Characterizing temporal variability in streams supports nutrient indicator development using diatom and bacterial DNA metabarcoding

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HIGHLIGHTS

- Temporal variability can confound stream assessments and management.
- High frequency sampling and DNA metabarcoding of stream periphyton quantified nutrient-biota relationships over time.
- Major precipitation events weakened nutrient-biota relationships for 1–3 weeks.
- Diatom and bacterial metrics represented seasonal mean nutrients better than short-term and same-day concentrations.
- Results strengthen inferences based on diatom and bacterial assemblages and inform monitoring strategies.

GRAPHICAL ABSTRACT
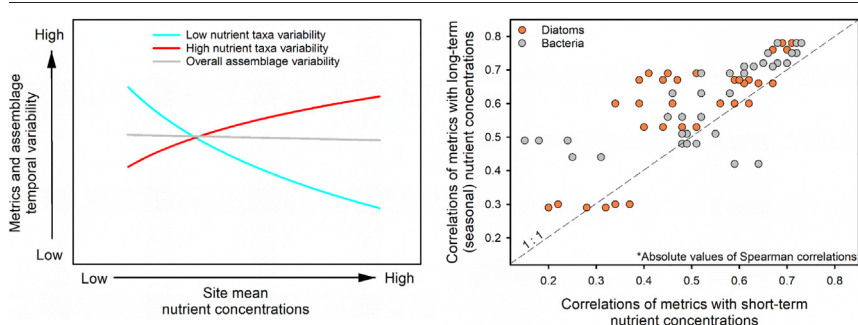
ABSTRACT

Interest in developing periphytic diatom and bacterial indicators of nutrient effects continues to grow in support of the assessment and management of stream ecosystems and their watersheds. However, temporal variability could confound relationships between indicators and nutrients, subsequently affecting assessment outcomes. To document how temporal variability affects measures of diatom and bacterial assemblages obtained from DNA metabarcoding, we conducted weekly periphyton and nutrient sampling from July to October 2016 in 25 streams in a 1293 km^2 mixed land use watershed. Measures of both diatom and bacterial assemblages were strongly associated with the percent agriculture in upstream watersheds and total phosphorus (TP) and total nitrogen (TN) concentrations. Temporal variability in TP and TN concentrations increased with greater amounts of agriculture in watersheds, but overall diatom and bacterial assemblage variability within sites—measured as mean distance among samples to corresponding site centroids in ordination space—remained consistent. This consistency was due in part to offsets between decreasing variability in relative abundances of taxa typical of low nutrient conditions and increasing variability in those typical of high nutrient conditions as mean concentrations of TP and TN increased within sites. Weekly low and high nutrient diatom and bacterial metrics were more strongly correlated with site mean nutrient concentrations over the sampling period than with same day measurements and more strongly correlated with TP than with TN. Correlations with TP concentrations were consistently strong throughout the study except briefly following two major precipitation events. Following these events, biotic relationships with TP reestablished within one to three weeks. Collectively, these results

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can strengthen interpretations of survey results and inform monitoring strategies and decision making. These findings have direct applications for improving the use of diatoms and bacteria, and the use of DNA metabarcoding, in monitoring programs and stream site assessments.

1. Introduction

Nutrient pollution from human activities remains a globally common and pressing issue because of its negative effects on stream ecosystems and water quality, specifically due to increased amounts of phosphorus and nitrogen associated with agriculture, growing populations, and watershed development (Carpenter et al., 1998; Stoddard et al., 2016; Sabo et al., 2019; Lin et al., 2021; Sabo et al., 2021). High nutrient loads contribute to changes in ecological communities, excessive algal growth, and altered biogeochemical cycles (Biggs, 2000; Stevenson et al., 2012; Woodward et al., 2012; Dodds and Smith, 2016), which subsequently can reduce biodiversity, affect food webs, and lead to negative ecosystem and socioeconomic outcomes farther downstream (Wang et al., 2007; Dodds et al., 2009; Weijters et al., 2009; Sobota et al., 2015; Wurtsbaugh et al., 2019). As a result, determining how organisms respond to increased phosphorus and nitrogen concentrations in streams is useful for understanding nutrient effects and for informing watershed and stream conservation and management efforts (Miltner, 2010; Chambers et al., 2012; Wagenhoff et al., 2017).

Algal and bacterial assemblages are especially useful indicators because they are directly affected by nutrients and have important roles in primary production, food webs, and nutrient and carbon cycling (Bunn et al., 1999; Peterson et al., 2001; Bernot et al., 2010; Woodward et al., 2012). Benthic diatoms, in particular, are very diverse, unicellular algae that are frequently used in stream monitoring and assessment programs around the world because of their responsiveness to changes in environmental conditions (Smol and Stoermer, 2010; Rimet, 2012; Stevenson, 2014; Wu et al., 2017; Pérez-Burillo et al., 2020; Charles et al., 2021), including nutrients (Potapova and Charles, 2007; Lavoie et al., 2008; Stevenson et al., 2008; Smucker et al., 2013). Likewise, bacteria, which coexist with diatoms in benthic periphyton, are highly diverse and are affected by nutrients. Although less commonly used than diatoms, bacterial assemblage structure can be a useful indicator of stream conditions (Washington et al., 2013; Lau et al., 2015; Simonin et al., 2019; Pilgrim et al., in review).

Using these species rich assemblages as indicators that can be used in monitoring and assessment programs first requires identifying and enumerating species in samples and summarizing how they change along nutrient gradients. With ongoing advancements in high throughput sequencing and bioinformatics, DNA metabarcoding that targets the chloroplast rbcL gene for diatoms and the rRNA 16S gene for bacteria is providing an increasingly effective way to analyze and quantify diversity in environmental samples (Liu et al., 2007; Hamsher et al., 2011; Werner et al., 2012; Kermarrec et al., 2013; Hall et al., 2018; Pawlowski et al., 2018). For both diatoms and bacteria, assemblage responses to increasing nutrients are driven by a change from dominance by taxa that thrive under low nutrient conditions to dominance by those with faster and higher maximal growth rates at higher nutrient concentrations (Manoylov and Stevenson, 2006; Shatwell et al., 2014; Simonin et al., 2019). Quantification of these changes and the nutrient concentrations at which they occur is used to develop indicators for stream monitoring and to provide support for possible nutrient management targets (Stevenson et al., 2008; Simonin et al., 2019; Smucker et al., 2020; Pilgrim et al., in review). These types of indicators and their responses to nutrients can be especially useful for effectively communicating complex assemblage data and results to stakeholders, policymakers, and the public.

