Quantifying spatial and temporal relationships between diatoms and nutrients in streams strengthens evidence of nutrient effects from monitoring data

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Abstract: Observational data are frequently used to better understand the effects of changes in P and N on stream biota, but nutrient gradients in streams are usually associated with gradients in other environmental factors, a phenomenon that complicates efforts to accurately estimate the effects of nutrients. Here, we propose a new approach for analyzing observational data in which we compare the effects of changes in nutrient concentrations in time within individual sites and in space among many sites. Covarying relationships between other, potentially confounding environmental factors and nutrient concentrations are unlikely to be the same in both time and space, and, therefore, estimated effects of nutrients that are similar in time and space are more likely to be accurate. We applied this approach to diatom *rbcL* metabarcoding data collected from streams in the East Fork of the Little Miami River watershed, Ohio, USA. Changes in diatom assemblage composition were consistently associated with changes in the concentration of total reactive P in both time and space. In contrast, despite being associated with spatial differences in ammonia and urea concentrations, diatom assemblage composition was not associated with temporal changes in these nitrogen species. We suggest that the results of this analysis provide evidence of a causal effect of increased P on diatom assemblage composition. We further analyzed the effects of temporal variability in measurements of total reactive P and found that averaging periods greater than ~1 wk prior to sampling best represented the effects of P on the diatom assemblage. Comparisons of biological responses in space and time can sharpen insights beyond those that are based on analyses conducted on only 1 of the 2 dimensions.

Key words: phosphorus, nitrogen, DNA metabarcoding, algae, periphyton, agriculture, temporal variability, *rbcL*, biomonitoring, Bayesian hierarchical model

Elevated nutrient concentrations are one of the most pervasive effects of human activities in freshwater ecosystems (Dodds et al. 2009, Mekonnen and Hoekstra 2018). Excess N and P, arising from a broad variety of human activities, are among the primary causes of degradation to streams in the USA (USEPA 2016). However, quantifying the effects of excess nutrients is challenging. In natural settings, changes in nutrient concentrations and in other environmental factors (e.g., flow, conductivity) often occur simultaneously, so specifically attributing changes in biota to elevated nutrients is difficult. That is, effects of environmental factors that are correlated with nutrient concentrations can introduce bias, or confound, estimates of nutrient effects. These difficulties are compounded in streams because of the transient nature of the chemical, physical, and biological attributes of these systems (Baker and Webster 2017) and because the stream food web is fueled by both autochthonous and allochthonous carbon sources. The dominant autochthonous source in wadeable streams is attached algae (periphyton) on the stream bed, but biogeochemical mechanisms for nutrient turnover and uptake in periphyton are often decoupled from water column nutrient concentrations. For example, elevated concentrations of nutrients during storm events often exceed the capacity for uptake by periphyton (Griffith et al. 2009, Smucker et al. 2013, Wood et al. 2015, Vadeboncoeur and Power 2017, Costello et al. 2018). Likewise, effects of allochthonous sources that wash into stream reaches from upstream or adjacent environments may not be directly related to nutrient loads or concentrations estimated from grab sampling at one point in time. Different macronutrient

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species (e.g., reactive P and N) can also covary with one another, further increasing the uncertainty of analyses targeted toward estimating the effects of each species (Jones et al. 2001).

Relationships between increased nutrients and biological responses can be quantified through experimental manipulations or analysis of observational data. In experimental manipulations, the ecosystem is modified to accommodate the introduction of treatment and control groups. Streamside mesocosms (Quinn et al. 1997, Rier and Stevenson 2006) and laboratory meso- and microcosms (Horner et al. 1990, Manoylov and Stevenson 2006, Wagenhoff et al. 2013, Shatwell et al. 2014) simplify the ecosystem so that it can be replicated and nutrient concentrations altered. Nutrient-enriched substrates (Grimm and Fisher 1986, Lowe et al. 1986, Bushong and Bachmann 1989, Chessman et al. 1992, Capps et al. 2011) can locally increase N and P concentrations in natural settings, but the method requires the introduction of artificial substrates that can also alter periphyton assemblage composition. Conducting these experiments is often resource and labor intensive, and, therefore, they usually can be implemented only in a small number of locations. Hence, experimentally identified relationships may not be widely generalizable to other locations.

Observational field data can be more easily collected from a wide variety of streams, and analyses of these data can potentially yield more broadly applicable relationships (Dodds et al. 2002), but causal relationships are difficult to establish through the use of observational data. Most relationships between nutrient concentration gradients and biological effects estimated from observational data can only corroborate or contradict known causal relationships because of the potential bias introduced by confounding variables. That is, any estimates of relationships between nutrients and biota may be altered by other environmental factors that covary with nutrient concentrations and that cannot be directly controlled in observational studies. For example, conductivity often covaries with nutrient concentrations and, on its own, can also alter biological assemblages (Smucker and Vis 2011a); therefore, a statistical estimate of nutrient effects may be biased if the analysis does not explicitly control for the effects of conductivity. Several methods for addressing the effects of known confounding variables and improving the accuracy of the estimated effects have been proposed, ranging in complexity from multiple linear regression to propensity score matching and machine learning (Yuan 2010, Smucker et al. 2013, Wagenhoff et al. 2017, Waite et al. 2020). However, these methods require that measurements of potential confounding variables are available, and they do not address the possible effects of unknown confounders.

