Nutrient limitation of primary production in rivers along a land use gradient in the Lake Biwa Basin, Shiga Prefecture, Japan

Christine L. Weilhoefer · Shin-ichi Nakano · Souyma Deb · Keitaro Fukushima

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Abstract Benthic microalgae (BMA) biomass and community structure in freshwater lotic systems are often limited by inorganic nutrient availability. We examined nutrient limitation of benthic algae biomass and community structure using nutrient diffusing substrates in four rivers along a land use gradient in the Lake Biwa basin, Japan. Ambient in-stream nutrient concentrations were correlated to catchment land use, with the highest nitrogen and phosphorus concentrations being found in rivers draining more forested catchments. Nutrient limitation of primary producer biomass and nutrient-driven changes in community structure were evident in all four rivers, regardless of in-stream nutrient concentrations and surrounding land use. BMA biomass (measured as chlorophyll log-response ratio) exhibited the greatest nutrient limitation in rivers with higher in-stream nutrient concentrations. The relationship to catchment land use was less clear, with the highest nutrient limitation being observed in the catchment with moderate amounts of agricultural and forested land use. Nutrient additions resulted in a shift from dominance by Bacillariophyceae (diatoms) to a mixed Bacillariophyceae-Chlorophyceae (chlorophyte) community in all rivers and this shift was most pronounced in the forested catchments. Changes within the diatom community with nutrient additions were also observed, although the shifts in diatom community within a river in response to nutrient additions were much smaller than the differences in diatom community composition among rivers. Diatom taxa classified as highly motile increased with nutrient additions in all rivers. Our results suggest that primary producer community in rivers may be sensitive to nutrient inputs even in areas with elevated nutrient concentrations and catchments dominated by agricultural land use. There is likely widespread nutrient limitation in rivers of Japan, across both in-stream nutrient and land use gradients and any increases in nutrient loading will likely stimulate benthic algal growth. Our findings highlight the importance of looking at both biomass and species composition to assess ecosystem-level impacts of elevated nutrient levels.

Keywords Benthic microalgae · Nutrient limitation · Primary production · Nitrogen · Phosphorus · Nutrient-diffusing substrates

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C. L. Weilhoefer
Department of Biology and Environmental Studies, University of Portland, 5000 Willamette Blvd, Portland, OR 97203, USA
e-mail: weilhoef@up.edu

S. Nakano · S. Deb · K. Fukushima
Center for Ecological Research, Kyoto University, Hirano 2-509-3, Otsu, Shiga 520-2113, Japan
e-mail: nakano@ecology.kyoto-u.ac.jp

S. Deb
e-mail: sdebburman81@gmail.com

K. Fukushima
e-mail: fmktaro@ecology.kyoto-u.ac.jp
Introduction

Excess nutrient input to aquatic ecosystems from anthropogenically-derived sources is a widespread global phenomenon, with impacts being documented from the poles to the tropics (Smith 2003; Smith et al. 2006; Smith and Schindler 2009) and despite efforts to reduce nutrient loads, many aquatic ecosystems continue to be impacted by excess nutrients (Bennett et al. 2001). In aquatic ecosystems, benthic microalgae (BMA), which includes all microscopic, unicellular photoautotrophs occupying the stream biofilm, are important primary producers that contribute to the cycling of carbon and nutrients. Over the past seventy years, the relationship between nutrients and BMA growth and community structure in streams and rivers has garnered much attention from aquatic ecologists (see reviews in Francoeur 2001; Larnd 2010). Positive relationships between both micro- and macronutrient concentrations and BMA biomass have been documented in both experimental and larger-scale survey studies. In regards to macronutrients, while initially phosphorus was thought to limit primary production in freshwater ecosystems (e.g., Correll 1998), continued study has demonstrated that primary production can be limited by either phosphorus or nitrogen, or co-limited by both nitrogen and phosphorus, or lack nutrient limitation all together (Pringle et al. 1986; Winterbourn 1990; Corkum 1996; Francoeur 2001; Elser et al. 2007; Tank et al. 2017). BMA community composition has also been related to nutrient concentrations in streams, with nutrient additions causing shifts in species (e.g., Lowe et al. 1986; Biggs et al. 1998) and with differing nutrient concentrations explaining a proportion of the variability in BMA assemblages across geographic areas (e.g., Griffith et al. 2002; Hill et al. 2003; Pan et al. 2004).

Nutrient limitation of BMA should be viewed in concert with other environmental factors that limit algae growth. As BMA are primary producers, light can be viewed as the ultimate factor limiting growth. Light limitation of the BMA community has been observed in many streams, particularly with high canopy coverage (e.g., Hill and Harvey 1990; Steinman and McIntire 1987; Steinman 1992). It follows that light and nutrients would interact to limit BMA growth. Nutrient limitation of BMA biomass and shifts in community structure are documented most often under high-light conditions (e.g., Lowe et al. 1986; Hill and Knight 1988; Bothwell 1989; Greenwood and Rosemond 2005). However, while field experiments have shown independent effects of light and nutrients on BMA, interactive effects are rarely detected (Marks and Lowe 1993; see review in Larnd 2010). This may be due an increased competition for space and light within the BMA matrix as increased cell densities in response to nutrients result in self-shading (Dodds et al. 1999; Azim and Asaeda 2005), regardless of canopy coverage. Nutrient limitation may also be influenced by temperature, as BMA species display a range in temperature optima and tolerances. Warmer temperatures favor cyanobacteria and chlorophytes over diatoms (Squires 1979; Hawes 1988; DeNicola 1996), which may in turn alter seasonal patterns in nutrient limitation as different algal groups will dominate the BMA at different times of the year. Several studies have shown that nutrient limitation of BMA varies across seasons (Francoeur et al. 1999; Wold and Hershey 1999; Beck et al. 2017, 2019), attributable in part to shifts in community composition with temperature (Olesky et al. 2021).

Nutrient loading and nutrient limitation vary across multiple spatial and temporal scales. Land-use, both within the near stream buffer and surrounding catchments, has been shown to influence in-stream nitrogen and phosphorus concentrations (Paul and Meyer 2001; Sonoda et al. 2001; Allan 2004; Taylor et al. 2004; Carr et al. 2005; Klose et al. 2012). In general, streams and rivers flowing through urban and agricultural lands have elevated nutrient concentrations compared to those in more pristine catchments (e.g., Paul and Meyer 2001; Hayakawa et al. 2006; Mulholland et al. 2008; Ponader et al. 2008; Woli et al. 2008; O’Brien and Wehr 2010). BMA biomass and community structure have been shown to vary with land use (Blinn and Bailey 2001; Dodds et al. 2002; Carr et al. 2005; Walker and Pan 2006; O’Brien and Wehr 2010) and response to nutrient additions may also vary with surrounding land-use (Tank and Dodds 2003; Hagy et al. 2020). In agricultural watersheds, nutrients can continue to be released decades after inputs decline (“legacy pollution”, Meals et al. 2010; Powers et al. 2016; Goyette et al. 2018). Others have demonstrated that the spatial variability of nitrogen and phosphorus within stream water varies with current and past land use, affecting patterns of nutrient limitation of BMA (Irvine and Jackson 2006; Hoellein et al. 2011). In addition to spatial patterns
in nutrient limitation of BMA biomass, recent studies have demonstrated temporal shifts in nutrient limitation of BMA biomass within streams (Reisinger et al. 2016; Yadav et al. 2018). These findings point to a need for localized studies to understand how primary producers within an individual river might respond to shifting nutrient loads.

Nutrient diffusing substrates (NDS) are an experimental tool commonly used to study nutrient limitation in streams and rivers with the general idea that a significant increase in BMA biomass to a specific nutrient indicates limitation by that nutrient (see reviews in Francouer 2001; Larnard 2010). Most NDS studies focus on the response of BMA biomass to nutrient additions (Beck et al. 2017). While several early studies did focus on shifts in BMA community composition in response to nutrient additions (e.g., Pringle and Bowers 1984; Fairchild et al. 1985; Lowe et al. 1986; Hill and Knight 1988; Pringle 1990; Biggs et al. 1998), recently less attention has been given to the overall community response to nutrient additions (but see Tromboni et al. 2019; Beck et al. 2019; Hagy et al. 2020). While individual species may be limited by a single nutrient and physiological differences among species will influence how they respond to nutrient additions (Scott et al. 2009), the BMA community is more likely influenced by both nitrogen and phosphorus (see review in Francouer 2001; Larnard 2010). Understanding how BMA biomass responds to nutrients is important for predicting nuisance algae growth and setting nutrient criteria; however, it provides an incomplete picture of the ecosystem-level effects of eutrophication. An understanding of how excess nutrients shift BMA taxonomic groups is essential to predict cascading impacts to the aquatic foodweb. Different algal group confers different nutritional value, both in terms of nutrients and fatty acids, to higher trophic levels (Brett and Müller-Navarra 1997; Stelzer et al. 2001; Cross et al. 2003; Martin-Creuzburg et al. 2008; Martin-Creuzburg and von Elert 2009; Guo et al. 2016a; Guo et al. 2016b).

