Nitrogen-fixing cyanobacteria (free-living and diatom endosymbionts): Their use in southern California stream bioassessment

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Abstract

A weight-of-evidence approach was used to examine how nutrient availability influences stream benthic algal community structure and to validate nutrient-response thresholds in assessing nutrient limitation. Data from 104 southern California streams spanning broad nutrient gradients revealed that relative abundance of N_2-fixing heterocystous cyanobacteria (*Nostoc, Calothrix*), and diatoms (*Epithemia, Rhopalodia*) containing cyanobacterial endosymbionts, decreased with increasing ambient inorganic N concentrations within the low end of the N gradient. Response thresholds for these N_2 fixers were 0.075 mg L^{-1} NO_3-N, 0.04 mg L^{-1} NH_4-N, and an N:P ratio (by weight) of 15:1. The NO_3-N threshold was independently validated by observing nitrogenase gene expression using real-time reverse transcriptase PCR. Morphometric analysis of cyanobacterial endosymbionts in *Epithemia* and *Rhopalodia* indicated that endosymbiont biovolume per diatom cell decreased with increasing NO_3-N (for levels <0.02 mg L^{-1}). Our findings indicate that abundance of heterocyst-containing cyanobacteria and endosymbiont-containing diatom cells are good indicators for rapid nutrient biomonitoring. Because heterocystous cyanobacteria and *Epithemia/Rhopalodia* were not always recorded together at N-limited sites, examining both assemblages jointly may provide a more comprehensive assessment of stream nutrient limitation than using either assemblage alone.

Introduction

Nutrients are a common cause of stream impairment and therefore a high-priority water-quality concern (Smith *et al.* 1999). Traditional monitoring of nutrients involves discrete sampling of ambient concentrations, but these data are rarely wholly indicative of the potential for ecosystem impacts. A more meaningful indicator is needed based directly on organisms and communities themselves, as they may represent the sum total of effects of nutrient loads over time and space (Cairns *et al.* 1993). Recently, indices based on diatoms and non-diatom benthic algae were developed to characterize trophic status and nutrient impairment of freshwater ecosystems (for reviews see Stevenson *et al.* 2010, Schneider and Lindstrøm 2011).

The fixation of atmospheric nitrogen gas (N_2) is an important mode of nutrient input into streams at both the organism and ecosystem levels (Marcarelli *et al.* 2008). The conversion of atmospheric N_2 to ammonia (NH_4-N) requires high levels of both energy and reductant and thus availability of preferred inorganic nitrogen (N) sources such as nitrate

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(NO_3-N) or (NH_4-N) in the environment inhibits the process (Bhaya *et al.* 2000). Cyanobacteria are the only photosynthetic microorganisms capable of nitrogen fixation (N_2 fixation) in the free-living state and in symbiosis (Bothe 1982). N_2 fixation is carried out in morphologicaly modified cells (heterocysts or heterocytes) formed in some filamentous cyanobacteria, but the process is also observed in nonheterocystous filamentous and unicellular forms (Philips *et al.* 1992). The symbiosis between unicellular coccoid cyanobacteria and freshwater diatoms *Epithemia* and *Rhopalodia* has been well documented (for reviews see DeYoe *et al.* 1992, Prechtl *et al.* 2004). Prechtl *et al*. (2004) demonstrated that endosymbionts in *R. gibba* fix N_2 under light conditions. In addition, field experiments and laboratory culturing have shown the number of endosymbionts in *R. gibba* and *E. turgida* cells is a function of N availability (DeYoe *et al.* 1992).

In streams, the dominant autotrophic nitrogen fixers (N_2 fixers) belong to heterocystous filamentous cyanobacteria, such as *Nostoc, Calothrix, Anabaena*, *Rivularia* and unicellular cyanobacterial endosymbionts of diatoms *Epithemia* and *Rhopalodia* (Komárek and Komárkova 2003). However, in most stream periphyton assessment programs, diatoms and non-diatom algae are analyzed separately, and therefore comprehensive studies including free-living heterocystous and endosymbiont cyanobacteria in diatoms in a common environment are lacking. For these reasons, only particular cyanobacterial or diatom genera have been proposed as indicators of nutrient status in freshwater ecosystems, such as *Epithemia* and *Rhopalodia* (Hill *et al.* 2001, Stevenson *et al.* 2008, Reavie at al., 2008), *Rivularia* (Mateo *et al.* 2010), and heterocystous cyanobacteria (Porter *et al.* 2008).

In this study, we collected data at multiple scales (community, molecular, and cellular), using different types of measures on free-living heterocystous and endosymbiont cyanobacteria in order to demonstrate mechanistic underpinnings of N_2 fixers community composition response to stream nutrient concentrations and limitation in southern California. However, while it is reasonable to infer linkages between the abundance of N_2 fixers and stream nutrient concentrations, causative relationships between the two have not been established experimentally *in situ* on a large scale. Any metric or index of water quality will be more defensible if its responsiveness to anthropogenic stress is proven causally, rather than just by association. Moreover, the suitability of tools to infer condition of natural resources is becoming increasingly important as regulations based on monitoring results are implemented. Finally, the majority of studies on nutrient limitation of these algae have been conducted in temperate, non-arid climates in the United States (Borchardt 1996). There is insufficient information to make generalizations about N_2-fixing benthic algae in more arid areas and, in particular, for those of the southern California Mediterranean climatic region.

We hypothesized that the abundance of heterocystous cyanobacteria and diatoms that host cyanobacterial endosymbionts would serve as integrative indicators of stream nitrogen status and nutrient limitation because of their ability to fix atmospheric nitrogen. In order to evaluate this hypothesis, we examined three lines of evidence using specimens collected from natural stream environments in southern California across nutrient concentration gradients examining the relationship between nutrient concentrations and 1) nitrogen-fixer relative abundance (biovolume) in streams; 2) total biovolume of endosymbionts per diatom cell; and 3) the induction of nitrogen fixation as revealed by real-time molecular analysis of fresh, field-collected specimens. Ultimately, our objective was to provide mechanistically supported evidence for the utility of nitrogen fixers in addressing bioassessment questions focused on stream nutrient conditions.

Methods

Study Design and Selection of Study Sites

The study region consisted of wadeable streams draining coastal watersheds along southern California, USA, from Point Conception in Santa Barbara County to the Mexican border (32°7′ N to 34°7′ N and 116°4′ W to 120°2′ W). Stream reaches employed in the study were selected in order to represent a broad range of factors known or hypothesized to influence periphyton community composition and biomass. These factors included varying degrees of human influence in the contributing watershed in terms of the amount and nature of development (i.e., residential/commercial, industrial, agricultural, and grazing, as well as pristine or essentially undeveloped open space), which in conjunction with basin geology, influenced water chemistry parameters downstream. This analysis resulted in a broad range of nutrient concentrations across sampling

sites. Our study design also captured variation in on-site factors, such as dominant substratum type, amount of canopy cover, gradient, flow, and channel dimensions.

