Quantifying stream periphyton assemblage responses to nutrient amendments with a molecular approach

James D. Hagy III[1,2], Katelyn A. Houghton[1,3], David L. Beddick Jr[1,4], Joseph B. James[1,5], Stephanie D. Friedman[1,6], and Richard Devereux[1,7]

[1]United States Environmental Protection Agency, Center for Environmental Measurement and Modeling, Gulf Environmental Measurement and Modeling Division, 1 Sabine Island Drive, Gulf Breeze, Florida 32561 USA

Abstract: Nutrient (nitrogen [N] and phosphorus [P]) pollution is a pervasive water quality issue in the USA for small streams and rivers. The effect of nutrients on the biotic condition of streams is often evaluated with biological indicators such as macroinvertebrate assemblages or periphyton assemblages, particularly diatoms. Molecular approaches facilitate the use of periphyton assemblages as bioindicators because periphyton is diverse and its composition as a whole, rather than just diatoms, soft-bodied algae, or any single group, may convey additional information about responses to nutrients. To further develop the concept that a taxonomically-broad evaluation of periphyton assemblages could be useful for developing stream bioindicators, we examined microbial assemblage composition with both 16S and 18S rRNA genes, enabling us to evaluate composition in 3 domains. We measured otherwise unknown nutrient responses of different periphyton groups in situ with experiments that used glass fiber filters to allow diffusion of amended nutrients into a stream. We deployed these experimental setups in 2 streams that differ in the extent of agricultural land-use in their catchments in the southeastern USA. Experiments consisted of controls, N amendments, P amendments, and both N and P amendments. Periphyton assemblages that grew on the filters differed significantly by stream, date or season, and nutrient treatment. Assemblage differences across treatments were more consistent among Bacteria and Archaea than among eukaryotes. Effects of nutrient amendments were more pronounced in the stream with less agricultural land use and, therefore, lower nutrient loading than in the stream with more agricultural land use and higher nutrient loading. Combined N and P amendments decreased species richness and evenness for Bacteria and Archaea by ~36 and ~9%, respectively, compared with controls. Indicator species analysis revealed that specific clades varied in their response to treatments. Indicators based on the responses of these indicator clades were related to nutrient treatments across sites and seasons. Analyses that included all the taxa in a domain did not resolve differences in responses to N vs P. Instead, better resolution was achieved with an analysis focused on diatoms, which responded more strongly to P than N. Overall, our results showed that in situ nutrient-diffusing substrate experiments are a useful approach for describing assemblage responses to nutrients in streams. This type of molecular approach may be useful to environmental agencies and stakeholders responsible for assessing and managing stream water quality and biotic condition.

Key words: streams, indicators, nutrient-diffusing substrates, DNA, microbial assemblage, indicator species analysis

Nutrient (nitrogen [N] and phosphorus [P]) pollution is a worldwide major cause of water quality degradation. Consequently, management actions focus on reducing nutrient loadings to improve biotic condition and protect against anticipated future degradation (Vitousek et al. 1997, WHO 2005, Davidson et al. 2012). Nutrients can enter waterways from a variety of sources. Inputs to small streams are often dominated by non-point sources associated with shallow groundwater transport and local surface water run-off. Agricultural land uses, including row crops and pasture, are significant sources of nutrients in the southeastern coastal plain of the USA and in other regions (Hoos and McMahon 2009, García et al. 2011).

Effective management of nutrient pollution requires nutrient exposure and effects to be adequately measured.

E-mail addresses: [2]hagy.jim@epa.gov; [3]Present address: Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, Georgia 30329 USA, houghton.katelyn@gmail.com; [4]beddick.david@epa.gov; [5]james.joe@epa.gov; [6]friedman.stephanie@epa.gov; [7]devereux.richard@epa.gov

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Nutrient concentrations are the most obvious indicator, but they can also be highly variable, so biotic indicators (bioindicators) have been developed and used widely for stream condition assessment (Goodnight 1973, Stevenson 2014). Stream bioindicators were first developed prior to the mid-1970s (e.g., Goodnight 1973, Karr 1981) and have been improved continuously through research and use in management programs (Barbour et al. 1996, Stevenson 2014). Both stream macroinvertebrate and periphyton algal assemblage composition have been widely used as stream bioindicators (e.g., DEP 2002, Stevenson 2014). Diatoms are a particularly useful part of periphyton assemblages because they are diverse, ubiquitous, sensitive to nutrients, and relatively easy to preserve and identify (Stevenson et al. 2010, Smucker et al. 2013). Diatoms and other algal indicators have been used worldwide (e.g., Gómez and Licursi 2001) and are a component of bioassessment protocols in at least 23 USA states (Paul et al. 2017), the European Union (Stevenson et al. 2010), and Australia (Chessman et al. 2007).

The rapid development of molecular phylogenetic techniques has the potential to augment existing bioassessment methods, which are successful and have been widely adopted. For example, molecular methods that involve sequencing of targeted regions of specific genes and referencing those sequences to online genetic databases provide a new means of identifying aquatic species. These methods can be used to characterize the composition of macroinvertebrate and algal taxa that have historically been identified only microscopically as well as bacterial and archaeal taxa that previously could not be identified at all. For example, molecular approaches have been used to quantify soil microbiomes and their responses to nutrients (Fierer et al. 2007, Leff et al. 2015, Astudillo-Garcia et al. 2019, Babin et al. 2019) as well as relationships between microbial assemblage structure and nutrient processing in aquatic ecosystems (Lisa et al. 2015). Thus, microbial assemblages may be sensitive and specific indicators of aquatic ecosystem responses to nutrient enrichment. In this context, specificity refers to an indicator changing substantially in response to differences in nutrient enrichment while changing less or not at all in relation to other stressors, which makes the indicator useful for diagnosis of nutrient effects. Further proof of concept comes from the application of molecular methods to a bacterial assemblage index that relates to a macroinvertebrate assemblage index across several hundred sites in New Zealand (Lau et al. 2015) as well as validation of a molecular approach to river biomonitoring with benthic diatoms (Kermarrec et al. 2014, Rivera et al. 2020).

One way that molecular-based bioindicators could augment existing bioassessment methods is by increasing the number of taxa identified. For example, if each taxon responds differently to a vector of possible stressors, abundances for many taxa could be combined in an index that responds most specifically to a stressor of interest, which in this study is nutrients. The high functional diversity of the Bacteria and Archaea may make it possible to develop indicators that have nutrient-specific responses or that diagnose exposure to other stressors. One application of such indicators could be to diagnose nutrient-related biological changes and subsequently suggest appropriate management thresholds for nutrient concentrations or corroborate evidence of nutrient impairment based on nutrient concentrations. Further, molecular indicators could reduce bioassessment costs associated with the time and expense required to comprehensively identify species from all domains. Stein et al. (2014) concluded that next-generation sequencing could be a cost-effective alternative to traditional methods, even before sequencing costs subsequently decreased nearly 10× (Wetterstrand 2013). Existing indicators can potentially include information from several types of biota, such as diatoms, non-diatom algae, cyanobacteria, and macroinvertebrates. Currently, monitoring programs that sample multiple types of biota are limited to fewer sites or dates than would be ideal because of the high cost of sample analysis (Fetscher et al. 2013). Because molecular approaches can sample across multiple types of biota, more sites and dates could be sampled, providing a benefit to environmental assessments.

Some studies have compared data collected using microcopy with data from molecular methods to further develop water quality bioindicators based on a molecular approach (Kermarrec et al. 2014, Lau et al. 2015, Rivera et al. 2020). We lacked data for traditional nutrient-related bioindicators at our study sites and expected to identify many taxa that could not be identified via microscopy. Further, we lacked information on nutrient sensitivity for many taxa. To quantify how each of the taxa identified in the molecular data respond to nutrient amendments, we used an experimental approach involving nutrient-diffusing substrates (NDS). NDS release nutrients into the water over time through a porous substrate upon which algae and microbes can grow (Capps et al. 2011). NDS offer simplicity and replicability relative to whole-stream nutrient enrichment experiments (Bumpers et al. 2017) and may accurately quantify periphyton responses to nutrients, even if they are insufficient for assemblages with more complex habitats such as insects or benthos. Laboratory incubations offer considerable experimental control (Latham et al. 2011) but require extensive and expensive apparatus to replicate natural growing conditions. In contrast, NDS offer in-situ exposure to natural populations, a natural pattern of external drivers (e.g., irradiance, temperature), and the potential for fewer artifacts associated with laboratory isolation. A variety of NDS can be used, including clay pots, plastic cups, and periphytometers (Capps et al. 2011), the latter of which have a variety of designs (e.g., Matlock et al. 1998). NDS have been used to examine N vs P limitation as well as the effect of other ions that may be limiting in freshwater environments (e.g., calcium; Lowe et al. 1986).