While many effective biotic indicators have been developed from individual watershed to national scales (Stevenson et al., 2008; Danielson et al., 2012; Becker et al., 2019; Kelly et al., 2020), unknown effects of temporal variability and antecedent nutrient conditions could confound nutrient-biota relationships and subsequently affect monitoring and assessment outcomes. Rainfall and runoff events throughout a sampling season

cause pulsed, transient increases in stream phosphorus and nitrogen concentrations from nonpoint sources (Sharpley et al., 2001; Hart et al., 2004; Alexander et al., 2008; Withers and Jarvie, 2008; Loecke et al., 2017). Biological uptake of nutrients during these events and throughout the summer can decouple relationships between benthic assemblages and water column nutrient concentrations when based on single, same-day observations (Jarvie et al., 2013; Wood et al., 2015; Vadeboncoeur and Power, 2017; Costello et al., 2018). Given the effects of temporal variability, sampling for nutrients and biota repeatedly over time could inform sampling strategies and improve the effectiveness of using diatom and bacterial indicators in monitoring programs, which often depend on only one, occasionally two, sampling events in a season.

A small but growing number of studies have examined effects of temporal variability on diatom-nutrient relationships and monitoring results by using experimental nutrient dosing (Pearce et al., 2020), transplanting substrata with established periphyton between low and high nutrient conditions (Lacoursière et al., 2011; Taylor et al., 2018), or using field observations from stream samples collected at monthly to quarterly or yearly intervals (Soininen and Eloranta, 2004, Taylor et al., 2007, Smucker and Vis, 2011, Virtanen et al., 2011, Snell et al., 2014, Virtanen and Soininen, 2016, Taylor et al., 2018), and less commonly every week or two (Lavoie et al., 2008; Smucker and Vis, 2011; Korhonen et al., 2013; Huttunen et al., 2020). These studies examined the durations over which diatom assemblages represent or integrate nutrient conditions and rates at which they change in response to increases or decreases in nutrient concentrations. Collectively, they support the conclusion that diatoms are representative of, and responsive to, nutrient conditions over time and are therefore useful indicators of nutrient condition. However, to our knowledge, no similar studies have been done for bacteria except for one examining assemblage structure in only two streams over ten weeks (Lear et al., 2008), and no studies have examined effects of temporal variability on diatoms for many streams at a watershed scale or on the use of DNA metabarcoding for applications of diatom and bacterial indicators in biomonitoring.

Here, we document the effects of temporal variability on diatom and bacterial assemblages, metrics, and monitoring results by using DNA metabarcoding and samples collected mostly weekly over four months from 25 streams in a 1293 km^2 watershed with mixed land use. Regarding metrics, we examined how relative abundances of gene sequence reads for four groups of indicator taxa changed in response to nutrient concentrations and environmental variability over time at sites. These metrics were based on taxa associated with low phosphorus, high phosphorus, low nitrogen, or high nitrogen concentrations (Smucker et al., 2020; Pilgrim et al., in review). Our objectives were (1) to describe variability in nutrients and assemblages during a sampling season, (2) to identify what factors corresponded with temporal variability (e.g., precipitation and agricultural land use), and (3) to document how this variability might affect nutrient-biota relationships. Characterizing how diatom and bacterial assemblages integrate nutrient effects and variability over time can strengthen interpretations of monitoring results, inform decision making, and have direct applications to improving their use, and the use of DNA metabarcoding, in monitoring and assessment programs.

2. Methods

2.1. Study design and description of existing data

To examine temporal variability in nutrients and gene sequence reads, we used publicly available diatom rbcL, bacterial 16S, and nutrient data (https://doi.org/10.23719/1504034; https://www.ncbi.nlm.nih.gov/

bioproject/592969). These data were generated from a study of 25 second- and third-order wadeable stream sites distributed throughout the East Fork of the Little Miami River watershed (1293 km^2), which is located in southwest Ohio, USA and experiences a temperate seasonal climate. No sites were downstream of another site; sites were selected to minimize confounding effects of non-nutrient factors, and historical monitoring data were used to ensure sites spanned gradients of total phosphorus (TP) and total nitrogen (TN) concentrations observed in streams within this watershed (Smucker et al., 2020). Agricultural land cover comprises 54% of the total watershed area, and watershed land cover upstream of each site, which was determined using the 2011 National Land Cover Database (Homer et al., 2015), ranged from 9–59% forest, 0–88% agriculture, and 0–69% urban among the 25 stream sites.

At each site, unfiltered water samples and epilithic periphyton were collected weekly from mid-July to late October 2016, except for the final two sampling events of the season which were two weeks apart. Acid persulfate wet digestion was performed prior to measuring TP with the standard ammonium molybdate and antimony potassium tartrate reaction and ascorbic acid reduction method (United States Environmental Protection Agency, 1993; Tucker, 2008). An alkaline wet oxidation persulfate method was used prior to cadmium reduction for measuring TN (Patton and Kryskalla, 2003; Smith and Bogren, 2003). Both TP and TN were measured using a QuikChem 8500 nutrient autoanalyzer system (Lachat Instruments, Milwaukee, WI, USA). Diatoms and bacteria were collected by removing periphyton from a 6.7 cm^2 area on the top surfaces of 5 rocks (total area of 33.5 cm^2) equally spaced throughout a 75-m stream reach. Diatom data consisted of operational taxonomic units (OTUs) identified based on sequence clustering at ≥97% similarity while bacterial taxonomic data consisted of amplicon sequence variants (ASVs). Further details of laboratory methods, best available taxonomic information, and bioinformatic analyses can be found in Smucker et al. (2020) for diatoms and Pilgrim et al. (in review) for bacteria. We focused on variability of metrics and assemblages as a whole given the potential for their direct applications and use in monitoring programs and assessments. As such, we took a taxonomy-free approach (Apothéloz-Perret-Gentil et al., 2017; Cordier et al., 2018; Feio et al., 2020) because attaching species names to OTUs and ASVs is limited by coverage in reference barcode libraries and accuracy of assignment algorithms, and ultimately this addition would not affect the interpretations of our study's results. Over the course of this study, each site had 13–14 periphyton samples ($n = 342$ among sites) and 10–12 nutrient samples collected (TP $n = 281$, TN $n = 280$ among sites).