While there is a strong history of studying the relationships between phytoplankton and water quality in lakes of Japan, much less work has been done on the benthic algae in rivers and streams. Relationships between water quality and BMA biomass in rivers and streams of Japan have been documented through survey studies (e.g., Tezuka et al. 1974; Tanaka 1975; Watanabe et al. 1975; Aizaki 1978, 1979; Aizaki and Sakamoto 1988) and more recently, spatiotemporal patterns of riverine BMA biomass and response to watershed phosphorus loading have been modeled using combined hydrothermal and nutrient runoff models (Kazama and Watanabe 2018). Many rivers in Japan are influenced by anthropogenic nitrogen loading, and the impacts to riverine biota have been noted in several studies (e.g., Lobo et al. 1995; Toda et al. 2002; Ogura et al. 2009; Ishikawa et al. 2016). However, the use of in situ NDS to document nutrient limitation of the BMA has not been implemented in Japan. The climate, geology, topography, and cultural history of Japan interact to create riverine conditions not common in other parts of the world, warranting further study of the response of BMA to nutrients. In their natural state, rivers in Japan have been characterized as short, steep, flashy, and high in sediments (Yoshimura et al. 2005). The concentration and delivery of nitrogen to rivers of Japan has been shown to vary with land use, geology, and season (Tabuchi et al. 1995; Woli et al. 2008; Kazama and Watanabe 2018).

Our study took place in the Lake Biwa region of Japan. Lake Biwa is the largest freshwater lake in Japan, with a surface area of 670 km² and a catchment area of 3848 km² and has one of the largest alluvial plains in Japan (Uemura 2012). Approximately 400 tributaries flow into Lake Biwa, while there is one outlet (Seta river) at its southern end. Lake Biwa is the oldest lake in Japan (~4 million years) and considered an “ancient lake,” having existed for at least one glacial cycle. The history of human use of its floodplain dates to at least 20,000 years ago (Yoda 2012), and it currently supports economically and culturally important fisheries, as well as recreation. It serves as the water source for people of the Keihanshin area, supplying drinking water for 14 million people (Kira et al. 2006; Nakano et al. 2008). Human population in the Lake Biwa basin doubled in the last 50 years of the twentieth century, to approximately 1.3 million people, resulting in widespread deforestation and increased lakeshore development (Kawanabe 1996; Kira et al. 2006; Nakano et al. 2008). These changes have resulted in anthropogenic eutrophication and degradation of various ecosystem services, including water supply and fisheries (Kawanabe 1996; Hseih et al. 2010; Hseih et al. 2011). As a result of stricter controls on industrial pollution and sewage treatment, concentrations of nitrogen and
phosphorus flowing into Lake Biwa have decreased since the 1990s (Okubo 2012).

The goal of this study was to examine nutrient limitation of BMA biomass and community structure during the baseflow period in four rivers in Japan along a land use gradient. We predict that primary production in all rivers would be nutrient limited. In addition, we predict primary production would exhibit the greatest nutrient limitation in rivers draining more forested watersheds, as there tend to be lower levels of background nutrients in these systems.

**Methods**

**Study area**

We studied four rivers in the Lake Biwa basin (Fig. 1) located in Shiga Prefecture in the south-central area of Honshu Island, Japan. Annual temperature in Shiga is between 3 °C and 27 °C and highest precipitation happens in the summer months (June–July; ~220 mm). Temperature and precipitation within the Basin exhibit a gradient, with the mountainous northern region being colder and wetter (rainfall: 2000–2500 mm/year) than the southern part of the basin (rainfall: <1500 mm/year; ILECF 2014). Streamflow is highest during the summer monsoon season (http://www1.river.go.jp/). The watershed of Lake Biwa is 51% forest-covered hills and mountains, 18% urban and agricultural areas, and 15% rice paddies and farmland (Kira et al. 2006) and there is a gradient of land use from the southern area near the city of Kyoto to the northern section (Yamamoto 2014). The highest population densities and rates of urbanization are at the southern end and along the lake margin (Nakano et al. 2008; Yamamoto 2014). Most rivers in the Lake Biwa basin have upper reaches dominated by pristine forests and lower reaches dominated by residential and rice paddy field land use (Nitzsche et al. 2020).

Four rivers of similar watershed size (~30–100 km²) but different dominant land uses were selected for this study (Table 1; Fig. 1). The Kusatsu (KUS; 35.01333 N 135.9349 E), Hayama (HAY; 35.03781 N 135.9556 E), and Uso (USO; 35.22544 N 136.2062 E) river catchments have similar proportions of rice paddy (~40%). Residential land use is higher in the Kusatsu river catchment (46%), while the HAY and USO river catchments have 25–30% residential land use. The Inukami (INU; 35.26192 N 136.2234 E) river catchment is dominated by forested land use (82%), with minimal agriculture and residential land uses. The lowland areas of the watersheds for all four rivers are underlain primarily by Kobiwako Group Quaternary rocks. The upland portion of the KUS and HAY River watersheds are underlain primarily by granitic rocks, while the USO river watershed is underlain primarily by rhyolite, and the INU river is underlain primarily by clastic sedimentary rocks (Nakano et al. 2008). Permission for BMA sampling is not required according to Japanese law; however, our field experiment was approved by the Shiga Fishermen’s Cooperative.
Field methods

Nutrient diffusing substrates: Nutrient diffusing substrates were used to test for nutrient limitation (nitrogen (N), phosphorus (P), and nitrogen + phosphorus (NP)) in the four rivers. NDS construction and deployment followed the methods outlined in Tank et al. (2017). NDS controls and nutrient amendment treatments consisted of small plastic polycon cups (30-ml) filled with a 2% agar solution and topped with a porous inorganic fritted glass disk (5.7 cm²) to serve as surface for BMA attachment. Prior to pouring into the cups, the agar solution was amended with nutrients as follows: P treatment received 0.5 M KH₂PO₄, the N treatment received 0.5 M NaNO₃, the NP treatment received 0.5 M KH₂PO₄ plus 0.5 M NaNO₃, and the control was agar-only. One control and one cup of each treatment was attached in a randomized fashion on a metal L-bar. In each river, four L-bars were placed near one another in the same riffle at a depth of 20 cm. NDS were oriented parallel to stream flow to help prevent sedimentation and secured to the stream bed with stakes. Riffle substrate consisted primarily of small to medium sized cobbles. NDS were incubated in the rivers for 20 days, from 22 November to 11 December 2019. After 20 days, NDS were collected; L-bars were gently removed, and disks were removed from polycon cups and stored on ice in the dark until returning to the laboratory.

Water chemistry samples: Water samples (n = 3) were taken on the day NDS were deployed in November and on the day NDS were retrieved in December to determine background in-stream concentrations of dissolved phosphate (SRP), nitrate + nitrite, and ammonium in the rivers. Samples were stored on ice in a cooler, filtered upon return to the laboratory, and frozen at −20 °C until analysis. Concentrations of nitrate, nitrite, ammonium, and phosphate were determined on a QuAAtro39 autoanalyzer (BL TEC), and concentrations of silica were determined using spectrophotometric methods (UVmini-1240, Shimadzu).

Baseline BMA collection: To examine spatial heterogeneity of the BMA biomass and community composition and determine whether the BMA assemblage colonizing NDS was reflective of the BMA community within the stream, BMA samples were collected at the time of NDS deployment and retrieval (in the same riffle but upstream of the NDS to avoid any nutrient leaching). BMA sampling consisted of scraping a known area (7.1 cm²) of BMA from a representative cobble from each sample reach using a stiff-bristled toothbrush. Five representative cobbles within each stream were sampled, and these samples were kept separate to analyze the heterogeneity of BMA within each river.