During the 2007-2008 timeframe, a total of 104 stream sites across 29 watersheds were sampled for benthic algae under dry-season (low-flow) conditions (for map see Stancheva *et al.* 2012). All sites were sampled at least once during the summer, and a subset of the sites spanning a broad range of human influence, and across multiple watersheds, was resampled in the fall. All 147 of the resulting samples were included in present study in order to capture the broadest gradient of nutrient conditions and periphyton communities possible.

For nitrogenase gene expression profiling, 16 streams in the study region were resampled to collect fresh algal material in September-October 2010 and May-June 2011 (Supplemental Information (SI) SI Resource 1; ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_205_222SI.pdf). Streams were selected in order to represent the entire range of the NO_3-N gradient where N_2-fixing algae had been recorded from the previous field survey (2007-2008).

Field Sampling of Periphyton

All 147 periphyton samples included in this study were collected using the "multi-habitat" sampling method as described by Fetscher *et al*. (2009). This technique entailed collection of material at 11 objectively selected locations spaced evenly across a 150 m or 250 m long stream reach (depending upon whether the average width of the stream was less or greater than 10 m). Within each reach, samples were obtained from variable sampling area depending on the substrate present at each of the 11 locations (e.g., cobble, silt/sand, gravel, bedrock, wood, concrete) using various sampling tools for removing the algae, such as a rubber or PVC delimiter, toothbrush, syringe scrubber (Fetscher *et al.* 2009). These subsamples were combined into a single well-mixed composite sample from which aliquots with equal volume (45 ml) were drawn for identification and quantitative analyses of diatoms and non-diatoms algae, including cyanobacteria. For best cellular preservation, all samples were fixed immediately upon collection in 2.5% histological grade glutaraldehyde and kept cold and in the dark until laboratory analysis.

To measure nitrogenase gene expression, a representative qualitative composite benthic algal sample was collected from each stream. All types of benthic algae visible within the stream reach were collected proportionally to their cover of the bottom. Algal thalli were removed very gently by hand in order to preserve their integrity and stored in Whirlpak bags containing native stream water. The algal samples, collected in the morning, kept cool and in the dark, were delivered to the laboratory for examination as quickly as possible (usually within two hours of their collection). Physical habitat data were recorded for each stream reach as described by Fetscher *et al*. (2009).

Laboratory Analyses

Chemical

For filterable inorganic nutrients such as nitrate (NO_3-N), nitrite (NO_2-N), ammonium (NH_4-N), orthophosphate (PO_4-P), total nitrogen (TN) and total phosphate (TP), stream water samples were passed through 0.45 µm pore size MCE membrane filters (Thermo Fisher Scientific Inc., Pittsburgh, PA) in the field immediately following collection. Filtered samples were immediately placed on ice, and frozen within 4 hours of collection. Frozen samples were stored at -20ºC for up to 4 weeks prior to analysis. NO_3-N + NO_2-N was analyzed by the cadmium reduction method (SM 4500-NO_3 F), and NO_2-N was analyzed by the colorimetric method (SM 4500-NO_2 B). NH_4-N was analyzed by distillation and the automated phenate method (SM 4500-NH_4 G) and PO_4-N was analyzed by the automated ascorbic acid reduction method (SM 4500-P F). Water chemistry analyses were carried out in accordance with APHA (2006). Analyses were carried out with a Lachat Instruments (division of Zelweger Analytics) Model QuickChem 8000 Flow Injection Analyzer.

Diatom Processing

For the species identification and quantification of diatoms, samples were cleaned with 30% hydrogen peroxide (H_2O_2) and the reaction was catalyzed by adding several crystals of potassium dichromate ($K_2Cr_2O_7$) following the method of Van Der Werff (1955). The cleaned material was rinsed multiple times with distilled water and processed into permanent microscope slides, using Naphrax as the mounting medium (refractive index 1.65; Brunel Microscopes Ltd., Chippenham, Wiltshire, UK). For each sample, at least 600 valves were identified and

enumerated with an Olympus BX-51 light microscope (Olympus Imaging America, Center Valley, PA, USA), using a 100 x oil immersion objective with a N.A. of 1.40.

Of the diatom taxa capable of nitrogen fixation recorded in the study, the four most abundant (i.e., *Epithemia adnata*, *E. sorex, E. turgida,* and *Rhopalodia gibba*) were selected for detailed morphometric analyses of their cyanobacterial endosymbionts. Twelve natural populations from each species, collectively sampled across 19 sampling sites (SI Resource 1), were utilized in the analysis. One set of measurements for each species originated from a sample (DGSY1, fall 2010) for which nitrogenase gene expression was also assessed, and nitrogenase activity was detected for cyanobacterial endosymbiont in *Rhopalodia gibba* and presumably in *Epithemia* species.

The populations used for the morphometric analyses were selected based on fulfillment of the following criteria: 1) a sufficient abundance of studied diatoms (at least 10 intact cells) in each sample; 2) a quality of cell preservation deemed adequate for analysis of cyanobacterial endosymbionts; and 3) coverage of a sufficiently broad and representative nitrate gradient across samples. All measurements were taken using material fixed with 2.5% histological grade glutaraldehyde. For each diatom species, all the endosymbionts detected in each of 10 host cells within each of 12 populations were measured. The following features were recorded: 1) number of endosymbionts per diatom host cell; and 2) diameter and length of each endosymbiont cell. The cell shape of cyanobacterial endosymbionts was approximated as "prolate sphaeroid" and based on this shape, biovolume was calculated according to Hillebrand *et al*. (1999).

Non-diatom Algae Processing

For proper identification and enumeration of non-diatom algal taxa, including cyanobacteria, we processed macroalgae (definition after Sheath and Cole 1993) separately from the microscopical algal fraction of each quantitative sample as described by Stancheva *et al*. (2012). First, the total volume of macroalgal fraction was determined by the increase in volume (ml) of distilled water in graduated centrifuge tube. The biovolume of each macroalgal species was estimated as proportion of the total volume of macroalgal fraction under stereo microscope. For the microalgal fraction, at least 300 natural algal counting entities were identified, enumerated and individual microscopical measurements were collected for each species along a known number of optical transects across the microscope slide at magnification 400x. The biovolume of each encountered algal taxon was calculated as individual biovolume (μm^3) per 1 cm^2 of area sampled. In this way the biovolume of each species was comparable across the samples. For this work, individual biovolume of each non-diatom algal taxa was converted to relative abundance (relative biovolume), which was calculated as a percentage of total algal biovolume per sample represented by each algal taxon. Specimen observation and photomicrography were performed with an Olympus BX41 microscope and an Olympus SZ-40 stereo microscope with an attached Olympus MicroFire S99809 digital camera (Olympus Imaging America, Center Valley, PA, USA).