2.2. Statistical analyses

2.2.1. Characterizing assemblages and relationships with environmental variables

We analyzed diatom and bacterial datasets separately using the same statistical approaches for each to summarize data, describe temporal variability, and identify relationships with nutrients. Relative abundances of gene sequence reads for taxa in each sample were used in statistical analyses. Previous work used threshold indicator taxa analysis (Baker and King, 2010) with 1000 bootstraps to identify diatom OTUs and bacterial ASVs that decreased or increased with increasing TP or TN concentrations (Smucker et al., 2020; Pilgrim et al., in review). Taxa that decreased are subsequently referred to as low P or low N diatoms or bacteria (52 low P diatom OTUs, 50 low N diatom OTUs, 105 low P bacterial ASVs, 63 low N bacterial ASVs), and taxa that increased are subsequently referred to as high P or high N diatoms or bacteria (49 high P diatom OTUs, 30 high N diatom OTUs, 138 high P bacterial ASVs, 74 high N bacterial ASVs). Relative abundances of all indicator taxa within each group were summed for each sample and used as response variables in analyses. We subsequently refer to these four indicator groups as biological metrics in a manner similar to those used in traditional biotic indices, such as relative abundances of

sensitive or tolerant species (Karr, 1981; Bahls, 1993; DeShon, 1995; Hill et al., 2000; Wang et al., 2005; Potapova and Charles, 2007; Zalack et al., 2010).

We used nonmetric multidimensional scaling (NMDS) to visualize differences in assemblage structure among sites and samples and to examine if axis scores were correlated with environmental variables. For the diatom NMDS, we included OTUs with ≥1% relative abundance in ≥1% of samples (137 of 650 OTUs). For the bacteria NMDS, this criterion left us with <60% of the total relative abundance of bacteria because of the high number of rare ASVs, so to be more inclusive and consistent with previous indicator species analyses, we used all ASVs meeting two criteria: the first being that they were observed in ≥5 samples, and the second being that they comprised 75% of the cumulative relative abundance from all samples when rank ordered from greatest to least average relative abundance (429 of 18,406 ASVs). For both NMDS ordinations, we used square-root transformed relative abundances and the Bray-Curtis dissimilarity coefficient. NMDS was conducted, with rotation to maximize variation along the first axis, using the vegan package and the metaMDS function (Oksanen et al., 2020) in R v. 4.0.3 (R Development Core Team, 2020). Stress was 0.162 for diatoms and 0.159 for bacteria, both indicating good support for meaningful interpretations of results, and 3-axis solutions were identified for both though we focus our results on the two axes that had strong and clear correlations with environmental variables. We used Spearman correlations to show relationships among axis scores, metrics, and environmental variables.

2.2.2. Examining temporal variability of assemblages and environmental conditions

For descriptive purposes, we used scatter plots of raw data (TP and TN concentrations and metrics) to show variability among dates within each site and variability among all sites on each date. We also used site specific z-scores of TP and TN concentrations and diatom and bacterial metrics to supplement interpretations of variability and changes over time across sites (i.e., this emphasized the within-site direction and magnitude of change throughout the study). Multivariate dispersion in a principal coordinates ordination with Bray-Curtis dissimilarity was calculated based on the mean distance of samples to their corresponding site centroids. This is a common approach to summarizing temporal variability and β-diversity; some studies of macroinvertebrates and diatoms indicate that increasing eutrophication can reduce distances to centroids due to an associated increase in taxonomic homogenization at sites (Maloney et al., 2011, Cook et al., 2018, Huttunen et al., 2020). Regarding our study, the mean distance of all samples within a site to their site centroid in ordination space represents a measure of within site variability of assemblages over time (e.g., distances increase with greater temporal variability in assemblage structure). We used the vegan package and the betadisper function in R v. 4.0.3 to calculate distances to site centroids (Oksanen et al., 2020; R Development Core Team, 2020).

Within site standard deviations of TP and TN concentrations, of NMDS axis scores, and of metrics provided additional measures of temporal variability at each site. Sites with larger standard deviations indicated greater variability over time. We used Spearman correlations to examine if variability among biota and nutrients were related to each other and if they were associated with eutrophication as represented by watershed percent agriculture and mean TP and TN concentrations at each site. Variability in diatom and bacterial metrics (standard deviations) and distance to centroids were plotted against site means of total phosphorus and total nitrogen ($n = 25$) to compare how variability of overall assemblage structure and variability of different components of assemblages (i.e., metrics) might change along a eutrophication gradient. To examine possible effects of precipitation events on nutrient concentrations and assemblages, we used data from the Parameter-elevation Regressions on Independent Slopes Model for a location near the center of the watershed to estimate daily rain amounts (PRISM Climate Group, Oregon State University, http://prism.oregonstate.edu, created 8 Mar 2019).

2.2.3. Documenting how temporal variability could affect monitoring and assessments

We took multiple approaches to document how temporal variability might affect the strength of diatom- or bacteria-nutrient relationships (i.e., metric performance). We first compared diatom- and bacteria-nutrient relationships using separate linear regressions for each week with metrics being response variables and same-week TP or TN concentrations being predictor variables. This quantifies how the strength of diatom and bacterial relationships with same day nutrient measurements might vary over a sampling season. Likewise, we conducted linear regressions for each week using each site's mean TP or TN concentrations from all dates as the predictor variable. The reasoning for this was to examine if diatom and bacterial metrics better reflected the "typical" seasonal conditions at sites than the same day nutrient concentrations. If this were the case, then the biology would better indicate nutrient effects and site conditions than infrequent measurements of the nutrients themselves. We plotted the weekly R^2 values from the same week regressions against the corresponding R^2 values from the study mean regressions to compare them and to visualize variability in metric-nutrient correlations. We also plotted these two sets of R^2 values by date with daily precipitation overlaid to examine possible effects of precipitation events on metric-nutrient relationships. To examine how antecedent nutrient conditions affect diatoms and bacteria, we used all available data from all weeks to compare Spearman correlations of metrics and NMDS axis scores with same week, 1-week lag (i.e., nutrient concentrations one week prior), 2-week averaged (i.e., mean concentrations based on the day of, the week prior, and two weeks prior to sampling), and study mean concentrations.
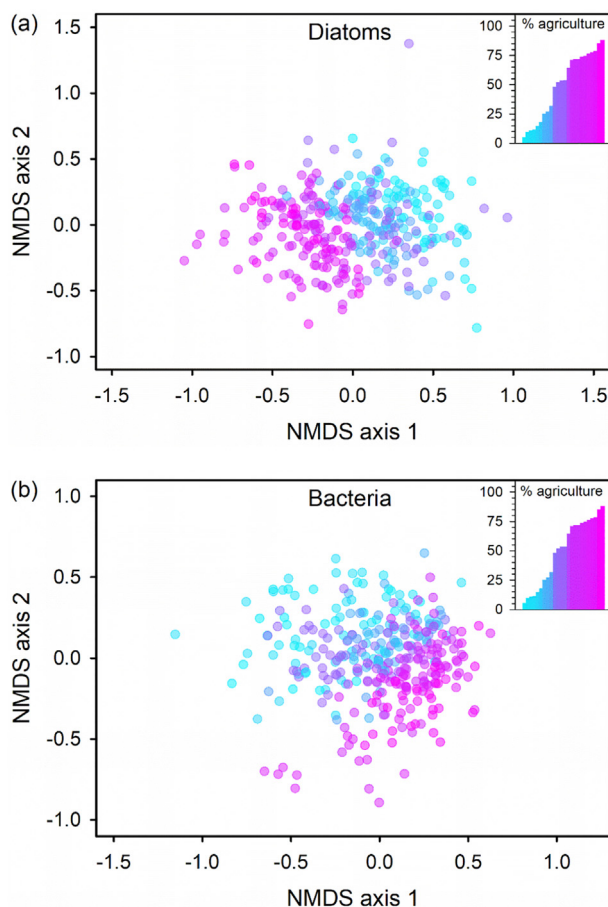
Table 1

