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Nitrogen and Phosphorus Uptake Stoichiometry Tracks Supply Ratio During 2-year Whole-Ecosystem Nutrient Additions

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Abstract

Nutrient uptake, storage, and release are critical ecosystem functions that affect carbon processing and food web dynamics. Yet, mechanisms controlling when ecosystems are net sinks or sources of nutrients are uncertain. Specifically, how nutrient supply ratios alter rates and ratios of net nutrient uptake and release is unclear. To assess whether net nitrogen (N) and phosphorus (P) uptake and release are linked to supply N:P, we experimentally enriched five forest streams at different N:P (target molar N:P range 2:1–128:1) for 2 years. We quantified net nutrient exchange (NNE) as the difference between the expected N and P fluxes assuming conservative transport (background concentrations plus experimental inputs) and the observed nutrient fluxes at the downstream end of each experimental stream reach. Supply N:P did not affect the magnitude of NNE for either N or P, but the likelihood of net N and P uptake was greatest at intermediate N:P supply (N:P = 99:1 and 55:1, respectively). Streams appeared to be highly flexible in their N:P uptake and release; the slopes between NNE_N and NNE_P within each stream increased with supply N:P. Furthermore, slopes comparing supply N:P to uptake and release N:P were near one (0.98 ± 0.06 SE and 0.82 ± 0.13 SE, respectively), indicating a high degree of flexibility. Overall, we found greater stoichiometric flexibility than has been shown in short-term nutrient-addition experiments. We suggest that this flexibility results from changes in nutrient recycling within biofilms or changes in community structure, which may take longer to manifest than the duration of shorter-term experiments.

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Highlights

- We tested the flexibility of N:P uptake during long-term ecosystem N and P additions.
- N and P were used at nearly the same ratio at which they were supplied to each stream.
- We found higher N:P flexibility than observed during short-term nutrient additions.

Introduction

The cycling of elements in ecosystems is often regulated by the availability of other elements (Schlesinger and others 2011; Helton and others 2015). The coupled cycling of N and P has received particular attention because both N and P often limit biological growth but, when present in excess, can cause severe ecosystem impairment (Conley and others 2009). The retention of N and P is an important ecosystem function that may be mediated by their relative availability (Schade and others 2011; Finlay and others 2013). Anthropogenic nutrient loading leads to ratios of nutrient supply (N:P) that are increasingly unbalanced relative to biological demand (Peñuelas and others 2013). As such, the degree to which nutrient demand can track supply or is constrained by the stoichiometry of organisms is important for predicting rates of net nutrient uptake or release (Sterner and Elser 2002). Ecosystem-level responses to unbalanced ratios of supply will depend on the responses of individual organisms to changes in supply ratios (Güsewell 2004; Scott and others 2012). There may also be emergent properties at the ecosystem level that promote higher stoichiometric flexibility in nutrient uptake relative to supply than is demonstrated by individual species (Cross and others 2005; Thrane and others 2017). Thus, predicting long-term ecosystem responses to increasing nutrient loads requires understanding the stoichiometric flexibility of entire communities and ecosystems.

Headwater streams provide an ideal ecosystem in which to study the effects of nutrient supply on nutrient cycling, as their directional, longitudinal nature simplifies quantification of nutrient supply and demand of the entire ecosystem (Stream Solute Workshop 1990). Nutrient cycling in streams is dynamic and comprised of fluxes between the water column and the benthic environment. The sum of gross nutrient uptake and gross nutrient release represents net nutrient exchange (NNE) within the ecosystem. When gross uptake exceeds gross nutrient release, NNE represents net uptake, and when gross release exceeds gross uptake NNE represents net release. While the phrase ''net uptake'' has previously been used to describe NNE (for example, Trentman and others 2015), in this paper we use the directionally neutral phrase NNE when describing all net fluxes and reserve the use of the phrase ''net uptake'' for situations in which gross uptake exceeds gross release. Most previous research on nutrient cycling in stream ecosystems has focused on the gross fluxes of nutrients, leaving our understanding of NNE much less developed (Brookshire and others 2009; von Schiller and others 2015).

The N:P stoichiometry of ecosystem NNE should be linked to the biomass N:P of the community of organisms that drive net uptake (for example, plants, algae, heterotrophic microorganisms). In an idealized system, ecosystem net uptake N:P from the soluble pool should equal the growth rate-weighted N:P of community biomass, under the assumption that uptake is predominantly biological, turnover times of N and P are similar, and the soluble pool is the primary source of nutrients to organisms (Cross and others 2005; Fanin and others 2013). If the N:P of net uptake deviates from the N:P of supply, then the limiting nutrient should become increasingly scarce relative to the non-limiting nutrient (Cross and others 2005; Small and others 2009). The N:P of net uptake is more likely to deviate from the N:P of supply if the community has fixed stoichiometry (homeostatic N:P content) and less likely if the community can alter its N:P to be more similar to supply (flexible N:P content; Sterner and Elser 2002, Cross and others 2005). Flexibility in the N:P of ecosystem *NNE* may arise from several mechanisms. At the species level, organisms display physiological flexibility in cell nutrient quotas (Hall and others 2005; Gulis and others 2017). Over longer time scales, changes in the ratio of nutrient supply may alter nutrient recycling efficiency and community structure in ways that can align nutrient demand with nutrient supply (Tilman 1982; Schade and others 2011; Thrane and others 2017). In forest streams, bacteria and fungi associated with detrital carbon drive biological nutrient uptake (Tank and others 2018), and both groups have a moderate degree of stoichiometric flexibility (Makino and others 2003;

Scott and others 2012; Gulis and others 2017). However, whether shifts in N:P supply produce changes in community structure or nutrient recycling over longer time scales that promote stoichiometric flexibility and allow the N:P of net nutrient uptake to reflect nutrient supply remains unclear.

Previous experimental studies on the effects of nutrient enrichment in headwater streams have found stark changes in ecosystem structure and function that may alter net nutrient exchange. For instance, water column nutrient enrichment leads to increases in nutrient content of benthic organic matter (Gulis and Suberkropp 2003; Suberkropp and others 2010; Manning and others 2015) and increases in microbial respiration and litter decomposition rates (Rosemond and others 2015; Kominoski and others 2018). Microbial colonization simultaneously depletes benthic organic matter stocks and increases the nutrient content of the organic matter that remains, with a net result of reduced storage of nutrients, particularly in the summer (P. M. Bumpers and others, *unpublished data*). Although nutrient enrichment reduces nutrient storage, how NNE is affected by enrichment remains unclear. Nutrients taken up in the stream can be transferred up the food web, exported as fine particles, or lost through dissimilatory processes, which can all influence NNE independent of a stream's ability to store organic matter.