Species Optima with Respect to Nutrients

To characterize nutrient concentration affinities of N_2-fixing heterocystous filamentous cyanobacteria and diatom taxa that host unicellular endosymbionts, their weighted average (WA) indicator values for NO_3-N, filterable TN and filterable TP were calculated by weighting the mean of each selected environmental variable by the species relative abundance using the R language and environment for statistical computing (R Development Core Team 2008). Weighted averaging is a technique commonly used to estimate species indicator values or optima (ter Braak and Looman 1986). This method does not assume any particular shape of species response curve, but in cases where distributions are unimodal, the weighted average is expected to approximate the regression-estimated species optimum (ter Braak and Looman 1995).

Data Analysis

N_2-fixer communities and their relationship with nutrients and physical habitat conditions were examined by canonical correspondence analysis (CCA). CCA was chosen after a preliminary detrended correspondence analysis showed a value for the length of the longest gradient larger than 4.0 (7.32; Lepš and Šmilauer 2003). Nutrient concentration and N:P ratio data were $\log_{10}(y+1)$ transformed to "normalize" their distribution prior to analysis. Species proportions and physical habitat percentage data were square-root transformed. A set of environmental variables that significantly relate with species communities was

identified by means of manual forward selection and tested for their significance by 499 Monte Carlo permutations ($p = 0.05$). Unrestricted global Monte Carlo permutation tests were used to test the significance of the first two CCA axes (999 permutations). A total of 33 N_2-fixer taxa (Table 1) and nutrient and physical habitat data (SI Resource 1) from 78 samples were used in CCA. The multivariate statistics were conducted using computer program CANOCO (v. 4) (ter Braak and Smilauer 1998). Differences in environmental conditions between the stream sites where either heterocystous cyanobacteria or diatoms *Epithemia/Rhopalodia*, but not both, were recorded were tested by one-way analysis of variance (ANOVA; Zar 1996).

N:P Ratio

Redfield (1958) proposed an atomic ratio of 16:1 as a transition point from N to P limitation based on cellular proportions of N and P in marine phytoplankton. However, ratios of N:P can be expressed in different ways when describing conditions for

Table 1. List of heterocystous filamentous cyanobacteria and diatom species of *Epithemia* and *Rhopalodia* with their ranges and weighted average (WA) indicator values for NO_3-N, filterable TN and filterable TP. n = indicates the frequency with which taxa were recorded in 147 stream samples. Name abbreviations in parenthesis were applied in Figure 5.

Taxon	n	NO_3-N (mg L^{-1})			TN (mg L^{-1})			TP (mg L^{-1})		
		WA	Min	Max	WA	Min	Max	WA	Min	Max
Heterocystous cyanobacteria	69									
Anabaena oscillatorioides (Ana_osc)	6	0.0088	0.0007	1.3662	0.144	0.0199	1.2964	0.0162	0.0011	0.0988
Anabaena inaequalis (Ana_ina)	1			0.003			0.0591			0.0152
Anabaena sp. 1 (Ana_1)	6	0.0043	0.0007	0.1163	0.191	0.0619	0.9205	0.0375	0.0011	0.0935
Anabaena sp. 2 (Ana_2)	4	0.003	0.003	0.007	0.0014	0.0014	0.2226	0.0012	0.0011	0.0186
Calothrix fusca (Cal_fus)	19	0.0137	0.0007	0.2031	0.1005	0.0014	0.6734	0.0073	0.0011	0.0988
Calothrix epiphytica (Cal_epi)	9	0.0028	0.0007	0.0065	0.0602	0.0014	0.1735	0.0218	0.0011	0.0311
Calothrix parietina (Cal_par)	5	0.0018	0.0007	0.2031	0.1324	0.0014	0.1894	0.0023	0.0011	0.004
Calothrix clavata (Cal_cla)	1			0.0639			0.0538			0.0011
Calothrix cristacea (Cal_cru)	1			0.0007			0.1331			0.0023
Dichothrix orsiniana (Dic_ors)	1			0.003			0.0199			0.0011
Nodularia spumigena (Nod_spu)	3	0.0056	0.0024	0.0108	0.1006	0.0591	0.3486	0.0169	0.0152	0.039
Nostoc verrucosum (Nos_ver)	39	0.0113	0.0007	0.409	0.1312	0.0014	0.9205	0.0202	0.0011	0.0988
Nostoc sp. 1(Nos_1)	9	0.0062	0.0025	0.8424	0.1438	0.0841	2.3192	0.0103	0.0011	0.2809
Nostochopsis lobatus (Noh_lob)	3	0.0011	0.0007	0.0056	0.1351	0.0634	0.1735	0.0022	0.0011	0.0033
Rivularia minutula (Riv_min)	6	0.1492	0.0168	0.2031	0.1794	0.0503	0.2174	0.0012	0.0011	0.0044
Rivularia haematites (Riv_hae)	2	0.0056	0.0042	0.0056	0.0778	0.0266	0.9205	0.0048	0.0011	0.0778
Rivulariaceae (Riv)	3	0.6719	0.005	1.3306	2.3715	0.6133	4.1099	0.2269	0.0481	0.3915
Scytonema crispum (Scy_cri)	1			0.0007			0.1331			0.0023
Tolypothrix distorta (Tol_dis)	3	0.0041	0.003	0.0107	0.0253	0.0014	0.076	0.0067	0.0011	0.0407
Trichormus variabilis (Tri_var)	1			0.0007			0.1331			0.0023
Diatoms	61									
*Epithemia sorex (E*sor)*	39	0.0148	0.0007	0.8177	0.1316	0.0014	0.992	0.0151	0.0011	0.1276
*Epithemia adnata (E*adn)*	29	0.006	0.0007	0.1325	0.0858	0.0014	0.81	0.007	0.0011	0.0935
*Epithemia turgida (E*tur)*	6	0.0059	0.0007	0.007	0.1767	0.0199	0.4383	0.0257	0.0011	0.0988
*Epithemia spp. (E*spp)*	3	0.1004	0.003	0.2395	0.1426	0.0395	0.292	0.0017	0.0011	0.0044
*Epithemia argus (E*arg)*	1			0.0042			0.0399			0.0011
*Epithemia frickei (E*fri)*	1			0.003			0.0886			0.0118
*Epithemia westermannii (E*wes)*	1			0.003			0.0889			0.0072
*Rhopalodia gibba (R*gib)*	32	0.012	0.0007	0.5659	0.1081	0.0014	1.0291	0.0126	0.0011	0.1276
*Rhopalodia musculus (R*mus)*	8	0.1126	0.0024	1.211	0.3269	0.0396	1.4805	0.0274	0.0072	0.063
*Rhopalodia acuminata (R*acu)*	5	0.2558	0.0007	0.6786	0.4289	0.0634	0.7934	0.0999	0.0033	0.3087
*Rhopalodia brebissonii (R*bre)*	2	0.286	0.0177	0.5577	0.4788	0.1623	0.7995	0.0755	0.063	0.0883
*Rhopalodia gibba var. minuta (R*gibm)*	2	0.0862	0.003	0.3674	0.206	0.0841	0.6174	0.0299	0.0011	0.1276
*Rhopalodia gibberula (R*gibr)*	1			0.0042			0.0494			0.0454