Here, we describe an approach for analyzing observational data to provide stronger evidence of a causal link between increased nutrients and diatom assemblage composition by comparing relationships estimated in time at individual sites and estimated in space among many sites. Diatoms are commonly used as indicators of nutrient effects in streams (Potapova and Charles 2007, Stevenson et al. 2008, Rimet 2012), but they also are known to be affected by other environmental factors and exhibit compositional variability due to other stochastic factors (e.g., immigration; Potapova and Charles 2002, Soininen 2007, Smucker and Vis 2011c, Taylor et al. 2018). As described above, effects of environmental factors that covary with nutrients can introduce bias, or confound, estimates of nutrient effects. However, confounding variables are unlikely to covary with nutrients in the same way in both temporal and spatial dimensions; therefore, relationships that are similar when estimated across both dimensions are more likely to arise from a causal relationship. Moreover, with this approach, direct measurements of possible confounders are not necessary, increasing the robustness of the method. Similarity in effects estimated in space and in time also satisfy the consistency criterion, as proposed by Hill (1965) in his set of criteria for establishing causal relationships. We hypothesized that the quantitative relationships between reactive nutrients on the occurrence of periphytic diatom species in small streams converge in spatial and temporal analyses, and, therefore, in combination, observed variation in species composition can more likely be causally attributed to increased nutrient concentrations. Data collected to test this hypothesis also allowed us to examine how variability in antecedent nutrient concentrations affects diatom assemblages, thus improving estimates of nutrient effects on diatom assemblages.

METHODS

Study design

We collected a dataset with sufficient samples to estimate relationships between diatom assemblage composition and nutrient concentrations in both spatial (25 different streams) and temporal dimensions (10–12 sampling visits/stream). During each visit, we collected measurements of the concentrations of different nutrient species and periphyton samples. We conducted DNA metabarcoding analyses of each sample to identify diatom taxa that were present in each sample and estimated relationships between different nutrient species and diatom assemblage composition over time for each site. We compared the temporal relationships with a relationship estimated between average nutrient concentrations and diatom assemblage composition in space among all sampled sites.

The dataset we analyzed is unique in 2 ways. First, the study was designed to ensure that sufficient samples were available to estimate both temporal and spatial effects: we collected weekly samples, except for the last 2 sampling events in October that occurred 2 wk after the previous ones, from 25 wadeable stream sites during mid-July to

October 2016. Second, we conducted DNA metabarcoding analyses of periphyton samples, which 1) provide a more comprehensive estimate of the diatom species present within a sample than would be possible with microscopy-based identification and enumeration (Tapolczai et al. 2019a, Pérez-Burillo et al. 2020, Kahlert et al. 2021) and 2) reduce possible bias and variability associated with taxonomist error (Lee et al. 2019).

Sampling methods

The study sites were located in 2nd- and 3rd-order streams within the East Fork of the Little Miami River (EFLMR) watershed (area = 1293 km^2) in southwest Ohio, USA. The watershed experiences a temperate seasonal climate and has mixed land use with 54% in agriculture. Based on insights from historical monitoring data, we selected 25 non-nested stream sites (i.e., no site was downstream of another) to represent a continuous and ~evenly distributed gradient spanning the range of nutrient concentrations experienced in the watershed (Smucker et al. 2020). Catchment areas for the sampled site ranged from 16 to 82 km^2.

Water chemistry measurements We collected water for nutrient analyses at each site in a 1-L acid-washed polypropylene bottle. These samples were stored on ice in the dark until being returned to the lab where they were kept at 4°C in the dark until being analyzed within 24 h (6% of samples) or were kept frozen (−20°C) until analyzing within 21 d (94% of samples). We used automated wet chemistry methods and a QuikChem® 8500 nutrient autoanalyzer system (Lachat Instruments, Milwaukee, Wisconsin) to measure total ammonia (TNH_4), urea, and total reactive P (TRP). We measured TNH_4 with a modified phenolate method (Smith 2001), and urea with an adjusted brackish water method using diacetyl monoxime and thiosemicarbazide in an acid solution (Nelson 2007). To measure TRP we used a high throughput method with the standard ammonium molybdate and antimony potassium tartrate reaction and ascorbic acid reduction (USEPA 1993, Tucker 2008). To expedite sampling of all 25 sites closely in time and to coordinate with an existing monitoring effort, we did not filter grab samples and reported all nutrient data as total species. We focused our analyses on the most biologically available nutrient species: TRP, TNH_4, and urea. Nitrate–nitrite was excluded from the analyses because it is taken up less readily than ammonia and urea (Twomey et al. 2005), and initial exploratory analysis showed that NO_3^- was not associated with diatom assemblage composition.

We acknowledge that the decision not to filter and to freeze samples may have introduced both positive and negative biases to measured nutrient concentrations, especially for P species (Gardolinski et al. 2001). Positive biases may arise if organic nutrients incorporated into biomass are released by freezing, thawing, and remineralizing, whereas negative biases may arise from complexation with mineral precipitates during the freeze–thaw cycle. However, the streams in this study have little suspended algal biomass, and we minimized potential for remineralization by analyzing samples immediately after thawing. The protocols of this monitoring effort were developed from extensive observations that quantified the effects of freezing and filtering on measurements of inorganic nutrient species (see Fig. S1).

Quantitative estimates of the temporal variability of nutrient concentrations over different time intervals can be used to help interpret estimates of the relationships between diatom assemblage composition and nutrient concentrations (see below). The weekly samples collected in the EFLMR were not frequent enough to estimate variability at time intervals shorter than a week, so we used daily measurements of total P (TP; with more frequent measurements during storm events) for 2 Ohio streams draining areas similar in size to the streams sampled in this study (Rock Creek catchment = 90 km^2 and Chickasaw Creek catchment = 43 km^2) to estimate the average temporal variability of P concentrations over time intervals ranging from 2 to 20 d. These data were collected by the National Center for Water Quality Research (https://ncwqr.org/monitoring/), starting in 1983 for Rock Creek and 2008 for Chickasaw Creek up through the present.

Diatom sampling and DNA analysis On each date at each site, we composited a periphytic diatom sample from 5 rocks retrieved equidistantly along a 75-m stream reach. On the surface of each rock, we positioned a 6.7-cm^2 plastic guide and removed the periphyton within the guide by scrubbing with a firm-bristled brush attached to a cordless drill. We then used a rinse bottle filled with creek water to spray the periphyton dislodged by the brush into a slurry. We combined the periphyton slurries collected from each of the 5 rocks into a new 500-mL low-density polyethylene bottle. Samples were kept on ice in the dark until returning to the lab, where they were frozen at −80°C until being thawed immediately prior to DNA extraction. After thawing, we filtered samples through sterile, 0.8-μm polycarbonate filters. We transferred an ~50-mg subsample of filtered periphyton into a 1.5-mL microcentrifuge tube where it was exposed to liquid N and then ground with a disposable pestle.