The key objective of this study was to use NDS to classify taxa or groups of taxa with respect to how they are affected by nutrients so that this information can be used to define a

water quality bioindicator. We hypothesized that 1) nutrient amendments cause characteristic, reproducible, and quantifiable shifts in the periphyton assemblage that grows on NDS and 2) the large number and diverse taxonomic groups that can be resolved with a molecular approach is useful for developing nutrient indicators. We conducted NDS experiments in 2 southeastern coastal plain streams and characterized periphyton microbial responses with 16S and 18S rRNA gene sequences. Our study demonstrates a method for implementing NDS experiments, illustrates data analysis methods that can be used with the resulting molecular data, and provides information on responses of various taxonomic groups to nutrient amendments in southeastern USA streams. This information could contribute to development of bioindicators of nutrient effects in streams based on a molecular approach.

METHODS

Study site and NDS experiments

We conducted NDS experiments at 2 stream sites, Fish River and Corn Branch, both of which are in the Fish River watershed on the southeastern coastal plain of southern Alabama, USA (Figs 1, (S1). These sites were selected to establish a contrast in land-use and expected water quality, although the study design did not depend on this contrast. The Fish River site (lat 30°39′12″N, long 87°47′32″W) has a catchment size of 27 km^2. This catchment has 11% agricultural land use, 87% natural land use, and 2% other uses. In contrast, the heavily agricultural Corn Branch site (lat 30°37′7″N, long 87°47′ 5″W) drains a smaller, 5.7-km^2 catchment that has 71% of its land use devoted to agriculture, 25% to natural land use, and 4% to other uses. The sites are 8 km apart and likely experience similar precipitation and other climatological drivers. The regional climate is sub-tropical with average rainfall of 174 cm/y and average air temperatures between 10°C in winter and 28°C in summer (1981–2010; http://prism.oregonstate.edu). The Corn Branch site is wadeable and heavily shaded by vegetation, whereas the Fish River site is 1 to 2 m deep and partially shaded by a highway overpass and some vegetation (Fig. S1). Sediments at both sites are silty.

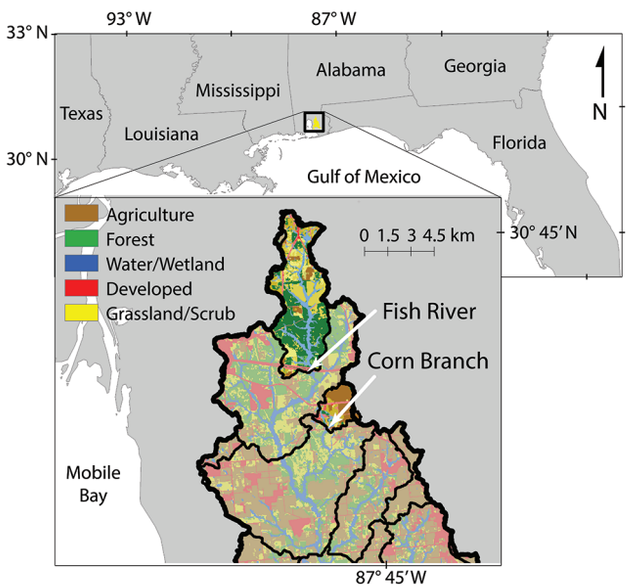

Figure 1. Station locations and associated land use within the catchments. Land use data is from the 2011 national land cover database (Homer et al. 2015).

We deployed the NDS experiments at each stream site for 14 d 6× between July 2015 and July 2016 and identified each experiment with date of recovery from the stream. The NDS experimental design included triplicates of each of 4 treatments: control, phosphate addition (+P), nitrate addition (+N), and addition of both nitrate and phosphate (+NP; Fig. 2A–C). The NDS were deployed on floating frames tethered to a weight so that substrates remained just below the water surface, exposing the filter to the stream water and providing a surface for periphyton growth as nutrients diffused through the filter (Figs S1–S3). We deployed a separate set of periphyton collectors with a larger surface area (Fig. S2) near the water surface at each site to obtain periphyton bulk characteristics. Periphyton was scraped from 2 plates onto a pre-combusted 47-mm GF/F glass microfiber filter (Whatman, Maidstone, United Kingdom) and processed for total and volatilizable solids following US EPA (2001). Periphyton chlorophyll-*a* was analyzed fluorometrically (Welschmeyer 1994) after methanol extraction (Jeffrey et al. 1997).

We prepared the NDS by mixing 2% noble agar with equal volumes of sterile 0.5 M potassium nitrate (+N), 0.5 M sodium phosphate (+P), or a solution containing 0.5 M potassium nitrate and 0.5 M sodium phosphate (+NP), to obtain final N and P concentrations of 0.25 M in 1% agar. The control substrate was 2% agar prepared with an equal volume of Milli-Q® water. Nutrient solutions and agar were autoclaved separately, cooled to between 45 and 50°C in a water bath to avoid formation of potentially toxic substrates (Tanaka et al. 2014) then mixed together. The still-molten solutions were poured into 130-mL capacity snap-seal plastic sample containers (model 1730-10, 100 mL; Corning®, Corning, New York) and allowed to harden, leaving 10 mL unfilled to accommodate a 2% agar cap on top of the hardened nutrient substrates. The agar cap prevented excessive nutrient diffusion at the beginning of the deployment. Diffusion rates from the NDS were evaluated prior to the study with NaCl as a tracer and measuring the change in conductivity in a headspace (see Appendix S1). A sterile 47-mm glass fiber filter was placed onto the agar surface and held in place with the cap, from which a 38-mm diameter opening was cut. The assembled NDS were stored in a sterile bag overnight at 4°C and deployed the following day. Upon recovery, filters were carefully removed from the agar surface and placed into sterile petri dishes that were then wrapped in

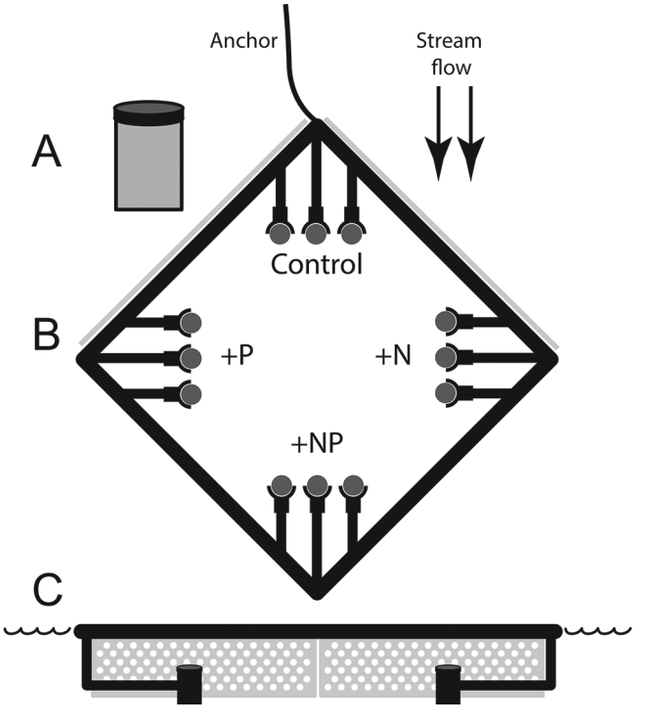

Figure 2. The nutrient diffusing substrate platform, including (A) agar-filled cups topped with a glass-fiber filter, (B) the floating frame holding the 12 NDS units with the control oriented upstream as indicated, and (C) the view looking upstream, showing the screen that deflects large debris to prevent it from physically impacting the periphyton.

aluminum foil, placed inside a zipper-seal bag, and transported on ice before freezing at –70°C until analysis.

Water quality measurements

We evaluated ambient water quality monthly at both sites in the context of a larger study of water quality at 30 other stations in the region (Hagy et al. 2018). We deployed a Satlantic (Sea-Bird Scientific, Bellevue, Washington) SUNA V2 ultraviolet sensor at each site during the NDS experiments to measure nitrate every 30 min. We also deployed water quality sondes (Model 6600 V2; Yellow Springs Instruments®, Yellow Springs, Ohio) to measure water temperature and conductivity every 30 min.

We collected water samples from the middle of the streams with a Niskin sampler and transferred them to dark, high-density polyethylene bottles, then transported the samples to the lab in an iced cooler and processed them within 4 to 6 h. Water samples were processed for particulate carbon, particulate nitrogen, dissolved organic carbon, total dissolved nitrogen, total dissolved phosphorus, particulate phosphorus, and dissolved inorganic nutrients (NH_4^+, NO_x, PO_4^{3-}). To measure particulate carbon, particulate phosphorus, and particulate nitrogen, we filtered sample water onto a pre-combusted (450°C, 3 h) GF/F filter until the filter nearly clogged and recorded the final filtered volume. We then analyzed the filters for particulate carbon and particulate nitrogen on a FlashEA elemental analyzer (CE Elantech, Lakewood, New Jersey). Particulate phosphorus filters were analyzed with the ash-hydrolysis method (Solórzano and Sharp 1980). We measured dissolved nutrients by dispensing the GF/F filtrate into vials and freezing them at –70°C until analysis. Dissolved organic carbon and total dissolved nitrogen were analyzed on a TOC-VCSN carbon analyzer (Shimadzu Scientific Instruments, Columbia, Maryland) with a nitrogen module that utilized a 720°C combustion catalytic oxidation coupled with nondispersive infrared carbon and chemiluminescent nitrogen detectors. Total dissolved phosphorus was quantified as PO_4^{3-} on a segmented flow auto-analyzer (Astoria-Pacific, Clackamas, Oregon) after persulfate oxidation. NH_4^+ concentrations were estimated fluorometrically following Holmes et al. (1999). NO_x and PO_4^{3-} concentrations were estimated with an Aquakem™ 200 discrete analyzer (Thermo Fisher Scientific™, Waltham, Massachusetts). NO_x and PO_4^{3-} samples <5× the method detection limit were re-analyzed with the segmented flow auto-analyzer, which is more sensitive. The NO_x and PO_4^{3-} analyses with both instruments used standard colorimetric methods (APHA 1989). Reduction of NO_3^- to NO_2^- prior to analysis was accomplished with an enzymatic reduction method (Patton et al. 2002) on the Aquakem 200 or cadmium reduction on the Astoria instrument.