nutrient limitation. For instance, Grimm and Fisher (1986) quantified nutrient limitation based on the ratio of NO_3-N+NH_4-N to soluble reactive P, while Lohman *et al*. (1991) based nutrient limitation on the ratio of TN to TP (both ratios by weight). Both Grimm and Fisher's and Lohman et al.'s stream nutrient enrichment experiments agreed in their conclusion that when the N:P ratio is <16:1, N is the limiting nutrient for algal growth, whereas when the N:P >16, algal growth is P-limited (Grimm and Fisher 1986, Lohman *et al.* 1991). In order to make inferences about the type of nutrient limitation at play in our study streams, we calculated N:P ratio as NO_3-N+NO_2-N+NH_4-N:PO_4-P by weight. The same selection of inorganic combined nitrogen measures was used by Hill and Knight (1988) in their calculation of N:P ratio for streams in California.

Molecular Analysis

To determine what nutrient conditions (in terms of NO_3-N concentrations) promote the process of N_2 fixation in stream benthic cyanobacterial communities, nitrogenase gene expression was examined via real-time PCR in field-collected specimens of *Nostoc, Calothrix*, and the *Rhopalodia* cyanobacterial endosymbiont. In the laboratory, algal species composition of freshly collected qualitative algal samples was microscopically determined and three subsets of 1 to 3 g of representative composite fresh algae samples from each stream was prepared for RNA extraction within four hours (typically between 11 a.m. and 2 p.m.) of field collection of material. Voucher algal specimens were preserved in 2.5% histological grade glutaraldehyde.

Primer Design

Primers specific to nitrogenase (nitrogenase molybdenum-iron protein *nifK*) and *16S rRNA* genes were designed for *Nostoc, Calothrix*, and the *Rhopalodia* cyanobacterial endosymbiont using the Primer3 (v.0.4.0.) software (Rozen and Skaletsky 2000), and are shown in Table 2. For *Nostoc* and *Calothrix*, the consensus sequence between two different species belonging to the same genus was used to design primers targeting the beta subunit of the nitrogenase gene sequence. These primers are therefore not considered to be species- or strain-specific. For *Nostoc*, consensus was built using *Nostoc* sp. *PCC 7120* and *Nostoc azollae 0708*; for *Calothrix, Calothrix desertica PCC 7102* and *Calothrix* sp. *PCC 7507* were used. Primers specific to the nitrogenase gene from *Anabaena variabilis* (synonym of *Trichormus variabilis*; Komárek and Anagnostidis 1989) were designed using the genomic sequence of *Anabaena variabilis ATCC 29413*. No amplification signal, however, was evidenced when these primers were used to detect nitrogenase expression in environmental samples containing *Anabaena* sp. and *Trichormus* sp. Nitrogenase genes sequences were not available for *Anabaena oscillatorioides* or *A. inaequalis* in NCBI (Geer *et al.* 2010). Primers specific to nitrogenase and *16S rRNA* genes were also designed based on the genomic sequence of the *Rhopalodia* endosymbiont *nifK* gene. In the absence of sequence information for the *Epithemia* endosymbiont *nifK* gene, and because some believe the *Rhopalodia* and *Epithemia* endosymbionts are the same species, with*16S rRNA* genes that are 98% identical (Nakayama *et al.* 2011), the same *nifK* and *16S rRNA* primer sets for were used for both species. Three additional 18S rRNA primer pairs were designed for the diatom *Cocconeis* and the green alga *Cladophora,* and *rbcL* primer for green alga *Ulva* (which were very common in the studied streams). Real-time RT PCR analysis was performed using these primer pairs to confirm the presence of algae in the samples in which N_2-fixing algae were not recorded.

RNA Extraction

Total RNA was isolated from the periphyton samples using a standard guandidium isothiocyanate procedure (Strommer *et al. 1993*). The integrity of the isolated RNA was assessed by denaturing agarose gel electrophoresis, and the concentration and purity were measured spectrophotometrically. First-strand cDNA synthesis was performed using a Verso™ RT cDNA synthesis kit (Fisher Scientific). For cDNA synthesis, 1 µg template RNA was combined with 500 ng of an Oligo-dT primer, 500 ng of random hexamer primers, and water to 13 µl. This template:primer mixture was incubated at 70°C for 5 minutes before 2.5 U reverse transcriptase was added. The complete reaction mixture was incubated for 30 minutes at 42°C.

Real-time PCR

With high specificity and sensitivity, real-time (RT) PCR offers a fast, accurate and culture-independent method for the detection of microbes in environmental samples (Kavanagh *et al.* 2010, He *et al.* 2011). Herein, RT PCR amplification of

Table 2. Primers used in RT PCR.