We quantified the influence of nutrient enrichment across a range of supply N:P on the long-term dynamics of dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) NNE in five forested headwater streams. Our experimental design included adding both N and P to the five streams at different concentrations to create a gradient of supply N:P (2:1–128:1). We used NNE estimates from these streams to answer two questions: (1) What drives variation in the NNE of N and P in nutrient-enriched streams? (2) To what degree does the NNE of N and P in streams match supply N:P (that is, exhibit non-homeostatic behavior)? We hypothesized that net uptake of both N and P would be maximized near supply N:P of 8:1 to 16:1, based on cellular stoichiometry of heterotrophic stream microorganisms. However, we expected considerable intra-annual variation in NNE rates due to seasonal changes in drivers of ecosystem metabolism (for example, temperature, microbial biomass, light; Kominoski and others 2018). Further, we predicted that the streams would exhibit a similar degree of homeostasis to that of the fungi and bacteria that dominate biogeochemical processes in these heterotrophic ecosystems (Schade and others 2011; Scott and others 2012; Gulis and others 2017). Specifically, we hypothesized that the inverse homeostasis coefficient (H^{-1}, Sterner and Elser 2002), interpreted as the log–log slope of the relationship between the N:P of nutrient supply and the N:P of net nutrient uptake or release, would reflect the degree of homeostasis of the bacteria and fungi that dominate these systems (for example, $H^{-1} \approx 0.5$; Makino and others 2003; Danger and Chauvet 2013; Gulis and others 2017). Alternatively, the slope of this relationship between supply and NNE might be closer to one due to changes in community composition that align demand with supply (Makino and others 2003; Danger and others 2008). To address these questions, we used a mass-balance approach to estimate NNE of DIN and SRP over a 2-year period in the five stream reaches that were experimentally enriched to create a gradient in water column N:P.

Methods

Overview

We estimated the NNE of N and P using an unconventional mass-balance approach during a long-term whole-stream nutrient enrichment experiment. This experiment was conducted primarily to assess the impact of nutrient enrichment on the processing rates and fates of organic matter and attendant effects on energy flow pathways and growth and production of consumers. While nutrient enrichment increased microbially-driven changes in organic matter processing rates and standing stocks, and production and growth of consumers (Bumpers and others 2015, 2017; Manning and others 2015; Demi and others 2018), the effects of enrichment at different N:P ratios on nutrient uptake dynamics has not been previously tested. Here, we quantified NNE based on the difference between the downstream nutrient concentrations and our estimate of nutrients added to the stream in experimental enrichments plus the measured upstream background concentrations (calculation details provided below). When we observed lower downstream nutrient concentrations, we interpreted the difference as net uptake. Similarly, we characterized a stream as having net nutrient release when downstream nutrients exceeded added concentrations. We used a bootstrapping technique to generate confidence intervals for our estimates and incorporate uncertainty in our nutrient concentration predictions in

each estimate. When evaluating the potential drivers of NNE, we used statistical models to test the effects of supply N:P, discharge, temperature, light, fungal biomass, and algal biomass (see below for sampling and analysis methods).

Study Site and Nutrient Manipulations

This study was conducted at the United States Department of Agriculture Forest Service Southern Research Station Coweeta Hydrologic Laboratory (hereafter, Coweeta) in the southern Appalachian Mountains, Macon County, North Carolina, USA (see Swank and Crossley (1988) for site information). We studied 70-m reaches of five low-order streams that we enriched with N and P for 2 years. The streams were similar physically, chemically, and biologically prior to nutrient addition (Manning and others 2016). We monitored ambient nutrient concentrations for 1 year before the continuous nutrient additions began on 11 July 2011 using solutions of ammonium nitrate (NH_4NO_3) and phosphoric acid (H_3PO_4). We programmed the nutrient-addition systems to maintain constant target enrichment concentrations and N:P by using metering pumps (LMI Milton Roy, Ivyland, Pennsylvania, USA) to adjust the rate of nutrient addition proportionally with discharge, which we measured continuously using pressure transducers (Keller America, Newport News, Virginia, USA). We used CR800 dataloggers (Campbell Scientific, Logan, Utah, USA) to control the nutrient dosing and to record the number of strokes the pumps made per 15 min. We distributed nutrient inputs along each experimental reach by releasing a nutrient-enriched solution using irrigation lines with spigots placed about 5 m apart along the experimental reach from 0 to 65 m from the top of the reach. We targeted different combinations of N and P concentrations in each stream (DIN added + background = 81, 244, 365, 488, and 650 μg L^{-1}; SRP added + background = 90, 68, 51, 33, and 11 μg L^{-1}). These additions correspond to an exponential gradient in targeted molar N:P of 2:1, 8:1, 16:1, 32:1, and 128:1, respectively.

Sample Collection and Analysis

We collected water samples every two weeks upstream and downstream of the nutrient inputs (n = 1, at 0 m and at 70 m). We filtered water samples in the field (0.45-μm nitrocellulose membrane filters; Millipore, Billerica, Massachusetts, USA) and froze them within 24 h of collection. We analyzed samples for DIN (NH_4–N + NO_3–N) and SRP concentrations within 28 days (Alpkem Rapid Flow Analyzer 300 for DIN, spectrophotometric method with UV-1700 spectrophotometer, Shimadzu, Kyoto, Japan, for SRP). Further details about the experimental design, infrastructure, and stream physicochemical characteristics can be found in Manning and others (2015, 2016) and Rosemond and others (2015). Following previous studies from this experiment (Manning and others 2015, 2016), we considered water chemistry values greater than 1.96 standard deviations from the mean values for a given stream to be outliers, which excluded 7% of DIN values and 4% of SRP values (mean values presented in Table 1).