DNA extraction and sequencing

We extracted DNA from the NDS filters with a PowerMag® Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California) with a KingFisher™ Duo (Thermo Fisher Scientific). We obtained partial 16S rRNA gene sequences (2 × 250 bp paired end) from a MiSeq sequencer (illumina®, San Diego, California) from the Argonne National Laboratory Environmental Sample Preparation and Sequencing Facility (http://ngs.igsb.anl.gov/) with the forward primer 515fB and the reverse primer 806rB targeting the V4 region of the 16S rRNA gene in Bacteria and Archaea (Caporaso et al. 2012, Apprill et al. 2015). We obtained partial 18S rRNA gene sequences (2 × 151 bp paired end) of eukaryotes in the same manner, with forward primer 1391f and reverse primer 1510R as modified by the Argonne National Laboratory (Amaral-Zettler et al. 2009). We determined with mothur that the sequence error rate was 0.01% based on a DNA cocktail of 20 known bacterial strains as the standard (Microbial Mock Community B, HM-783D; BEI Resources, Manassas, Virginia). All DNA sequences were deposited in the GenBank database and are accessible with the sequence read archive accession number PRJNA498721.

Processing DNA sequences

DNA sequences were processed with mothur software version 1.39.5 (Schloss et al. 2009) following the mothur MiSeq standard operating procedure (Kozich et al. 2013).

Sequences were assigned to operational taxonomic units (OTUs) based on 97% nucleotide sequence identity. 16S rRNA gene sequence contigs assembled from forward and reverse sequences had an average length of 254 bp. 18S rRNA gene sequence contigs assembled from paired end sequences were ~125 nucleotides in length. We obtained a total of ~2 million 16S rRNA gene sequences that averaged 16,000 sequences/library (a library is a group of sequences obtained from a single environmental sample) and a total of ~4 million 18S rRNA gene sequences that averaged 30,000 sequences/library (Table S1). The 16S rRNA gene sequences clustered into 322,792 OTUs, whereas the 18S rRNA gene sequences clustered into 50,463 OTUs. We subsampled sample libraries to 10,000 sequences and excluded those with fewer than 10,000 sequences from further analysis.

We used the SILVA non-redundant sequence database release 132 as the template for aligning and identifying both 16S and 18S rRNA gene sequences (Quast et al. 2013). SILVA classified Bacteria and Archaea where possible to domain, phylum, class, order, family, and genus. Eukaryote OTUs were classified differently because of variation in SILVA nomenclature between Eukarya lineages. Instead, SILVA assembled the Eukarya into levels representing clades at varying levels of taxonomic organization. Level 1 reflects all Eukarya, whereas the type of taxonomic group varied within levels 2 and higher. We labeled the sequence with the most reads associated with each OTU as the representative sequence. Representative sequences for 6 abundant OTUs that were classified as diatoms were submitted to BLAST (Zhang et al. 2004, Morgulis et al. 2008) to obtain additional information regarding possible identifications.

Periphyton assemblage analysis

We quantified species richness as the number of OTUs present in our rarefied samples (i.e., 10,000 sequences) and evenness via Pielou's evenness with the *vegan* package (Oksanen et al. 2017) in R (version 3.5.0; R Package for Statistical Computing, Vienna, Austria). Site differences among control treatments (no amendments) were quantified with linear mixed models that included sample date as a random effect. Differences resulting from nutrient treatments were evaluated with linear mixed models that quantified the effect of nutrient amendments with a block effect for sample location within sample date.

We used matrices of the relative abundance of sequences from each OTU to analyze assemblage composition and used non-metric multidimensional scaling to visualize differences among sites, dates, and nutrient treatments. We $\log(x+1)$-transformed taxon abundance prior to calculation of Bray–Curtis dissimilarities to increase the influence of differences in abundances of relatively less abundant OTUs and decrease the effect of differences associated with most abundant OTUs, which might otherwise dominate the analysis. Two-dimensional ordinations (i.e., $k = 2$) with random starts were implemented with the metaMDS function in the *vegan* package. Ordinations were computed separately for Bacteria and Archaea vs eukaryotes, but a single ordination was computed with data from all dates and sites. We then used a permutational multivariate analysis of variance (PERMANOVA), implemented with the adonis2 function in the *vegan* package, to quantify nutrient treatment differences separately by site, stratified by date. We used PERMANOVA with the *pairwiseAdonis* package in R (version 0.0.1; Arbizu 2017) to examine pairwise differences between nutrient treatments within sites, applying a Bonferroni correction to obtain p-values adjusted for multiple comparisons.

Indicator species analysis

We aggregated OTU abundances in each domain into clades following the hierarchical consensus taxonomy generated for each OTU. Bacteria and Archaea were aggregated at the class level, whereas SILVA's level 3 classification was used for eukaryotes. We also aggregated the subset of eukaryote OTUs that were classified as diatoms at SILVA's level 6, which is the genus level for diatoms. Associations between the relative abundance of clades and nutrient treatments or groups of nutrient treatments were evaluated with indicator species analysis (De Cáceres and Legendre 2009), which was implemented with the multipatt function (with func="r.g") from the *indicspecies* package in R (De Cáceres and Legendre 2009) applied to $\log_{10}(x+1)$-transformed abundances. Indicator species analysis was applied separately to data for Bacteria and Archaea, all eukaryotes, and the subset of eukaryotes identified as diatoms.

To isolate effects of nutrient amendments from environmental variability, we first classified clades with indicator species analysis according to their associations with nutrient amended (+N, +P, or +NP) treatments, controls or groups of either (De Cáceres and Legendre 2009). Classifications were made within individual experiments and subsequently aggregated. We defined a clade as a nutrient responder if it was consistently associated with any nutrient treatment or group of treatments, including controls. We defined a clade as a nutrient-nonresponder if it was not associated with any nutrient treatment, group of nutrient treatments, or control. Nutrient-responders were further classified based on whether they were associated with nutrient-amended treatments or with controls. We defined nutrient-increasers as clades that were associated with nutrient-amended treatments, which also means that their relative abundance was higher in nutrient-amended treatments than controls. We defined nutrient-decreasers as those that were consistently associated with controls, which also means that their relative abundance in nutrient-amended treatments was lower than controls. When referencing responses to a specific nutrient, we replace 'nutrient' with the specific nutrient, as in 'N-increaser' or 'P-decreaser'. Accordingly, we define an N-increaser as a clade that was consistently associated with an N-amended treatment or group of treatments that includes N amendment but not controls (there are 6 groups: +N;

+NP; +N and +NP; +P and +NP; +N and +P; +N, +P, and +NP). Similarly, an N-decreaser is a clade that was consistently associated with controls only or with a group including controls and a treatment lacking N-amendment (there are 2 groups: control, control and +P). P-increaser and P-decreaser were defined analogously. Classifications were included if $p < 0.05$ for the indicator value.

Analyses of each NDS experiment created lists of indicator clades for each of 11 completed experiments. We combined the lists from all the experiments to produce a single master list as follows. If a clade was identified as a nutrient-responder in any experiment, it was included on a global list of responsive clades. Then, if that clade was identified as a nutrient-decreaser in any experiment but a nutrient-increaser in another experiment, we removed it from the global list. However, we retained clades identified as nutrient-responders in 1 or more experiments but nutrient-nonresponders in 1 or more others.

Calculation of a nutrient response indicator

We calculated a nutrient response indicator for each library by summing the products of the log(x+1)-transformed relative abundances and corresponding trait values, which reflects the nutrient response inferred via indicator species analysis. We adapted this calculation from Stevenson's (2014) taxonomic metrics of stressors. In our case, the matrix of traits, θ_{ij} has a value for each nutrient j (i.e., N or P) for each clade i. Values are −1 for nutrient-decreasers, +1 for nutrient-increasers, and 0 for nutrient-nonresponders. The indicator for nutrient j is therefore M_j, which can be expressed as

$$M_j = \sum p_i \theta_{ij} \quad \text{(Eq. 1)},$$

where p_i is the log(x+1)-transformed abundance of sequences assigned to each clade.