Species/Genus Name	Gene Name	Forward Primer 5' to 3' Reverse Primer 3' to 5'	Estimated/Actual Product Tm*	Reference Organism Gene	Gene Bank #
Nostoc (consensus)	*nifK*	ACA GGT TCA GCA CCC ATT TC AAG GGT ACT GAC GAG TTC TTG A	81.2/82.5	Nitrogenase molybdenum-iron protein beta chain [Nostoc sp. PCC 7120] *Nostoc azollae*' 0708, complete genome	BAB73397.1 CP002059.1
Nostoc verrucosum	16S rRNA	TGT AGC GGT GAA ATG CGT AG TCG TCC CTC AGT GTC AGT TG	82.5/84.5	*Nostoc verrucosum* gene for 16S rRNA, partial sequence, note: uncultured *Nostoc verrucosum* Ashitsuki	AB511947.1
Calothrix (consensus)	*nifK*	GGA AAA GAA CTT CGC TCG TG GCT GCA TCT TCT GTC ATG GA	82.5/83.4	*Calothrix desertica* PCC 7102 dinitrogenase beta subunit (nifK) gene, complete cds *Calothrix* sp. PCC 7507, complete genome	EU358078.1 CP003943.1
Calothrix parietina	16S rRNA	CAG CCA CAC TGG AAC TGA GA ATC ATT CCG GAT AAC GCT TG	82.9/84.0	*Calothrix parietina* 2T10 partial 16S rRNA gene, strain 2T10	FR798917.1
Cyanobacterium endosymbiont of *Rhopalodia gibba*	*nifK*	AGG AAT GCC TTC TGC TGA GA GGG TAC GGA AGT AAG CCA CA	82.9/83.6	Cyanobacterium endosymbiont of *Rhopalodia gibba,* genomic sequence	AY728387.1
Cyanobacterium endosymbiont of *Rhopalodia gibba*	16S rRNA	CTT GAC ATG TCC CGA ACC TT CAC CGG CAG TCT CCT TAG AG	84.8/86.0	Cyanobacterium endosymbiont of *Rhopalodia gibba* gene for 16S rRNA, partial sequence, isolate: RGSB Namiki park RG	AB546730.1
Cocconeis cf. *molesta*	18S rRNA	TGG CCT ACC ATG GCT TTA AC ACC TCC CTG TGT CAG GAT TG	84/85.7	*Cocconeis* cf. *molesta* 18S rRNA gene, clone p800	AJ535148.1
Cladophora	18S rRNA	AAA AGC CCG TAG CTG AAC CT ACA CCG AGA TAC GGT CTT GC	82.9/84.0	Uncultured *Cladophora* clone *Cladophora*7 18S ribosomal RNA gene, partial sequence	GQ371038.1
Ulva flexuosa	*rbcL*	AAT TCG GTG GTG GTA CAT TAG G TTC AGG ACT CCA TTT ACA AGC A	82.0/83.6	*Ulva flexuosa* voucher GWS008545 ribulose-1,5-biphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast	HQ603532.1

*Tm refers to melting temperature.

small regions of the nitrogenase, rRNA, and *rbcL* genes was carried out using SYBR green chemistry for amplicon detection (Life Technologies, Grand Island, NY, USA). Reactions were performed in a total volume of 25 µl containing 5 µl cDNA template (diluted 1:20), 7.1 µl 2X SYBR green master mix and 0.24 µM each forward and reverse primers. Plates were loaded using a Biomek® 2000 automation workstation robot (Beckman Coulter, Inc., Fullerton, CA, USA). Each sample was run twice in triplicate using template cDNA synthesized from RNA extracted from periphyton samples. Negative no-template controls were included on every plate for each set of primers. The BioRad iCycler iQ system was used for thermocycling and to record changes in fluorescence. Amplification of target genes was performed in triplicate in a 96 well plate along with no-template controls to monitor potential contamination. The PCR reaction was initiated with a preincubation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and elongation at 82°C for 30 seconds. Melt curve analysis was performed immediately after the final PCR cycle to determine the specificity of the reaction. Amplification products were denatured at 95°C for 1 minute, annealed at 55°C for 1 minute, followed by a ramping of the temperature 0.5°C every 10 seconds until a final temperature of 95°C was reached. The cycle threshold (C_t), defined as the PCR cycle at which the SYBR green fluorescence exceeded background levels, and was set at a selected threshold of 100.

Results

Distribution of Heterocystous Cyanobacteria and Diatoms *Epithemia* and *Rhopalodia* along a NO_3-N Gradient

We identified 17 species of heterocystous filamentous cyanobacteria, which belong to the following genera: *Calothrix* (4 species), *Nostoc, Rivularia*, *Anabaena* and *Nodularia* (2 species each), *Nostochopsis, Dichothrix, Scytonema, Tolypothrix,* and *Trichormus* (1 species each). Four taxa were assigned to the genera *Nostoc* and *Anabaena* or to other broad taxonomic categories due to insufficient material for species identification. Table 1 presents a list of the heterocystous cyanobacteria detected in the study area with their nutrient species optima and ranges. The most frequently observed and abundant taxa were *Nostoc verrucosum, Calothrix fusca* and *Rivularia minutula.* The morphology of these taxa is illustrated on Figure 1 and their distribution along the NO_3-N gradient is presented on Figure 2. *Calothrix epiphytica* was also common species, but presented in very low abundance due to its small size and epiphytic development (SI Resource 3). *N. verrucosum* was recorded in 58% of the sites with N_2-fixing heterocystous cyanobacteria. This large, macroscopic cyanobacterium often dominated quantitatively the benthic algal community at NO_3-N levels below 0.01 mg L^{-1} (86% of its occurrences), and reached its maximum relative biovolume (99.9%) at even lower NO_3-N concentrations (<0.007 mg L^{-1}; Figures 1d and 2a). *C. fusca,* although not recorded as frequently as *N. verrucosum*, was abundant at some sites (reaching up to 23.5% relative biovolume), particularly when NO_3-N levels <0.03 mg L^{-1} (84% of its occurrences; Figures 1c and 2b). In contrast, *R. minutula* reached its maximum relative biovolume (96.5%) in sites with higher ambient NO_3-N concentrations (0.1 - 0.25 mg L^{-1}; Figures 1b and 2c) and its biovolume was positively correlated with NO_3-N concentrations (Spearman rank $\rho = 0.82$, $p = 0.04$).

Twelve diatom species and varieties belonging to the genera *Epithemia* and *Rhopalodia*, which contain N_2-fixing cyanobacterial endosymbionts were identified in the study area and were included with their nutrient species optima and ranges in Table 1. The most common and abundant species were: *E. adnata* (up to 10.5% relative abundance at 0.003 mg L^{-1} NO_3-N), *E. sorex* (up to 13% relative abundance at 0.0007 mg L^{-1} NO_3-N, Figure 1e), *E. turgida* (up to 17% relative abundance at 0.007 mg L^{-1} NO_3-N, Figure 1f), and *R. gibba* (reaching maximum relative abundance of 20% of valves in a sample with a NO_3-N concentration of 0.01 mg L^{-1}, Figure 1a). The obligate presence of cyanobacterial endosymbions in each intact cell of *Epithemia* and *Rhopalodia* was confirmed by additional scanning of all preserved samples in which these taxa were recorded. The morphometric study conducted on cyanobacterial endosymbionts in *E. adnata, E. sorex, E. turgida,* and *R. gibba* (SI Resources 2) showed that for all four species the mean total biovolume of cyanobacterial endosymbionts significantly negatively correlated with ambient water NO_3-N concentrations at the lower end of its range (0.0007 to 0.02 mg L^{-1}; Figure 3).