Next, we processed ground periphyton samples through our laboratory workflow for DNA metabarcoding consisting of DNA extraction, PCR amplification, and DNA sequencing. We used DNeasy® PowerLyzer® PowerSoil® kits (Qiagen, Hilden, Germany) and followed manufacturer's instructions to extract DNA, but with an added initial digestion with proteinase K at 56°C for at least 2 h. We used PicoGreen® dsDNA Quantitation Reagent (Molecular Probes, Eugene, Oregon) to quantify DNA extractions on a microplate reader (model HT1; BioTek®, Winooski, Vermont), which

we normalized to 10 ng/μL for polymerase chain reaction (PCR). We used PCR and previously described primers and reaction conditions (Vasselon et al. 2017) to amplify a portion of the chloroplast gene *rbcL*. These primers target diatoms, but occasional amplification of non-target taxa closely related phylogenetically to diatoms can occur. These uncertain taxon designations were <0.7% of all sequences. We conducted 20-μL PCR reactions consisting of 2 μL 10X PCR buffer (with $MgCl_2$), 0.6 μL 25-mM $MgCl_2$, 1 μL each of the forward and reverse primer cocktails (10 mM; Table 1), 4 μL 1X BSA, 0.4 μL 10-mM dNTPs, 0.1 μL Taq® polymerase (Qiagen), 8.9 μL sterile water, and 2 μL template DNA. Reaction conditions were 94°C for 150 s, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min with a final extension of 72°C for 10 min. The PCR primers in this round of PCR had 5' adapter sequences (5'-ACACTGACGACATGGTTCTACA-3' and 5'TACGGTAGCAGAGACTTGGTCT-3') for use in the 2nd round of PCR (dual indexing). We ran these PCRs in triplicate for each template and then pooled and cleaned them (QIAquick® 96 PCR Purification Kit; Qiagen) prior to a 2nd PCR where we added indexing primers for sequencing on a MiSeq™ sequencer (Illumina, San Diego, California). The 2nd round of PCR had reaction conditions with 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by a final extension step of 72°C for 5 min. We then used the AMPure XP kit (Beckman Coulter Life Sciences, Indianapolis, Indiana) to purify index PCR amplicons, quantified them with PicoGreen as above, and normalized them in EB buffer (Qiagen). We pooled index PCR plates into a single sample by combining 3 μL from each well into a 1.5-mL microcentrifuge tube. We then used a 500-cycle Illumina MiSeq sequencing kit (2 × 250) to sequence amplicons following manufacturer's protocols.

In order to generate operational taxonomic unit (OTU) DNA consensus sequences and the number of reads per OTU per sample, we performed bioinformatic analyses of the raw data from the MiSeq run. For bioinformatic analyses, we used USEARCH (version 9.2; https://www.drive5.com/usearch/manual9.2/; 64-bit; Edgar 2010) on demultiplexed reads from a single MiSeq DNA sequencing run. We used Cutadapt (version 1.14; Martin 2011) to merge paired reads and to remove primers. We excluded full length sequences shorter than 230 base pairs or with higher than expected errors based on Phred quality scores. We dereplicated the remaining sequences and identified unique sequences. We excluded sequences with <4 observations in the total sequencing run to reduce the number of OTUs created by possible sequencing artifacts. We screened the remaining data for chimeric sequences and clustered OTUs at ≥97% similarity, which is commonly used and has good discrimination power and performance similar to other thresholds ≥92% (Tapolczai et al. 2019b). We mapped all quality-filtered sequence reads onto these OTUs. The sequence data has been deposited in GenBank (BioProject ID: PRJNA592969, http://www.ncbi.nlm.nih.gov/bioproject/592969).

Ordination of diatom assemblage

We applied non-metric multidimensional scaling (NMDS) to the diatom OTU × sample matrix to quantify how diatom assemblage composition varied across samples and with respect to nutrient gradients. We expressed reads for each sample as the presence or absence of each OTU identified across all samples and quantified differences between the assemblage composition of different samples with the Simpson pairwise dissimilarity index, a metric that measures turnover among samples (Baselga 2010). Sorensen dissimilarities measure both nestedness and turnover, and analyses using Sorensen were nearly identical to those reported below. We then mapped diatom assemblages to 4 axes with NMDS as provided by the function metaMDS in the *vegan* package (Oksanen et al. 2012) in R (version 4.0.3; R Project for Statistical Computing, Vienna, Austria). The function metaMDS fits many ordinations with random restarts to reduce the possibility that the final ordination solution represents a local minimum. The final ordination is also rotated such that the 1st axis accounts for the greatest proportion of variance in ordination space (Oksanen et al. 2012). We specified 4 axes in the ordination which yielded a stress of 0.18. Ordinations computed with fewer axes yielded stresses that exceeded the recommended threshold of 0.2 (Clarke 1993).

Table 1. Polymerase chain reaction primer sequences (Vasselon et al. 2017) used to characterize diatom assemblage composition in the East Fork of the Little Miami River watershed, southwest Ohio, USA.

Primer name	Sequence	Cocktail
Diat_rbcL_708F_1	AGGTGAAGTAAAAGGTTCWTACTTAAA	Forward
Diat_rbcL_708F_2	AGGTGAAGTTAAAGGTTCWTAYTTAAA	Forward
Diat_rbcL_708F_3	AGGTGAAACTAAAGGTTCWTACTTAAA	Forward
R3_1	CCTTCTAATTTACCWACWACTG	Reverse
R3_2	CCTTCTAATTTACCWACAACAG	Reverse

Exploratory analysis indicated that position on the 1st NMDS axis was strongly correlated with nutrient concentrations, so subsequent analyses focused on factors that predicted position on this axis.