We developed the response or trait vector from our experiments and applied them back to the same samples, so we used a jackknife cross-validation approach wherein the matrix θ_{ij}, which is used to calculate M_j for the k^{th} library, was derived with library k excluded from the analysis. To facilitate explanation of the results, we centered the indicator by subtracting the average value of M_j obtained for all controls. This centering facilitates interpretation of positive vs negative values of the indicator but does not change differences between values. Specifically, positive values indicate an assemblage with more nutrient-increasers than controls, less nutrient-decreasers than controls, or some combination of both. The opposite is true for negative values.

We classified eukaryote clades that were P-responders into 3 functional groups, autotrophs, saprotrophs, and heterotrophs, to analyze how P-responses related to functional role. Classifications were based on descriptions of taxa from Maddison et al. (2007) and other widely available information, wherein algal taxonomic groups were classified as autotrophs, fungi were classified as saprotrophs, and other groups were classified as heterotrophs (Table S2E, F).

RESULTS

Site characteristics

Average specific conductivity, total nitrogen, and ammonium were 228, 84, and 1000% higher, respectively, at Corn Branch than at Fish River (Table 1), reflecting the much

Table 1. Mean ± standard deviation of key water quality variables and periphyton bulk properties at the study sites and relative % difference of Corn Branch compared to Fish River. *P*-values and numerator and denominator degrees of freedom are for tests for mean concentration differences by station and were based on linear mixed models on log concentration with quarterly sampling dates as a block effect. A single estimate of periphyton C:Chl-*a* was calculated from means of both parameters. AFDW = ash-free dry weight, df = degrees of freedom.

Parameter (units)	Fish River	Corn Branch	% difference in mean	P(H_0: Fish River = Corn Branch)
Water Quality				
Conductivity (mS/cm)	29 ± 4	95 ± 13	228	$p < 0.001$, df = 1,11
Total nitrogen (mg N/L)	0.44 ± 0.28	0.81 ± 0.50	84	$p = 0.023$, df = 1,9
Nitrate (mg N/L)	0.095 ± 0.051	0.20 ± 0.28	111	$p = 0.97$, df = 1,12
Ammonium (μg N/L)	10 ± 6.5	110 ± 130	1000	$p < 0.001$, df = 1,10
Total phosphorus (μg P/L)	114.3 ± 178.7	121.5 ± 88.9	6	$p = 0.136$, df = 1,10
Orthophosphate (μg P/L)	32.2 ± 52.8	12.4 ± 6.08	−61	$p = 0.664$, df = 1,12
Total organic carbon (mg C/L)	6.4 ± 2.8	7.0 ± 2.5	9	$p = 0.476$, df = 1,9
Dissolved organic carbon (mg C/L)	6.1 ± 2.7	4.9 ± 1.7	−20	$p = 0.302$, df = 1,10
Periphyton				
AFDW accumulation rate (g m^{-2} d^{-1})	37 ± 36	58 ± 28	57	$p = 0.274$, df = 1,3
Periphyton organic matter (%)	38 ± 14	20 ± 4	−47	$p = 0.073$, df = 1,3
Periphyton C:Chl-*a*	211	110	−48	–

higher percentage of agriculture (71%) in the Corn Branch catchment than in the Fish River catchment (11%). In contrast, the other water quality variables were similar (Table 1). Continuous nitrate measurements showed that nitrate at both sites increased when rain events occurred and then declined following those events (Fig. S4). Most of our laboratory-analyzed nitrate measurements were collected during low flow, so the average exposure to nitrate was sometimes much higher than our grab samples indicate.

Periphyton assemblages

We obtained periphyton assemblage composition data from 6 NDS experiments at Fish River and 5 NDS experiments at Corn Branch because the experiment that ended on 10 July 2015 was lost during a high-flow event. Altogether, we obtained 65 16S and 67 18S rRNA gene libraries from the Fish River site and 51 16S and 53 18S rRNA gene libraries from Corn Branch. The sequences were classified into 45,915 OTUs of Bacteria, 203 OTUs of Archaea, and 47,164 OTUs of Eukarya (Table S1). Phyla that accounted for >1% of bacterial abundance include Proteobacteria (64%), Bacteriodetes (19%), Verrucomicrobia (4%), Actinobacteria (3%), Acidobacteria (2%), and Cyanobacteria (2%). A median of 1% of the 16S rRNA gene sequences in each library were identified only to domain (i.e., as Archaea or Bacteria). The level 2 clades to which the greatest number of sequences were classified include Chlorophyta (30%), Ochrophyta (18%), Euglenozoa (9%), Ciliophora (5%), Ascomycota (5%), and Heterolobosea (4%). An additional 18% of eukaryotes were classified only to the domain level (i.e., Eukarya).

SILVA identified 514 OTUs as diatoms from 18 genera, of which 14 were pennate diatoms (Bacillariophyceae). The most abundant clade classified to genus (*Gomphonema*) accounted for 6% of the diatom sequences. Other abundant genera included *Eunotia*, *Navicula*, *Pinnularia*, and *Surirella*. Nearly all the diatom sequences (83%) were identified by SILVA only as diatoms (17%) or pennate diatoms (66%). Of these, 3 abundant diatom OTUs (12, 41, 220) accounted for 75% of the unclassified diatoms (45, 11, and 2% of the diatom sequences overall, respectively). BLAST analysis suggested that all 3 were likely members of either the genus *Pinnularia* or *Eunotia*, but they could not be definitively identified to genus. Another abundant but unclassified OTU (OTU 8) accounted for an additional 6% of diatom sequences and was likely in the genus *Nitzschia*, and another (OTU 198) that accounted for 3% of the sequences was probably in either the *Frustulia* or *Sellaphora* genera.

Periphyton assemblage structure, species richness, and evenness

In many of the experiments, assemblage structure in the nutrient treatments differed from that in the control treatments (Fig. 3). The magnitude of shifts in ordination space was often largest for treatments that included P and of intermediate magnitude for treatments that only included N (Fig. 3). In addition, the direction of the shift in ordination space was often similar in each of the 3 domain-specific ordinations for all nutrient-amended treatments and across all dates (Fig. 3). This pattern occurred for both groups of taxa but was more distinct for Bacteria and Archaea than eukaryotes and for Fish River compared with Corn Branch (Fig. 3). Shifts in position in the ordination resulting from nutrient amendments were not always apparent at Corn Branch, and shifts were not always in the same direction as they were for Fish River or even in the same direction for all nutrient treatments. For eukaryotes at Corn Branch, clear differences between the treatments and controls were only apparent in the experiment recovered on 5 April 2016

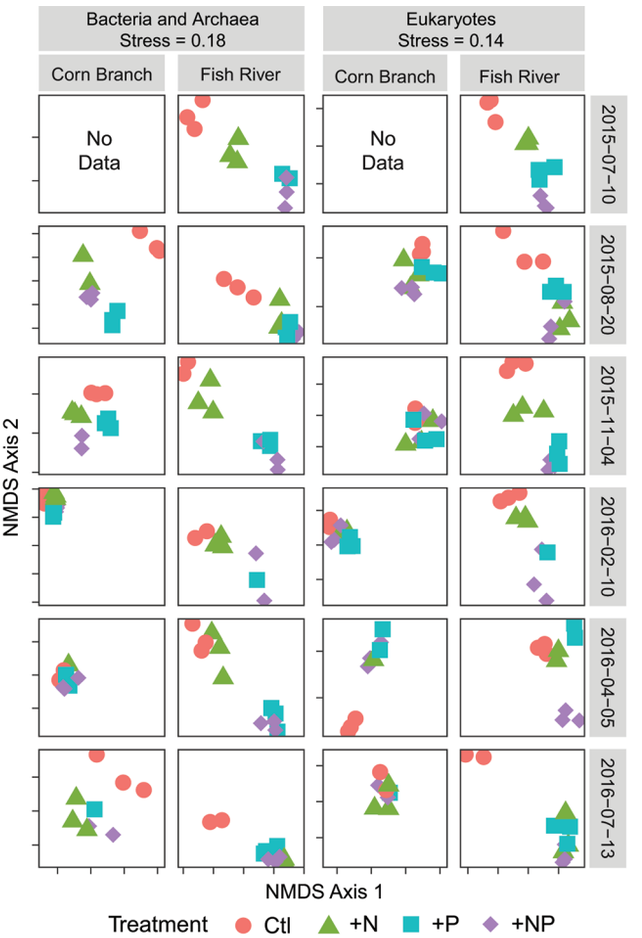

Figure 3. Non-metric multidimensional scaling (NMDS) ordinations illustrating shifts in Bacteria and Archaea and eukaryote community structure (based on 16S and 18S rRNA gene sequences) associated with nutrient enrichment treatments in up to 6 experiments per site at Corn Branch and Fish River. Dates at right indicate the date each experiment ended and was recovered from the stream. Identical symbols in each panel indicate replicate treatments. Ctl = control, +N = amended with nitrogen, +P = amended with phosphorus, +NP = amended with both nitrogen and phosphorus.