Estimating Net Nutrient Uptake and Release

Estimating NNE requires knowledge of the concentrations of nutrients expected to occur in the absence of NNE by the stream community (that is, the conservative concentration, $[C]_{cons}$). Typically, estimates of the expected concentration under conservative transport are derived from a conservative tracer such as chloride (Stream Solute Workshop 1990). Here, we used an alternative approach in which we estimated $[C]_{cons}$ of nutrients added to each stream reach and used bootstrapping to propagate the uncertainty in conservative concentrations as well as NNE (Figure 1). Specifically, we incorporated uncertainty around stage-discharge relationships, volume of nutrient solution moved per stroke of the dosing pump, and the relationship between discharge and the width of the stream channel. We calculated the enriched in-stream concentration of added nutrients (I; μM), which represents the cumulative sum of nutrients added along the entire experimental reach, as the product of the pump rate (n; strokes s^{-1}), the volume of nutrient solution per pump stroke (p_v; L $stroke^{-1}$), the concentration of the nutrient stock solution ($[C]_s$; μM) divided by the discharge of the stream (Q, L s^{-1}):

$$I = \frac{n * p_v * [C]_s}{Q} \quad (1)$$

The conservative concentration of nutrients ($[C]_{cons}$, μM) was then calculated as a function of the background nutrient concentration at 0 m ($[C]_0$, μM) plus I (time series of nutrient supply presented in Appendix S1):

$$[C]_{cons} = [C]_0 + I \quad (2)$$

We measured discharge in each stream using salt-dilution gauging on more than 20 dates during the experiment. We then used relationships be-

Table 1. Average Annual Concentrations of Dissolved Inorganic Nitrogen (DIN) and Soluble Reactive Phosphorus (SRP) Measured in μg L^{-1} and the Average N:P in Each Stream

Stream target N:P	Pretreatment			Year 1			Year 2		
	N:P	DIN (± SE)	SRP (± SE)	N:P	DIN (± SE)	SRP (± SE)	N:P	DIN (± SE)	SRP (± SE)
2	12.5	17 (2.0)	3.0 (0)	3.0	120.5 (15.5)	90.1 (6.5)	2.6	80.4 (7.9)	69.4 (6.5)
8	127.6	173.0 (10)	3.0 (0.3)	14.3	302.8 (26.2)	46.9 (4.1)	8.6	149.1 (10.7)	38.6 (2.7)
16	27.1	49.0 (8.0)	4.0 (1.0)	18.0	429.5 (51.2)	52.8 (7.3)	16.0	409.1 (85.3)	56.7 (11.9)
32	125.1	238 (22.0)	4.0 (0.4)	42.7	362.8 (26.5)	18.8 (1.9)	30.6	388.1 (12.0)	28.1 (1.2)
128	57.5	78.0 (9.0)	3.0 (0.3)	103.3	366.9 (43.1)	7.9 (1.0)	105.6	494.1 (32.6)	10.4 (0.5)

Averages and standard errors (SE) from the year before enrichment started and the 2 years of enrichment are presented. Streams are identified by their target N:P enrichment ratios.

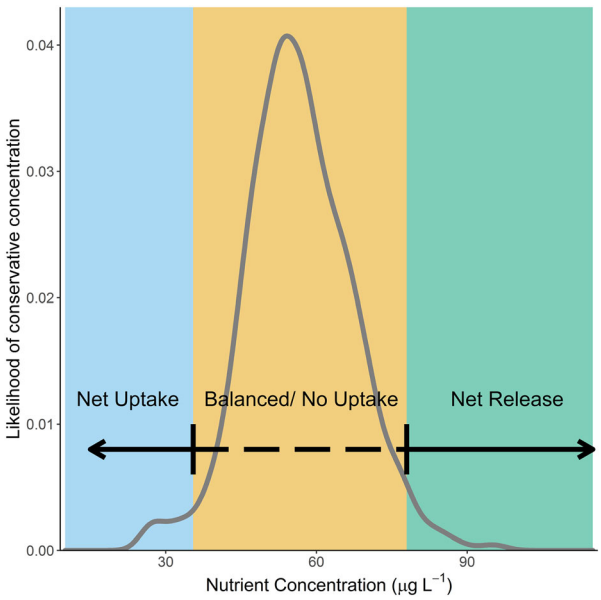

Figure 1. Example distribution of estimated P supply (added + background) concentrations (black line) from the stream enriched at a 16:1 N:P on 1 July 2012. Measured nutrient concentrations within the 95% CI of the estimated supply concentration (orange area) were classified as having no net uptake or release. Measured nutrient concentrations below the 95% CI of the supply concentration (blue area) were classified as net uptake, while greater measured concentrations (green area) were classified as net release.

tween discharge estimates and water depth (recorded by the CR800 data loggers) to develop a rating curve for each stream to infer discharge on all sampling dates by fitting power–law relationships to the water depth and discharge data. In one stream, the water depth logger was buried for an extended period, affecting the pressure transducer and leading to unreliable measurements, so we interpreted flow based on a multiple linear regression with the dilution-gauging estimates from two of the other streams (rating curves presented in Appendix S2). To account for uncertainty in depth–discharge relationships, for each bootstrapped iteration we resampled the water depth and discharge data used to generate the rating curve. Measured p_v was used when taken on the same day as measurements of nutrient concentration ($[C]_x$). When there was no measurement of p_v for a given day, we randomly sampled a value of p_v that was made on another date in the same stream.

We estimated NNE for N and P using a mass-balance calculation. We calculated NNE (μmol m^2 s^{-1}) as the difference between the measured nutrient concentration at the downstream end of each experimental reach ($[C]_x$; μM) and $[C]_{cons}$ multiplied by discharge (Q; L s^{-1}) and divided by the streambed area (A; m^2) within the 70-m reach:

$$NNE = \left([C]_{cons} - [C]_x\right) * Q/A \quad (3)$$

We considered an estimate of NNE to indicate net uptake if the 95% confidence intervals of the bootstrapped simulations were greater than zero, net release if the 95% confidence intervals were less than zero, and in balance (that is, gross uptake equals gross release) if the 95% CI overlapped zero. While we treat measured nutrient concentrations as error-free in this analysis, we conducted a sensitivity analysis to explore the effect of measure-

ment error (see Appendix S3). Additionally, we quantified the effect of adding nutrients along the entire reach, as opposed to adding them at a single point at the top of the reach, on our estimates of NNE (see Appendix S4).

Quantifying Ancillary Variables

We also examined the controls on NNE using covariate site data. We recorded light levels (photosynthetically active radiation) and water temperature using the CR800 data loggers. We estimated algal biomass monthly by measuring the chlorophyll-*a* content of biofilms scraped from five 225-cm^2 tiles from each stream, each of which was deployed for 2 months. We quantified fungal biomass based on the ergosterol concentrations of leaves and wood (collected eight times during the experiment) and estimates of the mass of leaves (monthly collections) and wood (single estimate) in each stream. We used bootstrapping to account for uncertainty in both fungal and algal biomass, and used LOESS regressions to interpolate values on all dates during the study (see Appendix S5 for more detail).