The distributions of heterocystous cyanobacteria and diatoms *Epithemia* and *Rhopalodia* along a NO_3-N gradient are presented on Figure 4. They were recorded at ambient NO_3-N concentrations

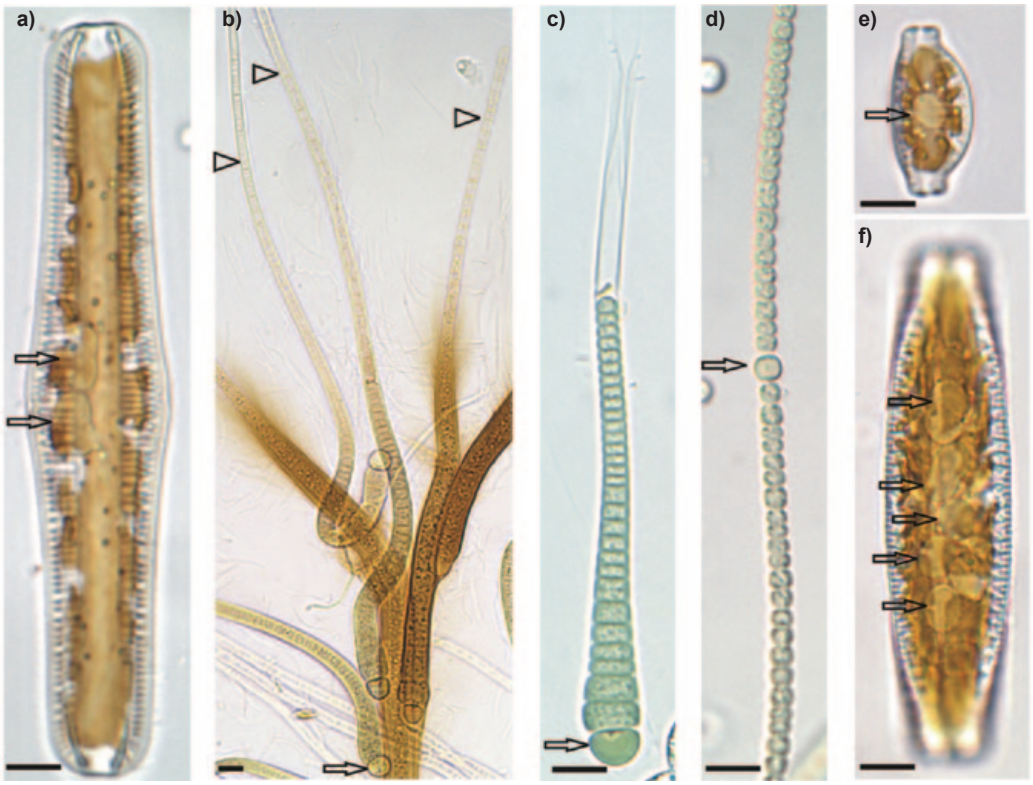

Figure 1. Light microscopic images of N_2-fixing heterocystous cyanobacteria and diatoms *Epithemia* and *Rhopalodia*, bearing cyanobacterial endosymbionts from southern California streams. *Rhopalodia gibba*, note the cyanobacterial endosymbionts (arrows; a); (B) *Rivularia minutula*, arrow shows the basal heterocyst; arrowheads show apical hairs (b); *Calothrix fusca*, arrow shows the basal heterocyst (c); *Nostoc verrucosum*, filament with intercalary heterocyst (arrow; d); *Epithemia sorex*, note the cyanobacterial endosymbiont (arrow; e); and *Epithemia turgida*, note the cyanobacterial endosymbionts (arrows; f). Scale bar for all panels = 10 µm.

not exceeding 1.36 mg L^{-1}, which was well below the upper end of the NO_3-N gradient in the studied stream dataset (21.5 mg L^{-1}; SI Resources 1 and 3). Three samples with uncertain species identification due to the poorly preserved single specimens were excluded from further analysis as outliers (*Nostoc* sp. at 6.4 mg L^{-1} NO_3-N, *Epithemia* sp. at 5.7 mg L^{-1} NO_3-N, *Nodularia harveyana* and *Nostoc linckia* at 15.5 mg L^{-1} NO_3-N).

Heterocystous cyanobacteria and diatoms *Epithemia* and *Rhopalodia* were most often recorded in abundance in streams with ambient NO_3-N <0.075 mg L^{-1}, NH_4-N <0.04 mg L^{-1} and N:P ratios <15:1 (over 79% of their occurrences). These N-limiting conditions were characteristic for half of the sites across the project dataset (n = 73 out of 147, SI Resource 1). N_2-fixing free-living cyanobacteria and in diatoms *Epithemia* and *Rhopalodia* were recorded in a total of 84% of these sites, but only in half of them together. In this low-NO_3-N range, heterocystous cyanobacteria dominated benthic algal communities, typically due to an abundance of *N. verrucosum* (Figures 2a and 4a). In addition, diatoms *Epithemia* and *Rhopalodia* reached their combined maximum relative abundance of 26.3% under low-NO_3-N conditions (Figure 4b). In stream sites with higher ambient NO_3-N concentration (>0.075 mg L^{-1}), diatoms from these genera, if present, were recorded as only a single valve per site (Figure 4b). *N. verrucosum* was relatively abundant at a single site with a higher NO_3-N concentration (0.11 mg L^{-1}), but low N:P ratio (1.9:1). High-biovolume growth of *N. verrucosum* was characteristic for N-limited streams and its rare presence in streams with a N:P ratio >30:1 corresponded to very low relative abundance (<1%). However, N_2-fixing heterocystous cyanobacteria exhibited a second biovolume spike at NO_3-N ranging from 0.1 to 0.25 mg L^{-1}, NH_4-N ranging from 0.01 to 0.02 mg L^{-1}, and high N:P ratios of 30 to 70:1. This spike was attributable to the high abundance of *Rivularia minutula* (Figures 1b, 2c, and 4a).

