs responds to changes in fatty acids provided by the diet after only two weeks. The use of the RNA/DNA ratio as a proxy for periphyton growth rate allows a comparison of these growth rates even in simple experimental set-ups and thereby permits the inclusion of this important process in ecotoxicological or ecological experiments. The observed fast turnover rates of fatty acids in consumer tissues show that even short-term changes in available primary producers can alter the fatty acid composition of primary consumers with important implications for the supply of higher trophic levels with physiologically important polyunsaturated fatty acids. With the results of the field study, I revealed gaps in the understanding of the linkages between catchment and in-stream phosphorus availability under near-natural conditions and demonstrated that seemingly comparable headwater streams had significantly different benthic communities. These differences most likely affect stream responses to environmental changes.

Zusammenfassung

Kleine Bäche umfassen den größten Teil der Fließstrecke und des Einzugsgebiets in Gewässernetzen. Sie sind eng mit ihrem Einzugsgebiet verbunden und reagieren empfindlich auf Änderungen des Wasserhaushalts und der Landnutzung. Trotz ihrer geringen wirtschaftlichen Bedeutung sind diese Bäche entscheidend für den ökologischen und chemischen Zustand größerer Gewässer. Obwohl zahlreiche Studien die Auswirkungen anthropogener Stressoren oder Umweltveränderungen untersuchen, mangelt es am Verständnis der natürlichen Bedingungen und Prozesse, welche jedoch die Reaktion eines Baches auf anthropogene oder klimatische Veränderungen entscheidend beeinflussen. Eine der größten Bedrohungen für aquatische Ökosysteme ist der übermäßige anthropogene Eintrag von Nährstoffen. Nährstoffe steuern Nahrungsnetze in erster Linie über die Primärproduzenten und ihre Konsumenten, in kleinen Bächen Periphyton und Grazer. Die Periphyton-Grazer-Interaktion ist der Hauptpfad der autochthonen Produktion in das Nahrungsnetz, und die Stärke dieser Interaktion bestimmt weitgehend die ökologischen Auswirkungen von Nährstoffeinträgen. Daher zielt diese Arbeit darauf ab, wichtige biologische Prozesse der Periphyton-Grazer-Interaktionen zu beleuchten. Ich untersuchte verschiedene Aspekte der Periphyton-Grazer-Interaktionen anhand von Laborexperimenten mit Fokus auf methodischen Fragen und einer Feldstudie, in der ich die benthischen Gemeinschaften drei morphologisch ähnlicher, Phosphor limitierter, natürlicher kleiner Bächen verglich. Mit dem Laborexperiment zur Periphyton-Wachstumsrate konnte ich zeigen, dass das RNA/DNA-Verhältnis von Periphyton als Proxy für dessen Wachstumsrate in kontrollierten Experimenten verwendet und somit dieser wichtigen Prozesses in ökotoxikologische oder ökologische Experimente einbezogen werden kann. Des Weiteren konnte ich zeigen, dass die Fettsäurezusammensetzung von Eintagsfliegenlarven auf Veränderungen der in der Nahrung enthaltenen Fettsäuren innerhalb von zwei Wochen reagiert. Diese schnelle Anpassung der Fettsäurezusammensetzung der Konsumenten zeigt, dass selbst kurzfristige Änderungen der verfügbaren Primärproduzenten wichtige Auswirkungen auf die Versorgung höherer trophischer Ebenen mit physiologisch wichtigen mehrfach ungesättigten Fettsäuren haben. Mit den Ergebnissen der Feldstudie habe ich Lücken im Verständnis der Zusammenhänge zwischen der Phosphorverfügbarkeit im Einzugsgebiet und im Bach unter naturnahen Bedingungen aufgedeckt und gezeigt, dass scheinbar vergleichbare Bäche signifikant unterschiedliche benthische Gemeinschaften aufweisen. Diese Unterschiede wirken sich höchstwahrscheinlich auf die Reaktion der Bäche auf zukünftige Änderungen aus.

General Introduction

In fluvial networks, headwater streams comprise the largest proportion of total channel length and watershed area (e.g. Benda *et al.*, 2005; Wipfli *et al.*, 2007). These small streams are most closely linked to their catchments, both with regard to hydrological processes and matter fluxes. They are, therefore, vulnerable to natural and anthropogenic changes in catchment hydrologic budget and land cover (Valett *et al.*, 1996; Wipfli *et al.*, 2007; Nelson *et al.*, 2009). Under natural conditions, lithology and soil characteristics strongly affect catchment runoff characteristics and stream water chemical composition due to bedrock chemical composition and weatherability, soil permeability, ion and nutrient budgets and surface-groundwater connectivity (Dillon & Kirchner, 1975; M. C. Feller & J. P. Kimmins, 1979; Mulholland, 1992; Valett *et al.*, 1996; Mattsson *et al.*, 2003). In anthropogenically impacted catchments, stream runoff regimes are often determined by deforestation, soil consolidation and sealing, urban and agricultural runoff, in addition to wastewater inputs. These anthropogenic impacts also largely determine stream sediment and chemical loads, i.e. ions, nutrients, heavy metals, pesticides, and drug residues (Allan *et al.*, 1997; Hancock, 2002; Allan, 2004; Walsh *et al.*, 2005). However, in most cases, a clear separation of natural and anthropogenic factors affecting stream communities is extremely difficult due to overlying effects (Allan, 2004). To assess the sole influence of single or defined groups of anthropogenic stressors, ecological and ecotoxicological experiments are usually conducted with standard test organisms or selected members of natural headwater stream communities (Dodson & Hanazato, 1995; Folt *et al.*, 1999; Wilson *et al.*, 2003; Wagenhoff *et al.*, 2012; Alexander *et al.*, 2013). However, the transferability of experimental results to real ecosystems is often low because of the vast array of factors influencing natural communities and their vulnerability to external stressors (Heugens *et al.*, 2001). Independent of anthropogenic influences, headwater streams are highly variable ecosystems, e.g. in terms of hydrology, temperature, and resource availability with partially strong seasonal patterns, which translate into high and diverse requirements for stream biota and consequently to highly variable stream communities (Wallace & Webster, 1996). Together with the regional species pool, these different environmental impacts largely determine the diversity and functional trait composition of local stream communities (Statzner *et al.*, 2004; Thompson & Townsend, 2006; Worischka *et al.*, 2015). The diversity and composition of species and their traits have been shown to influence the vulnerability of an ecosystem to anthropogenic stressors (Clements & Rohr, 2009; Mondy *et al.*, 2016). To successfully predict ecosystem responses to

expected future environmental changes (climate change or direct anthropogenic impacts) in order to develop and implement protection strategies, an improved understanding of existing ecosystem communities and processes is needed (Woodward *et al.*, 2010).

The food web of headwater streams is based on allochthonous resources provided by terrestrial ecosystems, e.g. leaf litter as a major carbon source, and autochthonous (in-stream) production, e.g. from algae. Phytoplankton is generally absent and the main primary producers are periphyton assemblages. These are complex, multi-species biofilm communities of algae, fungi, protozoa and bacteria growing on submerged surfaces; in this sense, 'periphyton' is synonymous with the German term '*Aufwuchs*' (Ruttner, 1953; Elwood & Nelson, 1972). Depending on the substrate, periphyton can be subdivided in epilithon, i.e. periphyton growing on rocks, epiphyton, i.e. periphyton growing on submerged plants, epixylon, i.e. periphyton growing on wood, and episammon/epipelon, i.e. periphyton growing on sand or mud (Vadeboncoeur *et al.*, 2006). For convenience, the general term 'periphyton' will be used throughout this thesis, independent of potential differences in substrate. From the whole periphyton community, especially algae strongly respond to changes in the availability of nutrients and light which may be mediated by changes in land use (Hill & Fanta, 2008; Hill *et al.*, 2009; Artigas *et al.*, 2013). Depending on the availability of these resources, periphyton assemblages can vary between thin diatom-dominated periphyton of oligotrophic (nutrient-poor) forest streams and thick multi-layer periphyton often dominated by filamentous green algae in open, eutrophic (nutrient-rich) urban or agricultural streams (Bunn *et al.*, 1999).

The most important macronutrients limiting primary production are phosphorus and nitrogen. In Germany, where aerial nitrogen deposition is high and soil nitrogen critical loads are often exceeded (Russow *et al.*, 2001; Watmough *et al.*, 2005), nitrogen inputs to surface water and groundwater are high. As a consequence, water nitrogen concentrations mostly exceed 200 μg dissolved inorganic nitrogen (DIN) L^{-1} (Keppner *et al.*, 2017), a concentration above which periphyton growth rate and biomass accrual was found to be saturated in laboratory experiments (Rier & Stevenson, 2006). Consequently, phosphorus is considered the limiting macronutrient to stream periphyton in German running waters. Laboratory and streamside flume experiments revealed a limitation of periphyton growth at phosphorus concentrations below 20 μg soluble reactive phosphorus (SRP) L^{-1} (Rier & Stevenson, 2006; Hill & Fanta, 2008; Winkelmann *et al.*, 2014). SRP concentrations in that range are typical for oligotrophic

headwater streams with (near-)natural catchments, so that a strong bottom-up control of periphyton growth and biomass accrual in these P-limited streams can be expected.

In headwater streams, the grazer community which consumes the periphyton is generally dominated by benthic macroinvertebrates, such as snails and insect larvae, although vertebrate grazers, i.e. tadpoles, can be dominant at specific sites (Hill & Knight, 1987; Hart & Robinson, 1990; Mallory & Richardson, 2005; Hill & Griffiths, 2017). Most grazers (especially insects and tadpoles) are merolimnic with aquatic larval stages and terrestrial or aerial adult forms. While grazers with strictly aquatic life-cycles, such as aquatic snails, exert a constant grazing pressure on the periphyton, the grazing pressure of merolimnic grazers is reduced in the period of metamorphosis and emergence. However, due to differing life-cycle strategies, emergence periods of different taxa vary, so there is generally no sudden relief of periphyton from grazing by merolimnic taxa altogether (Corbet, 1964; Brittain, 1982; Vilenica *et al.*, 2016).

The periphyton-grazer interactions are generally strong trophic links and play a key role in stream nutrient cycling (Hill & Griffiths, 2017). While nutrient and light availability determine periphyton primary production (bottom-up control), benthic grazers are capable of controlling periphyton standing stocks and composition (top-down control; Wallace & Webster, 1996; Bourassa & Cattaneo, 1998; Eckert & Carrick, 2014; Winkelmann *et al.*, 2014). In natural systems these two processes occur simultaneously and they are often balanced (Oksanen *et al.*, 1981; Hunter & Price, 1992). Therefore, nutrient uptake and conversion to biomass in streams is often tightly coupled between primary producers and higher trophic levels via the periphyton-grazer trophic link (Feminella & Hawkins, 1995; Evans-White & Lamberti, 2006). Consequently, positive nutrient effects on primary production are often apparent in increased grazer biomass or abundance rather than in increased periphyton standing stocks (Hart & Robinson, 1990; Biggs & Lowe, 1994; Huryn, 1998). On the one hand, periphyton quality and quantity determine how many grazers can be maintained and how well they may perform (Hill *et al.*, 1992b; Stelzer & Lamberti, 2002). Periphyton stoichiometry (molar C:N:P ratios) greatly influences grazer performance with respect to growth rate and the acquisition of energy reserves (Wagner *et al.*, 1998; Vrede *et al.*, 2002). Independent of stoichiometry, which to a large extent depends on nutrient availability, algal taxonomic composition largely affects food quality and thereby grazer performance. Diatoms are high quality food, whereas green algae are of intermediate and cyanobacteria of low food quality (Brown *et al.*, 1997; Brett *et al.*, 2000; Caramujo *et al.*, 2007; Martin-Creuzburg *et al.*, 2008). On the other hand, grazers can affect periphyton stoichiometry, biomass and

composition (Rosemond *et al.*, 1993; Feminella & Hawkins, 1995; Alvarez & Peckarsky, 2005; Evans-White & Lamberti, 2005). The respective effect of grazers on periphyton is partially taxon-specific and depends on foraging behaviour and grazer nutrient demand vs. nutrient availability; grazer excretions may contain high concentrations of the nutrient which is available in excess of grazer physiological demand (Lamberti *et al.*, 1995; Poff *et al.*, 2003; Hillebrand *et al.*, 2008). Nonetheless, a great number of grazing effects are reported independent of grazer taxon, with usually negative effects of high grazer densities on periphyton biomass (Feminella & Hawkins, 1995; Lamberti *et al.*, 1995; Mallory & Richardson, 2005).

Besides playing a central role in structuring aquatic ecosystems, some benthic grazers are important for the trophic coupling of aquatic and terrestrial ecosystems. The connection between the two ecosystem types is not limited to inorganic matter fluxes. It additionally includes cross-ecosystem food web links, such as the transfer of terrestrial derived carbon sources to aquatic systems (e.g. leaf litter) or the transfer of aquatic derived physiologically active long-chain polyunsaturated fatty acids (LC-PUFAs with a chain greater than or equal to 20 carbon atoms) to adjacent terrestrial systems (Marcarelli *et al.*, 2011; Greig *et al.*, 2012; Schulz *et al.*, 2015; Moyo *et al.*, 2017). A large proportion of the latter transfer is accomplished by emerging mayflies and caddisflies with grazing larval stages, which are dominant grazers in many headwater streams. Thereby, they provide a direct link between stream primary producers and terrestrial/aerial insectivores (Price *et al.*, 2003; Martínez del Río & McWilliams, 2016). Physiologically active LC-PUFAs, especially eicosapentaenoic acid (EPA, 20:5n-3), arachidonic acid (ARA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), are vital for most animals and humans, but are less abundant in terrestrial organisms than in aquatic organism (Parrish, 2009). In contrast to higher plants, many microalgae, such as diatoms, euglenoids, red algae, marine brown algae and dinoflagellates, synthesize considerable quantities of these physiologically active LC-PUFAs *de novo* (Kelly & Scheibling, 2012; Taipale *et al.*, 2013). These physiologically important fatty acids are enriched and conserved during their way through the food web and are synthesized from precursor fatty acids, mainly linoleic acid, 18:2n-6, LIN and alpha-linolenic acid, 18:3n-3, ALA, by higher trophic levels. This process is called trophic upgrading of the fatty acid composition (Gladyshev *et al.*, 2013). Many terrestrial consumers rely on the supply of these fatty acids from aquatic sources, consequently the trophic upgrading within aquatic ecosystems and their export to terrestrial systems is an important ecosystem service of aquatic habitats (Bell & Tocher, 2009; Gladyshev *et al.*, 2009; Volk & Kiffney, 2012). At the very base of this process are those organisms that

directly feed on algae capable of producing these important, physiologically active LC-PUFAs: the grazers. The trophic linkage between aquatic and terrestrial habitats in headwater catchments is established mainly via emerging aquatic insects, e.g. benthic grazers, (Gladyshev *et al.*, 2009; Lam *et al.*, 2013) because they are an important food source for terrestrial insectivores due to their high content of EPA, ARA and DHA (Twining *et al.*, 2016; Martin-Creuzburg *et al.*, 2017). These specific fatty acids are necessary for a healthy development and successful reproduction because they are key constituents of membranes and tissues; moreover, ARA and EPA are precursors to eicosanoids which play an important role in reproduction cycles and immune responses (Brett & Müller-Navarra, 1997; Stanley, 2006; Arts & Kohler, 2009). However, these LC-PUFAs cannot be synthesised *de novo* and their synthesis from precursor fatty acids is often too low to fully meet physiological demands (Arts *et al.*, 2001; Twining *et al.*, 2018). Therefore, an insufficient supply of these fatty acids with the diet can lead to reduced health and fitness.

Methods used to describe periphyton-grazer interactions at varying nutrient availabilities are numerous and both, state variables (biomass, abundance, elemental and fatty acid composition) and rates (growth rates, feeding rates, primary and secondary production rates) can be used as response variables to describe these interactions (Feminella & Hawkins, 1995; Bourassa & Cattaneo, 1998; Alvarez & Peckarsky, 2005; Hillebrand *et al.*, 2008; Guo *et al.*, 2018). These response variables can be determined for organism groups, e.g. all grazing taxa within a studied stream reach, for individual taxa or individuals of specific taxa. Periphyton biomass and growth rate is generally determined for the whole assemblage, although the algal composition is often analysed too. In addition, physiological indicators of consumer fitness, such as the amount of energy reserves as glycogen or triglyceride (storage lipid) content, can be determined to evaluate consumer performance (Hill *et al.*, 1992b; Koop *et al.*, 2008; Koop *et al.*, 2011). Changes in biomass and abundance are the most frequently determined response variables in the analysis of nutrient effects on periphyton-grazer interactions (Feminella & Hawkins, 1995). A complementary analysis of elemental and fatty acid composition of periphyton and grazers can yield a deeper understanding of underlying processes than the analysis of biomass alone, because these measures provide additional information on the quality of resources, resource usage by grazers, potential imbalances in grazer nutrition as well as information on the limiting nutrient for both resource and consumer growth (Hillebrand *et al.*, 2008; O'Brien & Wehr, 2010; Belicka *et al.*, 2012; Guo *et al.*, 2018). These measures of state variables can be easily applied in controlled experiments and field studies, e.g. by samplings at defined points in time, whereas the determination

of rates is more challenging due to the requirement of measurements over a defined time interval and the multitude of factors potentially influencing the measured rates during this interval *in situ* (Elwood & Nelson, 1972). Therefore, rates are mainly determined in controlled experiments (McIntire *et al.*, 1964; Hill *et al.*, 1992b; Winkelmann *et al.*, 2014).

In streams with sufficient grazer abundances to exert a high grazing pressure, periphyton growth rate is a better indicator of periphyton productivity than standing stocks. Owing to technical difficulties in applying standard measurement methods from lentic (still water) systems to lotic (riverine) systems without massively altering environmental conditions, e.g. by using measurement chambers, the growth rate of stream periphyton is difficult to measure *in situ* (McIntire *et al.*, 1964; Peterson *et al.*, 1993b; Bunn *et al.*, 1999). A promising approach to circumvent these difficulties might be the use of the RNA/DNA ratio as a proxy for growth rate. This proxy is applied successfully in marine fisheries and has more recently been tested successfully for plankton and aquatic invertebrates (Madariaga & Joint, 1992; Chícharo & Chícharo, 2008; Nicklisch & Steinberg, 2009; Grimm *et al.*, 2015). The use of the RNA/DNA ratio as a proxy for growth is based on the observation that the DNA content per cell is relatively constant, whereas the RNA content increases with increased protein synthesis, e.g. required for growth (Price, 1952; Dortch *et al.*, 1983; Buckley, 1984). If the RNA/DNA ratio is a suitable proxy for periphyton growth rate, its use could facilitate process oriented studies in stream benthic ecosystems because the RNA/DNA ratio is fast and easy to measure from samples obtained at defined points in time.

Dietary tracing via stable isotope and fatty acid composition is used to identify trophic links, i.e. resource usage of species within a common food web, resource-related biotic interactions such as competition and key sources of carbon or physiologically important fatty acids (Füreder *et al.*, 2003; Belicka *et al.*, 2012; Jackson *et al.*, 2012; Guo *et al.*, 2016). The tracing of resources within a food web is possible due to their distinct differences with respect to their $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ value, and with respect to their fatty acids composition. The $\delta^{13}\text{C}$ value is the ratio of the heavy ^{13}C isotope to the lighter ^{12}C isotope of the sample in relation to the ratio of the heavy ^{13}C isotope to the lighter ^{12}C isotope of the international standard Pee Dee Belemnite and the $\delta^{15}\text{N}$ value is the ratio of the heavy ^{15}N isotope to the lighter ^{14}N isotope of the sample in relation to the ratio of the heavy ^{15}N isotope to the lighter ^{14}N isotope of air. These distinct resource-specific values result from spatial and temporal differences in the availability of these stable isotopes and differences in biochemical processes and pathways in primary producers

which favour the enrichment of one isotope over the other and enable the *de novo* synthesis of a specific set of fatty acids (Farquhar *et al.*, 1989; Cook & McMaster, 2002; Goss & Wilhelm, 2009). With increasing trophic level, a trophic enrichment of selected isotopes and fatty acids takes place resulting in an increased $\delta^{15}\text{N}$ value and higher contents of physiologically important LC-PUFAs, especially EPA and DHA (Minagawa & Wada, 1984; Lau *et al.*, 2012; Guo *et al.*, 2018). Although, much knowledge has been gained on the time needed for a shift in resource composition to translate into consumer tissue composition with regard to stable isotope values, i.e. isotopic turnover rates (Tieszen *et al.*, 1983; Hobson & Clark, 1992; Sakano *et al.*, 2005; Hellmann *et al.*, 2015), little is known on the time needed for the translation of fatty acid pattern from resources into consumer tissue for benthic macroinvertebrates after a shift in feeding habit or resource availability. Nonetheless, recent studies demonstrate that seasonal differences in periphyton fatty acids translate into changes in grazer fatty acid composition (Lau *et al.*, 2012; Guo *et al.*, 2018). However, further research is needed to evaluate how fast and to which extent a shift in resource fatty acid composition is reflected by consumer fatty acids.

As presented above, there is a wide methodological palette available to assess and compare periphyton-grazer interactions. In the work contributing to this thesis, I combined both traditional methods and comparatively new approaches to characterize stream benthic communities with a selected focus on periphyton and benthic macroinvertebrate grazers.

The goals of the PhD thesis were 1) to evaluate whether the RNA/DNA ratio is a suitable proxy for periphyton growth rate (**study 1**); 2) to analyse how strongly short-term changes in food supply alter consumer fatty acid composition and whether membrane or storage lipids are more susceptible to these changes (**study 2**); 3) to determine if differences in catchment soil and bedrock phosphorus are reflected by in-stream soluble reactive phosphorus concentrations and to evaluate the importance of phosphorus availability for the structure and composition of the benthic communities of three near-natural, P-limited headwater streams (**study 3**).

Top-down control of periphyton biomass by benthic grazers is a key aspect of periphyton-grazer interactions. In situations in which periphyton is top-down controlled, periphyton growth rate rather than standing stocks may reveal the degree of food limitation benthic grazers are facing. Therefore, the aim of the first study was to develop a method for the fast determination of periphyton growth rates. To this aim, I tested if the RNA/DNA ratio is a suitable proxy for periphyton growth rate and if the

relationship between RNA/DNA ratio and growth rate is comparable between seasons and streams.

Owing to the importance of merolimnic grazers in aquatic food webs and for the transfer of LC-PUFAs to adjacent terrestrial food webs, the effect of a short-term shift in diet on the fatty acid composition of two mayfly grazers was determined in the second study. Late instars of the mayflies *Rhithrogena* sp. and *Baetis* sp. were reared on either a diatom biofilm (*Navicula pelliculosa*) or a green algae biofilm (*Stigeoclonium farctum*) for two weeks. The effect of the different diets on consumer fatty acid composition was analysed for consumer membrane and storage lipids to reveal potentially different response times of those lipid pools. Total fatty acid composition and selected marker fatty acids for both food sources were compared between consumers fed on either of the two food sources to assess the sensitivity of these measures to short-term changes in the fatty acids provided by the diet.

In the third study, I examined the influence of environmental factors on periphyton-grazer interactions. Therefore, the study consists of a comparison between the benthic communities of three morphologically similar headwater streams of German low mountain ranges with siliceous bedrock. While stream morphology and light availability between sites was similar, geogenic phosphorus availability differed between the three study sites (Lang *et al.*, 2017). I investigated if these differences in catchment phosphorus translate into corresponding in-stream concentrations of soluble reactive phosphorus (SRP) and if a bottom-up control based on the observed difference in phosphorus availability between the sites is the dominant factor shaping the stream benthic communities. With regard to the latter question, I focused on possible nutrient-related bottom-up effects at the base of the benthic food web.