Data Analysis and Modeling

To evaluate the biotic and abiotic drivers of *NNE*, we fit two sets of models to the data. First, we used linear mixed-effects models to identify drivers of *NNE*. We modeled *NNE* from each date and stream as a function of the N:P of nutrients supplied, a quadratic effect of N:P of nutrients supplied, stream discharge, temperature, light, chlorophyll-*a*, and fungal biomass. We used random effects of stream identity to address the non-independence of our estimates in the models of continuous rates. Before selecting these predictors, we evaluated their collinearity, and found no strong correlations among predictors (maximum Pearson's correlation coefficient < 0.50, Table A1). We normalized predictor variables by log-transforming then subtracting mean values and dividing by the standard deviation, which allowed us to evaluate the relative magnitude of each variable's effect (Gelman and Hill 2007). To meet the assumption of residual heterogeneity we also transformed the continuous NNE values using a Yeo-Johnson transformation (Yeo and Johnson 2000) in which positive values were transformed as $\log(x + 1)$ and negative values were transformed as $-\log(-x + 1)$. To incorporate uncertainty into our analysis we bootstrapped the model-selection and model-fitting process. For each bootstrapped iteration, we used stepwise model selection, which sequentially eliminated variables from the full model if their elimination reduced Akaike information criteria (AIC) scores. We then selected the model that was most often retained as the best model across the 1000 bootstrapped iterations and fit this model to each of the 1000 bootstrapped iterations to estimate confidence intervals around the fixed effects. The second set of models were logistic regressions that included the same candidate variables to predict the likelihood of net uptake (positive NNE) or net release (negative NNE). For the logistic regressions, the model selection did not include random effects, but the final model included a random effect of stream identity when estimating parameter values for the best model. We used the bootstrapped estimates of the predictor variables in this analysis, but the response variables remained constant as the classification scheme for determining the likelihood of net uptake or release had already taken the uncertainty around *NNE* estimates into account.

We then used two approaches to evaluate whether nutrient cycling was homeostatic in these streams. First, we evaluated the slope of the relationship between DIN NNE (NNE_{din}) and SRP NNE (NNE_{srp}) within each stream using a simple linear model in which NNE_{din} was modeled as a function of the interaction between stream identity and NNE_{srp}. If NNE was homeostatic across the streams, we expected each stream to have the same slope (non-significant interaction term; Sterner and Elser 2002). If NNE was flexible in regard to the N:P of supply, we expected that the interaction would be significant and the slopes of the NNE_{din} to NNE_{srp} relationship would vary across enrichment N:P. Second, we fit models to the subset of the data in which there was net uptake of both DIN and SRP for a given stream and date, or net release of both DIN and SRP. We fit models to the log–log relationship between the molar supply N:P (background plus added concentration) and NNE_{din}:NNE_{srp} to these two subsets of data (net uptake or net release). The slope of the relationship between log-transformed N:P supply and NNE_{din}:NNE_{srp} represents the inverse of the homeostasis coefficient, H (H^{-1}; Sterner and Elser 2002). If net uptake or release was homeostatic, we expected a slope of zero, with the *y*-intercept near the N:P of microbial biomass about 8:1–16:1. Alternatively, an increase in NNE_{din}:NNE_{srp} of net uptake or net release with increasing N:P supply would indicate stoichiometric flexibility, with a slope of one if NNE was completely flexible in regards to supply stoichiometry. We estimated the slope of this relationship and compared the slope to

null models with a slope of zero (completely homeostatic) and one (fully flexible).

Results

Approximately three-quarters of our estimates showed net uptake or release of nutrients within the stream channel (Figure 2). We found net uptake of DIN in 47% and net uptake of SRP in 45% of our measurements (Figure 2). We observed net release of DIN in 30% and net release of SRP in 23% of our measurements. Furthermore, individual streams switched between net uptake and net release throughout the experiment (Figure 3). Despite net uptake being more common than net release, confidence intervals of NNE_{din} and NNE_{srp} averaged over the entire course of the experiment overlapped with zero in most streams (Table A2). This suggests that dissolved nutrient inputs and exports were approximately balanced during the enrichment at the stream-reach scale, and that although net release events were less frequent, their higher magnitude compensated for the more frequent periods of net uptake.

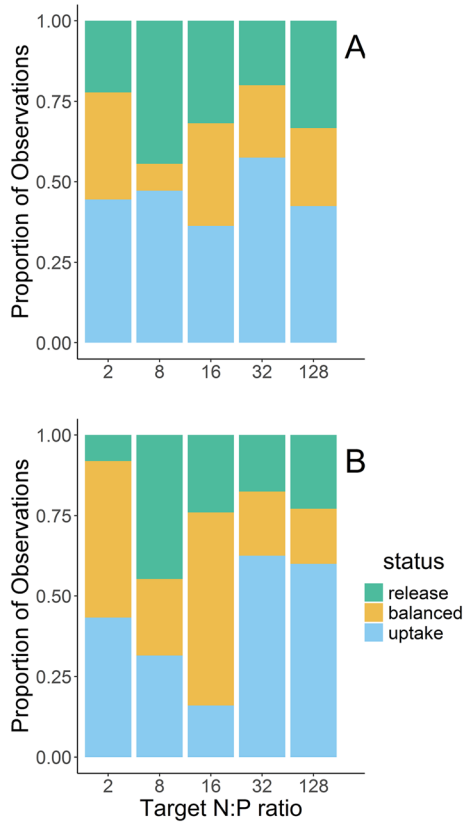

Figure 2. Relative frequency of net uptake, net release, and balanced uptake conditions of dissolved inorganic nitrogen (DIN, A) and soluble reactive phosphorus (SRP, B) across the five study streams. Streams are identified by their target supply N:P.

The N:P of supply did not affect rates of NNE_{din} or $NNE_{srp,}$ but did affect the likelihood of net DIN and SRP uptake or release. The mean quadratic terms for supply N:P in the models of both net DIN and SRP uptake were negative, with the greatest likelihood of net DIN uptake at a supply N:P of 99:1 (CI: 37:1− > 128:1) and the greatest likelihood of net SRP uptake at a supply N:P of 55:1 (CI: 29:1− > 128:1) (Figure 4, Table 2). The effect of supply N:P on net N and P release mirrored the effect on net uptake, with positive quadratic terms implying a greater likelihood of net release at high or low supply N:P. Beyond supply N:P, there were several other variables that predicted the net rates and likelihood of net nutrient uptake (Table 2). Chlorophyll-*a* was consistently linked to higher net uptake rates, a greater likelihood of net uptake, and a lower likelihood of net release (Table 2). Higher discharge was associated with a greater likelihood of net DIN uptake. Light was also a predictor of the likelihood of net DIN uptake and net release, with net uptake being more likely when light was low, and net release more likely when light was high (Table 2).