Laboratory methods

Sample preparation: Baseline BMA community samples (n = 5) were prepared for analysis of biomass as chlorophyll-α content and community composition (microscopic identification). Within 24 h after returning to the laboratory, BMA samples were filtered (GF/F filter, 0.7 µM pore size) and frozen until analysis of chlorophyll. The remaining sample was preserved with glutaraldehyde (final concentration 0.5%) for soft-bodied algae and Bacillariophyceae (diatom) species identification. The NDS BMA samples were prepared for biomass and community composition analysis. Each disk (n = 16 per river) was scraped clean using a stiff bristled brush to ensure removal of attached algae and then subsampled for chlorophyll

| Land cover information: KUS: City of KUS 2019/20; HAY: Ohte et al. (2010); USO: Integrated Planning of Water Resource Quality, Department of Environmental Engineering, Kyoto University 2000; INU: Ohte et al. (2010) |
|---|---|---|---|---|---|
| AREA km² | POP km⁻² | AGR % | PADDY % | FOR % | RES % |
| KUS | 38.3 | 433 | 44.5 | 40.3 | 3.2 | 46.2 |
| HAY | 33.7 | 2048 | 44.3 | 43.7 | 7.5 | 29.7 |
| USO | 84.2 | 411 | 44.8 | 40.5 | 23.8 | 25.9 |
| INU | 101.6 | 109 | 7.6 | 6.7 | 82.2 | 2.8 |
analysis and microscopic identification as per the above methods of baseline BMA.

Biomass determination: Within 1 week of filtering, filters for chlorophyll analysis were extracted in acetone (90%) at 4 °C for 24 h (APHA 2002). Phaeopigment corrected chlorophyll-a concentrations were calculated from absorbance measurements using a spectrophotometer (Hitachi U-1100 ratio beam spectrophotometer) according to the equations of Lorenzen (1967).

BMA species identification: The sample for soft-bodied algae species identification was scanned under 40× until 300 algae cells were identified and enumerated. When possible, Chlorophyceae (chlorophytes) and Cyanophyceae (cyanophytes) were identified to the genus-level. However, some small, round, non-descript chlorophytes were left at the class-level. Diatoms in the soft-bodied algae sample were identified to class Bacillariophyceae. The primary references for soft-bodied algae taxonomy were Hirose et al. (1977), Wehr et al. 2002, Ichinose and Wakabayashi (2008), and Bellinger and Sigee (2015). The sample of material for diatom identification was heat digested with concentrated hydrochloric acid. Cleaned diatom samples were mounted on slides with Naphrax high-resolution mounting medium. Transects were scanned until at least 600 diatom valves were identified and enumerated to the species level using a Leica DM 2500 microscope at 1000× magnification. The primary references for diatom taxonomy were Krammer and Lange-Bertalot (1986; 1988; 1991a, 1991b; 2000), and Watanabe et al. (1986). Shannon–Weaver diversity (H’), dominance, and taxa richness were calculated for diatom counts for each sample. Taxon dominance was calculated as the relative abundance of the most common taxa in each sample. Diatom taxa were classified according to their motility (Tyree et al. 2020), and morphological guild (Molloy 1992) and the relative abundance of each of these metrics were calculated for each NDS treatment.

Data analysis

Water chemistry

We assessed whether in-stream water chemistry varied between the rivers and the sampling dates using 2-way analysis of variance (2-ANOVA). Significant 2-ANOVA main effects of river and sampling date and interaction terms were examined with Bonferroni-corrected pairwise comparisons.

Baseline conditions and comparison to NDS

We assessed whether baseline BMA biomass and algal community structure changed over the course of the study and whether BMA biomass and algal community structure on control NDS was similar to baseline conditions. Two-way ANOVA was used to examine whether in-stream BMA biomass differed across sample type (NOV baseline sample, DEC baseline sample, DEC NDS control) and river. Significant 2-ANOVA main effects and interaction terms were examined with Bonferroni-corrected pairwise comparisons. Two-way ANOVA was used to test for differences in the relative abundance of diatoms in the soft-bodied algae count across sample types NOV baseline sample, DEC baseline sample, DEC NDS control) and river. Significant 2-ANOVA main effects and interaction terms were examined with Bonferroni-corrected pairwise comparisons.

Unconstrained ordination (NMDS, non-metric multi-dimensional scaling using Bray–Curtis distance measure) was used to examine variations in diatom community composition (arcsine-transformed relative abundance data) between sample type (NOV baseline sample, DEC baseline sample, DEC NDS control). Taxa with a relative abundance greater than 1% in one or more samples were included in the ordination analysis. ENVFIT analysis was used to test for associations between NDMS site scores and sample type (baseline or control NDS), river, in-stream water chemistry, and catchment land use.

Response to nutrient additions

The effects of nutrient addition on several BMA response variables were examined using 2-ANOVA (independent variables: presence/absence of N and P). A unique 2-ANOVA was run for each dependent variable (dependent variables: BMA biomass, % algae as diatoms, diatom metrics) for each river. For BMA biomass, nutrient limitation was indicated by a significantly greater response in a nutrient group relative to the control group. Interpretation of nutrient limitation followed Tank and Dodds (2003). A significant response to a single nutrient indicated limitation by that nutrient alone, co-limitation by
both N and P was indicated by a significant effect of both N and P or a significant N x P interaction, and primary limitation by one nutrient with secondary limitation by the other was indicated by a significant N x P interaction and a significant main effect of either N or P. For % algae as diatoms and diatom metrics, a significantly greater response in a nutrient group compared to the control indicated that this nutrient had an effect on this parameter.

To compare the magnitude of the BMA response to nutrient additions across treatments and rivers relative to the control, the log response ratio (LRR) was calculated for nitrogen, phosphorus, and nitrogen + phosphorus treatments as:

\[ LRR = \log_{10} \left( \frac{X_i}{C} \right) \]

where \( X_i \) is \( i \)th replicate NDS disk for nutrient treatment \( X \) (N, P, NP) and \( C \) is the average for all control disks in a river (Reisinger et al. 2016). LRR has been used as a measure of effect size, with an LRR greater than zero indicating a positive effect and an LRR less than zero indicating a negative effect. Two-way ANOVA was used to test for differences in the LRR among nutrient treatments and rivers. Significant 2-ANOVA main effects and interaction terms were examined with Bonferroni-corrected pairwise comparisons.

Unconstrained ordination (non-metric multidimensional scaling using Bray–Curtis distance measure) was used to examine variations in diatom composition (arcsine-transformed relative abundance data) between fertilization treatments on NDS. Taxa with a relative abundance greater than 1% on one or more NDS treatment were included in the ordination analysis. Ordination analysis was performed on all rivers together to examine variability in response to nutrient additions among rivers. PERMANOVA (Permutational multivariate analysis of similarity) was used to test for the differences in the degree of community similarity within and among groups across the four rivers. To examine community similarity across nutrient treatments across rivers, PERMANOVA was performed on data from all rivers combined. To examine community similarity across treatments within a river, separate PERMANOVA was performed for each river.

All data analyses and visualizations were performed using R-version 3.6.3 (Holding the Windsock, R Development Core Team 2018), using packages “ggplot2” (Wickham 2009), “MASS” (Ripley et al. 2018), and “vegan” (Oksanen et al. 2013). Prior to analysis, all variables were tested to determine if they met the normality and equal variance assumptions of ANOVA. When variables did not meet these assumptions, they were ln-transformed (e.g., water chemistry, BMA biomass) or arcsine-transformed (e.g., diatom and soft-algae relative abundances) to meet the assumptions of ANOVA. ANOVA model residuals were examined for normality and homogeneity of variance using Q-Q plots.