Study 1

RNA/DNA ratio as a growth indicator of stream periphyton

Summary

1. The quantification of periphyton growth in situ poses numerous difficulties.
2. The RNA/DNA ratio is widely used as a growth indicator in marine ecology. We tested its applicability as a growth indicator of periphyton in streams of different sizes.
3. Periphyton-covered stones sampled from two different watercourses during two seasons were exposed in laboratory flumes to different light levels to induce different growth rates. The relationship between rate of biomass accrual and RNA/DNA ratio was analysed by measuring the chlorophyll-a content and the RNA and DNA content of total nucleic acid extract of the periphyton respectively.
4. The RNA/DNA ratios showed a linear relationship with the biomass accrual at all sampling times. The slopes of these relationships varied significantly between the two watercourses, but not between seasons within the same watercourse.
5. These results indicate that the RNA/DNA ratio can be used as a growth indicator for the periphyton studied here. We recommend that it be used to detect differences in growth rate of the same periphyton community either over time or between different treatments in ecological or ecotoxicological experiments. However, for long-term monitoring studies, we recommend taxonomic analyses of the assemblages because the observed differences in the relationship between the RNA/ DNA ratio and growth rate might be attributed to community structure differences in the assemblages.

Due to copyright issues, the text of this chapter was replaced by the reference information. The interested reader is kindly asked to read the published paper via the following reference:

Daniela Mewes, Sandra Spielvogel Carola Winkelmann (2017), *Freshwater Biology*, 62(4), 807–818. © 2017 John Wiley & Sons Ltd

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Study 2

Mayfly nymph fatty acids respond to short term changes in food supply: Diet tracing by membrane and storage fatty acids

Submitted article authored by:

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Summary

We tested how the fatty acid composition of grazing mayfly nymphs (*Rhithrogena* sp. and *Baetis* sp.) respond to diets with distinct fatty acid compositions (diatoms or green algae) after two weeks. Food source effects were detected for alpha-linolenic acid, linoleic acid, eicosapentaenoic acid and arachidonic acid which also differed between grazer species. Although, storage lipids contained a lower proportion of polyunsaturated fatty acids than phospholipids, the amounts per dry weight were higher.

Introduction

Fatty acid profiles are suitable to trace resource usage in food webs, often complementing stable isotope analysis (Brett & Müller-Navarra, 1997; Belicka *et al.*, 2012; Lam *et al.*, 2013; Kainz *et al.*, 2017). Comparable to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, fatty acids of consumers integrate the composition of ingested food sources over a period which depends on preferential retention, storage size and growth rate (Chamberlain *et al.*, 2005; Brett *et al.*, 2006). We tested 1) how strongly the limitation to a defined food source alters the fatty acid composition of late instars of grazing nymphs of two mayfly species (*Rhithrogena* sp. and *Baetis* sp.) within a short period of two weeks; and 2) whether such alterations would be more readily detectable in storage lipids (neutral lipids, NL) or membrane lipids (phospholipids, PL).

Methods

We used two food sources: a diatom biofilm (*Navicula pelliculosa*; SGA strain number 1050-3, SAG Culture Collection of Algae, Göttingen, Germany), and a green algae biofilm (*Stigeoclonium farctum*; SAG strain number 477-19d). Diatoms and green algae differ strongly in their fatty acid composition, e.g. in the C16 fatty acids (16-carbon chain) suggested as biomarkers for these specific taxonomic groups (Diatoms: 16:1n-7, 16:2n-4, 16:3n-4; Green algae: 16:2n-6, 16:3n-3, 16:4n-3). Further differences occur in their physiologically important C18–C22 (18–22-carbon chain) Omega-3 (n-3) and Omega-6 (n-6) polyunsaturated fatty acids (PUFAs; Diatoms: Arachidonic acid, ARA, 20:4n-6, Eicosapentaenoic acid, EPA, 20:5n-3; Green algae: Alpha-Linolenic acid, ALA, 18:3n-3, Linoleic acid, LIN, 18:2n-6; Taipale *et al.*, 2013). The feeding experiment was split into two runs. For the first run only nymphs of *Rhithrogena* sp. were sampled and for the second run nymphs of *Rhithrogena* sp. and *Baetis* sp. were sampled and both species were included in the experiment. Each run comprised 6 containers per food source and species, i.e. a total of 12 containers for run 1 (*Rhithrogena* sp.), and a total of 24 containers for run 2 (both species). At the start of each run, two individuals of one of the two species and one tile (50 × 50 mm) covered with one of the two biofilms were placed into each container. All containers had wall openings covered by 1 mm-mesh and were placed into a single flume with circulating water current. The tile was replaced with a new biofilm-coated tile as needed so that the mayflies could feed *ad libitum*. After two weeks, all individuals were collected (total losses: 42% and 23% in run 1 and 2, respectively) and shock-frozen in liquid nitrogen.

Per run, food source and mayfly species, 2–3 individuals were combined to a single sample for the fatty acid analysis. These samples were analysed in duplicate (overall $n = 12$, supplementary material M1). Additionally, one sample of each food source was analysed per experimental run ($n = 4$).

Prior to extraction, two lipid fraction-specific internal standards were added to each lyophilized sample (19:0 PL, di-nonadecanoyl-glycerol phosphatidylcholine; Larodan, Solna, Sweden, and 19:0 NL, tri-nonadecanoyl-triacylglycerol, Sigma-Aldrich). Fatty acids were extracted and purified as in Gunina *et al.* (2014) adapted to smaller sample sizes. Each sample was extracted twice with a monophasic mixture of chloroform:methanol:0.15 M citric acid pH 4.0 (1:2:0.8, by vol.; Frostegård *et al.*, 1991). After phase separation, the lower chloroform phase was collected, and the upper phase was extracted a second time with chloroform. The total lipids in the combined chloroform phases were separated into neutral lipids, glycolipids, and phospholipids on a SPE column filled with activated silica gel using a sequential elution with chloroform:acetone (95% : 5%), acetone, and methanol. The single fractions were dried under nitrogen. Lipids were hydrolysed with 0.5 M NaOH in dried methanol to release fatty acids. These free fatty acids were then methylated with BF₃ (14% in methanol, Sigma-Aldrich) to yield fatty acid methyl esters (FAMES). Saturated NaCl was added to hydrolyse the toxic BF₃, and FAMES were extracted three times with n-hexane. The three hexane extracts per sample were combined and evaporated under nitrogen. FAME 13:0 (Sigma-Aldrich) was added to each sample as a second internal standard, and FAMES were re-suspended in toluene for GC-measurement. Additionally, five concentrations of three external standard mixtures (supplementary Table S1), used in the quantification of phospholipid, glycolipid and neutral lipid fatty acids respectively, were derivatized. Fatty acids were separated and measured on a GC-MS.

Chromatograms were analysed using OpenChrom (Wenig & Odermatt, 2010) and all peak areas were normalized by dividing by the area of the FAME 13:0 internal standard. The recovery of storage lipids and membrane lipids was calculated using the recovery of 19:0 NL and 19:0 PL, respectively. Due to difficulties in recovering 19:0 NL in some of the samples, mean recoveries were calculated from samples in which both fraction-specific internal standards were measured quantitatively ($n = 9$). The resulting mean recovery rate for either storage or membrane lipids was used to calculate the fatty acid contents per sample. The external standards were used for linear calibration of each fatty acid contained in the standard mixtures. Fatty acids in samples were identified by comparing mass spectra and retention times to those of the fatty acids contained in the

standards and FAME mass spectra provided online by W.W. Christie (2018). Fatty acids not contained in the standards were quantified using the calibration equation calculated for the fatty acid(s) with the most similar chain length and degree of unsaturation (for a detailed method description see supplementary material M2).

Fatty acid compositions of food sources and of storage and membrane lipids of mayfly nymphs were visualised using a non-metrical multidimensional scaling (NMDS) based on Bray-Curtis distances and fatty acid contents in $\mu\text{g mg}^{-1}$ dry mass (square root transformed). Differences between mayfly fatty acid signatures were assessed using Adonis, a permutational multivariate analysis of variance using a Bray-Curtis matrix. A similarity percentage analysis (Simpser) identified the fatty acids that contributed most to the differences identified by the Adonis analysis. The amount and the ratio of food source specific fatty acids were compared between mayfly nymphs fed on either food source using a two-way ANOVA (factors, mayfly species; food source) for each lipid fraction (total, NL and PL).

Results and Discussion

Fatty acid composition of the two biofilms conformed with literature and was well separated in the NMDS (Fig.1; e.g. Taipale *et al.*, 2013). In the diatom biofilm, monounsaturated fatty acids (MUFA) were the most abundant, but within the PUFAs ARA and EPA were dominant. The fatty acids of the green algae biofilm consisted mainly of PUFAs, wherein ALA and LIN were dominant. Based on all quantified fatty acids, significant differences in fatty acid profiles between membrane and storage lipids ($p = 0.001$) of mayfly nymphs were detected (Fig.4). The fatty acids contributing most to these differences, 16:0 and 16:1n-7, were more abundant in storage than in membrane lipids. The comparison of major fatty acid groups revealed additional deviations between the fatty acid profiles of the mayfly nymphs fed on the two food sources. Comparable to the food source, MUFAs (especially 16:1n-7) contributed most to fatty acids in the storage lipids of mayfly nymphs fed on diatom biofilm. The storage lipids of mayfly nymphs fed on green algae biofilm showed an even distribution of saturated fatty acids (SAFAs), MUFAs and PUFAs, which deviated from both the lipid composition of their food source and of the mayfly nymphs fed on diatoms. Independent of the food source, PUFAs contributed most to membrane fatty acids. Despite lower proportions of PUFAs in mayfly storage lipids, the amount of PUFAs per dry weight was higher in storage lipids than in membrane lipids of mayfly nymphs (Fig. 5B-C, E-F; for detailed information see supplementary Tables S2&S3).

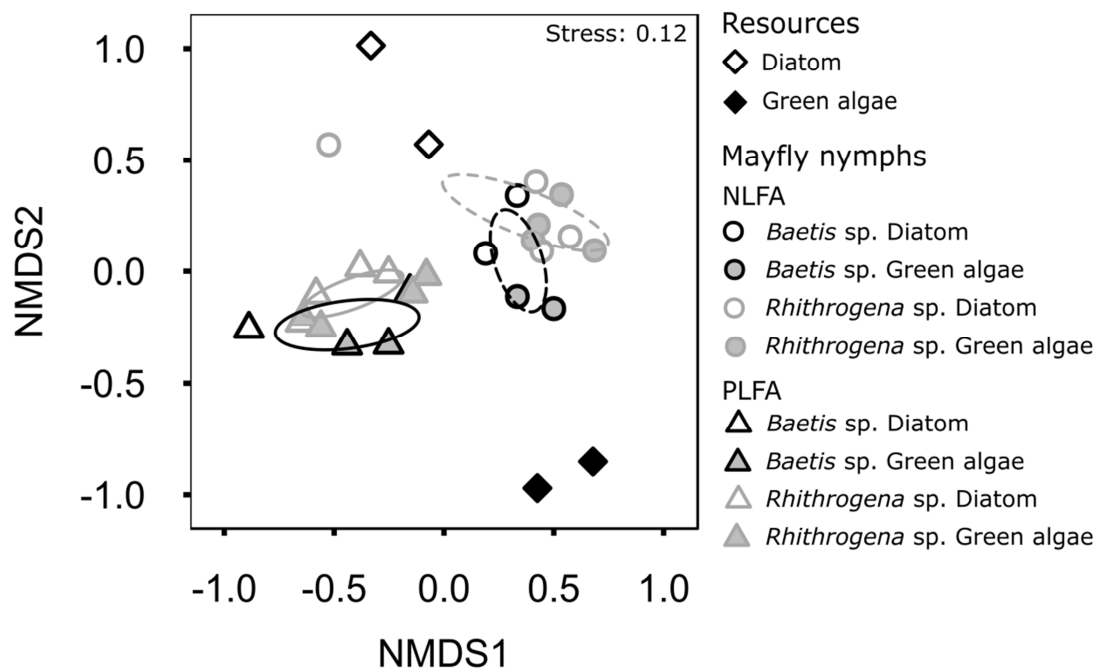


Fig. 1 Fatty acid composition of mayfly nymphs and their food sources (NMDS). Non-metric multidimensional scaling (NMDS) plot of the fatty acid composition of food sources (biofilm total fatty acids) and of storage (NLFA) and membrane (PLFA) lipids of mayfly nymphs fed on either resource. Standard error ellipses are shown for membrane (—) and storage (---) lipids of each mayfly species (black, *Baetis* sp., n = 4; grey, *Rhithrogena* sp., n = 8).

The in-depth analysis of food source specific fatty acids revealed effects of food source and species on the fatty acid composition of mayfly nymphs (Fig. 5, Table 7). All significant ANOVA results were related to the physiologically important polyunsaturated fatty acids ALA, LIN, ARA and EPA, probably owing to a preferential retention and conservation of these fatty acids in consumer membranes and tissue (Brett *et al.*, 2006; Gladyshev *et al.*, 2011). These fatty acids were the most abundant PUFAs in membrane and storage lipids. In contrast, the C16 PUFAs suggested as food source specific biomarkers (Taipale *et al.*, 2013) were detected only in low quantities and inconsistently among samples, suggesting a low transfer efficiency (Gladyshev *et al.*, 2011). Especially the C16 biomarkers for green algae were detected exclusively in storage lipids (Fig. 5 C&F). The insect fat body is an organ analogous to vertebrate adipose tissue and liver and therefore, not a place of mere storage but also of highly active metabolism (Canavoso *et al.*, 2001). Consequently, these C16 fatty acids may be useful in assessing the composition of complex mixtures of primary producers, e.g. periphyton or phytoplankton, but they are less useful in tracing specific diet components within the food web. They appear to be either rapidly metabolised or absorbed less efficiently so that their signature is lost in higher trophic levels. Only the mono-

unsaturated C16 fatty acid, 16:1n-7 (diatom marker), could be detected in high abundances in all mayfly samples and in both lipid fractions.

Table 1 Significant effects ($p < 0.05$) based on the ANOVA (factors: species, food source). Response variables were the food source specific fatty acids: 16:1n-7 + 16:2n-4 + 16:3n-4 (Diatom); ARA+EPA; 16:2n-6 + 16:3n-3 + 16:4n-3 (Green); ALA + LIN; either as amount ($\mu\text{g mg}^{-1}$ dry mass, DM) or proportion (%) of the different lipid fractions: membrane lipids (PLFA), storage lipids (NLFA) or total lipids (PLFA + NLFA). Sample number per species were $n = 8$ and $n = 4$ for *Rhithrogena* sp. and *Baetis* sp., respectively, and $n = 6$ per food source.

Response	Lipid fraction	Transformation	Factor	F	p
ALA+LIN	total lipids ($\mu\text{g mg}^{-1}$ DM)	none	species	10.541	0.012
ALA+LIN	total lipids ($\mu\text{g mg}^{-1}$ DM)	none	interaction	6.102	0.039
ALA+LIN	% total lipids	square root	species	15.632	0.004
ALA+LIN	% total lipids	square root	food source	8.440	0.020
ALA+LIN	% total lipids	square root	interaction	6.019	0.040
ARA+EPA	total lipids ($\mu\text{g mg}^{-1}$ DM)	none	species	9.577	0.015
ARA+EPA	% total lipids	none	species	23.720	0.001
Diatom+ARA+EPA	% total lipids	none	species	10.708	0.011
Green+ALA+LIN	total lipids ($\mu\text{g mg}^{-1}$ DM)	none	species	9.050	0.017
Green+ALA+LIN	total lipids ($\mu\text{g mg}^{-1}$ DM)	none	interaction	6.251	0.037
Green+ALA+LIN	% total lipids	square root	species	9.858	0.014
Green+ALA+LIN	% total lipids	square root	food source	6.791	0.031
$\left[\begin{array}{l} \text{diatom marker} \\ \text{green algae marker} \end{array} \right]$	PLFA	none	species	10.244	0.013
$\left[\begin{array}{l} \text{diatom marker} \\ \text{green algae marker} \end{array} \right]$	PLFA	none	food source	6.614	0.033
ALA+LIN	PLFA ($\mu\text{g mg}^{-1}$ DM)	none	species	10.040	0.013
ALA+LIN	% PLFA	none	species	27.479	0.001
ALA+LIN	% PLFA	none	food source	14.668	0.005
ARA+EPA	PLFA ($\mu\text{g mg}^{-1}$ DM)	none	species	5.710	0.044
ARA+EPA	% PLFA	none	species	62.807	<0.001
ARA+EPA	% PLFA	none	food source	6.726	0.032
Diatom+ARA+EPA	% PLFA	none	species	58.558	<0.001
Diatom+ARA+EPA	% PLFA	none	food source	14.634	0.005
Green+AL+LIN	PLFA ($\mu\text{g mg}^{-1}$ DM)	none	species	10.040	0.013
Green+AL+LIN	% PLFA	none	species	27.479	0.001
Green+AL+LIN	% PLFA	none	food source	14.668	0.005
ALA+LIN	% NLFA	square root	species	11.518	0.009
ALA+LIN	% NLFA	square root	food source	6.128	0.038
ARA+EPA	NLFA ($\mu\text{g mg}^{-1}$ DM)	none	species	5.463	0.048
ARA+EPA	% NLFA	none	species	10.792	0.011
Diatom+ARA+EPA	% NLFA	none	species	7.724	0.024
Green+AL+LIN	NLFA ($\mu\text{g mg}^{-1}$ DM)	none	species	6.261	0.037
Green+AL+LIN	NLFA ($\mu\text{g mg}^{-1}$ DM)	none	interaction	5.842	0.042
Green+AL+LIN	% NLFA	none	species	7.176	0.028

Significant food source related changes were only detected if fatty acids were analysed as proportion of the respective lipid fraction (Table 7), probably due to the large variability of lipid content in mayfly nymphs per dry weight (Fig 5 A–C, see also Winkelmann & Koop, 2007). Fatty acid trophic markers (resource specific, conserved fatty acids, e.g. ALA, LIN for green algae, and 16:1n-7, ARA, EPA for diatoms) in invertebrate consumers detected in either membrane or storage lipids, likewise, were influenced by changes in diet, at least if whole individuals are analysed (Table 1, Fig. 2B–C&E–F). Fatty acid trophic markers for diatoms were more abundant in mayfly nymphs fed on diatom biofilm and green algae trophic markers were more abundant in mayfly nymphs fed on green algae biofilm (Fig. 5).

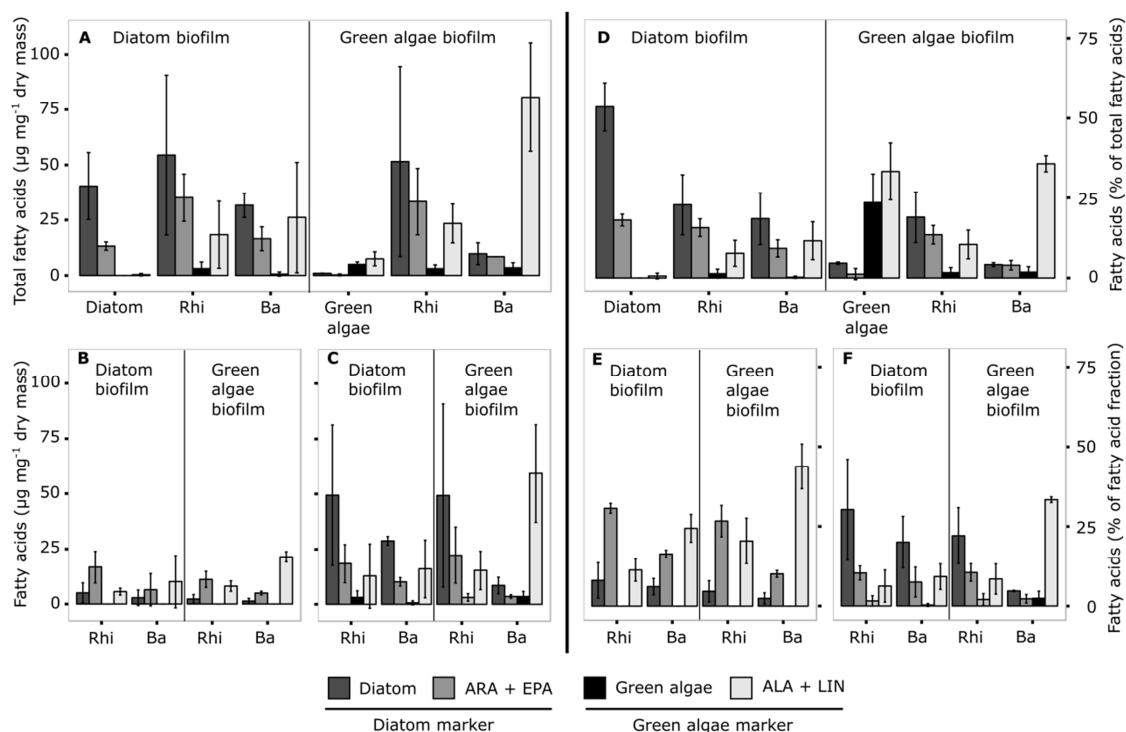


Fig. 2 Marker fatty acids used to trace the food sources. Plots on the left (A–C) show the amount of fatty acids in μg per mg dry mass and plots on the right (D–F) show the amount of fatty acids as percent of fatty acids per lipid fraction. Upper plots (A and D) show the amount of marker fatty acids based on total fatty acids (i.e. sum of all analysed fractions) for the food sources (*Navicula pelliculosa* biofilm = Diatom, *Stigeoclonium farctum* biofilm = Green algae) and the mayfly nymphs *Rhithrogena* sp. (Rhi) and *Baetis* sp. (Ba) fed on either of these resources (left side of each plot = Diatom biofilm, right side of each plot = Green algae biofilm). Plots (B) and (E) show the amount of marker fatty acids in mayfly membrane lipids, and the plots (C) and (F) show the amount of marker fatty acids in mayfly storage lipids. Marker fatty acids ‘Diatom’ were 16:1n-7 + 16:2n-4 + 16:3n-4, and the marker fatty acids ‘Green algae’ were 16:2n-6 + 16:3n-3 + 16:4n-3. Diatom: n = 2, Rhi: n = 4, Ba: n = 2, Green algae: n = 2.

The relation between saturated, mono-unsaturated and polyunsaturated fatty acids differs between membrane and storage lipids (Ghioni *et al.*, 1996, this study), so that ratios or absolute contents are only comparable between results obtained from the same lipid fraction. Owing to significant differences in the recovery rates of membrane and storage lipids ($53\% \pm 18\%$ and $26\% \pm 9\%$, respectively; mean \pm SD, $p = 0.002$, Welsh t-

test, n= 9), a fractionation of the lipid classes and the recovery of lipid fraction-specific internal standards seems to be required for quantitative analysis. Such accurate quantification is necessary, if exact export rates or consumption requirements are to be defined.

The significant difference in the content of food source-specific fatty acids between the two mayfly species (Table 7) might indicate different proportions of these resources in the natural diet of nymphs of *Baetis* sp. and *Rhithrogena* sp. in their original habitat. Since late instars of both species were used, the nymphs had most probably accumulated considerable amounts of lipids in their natural habitat prior to the experiment (Cavaletto *et al.*, 2003). Differences in growth and consumption rates between the species during the experiment possibly added to this species-specific response in fatty acid pattern. These rates, however, could not be determined in the chosen experimental set-up. As an active selection of preferred food sources would influence the fatty acid composition of grazing invertebrates, further research is needed to determine possible species-specific food preferences in invertebrate grazers from their natural habitat.