Estimating diatom–nutrient relationships in space

We used linear regression models to estimate diatom–nutrient relationships among sites (i.e., in space). First, we calculated site mean values for all nutrient measurements (TRP, TNH_4, and urea) and for the mean position along NMDS axis 1 for diatom assemblages observed at each site. Then, we fit 3 linear regressions with mean NMDS axis 1 scores as the dependent variable and site mean values of TRP, THN_4, and urea as predictors in each of the regressions. To illustrate the effects of including a covariate in the models, we also fit 3 additional multiple linear regressions with conductivity included as an additional predictor variable with each of the 3 nutrient species. Conductivity was selected as an example covariate in the models because of its known effects on diatom composition (Potapova and Charles 2003, Pajunen et al. 2017). Conductivity was not measured during this study, so we used mean values of historical data collected at each site for this illustrative example.

Estimating diatom–nutrient relationships in time

To quantify relationships in time, we estimated linear relationships between weekly measurements of each nutrient concentration and NMDS axis 1 scores within each site. Two additional aspects of this phase of the analysis distinguish it from the among-site models. First, we specified a hierarchical structure such that parameters describing the relationship for each site (slope and intercept) were assumed to be drawn from common normal distributions consisting of slopes and intercepts from all 25 sites (Gelman and Hill 2007). We assumed residual variance was the same for all sites, as is typical for hierarchical models. The hierarchical structure reflects the assumption that temporal relationships estimated in each site were not identical but that some similarity in responses among sites was expected.

Second, we accounted for differences between the measured, instantaneous nutrient concentration and the average nutrient concentration that was relevant to temporal changes in diatom assemblage composition. More specifically, weekly grab samples reflect concentrations at the time of sampling, but the observed diatom assemblage reflects a history of nutrient concentrations over some period of time (Δt) prior to sample collection. The relevant duration of Δt is not known but may be related to the doubling time of different diatom species, which has been estimated in 1 lab study as 2 to 16 d, depending on species characteristics (e.g., Morin et al. 2008). Other studies of the turnover in aggregate measures of diatom composition or structure have observed a similar range of values for Δt and suggested that Δt may also vary with environmental conditions (Lavoie et al. 2008, Smucker and Vis 2011b, Huttunen et al. 2020).

To account for the difference between each instantaneous nutrient measurement and the average nutrient concentration computed over Δt, we applied statistical techniques typically used to account for measurement error. Each instantaneous measurement was assumed to represent the average concentration plus a random error (Carroll et al. 2006, Yuan 2007). That is, each instantaneous nutrient concentration was assumed to be drawn from a log-normal distribution that was centered on the average nutrient concentration over Δt. The width of this distribution can be characterized as the standard deviation of log-transformed instantaneous nutrient concentrations (SD_N), and this width varies with Δt.

We were interested in identifying a relevant range of values of SD_N that could be used in our model for temporal relationships between diatoms and nutrient concentrations. To that end, we estimated the relationship between SD_N and Δt with the temporally intensive measurements of TP collected from Rock Creek and Chickasaw Creek. TP measurements in these sites were collected at least daily, so SD_N could be estimated for shorter Δt than the weekly data from EFLMR. We estimated the SD_N of TP for Δt ranging from 2 to 21 d by dividing the daily measurements into windows corresponding to each value of Δt and computing the SDs of log-transformed TP (SD_N) within each window. Then, we calculated the mean value of SD_N for all windows specified for a value of Δt. Exploratory analysis indicated that SD_N varied systematically with time of year, so we limited data from both streams to July to October to match the sampling period for the sites in the EFLMR. Daily data were not available for TRP, TNH_4, or urea in the Chickasaw Creek data set, so for this analysis we assumed that the range of SD_N of these other nutrient species was comparable to that of TP.

In contrast to the simple among-site model, a more complex Bayesian model was needed to represent the hierarchical structure of the data and to account for the temporal variability of nutrient concentrations. The complete model for temporal changes in NMDS axis 1 scores can be expressed mathematically as follows:

$$NMDS1_i = a_{j[i]} + b_{j[i]}\overline{TRP}_i + e_i, \qquad \text{(Eq. 1)}$$

where $NMDS1_i$ is the 1st NMDS axis score for sample i; $a_{j[i]}$ and $b_{j[i]}$ are site-specific model coefficients for site j, corresponding to sample i; $\overline{TRP}_i$ is the average value of TRP for sample i; and e_i is a normally distributed residual error. The site-specific model coefficients are related to one another by assuming they are drawn from common, normal distributions:

$$a_j \sim \text{Normal}(\mu_a, \sigma_a) \quad \text{and} \quad b_j \sim \text{Normal}(\mu_b, \sigma_b), \qquad \text{(Eq. 2)}$$

where the distributions are parameterized by the mean values, μ_a and μ_b, and standard deviations, σ_a and σ_b. The

Table 2. Pearson correlation coefficients between nutrient species concentrations and conductivity among samples taken at different times in the East Fork of the Little Miami River watershed, southwest Ohio, USA. Correlations among site means shown in parentheses. Only site mean correlations are shown for conductivity because conductivity measurements for each sample were not available (NA). – indicated no data.

	TRP	TNH_4	Urea
TNH_4	0.49 (0.80)	–	–
Urea	0.33 (0.66)	0.47 (0.81)	–
Conductivity	NA (−0.65)	NA (−0.56)	NA (−0.47)

distribution of instantaneous measurements of TRP is modeled as a log-normal distribution:

$$\ln(TRP_i) \sim \text{Normal}[\ln(\overline{TRP_i}), \text{SD}_N], \qquad \text{(Eq. 3)}$$

where TRP_i is the instantaneous TRP measurement for sample $_i$. Based on the results of analyses of data from Rock Creek and Chickasaw Creek, we fit the model for values of SD_N ranging from 0.1 to 0.5. We fit identical models using TNH_4 and urea as the nutrient species of interest. We used Rstan (version 2.14.0; Stan Development Team, Cocoa, Florida) with weakly informative prior distributions specified for all parameters to fit all Bayesian models. All other statistical calculations were performed in the R software.