Results

In-stream water chemistry concentrations varied among the four rivers and between the NOV and DEC sampling dates (Fig. 2; Table 2). To an extent, water chemistry correlated with catchment land use. The KUS (range: 6.0–16.3 µg L\(^{-1}\)) and HAY (range: 7.7–15.1 µg L\(^{-1}\)) rivers had relatively consistent SRP concentrations throughout the study. The SRP concentrations in the USO (range: 17.4–42.5 µg L\(^{-1}\)) showed higher variability among replicates within a sampling date. In the INU, SRP concentrations increased between the NOV and DEC sampling dates (range: 7.2–59.3 µg L\(^{-1}\)). The SRP was higher in the most forested river (INU) compared to the other rivers (2-ANOVA River x Date \( F_{3,16} = 5.55, p = 0.008, \) River main effect \( F_{3,16} = 8.54, p < 0.001, \) Date main effect \( F_{1,16} = 9.51, p = 0.007 \)). Nitrate + nitrite concentrations were high in all rivers and exhibited consistent patterns across the rivers for the two sampling dates, being highest in the rivers with more forested catchments (USO range: 3430.1–3832.2 µg L\(^{-1}\), INU range: 2292.8–2617.5 µg L\(^{-1}\)) compared to less forested catchments (KUS range: 1367.8–1868.7 µg L\(^{-1}\), HAY range: 225.7–894.8 µg L\(^{-1}\); 2-ANOVA River x Date \( F_{3,16} = 496.03, p < 0.001, \) River main effect \( F_{3,16} = 9.51, p = 0.007 \)). Concentrations of AM did not vary among the rivers but was higher for the DEC sampling date (2-ANOVA River x Date \( F_{3,16} = 3942.44, p < 0.001, \) Date main effect \( F_{1,16} = 77.33, p < 0.001 \)). Concentrations of AM did not vary among the rivers but was higher for the DEC sampling date (2-ANOVA River x Date \( F_{3,16} = 37.4, p = 0.05, \) River main effect \( F_{3,16} = 0.91, p = 0.46, \) Date main effect \( F_{1,16} = 6.35, \))
Silica concentrations varied across the rivers, being lowest in the most forested catchment (INU range: 4.4–6.5 g L⁻¹) and highest in the least forested catchment (KUS range: 9.1–11.0 g L⁻¹; 2-ANOVA River x Date \( F_{3,16} = 0.19 \), \( p = 0.91 \), River main effect \( F_{3,16} = 17.35 \), \( p < 0.001 \).

**Baseline conditions and comparison to NDS**

In-stream BMA biomass was highly variable within a sampling reach in all rivers and varied significantly between sampling dates and among rivers (Fig. 3; Table 2). BMA biomass was higher in the high-nutrient, forested catchment river (INU) compared to the
lower-nutrient, more agricultural rivers (KUS, HAY; 2-ANOVA River x Date  \( F_{6, 44} = 1.55 \), \( p = 0.19 \), River main effect \( F_{3, 44} = 6.01, p = 0.002 \), Type main effect \( F_{2, 44} = 8.17, p = 0.01 \)). While in-stream BMA biomass was higher in NOV, in-stream biomass in DEC was not different from biomass on the control NDS.

Baseline algal community structure did not vary between the sampling dates or between baseline samples and NDS control (Fig. 4; 2-ANOVA River x Date \( F_{6, 44} = 0.88 \), \( p = 0.52 \), River main effect \( F_{3, 44} = 0.92, p = 0.44 \), Type main effect \( F_{2, 44} = 2.97, p = 0.06 \)). Diatoms dominated both in-stream samples and the control NDS samples, with median % diatoms ranging between 89 and 100% throughout the study. While the relative abundance of diatoms was lower in DEC compared to NOV and control NDS samples, these differences were not significant. A few cells from a variety of other algae groups were found in some of the samples. Lyngbya filaments were present in all rivers, reaching abundances greater than 1% in the HAY and INU rivers in DEC.

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### Table 2

Results of 2-way ANOVA analyses comparing water chemistry variables between samples taken in November and December (Type) among rivers (River) and results of the 2-way ANOVA analysis comparing BMA variables between NDS control samples and baseline samples taken in November and December (Type) among rivers (River). Asterisks indicate significant main effects and interactions of 2-way ANOVA analysis ---(*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \))

| Water chemistry | River | Type | River × Type |
|-----------------|-------|------|--------------|
| Ammonium (µg L\(^{-1}\)) | \( F_{3, 16} = 0.91 \) | \( F_{1, 16} = 0.02^* \) | \( F_{3, 16} = 0.05 \) |
| Phosphate (µg L\(^{-1}\)) | \( F_{3, 16} = 8.54^{***} \) | \( F_{1, 16} = 9.51^{**} \) | \( F_{3, 16} = 5.55^{**} \) |
| Nitrate + Nitrite (µg L\(^{-1}\)) | \( F_{3, 16} = 3942.44^{***} \) | \( F_{1, 16} = 577.33^{***} \) | \( F_{3, 16} = 496.03^{***} \) |
| SiO\(_2\) (mg L\(^{-1}\)) | \( F_{3, 16} = 17.35^{***} \) | \( F_{1, 16} = 1.78 \) | \( F_{3, 16} = 0.19 \) |

| BMA | | | |
|-----------------|-------|------|--------------|
| Chlorophyll-a (µg L\(^{-1}\)) | \( F_{3, 16} = 6.01^{**} \) | \( F_{1, 16} = 8.17^{*} \) | \( F_{3, 16} = 1.55 \) |
| Diatoms (% of algae) | \( F_{3, 16} = 0.92 \) | \( F_{1, 16} = 2.97 \) | \( F_{3, 16} = 0.88 \) |

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**Fig. 3** Baseline BMA biomass (µg chlorophyll-a cm\(^{-2}\)) in the Kusatsu, Hayama, Uso, and Inukami rivers in November and December and on the control treatment of the NDS at the end of the experiment. Lines below boxes indicate rivers with similar biomass based on 2-way ANOVA. Letters below boxes indicate significant differences in biomass within a river between the November and December samples and on the control treatment of the NDS at the end of the experiment based on 2-way ANOVA.

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The in-stream diatom community did not vary between the NOV and DEC sampling dates at any river (Fig. 5). The control NDS colonized similar types of algae as baseline river samples, with Bray–Curtis dissimilarities ranging between 0.26 ± 0.21% for the KUS river, 0.35 ± 0.23% in the HAY river, 0.19 ± 0.12% in the USO river, and 0.28 ± 0.17% for the INU river (Fig. 5). Ordination site scores were associated with river and not sample type (ENVFIT River $R^2 = 0.98$, $p = 0.001$, ENVFIT sample type $R^2 = 0.010$, $p = 0.99$), indicating that diatom assemblages were more distinct among rivers than between baseline samples and NDS control samples. Baseline and control NDS diatom assemblages were correlated to both in-stream water chemistry (SRP, nitrate + nitrite, SiO$_2$) and catchment land use (% agriculture, % forest, % residential; Table 3).

**Response to nutrient additions**

BMA biomass: BMA biomass was nutrient limited in the three rivers (HAY, USO, INU), but not the KUS river (Fig. 6). The KUS had lower in-stream nutrients and the lowest proportion of forested land use in its catchment. The magnitude of the BMA response to nutrient additions (LRR) was highest in the rivers with higher in-stream nutrients and % forested
catchment (USO, INU), although only significantly higher in the USO river (Fig. 7; 2-ANOVA River x Nutrient amendment $F_{6, 36} = 2.38, p = 0.049$, River main effect $F_{3, 36} = 5.76, p = 0.003$, Nutrient amendment main effect $F_{2,36} = 10.44, p = 0.0003$). The limiting nutrient varied among the rivers and there were no clear patterns between which nutrient was limiting and either in-stream nutrient concentrations or catchment land use (Fig. 6). BMA biomass was limited by P alone in the USO (2-ANOVA N x P $F_{1,12} = 3.23, p = 0.097$, P main effect $F_{1,12} = 17.49, p = 0.001$, N main effect $F_{1,12} = 1.76, p = 0.21$). Primary nitrogen limitation with secondary P limitation was observed in the HAY (2-ANOVA N x P $F_{1,12} = 8.07, p = 0.02$) and INU rivers (2-ANOVA N x P $F_{1,12} = 5.02, p = 0.045$).
Fig. 7 Log-response ratio (mean ± standard error) of BMA biomass (µg chlorophyll-a cm$^{-2}$) for phosphorus (P), nitrogen (N), and nitrogen + phosphorus (NP) treatments in the Kusatsu, Hayama, Uso, and Inukami rivers. Lines below bars indicate rivers with similar LRR based on 2-way ANOVA. Letters below bars indicate significant differences in LRR between NDS treatments based on 2-way ANOVA.