Spearman correlations among diatom and bacteria nonmetric multidimensional scaling (NMDS) axes and metrics, percent watershed agriculture and forest, and total phosphorus (TP) and total nitrogen (TN) concentrations. Results are based on all samples (TP $n = 281$, TN $n = 280$, agriculture and forest $n = 342$ [all diatom and bacteria samples but only 25 site values of % agriculture and % forest]). NS = $p > 0.05$.

	NMDS 1	NMDS 2	% agriculture	% forest	TP	TN
Diatoms						
High P	−0.90	0.11	0.59	−0.51	0.62	0.46
Low P	0.84	0.09^{NS}	−0.70	0.64	−0.70	−0.47
High N	−0.79	0.16	0.44	−0.38	0.51	0.51
Low N	0.80	0.21	−0.61	0.56	−0.61	−0.62
NMDS 1	–	–	−0.77	0.65	−0.73	−0.51
NMDS 2	–	–	−0.30	0.26	−0.20	−0.22
Bacteria						
High P	0.59	−0.51	0.75	−0.64	0.72	0.61
Low P	−0.63	0.42	−0.67	0.63	−0.70	−0.58
High N	0.56	−0.43	0.75	−0.64	0.72	0.63
Low N	−0.41	0.07^{NS}	−0.46	0.45	−0.48	−0.64
NMDS 1	–	–	0.51	−0.40	0.49	0.31
NMDS 2	–	–	−0.60	0.56	−0.52	−0.24
Nutrients						
TN	–	–	0.43	−0.39	0.65	–
TP	–	–	0.76	−0.64	–	–

3. Results

3.1. Diatom and bacterial relationships with nutrients

Nonmetric multidimensional scaling ordinations of diatoms and bacteria showed that their assemblage structures were strongly associated with

Table 2

Spearman correlations of site standard deviations (SD) of total phosphorus (TP) and total nitrogen (TN) with watershed percent agriculture, site means of TP and TN, and with site standard deviations and site means of diatom and bacterial metrics and nonmetric multidimensional scaling (NMDS) axis 1 scores ($n = 25$ for each pairwise comparison in the table). Standard deviations represent the within site temporal variability over time.

	TP SD	TN SD	% agriculture	TP (mean)	TN (mean)
Environment					
% agriculture	0.55***	0.37*	–	–	–
TP (mean)	0.72***	0.65***	–	–	–
TN (mean)	0.56***	0.75***	–	–	–
Diatoms					
NMDS 1 SD	−0.06	−0.09	0.02	−0.01	−0.03
High P SD	0.06	0.21	0.33	0.37*	0.34*
Low P SD	−0.62***	−0.50**	−0.78***	−0.85***	−0.69***
High N SD	0.34*	0.39*	0.50**	0.56***	0.43**
Low N SD	−0.50**	−0.43**	−0.53***	−0.69***	−0.61***
Centroid distance	0.31	−0.06	−0.15	−0.10	0.04
NMDS 1 (mean)	−0.53***	−0.48**	−0.84***	−0.84***	−0.75***
High P (mean)	0.46**	0.42**	0.71***	0.79***	0.69***
Low P (mean)	−0.60***	−0.53***	−0.83***	−0.91***	−0.77***
High N (mean)	0.39*	0.44**	0.58***	0.70***	0.68***
Low N (mean)	−0.55***	−0.49***	−0.75***	−0.82***	−0.73***
Bacteria					
NMDS 1 SD	−0.38*	−0.47**	−0.45**	−0.49**	−0.46**
High P SD	0.16	0.34*	0.48**	0.48**	0.52***
Low P SD	−0.68***	−0.53***	−0.66***	−0.81***	−0.67***
High N SD	0.38*	0.52***	0.74***	0.73***	0.77***
Low N SD	−0.54***	−0.44**	−0.70***	−0.71***	−0.71***
Centroid distance	0.29	0.03	0.21	0.17	−0.04
NMDS 1 (mean)	0.52***	0.42**	0.75***	0.73***	0.61***
High P (mean)	0.56***	0.54***	0.87***	0.87***	0.80***
Low P (mean)	−0.59***	−0.51***	−0.81***	−0.89***	−0.79***
High N (mean)	0.61***	0.57***	0.88***	0.90***	0.81***
Low N (mean)	−0.59***	−0.45**	−0.76***	−0.82***	−0.71***

* $p < 0.10$.
** $p < 0.05$.
*** $p < 0.01$.