(Fig. 3). Overall, however, these ordinations suggested that nutrient amendments affected either the composition, the structure (relative abundances), or both of microbial assemblages.

PERMANOVA tests showed that nutrient treatments explained 14% of the variation in distances among assemblages of Bacteria and Archaea at Fish River ($p < 0.001$) and 12% at Corn Branch ($p < 0.001$). For eukaryotes, nutrient treatments explained 13% of variation in distances among assemblages at Fish River ($p < 0.001$) but only 7% at Corn Branch ($p = 0.094$). For Bacteria and Archaea, pairwise contrasts identified the largest effects, similarly based on R^2, at Fish River for +NP (0.171) and +P (0.158; Table 2A, B). Effects of +N were less, as suggested by the smaller magnitude of effects of +N vs controls, +N vs +P, and +NP vs +P (Table 2A). The effects of +P and +NP vs controls were smaller at Corn Branch than at Fish River, whereas differences by site were less pronounced for other contrasts (Table 2A). +NP had the largest effect at Corn Branch (0.115), like at Fish River, but the effect of +P vs controls was not larger than +N vs controls (~0.8). Differences between +N vs +P indicate that +N and +P effects were of similar magnitude compared to controls but involved different changes (Table 2A, B). Eleven of 12 pairwise contrasts for eukaryotes had smaller effect sizes than the corresponding contrasts for Bacteria and Archaea (Table 2B), meaning that nutrient treatments explained less of the overall variability among eukaryote assemblages. Otherwise, the response patterns were similar. The largest effect on eukaryote assemblages also resulted from +NP (0.147) and +P (0.136) treatments, whereas nutrient treatments with only N-amendment caused smaller effects (Table 2B). Nutrient effects on eukaryote assemblages were mostly smaller at Corn Branch compared with those observed at Fish River (Table 2B).

Species number and Pielou's evenness were positively correlated in both domains (Fig. 4). For Bacteria and Archaea in control treatments, species number averaged 1940 (95% confidence interval [CI] = 1706–2173), and Pielou's evenness averaged 0.78 (95% CI = 0.76–0.80) with no differences between the 2 study sites (Table S3). For eukaryotes, species number was 23% higher at Corn Branch (1629; 95% CI 1322–1936) than at Fish River (1324, 95% CI = 1031–1617) and Pielou's evenness was 8% higher at Corn Branch (0.77, 95% CI = 0.73–0.81) than at Fish River (0.715, 95% CI = 0.68–0.75).

Nutrient amendments reduced species number and evenness compared to controls, with the largest effects associated with +P and +NP treatments (Table S3). Species number for Bacteria and Archaea decreased by an average of 33% (mean ± SE decrease of 639 ± 85) in +P treatments and similarly (36%; 696 ± 82) in +NP treatments. +N treatments caused a smaller 14% decrease in species number

Table 2. Pairwise contrasts showing effect (R^2) of nutrient treatments on periphyton assemblage structure. Contrasts based on permutational multivariate analysis of variance tests by domain and site. $p < 0.001$ for all contrasts. Ctl = control, +N = amended with nitrogen, +P = amended with phosphorus, +NP = amended with both nitrogen and phosphorus.

	Ctl/ +N	Ctl/ +P	Ctl/ +NP	+P/ +N	+NP/ +N	+NP/ +P
A.—Bacteria and Archaea						
Fish River	0.071	0.158	0.171	0.074	0.080	0.044
Corn Branch	0.083	0.080	0.115	0.079	0.059	0.066
B.—Eukaryotes						
Fish River	0.080	0.136	0.147	0.054	0.061	0.037
Corn Branch	0.048	0.060	0.060	0.046	0.040	0.039

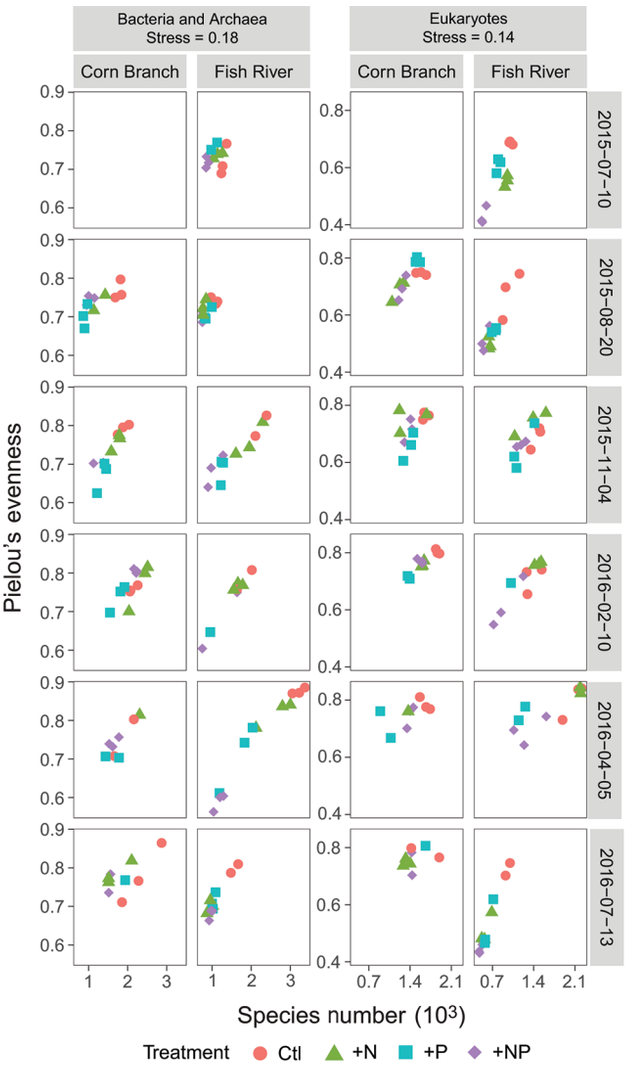

Figure 4. Number of species and Pielou's evenness as affected by nutrient amendments in NDS experiments. Dates at right indicate the date each experiment ended and was recovered from the stream. Identical symbols in each panel indicate replicate treatments.

(Table S3). Pielou's evenness for Bacteria and Archaea decreased by 9% (0.07 ± 0.013) in +P and +NP treatments compared with controls, whereas no clear change in evenness was observed for +N treatments. For eukaryotes, +NP and +P treatments decreased species number by 26 (385 ± 46) and 23% (336 ± 49), respectively, which was less than the decrease for Bacteria and Archaea. Pielou's evenness for eukaryotes decreased by 14% (0.10 ± 0.015) with +NP and 9% (0.065 ± 0.015) with +P. +N treatments decreased Pielou's evenness by 7% (0.051 ± 0.015), about the same as for +P (Table S3).

Indicator species analysis

Pooling the results across all experiments for the Bacteria and Archaea, 84 class-level clades were identified as P-responders, of which 69 clades were P-decreasers and 15 clades were P-increasers (Table S2A, B). No clades had the same response in all 11 NDS experiments, suggesting seasonal and spatial variability in assemblage composition or P-response at the taxonomic class level. The mean value of the P-indicator for +NP treatments was 20.5 more than controls, which is 15× the standard error for controls (Fig. 5A, B, Tables 3A, S4A). The effects of +P and +NP treatments were of similar magnitude, whereas the effect of +N was about half as large (52%) as the effect of +NP. Although variability in responses of the P-indicator was evident among experiments (Fig. 5A, B), systematic interactions between nutrient treatment and site or nutrient treatment and date were not detected.

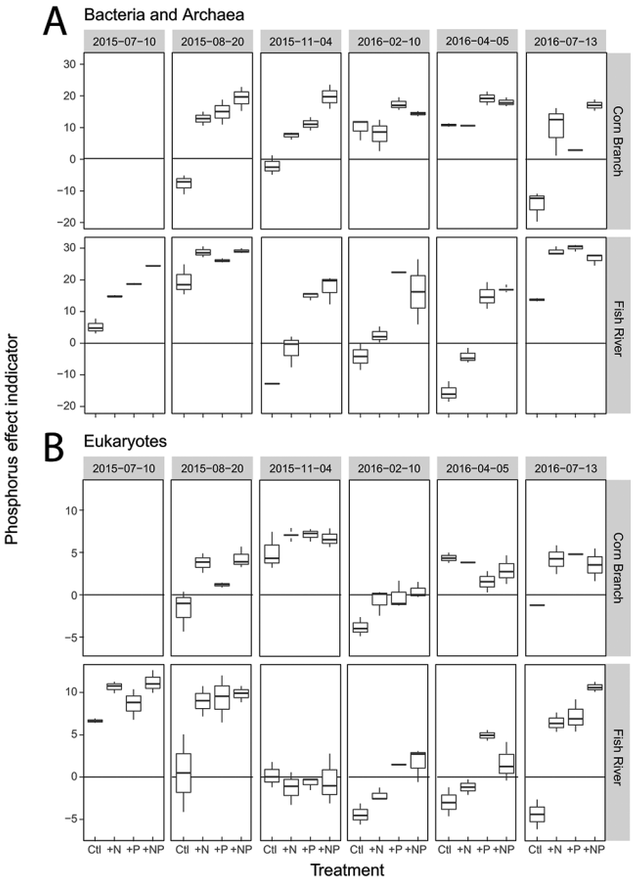

Figure 5. Values of P-indicator for Bacteria and Archaea (A) and eukaryotes (B) based on indicator species analysis.