Fig. 8 Abundance of diatoms relative to other major algal groups on NDS substrates (control (n = 4), P: phosphorus addition (n = 4), N: nitrogen addition (n = 4), NP: nitrogen + phosphorus addition (n = 4)) in the Kusatsu, Hayama, Uso, and Inukami rivers at the end of the experiment. * indicates fertilization treatments that were significantly different from the control. ‘I’ indicates a significant N x P interaction as indicated by 2-way ANOVA.
Algal community response to nutrient additions:
The relative abundance of diatoms in relation to other algal groups was significantly impacted by nutrient additions in all rivers, regardless of in-stream nutrient conditions or catchment land use (Fig. 8). The relative abundance of diatoms was significantly lower in the N and P treatments in all rivers and there was no interactive effect of N and P in any river (Table 4).

The relative abundance of chlorophytes was affected by nutrient additions in all rivers (Table 4). The relative abundance of chlorophytes increased in response to both nutrients in the KUS, HAY, USO, and INU, with a median abundance of 10.0% (KUS), 22.9% (USO), and 26.7% (INU) on the +NP treatment. In the HAY, chlorophyte abundance increased with N addition only. The increase in chlorophytes was driven by increases on all nutrient addition substrates in Chlamydomonas taxa in the KUS, HAY, USO, and INU, and increases in Tetraspora in the INU river.

Diatom community structure response to nutrient additions: Diatom diversity on NDS substrates varied among the rivers (Table 4) and while some diversity metrics responded to nutrient additions, there were no consistent patterns within and/or across rivers (Table 4). Taxa richness and diversity were higher in the HAY River compared to the other rivers (Table 5). The USO River had lower diversity (H'),

### Table 4: Results of the two-way ANOVA analysis on phosphorus (P) and nitrogen (N) additions for relative abundance of algal groups and diatom community metrics in the Kusatsu (KUS), Hayama (HAY), Uso (USO), and Inukami (INU) rivers

| Metric                  | KUS       | HAY       | USO       | INU       |
|-------------------------|-----------|-----------|-----------|-----------|
| Diatom abundance (%)    | P: F_{1,12} = 37.74*** N: F_{1,12} = 7.08* N × P: F_{1,12} = 1.98 | F_{1,12} = 6.02* F_{1,12} = 38.71*** F_{1,12} = 0.25 | F_{1,12} = 0.01 F_{1,12} = 28.19*** F_{1,12} = 0.01 | F_{1,12} = 4.03 F_{1,12} = 24.81*** F_{1,12} = 0.01 |
| Chlorophyte abundance (%)| P: F_{1,12} = 68.92*** N: F_{1,12} = 20.76*** N × P: F_{1,12} = 0.35 | F_{1,12} = 4.03 F_{1,12} = 15.83** F_{1,12} = 0.01 | F_{1,12} = 0.01 F_{1,12} = 13.77** F_{1,12} = 0.87 | F_{1,12} = 6.27 F_{1,12} = 18.31** F_{1,12} = 0.39 |
| Diatom taxa richness     | P: F_{1,12} = 0.78 N: F_{1,12} = 0.14 N × P: F_{1,12} = 0.25 | F_{1,12} = 2.60 F_{1,12} = 5.09* F_{1,12} = 1.27 | F_{1,12} = 0.17 F_{1,12} = 0.47 F_{1,12} = 0.08 | F_{1,12} = 0.08 F_{1,12} = 5.09* F_{1,12} = 2.37 |
| Diatom H’                | P: F_{1,12} = 0.00 N: F_{1,12} = 4.75 N × P: F_{1,12} = 1.87 | F_{1,12} = 0.18 F_{1,12} = 6.81* F_{1,12} = 0.82 | F_{1,12} = 0.20 F_{1,12} = 0.02 F_{1,12} = 0.08 | F_{1,12} = 0.08 F_{1,12} = 2.37 F_{1,12} = 0.78 |
| Diatom taxa evenness     | P: F_{1,12} = 1.87 N: F_{1,12} = 9.6** N × P: F_{1,12} = 19.402** | F_{1,12} = 0.43 F_{1,12} = 1.99 F_{1,12} = 0.06 | F_{1,12} = 15.58* F_{1,12} = 0.02 F_{1,12} = 7.25* | F_{1,12} = 0.02 F_{1,12} = 2.94 F_{1,12} = 0.78 |
| Maximum abundance        | P: F_{1,12} = 0.19 N: F_{1,12} = 4.79* N × P: F_{1,12} = 11.751** | F_{1,12} = 0.00 F_{1,12} = 0.92 F_{1,12} = 0.21 | F_{1,12} = 8.95* F_{1,12} = 0.01 F_{1,12} = 10.37** | F_{1,12} = 0.01 F_{1,12} = 2.94 F_{1,12} = 0.78 |
| Motile                   | P: F_{1,12} = 8.55* N: F_{1,12} = 3.65* N × P: F_{1,12} = 2.86 | F_{1,12} = 7.19* F_{1,12} = 3.19 F_{1,12} = 0.67 | F_{1,12} = 13.13** F_{1,12} = 0.00 F_{1,12} = 3.19 | F_{1,12} = 3.19 F_{1,12} = 7.19* F_{1,12} = 0.78 |
| Stalked                  | P: F_{1,12} = 0.85 N: F_{1,12} = 2.14 N × P: F_{1,12} = 0.93 | F_{1,12} = 0.74 F_{1,12} = 1.27 F_{1,12} = 0.93 | F_{1,12} = 0.28 F_{1,12} = 5.54* F_{1,12} = 4.32 | F_{1,12} = 0.28 F_{1,12} = 0.35 F_{1,12} = 0.79 |
| Adnate                   | P: F_{1,12} = 1.76 N: F_{1,12} = 2.93 N × P: F_{1,12} = 4.06 | F_{1,12} = 0.09 F_{1,12} = 2.27 F_{1,12} = 0.85 | F_{1,12} = 22.95*** F_{1,12} = 2.73 F_{1,12} = 16.60** | F_{1,12} = 0.09 F_{1,12} = 3.38 F_{1,12} = 0.46 |

Asterisks indicate significant main effects and interactions of two-way ANOVA analysis (*p < 0.05, **p < 0.01, ***p < 0.001)

P main effect $F_{1,12} = 1.55, p = 0.25$, N main effect $F_{1,12} = 19.49, p = 0.008$.

Algal community response to nutrient additions: The relative abundance of diatoms in relation to other algal groups was significantly impacted by nutrient additions in all rivers, regardless of in-stream nutrient conditions or catchment land use (Fig. 8). The relative abundance of diatoms was significantly lower in the N and P treatments in all rivers and there was no interactive effect of N and P in any river (Table 4). The relative abundance of chlorophytes was affected by nutrient additions in all rivers (Table 4). The relative abundance of chlorophytes increased in response to both nutrients in the KUS, USO, and INU, with a median abundance of 10.0% (KUS), 22.9% (USO), and 26.7% (INU) on the +NP treatment. In the HAY, chlorophyte abundance increased with N addition only. The increase in chlorophytes was driven by increases on all nutrient addition substrates in Chlamydomonas taxa in the KUS, HAY, USO, and INU, and increases in Tetraspora in the INU river.