Fig. 1. Nonmetric multidimensional scaling (NMDS) ordination for diatoms with site colors indicating gradients of low (cyan) to high (pink) watershed percent agriculture (a). NMDS for bacteria with site colors indicating gradients of low to high watershed percent agriculture (b).

watershed agriculture and forested land cover and with TP and TN concentrations (Figs. 1, S1, Table 1). For diatoms, increasing axis 1 scores were associated with less agriculture, more forested land cover, and lower TP and TN concentrations. Increasing axis 1 scores also were correlated with increasing relative abundances of low P and low N diatoms and with decreasing relative abundances of high P and high N diatoms (Table 1). For bacteria, decreasing axis 1 scores and increasing axis 2 scores were associated with less agriculture, more forested land cover, and lower TP and TN concentrations. Decreasing axis 1 scores and increasing axis 2 scores also were correlated with increasing relative abundances of low P bacteria and with decreasing relative abundances of high P and high N bacteria (Table 1). Relative abundances of low N bacteria increased with decreasing axis 1 scores. Based on all samples, diatom and bacterial metrics also were directly correlated with TP and TN concentrations, with low nutrient taxa decreasing and high nutrient taxa increasing in relative abundances with greater nutrient concentrations (Table 1). Based on site means, diatom and bacterial metrics and NMDS axis scores were even more strongly correlated with watershed percent agriculture and TP and TN concentrations (Table 2).

3.2. Temporal variability of assemblages and environmental conditions

Both TP and TN concentrations increased and became more variable as watershed percent agriculture increased (Fig. 2, Table 2). TP and TN variability also were correlated with site means of diatom and bacterial NMDS axis 1 scores and metrics and with variability in most diatom and bacterial metrics (high P diatom and bacteria standard deviations being exceptions). Variability of diatom metrics was greater than that of bacterial metrics (Figs. 3, S2–S3). Within site variability of low P and low N diatom and bacterial relative abundances decreased with greater watershed

percent agriculture, whereas variability in relative abundances of high P bacteria and high N diatoms and bacteria increased (Table 2, Figs. 3, S2–S3). Distances to site centroids were not significantly correlated with nutrient concentrations or watershed percent agriculture for diatom and bacterial assemblages (Fig. 3, Table 2), but greater standard deviations of NMDS axis 1 scores for bacteria were associated with greater percent agriculture in watersheds and with greater TP and TN concentrations (Table 2).

Two large precipitation events at the end of July and mid-August were associated with short-term increases in TP concentrations, but TN concentrations only increased with the first event before steadily decreasing during the remainder of the study (Fig. 2). High P and high N diatoms and bacteria had temporary increases in relative abundances corresponding with these two precipitation events as well (Figs. S4–S5). Low P and low N diatom relative abundances remained lower than average between these two precipitation events. Low P and low N bacterial relative abundances were less affected by the precipitation events, but relative abundances of low N bacteria increased while those of high N bacteria decreased during the remainder of the study, which also corresponded with the decreasing TN trend (Figs. 2, S5).

3.3. Effects of temporal variability on monitoring and assessments

Weekly diatom and bacterial metrics were almost always more strongly correlated with site mean TP or TN concentrations than with same week concentrations (Figs. 4, S6–S7). This was even clearer when comparing the mean R^2 values across weeks (large symbols in Fig. 4). Correlations of low P and high P diatom and bacterial metrics with TP concentrations were much stronger than those of low N and high N metrics with TN concentrations. The first major precipitation event during the third week of the study temporarily reduced the strength of diatom-TP relationships,
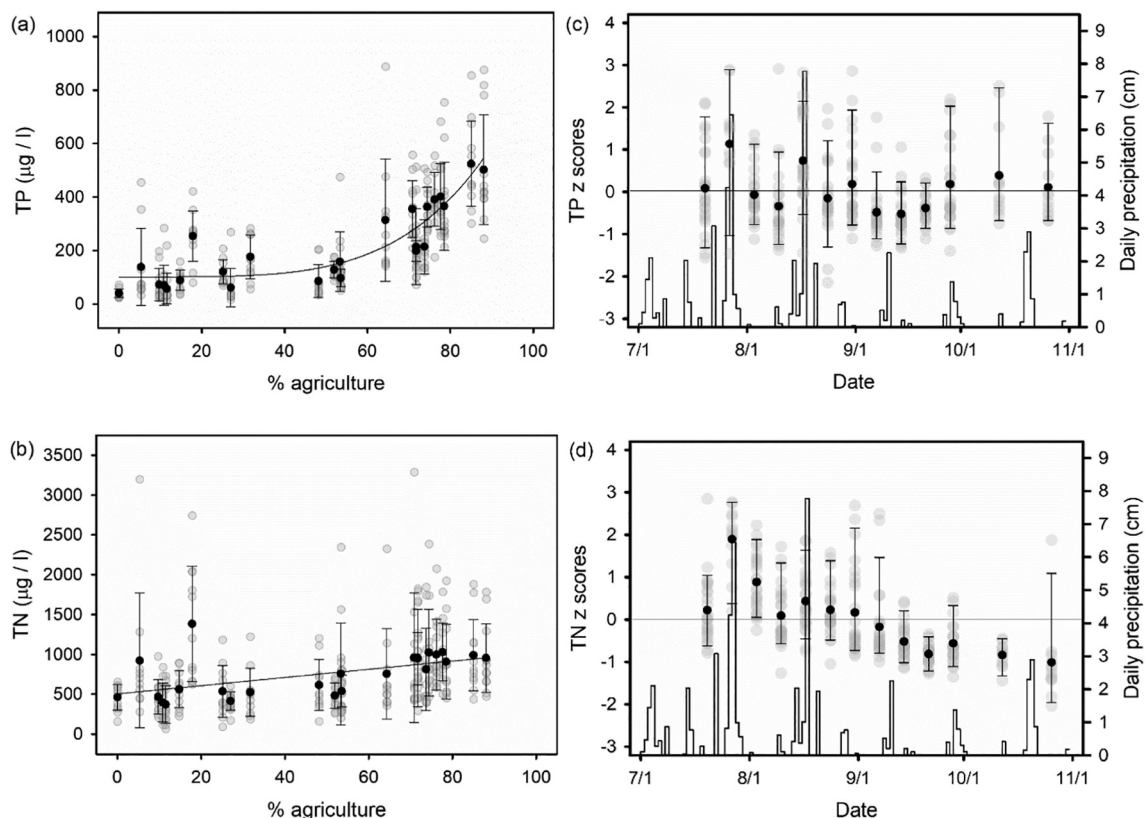