Among the 15 clades in the 16S rRNA gene data that were identified as P-increasers, the most abundant were 2 classes of Proteobacteria (Betaproteobacteria and Gammaproteobacteria), the class Flavobacteriia, and 2 clades within the phylum Verrucomicrobia, particularly the class Verrucomicrobiae (Table S2A). The P-decreasers included 69 clades of which 8 were from the domain Archaea, despite the very low overall relative abundance of archaeal sequences (Table S2B). None of the Archaea were P-increasers (Table S2A). Several bacterial phyla included multiple classes that were P-decreasers, including the Acidobacteria, Actinobacteria, Bacteroidetes, Planctomycetes, and Verrucomicrobia. The most abundant P-decreaser classes included the Acidimicrobia, Actinobacteria, and Thermoleophilia (phylum Actinobacteria). Other abundant classes included Bacteroidia (phylum Bacteroidetes), Clostridia (phylum Firmicutes), Planctomycetacia (phylum Planctomycetes), Deltaproteobacteria (phylum Proteobacteria), and the OPB35_soil_group (phylum Verrucomicrobia). The groups identified as unidentified Bacteria and unidentified Archaea were both characterized as P-decreasers (Table S2B).

Seventy-four bacterial or archaeal clades were N-responders, and of those, 17 were N-increasers and 56 were N-decreasers (Table S2C, D). The pattern of microbial assemblage response to N-enrichment was similar to that of P-enrichment, such that 1) more clades were decreasers than increasers, 2) the status of clades as N-responders vs N-nonresponders varied among experiments, and 3) there was nonetheless a strong and predictable response of the resulting N-indicator to nutrient amendments (Fig. 6A, B, Table 3A). The mean value of the N-indicator for the +N treatment was +9.2, which is 6.6× the standard error for controls (Table S4D). The effect of +P and +NP treatments on the N-indicator was 30 and 75% larger, respectively, than the effect of +N, implying that the N-indicator was not specific to N (Fig. 6A, B). The N-indicator for Bacteria and Archaea responded less to nutrient amendments than did the P-indicator (Figs 5, 6A). Also, the magnitude of +N and +P effects on the N-indicator overlapped substantially, whereas the same effects for the P-indicator overlapped minimally (+P was larger; Table 3A). Similar response patterns for the N- and P-indicators reflect the fact that the major phyla and classes that were identified as N-responders were largely the same as those that were P-responders (Table S2A–D).

Pooling the results across all experiments for the eukaryotes, 33 level-3 clades were identified as P-responders, of which 21 clades were P-decreasers and 12 clades were P-increasers (Table S2E–H). The eukaryote P-indicator responded the most to the +NP treatment, with a mean response of 4.6 (Table 3B), which is 4× the standard error for controls

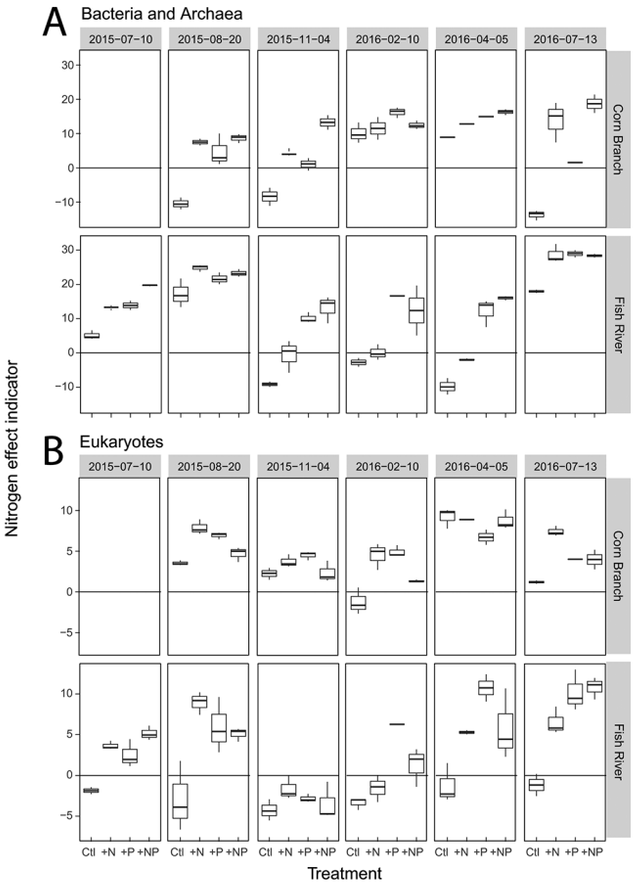

Figure 6. Values of N-indicator for Bacteria and Archaea (A) and eukaryotes (B) based on indicator species analysis.

(Fig. 5B, Table S4B). The eukaryote P-indicator was also higher than controls for +N and +P treatments, but the effect sizes were 20 and 15% less than +NP, respectively, and were apparently equal to each other ($p = 0.988$; Table 3B). The similarity of +N and +P effects contrasted with results for Bacteria and Archaea, where they were different (Tables 3A, (S4B). Relatively abundant eukaryote taxa that were P-increasers include the macroinvertebrate arthropod class Maxillopoda and the class Kinetoplastea, which are flagellated protists, often parasites, in the phylum Euglenozoa (Table S2E). Six fungal clades from 3 phyla (Ascomycota, Basidiomycota, and Cryptomycota) were also among the P-increasers. Perhaps surprisingly, few algal taxa were P-increasers. One algal clade that was a P-increaser is the yellow-green algae Xanthophyceae (Table S2G). In contrast, algal taxa were more common among the P-decreasers, especially the abundant Chrysophyceae (golden-brown algae) and other unclassified members of the phylum Ochrophyta (Table S2F). Abundant non-algae P-decreasers included the ciliate class Intramacronucleata, the parasitic water molds in the order Peronosporales, and the roundworm (Nematoda) class Chromadorea (Table S2F). The clade identified as unidentified eukaryotes was also characterized as a P-decreaser.

Thirty-two level-3 eukaryote clades were identified as N-responders, of which 14 were N-increasers and 18 were N-decreasers (Table S2G, H). The N-indicator derived from the eukaryote data responded to nutrient amendments in a pattern similar to the corresponding P-indicator (Tables 3B, 4E). The response of the N-indicator to the +NP treatment (+4) was 3.6× the standard error for controls (vs 4 for the P-indicator). The effects of +P and +N treatments were not distinguishable from either +NP or from each other (Tables 3B, 4E). Many of the eukaryote clades that were P-responders were also N-responders (Table S3), which may reflect overlap in the treatment associations included in the N- and P-indicators. For example, association with +NP was considered in both N and P responses. One notable difference between N- and P-responses was that the abundant clade Tetramitia (Table S2G), which was dominated by the genus *Naegleria*, was a P-decreaser but an N-increaser.

Nine diatom genera and 1 unidentified genus-level diatom clade were identified as nutrient-responders, with 6 of the 10 clades responding the same way to both N and P (Table 4). Changing the classification of several abundant OTUs with uncertain SILVA identifications to either *Pinnularia* or *Eunotia* based on BLAST results caused the genus to which they were assigned to be reclassified from P-decreaser to P-nonresponder. This result suggests that these abundant OTUs were nutrient-nonresponders, and that adding their abundances to abundances for nutrient-responsive genera overwhelmed and obscured their observed responses to nutrients. The most abundant genera within the nutrient-indicative diatoms were *Gomphonema*, which was a P-decreaser, and *Nitschia*, which was a P-increaser. Neither *Gemphonema* nor *Nitschia* were N-responders (Table 4).

Responses of the diatom indices were more varied by station and sample date than the taxonomically broader indices (Figs S1, 5, 6). Diatom P-indicator values were 1.24× higher for +P treatments, or 2.5× the standard error for controls (Tables 3C, (S4F). The effect of +NP was effectively the same, whereas indicator values for +N treatments were similar to controls (Table 3A–C). The N-indicator for diatoms did not respond to +N treatments in the way we expected, nor were there any clear effects of nutrient treatments on the N-indicator (Table 3C). Differences between the P and N diatom indicators were likely affected most by dominant genera. These differences included *Gomphonema,* which was a P-decreaser but an N-nonresponder, *Nitzschia,* which was a P-increaser but an N-nonresponder, and *Frustulia*, which was an N-increaser but a P-nonresponder.

DISCUSSION

Previously published research has used molecular methods to quantify stream periphyton assemblage composition, which could be used to create stream bioindicators of water quality (e.g., Kermarrec et al. 2014, Lau et al. 2015, Valentin et al. 2019, Rivera et al. 2020). Our successful application

Table 3. Least-squares means and 95% confidence intervals (CI) for values of the P- and N-indicator based on (A) Bacteria and Archaea, (B) all eukaryotes, and (C) diatoms. Values greater than zero indicate a higher than average relative abundance of P- or N-increasers, a lower than average relative abundance of P- or N-decreasers, or both. Additional statistical details are in Table S4. SE = standard error, Ctl = control, +N = amended with nitrogen, +P = amended with phosphorus, +NP = amended with both nitrogen and phosphorus.