In conclusion, the fatty acid composition of late instars integrates probably over the entire larval development with a high retention of ALA, LIN, ARA, and EPA in both membrane and storage lipids (Sushchik *et al.*, 2003). Nonetheless, a short term shift in the availability of trophic marker fatty acids can be reflected by the fatty acid composition of mayfly nymphs, if the amount of these fatty acids already retained within the organism is low enough for the change to have significant effects. Distinct compositions of lipid classes suggest that only identical fractions should be compared between studies.

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Study 3

Differences in catchment soil and bedrock phosphorus do not translate into strong bottom-up effects in headwater streams

Manuscript authored by:

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Summary

Technological progress has led to a drastic impact of human activities on lotic ecosystems. One of the most severe threats is the excessive input of nutrients of anthropogenic origin. Headwater streams are highly vulnerable to pollution as they comprise much of the channel length and watershed area in fluvial networks. For this reason, concentration-effect relationships for single or defined groups of anthropogenic stressors are frequently studied in near-natural headwater streams or with selected members of their community. Such studies showed that the limiting nutrients have strong bottom-up effects on benthic communities, but little is known about the dominant factors that shape these systems in their (near-)natural condition, such as geology, which has been shown to greatly influence stream nutrient concentrations, especially in low-impacted catchments. Here we analysed the effect of geogenic phosphorus (P) availability on the benthic communities of three near-natural, morphologically similar headwater streams in German low mountain ranges with differing catchment P availability. We tested for bottom-up effects on stream benthic communities using whole community measures and measures related to the performance of individual grazer taxa. Contrary to our expectations, stream water soluble reactive phosphorus (SRP) did not mirror catchment P availability; sites ranked differently depending on the potential source of available P in stream (water SRP or soil inorganic P). SRP concentrations of the streams were low but similar (8 - 13 $\mu\text{g SRP L}^{-1}$), i.e. general catchment characteristics and water SRP concentrations were comparable. Despite similar environmental conditions, the benthic communities and some measures related to grazer performance (overlap of isotopic niche area and grazer N:P ratio) significantly differed between the streams and were likely partly related to bottom-up effects. Environmental factors, biotic interactions and regional species pool probably played

major roles in shaping the benthic communities of the studied streams. Thus, natural differences between field sites in addition to systemic differences between field and experimental conditions need to be addressed when transferring experimental results to seemingly comparable ecosystems.

Introduction

Although phosphorus (P) and nitrogen (N) are essential nutrients for all organisms in freshwater ecosystems, the excessive anthropogenic input of these nutrients, which leads to eutrophication, is one of the major threats to aquatic biodiversity (Millennium Ecosystem Assessment, 2005). The development of protection strategies and chemical and ecological water quality threshold values of nutrients, heavy metals, pesticides and drug residues require knowledge of the effects of these stressors on the community and food web structure of running waters. This knowledge is usually achieved in either nutrient enrichment or ecotoxicological laboratory experiments, depending on the stressor of interest, and in field experiments in headwater streams or with selected members of their community, e.g. periphyton and selected macroinvertebrate taxa (Lugthart & Wallace, 1992; Rier & Stevenson, 2006; Alexander *et al.*, 2013). Headwater streams are chosen for these experiments owing to their particular vulnerability to pollution as they make up a large proportion of the total channel length and watershed area of fluvial networks (Wipfli *et al.*, 2007; Nelson *et al.*, 2009). Especially in forested catchments, headwater streams provide good experimental conditions for assessing the sole impact of single or defined groups of anthropogenic stressors on real benthic communities because many are still only minimally affected by anthropogenic influences (Charvet *et al.*, 2000; Allan, 2004). In addition, large fish species and plankton are usually not present, which limits the food web complexity. However, there is still a considerable gap in the knowledge on the processes and factors shaping these systems in their natural state.

Under (near-)natural conditions, nutrient concentrations in headwater streams are strongly influenced by catchment geology and soils (Dillon & Kirchner, 1975), and these streams are often ranked at the lower limb of the nutrient response curve. At this lower limb, e.g. 0–20 μg soluble reactive phosphorus (SRP) L^{-1} or 0–200 μg dissolved inorganic nitrogen L^{-1} , nutrient response curves fitted to experimental data (e.g. efficiency loss, Monod or Michaelis-Menten kinetics) predict the strongest response of periphyton biomass and growth rate to increasing nutrient concentrations (e.g. Rier & Stevenson, 2006; O'Brien *et al.*, 2007; Hill & Fanta, 2008; Winkelmann *et al.*, 2014). In enrichment experiments, increased nutrient availability directly affects biomass, stoichiometry, and community composition of periphyton (Stelzer & Lamberti, 2001;

Hill *et al.*, 2011), thereby facilitating higher trophic levels through increased amounts or higher quality of food resources (bottom-up effects; e.g. Peterson *et al.*, 1993a; Sterner & Hessen, 1994; Hillebrand & Kahlert, 2001; Hillebrand, 2002). Changes in food quantity and quality induced by higher nutrient availability determine the fitness of primary consumers, which might be observed even prior to an effect on the community level (Rosillon, 1988; Hart & Robinson, 1990; Sterner & Hessen, 1994).

When experimental results are extrapolated to streams under (near-)natural conditions, nutrients appear to be among the dominant factors that shape the communities, which implies strong nutrient-related bottom-up effects, especially within a low range of nutrient concentrations. However, field surveys attempting to validate the response of lotic communities to increasing nutrient availability observed in experimental studies have yielded inconsistent results, especially at low nutrient concentrations, because other factors, such as altitude, slope, and light, also might shape the communities and thereby intensify or blur nutrient effects (e.g. Bourassa & Cattaneo, 1998; Chételat *et al.*, 1999; Evans-White *et al.*, 2009; Lewis & McCutchan, 2010). Therefore, the question remains whether differences in P availability in streams with comparable catchment characteristics would lead to bottom-up responses, which despite their different intensities are comparable to the responses predicted for lotic communities at the lower limb of the nutrient response curve.

Strong bottom-up effects on stream benthic communities can be detected by measuring standing stocks of primary producers and primary consumers (Biggs & Lowe, 1994; Hillebrand, 2002). More subtle effects can be revealed by determining physiological indicators of fitness, such as the content of storage lipids (triglycerides) and the RNA/DNA ratio, which reflect nutritional status or growth of consumers (e.g. Buckley *et al.*, 1999; Vrede *et al.*, 2002; Koop *et al.*, 2011; Normant-Saremba *et al.*, 2015). Stable isotope analysis can provide valuable insights into the utilization of specific food resources (Mulholland *et al.*, 2000; Zah *et al.*, 2001; Bergfur *et al.*, 2009), and the isotopic niches occupied by different consumer taxa might provide insights into the trophic structure of benthic communities (Layman *et al.*, 2007a; Layer *et al.*, 2013), which especially facilitates the detection of bottom-up effects.

Higher P availability can enhance the quantity and diversity of primary producers and thus potentially increase the quantity and quality of food for primary consumers, such as

grazers (Stelzer & Lamberti, 2001; Liess *et al.*, 2009). This in turn can increase the lipid content in different grazer taxa (Hill *et al.*, 1992b) and stimulate grazer growth (Hill *et al.*, 1992b; Stelzer & Lamberti, 2002).

The quantity and diversity of primary resources also affects resource utilisation of consumers (e.g. Layman *et al.*, 2007b; Layer *et al.*, 2013). For instance, at higher periphyton biomass and diatom species richness and density the isotopic niches of different functional feeding groups of primary consumers considerably overlapped, and primary consumers (particularly grazers) relied more on periphyton and a higher number of diatom species was detected in the gut contents of grazing mayfly nymphs (Layer *et al.*, 2013). This indicates that mayfly nymphs diversify their resource use with higher diversity of primary producers, which should lead to an increased isotopic niche width. As grazing nymphs of different mayfly taxa probably compete for similar resources, a low quantity and diversity of available primary resources might not only be reflected in a narrow isotopic niche width of a single mayfly taxon, but also might force coexisting mayfly taxa to segregate their resource usage to reduce competition. Such segregation in resource usage will probably become less important when primary production increases with higher P availability, thus resulting in more similar resource utilisation by nymphs of different mayfly taxa.

In this study, we used a comparative approach to assess whether a natural difference in P availability is the dominant factor shaping the stream benthic communities of three morphologically similar low mountain headwater streams. We expected that SRP concentrations of the headwater streams would mirror catchment soluble inorganic P budgets, and that if P is one of the dominant factors shaping the benthic communities in the studied streams, the P level would affect standing stocks of primary producers and/or primary consumers (total grazer biomass), physiological indicators of grazer fitness (lipid content and RNA/DNA ratio, a proxy for growth, would be higher with higher P availability) and the overlap of grazer isotopic niche (higher at higher SRP concentrations). We assumed that physiological indicators of grazer fitness and isotopic niche overlap are more sensitive to subtle bottom-up effects.

Material & Methods

Study sites

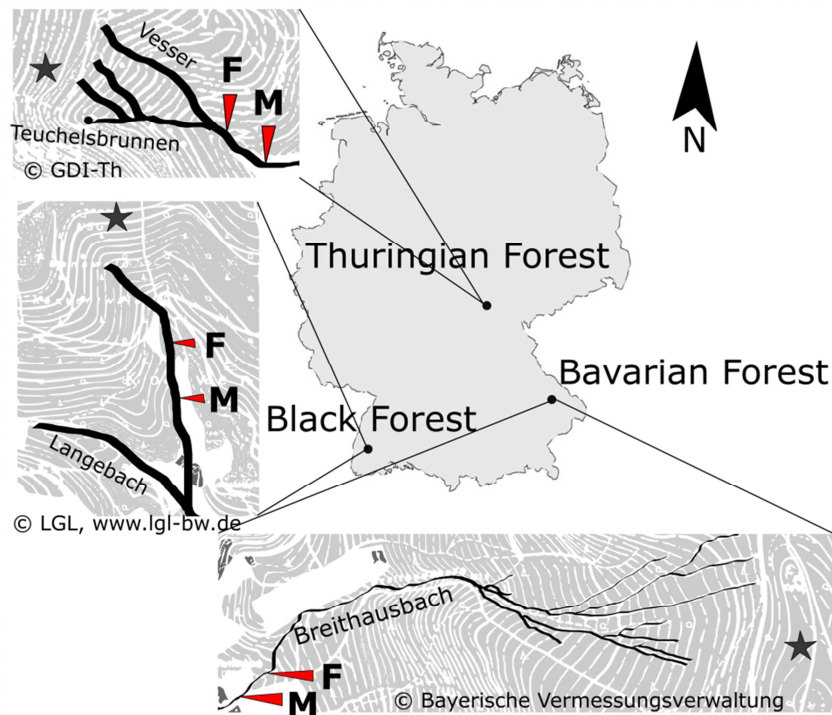


Fig. 3 Location of study and sampling sites in Germany. Detailed maps show streams (black), forest (grey) and meadow (white). Star, position of the core study site of each forest ecosystem of the research project SPP-1685. F, forest stream sampling site; M, meadow stream sampling site. Sampled streams were of first (Black Forest), second (Thuringian Forest), and third order (Bavarian Forest, sensu Strahler). Coordinates of the sites are given in Table 1.

We studied three headwater streams located in the German low mountain ranges of the central Black Forest, Thuringian Forest and Bavarian Forest (Fig. 6; coordinates are given in Table 8). All streams have anthropogenically low-impacted and forested catchments. The sampled stretches were located at the outer margin of the areas studied in a joint forest ecosystem research project (SPP-1685: Ecosystem Nutrition, funded by the German Research Foundation, DFG), in which the cycling of P in the soils of temperate forests on siliceous bedrock was studied (Bol *et al.*, 2016; Prietzel *et al.*, 2016; Zavišić *et al.*, 2016; Julich *et al.*, 2017; Lang *et al.*, 2017). The soil inorganic P at the sites is related to bedrock P (Zavišić *et al.*, 2016; Lang *et al.*, 2017). We assumed that the P availability of the three streams would differ because the study sites were within forested, low-impacted catchments whose siliceous bedrock material forms a geosequence in P content (Proder & Ramachandran, 2013; Lang *et al.*, 2017). Although the joint research project comprised five study sites, only three of these sites contained

streams that were classified as coarse-material-rich, siliceous low mountain streams (Type 5, Pottgiesser & Sommerhäuser, 2008), which were therefore considered comparable. Based on bedrock material (Proder & Ramachandran, 2013; Prietzel *et al.*, 2016), stocks of soil total P and soil plant available P (P_i ; Lang *et al.*, 2017), our study sites lie on a geogenic gradient of P availability (Table 8).

Table 2 Terrestrial parameters of the study sites. All data were collected at the specific SPP-1685 sites except for bedrock P in ppm (Porder & Ramachandran, 2012; raw data: EarthChem.org). ‘Stand age’ is the age of the forest stand on the specific site. Coordinates, elevation, and slope are given for the stream sampling sites, and not for the SPP core sites.

Site parameters	Black Forest	Bavarian Forest	Thuringian Forest
Geographical coordinates	48°01'01''N, 7°57'58.3''E	48°58'28.4''N, 12°51'11.6''E	50°36'16''N, 10°46'41''E
Elevation sampling site (m.a.s.l.)	740	740	720
Mean annual temperature (°C air) ^a	6.8	4.5	5.5
Precipitation (mm p. a.) ^b	1749	1299	1200
Slope (sampled stream stretch)	26%	11%	16%
Stand age (years) ^b	132	131	123
Tree species ^b	<i>Fagus sylvatica</i> , <i>Picea abies</i> , <i>Abies alba</i>	<i>Fagus sylvatica</i> , <i>Picea abies</i> , <i>Abies alba</i>	<i>Fagus sylvatica</i>
Bedrock ^b	Paragneiss	Paragneiss	Trachyandesite
Bedrock P ppm (mode) ^c	262 (Gneiss)	262 (Gneiss)	1920
Bedrock P (mg P g ⁻¹) ^d	0.29	1.38	n.a.
Soil type ^b	Dystic Skeletic Cambisol	Hyperdystic Chromic Folic Cambisol	Hyperdystic Skeletic Chromic Cambisol
Soil total P stock (g m ⁻²) up to 1 m soil depth ^b	231	678	468
Soil P_i stock (g m ⁻²) up to 1 m soil depth ^b	42	105	132
Soil total nitrogen stock (kg m ⁻²) up to 1 m soil depth ^b	0.8	1.4	1.1

^a<https://www.ecosystem-nutrition.uni-freiburg.de/standorte> (Black Forest = Conventswald; Bavarian Forest = Mitterfels; Thuringian Forest = Vessertal)

^bLang *et al.* (2017)

^cPorder & Ramachandran (2012)

^dPrietzel *et al.* (2016)

More precisely, the sites showed a deviating order along two terrestrial gradients in phosphorus availability: 1) soil P_i gradient: Black Forest < Bavarian Forest < Thuringian Forest, and 2) total P stock gradient: Black Forest < Thuringian Forest < Bavarian Forest. We expected that the gradient in soil P_i stocks is relevant for P availability in the stream because P_i is more readily water soluble and as such more likely to be leached from the soil and transported into the streams than total P (Sharpley *et al.*, 1994; Vadas *et al.*, 2005; Yu *et al.*, 2006). Therefore, only the soil P_i stocks were considered in the analysis.

Sampling was conducted in spring (April) and summer (July) 2014. As light can be considered an important factor shaping the benthic communities in streams (Steinman, 1992; Hill *et al.*, 1995; Cashman *et al.*, 2013), the stream reach leaving the forest was sampled at all sites to compare forest and meadow sections within a continuous stream reach. Therefore, at each study site, the sampled stream reach was subdivided into a forested section (upstream) and a meadow section (downstream); the sections were sampled separately in both seasons. These sections formed a continuous stream reach of up to 350 m total length without any inflowing watercourses within each stream. Therefore, we expected that differences in the benthic communities between the types of land use would be mainly based on changes in irradiance. Differing distances of the forest edge to the source region of each stream resulted in differences in the stream order. The distances of the sampled stream reaches to their source regions were approximately 335 m, 440 m and 1.7 km at the Black Forest site, Thuringian Forest site, and Bavarian Forest site, respectively. The streams of the Black Forest, Thuringian Forest and Bavarian Forest site were of first, second, and third order, respectively (Strahler, 1957; Fig. 6). All three streams were slightly carved into the landscape and were straight and narrow (< 1 m), except for the upper (forested) stream section at the Thuringian Forest site, which had a broader stream bed (up to 2 m). At all sites, the only macrophytes growing in the streams were aquatic mosses on large boulders or rocks that maintain a stable position within the streams. The meadows adjacent to the lower section were extensively used as pastures for cattle or horses but were not grazed during the sampling period. During summer sampling, the meadow sections of all streams were completely shaded by riparian vegetation, i.e. tall grasses and perennial herbs. All streams had an approximately neutral pH [pH 7.6 ± 0.2 , 7.2 ± 0.2 , and 7.2 ± 0.2 (mean \pm SD, n = 4), Black Forest, Thuringian Forest, and Bavarian Forest sites, respectively] and were nearly oxygen-saturated (>90% O₂). The study sites were sampled in each sampling period according to the mean annual temperature (Table 8) so that invertebrates sampled at all sites were in comparable developmental stages. Sampling started at the Black Forest site and ended at the Bavarian Forest site, with a time-lag of approximately ten days between samplings for each sampling period.

Periphyton

Periphyton samples were split into quantitative samples to determine area-related periphyton biomass, and qualitative samples to estimate periphyton composition and to provide material for stable isotope measurements (used as a baseline, see section on stable isotope analysis below) and for the analysis of periphyton P content. Overall, 120 quantitative samples (20 per study site and season) and 12 qualitative samples (2 per site and season) of periphyton were collected.

The quantitative periphyton samples were collected from ten randomly chosen stones per site, season and type of land use. Periphyton was brushed off the light-exposed parts of each stone as completely as possible using a plastic brush and stream water. The resulting periphyton suspension from each stone was collected in a separate container. The sampled area was determined by wrapping each stone in a single layer of aluminium foil. The sampled area was marked on the foil, the foil outside the marked area was cut off, and the remaining foil was washed, dried and weighed. In the laboratory, the total volume of each suspension was measured in a graduated cylinder. Four 2-ml aliquots of each suspension were centrifuged (5 min at 12,500 rpm; equivalent to $9,800 \times g$; Gusto High-Speed Minicentrifuge, Biozym Scientific GmbH, Hessisch Oldendorf, Germany), the supernatant was discarded and the pellets were shock-frozen in liquid nitrogen at the day of sampling. Pellets were stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. From these quantitative samples, periphyton biomass was estimated as chlorophyll *a* (Chl *a*) concentration per area. Chl *a* was determined from three aliquots per stone via cold ethanol extraction of the homogenized sample and measured photometrically at 665 nm (method described in detail in Mewes *et al.*, 2017).

For the qualitative periphyton samples another 8–12 stones comparable to those used for the quantitative samples were brushed off to form a single pooled suspension from the respective type of land use per site and season. From these suspensions, a sufficient amount of periphyton was centrifuged for subsequent analysis. Five aliquots per site, season and type of land use were taken for stable isotope analysis, two for the determination of periphyton P content, and two for microscopy analysis of periphyton composition. Periphyton pellets were shock-frozen in liquid nitrogen on the day of sampling and stored frozen at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Periphyton P was analysed as SRP using the molybdenum blue method (Legler, 1988) after alkaline digestion (Valderrama, 1981). For each sample, a duplicate of 4–6 mg lyophilized periphyton was suspended in 5 ml millipore water. Then 10 ml of oxidation reagent (1 L containing 50 g potassium peroxodisulfate, 30 boric acid and 350 ml 1M sodium hydroxide; Valderrama, 1981) was added and samples were boiled in a stainless-steel pressure cooker for 30 min. For the determination of SRP, samples were cooled to room temperature, transferred to graduated cylinders, and volumes were adjusted to a total volume of 15 ml using Millipore water. Periphyton %N was determined by stable isotope analysis. Mean %N and %P was calculated from replicates (three and two replicates were analysed per site, season and type of land use for %N and %P, respectively) and converted to molar units to obtain the periphyton N:P molar ratio.

To roughly estimate periphyton composition, the pellets from the qualitative samples were resuspended, and the mean percentage of each taxonomic group (green algae, red algae, diatoms, cyanobacteria and lichens) in the suspension was estimated relative to the total area covered by these groups on the slide (which was set to 100% in each microscopic field of view) using bright field microscopy (400 × magnification). The mean percentage of each group was estimated from 50 fields of view. From this mean percentage of each taxonomic group per site, season and type of land use their respective proportion of the total periphyton biomass at the specific site, season and type of land use was calculated as $\mu\text{g Chl } a \text{ cm}^{-2}$ of stone surface.

Benthic macroinvertebrate community

At each of the three study sites, quantitative samples of the macroinvertebrate community were collected from two pools and two riffles per type of land use (forest, meadow), resulting in eight samples per site and season. The two samples from each habitat type (pool or riffle) were pooled according to type of land use and season, resulting in four samples per site and season and 24 quantitative macroinvertebrate samples in total. Because size and sediment structure (large rocks) of the streams prohibited the appropriate use of a Surber sampler, macroinvertebrate samples were collected by kick sampling of a defined area with a hand net (25 cm × 18 cm, mesh size: spring: 1 mm; summer: 500 μm). A smaller mesh size was chosen in summer due to the higher occurrence of small, early developmental stages of merolimnic stream insects (species with aquatic larval stages) in that season. The area sampled depended on

stream width, substrate and flow characteristics to ensure that all individuals from the sampled area were flushed into the hand net by the current. The sampled area was usually about as wide as the hand net (25 cm) and approximately 30–60 cm in length, depending on the structure used to place the net. After each sampling, the width and length of the sampling area was measured to enable area-related calculations. On average, the sampled area of each pooled sample was $0.182 \pm 0.082 \text{ m}^2$. Coarse debris and sediment were removed from the pooled macroinvertebrate samples, which were then stored in 96% ethanol. At the Black Forest site, two taxa covered by species conservation law [dragonfly nymphs (Anisoptera) and fire salamander larvae (*Salalamdra salamandra*)] were found in some of the macroinvertebrate samples. The fire salamanders were immediately released into the stream, and the dragonfly specimens were sorted from the rest of the sample, placed in a white tray containing some water, measured with a ruler and then released into the stream.

In the laboratory, all macroinvertebrate individuals from each quantitative sample were identified to the lowest practical taxonomical level (mostly species or genus level) and counted and measured to the nearest 0.1 mm under a stereomicroscope (TSO Thalheim Spezialoptik GmbH, Pulsnitz, Germany). For highly abundant taxa, only 50–100 randomly chosen individuals of each taxon were measured and the mean body length was attributed to the remaining individuals of this taxon. The densities of taxa were calculated as individuals per m^2 based on the size of the area actually sampled. The biomass was calculated using published body length or head width per dry mass regressions (Smock, 1980; Meyer, 1989; Burgherr & Meyer, 1997; Benke *et al.*, 1999; Baumgärtner & Rothhaupt, 2003; Hellmann, 2010; Mährlein *et al.*, 2016). The distribution of functional feeding groups was calculated based on individual biomass and the classification provided by Schmidt-Kloiber & Hering (2015) in the freshwater ecology.info database. We calculated specific biomasses for the functional feeding groups ‘grazer’, ‘shredder’, ‘collector’, ‘filter feeder’ and ‘predator’, the remaining biomass belonging to other feeding groups was summarized as ‘other’.