RESULTS

A total of 276 samples with paired nutrient and metabarcode data collected at 25 sites were available for analysis. Across the available data, TRP ranged from 3 to 695 µg/L, TNH_4 ranged from 4 to 86 µg/L, and urea ranged from 1 to 157 µg/L. Ten samples with TNH_4 or urea measurements that were extremely high outliers outside of these ranges were excluded, leaving a total of 266 samples. In individual samples, TRP, TNH_4, and urea were weakly correlated, but when correlations were computed based on mean site concentrations, the correlations strengthened considerably (Table 2).

Mean position along the 1st NMDS axis for each site was strongly associated with mean TRP, TNH_4, urea, and conductivity (Fig. 1A–C). In the linear regression models, slopes (±SE) of the relationships based on standardized values of TRP, TNH_4, and urea were -0.13 ± 0.02, -0.12 ± 0.02, and -0.10 ± 0.02, respectively. The effect of conductivity on NMDS axis 1 scores was strongest for the multiple linear regression model that included urea and weakest for the model that included TRP (Table 3). Similarly, the slopes estimated for TRP changed the least between the simple 1-variable model and the multiple regression model, whereas the slope for urea changed by the greatest amount.

Analysis of data from Rock and Chickasaw creeks provided a distribution of SD_N of log(TP) for different windows of time. Mean SD_N on log(TP) increased from ~0.15 for a 2-d window to 0.35 for a 21-d window in Chickasaw Creek (Fig. 2). SD_N for log(TP) in Rock Creek followed the same pattern but reached values of ~0.40 for the longest windows. The increase in SD_N with larger values of Δt likely occurred because of autocorrelation of TP measurements over short time periods, such that TP measurements collected over short time intervals are more similar than TP measurements collected over longer time intervals. Uncertainty in estimates of mean SD_N also increased with Δt because the number of different windows that could be defined decreased as Δt increased. Because of the log transformation applied to TP data, estimates of SD_N can be approximately interpreted as proportions of the mean value, so an SD_N of 0.1 indicates that over a 2-d window, TP varied about the mean value by ~10%.

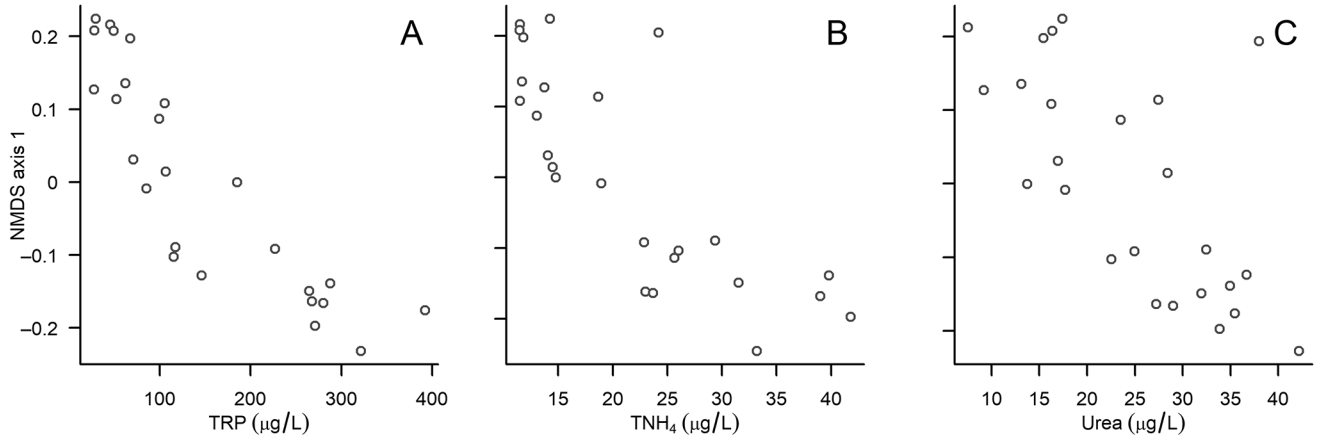

Figure 1. Relationships between mean position along the 1st non-metric multidimensional scaling (NMDS) axis, representing variation in diatom assemblage composition, and mean-site total reactive P (TRP; A), ammonium (TNH_4; B), and urea (C) concentrations in the East Fork of the Little Miami River watershed, southwest Ohio, USA.

Table 3. Mean standardized regression coefficients for site-mean models of relationships between diatom assemblage composition and nutrient species + conductivity in the East Fork of the Little Miami River watershed, southwest Ohio, USA. SE is the standard error on each of the coefficients.

Model	Nutrient	Conductivity	SE
TRP + conductivity	−0.110	0.035	0.019
TNH_4 + conductivity	−0.091	0.056	0.020
Urea + conductivity	−0.068	0.074	0.021

We estimated within-site temporal relationships between nutrient concentrations and position on the 1st NMDS axis and used an initial value of 0.3 for the SD_N of the nutrient measurements, corresponding to an averaging time of ~1 to 2 wk for soluble reactive P (Fig. 2). The slopes of the site-specific temporal relationships between standardized TRP concentrations and position on NMDS axis 1 were all <0, with a mean value of −0.08 ± 0.02 (Fig. 3). Standardized slopes for temporal relationships between TNH_4 and NMDS axis 1 and between urea and NMDS axis 1 both clustered around 0, with mean values of 0.004 ± 0.010 and 0.00 ± 0.007, respectively.