	P-indicator		N-indicator	
Treatment	Mean ± SE	95% CI	Mean ± SE	95% CI
A.—Bacteria and Archaea responses				
Ctl	−1.58 ± 1.33	−4.99, 1.84	−1.08 ± 1.40	−4.69, 2.52
+N	9.15 ± 1.34	5.71, 12.6	8.12 ± 1.41	4.49, 11.7
+P	15.9 ± 1.38	12.3, 15.5	11.2 ± 1.44	7.50, 14.9
+NP	18.9 ± 1.33	15.5, 22.3	15.0 ± 1.40	11.4, 18.6
B.—Eukaryote responses				
Ctl	0.07 ± 1.09	−2.74, 2.89	1.04 ± 1.14	−1.89, 3.96
+N	3.72 ± 1.10	0.896, 6.54	5.85 ± 1.14	2.91, 8.78
+P	3.93 ± 1.11	1.08, 6.78	6.18 ± 1.15	3.22, 9.14
+NP	4.60 ± 1.09	1.80, 7.41	5.10 ± 1.14	2.18, 8.02
C.—Diatom responses				
Ctl	0.139 ± 0.464	−1.05, 1.33	0.229 ± 0.230	−0.362, 0.820
+N	0.249 ± 0.465	−0.947, 1.45	−0.013 ± 0.231	−0.607, 0.581
+P	1.380 ± 0.471	0.170, 2.59	0.375 ± 0.237	−0.234, 0.984
+NP	1.313 ± 0.462	0.125, 2.50	0.280 ± 0.228	−0.307, 0.866

of molecular methods to analyze periphyton responses to nutrients in 2 coastal plain streams in the southeastern US reaffirms several benefits of using this rapidly advancing technology to develop water quality bioindicators. The benefits include 1) quantifying characteristic and replicated changes in periphyton assemblages in response to nutrient amendments, 2) resolving shifts in 3 domains (Bacteria, Archaea, and eukaryotes) and for diatoms, and 3) using methods that are reproducible and cost effective. We used a direct experimental approach to identify nutrient-responsive

Table 4. Diatom genera determined with indicator species analysis to be associated with N or P amendments. The + symbol indicates that the group is an N- or P-increaser, the – symbol indicates that the group is an N- or P-decreaser, and 0 indicates that the group is a non-responder to N or P. The proportion of diatom nutrient responders quantifies the contribution of each genus (or unclassified group) to the total of all diatoms identified as nutrient responders. The diatom taxa that did not respond significantly to nutrients include: *Achnanthes*, Bacillariophyceae (unclassified), *Eunotia*, *Placoneis*, *Surirella*, *Arcocellulus*, *Cyclotella*, Mediophyceae (unclassified), *Skeletonema*, *Ulnaria*, *Aulacoseira*, *Melosira*, Diatomea (unclassified).

Genus or group	Effect of P	Effect of N	Proportion of nutrient-responders (%)
Bacillariophytina, unclassified	+	+	3
Eunotia[a]	−/0	−/0	17
Fistulifera	+	+	0
Fragilaria	–	–	0
Frustulia	0	+	12
Gomphonema	–	0	24
Navicula	–	–	11
Neidium	+	+	3
Nitzschia	+	0	25
Pinnularia[a]	0/−	0/−	
Stenopterobia	–	0	4

[a] Results are presented with provisional taxonomic assignment of 2 abundant OTUs to *Pinnularia*. In the equally likely alternative scenario wherein these OTUs are *Eunotia*, *Pinnularia* responds as indicated (-) and *Eunotia* is non-responsive (0).

taxonomic groups because the effects of nutrients on many of the taxa we identified were unknown. These identified groups can be verified in future studies, enabling further development of microbial indicators of nutrient pollution in streams.

The ability of our study to resolve nutrient effects with the replication that we used (triplicate controls and treatments) supports the concept that nutrient effects on biological assemblages could be monitored using molecular methods. Had we been unable to resolve assemblage changes caused by our artificial nutrient effects it could suggest that either natural variability in assemblages is too high or that sampling variability associated with our methods is too high, either of which could limit the potential for monitoring programs to resolve natural changes resulting from nutrients. For example, founder effects on immigration of cells to artificial substrates might have caused strongly divergent assemblages within treatments (e.g., Kelly et al. 2014). Variation introduced during sample processing could be associated with DNA extraction and amplification. We did resolve nutrient effects, so we conclude that triplicate sampling is adequate and that it is likely practical and affordable for monitoring programs to support that level of sampling.

Effective biological monitoring of nutrient effects at an affordable cost is valuable because nutrient effects are widespread, yet variable on a variety of temporal and spatial scales, suggesting that monitoring could be improved by sampling more dates and sites. Sampling more dates would allow biomonitoring programs to better resolve responses of biological assemblages to naturally varying nutrient concentrations and temporal factors that modulate nutrient effects on biological assemblages (Knight et al. 2012). Sampling more sites can help resolve where effects are occurring within watersheds and help relate biological responses to causal factors, which is useful for water quality policy. Stein et al. (2014) evaluated costs of biological monitoring programs and found that molecular methods based on next-generation sequencing could be cost effective, and costs have continued to decline, suggesting that biological monitoring may be improved by further developing molecular procedures and applying them more frequently and at more locations.

One benefit of the large number and diverse taxonomic groups that we resolved with a molecular approach is that we were able to quantify a strong negative effect of nutrient amendments on periphyton diversity. Biological diversity can be understood across scales from genes to ecosystems, with high diversity often regarded as a desirable characteristic. Changes in diversity in streams have been associated with land use and implementation of best management practices (Moore and Palmer 2005, Hosen et al. 2017). Species diversity, which we examined by quantifying species number and evenness, may be important because of its potential association with high community productivity or resilience, which could be supported by an insurance effect of rare taxa (Kurm et al. 2019). Conversely, low or declining diversity, including few rare taxa, may indicate a stressed condition, a loss of production or functional potential, or greater vulnerability to future collapse (Shade 2017, Kurm et al. 2019).

We are interested in the possible mechanisms responsible for the observed effect of nutrients on diversity. Understanding these mechanisms is important because of the possible implications for translating our experimental results to expected effects in streams receiving different nutrient loads, which is what we ultimately want to understand. Our substrates were uncolonized at deployment, so diversity quantified at recovery reflects both rates of recruitment to the substrate and abiotic and biotic effects impacting recruits. Because added nutrients only affect the nutrient-amended substrate (i.e., not the whole stream), decreased diversity probably does not reflect decreased recruitment. Nutrients most likely affect taxa whose growth after recruiting to the substrate most benefits from the added nutrients. Because sampling effort is fixed, these nutrient-increasers ensure that some other taxa have lower relative abundance and makes it more likely that some taxa will not be part of the rarefied assemblage at all, decreasing both species number and evenness (Fig. 3). The relative abundance of taxa whose growth is actually reduced by either nutrients or by the presence of nutrient-increasers would be expected to decrease even more, with an even greater chance that these taxa are removed from the assemblage. Longer-term deployment of our substrates could be affected by community interactions beyond what we observed in 2 weeks, including additional competition for space on the substrate (ours were not always thickly colonized) and more developed trophic interactions. Sustained nutrient exposure and ecological changes in a whole stream and its watershed could lead to additional processes affecting periphyton assemblages, such as changes in sources of taxa and metapopulation dynamics (Leibold et al. 2004, Hosen et al. 2017). To further examine these differences between our experiments and expectations for nutrient effects in natural streams, it would be useful to examine if the magnitude of assemblage changes and the identities of the most responsive taxonomic groups vary with deployment duration. Validating the nutrient indicators on natural substrates like leaves, roots, and sticks across natural nutrient gradients would also be useful and important. Sampling these substrates is a common alternative to scraping rocks, which are usually not present in southeastern coastal plain streams.

In addition to the changes in diversity, we also observed characteristic and quantifiable changes in periphyton assemblage composition in response to nutrient amendments, as we hypothesized might occur. Our results provided information on the relative magnitude of N- vs P-responses, the effects of nutrients on taxa in different functional groups (i.e., autotrophs vs heterotrophs), and different domains (Bacteria and Archaea vs eukaryotes), which

we examine over the next several paragraphs. No previous study has examined microbial assemblage responses to nutrients in southeastern coastal plain streams with the molecular approach we used. Carey et al. (2007) used NDS experiments to show that light limitation was the most important factor in Georgia streams, followed by either P-limitation or N- and P- limitation together. We also observed a stronger response to +NP than to +P alone in our data, which occurs in primary producers in a range of aquatic ecosystems (Fisher et al. 1999, Elser et al. 2007). Our results probably reflect complex responses to both nutrient concentrations and the N:P ratio (Keck and Lepori 2012). Light limitation may have been important at our sites and could potentially have led to site differences in nutrient responses (Fig. S1). However, the effect of light cannot be separated from other site differences (e.g., watershed characteristics).