Diatom community structure response to nutrient additions: Diatom diversity on NDS substrates varied among the rivers (Table 4) and while some diversity metrics responded to nutrient additions, there were no consistent patterns within and/or across rivers (Table 4). Taxa richness and diversity were higher in the HAY River compared to the other rivers (Table 5). The USO River had lower diversity ($H'$),
| Table 5 | Median relative abundance of algal groups, the 15 most abundant diatom taxa, and diatom community metrics in the Kusatsu (KUS), Hayama (HAY), Uso (USO), and Inukami (INU) rivers on nutrient diffusing substrates (n = 4 for each treatment) |
|---------|---------------------------------------------------------------------------------------------------|
|         | KUS                                                  | HAY                                                  | USO                                                  | INU                                                  |
|         | cntrl  P  N  NP                                     | cntrl  P  N  NP                                     | cntrl  P  N  NP                                     | cntrl  P  N  NP                                     |
| Chlorophyceae – all | 0.0  4.0  1.2  10.0 | 0.0  1.7  4.5  7.2 | 1.0  6.9  5.5  22.9 | 0.0  3.0  10.5  26.7 |
| Bacillariophyceae – all | 99.2  96.0  98.7  89.9 | 99.7  98.4  95.0  92.8 | 99.5  93.0  94.5  77.0 | 100.0  96.5  88.0  73.0 |
| Achnanthidium pyrenaicum (Hustedt) H. Kobayasi | 0.0  0.0  0.4  0.2 | 0.8  0.5  0.8  0.4 | 3.5  4.2  2.7  6.9 | 0.2  1.8  3.0  3.3 |
| Cymbella turgidula Grunow | 0.2  0.2  0.0  0.0 | 0.2  0.0  0.0  0.0 | 3.6  4.2  3.4  2.7 | 3.2  1.9  2.8  1.5 |
| Encyonema silesiacum Bleisch | 6.2  6.2  7.4  6.5 | 2.6  1.2  1.6  1.4 | 1.4  2.3  1.6  0.8 | 14.8  16.4  20.8  15.4 |
| Fragilaria vaucheriae (Kütz.) Peterson | 6.3  5.5  7.2  7.9 | 7.9  4.6  6.6  4.4 | 1.8  2.8  2.2  5.5 | 5.6  9.0  11.1  11.6 |
| Gomphonema parvulum (Kützing) (Kützing) | 13.6  14.7  14.1  11.5 | 13.6  7.8  11.2  13.7 | 0.0  0.4  0.0  0.0 | 5.0  3.4  6.1  5.4 |
| Melosina varians C. Agardh | 15.6  16.1  26.0  18.0 | 13.2  16.0  13.3  14.7 | 3.7  5.2  4.5  5.8 | 34.3  23.3  19.5  23.2 |
| Navicula gregaria Donkin | 0.6  2.0  1.7  1.8 | 1.1  1.4  1.4  1.0 | 0.6  0.4  1.0  0.7 | 2.8  1.7  2.0  1.1 |
| Navicula perminuta Grunow | 13.4  21.8  14.4  16.3 | 3.9  4.9  3.1  5.0 | 2.0  2.5  0.7  1.2 | 1.6  1.7  1.0  1.7 |
| Navicula viridula (Kutzing) Ehrenberg | 0.9  0.4  0.6  0.6 | 2.2  5.4  4.2  4.9 | 0.2  0.8  0.3  0.3 | 1.5  0.5  1.8  1.3 |
| Nitzchia fonticola (Grunow) Grunow | 0.0  0.0  0.0  0.0 | 0.0  0.2  0.4  0.8 | 75.3  70.9  75.5  67.5 | 0.6  6.4  7.7  4.7 |
| Nitzchia palea (Kutzing) W. Smith | 1.6  1.2  2.1  2.1 | 2.3  2.8  3.3  2.4 | 0.4  0.0  0.4  0.0 | 1.0  0.8  1.1  1.3 |
| Nitzchia perminuta (Grunow in Van Heurck) H.Perag | 1.7  2.0  2.6  1.6 | 0.6  0.8  0.2  1.6 | 0.0  0.0  0.0  0.0 | 0.6  0.2  0.0  0.4 |
|                         | KUS |          |           |          |          | HAY |          |           |          |          | USO |          |           |          |          | INU |          |           |          |          |
|-------------------------|-----|----------|----------|----------|----------|-----|----------|----------|----------|----------|-----|----------|----------|----------|----------|-----|----------|----------|----------|----------|
|                         | cntrl | P | N | NP | cntrl | P | N | NP | cntrl | P | N | NP | cntrl | P | N | NP | cntrl | P | N | NP | cntrl | P | N | NP |
| *Reimeria sinuata* (W. Greg.) Kociolek and Stoermer | 5.6 | 4.7 | 3.1 | 5.8 | 3.4 | 1.9 | 1.8 | 2.9 | 0.2 | 0.0 | 0.0 | 0.8 | 0.6 | 0.8 | 0.4 | 0.4 |
| *Surirella angusta* Kutz | 2.9 | 2.7 | 1.9 | 6.9 | 2.4 | 4.1 | 1.8 | 3.1 | 0.0 | 0.0 | 0.0 | 0.0 | 1.4 | 2.7 | 1.2 | 1.8 |
| *Synedra ulna* (Nitzsch) Ehrenberg | 5.6 | 2.6 | 3.8 | 1.8 | 2.7 | 2.4 | 2.3 | 1.8 | 0.4 | 0.2 | 0.4 | 0.8 | 5.7 | 3.4 | 4.0 | 5.3 |
| Richness                | 31.5 | 34.0 | 33.0 | 32.5 | 44.5 | 43.5 | 41.5 | 38.0 | 20.0 | 19.5 | 20.0 | 36.0 | 36.0 | 33.0 | 30.5 | 32.5 |
| Shannon diversity       | 2.80 | 2.60 | 2.50 | 2.70 | 3.20 | 3.20 | 3.10 | 3.00 | 1.20 | 1.30 | 1.20 | 2.60 | 3.60 | 2.70 | 2.60 | 2.60 |
| Evenness                | 0.81 | 0.76 | 0.77 | 0.77 | 0.85 | 0.80 | 0.85 | 0.84 | 0.40 | 0.45 | 0.39 | 0.47 | 0.72 | 0.76 | 0.77 | 0.75 |
| Maximum abundance       | 15.8 | 21.8 | 26.0 | 18.0 | 15.0 | 16.0 | 15.0 | 15.5 | 75.3 | 70.9 | 75.5 | 67.5 | 34.3 | 23.3 | 20.8 | 23.7 |
| Highly motile taxa      | 11.5 | 12.5 | 12.5 | 18.8 | 17.9 | 21.8 | 21.0 | 26.9 | 7.8 | 7.3 | 7.9 | 6.9 | 9.8 | 16.4 | 18.9 | 14.1 |
| Stalked taxa            | 17.6 | 17.4 | 16.6 | 14.7 | 17.1 | 11.2 | 15.7 | 15.9 | 3.8 | 4.6 | 3.8 | 2.9 | 10.9 | 11.8 | 14.2 | 10.9 |
| Adnate taxa             | 3.7 | 2.3 | 2.1 | 2.4 | 4.6 | 4.0 | 2.9 | 3.4 | 4.3 | 4.7 | 3.1 | 7.7 | 3.0 | 4.8 | 5.2 | 5.7 |

cntrl: control, P: phosphorus addition, N: nitrogen addition, NP: nitrogen + phosphorus addition (NP)
taxa richness, and evenness (J) and higher dominance by a single taxon than the other rivers (Table 5). Some diatom morphological metrics did show consistent patterns with nutrient additions. The relative abundance of highly motile taxa increased with P fertilization in the HAY, USO, and INU rivers and with both P and N fertilization in the KUS river (Table 4).

Dominant diatom taxa were similar in the KUS, HAY, and INU rivers, with Melosira varians, Gomphonema parvulum, Encyonema silesiacum, and Fragilaria vaucheriae dominating the assemblages on the NDS (Table 5). The USO river was overwhelmingly dominated by Nitzschia fonticola, a taxon not common in the other three rivers, with Achnanthidium pyrenacum and Cymbella turgidula also being abundant. Despite overlap in common taxa, the overall diatom community structure was distinct among the four rivers, regardless of nutrient treatment. (Fig. 9; NMDS 2-dim solution, stress = 0.08; PERMANOVA River $F_{3,48} = 66.4, p = 0.001$).

The effect of fertilization on the overall diatom assemblage was pronounced in all rivers, regardless of in-stream water chemistry or catchment land use, with diatom assemblages on all fertilized treatments being distinct from the control (Fig. 10; all individual river PERMANOVAs significant at $p = 0.001$). In the KUS river, assemblages on control substrates were distinct from all fertilized NDS assemblages and this was driven by a higher abundance of Melosira varians, Navicula gregaria, and Nitzschia palea on control substrates (Table 6). Assemblages on the NP treatment were also distinct, driven by a higher abundance of Gomphonema parvulum, Surirella angusta, and Synedra ulna. In the HAY river, assemblages on control substrates were distinct from all fertilized NDS assemblages, driven by the higher abundance of Encyonema silesiacum and Fragilaria vaucheriae. In the USO river, diatom assemblages on the control and N treatment were distinct from the P and NP treatments, driven by a higher abundance of E. silesiacum, N. gregaria, Navicula perminuta, and Reimeria sinuata. The P and NP treatments were associated with higher abundances of Achnanthidium pyrenacum, F. vaucheriae, and M. varians. Assemblages on the control treatment were distinct from those on all fertilized treatments in the INU river, driven by a higher abundance of E. silesiacum, N. perminuta, and R. sinuata on these treatments (Table 6).