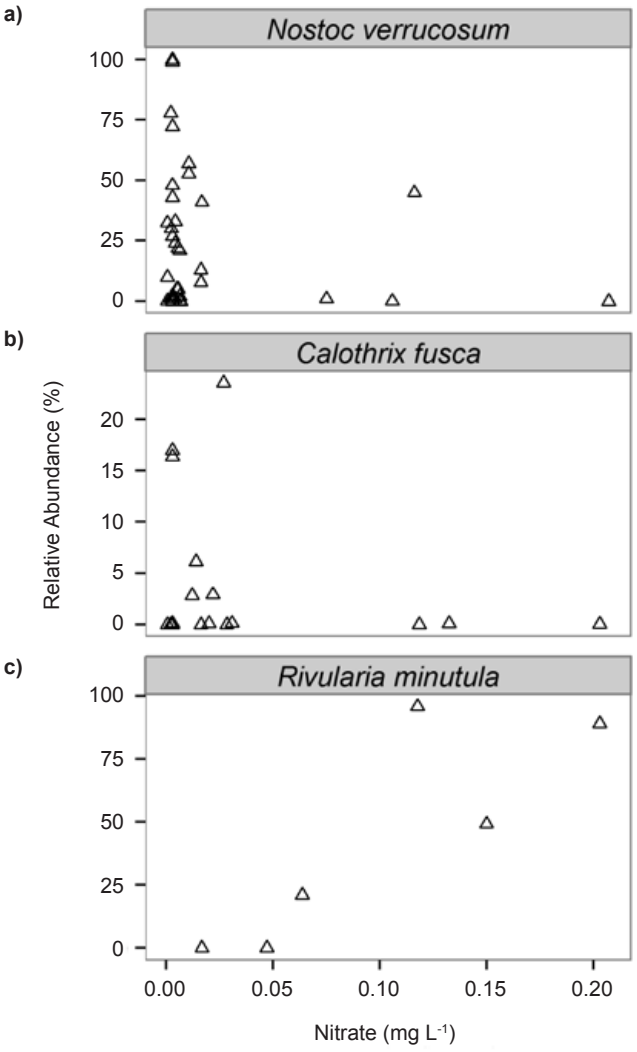

Figure 2. Distribution of the three most abundant N_2-fixing cyanobacteria along the NO_3-N gradient in study streams. Data shown are percent of total non-diatom algal biovolume comprised of heterocystous cyanobacteria of three species: *Nostoc verrucosum* (n = 39, of which one outlier record with relative biovolume 0.9% at 0.4 mg L^{-1} NO_3-N was excluded; a), *Calothrix fusca* (n = 18; b), and *Rivularia minutula* (n = 6; c), Spearman rank ρ = 0.82, p = 0.04. Each panel includes only sites where that taxon was recorded.

Relationship between Heterocystous Cyanobacteria and Diatoms *Epithemia* and *Rhopalodia* with Nutrients and Physical Habitat Conditions

Canonical correspondence analysis (CCA) showed five environmental variables were statistically significant (p = 0.05) and collectively explained 60% of the variation in distribution of N_2-fixing algae (Figure 5). The first two ordination axes accounted for 11.9% of variation in N_2-fixers species composition among sites. The species-environmental correlations for the first two axes were high (r = 0.81). Monte Carlo permutation tests showed that both axes were statistically significant (p <0.01). The first CCA axis was positively correlated with N:P ratio (r = 0.82), NO_3-N (r = 0.39) and NH_4-N (r = 0.22), and negatively with canopy cover (r = -0.30; SI Resource 4). The major group of N_2-fixers were ordinated on the left side of the first axis based on their relationship with environmental variables. *Rivularia minutula* was the only species ordinated on the far right side of the first axis. The second axis was positively correlated with filterable TN (r = 0.55) and NH_4-N (r = 0.31), and negatively with sand/silt substratum (r = -0.40). Most of the diatom species occupied the lower left side of the second axis in ordination space, a position corresponding to fine sediments and very low concentrations of filterable inorganic N forms, in contrast to the cyanobacteria *Rivularia haematites* and unknown Rivulariaceae positioned on the opposite upper side of second axis associated with elevated concentrations of filterable TN. Canonical correspondence analysis demonstrated a relationship between NH_4-N and the distribution of N_2-fixing algae. Both heterocystous cyanobacteria and *Epitemia* and *Rhopalodia* were recorded across a range of NH_4-N values from 0.0005 to 1.07 mg L^{-1}, but were most abundant at NH_4-N concentrations below 0.02 mg L^{-1}, where 88% of their occurences were recorded.

Although N_2-fixing cyanobacteria and diatoms *Epithemia* and *Rhopalodia* tend to coexist, they were found together only in 45% of the streams where any type of N_2-fixing algae were recorded. In the sites where cyanobacteria and diatoms coexisted, NO_3-N was <0.13 mg L^{-1}, NH_4-N was <0.03 mg L^{-1} and N:P was <8:1. In the remaining streams where either diatoms or cyanobacteria, but not both, were recorded, ANOVA showed differences in some environmental conditions. Canopy cover, silicate concentrations and percent soft substratum were significantly higher in diatom habitats, compared to cyanobacterial habitats (SI Resource 5).

Nitrogenase Gene Expression in *Nostoc*, *Calothrix*, and the *Rhopalodia* Cyanobacterial Endosymbiont along a NO_3-N Gradient

In the streams (re)sampled to measure nitrogenase gene expression levels, the ambient NO_3-N concentrations ranged from 0.0002 to 11.2 mg L^{-1} (Table 3), which represented the entire NO_3-N