Within each site, the correction from instantaneous TRP measurements to a mean TRP measurement (computed over a time interval, Δt) is manifested as a steeper relationship between TRP and position on the 1st NMDS axis (Figs 4, S2). The steeper relationship is a typical result of accounting for measurement error in the predictor variable because instantaneous measurements are distributed more widely than mean concentrations. Therefore, a line fit to mean TRP measurements is steeper than a line fit to the instantaneous values.

We examined the effects of different assumptions regarding the relevant value of Δt by recomputing within-site relationships for SD_N ranging from 0.1 to 0.4, corresponding to the range of SD_N estimated for values of Δt from 2 to 21 d. Standardized slopes estimated from temporal and spatial models were statistically indistinguishable when SD_N of TRP was ≥0.3 (Fig. 5), which is associated with a Δt of ≥1 wk (Fig. 2).

DISCUSSION

Our analysis of field observations of diatom assemblage composition indicated that variation in periphytic diatom composition was more strongly associated with TRP than with urea or TNH_4. Furthermore, the similarity in values of the parameters estimated from temporal, within-site models and a spatial, among-site model supports our interpretation that the observed relationship was likely to be causal. The analysis described here improves on efforts to distinguish between the effects of different nutrient species in observational data. Estimating the effects of different nutrient species in observational data is difficult because elevated concentrations of multiple nutrients often arise from the same activities in the watershed and are, therefore, strongly correlated. Here, though, we have demonstrated that differences in the correlational structure between temporally and spatially resolved data can be used to isolate the effects of different nutrient species. Differences in mean diatom assemblage composition among different sites (i.e., across space) were similarly related to gradients of mean TRP, TNH_4, and urea concentrations at each site, but in the temporal analysis, no relationship was observed between TNH_4 or urea and diatom assemblage composition. These findings suggest that the among-site relationships estimated for TNH_4 and urea most likely arose from the strong correlations between the 3 nutrient species concentrations. Because of the difficulties in isolating an effect of a particular nutrient, most previous analyses of observational data have modeled the effects of nutrients by representing a gradient of overall nutrient enrichment with a composite indicator (Hering et al. 2006) or by modeling only 1 nutrient with the understanding that it represents an overall nutrient gradient (Yuan 2010). The present analysis offers an approach that can be used to disentangle the relative effects of different nutrient species. We first discuss our findings in the context of current understanding of the effects of nutrients on diatoms in flowing waters and then consider the different sources of uncertainty in our analysis.

Effects of nutrients on diatoms

The present findings are broadly consistent with the idea that freshwater ecosystems are more limited by P than N, but much of the research examining the relative effects of P and N focuses on bulk algal growth rates (Francoeur et al.

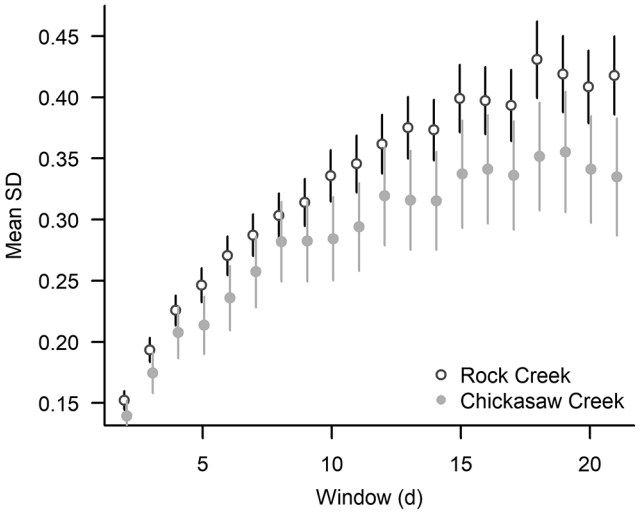

Figure 2. Mean SD of log-transformed total P (TP) estimated from daily TP measurements from Rock Creek (open circles) and Chickasaw Creek (filled circles), Ohio, USA, in the indicated window (d) size. The Rock Creek catchment area is 90 km^2 and the Chickasaw Creek catchment area is 43 km^2.

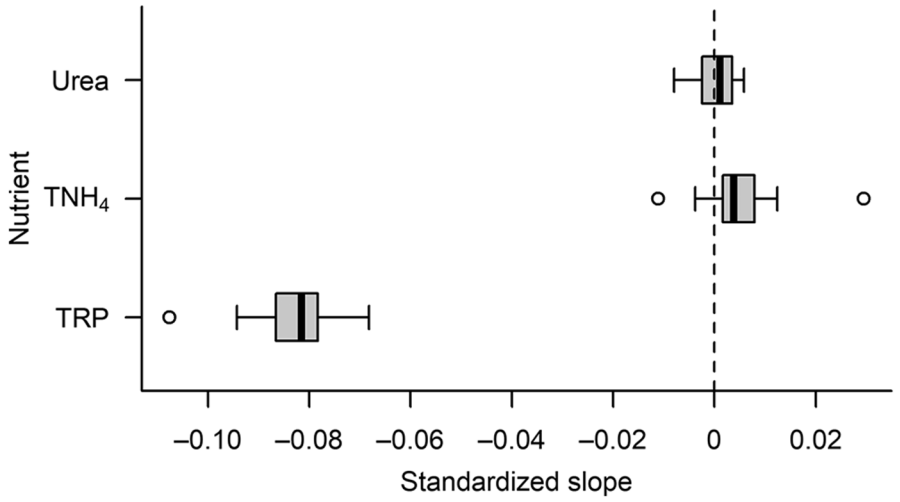

Figure 3. Distributions of slopes (Bayesian model) between standardized nutrient concentration and non-metric multidimensional scaling axis 1, representing variation in diatom assemblage composition, estimated for changes in time at each site in the East Fork of the Little Miami River watershed, southwest Ohio, USA, with the SD of log-transformed instantaneous nutrient concentrations = 0.3. Dashed vertical line shows slope = 0. Vertical line segments that define each box correspond to the 25th, 50th, and 75th percentiles of the plotted distributions. Left horizontal line segment (whisker) extends to smallest value that is within a distance of 1.5× the interquartile range from the left edge of the box. Right whisker extends to greatest value that is within a distance of 1.5× the interquartile range from the right edge of the box. Values outside the range defined by the whiskers are shown with open circles.