Physiological indicators

For the analysis of the physiological indicators RNA/DNA ratio and triglyceride content additional specimens of the grazer taxon *Baetis* sp., which were abundant in each of the streams, were sampled. These specimens were collected during both seasons (spring and

summer) at each study site and type of land use (forest and meadow) using a hand net. Samples were immediately frozen in liquid nitrogen and stored at $-196\text{ }^{\circ}\text{C}$ until analysis.

In the laboratory, samples were lyophilized, and four to ten individuals were pooled according to sampling site, land use and season to obtain a total biomass of 2.5–6.5 mg dry mass. The pooled samples were homogenized in a 2-ml reaction vial using a glass rod pre-cleaned with HPLC-grade acetone. Approximately 1 mg of each homogenate was used for RNA/DNA analysis; the remainder was used for the determination of triglyceride concentration. DNA and RNA were extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Wisconsin, USA) and measured using a Qubit® 2.0 Fluorometer with the associated kits Qubit™ dsDNA BR Assay and RNA Assay, respectively (Invitrogen™, Life Technologies, Darmstadt, Germany), as described in Normant-Saremba *et al.* (2015). The triglyceride concentration was determined photometrically after hexane extraction of the homogenate following the method described in Winkelmann & Koop (2007). Owing to differences in abundance between sites, type of land use and season, the number of analysed samples varied (see supplement Table S3).

Stable isotope analyses

Stable isotopes of samples of periphyton and specimens of the abundant grazer taxa *Baetis* sp. and *Rhithrogena* sp. were measured. For this purpose, additional specimens of these grazer taxa were sampled per site, season and type of land use and immediately shock-frozen in liquid nitrogen and stored at -20°C until analysis. Owing to differences in abundance between sites and seasons, the number of samples analysed varied (see Table 6). For the stable isotope analysis all samples were lyophilized for 24 h, homogenized using a mortar and weighed into tin capsules (5×9 mm, IVA Anylsentechnik e.K., Germany, animal material: 0.2–1.0 mg dry mass; periphyton: 5.8–9.9 mg dry mass) using a microbalance (Mettler Toledo XS 205 dual range, precision 0.01 mg, Columbus, Ohio, USA). Isotopic ratios of carbon and nitrogen isotopes were measured using a Delta Advantage Isotope Ratio Mass Spectrometer connected to a Flash HT element analyser (Thermo Fisher Scientific, Bremen, Germany). The stable carbon and stable nitrogen isotopic values are presented as δ -values relative to the international reference standards Vienna PeeDee belemnite for carbon and atmospheric

N₂ for nitrogen in units of per mille (‰). Repeated analyses of internal standards (Casein, IVA Analysentechnik, Germany) resulted in a typical measurement precision of $\pm 0.1\text{‰}$ for carbon and $\pm 0.2\text{‰}$ for nitrogen.

Grazer stoichiometry

Grazer %N was obtained from stable isotope analysis and %P was determined analogous to periphyton %P from individual nymphs of the mayflies *Baetis* sp. and *Rhithrogena* sp. ranging between 0.2 and 5.4 mg. Samples > 1 mg were diluted to 25 ml prior to SRP determination. Mean %N and %P were calculated from all samples of a single taxon per site, season and type of land use, and converted to molar units to obtain grazer N:P molar ratio (the number of samples used to calculate these means per taxon, site, season and type of land use is given in supplementary Table S1).

Water chemistry

At each sampling event, the water physico-chemical parameters pH, temperature, conductivity, oxygen concentration and oxygen saturation were measured (SG78–SevenGo Duo pro™ and SG9–SevenGo pro™, Mettler Toledo GmbH, Giessen, Germany) at each study site in the two sections with differing types of land use. In addition, 100 ml water samples (filtered over 0.45 µm cellulose acetate filters, Sartorius AG, Goettingen, Germany) were collected, frozen as soon as possible on the day of sampling, and kept frozen until analysis of phosphate and nitrate concentrations (measured photometrically as SRP using the molybdenum blue method, Legler, 1988, and as NO₃ after DIN EN ISO 13395, 1996 using a continuous flow analyser, CFA, AutoAnalyser 3; Seal Analytical GmbH, Norderstedt, respectively). Data on average nutrient concentrations of stream water from the sites Thuringian Forest (SRP) and Black Forest (SRP, NO₃) were kindly provided by participants of the joint forest ecosystem research project (see Table 9). To obtain an annual mean of stream SRP and nitrate concentrations at the Bavarian Forest site, additional water samples were collected every two weeks between April 2014 and April 2015 (n = 30), and their SRP and nitrate concentrations were analysed as described above.

Statistical analyses

All statistical analyses were conducted using the statistical software R, version 3.4.4 (R Core Team, 2018). To analyse the influence of the environmental factors on the distribution of macroinvertebrate taxa we calculated a distance-based redundancy analysis (dbRDA, *capscale* function, distance: Bray-Curtis, R package *vegan*, version 2.4-6; Oksanen *et al.*, 2016), i.e. a constrained ordination method that allows the use of non-Euclidean distances (Legendre & Anderson, 1999; McArdle & Anderson, 2001). Taxon-specific biomass and presence/absence data of macroinvertebrates were analysed separately. Biomasses were 4th-root transformed prior to the analyses to reduce the effect of very large and/or abundant taxa (Anderson *et al.*, 2011). Presence/absence data per site were corrected by the smallest sampled area (among all samples), and only taxa with an abundance > 0.9 individuals per m² were included so that data used for site comparison based on species occurrence are directly comparable among sites despite differences in the sampled areas. For the dbRDA, the means of absolute values of the environmental factors were standardized to a range between 0 and 1. This prevented a qualitative influence of factors based on differences in numerical ranges and units. In addition to mean soil P_i stocks and mean stream SRP concentration, the environmental factors mean water nitrate concentration, mean water conductivity, season, mean slope, type of land use, habitat, average annual precipitation and average mean annual temperature were included in the analyses. An analysis of similarities (ANOSIM) was calculated to test for significant differences between macroinvertebrate communities based on site, season, type of land use, and habitat (*anosim* function, distance: Bray-Curtis, R package *vegan*, version 2.4-6; Oksanen *et al.*, 2016).

Between-site differences were analysed using a non-parametric ANOVA and post-hoc test (R package *WRS2*, version 0.9-2; Mair *et al.*, 2017) for the variables total biomass of periphyton, total biomass of benthic macroinvertebrates and periphyton N:P molar ratio. Mean biomass per season and type of land use were included in the analysis for each site (n = 4, supplementary Table S2&S3). Differences in occurrence of periphyton primary producer groups and the group-specific biomass of the macroinvertebrate functional feeding groups (grazer, shredder and predator) between sites were analysed using the same method.

Qualitative bottom-up effects of the P availability on the level of individual taxa were analysed by comparing the isotopic niche area of two grazers (*Rhithrogena* sp. and *Baetis* sp.) and the overlap of their isotopic niches between sites. To account for variation between season and type of land use, $\delta^{13}\text{C}$ - and $\delta^{15}\text{N}$ -values were corrected for each grazer individual based on the isotopic values of periphyton, which can be considered their baseline resource, and plotted separately. For each grazer sample, the difference to the mean $\delta^{13}\text{C}$ - and $\delta^{15}\text{N}$ -values of periphyton originating from the respective type of land use per season at each study site was calculated. The maximum-likelihood-fitted standard ellipse areas (SEA_c) of *Baetis* sp. and *Rhithrogena* sp. were determined as a measure of their core isotopic niche, based on the transformed $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values using analyses of the stable isotope Bayesian ellipses in R, implemented in the package SIBER (version 2.1.3; Jackson *et al.*, 2011). The function *maxLikOverlap* within SIBER was used to determine the area of overlap between the SEA_c s of the two taxa. This function calculates the percentage of overlap at each study site as a proportion of the area enclosed by the combined outer lines of both standard ellipses.

Between-site differences in the N:P molar ratio of the mayfly nymphs and the physiological status of individual grazer taxa (body length of *Rhithrogena* sp., body length of *Baetis* sp., *Baetis* sp. triglyceride content, *Baetis* sp. RNA/DNA ratio) were analysed using non-parametric ANOVAs with subsequent post-hoc tests (R package WRS2, Mair *et al.*, 2017). Means per site, season and type of land use were used as replicates in the analyses (supplementary Table S2&S3). Prior to the non-parametric ANOVA, the %N and %P values of mayfly nymphs of the taxa *Rhithrogena* sp. and *Baetis* sp. were compared using a Mann-Whitney U-test. If the results were not significant, the %N and %P values of both taxa were combined to calculate the grazer N:P molar ratios per site, season and type of land use.

Results

P availability

Contrary to our expectations, stream water SRP (Table 9) was unrelated to catchment stocks of inorganic P (bedrock P and soil P_i stocks, Table 8). Therefore, the ranking of the study sites differed between the P sources under consideration. In the order of increasing stream water SRP, the sites were ranked as Bavarian Forest < Thuringian

Forest < Black Forest. The ranking by increasing soil P_i stocks was Black Forest < Bavarian Forest < Thuringian Forest.

Table 3 Aquatic parameters of the study sites. The N:P molar ratio was based on N and P from mean water nitrate and SRP concentrations.

Study site	SRP ($\mu\text{g L}^{-1}$)	NO_3 (mg L^{-1})	N:P (molar)	Conductivity ($\mu\text{S cm}^{-1}$)
Black Forest	12.9 ± 3.6^a	3.26 ± 1.03^c	125	77.3 ± 8.3
Thuringian Forest	10.9 ± 3.5^b	5.92 ± 1.05	269	59.5 ± 0.8
Bavarian Forest	8.1 ± 3.0	3.34 ± 0.86	209	32.1 ± 0.7

^aBol et al. 2016 (January 2014 – December 2014, biweekly sampling, n = 24)

^bJulich (May 2014 – February 2017, monthly sampling, n = 24; pers. comm.)

^cData provided by Forest Research Institute Baden-Württemberg, Departement Soil and Environment (as in Bol et al. 2016)

The range of SRP concentrations in stream water covered by the studied streams was small. Consequently, the observed differences in mean stream water SRP concentrations were small (Table 9). Despite a relatively high temporal variability of the water SRP concentrations, there was no clear seasonal pattern (data not shown). Conductivity of stream water, measured during the sampling of stream biota, followed water SRP concentrations (Table 9). Similar to water SRP, water nitrate concentrations (Table 9) appeared to be unrelated to soil total N stocks (Table 8).

Because the N:P molar ratio in stream water was much higher than the Redfield ratio of 16:1 (N:P) at all sites (Table 9), N was probably not limiting. Based on the SRP concentrations, the studied streams could be classified as oligotrophic; in terms of nitrate concentrations they could be classified as mesotrophic (Dodds *et al.*, 1998).

Periphyton

Periphyton total biomass did not significantly differ between sites (Fig. 7A, Table 10, supplementary Fig S1A), which indicated no bottom-up response to differences in P availability. However, site comparisons of the five dominant primary producer groups revealed site-specific differences in the occurrence of cyanobacteria and lichens ($p = 0.004$ and $p = 0.001$, respectively, Fig. 7A, Table 10&11). Cyanobacteria dominated the periphyton assemblage at the Thuringian Forest site and contributed the lowest proportion to total periphyton at the Black Forest site, which indicated that the dominance of cyanobacteria increased with increasing soil P_i stocks. Lichens were the dominant group at the Black Forest site but comprised the lowest proportion of total periphyton at the Bavarian Forest site, which indicated that the dominance of lichens

increased with increasing stream SRP (for details, see supplementary Figure S1). The other three groups did not significantly differ between sites ($p > 0.05$, Table 10). Periphyton N:P molar ratio was high but did not differ between sites (129 ± 26 , $n = 12$, Table 10).

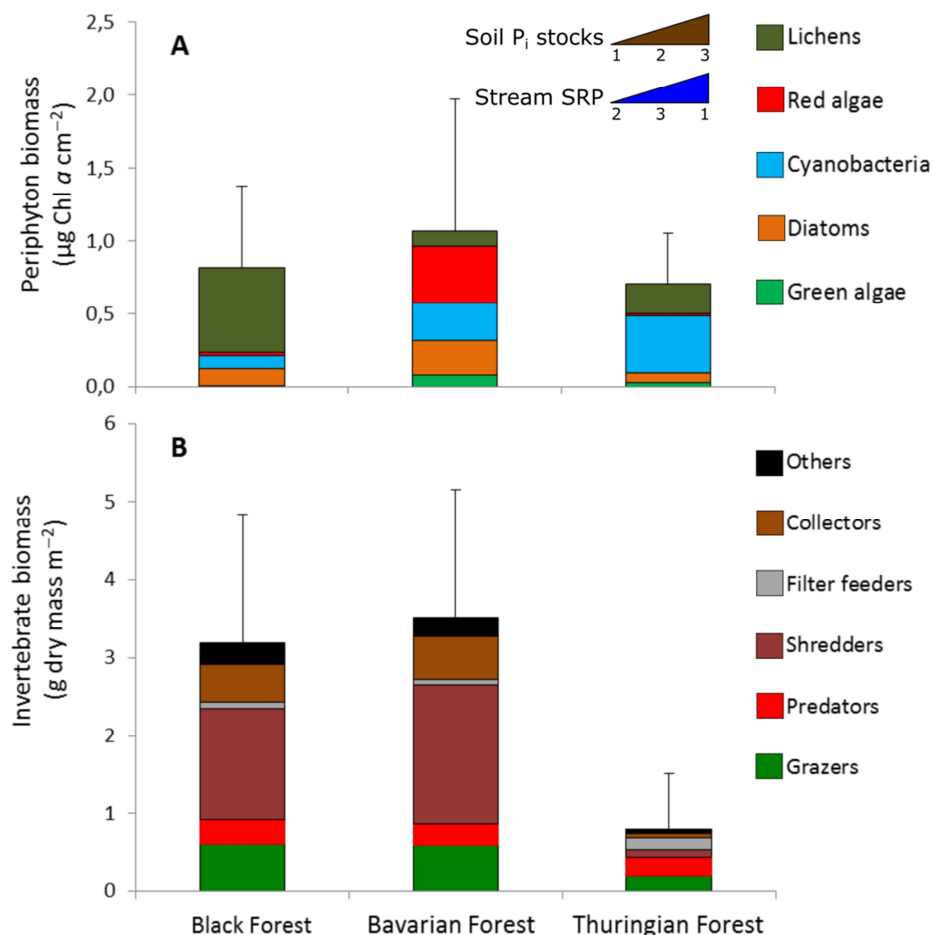


Fig. 4 Periphyton and macroinvertebrate group-specific biomass. (A) Periphyton and (B) macroinvertebrate group-specific biomass at the three study sites. (A) Biomass of benthic periphyton (mean \pm SD; $n = 4$) is shown distributed among the five dominant taxonomic groups of primary producers. Ranking of the study sites by either soil P_i stocks or stream water SRP is indicated at the top right. Site codes: 1, Black Forest; 2, Bavarian Forest; 3, Thuringian Forest. (B) Biomass of benthic macroinvertebrates (mean \pm SD; $n = 8$) is shown distributed across the functional feeding groups. Biomass attributed to ‘Other’ is the biomass that was not attributed to any of the specified feeding groups.

Benthic macroinvertebrate community

Significant differences in biomass for total macroinvertebrates, grazers and shredders were detected between sites (Fig. 7B, Table 10&11, supplementary Fig. S1H–J). The biomass at the Bavarian Forest site was significantly higher than the biomass at the Thuringian Forest site; total macroinvertebrate biomass and grazer biomass at the Black Forest site did not significantly differ from the other two sites. Shredder biomasses at

the Bavarian Forest site and the Black Forest site were similar; the significant difference to the Thuringian Forest site was mainly based on the absence of the elsewhere highly abundant amphipod shredder *Gammarus fossarum/pulex* at this site (supplementary Table S4). This indicated that differences in macroinvertebrate biomass were unrelated to the differences in either soil P_i stocks or water SRP. Likewise, species richness differed between the streams, but was unrelated to either stream SRP or soil P_i stocks. A total of 103 benthic macroinvertebrate taxa were found, 29 of which were present at all study sites. The benthic macroinvertebrate community at the Black Forest site had the highest number of taxa (85 taxa); the number of taxa found at the other two study sites was lower but similar to each other (Thuringian Forest: 50 taxa, Bavarian Forest: 51 taxa, for abundance data, see supplementary Table S4).

Table 4 Between-site comparison. Results of the non-parametric ANOVAs as p-value and effect size. Mean values per site, season, and type of land use were used as replicates in the analysis (n, for n per site see supplementary Table S1). Shredder biomass was square root transformed for the analysis, as indicated by (sqrt). Significant p-values (< 0.05) are shown in boldface.

Response	p-value	Effect size	n
Periphyton biomass ($\mu\text{g Chl a cm}^{-2}$)	0.783	0.39	12
Periphyton N:P (molar)	0.179	0.58	12
% diatoms	0.223	0.93	12
% green algae	0.446	1.79	12
% red algae	0.140	0.95	12
% cyanobacteria	0.004	1.04	12
% lichens	0.001	0.85	12
Invertebrate biomass ($\text{mg dry mass m}^{-2}$)	0.042	1.03	12
Grazer biomass ($\text{mg dry mass m}^{-2}$)	0.038	0.61	12
Shredder biomass (sqrt) ($\text{mg dry mass m}^{-2}$)	0.007	0.90	12
Predator biomass ($\text{mg dry mass m}^{-2}$)	0.787	0.30	12
Grazer N:P (molar)	0.079	0.66	11
<i>Baetis</i> sp. triglycerides ($\mu\text{mol g}^{-1}$ dry mass)	0.315	1.20	10
<i>Baetis</i> sp. RNA/DNA	0.155	1.27	10
<i>Baetis</i> sp. body length (mm)	0.317	0.38	12
<i>Rhithrogena</i> sp. body length (mm)	0.255	1.27	9

Table 5 Pairwise site comparisons (post hoc-test). Results of pairwise site comparisons for non-parametric ANOVAs with significant results at the level of $p < 0.1$. Shredder biomass was square-root transformed for the analysis, as indicated by (sqrt). Significant p-values (< 0.05) are shown in boldface.

Response	Post-hoc: site comparison	p-value
% Cyanobacteria	Black Forest vs. Bavarian Forest	0.019
% Cyanobacteria	Black Forest vs. Thuringian Forest	0.005
% Cyanobacteria	Bavarian Forest vs. Thuringian Forest	0.016
% Lichens	Black Forest vs. Bavarian Forest	0.001
% Lichens	Black Forest vs. Thuringian Forest	0.003
% Lichens	Bavarian Forest vs. Thuringian Forest	0.049
Invertebrate biomass	Black Forest vs. Bavarian Forest	0.755
Invertebrate biomass	Black Forest vs. Thuringian Forest	0.055
Invertebrate biomass	Bavarian Forest vs. Thuringian Forest	0.043
Grazer biomass	Black Forest vs. Bavarian Forest	0.953
Grazer biomass	Black Forest vs. Thuringian Forest	0.091
Grazer biomass	Bavarian Forest vs. Thuringian Forest	0.012
Shredder biomass (sqrt)	Black Forest vs. Bavarian Forest	0.637
Shredder biomass (sqrt)	Black Forest vs. Thuringian Forest	0.011
Shredder biomass (sqrt)	Bavarian Forest vs. Thuringian Forest	0.011
Grazer N:P (molar)	Black Forest vs. Bavarian Forest	0.176
Grazer N:P (molar)	Black Forest vs. Thuringian Forest	0.019
Grazer N:P (molar)	Bavarian Forest vs. Thuringian Forest	0.473

The environmental factors included in the dbRDA explained 59% and 56% of the total variance in the data set of macroinvertebrate taxon-specific biomass and taxon presence/absence, respectively (Fig. 8, Table 12). According to the analyses the site-related vectors water SRP concentration and soil P_i stock were redundant to all other vectors related to between site differences, i.e. water conductivity, water nitrate, precipitation, slope, and temperature (Table 12). Altogether, site-related vectors explained 30% and 26% of total variance of macroinvertebrate taxon-specific biomass and taxa presence/absence, respectively (Table 12). The vectors season, habitat and land use were independent of the other vectors and explained 29% and 31% of total variance of macroinvertebrate taxon-specific biomass and taxa presence/absence, respectively (Table 12). The vector land use least explained the variation in macroinvertebrate community composition (Table 12). Moreover, it did not produce significant differences between groups in the ANOSIM ($R = 0.054$, $p = 0.199$, taxon-specific biomass). Site had the strongest influence on macroinvertebrate community composition ($R = 0.658$, $p = 0.001$, ANOSIM), whereas season and habitat had a lower influence on taxa distribution ($R = 0.336$, $p = 0.001$, and $R = 0.246$, $p = 0.002$, respectively, ANOSIM).

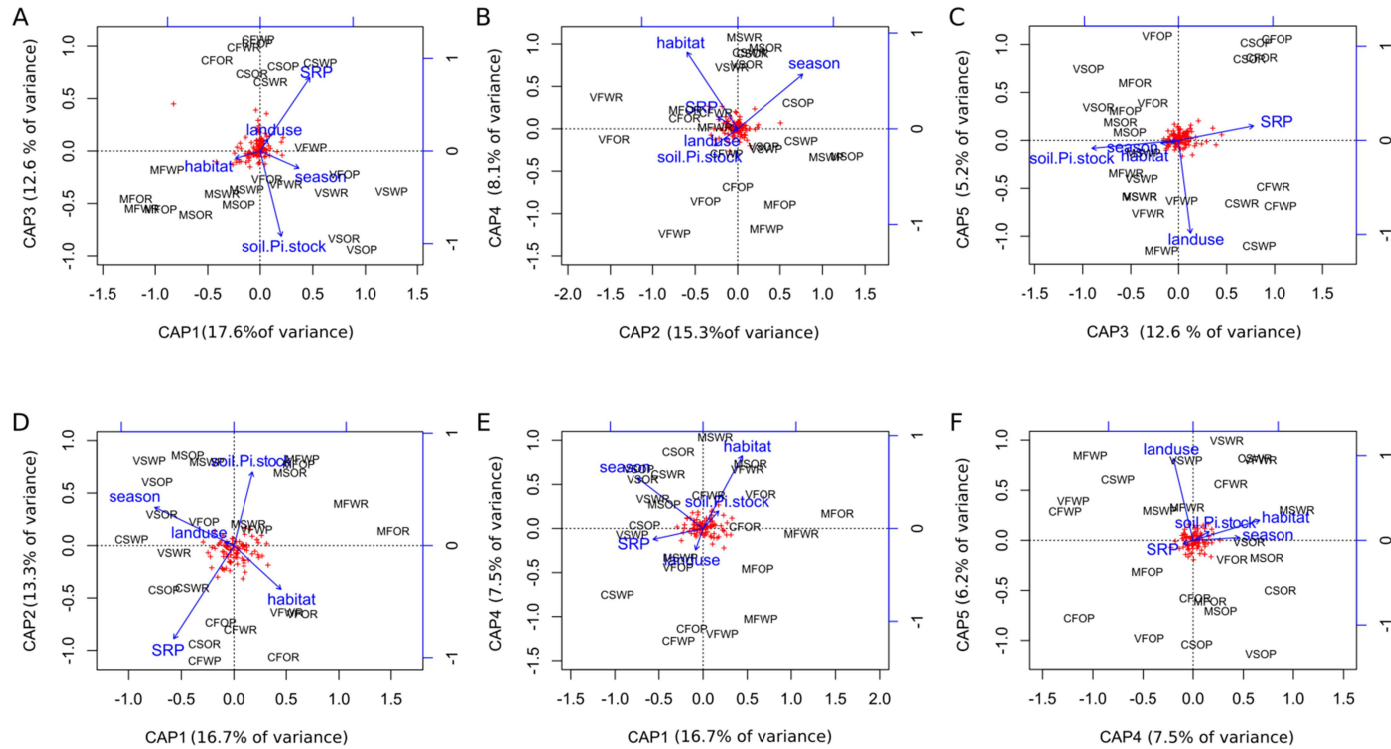


Fig. 5 Macroinvertebrate community distance-based redundancy analysis (dbRDA). Influence of environmental factors on the community composition of macroinvertebrates at the three studied sites, analysed by distance-based redundancy analysis (dbRDA, Bray-Curtis distance). (A-C) Shifts in the community composition based on taxon-specific biomass (4throot transformed). (D-E) Shifts in the community composition based on taxa presence/absence data. The axes were chosen to display the shift best explained by the environmental factors (A,D) SRP and soil Pi stock, (B,E) season and habitat, and (C,F) land use. Red crosses, positions of single taxa; abbreviations, position of the entire community sampled at each site (C, Black Forest; M, Bavarian Forest; V, Thuringian Forest), per season (F, spring; S, summer), type of land use (O, meadow; F, forest), and habitat (P, pool; R, riffle), e.g. CSWP = Black Forest, summer, forest, pool. The terms SRP and soil P_i stock were redundant to all other factors related to differences between sites, i.e. water nitrate, water conductivity, slope, mean annual temperature and annual precipitation.