We used two different analyses to evaluate whether the stoichiometry of NNE was flexible across streams. If stream NNE was strictly homeostatic, we expected the slope of the relationship between NNE_{srp} and NNE_{din} to be the same across the streams with different supply N:P. We found that the slopes of the relationship between NNE_{din} and NNE_{srp} varied among streams ($F_{4,142} = 15.0$, $P < 0.0001$), and slopes tended to be steeper in streams with higher supply N:P (Figure 5a, Table A3). Slopes of the relationship between NNE_{din} and NNE_{srp} ranged from 73 (se = 16.4) in the stream with an enrichment N:P of 128:1, to a slope of 1.38 (SE = 0.65) in the stream with an enrichment N:P of 2:1 (Figure 5A, Table A3). Further, we expected that if the stream NNE was homeostatic, the log–log slope between supply N:P and either net uptake or release NNE_{din}:NNE_{srp} would be indistinguishable from zero. When we examined the dates on which there was net uptake of both DIN and SRP, we found that the log–log slope of the relationship between supply N:P and NNE_{din}:NNE_{srp} was 0.98 (± 0.06 SE, Figure 5b). The slope of this relationship was different from zero ($F_{1,40} = 230$, $P < 0.0001$), but not different from one ($F_{1,40} = 0.12$, $P = 0.73$). When we examined only the dates when there was net release of both DIN and SRP the log–log slope of the relationship between supply N:P and

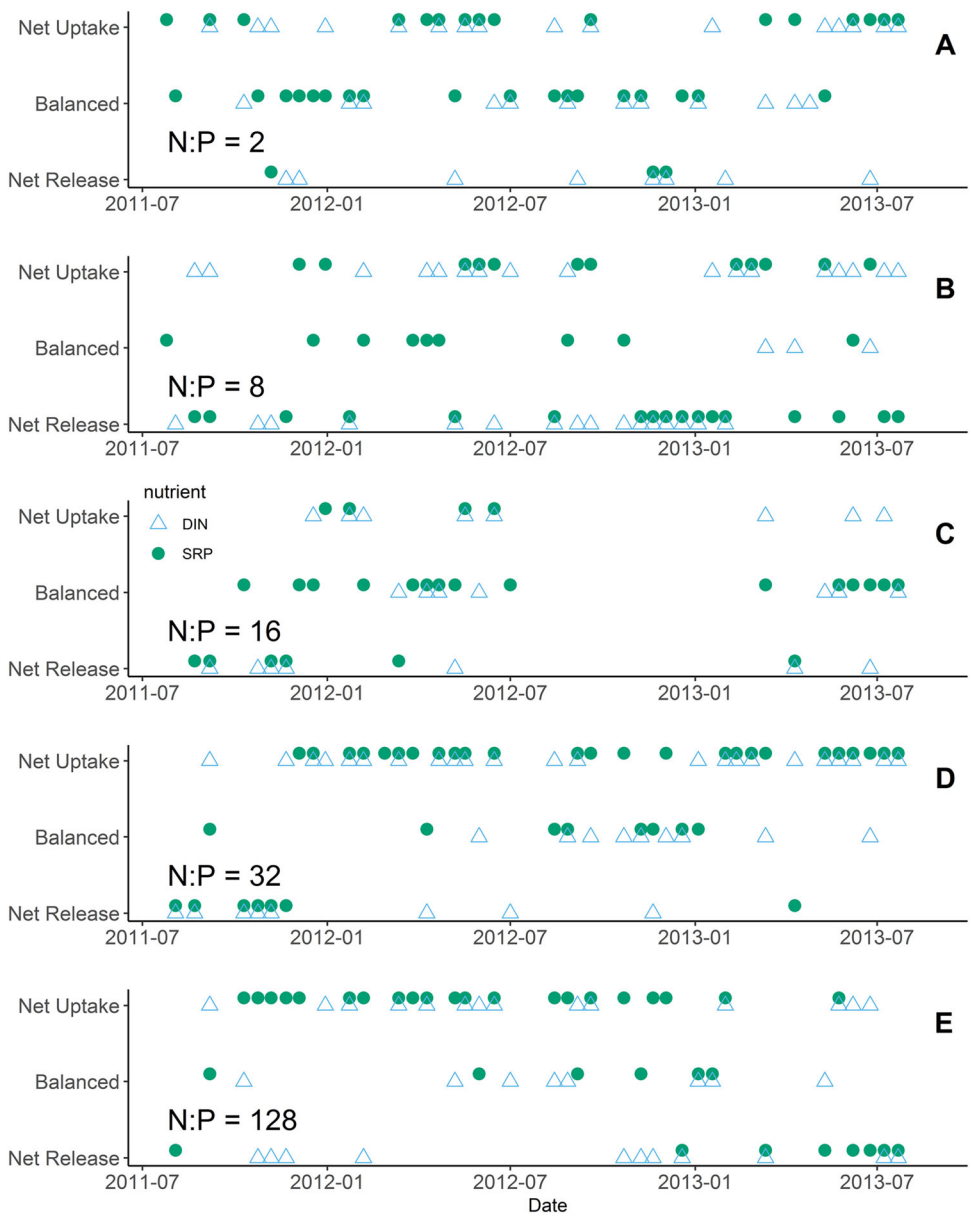

Figure 3. Time series of net nutrient exchange of dissolved inorganic nitrogen (DIN, open blue triangles) and soluble reactive phosphorus (SRP, closed green circles). Streams are identified by their target supply N:P.

NNE_{din}:NNE_{srp} was 0.82 (± 0.13 SE, Figure 5c). Again, the slope of this relationship was different from zero ($F_{1,19}$ = 37.6, $P < 0.0001$), but not different from one ($F_{1,19}$ = 1.74, P = 0.20). A slope of one characterizes an ecosystem that is completely flexible in the stoichiometry of net N:P uptake or release relative to N:P supply.