1999) rather than the species composition response considered here. The variation in species composition with changes in TRP observed in the present data set may reflect differences in the competitive balance among species (e.g., Passy 2007), but elucidating the mechanism by which TRP alters this balance is beyond the scope of the present study. Some possible mechanisms include differences in the efficiency of uptake kinetics among species (Smith and Kalff 1982) or differences in the morphological characteristics of diatoms (e.g., prostrate vs stalked forms) that affect mass transfer rates for nutrients (Dodds and Biggs 2002, Larned et al. 2004).

The weak temporal relationship between diatom assemblage composition and TNH_4 and urea concentrations may have occurred because rapid conversion and turnover of these N species limited our ability to detect a signal from grab samples (McCarthy et al. 2013, Hampel et al. 2019). In addition to direct biotic uptake of ammonia (Webster et al. 2003), extracellular enzymes can quickly convert urea to NH_4^+ and NH_4^+ to NO_3^- under oxic conditions (Sinsabaugh and Follstad Shah 2012). These processes occur in both the hillslope soils that drain to streams as well as the stream hyporheic and near-bed environments (Wymore et al. 2019). As a result, NO_3^- concentrations in streams draining impacted watersheds are often 2 orders of magnitude greater than NH_4^+ and urea, except when discrete sampling events coincide with a fertilizer runoff event or are affected by a point source discharge (Glibert et al. 2005). It is also possible that N saturation was occurring in the studied streams because of the relatively large N mass in these streams and the constant additions to the NO_3^- pool from lateral leaching flows and upwelling groundwater (Earl et al. 2006). These high concentrations would dilute the signal from biological uptake and account for the weak spatial and temporal effects of NO_3^- on diatom assemblage found in previous studies (Mulholland et al. 2008) and observed in early exploratory analyses conducted in this study.

N still likely affects stream biological communities, especially when considering larger-scale studies and responses other than diatom assemblage composition. For example, in a large mesocosm study, increased N concentrations were found to have a greater combined effect on stream periphyton composition and function than increased P (Costello et al. 2018). Also, in a bioassessment survey of the EFLMR, total Kjeldahl N was identified as the strongest correlate with biological index scores based on the stream fish community (Ohio EPA 2014). Indeed, the management plan for the EFLMR concluded that both P and N should be managed as causes of organic enrichment (Ohio EPA 2020).

Sources of uncertainty

The effect of variation in TRP on diatom species composition was consistent between temporal and spatial analyses, but uncertainties remain. First, our estimates of the effect of averaging time on the relationship between TRP and diatom species composition is comparable to other studies, but we accounted for averaging time by incorporating an estimate of the variance of TRP in our statistical model, and converting this variance to averaging time is uncertain. Temporal models that were fit using an SD_N associated

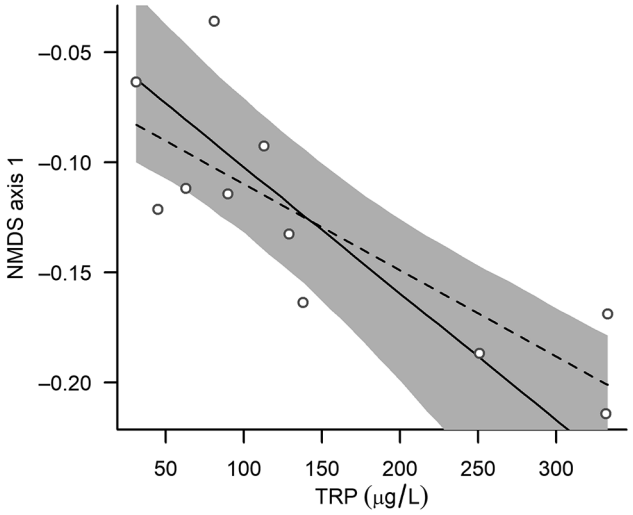

Figure 4. Example of the relationship between total reactive P (TRP) and non-metric multidimensional scaling (NMDS) axis 1 position, representing variation in diatom assemblage composition, for 1 site in the East Fork of the Little Miami River watershed, southwest Ohio, USA. Solid line represents the estimated relationship based on the Bayesian modeled mean TRP based on an SD of log-transformed instantaneous nutrient concentrations = 0.3. Gray shading represents the 90% credible interval on the estimated relationship. Dashed line represents a simple linear regression fit to data. Open circles represent the observed data.

with at least a 1-wk averaging time yielded relationships that were comparable to the among-site model. This averaging time is broadly similar to durations that have been estimated elsewhere, although the range of durations is wide (Larned 2010). For example, laboratory studies of the response of diatoms to P enrichment suggested that changes occur in 6 d (Pan and Lowe 1994). In field data, the strongest correlations between diatom indices or metrics and P concentrations were observed using a 5-wk average, with shorter integration times in less eutrophic rivers (Lavoie et al. 2008). Stronger correlations were also observed using summer mean nutrient concentrations rather than same-day concentrations (Smucker and Vis 2011b). Differences in response times associated with initial nutrient concentrations have also been observed in translocation experiments, in which diatoms from a high nutrient environment are moved to a low nutrient environment, and vice versa. In 1 of these experiments, diatom assemblage structure changed in 1 wk when nutrient concentrations increased across treatments but required 4 wk to respond when nutrient concentrations decreased across treatments (Lacoursière et al. 2011). In a similar study, diatoms from different high-nutrient streams were transferred to a low-nutrient stream, and differences in assemblage structure were quantified with a bioassessment index. Here, up to 60 d were required before the diatom index values in the transferred samples were comparable to those observed in the low-nutrient stream (Rimet et al. 2009). The structural changes in the diatom assemblage were characterized by changes in growth form from stalked diatoms (in high nutrient, dense biofilms) to adnate growth forms. Finally, analysis of high-frequency nutrient data suggested that 7 to 21 d prior to sampling is the relevant time scale for predicting diatom assemblage responses to changes in P concentration (Snell et al. 2014).