Table 6 Vector statistics of the distance-based redundancy analysis (dbRDA) for the benthic community measures macroinvertebrate taxon-specific biomass (Fig. 3 A–C) and macroinvertebrate taxa presence/absence (Fig. 3 D–F). Given are the proportions of explained variance by each vector and their p-value. For redundant vectors instead of the explained variance the redundant vector for which the explained variance is given is stated.

Vector	Macroinvertebrate taxon-specific biomass		Macroinvertebrate presence/absence	
	Explained variance	P	Explained variance	P
Season	13%	0.001	14%	0.001
Land use	5%	0.002	7%	0.003
Habitat	11%	0.001	10%	0.001
Water SRP	14%	0.001	15%	0.001
Soil P _i stock	16%	0.001	11%	0.001
Conductivity	SRP		SRP	
Water NO ₃	Soil P _i		Soil P _i	
Precipitation	Soil P _i		Soil P _i	
Slope	Soil P _i		Soil P _i	
Temperature	Soil P _i		Soil P _i	
Residuals	41%		44%	

Individual grazer taxa

None of the four physiological indicators analysed (*Rhithrogena* sp. body length, *Baetis* sp. body length, *Baetis* sp. RNA/DNA ratio, *Baetis* sp. triglyceride content) significantly differed between sites (Table 10). For *Baetis* sp. and *Rhithrogena* sp., the analysed %N and %P were not significantly different ($p > 0.5$) and thus these values were used to calculate grazer N:P molar ratios. Although the overall site comparison of the grazer N:P molar ratio was only significant at the $p < 0.1$ -level ($p = 0.079$), the site comparison revealed significant differences between the grazer N:P molar ratio at the sites Black Forest and Thuringian Forest (N:P 13 ± 2 , $n = 4$, and N:P 20 ± 3 , $n = 4$, respectively, $p = 0.019$, Table 10&11); grazers at the site Bavarian Forest were intermediate and did not significantly differ from any of the other sites (N:P 18 ± 3 , $n = 4$). The results indicate that grazer N:P is unrelated to the P sources considered.

Stable isotope analysis revealed changes in grazer trophic interactions between sites. The isotopic niche area, indicated by the maximum-likelihood standard ellipse area (SEA_c), was largest at the Black Forest site for both species, where SRP was highest, but showed no consistent trend for either species at the two other sites (Fig. 9, Table 13). However, for both taxa, differences between isotopic niche area at the Bavarian Forest site and the Thuringian Forest site were smaller than that at the Black Forest site.

The increase in isotopic niche overlap seemed to be based primarily on changes of the position of the SEAc of *Rhithrogena* sp. in the $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$ space because only this taxon showed a consistent increase in the isotopic niche area with increasing isotopic niche overlap. Similar to the change in isotopic niche area, the change in isotopic niche overlap was highest towards the Black Forest site (Fig. 9, Table 13).

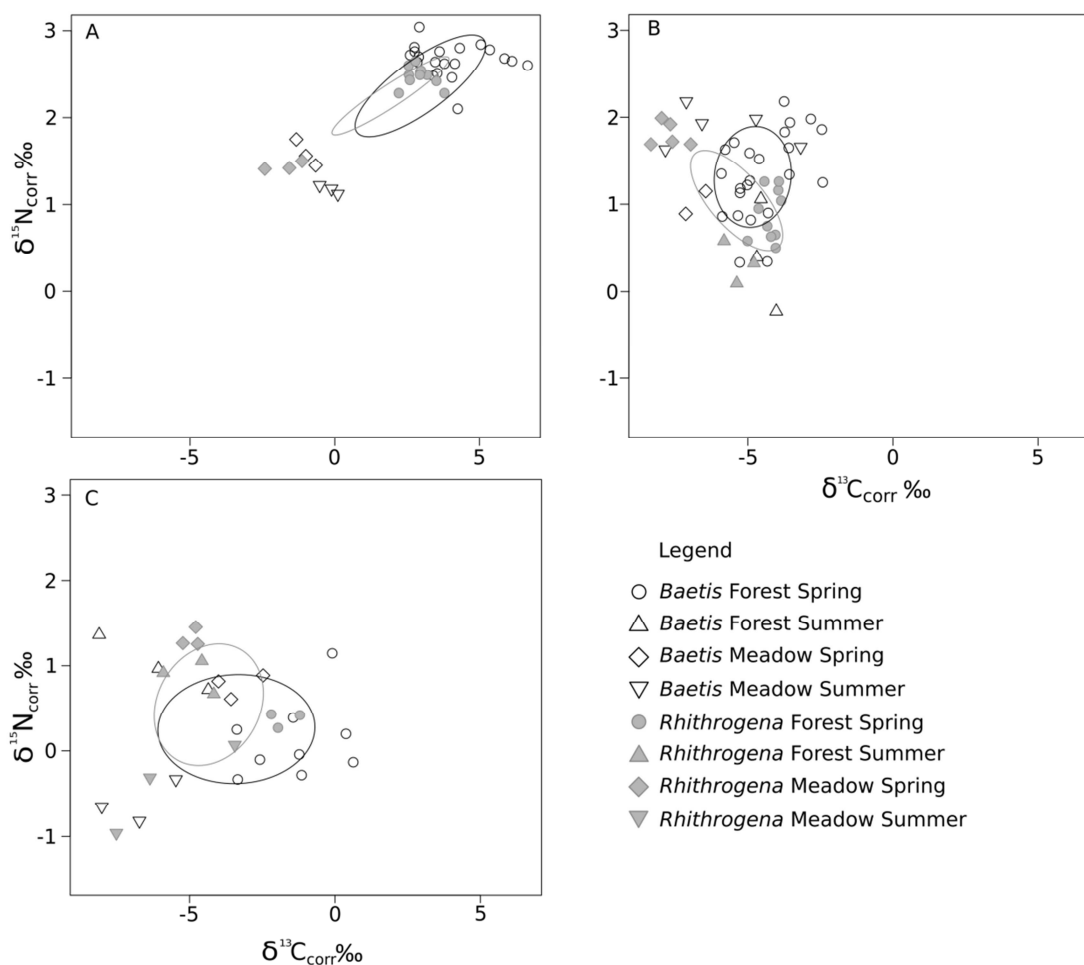


Fig. 6 Grazer isotopic niche overlap. Stable isotope values were transformed as described in the methods section to allow comparison between sites. The niche overlap was calculated using maximum likelihood fitted standard ellipse areas (SEAc). Isotopic niche overlap of grazing nymphs of *Baetis* sp. and *Rhithrogena* sp. at (A) Bavarian Forest 30.37%, (B) Thuringian Forest 32.7%, and (C) Black Forest 45.24%.

Table 7 Grazer isotopic niche overlap (statistics). Isotopic niche area (maximum-likelihood standard ellipse area, SEAc) of the abundant grazer taxa *Baetis* sp. and *Rhithrogena* sp. and the percent overlap of grazer SEAcS at the three study sites. The ratio of overlap is calculated as the area of overlap divided by the combined area of both SEAcS.

Site	Taxon	Season	Type of land use	n	SEAc [% ²]	Combined area of both SEAcS [% ²]	Area of overlap [% ²]	ratio overlap [%]
Bavarian Forest	<i>Baetis</i> sp.	spring	meadow	3	2.66	2.85	0.86	30.37
		summer	meadow	3				
		spring	forest	20				
		summer	forest	0				
	<i>Rhithrogena</i> sp.	spring	meadow	3	1.05			
		summer	meadow	0				
		spring	forest	11				
		summer	forest	0				
Thuringian Forest	<i>Baetis</i> sp.	spring	meadow	2	2.49	3.54	1.16	32.7
		summer	meadow	5				
		spring	forest	23				
		summer	forest	3				
	<i>Rhithrogena</i> sp.	spring	meadow	5	2.21			
		summer	meadow	0				
		spring	forest	10				
		summer	forest	3				
Black Forest	<i>Baetis</i> sp.	spring	meadow	3	5.74	7.07	3.20	45.24
		summer	meadow	3				
		spring	forest	9				
		summer	forest	3				
	<i>Rhithrogena</i> sp.	spring	meadow	3	4.54			
		summer	meadow	3				
		spring	forest	3				
		summer	forest	3				

Discussion

Although nutrient response models fitted to experimental data clearly predict a very strong positive response of periphyton growth rate to increasing nutrient concentrations in the range of 0–20 $\mu\text{g SRP L}^{-1}$ (Rier & Stevenson, 2006; Hill *et al.*, 2009; Winkelmann *et al.*, 2014), it remains unclear whether a phosphate gradient in that range would similarly induce strong bottom-up effects in real stream ecosystems. A simple extrapolation of the results gained in highly controlled laboratory experiments does not seem to be possible because in real and complex ecosystems, bottom-up responses can be attenuated by top-down control or masked by environmental variability (Biggs & Close, 1989; Biggs & Lowe, 1994; Artigas *et al.*, 2013). Nevertheless, large-scale enrichment experiments have shown bottom-up effects in complex lotic food webs (e.g. Peterson *et al.*, 1993b; Peterson *et al.*, 1993a; Davis *et al.*, 2010; Sabater *et al.*, 2011). However, although conducted on a large scale and over long times, these enrichment experiments still represent the observation of an artificially induced, abrupt, temporal trajectory of the ecosystem state. Therefore, we questioned whether such results are transferable to benthic communities that developed under differing natural nutrient conditions.

Because bedrock and soils from the catchment area greatly affect stream water nutrient levels (Dillon & Kirchner, 1975), we expected stream SRP concentrations to match the differences in availability of catchment inorganic P (bedrock and soil). Contrary to this expectation, stream SRP and catchment inorganic P were unrelated, which might partly be explained by the ability of periphyton and leaf-decomposing microorganisms to drastically reduce SRP concentrations in stream water during times of intensive growth (Mulholland, 1992; Mulholland & Hill, 1997; Winkelmann *et al.*, 2014). This strong effect of periphyton was evident from differences in SRP concentrations measured at a single site on the same day in stream sections with different amounts of light and therefore probably different photosynthetic rates. On a sunny day in spring, water SRP concentrations in forested and meadow sections of the Black Forest site strongly differed (16 $\mu\text{g SRP L}^{-1}$ in the shaded forested section and only 5 $\mu\text{g SRP L}^{-1}$ in the open meadow section), whereas the water SRP concentrations on a sunny day in summer with a closed vegetation cover in both sections were more similar (12 and 10

$\mu\text{g SRP L}^{-1}$ in the forested and meadow section, respectively). This indicates that the water SRP concentration in these small headwater streams might instead represent a response variable, which renders it unsuitable as a predictor. However, no seasonal pattern was observed in the water SRP concentration. Therefore, no strong P depletion by seasonally occurring maxima in biological demand was indicated. Moreover, the use of the terrestrial P_i stock as a proxy for P supply of stream ecosystems includes sources of error because a high P_i stock represents only the potential P import. The actual P import is controlled not only by the P_i stock size, but also by redox conditions, soil characteristics (e.g. texture, content of aluminium and iron hydroxides, and content of clay minerals), and by hydrological and climatic patterns (Mulholland, 1992; Kortelainen *et al.*, 2006; Sohr *et al.*, 2018 under review). However, since our aim was to analyse the influence of differences in geogenic P availability on benthic communities of small headwater streams, an elucidation of which processes predominantly determined P availability in the streams was beyond the scope of this study.

We could only include the intermediate sites from the P gradient studied in the forest ecosystem research project (SPP-1685), which might explain why stream water SRP varied only slightly between sites, covering a range of mean SRP concentrations only of 8–13 $\mu\text{g L}^{-1}$. Therefore, it is not surprising that we did not observe any consistent bottom-up regulation neither an increase in standing stocks of producers or consumers nor a shift in the benthic community towards consumers relying on autochthonous resources, especially benthic grazers or their predators (Peterson *et al.*, 1993b; Davis *et al.*, 2010; Artigas *et al.*, 2013).

On the contrary, from a larger perspective the lack of distinct differences in stream water SRP make the streams appear even more comparable because all three streams are headwaters with similar stream morphology in low-impacted, forested catchments of German low mountain ranges with siliceous bedrock. Nonetheless, significant differences in macroinvertebrate biomass and in the community composition of both macroinvertebrates and periphyton were detected between sites. Although the dbRDA implied that the included site-related environmental factors explain a considerable part of the variation in macroinvertebrate community composition (29%), the influence of single, site-related factors could not be determined. Such a weak connection between

biotic response variables and any single environmental factor might be explained by the multitude of factors controlling and affecting community composition and biotic interactions in the field (Feminella, 1996; Arbuckle & Downing, 2002; Heino, 2005; Herlihy *et al.*, 2005; Heino *et al.*, 2007). This multitude of interacting factors often results in inconclusive patterns owing to masking or opposing effects (Allan, 2004; Alexander *et al.*, 2013). Similar observations were made in the study of Lewis & McCutchan (2010), where P availability was only second to other factors in shaping the benthic communities at these low P concentrations. Unfortunately, like Lewis & McCutchan (2010), we were unable to fully identify those factors. Not only the environmental factors identified as potential predictors (season, habitat, mean water phosphate concentration, conductivity, mean water nitrate concentration, soil P_i stocks, mean annual precipitation, slope, and mean annual temperature) are expected to greatly influence the benthic communities of streams, but also the regional species pool, especially when the streams are located in different regions (Shurin & Allen, 2001; Heino *et al.*, 2003).

Another factor shaping community composition is the biotic interaction between organisms within an ecosystem (Menge, 1992; Jackson *et al.*, 2001). Indeed, in our study, both isotopic niche area and overlap of dominant grazer taxa appear to depend on both bottom-up related resource availability and biotic interactions. We observed an increase in the overlap of the isotopic niches of the mayfly nymphs of *Rhithrogena* sp. and *Baetis* sp. at the Black Forest site. This could be interpreted as a result of higher diversity and a higher quantity of available resources, i.e. periphyton (Layer *et al.*, 2013). However, the larger niche overlap might represent a stronger competition between the grazer taxa at the Black Forest site owing to strong food limitation. When we compared the food availability represented by the amount of ingestible periphyton and the grazer biomass, we identified a potentially severe food limitation at the Black Forest site. A high grazer biomass coincided with a low food supply despite a comparable total periphyton biomass because the periphyton was dominated by lichens. Although we did not find any literature comparing the feeding preferences of grazers for benthic algae and aquatic lichens, we suspect that lichens are of low food quality because of their growth form (crustose and very firmly attached, which prevents easy ingestion) and potentially of low nutritional quality. In comparison, the Bavarian Forest

site had the most even distribution between periphyton primary producer groups, with an overall macroinvertebrate and grazer biomass comparable to those of the Black forest site, such that a less severe food limitation appears likely. Although the periphyton community at the Thuringian Forest site was dominated by cyanobacteria, which have a low nutritional quality (Martin-Creuzburg *et al.*, 2008), the low macroinvertebrate biomass might prevent a severe food limitation of primary consumers at this site. Consequently, food limitation and therefore competition appears lower and similar at the Bavarian Forest and Thuringian Forest sites, which aligns with the inconsistent results for grazer isotopic niche area between taxa at these sites. The suggested varying degree of food limitation based on pronounced differences in periphyton composition rather than in biomass indicates a strong need for further information on food preferences of macroinvertebrate grazers and the nutritional quality of periphyton components such as algae, bacteria and lichens.

Both the combination of periphyton composition with the macroinvertebrate biomass and grazer isotopic niche overlap provide evidence that the periphyton at the Black Forest site experienced the highest grazing pressure. Especially the composition of heavily grazed periphyton might not reflect the composition of the resources actually ingested if grazers selectively fed, either actively by selecting prioritized resources or passively by sparing inedible components (Lamberti & Resh, 1983; Wellnitz & Rader, 2003; Evans-White & Lamberti, 2005, 2005).

A large proportion of the variability within our data remained unexplained, even though we assumed that the sites were comparable. Nevertheless, our results suggest a strong linkage between bottom-up and top-down controls in the studied benthic communities. These results underline the difficulty in inferring a specific benthic community structure from general environmental conditions.

Conclusions

In ecology and ecotoxicology, experiments and field studies complement each other by exposing underlying processes and mechanisms and revealing general patterns and their limits. When these approaches are combined by transferring experimental results to field ecosystems, two main aspects need to be considered: 1) the characteristics and origin of the communities used in experimental studies, and 2) the communities and

environmental characteristics of the field sites. If these aspects are found to be comparable, the results are likely to be transferable. The same applies in the development of protection and management strategies, which need to account for differences in vulnerability of particular ecosystems or ecosystem compartments.

Acknowledgements

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General Discussion

In the three studies contributing to this thesis, I successfully demonstrated that the RNA/DNA ratio is a suitable proxy for periphyton growth rate, that the fatty acid composition of grazing mayfly nymphs responds to changes in fatty acids provided by the diet after only two weeks, and that these changes are apparent in both membrane and storage lipids. I was able to reveal gaps in the understanding of the linkages between catchment and in-stream phosphorus availability under near-natural conditions because catchment phosphorus availability was found to be an inadequate predictor of stream SRP concentrations and benthic community composition. Furthermore, I showed that seemingly comparable headwater streams had significantly different benthic communities. These differences most likely affect in-stream processes and stream biotic responses to environmental changes, e.g. climate change, and newly introduced anthropogenic stressors, e.g. nutrients, pesticides and drug residues.

Although my results show that the RNA/DNA ratio is a suitable proxy for periphyton growth rate, the application appears to be limited to controlled experiments until a deeper understanding of the processes and factors leading to the observed differences in the relationship between RNA/DNA ratio and growth rate of different periphyton assemblages is achieved (**study 1**). Therefore, this measure was not included in the comparison of the three headwater streams in study 3. It is important to point out that the RNA/DNA ratio provides an estimate of periphyton growth useful in comparisons but cannot be used to measure exact growth rates. Nonetheless, even in this limited application, the RNA/DNA ratio as a proxy for periphyton growth can be a useful extension of the already applied endpoints, i.e. measures used to detect potential effects of the analysed substances or treatments, in ecotoxicological and ecological studies allowing the inclusion of this ecologically important process in simple experimental study designs. This can be illustrated by the following example:

In an experiment where invertebrate grazer biomass was indirectly controlled by the exclusion of fish, treatments with significantly higher grazer biomass in fish-exlosures resulted in significantly lower periphyton standing stock, measured as chlorophyll *a* (Chl *a*) concentrations per area (Gerke *et al.*, 2018). Unpublished data from this study on periphyton RNA/DNA ratios indicate the possibility of a positive effect of grazing on periphyton growth rate in spite of the reduction of periphyton biomass by grazing. However, due to the very high variability of the data especially in exclosures no significant difference was found (Fig. 10A, $p = 0.136$, Wilcoxon rank sum test).

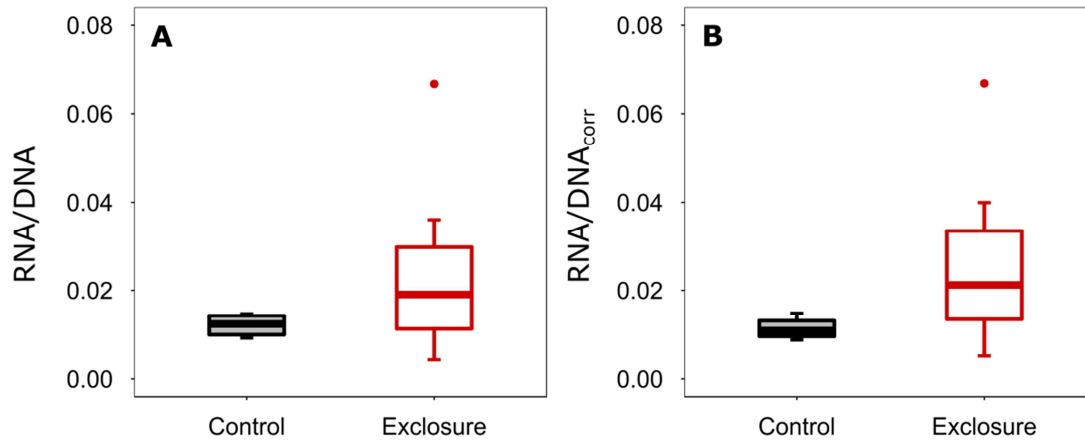


Fig. 7 RNA/DNA ratio of periphyton in experiment III in which a significantly higher grazer biomass and a significantly lower chl a concentration per area were determined in exclosures vs. controls (Gerke *et al.*, 2018). A) uncorrected RNA/DNA ratios, $p = 0.136$, $n = 9$, B) RNA/DNA_{corr} ratios with DNA corrected for proportional deviations from the mean Chl *a*/AFDM (ash free dry mass) ratio and the slope of the linear regression of Chl *a*/AFDW ratio vs. DNA (equation 2), $p = 0.063$, $n = 9$. Boxes: 75 and 25%, whiskers: 95 and 5%, dots: outliers. (Madlen Gerke, unpublished data)

It might be speculated that the effect of grazing pressure on periphyton RNA/DNA ratio was partially masked by the high variability of ash free dry mass (AFDM) in the periphyton from exclosures (Gerke *et al.*, 2018) because large amounts of detrital DNA lower the RNA/DNA ratio independently of growth rate. To reduce such effects, in study 1 I recommended a correction of the RNA/DNA ratio using the Chl *a*/AFDM ratio or Chl *a*/C ratio depending on availability. To test the feasibility of that correction, I reanalysed the data and corrected the DNA content by the proportional deviation of the sample Chl *a*/AFDM ratio from the overall mean of the Chl *a*/AFDM ratio within the data set. Since such a correction would assume a regression with a slope = 1 of Chl *a*/AFDW and DNA, this deviation was corrected by the calculated slope of the linear regression of $\mu\text{g Chl } a/\text{mg AFDW}$ vs. DNA (term *b* in eqn 3).

$$DNA_{corr} = DNA + DNA \times \left[\frac{\left(\frac{Chl\ a}{AFDM} \right)_{sample} - \left(\frac{Chl\ a}{AFDM} \right)_{mean}}{\left(\frac{Chl\ a}{AFDM} \right)_{mean}} \div b \right] \quad (3)$$

Prerequisite for the applicability of this correction is a linear correlation of DNA and Chl *a*/AFDW ratio. In the dataset from the experiment described in Gerke *et al.* (2018) this prerequisite was met ($p < 0.001$, $r = 0.534$, Pearson's product-moment correlation). The use of the recommended correction using the Chl *a*/AFDW ratio made the effect of increased macroinvertebrate grazing on periphyton RNA/DNA ratio clearer. This effect now becomes significant at the $p < 0.1$ -level indicating a trend within the data set (Fig.10B, $p = 0.063$, Wilcoxon rank sum test). The observed high variability in the exclosures probably derived from the high variability in grazer biomass in the exclosures compared to the controls (Gerke *et al.*, 2018).