Fig. 9 Non-metric multi-dimensional scaling ordination plots of diatom assemblages on NDS (control, + P: phosphorus addition, + N: nitrogen addition, + NP: nitrogen + phosphorus addition) for Kusatsu, Hayama, Uso, and Inukami rivers (sites in species space)
Nutrient limitation of primary producer biomass and nutrient-driven changes in community structure were evident in all four rivers in the Lake Biwa basin, regardless of in-stream nutrient concentrations and surrounding land use. We observed increases in primary producer biomass, shifts from a diatom-dominated to a mixed diatom-chlorophyte algal community, and differences in overall diatom community structure with nutrient additions. The important nutrient and magnitude of the response varied among the four rivers and depended, to an extent, on the response variable examined. Changes in both algal abundance and community structure in response to nutrient additions have been observed in many lotic ecosystems across in-stream nutrient and surrounding land use gradients (e.g., Pringle 1987; Tank and Dodds 2003; Larned 2010) and the magnitude of response we observed, as measured by LRR, was similar to those observed in short-term nutrient addition studies from around the world (Francouer 2001; Beck et al. 2019). BMA biomass was primarily limited by N and secondarily by P in two rivers, P-limited in one river, and not nutrient limited in one river, partially supporting our prediction of nutrient limitation of...
BMA biomass. A long history of NDS studies in lotic ecosystems has demonstrated that BMA biomass can be limited by N, P, or co-limited by N and P simultaneously. The limiting nutrient varied across the many NDS studies due to a variety of factors (NDS type, duration of the experiment, concentration of nutrients used, etc.) (e.g., Dodds and Welch 2000; Francoeur 2001; Tank and Dodds 2003), with NP usually having the largest effect size (Beck et al. 2017).

In our study, the magnitude of the BMA biomass response to nutrient additions was related to in-stream dissolved inorganic nutrient concentrations, but the response was counter to what was expected. The lowest LRR was observed in the river with the lowest nitrate+nitrite and SRP concentrations (HAY) and the greatest LRR observed in the river with the highest nitrate+nitrite and high SRP concentrations (USO). In addition, there were no clear patterns between limiting nutrient for BMA biomass and in-stream dissolved inorganic nutrient concentrations. For example, primary N-limitation was observed in the INU river, where in-stream nitrate+nitrite concentrations were high, and P-limitation was observed in the USO river, which had elevated concentrations

Table 6  Associations between diatom species scores and multidimensional scaling (NMDS) ordination axes for the 15 most abundant diatom taxa in the Kusatsu (KUS), Hayama (HAY), Uso (USO), and Inukami (INU) rivers

|               | KUS NMDS axis 1 | KUS NMDS axis 2 | HAY NMDS axis 1 | HAY NMDS axis 2 | USO NMDS axis 1 | USO NMDS axis 2 | INU NMDS axis 1 | INU NMDS axis 2 |
|---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Achnanthis pyrenaicum | +              | −              | +              | +              | +              | +              | +              | −              |
| Cymbella turgidula      | na             | na             | na             | na             | −              | +              | −              | +              |
| Encyonema silesciacum   | +              | −              | −              | −              | −              | −              | −              | +              |
| Fragilaria vauheriae    | na             | na             | −              | −              | +              | +              | +              | −              |
| Gomphosphaera parvula   | +              | +              | −              | −              | −              | −              | −              | +              |
| Melosira varians        | −              | −              | −              | +              | +              | +              | −              | +              |
| Navicula gregaria       | −              | −              | +              | −              | −              | −              | −              | −              |
| Navicula perminuta      | −              | +              | +              | +              | −              | −              | +              | +              |
| Navicula viridula       | +              | −              | +              | −              | +              | −              | −              | +              |
| Nitzchia fonticola      | na             | na             | +              | +              | −              | +              | +              | −              |
| Nitzchia palea          | −              | −              | +              | −              | −              | −              | −              | −              |
| Nitzchia perminuta      | −              | +              | −              | +              | na             | na             | −              | −              |
| Reimeria sinuata        | na             | na             | −              | +              | −              | −              | +              | +              |
| Surirella angusta       | +              | +              | +              | −              | +              | −              | +              | −              |
| Synedra ulna            | +              | +              | −              | +              | +              | −              | −              | −              |

“+” indicates positive association between the taxa and the ordination axis, “−” indicates a negative association between the taxa and the ordination axis, “na” indicates taxa not present in that river.
of both SRP and nitrate+nitrite. The lack of consistency between in-stream nutrient levels and BMA biomass response is not entirely surprising as previous research has demonstrated that a variety of factors, including background nutrient levels and N:P ratios, can be important in predicting which nutrient limits primary production. For example, the meta-analysis of Keck and Lepori (2012) found that in-stream nutrient concentrations were correlated with response to N and P treatments and that the N:P ratio was the best predictor of N-limitation but did not predict P-limitation. While across many experiments, Sanderson et al. (2009) found the greatest response in streams with low ambient N and higher ambient P. However, others have found that in-stream nutrient concentrations and N:P ratios are poor predictors of which nutrient is limiting (Francoeur et al. 1999; Wold and Hersey 1999; Klose et al. 2012). Our in-stream nutrient values are from a two-time synoptic sampling during baseflow and not a full characterization of the nutrient profiles of these rivers. Nutrient concentrations in rivers in the Lake Biwa basin have been shown to vary seasonally (Nakano et al. 2008; Ohte et al. 2010) and we observed variation in nutrient concentrations within our rivers between our two collection dates, particularly for SRP. While nutrient limitation of BMA biomass was clearly evident in three of the four rivers, a more detailed characterization of in-stream nutrients would be necessary to clarify the relationship between in-stream nutrients and nutrient limitation.

We also predicted that patterns of nutrient limitation would vary with catchment land use, being highest in rivers draining more pristine catchments. This prediction was partially supported; nutrient limitation of BMA biomass was not observed in the KUS river, which has the highest percentage of agricultural and residential land use in its catchment. And while a high LRR was observed in the river with the most forested catchment (INU), the greatest LRR was observed in the USO river, with its catchment comprised of 45% agricultural land use. Several NDS studies have documented greatest nutrient limitation in rivers draining pristine areas and rarely report nutrient limitation in agricultural catchments. For example, Beck et al. (2017) found that both the limiting nutrient and the magnitude of the response of BMA biomass varied with land use, with lower responses in urbanized and agricultural ecoregions compared to forested areas. Keithan et al. (1988) and Munn et al. (1989) reported no consistent changes in BMA biomass in response to nutrient additions in streams draining agricultural areas in the U.S. While developed and agricultural land use usually results in higher in-stream nutrient concentrations (Meybeck 1998; Paul and Meyer 2001; Allan 2004; Johnson et al. 2009; Klose et al. 2012), in our study nutrient concentrations were highest in the rivers draining more forested catchments (USO, INU). Land use practices within Japan may contribute to our findings differing from other published studies. Throughout much of the industrialized world, and in the USA in particular, large-scale industrialized agriculture in rural locations is common. However, agricultural practices in Japan are much less centralized, with approximately one third of Japan’s agricultural output generated by urban agriculture (Moreno-Penarando 2011). Widespread urban agricultural found throughout Shiga prefecture results in heterogeneous and patchy land use within the studied catchments. For example, the INU river had a predominately forested watershed, but the area near our sampling location was dominated by agriculture and residential land uses. While the USO river sampling site had a forested near stream buffer, despite its overall watershed being characterized by more residential and agricultural land uses. The importance of near stream land use relative to catchment land use in influencing stream water quality and BMA abundance and composition has been documented in several studies (Sonoda 2001; Walker and Pan 2006; Klose et al. 2012).