Fig. 2. Within site TP and TN variability plotted against watershed % agriculture (a, b) and within site variability plotted over time as site-specific z-scores by date (c, d). Regression lines are based on site means and are intended for descriptive visualizations of relationships between watershed % agriculture and (a) TP ($y = 43.7739 \times e^{(0.0272x)}$, $R^2 = 0.76$, $p < 0.01$) and (b) TN ($y = 505.4212 + 5.1405x$, $R^2 = 0.32$, $p < 0.01$). Black circles = site means ± 1 SD for all dates (a, b) and black circles = date means ± 10th and 90th percentiles for all sites (c, d). Gray circles = data from all dates at each site (a, b) or from all sites on each date (c, d). Z-scores were calculated for each site individually. TP $n = 281$, TN $n = 280$.

but correlations rebounded by the sixth week and remained mostly consistent despite the second precipitation event that also occurred during week six. (Figs. 5, S6). Bacteria-TP relationships showed the opposite pattern, remaining consistent following the first precipitation event and declining following the second (Figs. 5, S7). The R^2 values for low P and high P bacteria rebounded within one and three weeks, respectively, and remained consistently high for the remainder of the study. The R^2 values for low N and high N diatoms were relatively variable throughout the study period, whereas those for bacteria were relatively consistent except between weeks 3–8 (Fig. 5). Correlations of diatom and bacterial metrics with 1-week lags and 2-week means of TP and TN concentrations did not provide any benefit over simply using study mean, or even same week, concentrations to characterize diatom- or bacteria-nutrient relationships (Table 3).

4. Discussion

4.1. Summary of key findings informing the applications of diatom and bacterial indicators

Characterizing nutrient-biota relationships over time and documenting variability in assemblages and nutrients in these streams provides new perspectives and further support for using DNA metabarcoding to develop diatom and bacterial indicators that could be used in monitoring programs. Analysis of all samples and of individual weekly data showed that diatom and bacterial assemblages best represented site means of nutrient concentrations during the study period (i.e., typical conditions at these sites) though they also were correlated with concentrations on the day of, those one week prior to, and those averaged over two weeks prior to biological sampling. This finding contributes further evidence showing that diatoms

are good integrators of nutrient effects over time (Taylor et al., 2007; Lavoie et al., 2008; Smucker and Vis, 2011; Snell et al., 2014; Yuan et al., 2022) and that bacteria can be useful indicators of nutrient conditions as well (Simonin et al., 2019, Pilgrim et al., in review).

Except for the effects of two large precipitation events during our study, these weekly relationships were relatively consistent with mean TP over time, but relationships with mean TN were weaker and more variable. Complementary analyses similarly found that diatom assemblages integrated variation in phosphorus averaged over periods spanning at least two to four weeks prior to sampling (Yuan et al., 2022) and that diatom and bacterial assemblages were more strongly associated with TP than with TN concentrations in this watershed (Smucker et al., 2020; Pilgrim et al., in review). For simplicity and brevity, we subsequently focus our discussion mostly on TP, because of its stronger relationship with biota, and on nutrient effects in general. Although two large precipitation events temporarily weakened correlations, our results indicated that diatom and bacterial assemblages represented typical TP concentrations again within one to three weeks.

Lastly, diatoms and bacteria were robust indicators of spatial differences in nutrient conditions among sites when possible confounding effects of temporal variability were reduced by averaging, as evidenced by the strongest correlations being between site means of metrics and site means of nutrient concentrations. Regardless of temporal variability, assemblage structure and metrics were strongly associated with watershed percent agriculture and with TP and TN concentrations when using data from all sampling dates. These DNA-based relationships are qualitatively similar to studies based on diatom morphological identification and enumeration that have found nutrients and watershed agriculture to be important factors affecting diatom assemblage structure (Potapova and Charles, 2007;
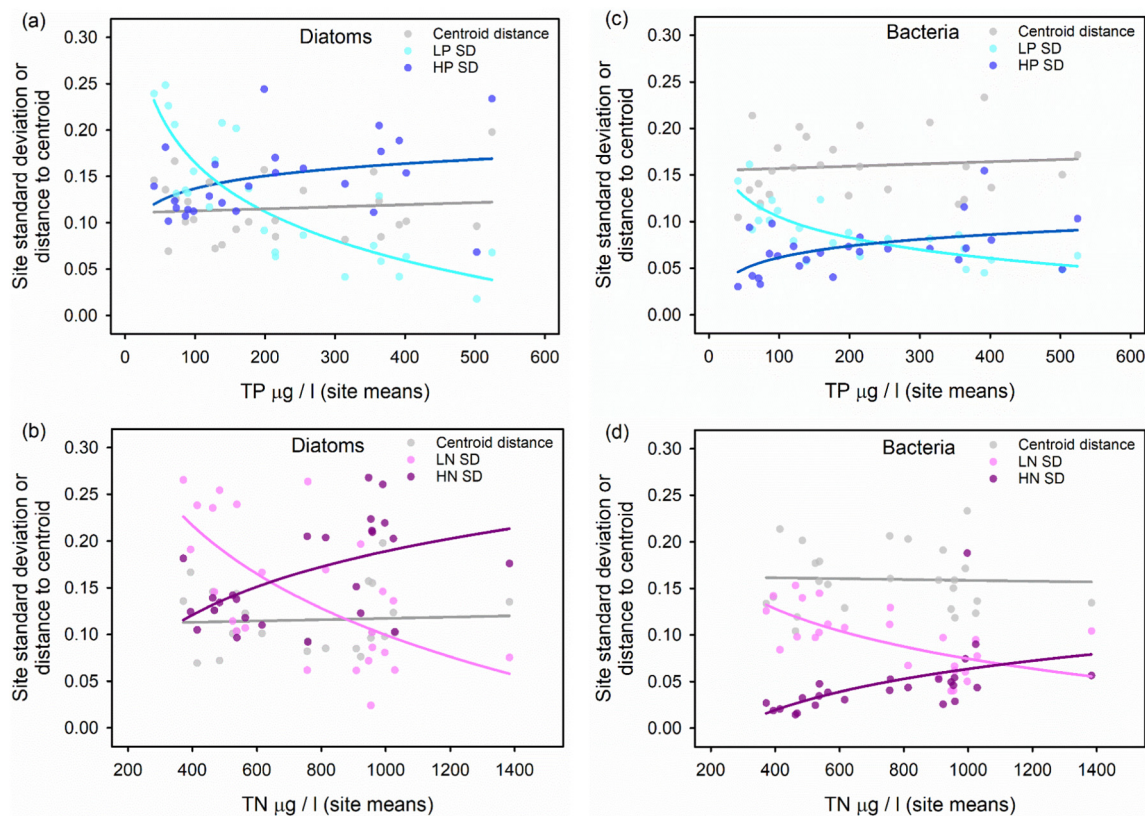