Averaging time is included in the current analysis only in terms of its effect on SD_N, and the relationship between averaging time and SD_N can vary. For example, we assumed that the average concentration over Δt characterized nutrient conditions, but pulsed nutrient loads and the timing of periods of elevated concentrations may be relevant to the diatom assemblage (Litchman et al. 2009), and nutrient concentrations collected more frequently to capture these shorter-term events may reveal greater levels of variability than observed with the daily data used here. We also observed that the relationship between SD_N and averaging time depends on the time of year (results not shown) and on the size of the stream (Fig. 2). Studies that more closely examine the effects of these factors and their effect on SD_N and averaging time would require temporally intensive nutrient measurements matched with periphyton samples.

The 2nd source of uncertainty we faced in this study was the influence of covariates on the analysis. We were

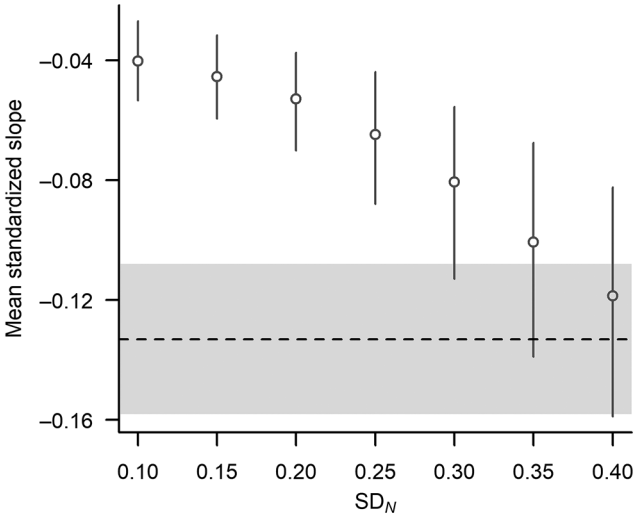

Figure 5. Estimates of within-site slopes of relationship between standardized total reactive P (TRP) and non-metric multidimensional scaling (NMDS) axis 1 for different SDs of log-transformed instantaneous nutrient concentrations (SD_N) at sites in the East Fork of the Little Miami River watershed, southwest Ohio, USA. Open circles: mean within-site slope for indicated SD_N. Line segments represent the 90% credible interval. Horizontal dashed line and gray shading represent estimated mean and 90% confidence limits for standardized slope based on the spatial model for TRP.

interested in accurately estimating the relationship between nutrient concentrations and position on NMDS axis 1, and for a covariate to bias the estimate of this relationship it must both covary with nutrient concentrations and exert its own effect on diatom composition. Factors that are correlated with nutrients but exert no influence on diatom composition would not alter the estimated relationship, whereas factors that are correlated with NMDS axis 1 but not with nutrients would reduce residual variability but would not change the estimated slope with nutrients. Conductivity provides one example of a potentially confounding variable because it may be correlated with nutrients both temporally and spatially (Biggs and Close 1989, Villa et al. 2019) and is known to influence diatom assemblage composition (Smucker and Vis 2011a). So, is it possible that the confounding effect of conductivity might alter our conclusion regarding the evidence for a causal relationship between TRP and diatom assemblage composition? Ideally, we would test for this possibility by including conductivity as a covariate in the spatial and temporal models relating diatom composition to TRP. However, in the present study, conductivity measurements were not collected, and we could only assess the effects of conductivity in the spatial model. Inclusion of historical mean values of conductivity in the spatial model indicated that conductivity only exerted a weak influence on the estimated relationship between diatom composition and TRP. In our study, the coefficient of variation among sites for mean TRP concentrations was ~4× the coefficient of variation of mean conductivities. Studies of temporal variation within sites have found that the difference in coefficients of variation between different P species and conductivity were substantially greater than the differences among-site observed here (Cattaneo and Prairie 1995, Haggard et al. 2007), hence, conductivity is less likely to alter relationships between TRP and diatom composition in the temporal model. From these insights we conclude that covariation between TRP and conductivity is not likely to weaken the evidence for a causal effect of TRP on diatom composition.

Other confounding variables are possible, but for these variables to influence our findings, they would have to meet the same requirements of being associated with TRP and independently affecting diatom composition. Elevated nutrients are correlated with increased agricultural land use in the EFLMR, so other factors that influence diatoms and that originate from increased agricultural land use are potential candidates for confounding, such as increased herbicide concentrations (Munn et al. 2018) and increased suspended sediment. Similarly, streamflow is often associated with TRP concentrations (Stenback et al. 2011) and can directly influence diatom composition (Cardinale et al. 2005). For these parameters, we lacked measurements to directly assess their effects; therefore, we cannot conclusively establish a causal relationship between diatom composition and TRP based only on this study.

The analysis approach used here is particularly important to better understanding the autecology of different diatom species, and it highlights a novel and effective application of DNA metabarcoding to understand nutrient effects on diatoms. Analyses of diatom autecology often begins by examining the relationship between assemblage composition and environmental gradients to determine which factors can be analyzed to determine species autecology. Here, we have described an approach to more specifically identify environmental variables that are causally associated with the occurrence of different diatom species—information that can help guide subsequent analyses of species–environment relationships. From a broader perspective, the results of this analysis highlight the possible insights that can be gained from carefully designed field observations, and we hope that this work stimulates similar studies. Acquiring a fuller understanding of the effects of different anthropogenic stressors in stream ecosystems likely requires a synthesis of laboratory and field observations. Here, we have shown that quantitative estimates of biological effects from field observations are possible, and these types of analyses may facilitate stronger comparisons between field and laboratory studies.

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