Besides showing the usefulness of the recommended correction, this example illustrates one possible application of the RNA/DNA ratio as proxy for periphyton growth rate in controlled ecological experiments. The inclusion of periphyton growth rate by using the RNA/DNA ratio can reveal periphyton-grazer interactions that would have remained hidden if solely periphyton biomass had been analysed. The trend towards a higher RNA/DNA ratio in exclosures with increasing grazing pressure by macroinvertebrate grazers indicates an increasing periphyton growth rate in response to grazing. At first glance, this finding seemingly contradicts other studies reporting reduced periphyton productivity in response to grazing. However, the negative effect of grazing on primary production is mainly derived from measurements of area-specific primary production (Jacoby, 1987; Hill *et al.*, 1992a; Rosemond *et al.*, 1993). The thus measured periphyton primary production is directly influenced by periphyton biomass per area so that this area-specific primary production is generally reduced by grazing due to the concomitant reduction of periphyton biomass (Feminella & Hawkins, 1995). The RNA/DNA ratio, on the contrary, is a biomass-specific measure because DNA per cell is relatively constant so that DNA can also be used as a measure of biomass (Dortch *et al.*, 1983). Therefore, the effects of grazing on the RNA/DNA ratio are more likely comparable to the effect of grazing on the biomass-specific primary production. The biomass-specific primary production can easily be calculated if periphyton biomass per area is determined subsequently to the area-specific primary production. On the biomass-specific primary production, a positive effect of grazing can be observed (Lamberti & Resh, 1983; Stewart, 1987; Gelwick & Matthews, 1992). This positive effect is more likely to occur under non-limiting growth conditions which usually result in thicker periphyton mats under low-grazing conditions (Feminella & Hawkins, 1995). In these thick periphyton mats the upper algal layers reduce nutrient diffusion and light penetration (self-shading) towards the lower algal layers and consequently limit their growth (Feminella & Hawkins, 1995). Therefore, increased periphyton growth rates under high grazing pressure if neither light nor nutrients are limiting, as observed for instance in the experiment of Gerke *et al.* (2018), are likely a consequence of increased light and nutrient availability throughout the periphyton matrix that is thinner due to grazing. Following this line of thought, an increased periphyton growth rate in response to high grazing pressure most likely also affects periphyton stoichiometry and potentially increases nutrient and matter cycling within the stream food web (Lamberti & Resh, 1983; Elser *et al.*, 1996; Ågren, 2004). In fact, by removing dead or senescent algal cells grazers appear to maintain periphyton in productive, early successional stages (Lamberti & Resh, 1983; Jacoby, 1987). These early stages are often composed

of large proportions of diatoms which are of high nutritional value to grazers (De Nicola *et al.*, 1990; Poff & Ward, 1995).

In **study 2**, I assessed the effect of short-term changes in available fatty acids within diets of different food quality (diatoms and green algae) on grazer fatty acid composition. The effect of food quality on mayfly nymphs was most apparent in the relative amounts of the physiologically important long-chain polyunsaturated fatty acids (LC-PUFAs) ARA and EPA and their precursor fatty acids LIN and ALA. Mayfly nymphs fed on green algae contained higher amounts of LIN and ALA in their body tissue, whereas mayfly nymphs fed on diatoms contained higher amounts of ARA and EPA (study 2). Based on these results, changes in periphyton composition due to changes in land use and nutrient availability favouring green algae could reduce the availability of LC-PUFAs in aquatic systems and their export to adjacent terrestrial habitats. Conditions favouring green algae include higher light and nutrient availabilities, as observed in most agricultural or urban streams (Bunn *et al.*, 1999; Allan, 2004). Although some consumers are capable of synthesizing these LC-PUFAs from their C18 precursor fatty acids by elongation and desaturation processes, the conversion efficiency is often too low to meet physiological requirements (Tocher, 2010; Twining *et al.*, 2018). Therefore, direct and indirect anthropogenic impacts may lead to a reduction in the availability of LC-PUFAs in aquatic ecosystems. Subsequently the ability of aquatic ecosystems to export these LC-PUFAs to adjacent terrestrial habitats would be reduced too. This reduced availability of LC-PUFAs could have far-reaching and unpredictable consequences for both aquatic and terrestrial food webs (Gladyshev *et al.*, 2009).

Exemplary, I quantified the trophic transfer of the physiologically important LC-PUFAs ARA and EPA (Fig. 11) based on the results of study 2. This trophic transfer was evaluated for the trophic link between the diatom biofilm and the storage (NL) and membrane lipids (PL) of mayfly nymphs. For the green algae biofilm the trophic transfer of ARA and EPA could not be evaluated, since *Stigeocolnium* sp. does not contain any LC-PUFAs (Liu *et al.*, 2016, study 2). The trophic transfer and enrichment of EPA was almost 7-times higher than that of ARA. This lower transfer or retention of ARA compared to EPA in consumer tissue seems related to the amounts of these fatty acids required to meet physiological demands. While EPA is an essential constituent of mayfly membranes (EPA content in membranes was on average 5-times higher than ARA content in the analysed mayfly nymphs; Sushchik *et al.*, 2003; study 2), ARA is the most important precursor of eicosanoids which play an important role e.g. in insect

reproduction but are active at very low concentrations (Bell & Sargent, 2003; Stanley, 2006). Consequently, EPA is required in large quantities to enable somatic growth while ARA is required in lower amounts.

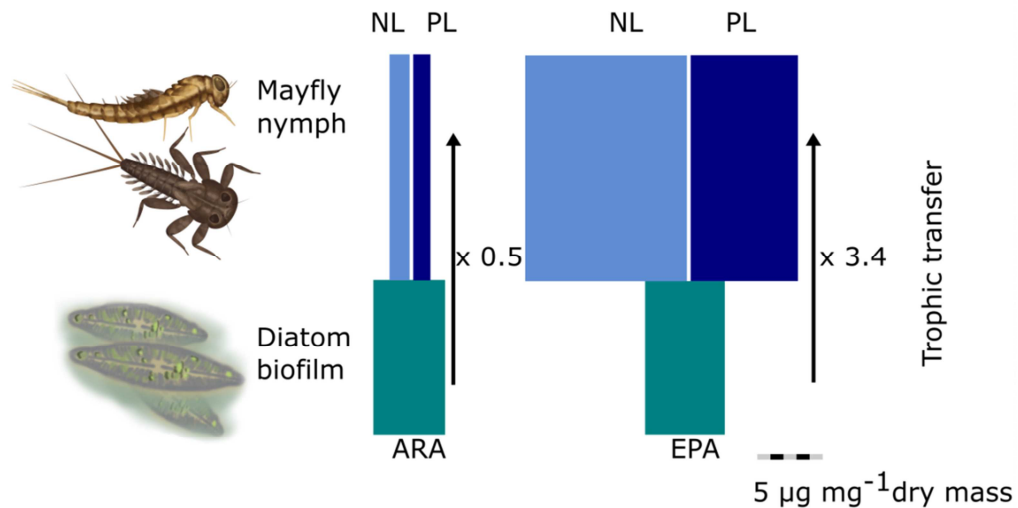


Fig. 8 Trophic transfer of the LC-PUFAs arachidonic acid (ARA) and eicosapentaenoic acid (EPA) from aquatic diatom biofilm ($n = 2$) to the storage lipids (neutral lipids, NL) and membrane lipids (phospholipids, PL) of aquatic mayfly nymphs (*Baetis* sp. + *Rhithrogena* sp., $n = 12$). Arrows show the factors by which ARA and EPA are retained in mayfly nymphs relative to their amount in the diatom biofilm. Presented values are means, which are relative to the scale at the bottom right.

With an enrichment of EPA, grazing mayfly nymphs are an important first step in the trophic upgrading by approximating the fatty acid demand of consumers at higher trophic levels more closely than the basal resource. Characteristic for most aquatic consumers is a higher demand for Omega 3 (n-3) fatty acids, such as EPA and DHA, compared to Omega 6 (n-6) fatty acids, such as ARA (Copeman *et al.*, 2002; Bell & Sargent, 2003; Hixson *et al.*, 2015).

However, besides affecting stream food webs via food quality, land use change and eutrophication can change benthic community composition directly or indirectly due to increased anthropogenic nutrient import (Huryn *et al.*, 2002; Chambers *et al.*, 2006). It is however difficult to quantify and predict these effects due to highly complex trophic interactions and environmental effects. Studies analysing the concentration-effect relationship of nutrients on periphyton and benthic grazers over a wide range of concentrations generally found a pronounced bottom-up effect on either periphyton or grazer biomass (Bourassa & Cattaneo, 1998; Francoeur, 2001). In addition, there is evidence for the influence of catchment geology on stream nutrient concentrations, which is also derived from studies investigating large gradients (Dillon & Kirchner, 1975; Krueger & Waters, 1983). In **study 3**, I analysed if predictions made from such

broad range and large-scale studies are adequate to predict stream soluble reactive phosphorus (SRP) based on catchment phosphorus availability and if stream SRP is a good predictor for stream benthic community structure and grazer performance. Despite general relationships between nutrient availability and the structure of stream benthic communities, and between catchment geology and stream nutrient availability, my results suggest that these relationships are much weaker when only a small range of these gradients is considered (Lammert & Allan, 1999). Study 3 included only catchments with siliceous bedrock containing similar amounts of phosphorus which resulted in similar phosphorus availabilities within the catchment soils (Zavišić *et al.*, 2016; Lang *et al.*, 2017) and within the studied streams (study 3). Although the SRP concentrations of the studied streams lay on the lower limb of the nutrient response curve ($< 20 \mu\text{g SRP L}^{-1}$), a range for which models predict a steep, nearly linear biomass response of periphyton, no consistent nutrient-related bottom-up effect was detected. My results align with the results of Lewis & McCutchan (2010), who found that nutrients play only a secondary role in shaping the benthic communities of mountain streams with such low nutrient concentrations. However, in contrary to the study by Lewis & McCutchan (2010), who studied streams at a broad range of elevations (1200-3600 m.a.s.l.), the streams examined in study 3 were similar with respect to altitude, catchment land use, and stream morphology. Since a dominant effect of the limiting nutrient (phosphorus) on the benthic community could not be detected, these streams would have been expected to have very similar benthic communities. However, benthic communities differed clearly in both the periphyton and macroinvertebrate community composition. This observation together with the analysis of grazer isotopic niche overlap, seem to indicate stream-specific differences in the strength of biotic interactions. These results demonstrate the caveats which concern the transferability of results even between streams with seemingly comparable catchment and morphological characteristics, especially when no information on the biotic community of these streams is available. Such information on community and food web structure appear crucial in the much needed assessment of the vulnerability of a stream community to expected future changes (Clements & Rohr, 2009; Thompson *et al.*, 2012).

Aquatic ecosystems in their natural state often exhibit a balance between simultaneous bottom-up and top-down controls because resources and consumers co-adapted in the framework of abiotic and biotic conditions and their natural variability (Oksanen *et al.*, 1981). In anthropogenically altered ecosystems, the natural balance between bottom-up and top-down controls is often disturbed. High nutrient inputs from agricultural and

urban sources, e.g. agricultural runoff and effluents of waste water treatments plants, often coincide with a reduced habitat quality for aquatic consumers and altered food web structures due the introduction of harmful chemicals. Co-occurring morphological alterations of the stream/river and the introduction of alien species may additionally decrease habitat quality and further alter the aquatic food web (Lake *et al.*, 2000). Even without the additional effects of morphological alterations and alien species, such a scenario typically leads to the phenomenon known as eutrophication. The term eutrophication describes the process in which high nutrient availabilities in aquatic systems result in a mass production of primary producers with partially devastating consequences for the entire system due to chain reactions which ultimately push the system over its tipping point (Smith *et al.*, 1999; Smith, 2003). Eutrophication is listed among the most severe threats to global biodiversity and impairs main functions of aquatic ecosystems, such as water quality for drinking water production, habitat quality, nutrient and matter cycles and assimilative capacity (Millennium Ecosystem Assessment, 2005; Ibsch *et al.*, 2009). As outlined above, the extent of the observed eutrophication is not only a result of nutrient availability. A decreased availability of light due to shading by riparian vegetation can reduce primary production and thereby eutrophication, and is suggested in several studies as countermeasure (Bunn *et al.*, 1999; Hill *et al.*, 2009; Burrell *et al.*, 2014). This approach to mitigate eutrophication only considers the local scale and does not account for the connectedness of lotic systems. Due to the connection of headwater streams to rivers and ultimately to the oceans, all nutrients which are not retained within the local food web will be transported downstream. Increased shading results in a reduced primary production at a given location and will thereby reduce the retention of phosphorus (Sabater *et al.*, 2000). Consequently, the problem of eutrophication will only be relocated to downstream regions which cannot be effectively shaded by riparian vegetation, e.g. large rivers, lakes, reservoirs, estuaries and coastal regions. Grazers may reduce eutrophication if their abundance is sufficient to consume the surplus primary production and thereby contribute to a higher overall productivity of the system (Worm *et al.*, 2000). A high grazing pressure on periphyton of a eutrophic stream or river could not only reduce periphyton biomass below critical levels but could also reduce the amount of filamentous green algae in favour of a higher proportion of diatom taxa (Lamberti & Resh, 1983; Jacoby, 1987). Such a grazer induced shift in periphyton algal composition would also be beneficial for the production, transfer and export of physiologically important LC-PUFAs, which are synthesised *de novo* by diatoms but not by filamentous green algae such as *Stigeoclonium* sp. (Liu *et al.*, 2016). The importance of abiotic

conditions, such as nutrient and light availability and the frequency of disturbances by flood events, for observed eutrophication effects is increasingly well understood (Biggs, 2000; Burrell *et al.*, 2014), whereas the role of biotic interactions in this context is understood less well and their potential in management strategies for running waters is largely unknown (Dodds, 2006). Therefore, a better understanding of these biotic interactions is required to protect our running waters from critical impairments and to implement adequate measures to enable and support their recovery from existing impairments caused by human activities.

Perspectives

By answering three basic questions on relevant links in lotic systems, my results also provide substrate for future research by raising questions about underlying processes. In the following, I will outline possible ways to address some of the questions which resulted from studies 2 and 3.

In **study 2**, I detected a higher EPA/ARA ratio in the tissue of grazing mayfly nymphs relative to the ratio of these fatty acids in the food source (diatom biofilm). The higher EPA/ARA ratio could either be a result of a selective absorption and retention of EPA by the consumer or of a metabolic pathway which ends in the synthesis of EPA and uses ARA as precursor. This pathway, including the desaturation of ARA by $\Delta 17$ desaturase to EPA, is described in eukaryotic algae and fungi (Guschina & Harwood, 2006; Xue *et al.*, 2013) but is, to my knowledge, not described in animals at the base of the food web. The analysis of metabolic pathways of specific compounds, such as ARA, to elucidate their absorption and fate can be achieved by the combination of position-specific ^{13}C labeling with compound-specific ^{13}C analysis, as shown for palmitate (16:0) by Dippold & Kuzyakov (2016). Comparable analysis for the trophic transfer of fatty acids in aquatic food webs could elucidate the process responsible for fatty acid-based trophic upgrading. In addition, insights on fatty acid turnover rates in storage and membrane lipids could be acquired from such studies.

Study 3 revealed gaps in understanding the link between the terrestrial and aquatic phosphorus cycle. Most studies target either the terrestrial or the aquatic phosphorus cycle but only few attempt to link these cycles to explain in-stream nutrient dynamics (Mulholland, 1992; Mulholland & Hill, 1997). However, in headwater streams with low-impacted catchments the combination of catchment and in-stream processes

appears to determine the phosphorus availability within the stream (Mulholland & Hill, 1997). To unravel the sources of the phosphorus available to aquatic primary producers, a combined approach including both geochemical and biological processes is required. For this purpose, various input-pathways and the form in which phosphorus is introduced should be considered. Potential pathways may include springs, groundwater, stream bed sediments, interflow, surface runoff and precipitation. Potential phosphorus forms that should be considered are dissolved inorganic phosphorus, dissolved organic phosphorus, particulate inorganic phosphorus, particulate organic phosphorus and phosphorus bound in plant matter, like leaf litter or riparian vegetation entering the stream in large quantities at the end of the growing season. In addition to the high number of necessary variables, their temporal variability in the light of hydrologic processes, temperature and light availability would need to be documented to identify temporal pattern and cause-effect relationships. Therefore, a well-coordinated, large-scale, and long-term, interdisciplinary research project seems necessary to tackle this question as a whole.

In the context of the questions outlined above, the research presented in this thesis is another single step towards understanding the driving processes in running waters which needs to be followed by many more to reach the ultimate goal of understanding and predicting land-use effects. Only with a profound understanding of these processes will we be able to protect and restore our running waters, the lifelines of this world.

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Supplementary Material

Study 2 — Supplementary material as referenced in the manuscript

Supplementary material M1 — Detailed sample information

The following tables contain detailed information on the number of individual mayfly nymphs included in the experiment (Supplementary Table 1), losses of these individuals during the experiment and causes to these losses (Supplementary Table 2). Moreover, information is given on the individuals included in the fatty acid analysis following the experiment (Supplementary Table 3).

Supplementary Table 1 Number of individuals per species and food source in each step of the experiment. For each species the same number of replicates was analysed per food source and experimental run. Due to the low number of remaining individuals of *Baetis* sp. fed on green algae the number of replicates in the fatty acid analysis per species, food source and experimental run was only two.

Step	Run	Species	Diatom	Green algae	Replicates	max. replicates possible
start	1	<i>Rhithrogena</i> sp.	12	12	6	
start	2	<i>Rhithrogena</i> sp.	12	12	6	
start	2	<i>Baetis</i> sp.	12	12	6	
end	1	<i>Rhithrogena</i> sp.	6	8		d: 3; g: 4
end	2	<i>Rhithrogena</i> sp.	12	11		d: 4; g: 4
start	2	<i>Baetis</i> sp.	8	6		d: 4; g: 2
FA analysis	1	<i>Rhithrogena</i> sp.	4	4	2	
FA analysis	2	<i>Rhithrogena</i> sp.	5	5	2	
FA analysis	2	<i>Baetis</i> sp.	4	5	2	

Supplementary Table 2 Causes for losses of individual mayfly nymphs during the experiment.

Run	Species	Death	Emergence	Escape from box*	Total loss
1	<i>Rhithrogena</i> sp.	4	6		10
2	<i>Rhithrogena</i> sp.	1			1
2	<i>Baetis</i> sp.	4	4	2	10

*Individuals found in the flume outside the boxes. These individuals could not be reassigned to a specific food source and were not analysed.

Supplementary Table 3 Detailed information on the samples analysed for the fatty acid composition of mayfly nymphs.

Food source	Run	Species	Animals per replicate	Dry mass (mg)	Mean body length (mm)
diatom	1	<i>Rhithrogena</i> sp.	2	9,28	11,8
green algae	1	<i>Rhithrogena</i> sp.	2	6,93	10,6
diatom	2	<i>Baetis</i> sp.	2	8,08	15,8
green algae	2	<i>Baetis</i> sp.	3	7,23	12,8
diatom	2	<i>Rhithrogena</i> sp.	3	6,88	9,1
green algae	2	<i>Rhithrogena</i> sp.	3	8,92	10,1
diatom	1	<i>Rhithrogena</i> sp.	2	6,2	10,2
green algae	1	<i>Rhithrogena</i> sp.	2	8,15	11,3
diatom	2	<i>Rhithrogena</i> sp.	2	4,99	9,4
green algae	2	<i>Rhithrogena</i> sp.	2	4,73	9,2
diatom	2	<i>Baetis</i> sp.	2	5,63	13,6
green algae	2	<i>Baetis</i> sp.	2	5,05	13,1

Supplementary material M2 — Detailed method description of the experiment and the fatty acid analysis

Late instar mayfly nymphs of *Rhithrogena* sp. (09.04.2015 and 28.04.2015, mean body length: 10 ± 2 mm) and *Baetis* sp. (28.04.2015, mean body length: 14 ± 2 mm) were collected on the specified dates in a second-order stream in Rhineland-Palatinate, Germany (Hohesteinsbach, N 50.315660, E 7.454931). Mayfly nymphs were kept for two weeks and supplied during that time with a biofilm of either the microalga (diatom) *Navicula pelliculosa* (SGA strain number 1050-3, SAG Culture Collection of Algae, Göttingen, Germany) or the green alga *Stigeoclonium farctum* (SAG strain number 477-19d) as food source. The biofilms were pre-cultured on 5 cm \times 5 cm ceramic tiles in the culture medium described in Mewes *et al.* (2017).

We set up two consecutive experiments conducted 10–24 April and 28 April–12 May in 2015. Transparent, square, 1-L, wide-neck PVC containers (Mould no. 310-32, Kautex Textron GmbH & Co. KG, Bonn, Germany) were each filled with an approximately 2 cm thick layer of clean, baked, coarse gravel for stability. In all four side walls of each container, circular holes (\varnothing 6.5 cm) were cut and covered with mesh (mesh size: 1 mm) to allow water to flow through the container. All containers of an experiment were placed in a single flume with a mixture of 120 L Hohesteinsbach stream water and 80 L deionized water at a water level of about 9 cm and with medium current (for details on flume design, see Mewes *et al.*, 2017). Evaporative loss during the experiment was compensated by adding deionized water. During the first experiment, the measured water parameters were: pH 8.6 ± 0.0 , conductivity $496 \pm 31 \mu\text{S cm}^{-1}$, temperature 15.3 ± 0.8 °C, and oxygen saturation $99.4\% \pm 0.7\%$ (mean \pm SD, n = 10). During the second experiment, the measured water parameters were: pH 8.3 ± 0.6 , conductivity $467 \pm 62 \mu\text{S cm}^{-1}$, and temperature 16.6 ± 1.2 °C (mean \pm SD, n = 7); oxygen saturation could not be measured for technical reasons. The higher average temperature during the second experiment resulted from a sudden change in weather between day four and day six of the experiment, during which the temperature increased from 15 °C to 18 °C, with high temperatures throughout the rest of the experiment (> 17 °C).

Each experiment comprised 6 containers per food source and species, i.e., 6 containers \times 2 biofilms \times 1 species (*Rhithrogena* sp.) = 12 containers for experiment 1, and 6 containers \times 2 biofilms \times 2 species = 24 containers for experiment 2. At the start of the

experiment, two individuals of one of the two species and one tile covered with one of the two biofilms were placed into each container. The tile was replaced with a new biofilm-coated tile as needed so that the animals could feed *ad libitum* during the experiment. At the end of the experiment, all individuals remaining in the containers were collected, placed on cellulose tissue to absorb the water, transferred into 2 ml safe-lock caps (Eppendorf, Eppendorf AG, Germany), and shock frozen in liquid nitrogen. The samples were stored in liquid nitrogen. Losses during the experiments were due to emergence (6 and 4, exp. 1 and 2, respectively), death (4 and 5, exp. 1 and 2, respectively), and escapes from containers (2, exp. 2 only) and added up to losses of 10 and 11 individuals for experiment 1 and 2, respectively.