Fig. 3. Site variability in metrics (standard deviations for each site using data from all weeks) and distances to centroids plotted against site mean TP and TN concentrations for diatoms (a, b) and for bacteria (c, d). HP = high phosphorus taxa, LP = low phosphorus taxa, HN = high nitrogen taxa, LN = low nitrogen taxa. Regression lines are intended for descriptive visualizations of relationships: LP diatoms $y = 0.5157–0.0762 \times \ln(x)$, $R^2 = 0.71$, $p < 0.001$; HP diatoms $y = 0.478 + 0.0194 \times \ln(x)$, $R^2 = 0.12$, $p = 0.091$; LN diatoms $y = 0.9830–0.1279 \times \ln(x)$, $R^2 = 0.42$, p < 0.001; HN diatoms $y = -0.3246 + 0.0744 \times \ln(x)$, $R^2 = 0.28$, $p = 0.006$; LP bacteria $y = 0.2521–0.0319 \times \ln(x)$, $R^2 = 0.64$, $p < 0.001$; HP bacteria $y = -0.0202 + 0.0177 \times \ln(x)$, $R^2 = 0.23$, $p = 0.016$; LN bacteria $y = 0.4789–0.0585 \times \ln(x)$, $R^2 = 0.42$, $p < 0.001$; HN bacteria $y = -0.269 + 0.0482 \times \ln(x)$, $R^2 = 0.27$, $p = 0.008$.
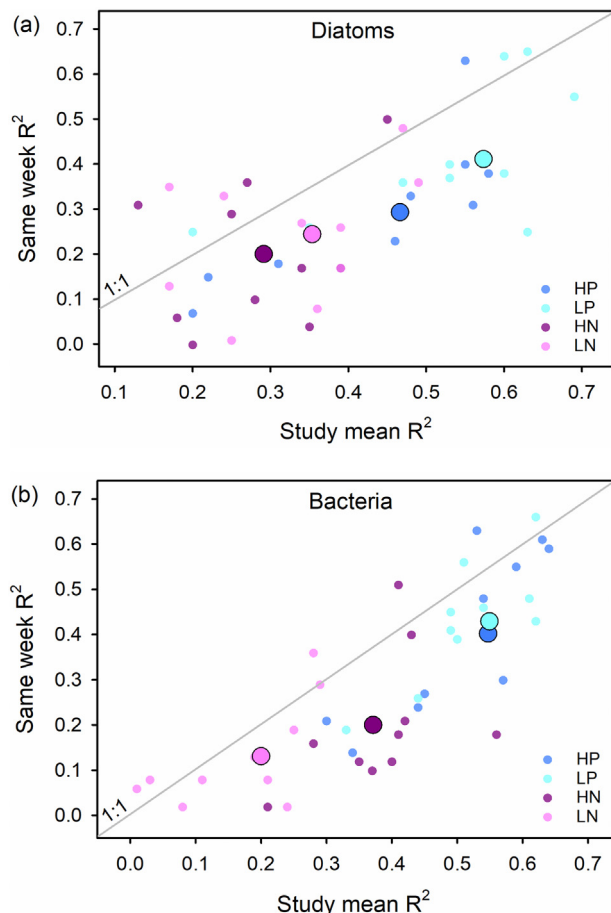
Fig. 4. Scatter plots for diatoms (a) and bacteria (b) comparing correlations between metrics and same week nutrient concentrations and correlations between metrics and site mean nutrient concentrations for each sampling week (linear regression R^2 values for each week). Mean R^2 values for each metric are shown as large symbols. HP = high phosphorus taxa, LP = low phosphorus taxa, HN = high nitrogen taxa, LN = low nitrogen taxa.

Porter et al., 2008; Smucker and Vis, 2009; Chambers et al., 2012; Pillsbury et al., 2019; Waite et al., 2021).

4.2. Ecological interpretations and implications of temporal variability

While diatoms and bacteria were effective indicators of nutrient conditions over time, temporal variability in nutrient concentrations still likely contributed to changes in the relative abundances of low and high nutrient taxa within sites. Other factors, such as trophic interactions, scouring and senescence, and population and community dynamics, quite likely affect diatom and bacteria variability over time as well (Humphrey and Stevenson, 1992; Biggs, 2000; Stevenson et al., 2006; Suplee et al., 2015), but we can only focus on nutrient-biota relationships given the scope of our study. Temporal variability in low P and low N diatoms and bacteria was much greater in streams with lower mean nutrient concentrations. This was despite both TP and TN concentrations becoming more variable as watershed percent agriculture and site means of nutrient concentrations increased. This might suggest that (1) in low-nutrient streams, low nutrient taxa are highly susceptible to small transient increases in nutrient availability because other taxa with higher nutrient demands temporarily increase their growth rates while more P or N is available, and (2) in high nutrient streams, variability of low nutrient taxa is much lower likely due to nutrients already being so high that other taxa that thrive under higher nutrient concentrations dominate assemblages. Temporal variability in relative
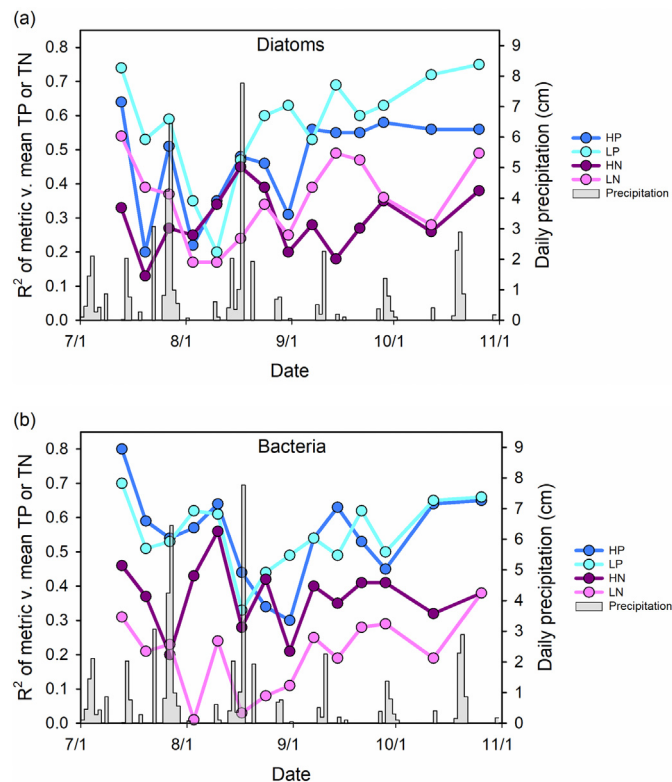