Discussion

Our overall goals of this study were to test how net nutrient uptake and release responded to nutrient enrichment, evaluate whether the stoichiometry of NNE in streams is homeostatic, and explore the drivers of NNE through time in five headwater streams enriched with dissolved nutrients at different N:P. We detected net uptake of N and P almost twice as often as net release. However, the average rate of NNE in each stream over the 2-year study was near zero, suggesting that the magnitude of release events compensated for much of the observed net nutrient uptake within each stream reach. Although we expected greater net uptake at intermediate levels of supply N:P, we found that the magnitude of NNE was not related to supply N:P. However, the likelihood of net uptake of SRP

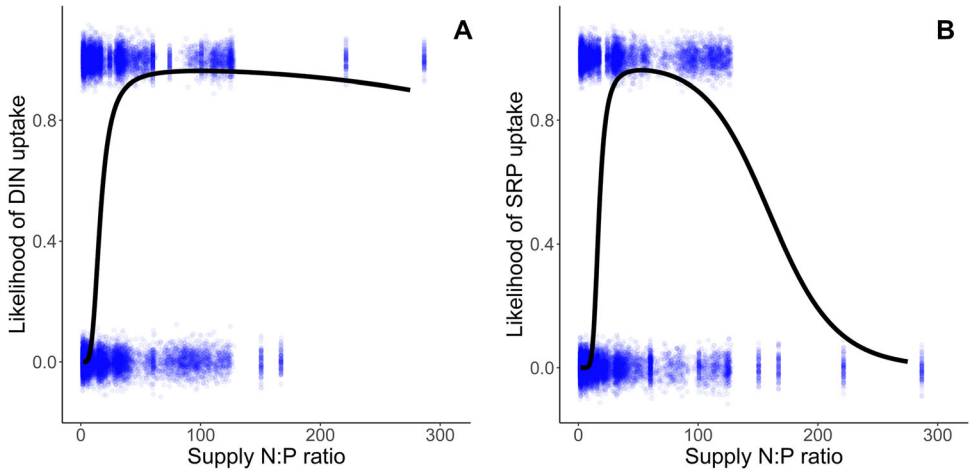

Figure 4. Likelihood of dissolved inorganic nitrogen (DIN, A) and soluble reactive phosphorus (SRP, B) net uptake as a function of N:P supplied to the experimental streams. The best model explaining both DIN and SRP net uptake likelihood included quadratic effects of N:P supply (Table 2). Blue points represent a subset of the bootstrapped estimates of net uptake, and black lines represent the mean effect of supply N:P on the likelihood of net uptake.

Table 2. Bootstrapped Parameter Estimates From Each Final Linear Mixed Effects Model of Soluble Reactive Phosphorus (SRP) and Dissolved Inorganic Nitrogen (DIN) Net Nutrient Exchange (*NNE*), and Logistic Regressions of the Likelihood of Either Net Uptake or Net Release

Response variable	Terms in final model	Coefficient mean and 95% CI	Marginal and conditional R^2 mean and 95% CI
DIN NNE rate	Chlorophyll-*a*	0.78 (0.54, 1.03)	Marginal 0.17 (0.08, 0.27) Conditional 0.20 (0.10, 0.32)
SRP NNE rate	Chlorophyll-*a*	0.30 (0.20, 0.41)	Marginal 0.12 (0.05, 0.19) Conditional 0.36 (0.19, 0.51)
DIN Net Uptake Likelihood	Supply N:P (linear)	4.86 (2.45, 7.24)	Marginal 0.24 (0.13, 0.35)
	Supply N:P (quad)	−1.85 (−3.65, 0.35)	Conditional 0.24 (0.13, 0.35)
	Chlorophyll-*a*	0.69 (0.37, 1.00)	
	Discharge	0.47 (0.28, 0.70)	
	Light	−0.61 (−0.83, −0.40)	
DIN Net Release Likelihood	Supply N:P (linear)	−5.89 (−8.93, −3.10)	Marginal 0.33 (0.18, 0.47)
	Supply N:P (quad)	2.62 (−0.24, 4.86)	Conditional 0.33 (0.18, 0.47)
	Chlorophyll-*a*	−0.93 (−1.30, −0.54)	
	Light	0.67 (0.39, 0.85)	
SRP Net Uptake Likelihood	Supply N:P (linear)	7.93 (4.99, 10.68)	Marginal 0.33 (0.22, 0.45)
	Supply N:P (quad)	−4.70 (−7.20, −2.26)	Conditional 0.37 (0.25, 0.50)
	Chlorophyll-*a*	0.78 (0.51, 1.30)	
SRP Net Release Likelihood	Supply N:P (linear)	0.16 (−4.03, 4.48)	Marginal 0.23 (0.12, 0.35)
	Supply N:P (quad)	6.78 (1.73, 13.10)	Conditional 0.45 (0.19, 0.70)
	Chlorophyll-*a*	−0.92 (−1.38, −0.52)	

Independent variables have been scaled to allow them to be interpreted as a relative effect size (Gelman and Hill 2007), and parameter estimates are reported along with their 95% confidence intervals from the bootstrapped model fitting. Marginal R^2 is reported for each model and represents the proportion of variation explained by the fixed-effects portion of the model, while the conditional R^2 represents the proportion explained by the fixed and random (stream identity) effects together. Full models included the variables (chlorophyll-a, discharge, fungal biomass, light, temperature, and supply N:P ratio).

and DIN was related to supply N:P, with the likelihood of net uptake peaking at intermediate values of supply N:P, though these N:P values were higher than we predicted. Despite these streams receiving little light, chlorophyll-*a* was an important driver of NNE across the five streams, with discharge and light often included in the model that best predicted daily rates of NNE or the likelihood of net