After each experiment, the fatty acids of two samples of two to three animals each per species and food source, and the fatty acids of one sample of each biofilm resource were analyzed. A total of eight *Rhithrogena* sp. samples, four *Baetis* sp. samples, two *Navicula pelliculosa* biofilm samples, and two *Stigeoclonium farctum* biofilm samples were analyzed. The samples were lyophilized prior to analysis.

Between 4.73 and 9.48 mg dry weight (weighed in on a XS205 DualRange Analytical Balance; Mettler Toledo-AG, Greifensee, Switzerland) of each sample was placed into a conical 5-ml glass centrifuge tube (Pyrex®, Corning Inc., USA) and coarsely minced using a glass rod. Then, 25 μl (1 mg ml^{-1}) 19:0 phospholipid (di-nonadecanoyl-glycerol phosphatidylcholine; Larodan, Solna, Sweden) and 25 μl (1 mg ml^{-1}) 19:0 neutral lipid (tri-nonadecanoyl-triacylglycerol, Sigma-Aldrich) were added to each sample as internal standards to calculate the recovery of phospholipids and neutral lipids. The samples were extracted using the liquid:liquid extraction method described in Gunina *et al.* (2014), with volumes adapted to the smaller sample sizes. Each sample was extracted twice, first with 2.3 ml and then with 0.8 ml of a monophasic mixture of chloroform:methanol:0.15 M citric acid pH 4.0 (1:2:0.8, by vol.) (Frostegård *et al.*, 1991). For the first extraction, the samples were shaken for 2 h (Program C1, 60 rpm, Intelli-Mixer, neoLab Migge GmbH, Heidelberg, Germany) and then centrifuged for 15 min at 2,000 rpm ($841 \times g$, Sigma 4-16, rotor 11650, round bucket No. 13450). The supernatant was transferred into a fresh 5-ml glass centrifuge tube. In the second extraction of the same initial samples, the samples were shaken for 30 min and then centrifuged (same settings as before). The supernatants of each sample were then

combined, 0.8 ml chloroform and 0.8 ml 0.15 M citric acid were added, and the mixtures were shaken for 15 min and then centrifuged for 7 min as above to separate the phases. The lower chloroform phase was collected in a fresh 5-ml centrifuge tube, and the upper methanol phase was extracted a second time with 0.8 ml chloroform. The lower chloroform phase of the second extraction was combined with that of the first extraction, and the volume was reduced to approximately 0.5 ml under a gentle stream of nitrogen.

The total lipids in the chloroform phase were separated into three fractions, namely neutral lipids, glycolipids, and phospholipids, using a 3-ml glass SPE column (Chromabond, Macherey-Nagel GmbH & Co. KG, Düren, Germany) filled with 1.5 cm activated silica gel (heated at 120 °C overnight, particle size 0.063–0.200 mm) in chloroform. The activated silica gel was bordered by glass fiber filters (Chromabond, Macherey-Nagel GmbH & Co. KG) to maintain uniform packing in the column. The raw extract in chloroform was loaded onto the column. Neutral lipids were eluted with 11 ml of chloroform:acetone (95:5 by vol). The addition of 5% acetone was necessary because previous fractionations showed that when only chloroform was used, a high proportion of neutral lipids did not elute and were carried over to the glycolipid fraction, as detected by the recovery of the neutral lipid internal standard in the glycolipid fraction. Glycolipids were then eluted with 11 ml acetone, followed by elution of phospholipids with 17.5 ml methanol. The flow rate for all three elutions was no more than 2 drops per second. Each of the three fractions was concentrated to approximately 0.5 ml under nitrogen, transferred to 5-ml reaction vials (33299 Supelco used with PTFE/silicon septa), and evaporated to dryness under nitrogen for subsequent derivatization.

The fatty acids in the lipid samples were released and derivatized in three steps. First, lipids were hydrolyzed with 0.5 ml 0.5 M NaOH in dried methanol at 100 °C for 10 min to yield free fatty acids. The free fatty acids were then methylated with 0.75 ml BF₃ (14% in methanol, Sigma-Aldrich) at 80 °C for 15 min to yield fatty acid methyl esters (FAMES). Finally, 0.5 ml saturated NaCl was added to reduce the toxicity of BF₃, and the FAMES were extracted three times with 1 ml n-hexane. The three extractions of each sample were combined, and hexane was evaporated under nitrogen. FAME 13:0 (15 µl 1 mg ml⁻¹ in toluene) was added to each sample as a second internal standard,

and an additional 185 μl of toluene was added (total volume = 200 μl) and the sample was ultra-sonicated for 10 min to re-suspend all FAMEs. The FAMEs were transferred to GC vials containing glass inlets and stored at $-20\text{ }^{\circ}\text{C}$ until measurement. In addition to the test samples, five concentrations of an external standard mixtures of 33 free fatty acids plus the phospholipid internal standard, 30 free fatty acids, or 29 free fatty acids plus the neutral lipid internal standard (Table 1), used in the quantification of phospholipid, glycolipid and neutral lipid fatty acids respectively, were derivatized. All solvents used during extraction, fractionation, and derivatization were of HPLC or GC grade, and all glassware was baked at $300\text{ }^{\circ}\text{C}$ for 3 h before use.

Fatty acids were separated and measured on an Agilent Technologies 7890A GC System coupled to a 7000 GC/MS Triple Quad (Agilent Technologies, Santa Clara, CA, USA) in a 10:1 split mode. Helium was the carrier gas, with a column flow rate of 1.18 ml min^{-1} . The inlet temperature was set to $270\text{ }^{\circ}\text{C}$; the injector temperature of $80\text{ }^{\circ}\text{C}$ was held isothermally for 1 min after each injection. Samples of $1\text{ }\mu\text{l}$ were separated on a 15 m DB-1MS GC column (ID 0.25 mm, film thickness $0.25\text{ }\mu\text{m}$, Agilent Technologies) in combination with a 30 m DB-5MS column (ID 0.25 mm, film thickness $0.25\text{ }\mu\text{m}$, Agilent Technologies) with the following temperature program: 80 to $164\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$, 164 to $175\text{ }^{\circ}\text{C}$ at $0.7\text{ }^{\circ}\text{C min}^{-1}$ and held for 2 min, 175 to $196\text{ }^{\circ}\text{C}$ at $0.7\text{ }^{\circ}\text{C min}^{-1}$ and held for 4 min, 196 to $215\text{ }^{\circ}\text{C}$ at $1\text{ }^{\circ}\text{C min}^{-1}$, and 215 to $300\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C min}^{-1}$ and held for 10 min.

Chromatograms were analyzed using OpenChrom (<http://www.openchrom.net>, Wenig & Odermatt, 2010). For each chromatogram (samples and external standards), all peak areas were normalized by dividing by the area of the FAME 13:0 internal standard. The recovery of storage lipids and membrane lipids was calculated using the recovery of neutral lipid and phospholipid internal standards, respectively. As no internal standard for glycolipids was available, we assumed that their recovery was comparable to that of the phospholipid internal standard, as both fractions are composed of polar lipids. Due to difficulties in recovering the neutral lipid internal standard in some of the samples, mean recoveries for both membrane lipids and storage lipids were calculated from samples in which both internal standards could be measured quantitatively ($n = 9$). The resultant mean recovery for either storage lipids or membrane lipids was used to calculate their fatty acid contents. The five concentrations of the external standards were

used for linear calibration of each fatty acid contained in the standard mixture. Fatty acids in samples were identified by comparing mass spectra and retention times to those of the fatty acids contained in the standards and FAME mass spectra provided by W.W. Christie in the archive section of 'The Lipid Web' (<http://www.lipidhome.co.uk/>). Fatty acids in samples that were not contained in the standards were quantified using the calibration equation calculated for the fatty acid(s) with the most similar chain length and degree of unsaturation; for example, 16:4n-3 was quantified using the calibration equation for 16:3n-3, and 18:2n-4 was quantified using the calibration equation for 18:2n-6.

Only the neutral lipids and phospholipids from the mayfly samples were analyzed because these are the major lipid classes in these insects (Cavaletto *et al.*, 2003). Neutral lipids, phospholipids, and glycolipids from algal biofilms were analyzed because the thylakoid membrane is composed mostly of glycolipids with a few mandatory phospholipids, and the other membranes are composed mainly of phospholipids (Garab *et al.*, 2016).

Supplementary Table S1 — External Standards

Supplementary Table 4 Composition of the three external standard mixtures used for quantification of fatty acids. If not otherwise stated fatty acids contained within the standard mixtures are free fatty acids.

Phospholipids	Glycolipids	Neutral lipids
14:0	14:0	14:0
a14:0	a14:0	
i14:0	i14:0	i14:0
15:0	15:0	15:0
a15:0	a15:0	
i15:0	i15:0	i15:0
16:0	16:0	16:0
10Me16:0		
16:1n-5	16:1n-5	16:1n-5
16:1n-7	16:1n-7	16:1n-7
16:2n-4	16:2n-4	16:2n-4
16:2n-6	16:2n-6	16:2n-6
16:3n-3	16:3n-3	16:3n-3
a17:0		
17:0	17:0	17:0
18:0	18:0	18:0
10Me18:0		10Me18:0
18:1n-9	18:1n-9	18:1n-9
18:1n-7	18:1n-7	18:1n-7
18:2n-6	18:2n-6	18:2n-6
18:3n-3	18:3n-3	18:3n-3
18:3n-6	18:3n-6	18:3n-6
18:4n-3	18:4n-3	18:4n-3
19:0 phospholipid		19:0 neutral lipid
20:0	20:0	20:0
20:1n-9	20:1n-9	20:1n-9
20:4n-6	20:4n-6	20:4n-6
20:5n-3	20:5n-3	20:5n-3
21:0	21:0	21:0
22:5n-3	22:5n-3	22:5n-3
22:6n-3	22:6n-3	22:6n-3
	22:0	22:0
23:0	23:0	23:0
24:0	24:0	24:0

Supplementary table 5 Fatty acid content of the analysed samples. Fatty acid content of total lipids ($\Sigma(\text{GLFA}+\text{NLFA}+\text{PLFA})$) of the diatom (*Navicula pelliculosa*) and green algae (*Stigeoclonium farctum*) biofilms. Biomarker FAs for diatoms (16:2n-4, 16:3n-4, 16:4n-1) and green algae (16:2n-6, 16:3n-3, 16:4n-3) are highlighted in the columns of the diatom biofilm and the green algae biofilm, respectively. FA composition of mayfly membrane and storage lipids for mayfly species and food source provided during the experiment. The fatty acid content is given in $\mu\text{g mg}^{-1}$ dry mass (Biofilms and *Baetis* sp.: n = 2, value1 | value2; *Rhithrogena* sp.: n = 4, mean \pm SD).

Fatty acid	Total lipids		Membrane lipid fatty acids				Storage lipid fatty acids			
	<i>Navicula pelliculosa</i>	<i>Stigeoclonium farctum</i>	<i>Baetis</i> sp.	<i>Baetis</i> sp.	<i>Rhithrogena</i> sp.	<i>Rhithrogena</i> sp.	<i>Baetis</i> sp.	<i>Baetis</i> sp.	<i>Rhithrogena</i> sp.	<i>Rhithrogena</i> sp.
	(Diatom biofilm)	(Green algae biofilm)	Diatom biofilm	Green algae biofilm	Diatom biofilm	Green algae biofilm	Diatom biofilm	Green algae biofilm	Diatom biofilm	Green algae biofilm
12:0	0 0	0 0	0 0	0 0	0 \pm 0	0 \pm 0	1,8 0	1,9 1,3	1,2 \pm 0,8	1,7 \pm 0,5
14:0	4 3,2	1,3 2,4	0 1,8	0 2	0,9 \pm 0,9	0,9 \pm 0,9	3,9 4,9	3,4 4,2	4,3 \pm 1,2	4,5 \pm 1,9
15:0	0 0	0 0	0 0	0 0	0 \pm 0	0,3 \pm 0,4	1,5 1,4	1,6 1,4	1,4 \pm 0,8	1,7 \pm 0,3
16:0	8,8 5,4	5 4,3	0,1 5,9	1,2 5,8	2,8 \pm 1,8	2,6 \pm 1,8	25,9 92,1	32,7 78,1	53,8 \pm 34,3	60,1 \pm 26,3
17:0	0 0	0 0	0 1	0,3 1,1	0,3 \pm 0,4	0,6 \pm 0,5	0 0	0 0,9	0,7 \pm 0,8	0,8 \pm 0,6
18:0	0 1,4	0,5 0	0,7 4,5	5 4,3	3,8 \pm 1	3 \pm 0,8	1,9 2,2	2,2 2	1,6 \pm 1	2,3 \pm 0,3
22:0	0 0	0 0,6	0 0	0 0	0 \pm 0	0 \pm 0	0 0	0 0	0 \pm 0	0 \pm 0
24:0	1,3 1,3	0 0,6	0 0	0 0	0 \pm 0	0 \pm 0	0 0	0 0	0 \pm 0	0 \pm 0
i13:0	0 0	0 0	0 0	0 0	0 \pm 0	0 \pm 0	0 0	0 0	0,5 \pm 0,9	1 \pm 0,6
i15:0	0 1,4	0 0	0 0	0 0	0 \pm 0	0 \pm 0	1,7 2,1	1,4 1,7	1,4 \pm 0,8	1,7 \pm 0,4
14:01	0 0	0 0	0 0	0 0	0 \pm 0	0 \pm 0	0 0	0 0	0,9 \pm 0,9	0,7 \pm 0,8
16:1n-5	0 0,2	0 0	0 0,7	0 0	0 \pm 0	0,3 \pm 0,4	1,8 1,9	1,5 1,2	1,7 \pm 1	2 \pm 0,4
16:1n-7	40,1 22	1,7 1,6	0,4 3,8	0,5 2,2	4,3 \pm 3,4	1,6 \pm 1,1	24,6 26,9	5,9 10,4	43,2 \pm 25,9	42 \pm 31,7
16:1n-9	0 0	0 0	0 0	0 0	0 \pm 0	0 \pm 0	0 0	1,7 2,5	0,7 \pm 1,2	1,1 \pm 1,1
18:1n-7	0,4 3,4	0 0,3	3,5 8	3,6 4,7	8,5 \pm 3	6,7 \pm 2,4	9,7 21,1	5,9 15,4	13,8 \pm 6,9	16,6 \pm 8,4
18:1n-9	4,2 3,6	1 1,1	0,5 7	4,9 7,5	7,2 \pm 2,1	6,1 \pm 1,9	4,9 14,6	10,8 21,3	11,2 \pm 5,3	14,6 \pm 6,5
14:2	0 0	0 0	0 0	0 0	0 \pm 0	0 \pm 0	0 0	0 0	0,3 \pm 0,5	0,8 \pm 0,8

Fatty acid	Total lipids		Membrane lipid fatty acids				Storage lipid fatty acids			
	<i>Navicula pelliculosa</i> (Diatom biofilm)	<i>Stigeoclonium farctum</i> (Green algae biofilm)	<i>Baetis</i> sp. Diatom biofilm	<i>Baetis</i> sp. Green algae biofilm	<i>Rhithrogena</i> sp. Diatom biofilm	<i>Rhithrogena</i> sp. Green algae biofilm	<i>Baetis</i> sp. Diatom biofilm	<i>Baetis</i> sp. Green algae biofilm	<i>Rhithrogena</i> sp. Diatom biofilm	<i>Rhithrogena</i> sp. Green algae biofilm
14:3	0 0	0 0	0 0	0 0	0±0	0±0	0 0	0 0	0±0	0,4±0,8
C16PUFA	1,8 0	0 0	0 0	0 0	0±0	0±0	0 0	0 0	0±0	0±0
16:2n-4	0,8 1	0 0	0 1,5	0 0	0,7±0,7	0,7±0,7	0,8 3,3	0 0,7	2,4±2	3±2
16:2n-6	0 0	1,5 0	0 0	0 0	0±0	0±0	0 0	0 0	0±0	0±0
16:3n-3	0 0	1,2 1,1	0 0	0 0	0±0	0±0	0 1,3	3,1 1,9	1,9±1,4	2,4±0,4
16:3n-4	1,5 3,4	0 0	0 0	0 0	0±0	0±0	2 0	0 0	3,9±1,7	4,3±2,6
16:3n-6	0 0	0 0	0 0	0 0	0±0	0±0	0 0	0 0	1,7±1	2,6±0,4
16:4n-3	0 0	3,1 3,1	0 0	0 0	0±0	0±0	0 0	2 0	1,2±1,2	0,7±1,1
C18PUFA	0 0	0 0	0 0	0 0	0±0	0±0	2,6 0,8	0 0	0±0	0±0
18:2n-4 (?)	0 0	0 0	0 1,2	0 1,3	3,2±4,3	0,2±0,3	0 0	0 0	0±0	0±0
18:2n-6	0 0	0,8 2,5	0 2,9	2,5 3,5	1,2±0,5	1,3±0,6	2,9 4,7	11,2 13,5	2,7±2,3	3,4±0,9
18:3n-3	0 0,9	4,1 6,8	1,8 15,6	17,2 19,5	4,4±1	6,8±1,4	3,8 20,6	32,4 61,5	10,1±10,5	12±7,1
18:3n-4(?)	0 0	0 0	0 0	0 0	0±0	0±0	2,3 0	0 0	0±0	0±0
18:3n-6	0 0	0 0	0 0	0 0	0,3±0,5	0±0	0 0	0 0	0,1±0,2	0,2±0,3
18:4n-3	0 0	0 0	0 1,8	0,8 2	0,3±0,6	0,7±0,7	3 3,7	6,6 8,3	1,6±1,3	2,9±0,8
20:4n-6	6,4 6	0 0	0 2	0,9 0	2,9±1,1	1,2±0,4	3,9 1,8	0 0	2,4±1,7	1,5±2,6
20:5n-3	7,9 5,9	0 0,6	1,3 9,6	3,5 5,5	13,8±5,3	9,9±3,2	7,6 6,9	4,1 3	16±6,6	20,8±10,1
22:6n-3	0,8 0	0 0	0 0	0 0	0±0	0±0	0 0	0 0	0±0	0±0

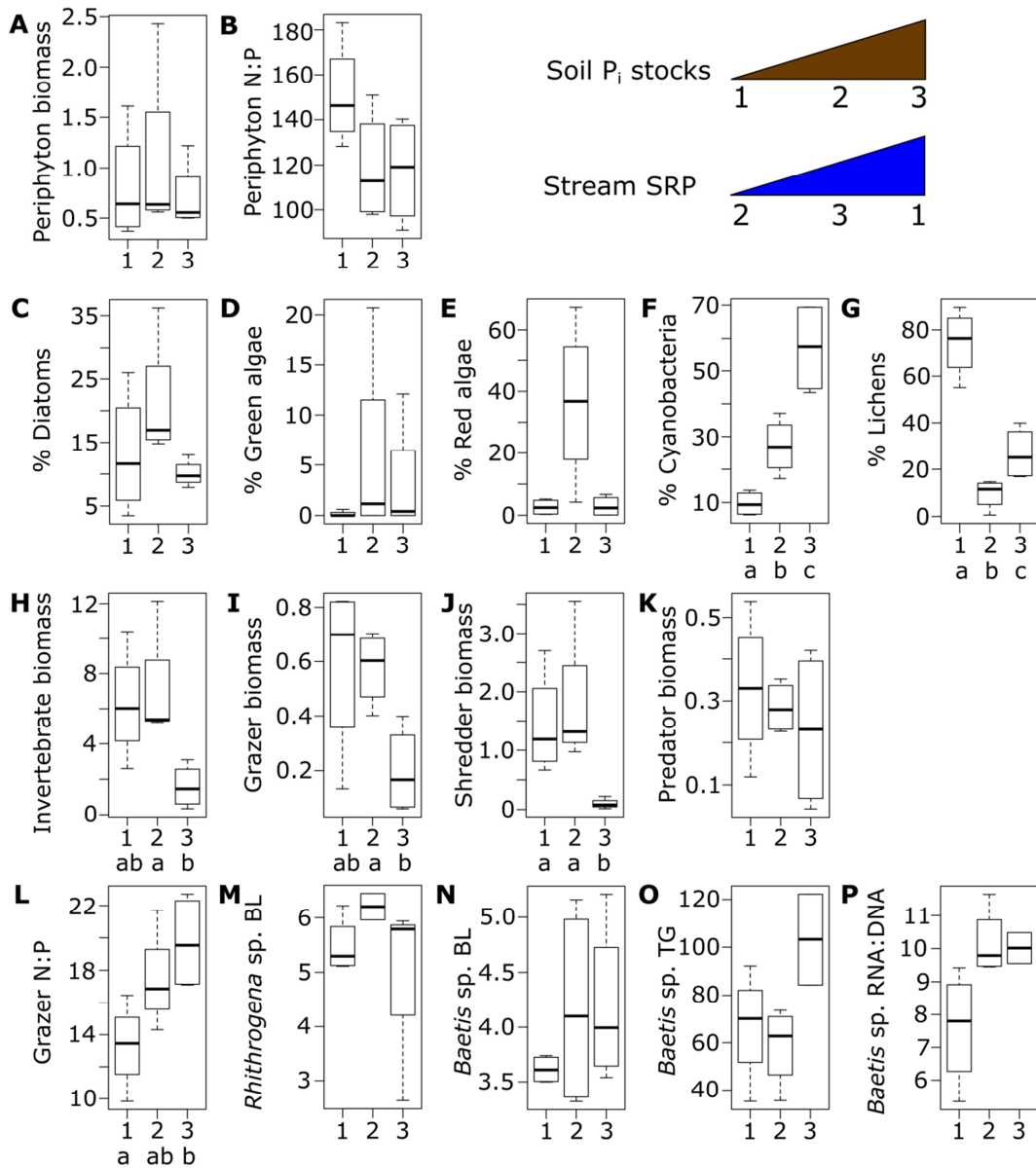
Supplementary Table 6 Contribution of fatty acid groups as % of fraction. Biofilm GLFA and PLFA are both fractions of membrane lipids (GLFA are major components of the thylakoid membranes). In mayflies PLFA = membrane lipid fatty acids and NLFA = storage lipid fatty acids. Biofilm total = (GLFA + NLFA + PLFA). Mayfly total = (PLFA + NLFA). SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EFA = (ALA+LIN+ARA+EPA+DHA).