Fig. 5. Weekly R^2 values from diatom (a) and bacteria (b) regressions between metrics and site means of TP and TN concentrations plotted versus dates with daily precipitation overlaid. HP = high phosphorus taxa, LP = low phosphorus taxa, HN = high nitrogen taxa, LN = low nitrogen taxa.

abundances of high P and high N taxa tended to be much less than that for low P and low N taxa, though their temporal variability did increase with greater TP or TN concentrations, with high N diatom variability increasing the most. This may suggest that above a certain threshold, TP or TN concentrations are great enough to consistently sustain the populations and temporal dominance of high P and high N taxa regardless of fluctuations in concentrations over time, though changes in relative abundances of non-indicator taxa could contribute to their

Table 3
Spearman correlations of diatom and bacterial metrics and nonmetric multidimensional scaling (NMDS) axis scores with nutrient concentrations on the same day of sampling, one week prior, the mean of same day measurements and those collected one and two weeks prior, and mean concentrations at each site (strongest TP and TN correlation for each response is bolded). NMDS axis scores and metrics were almost always most strongly correlated with study mean TP and TN concentrations. For all correlations, $p < 0.05$.

	Same week		1-week lag		2-week mean		Study mean	
	TP	TN	TP	TN	TP	TN	TP	TN
Diatoms								
NMDS 1	−0.73	−0.51	−0.69	−0.41	−0.71	−0.45	**−0.78**	**−0.69**
NMDS 2	−0.20	−0.22	−0.28	−0.34	**−0.32**	**−0.37**	−0.29	−0.30
High P	0.62	0.46	0.59	0.34	0.60	0.39	**0.67**	**0.60**
Low P	−0.70	−0.47	−0.67	−0.39	−0.70	−0.44	**−0.76**	**−0.67**
High N	0.51	0.51	0.48	0.40	0.48	0.44	**0.53**	**0.53**
Low N	−0.61	**−0.62**	**−0.67**	−0.56	−0.64	−0.59	−0.66	−0.60
Bacteria								
NMDS 1	0.49	0.31	0.48	0.25	**0.55**	0.31	0.51	**0.44**
NMDS 2	−0.52	−0.24	−0.48	−0.18	−0.45	−0.15	**−0.56**	**−0.49**
High P	0.72	0.61	0.66	0.52	0.71	0.58	**0.75**	**0.69**
Low P	−0.70	−0.58	−0.65	−0.46	−0.68	−0.52	**−0.72**	**−0.63**
High N	0.72	0.63	0.68	0.61	0.73	0.67	**0.78**	**0.71**
Low N	−0.48	**−0.64**	**−0.51**	−0.59	−0.49	**−0.64**	−0.48	−0.42

variability. These offsets in temporal variability between low nutrient taxa and high nutrient taxa along an increasing TP or TN gradient likely contributed to the mostly consistent temporal variability in overall assemblage structure along TP and TN gradients, as inferred from site means of distances to their centroids.

Two large precipitation events were associated with brief increases in TP concentrations, but only the first coincided with an increase in TN concentrations. These different patterns might reflect greater delivery of phosphorus via runoff and erosion during both high precipitation events at a watershed scale (Hart et al., 2004; Alexander et al., 2008; Withers and Jarvie, 2008), whereas the first increase in TN concentrations could be associated with a flush of accumulated nitrogen (Davis et al., 2014; Loecke et al., 2017) or possibly from mid-season applications of fertilizer (Ciampitti and Vyn, 2012; Momesso et al., 2022), though this would be crop dependent and would vary among agricultural practices (Jacinthe and Dick, 1997; Wagner-Riddle et al., 2007); measurements earlier in the season would have been required to confirm this. These nutrient pulses were followed by a longer-term seasonal decrease of TN during baseflow conditions, but relatively stable TP concentrations, which was associated with increases of low N and decreases in high N bacterial relative abundances. These results indicate that bacterial assemblages possibly were becoming more N-limited over time, despite the evidence of consistently stronger diatom and bacterial correlations with TP concentrations. Diatoms did not show a similar response to decreasing TN.

The increases in TP and TN concentrations associated with these large precipitation events corresponded with brief increases in relative abundances of high nutrient taxa (Figs. 2, S4, S5) but they counterintuitively were associated with a break down in correlations (Fig. 5). We interpret this as evidence that increased nutrients stimulated the growth of taxa with high nutrient demands but the relationship between metrics and nutrient concentrations possibly became decoupled because (1) effects of increased nutrient availability in low nutrient streams likely differ from those in higher nutrient streams with assemblages already being dominated by higher nutrient taxa (Lavoie et al., 2008), (2) temporary increases in nutrient availability possibly saturate maximal growth rates at some point (Hill and Fanta, 2008; Shatwell et al., 2014; Schmidt et al., 2019), beyond which the relationship would become decoupled because higher nutrient concentrations would no longer be associated with a corresponding increase in growth rates, and (3) differences among taxa in their abilities for luxury uptake of nutrients, including storage as polyphosphates, for later growth possibly adds further complexity (Seviour et al., 2003; Rier et al., 2016).

5. Conclusions

Although high frequency sampling of nutrients and biota likely would be impractical for routine monitoring programs, our results provide useful information that can strengthen inferences based on diatom and bacterial metrics and inform monitoring strategies. Diatom and bacterial indicators developed using DNA metabarcoding in this watershed integrated seasonal nutrient concentrations and performed consistently well throughout a summer monitoring period in a temperate climate despite temporal variability and a brief breakdown in correlations following disturbance. Additional studies of temporal variability in other regions would further contribute to better understanding how differences in nutrient concentration gradients, climate, and anthropogenic sources of nutrients affect stream conditions and how diatom and bacterial assemblages respond to nutrient conditions over time. Given that temporal variability inherently affects all trophic levels and biomonitoring programs, from an assessment perspective, diatoms and bacteria provide more reliable estimates of site conditions than infrequent nutrient measurements because they better integrate nutrient effects over longer durations of time, and because they provide the ecological context for understanding nutrient effects and for supporting possible management targets.

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