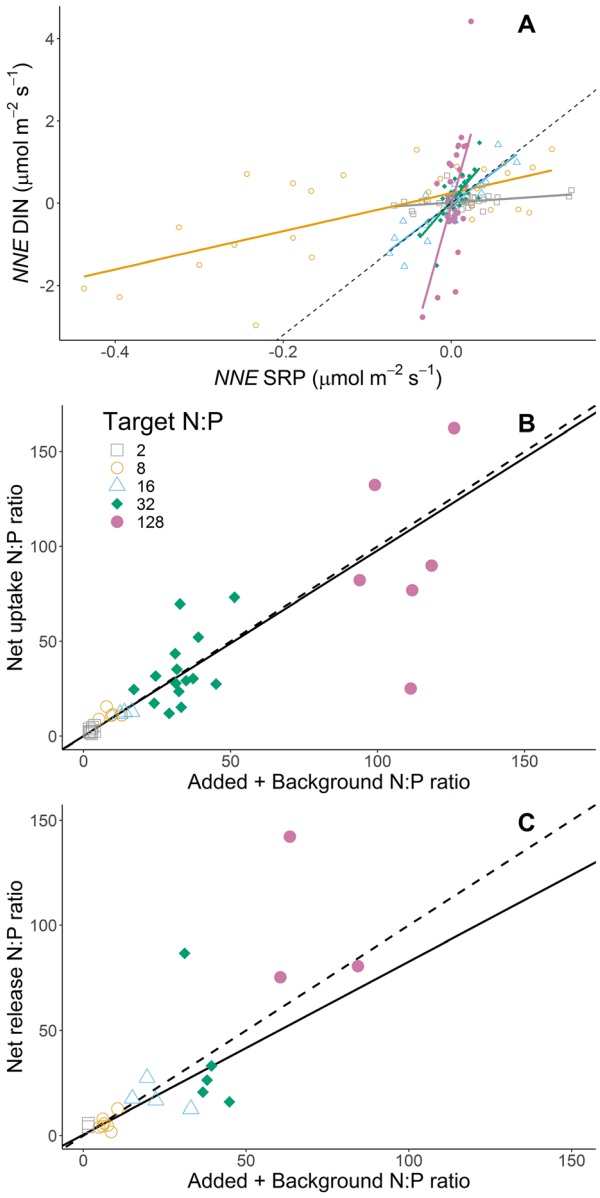

Figure 5. a Rates of net nutrient exchange (NNE) of soluble reactive phosphorus (SRP) compared to those of dissolved inorganic nitrogen (DIN) in each stream. **b** The molar net NNE N:P compared to the molar supply N:P on dates when both N and P had net uptake (95% confidence intervals of estimate did not overlap zero), or release (**c**). In panel (A) the dashed line indicates the Redfield ratio (N:P = 16) and the colors and shapes represent the different experimental streams. In panel (B and C), the dashed line indicates a 1:1 relationship, which would be our expectation based on stoichiometric flexibility, and the solid line indicates the best fit. Streams are identified based on their supply N:P. See Table A3 for slopes of panel A.

uptake or release. While we predicted that streams would exhibit a similar degree of homeostasis to that of fungi and bacteria studied under laboratory conditions (Makino and others 2003; Gulis and others 2017), both methods of assessing stoichiometric flexibility pointed toward our study streams being more flexible, at least across our moderate target enrichment concentrations (650–81 μg L^{-1} DIN, 90–11 μg L^{-1} SRP). Consequently, processes occurring at the community and ecosystem level may increase stoichiometric flexibility beyond that observed during shorter-term nutrient additions under controlled conditions.

The N:P of nutrient supply did not affect the magnitude of N or P NNE. We predicted that net uptake would be maximized near the N:P of microbial biomass, which we expected to be between 8:1 and 16:1 (Gulis and others 2017; Zhang and Elser 2017) and that net uptake of N and P at N:P supply greater or less than the N:P of microbial biomass would be limited by the element in short supply (Sterner and Elser 2002). Rather, we found no relationship between the N:P of supply and the NNE of DIN or SRP, which makes sense in light of our findings of high N:P NNE flexibility. However, we did find quadratic relationships between supply N:P and the likelihood of net DIN and SRP uptake, which were generally better constrained in our analysis than exact rates of NNE. For both N and P, the likelihood of net uptake peaked at an N:P ratio higher than median reported values for microbial biomass, and the lower end of the confidence intervals were at supply N:P of 29:1 and 37:1 respectively. Thus, the ranges of N:P supply that maximized the likelihood of net uptake were generally higher than we expected based on the stoichiometry of fungal and bacterial biomass. Although the streams we studied are very heterotrophic (Kominoski and others 2018), algae, which can have much higher biomass N:P than bacteria and fungi, may have raised the optimal supply N:P to maximize the likelihood of N and P uptake (Hall and others 2005). Alternatively, dissimilatory losses of N may have also raised the optimal N:P for uptake above that of the biomass in the stream (Cross and others 2005).

The results of our analysis suggest that, under conditions of chronic, low-level nutrient enrichment, heterotrophic stream ecosystems can have a high degree of stoichiometric flexibility in their ability to retain and mineralize nutrients. Gulis and others (2017) quantified the N:P homeostasis coefficients of pure cultures of aquatic fungi and an assembled community grown on submerged leaf litter and found that H^{-1} ranged from 0.31 to 0.53, indicating a moderate degree of flexibility. Schade and others (2011) conducted short-term (< 1 d) additions of nutrients to streams at different supply

N:P and calculated an H^{-1} of 0.77 in an autotrophic stream and an H^{-1} of 0.58 in a heterotrophic stream. Here, we calculated a net uptake H^{-1} of 0.98 (SE 0.06), and net release H^{-1} of 0.82 (SE 0.13) across all five streams. Previous theoretical works suggest that processes operating over a continuum of time scales, such as changes in community composition, or nutrient recycling within benthic biofilms, can align nutrient flux between the bed and the water column with the dissolved nutrient supply (Tilman and others 1981; Schade and others 2005; Danger and others 2008), and our data provide empirical support for those predictions. A meta-analysis of fungal fruiting body stoichiometry found that saprophytic fungi had N:P content ranging from 3:1 to 473:1 (Zhang and Elser 2017), and heterotrophic aquatic bacteria have been observed with N:P content ranging from 2:1 to 83:1 (Godwin and Cotner 2015). This degree of variation in body N:P suggests that there is enough variation among fungi and bacteria to produce the range in net uptake rate N:P we observed through changes in community composition alone. Experimental evidence from smaller-scale manipulations highlight that changes in community structure can promote an increase in apparent N:P stoichiometric flexibility (Danger and others 2008; Fanin and others 2013; Godwin and Cotner 2014). Additionally, the similar flexibility exhibited between net uptake and net release suggests that a parallel mechanism (for example, accumulation or mineralization of nutrients in biomass) is largely driving both processes.