One of the most striking observations in our study was the shift away from a diatom-dominated BMA community with nutrient additions in all streams. Diatoms tend to grow best under low nutrient conditions and replacement of diatoms by other algae groups has been observed in aquatic ecosystems with higher nutrient levels and in response to in situ nutrient additions (Rosmarin 1983; Bothwell 1989; Cámara and Alonso 2006; Lange et al. 2011; Chase et al. 2017; Timoshikin et al. 2018). Filamentous chlorophytes, capable of exploiting both water column and benthic nutrients, often dominate in rivers and lakes with elevated nutrient concentrations or on+P treatments of NDS studies (Lowe et al. 1986; Dodds 1991; Stevenson et al. 2006; Ozersky et al. 2018). The chlorophytes colonizing NDS in our study were primarily unicellular, non-filamentous taxa, less able to take
advantage of nutrients outside of the biofilm, indicating that they were most likely responding to the additional nutrients from the NDS. The shifts away from a diatom-dominated community corresponded to catchment land use, with the greatest decrease in diatoms found in the more forested catchments (INU, USO). Our findings highlight the importance of looking at both biomass and species composition to understand potential ecosystem-level impacts of elevated nutrient levels. Whereas measurement of algal abundance provides information on the quantity of food available, shifts in the types of algae can provide information as to habitat condition and quality of food available for secondary consumers. Algae are an essential source of certain poly-unsaturated fatty acids (PUFA; e.g., ω-3, ω-6) that cannot be synthesized by animals themselves. The PUFA content varies across algal groups, with diatoms and cryptophytes being considered the best food sources, chlorophytes being considered intermediate-quality food sources, and cyanophytes being considered low-quality food sources (Brett et al. 2006; Burns et al. 2011; Taipale et al. 2013). In lakes, the highest zooplankton biomass was associated with the fatty acid quality of phytoplankton and not phytoplankton biomass (Gladyshev et al. 2010). Overall periphyton community fatty acid profiles have been shown to shift with experimental nutrient additions and along increasing nutrient gradients (Hill et al. 2011; Cashman et al. 2013). Iannino et al. (2020) linked shifts in fatty acid profiles with phosphorus additions, as diatoms became outcompeted by chlorophytes and cyanophytes. In our study, the increase in chlorophytes with both N and P fertilization indicates a shift away from a high quality to an intermediate-quality food source.

We also observed shifts within the diatom community in all rivers in response to nutrient additions, with the overall diatom community on the control substrates being different from all fertilized treatments. Changes within the diatom community in response to nutrient additions have been documented in many NDS studies (e.g., Stockner and Shortreed 1976; Lowe et al. 1986; Keithan et al. 1988; Pringle and Bowers 1984; Pringle 1990). We did not see consistent patterns across rivers in individual taxa that came to dominate with nutrient additions. In fact, the shifts in diatom community within a river in response to nutrient additions were much smaller than the differences in diatom community composition among rivers (Fig. 9). We attribute this to differences in background nutrient concentrations and other habitat conditions (see below) among the rivers as baseline diatom assemblages were strongly correlated to both in-stream water chemistry and catchment land use (ENVFIT analysis). We did, however, observe increases in the relative abundance of highly motile diatom taxa in all rivers in response to nutrient additions. Several other studies have documented a shift from small, sessile taxa capable of exploiting low nutrient conditions to larger, more mobile taxa with nutrient additions (Pringle and Bowers 1984; Pringle 1990). Finally, while it has often been observed that nutrient additions result in dominance by erect, tall-growing taxa in streams without light limitation (Lowe et al. 1986; Bothwell 1989; Lohman 1991; Chase et al. 2017) due to high profile diatom guilds being favored in high nutrient conditions (Passy 2007), we did not observe an increase in stalked taxa in any river. Motile and high-profile taxa are able to remain in the euphotic zone of the biofilm even as nutrient additions stimulate algal growth and self-shading within the biofilm. While we did not quantify sediment conditions in the rivers, fine sediment loads in many Japanese rivers are high (Yamada and Nakamura 2002), which would favor low-profile, motile taxa who are both capable of exploiting excess nutrients and adapted to high sediment loads.

Several factors, related to both study design and geography of our study region, may have contributed to our findings being different from our predictions and to those reported in the literature. First, within the Lake Biwa basin, it is difficult to find rivers along a land use gradient that have similar climate and underlying geology. As you move northward, land use shifts from urban, to more agricultural, to more forested. There is also a noticeable climatic shift as you move north, with the northern region being colder and wetter. Bedrock geology was also variable among our sites, with the headwaters of the KUS and HAY being primarily granitic, the USO being silica-rich volcanic rhyolite, and the INU being clastic sedimentary. Second, we conducted our study during the winter, dry season on Honshu, when BMA biomass is lowest in Japan (Aizaki 1979), rivers were at baseflow, and watershed-nutrient delivery may have been reduced. While the majority of NDS studies have been conducted during peak BMA growth season, differences in nutrient limitation across seasons have been
observed by some (e.g., Francoeur et al. 1999; Wold and Hersey 1999; Beck et al. 2017; Beck et al. 2019), although it was unclear in these studies whether seasonal differences result from differences in temperature or nutrients or some combination of the two. Finally, the intense hydrologic management of rivers in populated areas of Japan may alter the timing and delivery of nutrients to these ecosystems, thus shaping how BMA respond to nutrient additions. The steep topography of Japan has resulted in population, industrialization, and agricultural being concentrated in coastal areas and along alluvial plains (Yoshikawa 2004). Consequently, most rivers in Japan are hydrologically regulated and channel works, such as dams, channelization, and concrete embankments, currently dominate the structure of most rivers and streams flowing through populated areas (Toda et al. 2002; Yamada and Nakamura 2002; Yoshimura et al. 2005). Channel works trap both sediments and nutrients which may be released during flooding, potentially resulting in a disconnect between land use and synoptic measurements of in-stream nutrients.

Finally, while our study is one of the first examples of using NDS to infer nutrient limitation of primary production in rivers of Japan, there are limitations to this method worth noting in the context of our study. A major shortcoming of NDS is that only a single stressor is assessed when it is commonly accepted that multiple abiotic and biotic stressors act in concert to determine BMA biomass and community structure (e.g., Baekkelie et al. 2017; Beck et al. 2019). We did not quantify the impacts of grazers or physical factors in our study. In rivers without light limitation, grazing can be an important top-down control affecting both algal biomass and community structure, thus confounding the effects of nutrient additions (Feminella and Hawkins 1995; Lamberti et al. 1995). Grazer communities have been shown to modify BMA community structure rivers in Shiga prefecture (Katano et al. 2007; Katano and Doi 2019). Physical factors, such as turbidity, flow, and scouring, can also impact the results of NDS experiments by reducing light reaching BMA or physically removing algal biomass (Dodds and Welch 2000; Beck et al. 2017). Near-bed water velocity has been shown to influence benthic algal biomass accumulation in rivers in Japan (Ateia et al. 2016). While we did not measure river flow or scouring, there were no significant rainfall or flooding events during the time of our study. Finally, we used NDS to measure nutrient limitation in one reach in the downstream area of each river during the dry, winter season, and it is likely that patterns of nutrient limitation could vary along the river continuum in these heterogeneous rivers. Nutrient concentrations in rivers can be spatially variable (Dent and Grimm 1999; Hoellein et al. 2011) and significant variability in nutrient limitation at the reach scale has been demonstrated (Irvine and Jackson 2006).

Despite the differences in our study region and issues inherent in the NDS approach, similar to many studies from other regions, our experiment conclusively demonstrated that BMA growth was stimulated by nutrient addition. There is likely widespread nutrient limitation in rivers of Japan, across both in-stream nutrient and land use gradients, and any increases in nutrient loading will likely stimulate benthic algal growth. The susceptibility to nutrients of BMA in source rivers has the potential to have profound impacts to the larger Lake Biwa system. River–lake linkages are often overlooked (e.g., Kling et al. 2000; Luecke and MacKinnon 2008), or when they are considered, the focus is primarily on how lakes impact downstream river segments (e.g., Jones 2010; Gillet et al. 2016). However, in-flowing rivers can be a source of carbon and nutrients (e.g., Cole et al. 2006; Jones 2010; Canham et al. 2012; Sadro et al. 2012) and algal seed colonizers to lakes (Conroy et al. 2008; Bridgeman et al. 2011). Rossiter (2000) noted that widespread channelization in the rivers feeding Lake Biwa led to reduced flora and fauna and reduced nutrient spiraling in these rivers, resulting in greater nutrient transport to the lake. As an ancient lake, Lake Biwa has high levels of biodiversity and endemism. Subsequently, it is thought to be less resistant to species invasions and more vulnerable to ecological impacts (Kawanabe 1996; Hampton et al. 2018). Lake Biwa itself has been subject to eutrophication in the latter half of the twentieth century (Hsieh et al. 2010; Tsugeki et al. 2010), but stricter controls on industrial pollution and sewage treatment have reduced concentrations of nitrogen and phosphorus in the lake (Okubo 2012). Our study took place during the winter low flow period, when catchment–river–lake linkages were reduced. Further experimentation during the summer rainy season (late June–early July) will aid in predicting how the Lake-Biwa basin may respond to changes in nutrient loading.
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Data availability  The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest  The authors have no financial or non-financial interests that are directly or indirectly related to the work submitted for publication.

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