Group	Type	Fraction	SAFA	MUFA	PUFA	C18 PUFA	C20-22 PUFA	EFA	$\frac{EFS}{PUFA}$	$\frac{(ALA + LIN)}{PUFA}$	$\frac{(ARA + EPA)}{PUFA}$	n
Diatom biofilm	Biofilm	GLFA	22.2 ± 0.9	51.0 ± 6.8	26.8 ± 6.0	1.8 ± 1.8	17.2 ± 1.3	19.0 ± 3.1	72.1 ± 4.6	5.5 ± 5.5	66.6 ± 10.1	2
Diatom biofilm	Biofilm	NLFA	16.5 ± 1.8	66.4 ± 5.5	17.1 ± 3.7	0 ± 0	12.8 ± 3.1	12.8 ± 3.1	74.1 ± 2.4	0 ± 0	74.2 ± 2.4	2
Diatom biofilm	Biofilm	PLFA	9.3 ± 6.8	28.9 ± 11.7	61.8 ± 18.5	0 ± 0	61.8 ± 18.5	61.8 ± 18.5	100 ± 0	0 ± 0	93.1 ± 7.0	2
Diatom biofilm	Biofilm	total	18.0 ± 1.8	578 ± 4.6	24.3 ± 2.8	0.6 ± 0.6	18.5 ± 0.8	19.1 ± 1.5	79.1 ± 2.9	2.3 ± 2.3	74.7 ± 3.1	2
Diatom biofilm	Mayfly	NLFA	36.9 ± 7.2	39.4 ± 10.4	23.2 ± 6.0	9.5 ± 5.1	9.4 ± 2.8	16.6 ± 4.3	71.4 ± 4.8	29.9 ± 16.8	41.5 ± 12.5	6
Diatom biofilm	Mayfly	PLFA	14.5 ± 3.6	38.3 ± 7.5	47.2 ± 5.9	20.2 ± 6.9	25.9 ± 6.9	41.6 ± 3.6	89.0 ± 9.6	34.2 ± 16.3	54.9 ± 13.2	6
Diatom biofilm	Mayfly	total	32.1 ± 7.2	37.7 ± 7.6	29.8 ± 4.1	12.6 ± 4.1	13.5 ± 3.8	22.5 ± 3.0	75.7 ± 4.0	30.3 ± 14.6	45.4 ± 11.4	6
Green algae biofilm	Biofilm	GLFA	27.4 ± 0.5	9.2 ± 1.8	63.4 ± 1.3	33.12 ± 4.7	0 ± 0	33.1 ± 4.7	52.1 ± 6.4	52.1 ± 6.4	0 ± 0	2
Green algae biofilm	Biofilm	NLFA	69.1 ± 30.9	7.0 ± 7.0	23.9 ± 23.9	16.0 ± 16.0	0 ± 0	16.0 ± 16.0	66.9 ± 0	66.9 ± 0	0 ± 0	2
Green algae biofilm	Biofilm	PLFA	13.5 ± 13.5	4.4 ± 4.4	82.1 ± 17.9	76.8 ± 23.2	5.3 ± 5.3	82.1 ± 17.9	100 ± 0	91.8 ± 8.2	8.2 ± 8.2	2
Green algae biofilm	Biofilm	total	32.2 ± 1.2	9.8 ± 0.1	58.1 ± 1.2	33.3 ± 6.3	1.2 ± 1.2	34.5 ± 7.5	59.3 ± 11.8	57.2 ± 9.7	2.1 ± 2.1	2
Green algae biofilm	Mayfly	NLFA	36.3 ± 2.2	30.5 ± 8.3	33.2 ± 8.4	19.4 ± 13.5	7.7 ± 4.4	24.5 ± 8.8	72.1 ± 11.8	44.8 ± 25.5	27.3 ± 18.0	6
Green algae biofilm	Mayfly	PLFA	17.0 ± 4.3	30.5 ± 6.4	52.6 ± 6.0	30.6 ± 13.1	21.1 ± 8.6	49.4 ± 7.5	93.6 ± 5.4	52.0 ± 18.1	41.6 ± 18.8	6
Green algae biofilm	Mayfly	total	325 ± 1.8	30.7 ± 7.7	36.8 ± 8.2	21.4 ± 13.6	10.3 ± 5.0	29.2 ± 8.4	78.3 ± 7.8	46.9 ± 22.9	31.3 ± 17.7	6

Supplementary Table S3 — Contribution of fatty acid groups

Sudy 3 — Supplementary material as referenced in the manuscript

Supplementary Figure S1 — Boxplots and ANOVA results



Supplementary Figure 1 (A–G) Variability of periphyton and (H–P) invertebrate response variables at the three study sites. Site codes: 1, Black Forest; 2, Bavarian Forest; 3, Thuringian Forest. Site ranking with increasing P availability based on soil P_i stocks and water SRP is given at the top right. Significant results of the non-parametric ANOVAs are shown below each plot and indicated by letters. Periphyton response variables: periphyton biomass (µg Chl *a* cm⁻², A), periphyton N:P molar ratio (B), proportion of diatoms (C), green algae (D), red algae (E), cyanobacteria (F), and lichens (G) of total periphyton autotrophs. Macroinvertebrate response variables are: Macroinvertebrate biomass (g dry mass m⁻², H), grazer biomass (g dry mass m⁻², I), shredder biomass (g dry mass m⁻², J), predator biomass (g dry mass m⁻², K), mayfly nymphs (*Baetis* sp. and *Rhithrogena* sp.) N:P molar ratio (L), body length (BL) of *Rhithrogena* sp. (mm, M), body length (BL) of *Baetis* sp. (mm, N), triglyceride content of *Baetis* sp. (µmol g⁻¹ dry mass, O), RNA/DNA ratio of *Baetis* sp. (P) Sites are presented in ascending order of water phosphate concentrations. Number of replicates are given in Table 10.

Supplementary Table 7 Mean values and sample number (n) of macroinvertebrate samples included in the analysis of variance (ANOVA, Table 10). Overall site means are shown in boldface. The subsamples contributing to this overall mean are presented below in regular type. These subsample means are included in the ANOVA providing the n given with the overall mean for each site.

Site	Season	Land use	Mean macro-invertebrate biomass	n	Mean grazer biomass	n	Mean shredder biomass	n	Mean predator biomass	n	Mean <i>Baetis</i> sp. RNA/DNA	n	Mean <i>Baetis</i> sp. TG	n	Mean body length <i>Baetis</i> sp.	n	Mean body length <i>Rhithrogena</i> sp.	n	Mean grazer N:P (molar)	n
Black Forest			6261.49	4	588.63	4	1434.37	4	329.17	4	7.59	4	66.93	4	3.62	4	5.48	4	13.30	4
Spring Meadow			6314.89	2	817.81	2	2700.53	2	296.36	2	9.42	3	72.03	4	3.51	2	5.11	2	9.84	1
Spring Forest			10407.84	2	580.77	2	1412.49	2	537.96	2	5.35	1	35.30	1	3.74	2	5.45	2	13.69	1
Summer Meadow			5724.12	2	134.10	2	665.08	2	118.90	2	7.19	4	92.18	5	3.50	2	6.22	2	13.22	1
Summer Forest			2599.10	2	821.84	2	959.39	2	363.47	2	8.41	3	68.20	3	3.71	2	5.14	2	16.46	1
Bavarian Forest			7027.49	4	577.71	4	1787.54	4	284.21	4	10.17	4	58.70	4	4.17	4	6.21	2	17.63	3
Spring Meadow			12132.65	2	703.40	2	973.34	2	352.06	2	10.09	5	57.34	5	4.80	2	6.45	2	16.86	1
Spring Forest			5219.92	2	666.59	2	3550.21	2	321.46	2	11.65	5	35.60	5	5.16	2	5.96	2	21.71	1
Summer Meadow			5362.78	2	401.07	2	1337.96	2	236.62	2	9.49	6	68.23	6	3.32	2	0	0	14.31	1
Summer Forest			5394.63	2	539.79	2	1288.65	2	226.72	2	9.45	5	73.64	5	3.40	2	0	0	0	0
Thuringian Forest			1586.17	4	198.72	4	98.00	4	231.61	4	10.01	2	103.25	2	4.18	4	4.79	3	19.72	4
Spring Meadow			3093.08	2	259.56	2	74.76	2	420.26	2	0	0	0	0	3.75	2	5.94	2	17.20	1
Spring Forest			2033.51	2	398.64	2	221.03	2	369.68	2	0	0	0	0	4.24	2	5.79	2	17.10	1
Summer Meadow			341.63	2	74.58	2	81.61	2	93.03	2	9.55	5	84.27	3	3.54	2	0	0	21.88	1
Summer Forest			876.48	2	62.11	2	14.62	2	43.47	2	10.48	5	122.24	5	5.21	2	2.63	1	22.72	1

*Supplementary Table S2 — Grazer stoichiometry: sample number***Supplementary Table 8 Number of samples included in the analysis of grazer stoichiometry.**

Site, land use, season	Species	n (%N)	n (%P)
Black Forest, meadow, spring	<i>Baetis</i> sp.	3	3
Black Forest, meadow, spring	<i>Rhithrogena</i> sp.	3	3
Black Forest, meadow, summer	<i>Baetis</i> sp.	3	3
Black Forest, meadow, summer	<i>Rhithrogena</i> sp.	3	3
Black Forest, forest, spring	<i>Baetis</i> sp.	7	NA ^a
Black Forest, forest, spring	<i>Rhithrogena</i> sp.	1	3
Black Forest, forest, summer	<i>Baetis</i> sp.	3	3
Black Forest, forest, summer	<i>Rhithrogena</i> sp.	3	3
Bavarian Forest, meadow, spring	<i>Baetis</i> sp.	3	3
Bavarian Forest, meadow, spring	<i>Rhithrogena</i> sp.	3	NA
Bavarian Forest, meadow, summer	<i>Baetis</i> sp.	3	3
Bavarian Forest, forest, spring	<i>Baetis</i> sp.	NA	3
Bavarian Forest, forest, spring	<i>Rhithrogena</i> sp.	1	NA
Bavarian Forest, forest, summer	<i>Baetis</i> sp.	NA	6
Thuringian Forest, meadow, spring	<i>Baetis</i> sp.	2	3
Thuringian Forest, meadow, spring	<i>Rhithrogena</i> sp.	2	3
Thuringian Forest, meadow, summer	<i>Baetis</i> sp.	5	3
Thuringian Forest, forest, spring	<i>Baetis</i> sp.	10	NA
Thuringian Forest, forest, spring	<i>Rhithrogena</i> sp.	2	2
Thuringian Forest, forest, summer	<i>Baetis</i> sp.	3	3
Thuringian Forest, forest, summer	<i>Rhithrogena</i> sp.	3	1

^aNot analysed

Supplementary Table 9 Mean values and sample number (n) periphyton samples included in the analysis of variance (ANOVA, Table 10). Overall site means are shown in boldface. The subsamples contributing to this overall mean are presented below in regular type. These subsample means are included in the ANOVA providing the n given with the overall mean for each site.

Site	Mean periphyton biomass		Mean periphyton N:P (molar)		Mean % diatom		Mean % green algae		Mean % red algae		Mean % Cyano-bacteria		Mean % lichen	
Season Land use		n		n		n		n		n		n		n
Black Forest	0.81	4	150.96	4	13.2	4	0.2	4	2.6	4	9.7	4	74.4	4
Spring Meadow	0.82	10	128.26	1	8.3	50	0.6	50	4.4	50	6.3	50	80.4	50
Spring Forest	0.37	10	183.16	1	3.5	50	0.0	50	0.3	50	6.7	50	89.5	50
Summer Meadow	1.61	10	150.98	1	26.0	50	0.0	50	5.2	50	13.7	50	55.1	50
Summer Forest	0.46	10	141.41	1	14.9	50	0.0	50	0.5	50	12.0	50	72.4	50
Bavarian Forest	1.06	4	118.64	4	21.2	4	5.8	4	36.3	4	27.0	4	9.6	4
Spring Meadow	0.56	10	125.47	1	36.2	50	20.7	50	4.2	50	23.7	50	14.7	50
Spring Forest	2.43	10	150.81	1	14.8	50	0.0	50	67.2	50	17.2	50	0.6	50
Summer Meadow	0.60	10	97.94	1	17.8	50	0.0	50	31.6	50	37.1	50	13.5	50
Summer Forest	0.67	10	100.32	1	16.1	50	2.3	50	42.0	50	30.0	50	9.6	50
Thuringian Forest	0.70	4	117.38	4	10.1	4	3.2	4	2.9	4	57.0	4	26.7	4
Spring Meadow	0.60	10	103.56	1	7.9	50	0.8	50	4.6	50	69.4	50	16.9	50
Spring Forest	0.50	10	134.71	1	13.0	50	0.0	50	0.1	50	69.3	50	17.6	50
Summer Meadow	1.22	10	140.28	1	9.9	50	0.0	50	6.7	50	43.4	50	39.9	50
Summer Forest	0.51	10	90.95	1	9.5	50	12.1	50	0.1	50	45.8	50	32.5	50

Supplementary Table S4 — Abundance of macroinvertebrate taxa

Supplementary Table 10 Abundance of macroinvertebrate taxa (mean \pm SD, n = 8) at the three studied streams. The values display the average abundance and its variability within each stream across sampled habitat types (pool, riffle), types of land use (forest, meadow) and season (spring, summer).

Taxon	Black Forest	Thuringian Forest	Bavarian Forest
<i>Agapetus</i> sp.	0 \pm 0	1 \pm 3	2 \pm 4
<i>Agapetus delicatulus</i>	0 \pm 0	4 \pm 11	0 \pm 0
<i>Agapetus fuscipes</i>	308 \pm 703	50 \pm 69	0 \pm 0
<i>Amphinemura</i> sp.	17 \pm 39	74 \pm 118	0 \pm 0
<i>Ancyclus fluviatilis</i>	1 \pm 4	0 \pm 0	0 \pm 0
<i>Anisus</i> sp.	1 \pm 1	0 \pm 0	0 \pm 0
<i>Asellus aquaticus</i>	2 \pm 4	0 \pm 0	0 \pm 0
<i>Atherix</i> sp.	4 \pm 8	36 \pm 34	0 \pm 0
<i>Baetis</i> sp.	8 \pm 14	14 \pm 28	47 \pm 64
<i>Baetis alpinus/melanonyx</i>	449 \pm 472	45 \pm 65	378 \pm 425
<i>Baetis fuscatus</i>	0 \pm 0	0 \pm 0	114 \pm 251
<i>Baetis muticus</i>	119 \pm 161	51 \pm 63	2 \pm 5
<i>Baetis rhodani</i>	26 \pm 50	0 \pm 0	106 \pm 142
Blephariceridae indet.	5 \pm 9	5 \pm 14	0 \pm 0
<i>Brachyptera seticornis</i>	1 \pm 4	55 \pm 99	6 \pm 11
<i>Bythinella</i> sp.	34 \pm 22	0 \pm 0	0 \pm 0
Ceratopogonidae indet.	128 \pm 173	8 \pm 14	9 \pm 16
<i>Chaetopteryx major</i>	0 \pm 0	0 \pm 0	1 \pm 2
<i>Chaetopteryx villosa</i>	0 \pm 0	0 \pm 1	9 \pm 22
Chironomidae indet.	703 \pm 945	216 \pm 119	136 \pm 96
<i>Chloroperla</i> sp.	4 \pm 10	0 \pm 0	0 \pm 0
Coleoptera indet.	0 \pm 0	0 \pm 0	2 \pm 3
<i>Cordulegaster boltonii</i>	5 \pm 8	0 \pm 0	0 \pm 0
<i>Crunoecia</i> sp.	4 \pm 5	4 \pm 11	0 \pm 0
<i>Dicranota</i> sp.	8 \pm 11	14 \pm 12	11 \pm 11
<i>Diplectrona felix</i>	5 \pm 9	0 \pm 0	0 \pm 0
Diptera indet.	2 \pm 4	0 \pm 1	0 \pm 0
Dixidae indet.	12 \pm 25	4 \pm 6	2 \pm 3
<i>Drusus annulatus</i>	9 \pm 14	1 \pm 3	0 \pm 0
<i>Drusus discolor</i>	0 \pm 0	12 \pm 23	5 \pm 13
<i>Drusus monticola</i>	1 \pm 4	0 \pm 0	0 \pm 0
<i>Dugesia</i> sp.	149 \pm 116	3 \pm 6	6 \pm 8
<i>Ecclisopteryx madida</i>	0 \pm 0	0 \pm 0	21 \pm 31
<i>Ecdyonurus submontanus</i>	0 \pm 0	0 \pm 0	10 \pm 8
<i>Eiseniella tetraedra</i>	23 \pm 24	7 \pm 14	3 \pm 4
<i>Ellipteroides</i> sp.	18 \pm 39	0 \pm 0	0 \pm 0
<i>Elmis</i> sp.	279 \pm 303	25 \pm 23	146 \pm 209
<i>Elodes</i> sp.	16 \pm 41	1 \pm 1	12 \pm 19
<i>Eloeoiphila</i> sp.	3 \pm 5	1 \pm 1	3 \pm 6
Empididae indet.	2 \pm 4	2 \pm 5	3 \pm 5

<i>Epeorus assimilis</i>	2 ± 5	0 ± 0	6 ± 10
<i>Ephemera danica</i>	11 ± 17	0 ± 0	0 ± 0
<i>Ephemerella mucronata</i>	1 ± 2	1 ± 1	0 ± 0
<i>Erpobdella octoculata</i>	3 ± 7	0 ± 0	0 ± 0
<i>Esolus</i> sp.	3 ± 6	8 ± 16	1 ± 2
<i>Gammarus fossarum/pulex</i>	1016 ± 352	0 ± 0	1692 ± 1167
<i>Glossosoma conformis</i>	0 ± 0	3 ± 4	19 ± 33
<i>Goera pilosa</i>	0 ± 1	0 ± 0	0 ± 0
Goeridae indet.	2 ± 4	0 ± 0	0 ± 0
<i>Habroleptoides auberti</i>	15 ± 26	21 ± 39	0 ± 0
<i>Habroleptoides confusa</i>	72 ± 75	10 ± 11	0 ± 0
<i>Halesus rubricollis</i>	0 ± 0	0 ± 0	1 ± 3
Heptageniidae indet.	11 ± 29	0 ± 0	0 ± 0
Hydracarina indet.	26 ± 35	32 ± 33	19 ± 28
Hydraena indet.	42 ± 69	11 ± 11	27 ± 40
<i>Hydropsyche</i> sp.	9 ± 16	10 ± 17	2 ± 5
<i>Hydropsyche cf. tenuis</i>	1 ± 4	1 ± 2	0 ± 0
<i>Hydropsyche fulvipes</i>	21 ± 27	0 ± 0	0 ± 0
<i>Hydropsyche siltalai</i>	8 ± 22	0 ± 0	0 ± 0
Hydroptilidae indet.	0 ± 1	0 ± 0	0 ± 0
<i>Isoperla</i> sp.	16 ± 13	20 ± 12	46 ± 50
<i>Leuctra</i> sp.	57 ± 80	66 ± 59	84 ± 64
<i>Leuctra braueri</i>	98 ± 90	26 ± 21	39 ± 57
<i>Leuctra nigra</i>	0 ± 0	51 ± 135	5 ± 12
Limnephilidae indet.	16 ± 32	24 ± 43	47 ± 64
<i>Limnius</i> sp.	287 ± 327	28 ± 31	287 ± 264
<i>Liponeura</i> sp.	0 ± 0	1 ± 3	0 ± 0
<i>Lithax niger</i>	21 ± 43	6 ± 9	35 ± 37
Lonchopteridae indet.	1 ± 4	0 ± 0	0 ± 0
<i>Lumbriculus</i> sp.	0 ± 0	16 ± 42	1 ± 3
<i>Melampophylax melampus</i>	0 ± 0	0 ± 0	2 ± 3
<i>Micrasema longulum</i>	0 ± 0	3 ± 9	0 ± 0
<i>Molophilus</i> sp.	2 ± 4	0 ± 0	0 ± 0
Nematoda indet.	7 ± 15	4 ± 7	0 ± 0
Nematomorpha indet.	3 ± 4	3 ± 6	2 ± 6
<i>Nemoura</i> sp.	38 ± 49	12 ± 19	3 ± 6
Nemouridae indet.	3 ± 7	1 ± 3	0 ± 0
<i>Neolimnomyia</i> sp.	44 ± 111	0 ± 0	0 ± 0
<i>Niphargus</i> sp.	1 ± 4	0 ± 0	0 ± 0
<i>Odontocerum albicorne</i>	33 ± 33	1 ± 3	1 ± 2
Oligochaeta indet.	283 ± 361	520 ± 603	114 ± 121
<i>Oulimnius</i> sp.	3 ± 4	2 ± 4	0 ± 0
<i>Paraleptophlebia</i> sp.	6 ± 12	0 ± 0	0 ± 0
<i>Pedicia</i> sp.	1 ± 2	1 ± 3	0 ± 0
<i>Philopotamus</i> sp.	1 ± 1	0 ± 0	0 ± 0

<i>Philopotamus montanus</i>	20 ± 22	43 ± 75	23 ± 49
<i>Philopotamus variegatus</i>	0 ± 0	1 ± 2	0 ± 0
<i>Pisidium</i> sp.	21 ± 27	1 ± 3	1 ± 2
Planorbidae indet.	2 ± 4	0 ± 0	0 ± 0
Plecoptera indet.	1 ± 4	0 ± 0	1 ± 2
<i>Plectrocnemia geniculata</i>	6 ± 8	4 ± 8	3 ± 4
<i>Polycelis felina</i>	119 ± 130	261 ± 218	191 ± 118
<i>Potamophylax cingulatus</i>	1 ± 2	0 ± 0	12 ± 15
<i>Potamophylax luctuosus</i>	0 ± 0	0 ± 0	6 ± 16
<i>Potamophylax nigricornis</i>	1 ± 4	0 ± 0	0 ± 0
<i>Prosimulium</i> sp.	39 ± 100	139 ± 355	39 ± 96
<i>Protonemura</i> sp.	67 ± 105	289 ± 567	106 ± 177
Psychodidae indet.	13 ± 12	31 ± 46	10 ± 15
<i>Psychomyia pusilla</i>	0 ± 0	0 ± 0	2 ± 5
<i>Ptilocolepus granulatus</i>	3 ± 5	0 ± 0	0 ± 0
Ptychopteridae indet.	3 ± 9	1 ± 1	0 ± 0
<i>Rhabdiopteryx</i> sp.	0 ± 0	0 ± 0	2 ± 6
<i>Rhithrogena semicolorata</i> -group	153 ± 132	44 ± 59	7 ± 10
<i>Rhyacophila</i> sp.	14 ± 17	9 ± 9	24 ± 19
<i>Rhyacophila evoluta</i>	0 ± 0	3 ± 5	0 ± 0
<i>Rhyacophila hirticornis</i>	0 ± 0	0 ± 0	3 ± 8
<i>Rhyacophila laevis</i>	1 ± 1	0 ± 0	0 ± 0
<i>Rhyacophila polonica/praemorsa</i>	0 ± 0	5 ± 12	0 ± 0
<i>Rhyacophila pubescens</i>	1 ± 3	0 ± 0	4 ± 8
<i>Rhyacophila tristis</i>	43 ± 62	8 ± 10	30 ± 34
Sciomycidae indet.	1 ± 4	0 ± 0	0 ± 0
Scirtidae indet.	49 ± 82	2 ± 5	0 ± 0
<i>Scleroprocta</i> sp.	15 ± 24	0 ± 0	0 ± 0
<i>Seratella ignita</i>	7 ± 18	1 ± 1	0 ± 0
<i>Sericostoma personatum/flavicorne</i>	43 ± 58	58 ± 68	15 ± 23
<i>Silo pallipes</i>	4 ± 9	0 ± 0	0 ± 0
<i>Simulium</i> sp.	50 ± 78	121 ± 194	68 ± 90
<i>Siphonoperla</i> sp.	1 ± 4	0 ± 0	1 ± 2
<i>Sisyra</i> sp.	1 ± 1	0 ± 0	0 ± 0
<i>Stactobia moselyi</i>	1 ± 4	0 ± 1	0 ± 0
<i>Stenelmis</i> sp.	11 ± 29	1 ± 2	99 ± 177
Tabanidae indet.	1 ± 3	0 ± 0	0 ± 0
Thaumaleidae indet.	11 ± 12	1 ± 1	4 ± 9
<i>Tinodes rostocki</i>	9 ± 16	0 ± 0	26 ± 33
<i>Tipula</i> sp.	5 ± 8	0 ± 0	0 ± 0
Trichoptera indet.	5 ± 11	4 ± 5	2 ± 4
<i>Wormaldia occipitalis</i>	3 ± 5	0 ± 0	0 ± 0

indet. = indeterminate