Biotic and abiotic variables other than the supply N:P also explained NNE during our 2-year whole-stream nutrient enrichment. Chlorophyll-*a* had positive effects on NNE (greater net uptake) and appeared to play a role in the processing of N and P (Table 2). This is relatively surprising given that the streams in this study are extremely heterotrophic, with rates of primary production often undetectable using whole-ecosystem measurement approaches (Benstead and others 2009; Kominoski and others 2018). Our results thus add to the body of work suggesting that phototrophic biofilms can play a disproportionate role in heterotrophic streams (Minshall 1978; Thorp and Delong 2002; Brito and others 2006). The positive effect of discharge on the likelihood of net DIN uptake was unexpected, as we expected net uptake to be greater at lower flows when water residence times are longer (Levi and McIntyre 2020). The positive effect of discharge might be caused by the seasonality of flow, which tends to be greater in the winter and thus overlaps with periods of microbial biomass accrual (Mulholland and others 1985; Suberkropp and others 2010), or could be a result of a reduced boundary layer thickness at higher flows and or altered hyporheic exchange. We did not measure abiotic sorption–desorption of nutrients directly in this experiment but it is possible that it played a role in nutrient retention and release, as it has in other large-scale nutrient additions (Small and others 2016). However, previous work at Coweeta examining the relative roles of biotic and abiotic processes in the gross uptake of NH_4 and SRP—by incubating intact and sterilized sediments—found that gross uptake of N and P at Coweeta was predominantly biological (Munn and Meyer 1990).

Broadly, our results support the idea that nutrient inputs and outputs tend to be balanced at the reach scale in small streams, while also suggesting that nutrient cycling in these ecosystems is dynamic over time (Brookshire and others 2009; von Schiller and others 2015). We added nutrients continuously over the 2 years of enrichment to achieve relatively constant target concentrations for the duration of our study. Extended enrichments estimate net nutrient dynamics (that is NEE) rather than gross nutrient fluxes between the water column and biological compartments because both removal and production processes have sufficient time to respond to the enriched conditions (Stream Solute Workshop 1990; Payn and others 2005). Results of an isotope-tracer study in one Coweeta stream estimated turnover times for N of between 1 and 2 months in different biological compartments (Tank and others 2000), implying that these streams take considerable time to reach an equilibrium with their nutrient inputs. However, when we explored the effect of time on the residuals of some of the models we ran, we did not detect systematic changes through time (Appendix S6). The apparent net balance of nutrients during the experiment was a result of periods of net uptake and release balancing one another out over time, which is similar to the results found for P cycling in Bear Brook, New Hampshire (USA; Meyer and Likens 1979).

We consider our NNE results as largely driven by microbial processes associated with benthic organic matter. We were not able to explore the direct roles of higher-order consumers on reach-scale nutrient dynamics, beyond their indirect effects of feeding on and affecting the biomass and production of basal food resources and associated microorganisms (Cross and others 2006). At our study sites, the direct effects of consumers would specifically include nutrient flows to production of macroinver-

tebrates and salamanders and export of nutrients via drift, migration, or emergence, which were not assessed as part of this study.

The approach used to quantify NNE in this experiment is unconventional, but represents an opportunistic approach to use data from an experiment designed to answer a different suite of questions to characterize the balance between processes that remove nutrients from transport and those that release nutrients back into the water column. Typical nutrient-addition experiments use a conservative tracer to account for dilution along the experimental reach and add all of the nutrients at a single upstream location. We did not use a conservative tracer in this experiment, which in theory could bias our results toward detecting net uptake more often, as dilution along the reach would lower the observed concentration of nutrients. However, the short (70-m) length of the stream reaches used in this experiment places some bounds on the magnitude of this bias, which we expect to be relatively small. In addition, because nutrients were added longitudinally along the reach, some of the nutrients were added only a few meters above the downstream sampling point. Hence there was only a very short area of stream in which they could be taken up during transport. We used simulations to evaluate how this longitudinal nutrient addition along the reach may have biased our estimates of NNE relative to additions only at the top of the reach (Appendix S4). When we simulated NNE as a constant rate along the reach, we found that the location of inputs did not bias estimates of NNE (Figure S12). When we simulated NNE as a constant proportional loss or a saturating function of concentration, we found that there was a strong correspondence between NNE calculated from the single-point addition and the longitudinal addition (R^2 = 0.96 and 0.98, respectively, Figures S13 and S14), though there was some bias, with NNE calculated from longitudinal additions always being closer to zero than from single-point additions. The absolute magnitude of the bias increased with the absolute magnitude of NNE, whereas the relative magnitude had a stronger relationship with the half-saturation constant used to simulate NNE, ranging from 4.4 to 43%. If the mechanisms underlying net uptake and net release differ, so might the degree of bias in these estimates, which could possibly lead to bias in our overall mean NNE estimates. Although the sources of bias we have outlined limit the direct comparison of rates calculated in this study with rates calculated using single-point nutrient-addition methods, our estimates of NNE are sufficiently robust for comparisons within and among our experimental streams.

The nutrient additions in this experiment represent a relatively low level of nutrient enrichment; in heavily urbanized or agricultural watersheds, both SRP and DIN can be an order of magnitude higher than the highest concentrations we tested. Furthermore, in human-altered streams dissolved nutrient N:P can vary from less than 0.01:1 to over 1000:1 (Manning and others 2020). Thus, although this study represents an unprecedented test of how NNE responds to differing supply N:P, streams experiencing higher concentrations or more unbalanced ratios of nutrient enrichment may respond differently from our experimental streams. Furthermore, although the experimental approach of this study allowed us to isolate the effects of nutrient input stoichiometry on NNE, nutrient pollution often happens simultaneously with other stressors in aquatic ecosystems, such as hydrologic alteration, sedimentation, and addition of other dissolved contaminants. Thus, studying nutrient cycling along diverse landscape gradients is important for understanding how the complex suite of stressors that often accompany nutrient pollution combine to affect nutrient cycling (Niyogi and others 2004; Covino and others 2012).

Our study highlights the importance of examining ecosystem processes and their responses to environmental change over longer time scales. Although studies of individual organisms (Güsewell 2004) and short-term studies of whole ecosystems (Schade and others 2011; Tromboni and others 2018) have found some degree of flexibility in the stoichiometry of nutrient use, these earlier studies did not predict the high-level of stoichiometric flexibility that we observed during this experiment. In our study, enhanced stoichiometric flexibility was likely caused by changes in microbial community composition or nutrient recycling within biofilms. We observed a high degree of flexibility during our 2-year study, similar to a study of microbes in terrestrial environments that found flexibility in biomass stoichiometry linked to changes in community structure in an experiment lasting 98 days (Fanin and others 2013). These types of responses likely take even longer to observe in communities with slower turnover in species composition, such as forests. A full understanding of ecosystem dynamics clearly requires examining processes at the ecosystem scale over time periods long enough that community dynamics and their ecosystem-level consequences can unfold (Carpenter and others 1995).

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DATA AVAILABILITY

Data and code used for this paper are published through Zenodo and open access at: https://doi.org/10.5281/zenodo.7057891

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