Our results suggest that changes in taxa that are not primary producers can be an important aspect of the response of periphyton assemblages to nutrients and that the ability of a molecular approach to quantify the diverse taxonomic groups that are involved is an important benefit of that approach. Conceptual and empirical models of nutrient effects in streams have often emphasized effects pathways that involve the stimulation of primary production (Dodds and Smith 2016), although some of these models also address algal species composition (Stevenson 2014) and the effects of nutrients on organic matter decomposition (Suberkropp and Chauvet 1995, Woodward et al. 2012, Krishna and Mohan 2017). In our study, heterotrophs such as arthropods, ciliates, rotifers, and amoeba tended to have higher relative abundance in nutrient-amended treatments. For example, the sequences classified as autotrophs tended to be P-decreasers, the opposite of what might be expected if the most important response to nutrients was increased primary production (Table 5). In contrast, heterotrophs were more likely to be P-increasers (Table 5). It is possible that nutrient amendments also stimulated algal productivity, but the data are consistent with a stronger response from heterotrophs. A strong response by heterotrophs could reflect changes in organic matter sources toward lower carbon:nitrogen or carbon:phosphorus that can benefit relatively nutrient-rich herbivores or decomposers (Elser et al. 2000), which is supported by research showing more rapid litter breakdown with elevated dissolved nutrients (Biasi et al. 2017). Many fungal clades were nutrient-responders (Table S2), suggesting another important category of nutrient-response besides than autotrophs. However, fungal indicator clades, which we characterize as saprotrophs (Table 5), were distributed between P-decreasers and P-increasers in roughly the same proportion as other indicator taxa (i.e., tilted toward nutrient-decreasers). Fungal assemblage changes have not been widely used to indicate water quality, but a molecular approach provides a practical means of quantifying a response and could be informative because fungi are both important as decomposers and responsive to nutrients (Suberkropp and Chauvet 1995, Biasi et al. 2017, Krishna and Mohan 2017). If analysis of fungal community composition is of interest, more detailed taxonomic results for fungi can be obtained by sequencing the Internal Transcribed Region rather than more generally applicable 18S rRNA gene sequences that we used (Frąc et al. 2018).

Table 5. The fraction of sequences in all control treatments of eukaryote clades identified as P-decreasers or P-increasers (%; Table S2E, F), summarized by trophic role inferred from general physiological or ecological descriptions.

Functional Role	P-decreasers	P-increasers
Autotrophs	97	3
Saprotrophs	81	19
Heterotrophs	67	33
Unclassified	100	0
All Identified Clades	82	18

We observed relatively larger nutrient responses for Bacteria and Archaea than for eukaryotes (Tables 2A, B, 3), which suggests one of the benefits of sampling diverse taxonomic groups with a molecular approach. We propose 2 explanations for these differential responses, although there could be others. One idea is simple and methodological: the 16S rRNA gene is a longer read and is better sequenced, providing more and higher quality sequences. Moreover, the SILVA database provides more reliable taxonomic identifications for Bacteria and Archaea based on 16S rRNA genes than for eukaryotes based on the 18S rRNA gene. Thus, Bacteria and Archaea may not be better nutrient-indicator taxa, but our molecular approach may be better able to resolve their nutrient responses. A second explanation for observing relatively larger effects in Bacteria and Archaea is predicated on the idea that microbes are numerous and small, fast growing, and well distributed, such that, at the extreme, "everything is everywhere but the environment selects" (Fenchel and Finlay 2004). Accordingly, relatively strong nutrient responses in the Bacteria and Archaea could reflect less variability due to immigration and founder effects than for eukaryotes and faster growth rates for some taxa. The prospect that Bacteria and Archaea might have strong nutrient responses motivated our research approach and our hypothesis that quantifying diverse taxonomic groups would be a benefit of using a molecular approach.

We observed that many clades that were nutrient-decreasers are known to be relatively slow growing, suggesting that periphyton successional dynamics could be an important consideration for understanding our data (McCormick and Stevenson 1991). For example, the nutrient-decreasers included several clades belonging to the Archaea, Acidobacteria, and Actinobacteria, which are often slow growers or are associated with oligotrophy or extreme environments (Fierer et al. 2007, 2012). Veach et al. (2016)

used a molecular approach to examine Bacterial succession in streams and observed that Acidobacteria abundance increased linearly during biofilm succession. We did not investigate temporal changes, but these previous studies suggest that at the end of our 2-wk periphyton collector deployments, neither biomass nor species composition was likely to be at equilibrium, especially because assemblages on the periphyton collectors were undergoing primary succession (Veach et al. 2016) rather than re-colonizing disturbed substrates. The transitional state of the assemblage is important if the rate and course of biomass increase and species composition during succession is altered by nutrient enrichment. We analyzed relative abundance of amplicons, so relief of nutrient limitation for fast-growing nutrient-increasers could make slower-growing species appear to be nutrient-decreasers during periphyton succession, even if nutrients actually increased their growth rates. Nonetheless, negative associations with nutrients implies that any benefit a clade derives from nutrient amendments is smaller or less consistent than the average for all taxa in the assemblage. Clades with no nutrient associations were likely either inconsistent or variable in their response, potentially because of inconsistent immigration to the substrate.

Our research suggests that the level of taxonomic aggregation used in the analysis is important and that the choice may involve a trade-off between high taxonomic resolution with associated functional specificity and lower taxonomic resolution (i.e., greater aggregation), allowing for broader applicability. In our initial analysis of these data (Hagy et al. 2018), we evaluated contributions to assemblage dissimilarity at the OTU level and derived a nutrient-response index based on thousands of OTU abundances. Although the index captured nutrient effects in each experiment very effectively, responses were remarkably site specific and date specific, making the resulting indicator ineffective for broader application. Moreover, applying an indicator based on OTUs from this study to a new data set with entirely different OTUs might not be possible. We therefore grouped OTUs into clades based on hierarchical taxonomy and evaluated nutrient responses of the clades. This approach could create improved transferability if other studies classified their OTUs with the same database, but this increased generality may come at the cost of lower functional specificity, perhaps especially in the Bacteria and Archaea where a class-level clade across different habitats may include different taxa at the species level. Moreover, evolutionarily-related taxa can differ in ecological function or traits, arguing for classification based on function rather than phylogenetic descent (Zhu et al. 2015). It might be possible to combine the transferability gained from taxonomic classification with the functional resolution of OTU-level analysis by classifying OTUs without grouping them, but the need to consider many nutrient-responding taxa could be a problem. Hosen et al. (2017) identified 312 OTUs that indicated particular site-types (e.g., urbanized vs forested), then focused on 5 OTUs that also correlated with dissolved nitrogen. The same authors also narrowed their discussion by focusing on the most abundant OTUs among indicator groups. These issues are important opportunities for future research in both basic microbial ecology and applications to microbial indicators.

Our analysis of the diatom assemblage was consistent with the known value of using diatoms as indicators, but it does not advance application of molecular methods to diatom indicators as more targeted research has done (e.g., Valentin et al. 2019). Diatoms are the component of the microbial assemblage that has been used the most for water quality indicators, including indicators of nutrient effects. We hypothesized that the key benefit of our molecular approach is the large number of taxonomic groups that can be resolved, so we did not seek to replicate existing diatom indicators with a molecular approach. As we expected, we found that our 18S rRNA gene sequences lacked the genetic variability needed to resolve the diatom assemblage at the level possible via light microscopy. Despite this shortcoming, the diatom assemblage composition that our data did resolve some nutrient responses. Our diatom data responded less consistently to nutrient treatments than did the broader periphyton assemblage, but the diatom index was more specific to P amendments. This specificity shows that diatoms are valuable as indicator taxa even with broad molecular approaches and that they should remain a key focus within a broader strategy for molecular-based stream indicators. However, it would be better to sequence genes that differentiate diatoms more effectively, such as *rbc*L, the gene that encodes the ribulose bisphosphate carboxylase large subunit (Zimmermann et al. 2011, 2015, Kermarrec et al. 2014, An et al. 2018, Valentin et al. 2019).

The field of water quality indicators is relatively well developed, and microscopic analysis is the key tool. Specifically, existing indicators hinge on relatively precise cell counts of a small number of taxa about which much is known. Our molecular approach provides less precise relative abundance of many taxa, about which less is collectively known. Thus, effective indicator development hinges on both further development and validation of indicators and broad growth in knowledge of microbial ecology. Molecular methods do not generally produce data such as cell counts by taxon required for traditional bioindicators so they are likely to be used as an additional tool rather than as a replacement for traditional indicators until additional knowledge is acquired. To move toward future applications of molecular methods in a stream bioassessment and water quality management context, we suggest additional research in several areas, including development of effective standard field, laboratory, and data processing protocols, broader development and validation in a variety of aquatic environments, and investigations to find meaningful linkages between periphyton assemblage composition and broader measures of biotic integrity and ecosystem function.

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