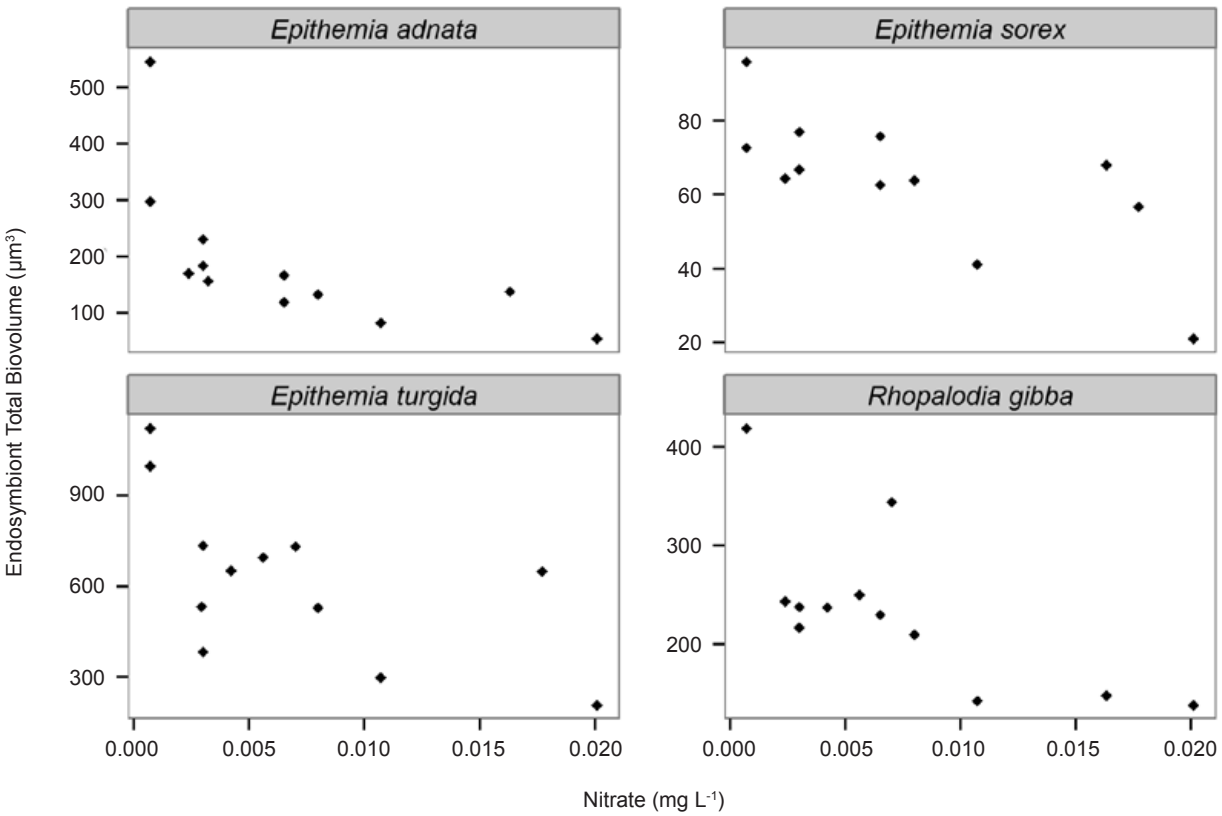

Figure 3. Cyanobacterial endosymbiont total biovolume per diatom host cell (n = 120) along the NO_3-N gradient. Each point corresponds to the mean cyanobacterial endosymbiont total biovolume per diatom host cell observed at single sampling site (n = 10 diatoms per site). *Epithemia adnata*, Spearman rank ρ = -0.91, p = 0.0001; *Epithemia sorex*, Spearman rank ρ = -0.72, p = 0.008; *Epithemia turgida*, Spearman rank ρ = -0.67, p = 0.01; *Rhopalodia gibba*, Spearman rank ρ = -0.74, p = 0.006.

gradient within which N_2-fixing algae were recorded in the field survey of 104 sites (SI Resource 1). To evaluate the specificity of the real-time PCR primers, melt curves acquired after each run were examined (Table 2). Melt curves showing single dissociation peaks within +/- 3°C of the expected temperature, indicated high specificity of the PCR primers. Nitrogenases specific to *Nostoc* and *Calothrix* were detected only in streams where those taxa were recorded and ambient NO_3-N was <0.074 mg L^{-1}. Nitrogenase expression specific to the *Rhopalodia* cyanobacterial endosymbiont was further restricted to streams where NO_3-N levels were <0.01 mg L^{-1} (Table 3). No nitrogenase expression was detected in streams when NO_3-N >0.14 mg L^{-1}, despite the presence of single *Epithemia* valves in some samples, and numerous filaments of *Calothrix* cf. *parietina* observed via light microscopy.

The *16S rRNA*, but not nitrogenase expression, from the *Rhopalodia* cyanobacterial endosymbiont was detected in four samples where NO_3-N was <0.025 mg L^{-1}. Diatoms were not observed in these samples via light microscopy, hence our inability to detect nitrogenase expression may simply be a function of insufficient cell numbers. *Calothrix* and *Nostoc 16S rRNA* expression in the absence of nitrogenase expression was also detected in a single sample collected from a stream with an ambient NO_3-N concentration of 0.32 mg L^{-1} (SGCT1; Table 3), where *Rivularia minutula* was previously recorded. While *R. minutula* was not collected during the 2011 sampling, multicellular filaments of *Calothrix* cf. *parietina* were recorded microscopically in that sample.

Discussion

N is typically the limiting nutrient in many freshwater ecosystems of southwestern United States (Grimm and Petrone 1997). Horne and Carmiggelt (1975) showed that N_2 fixation rates in northern Californian streams with NO_3-N levels of 0.002 mg L^{-1} can be quite high in periphyton communities dominated by *Nostoc*. Hill and Knight (1988) who

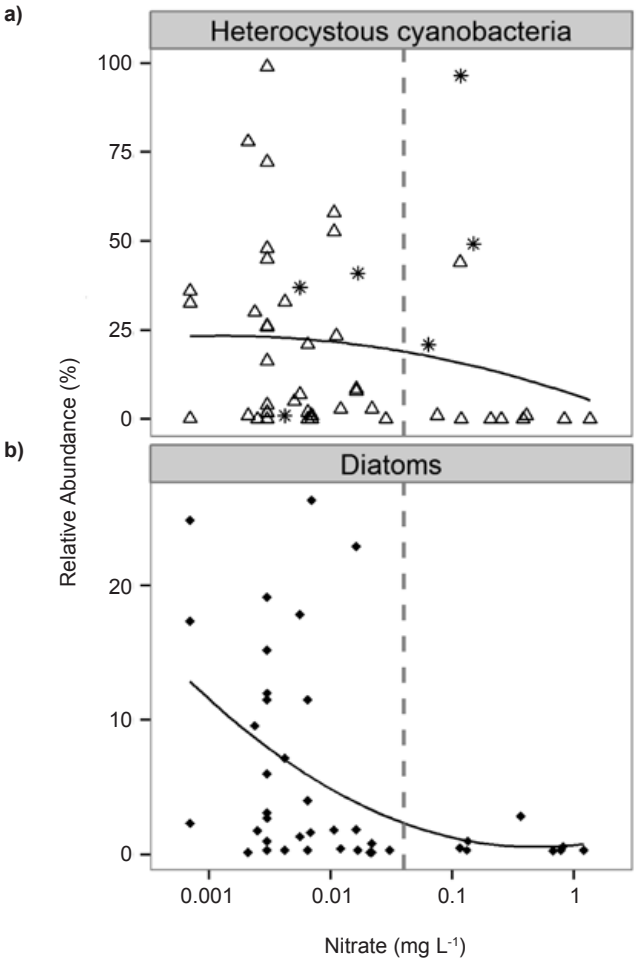

Figure 4. Distribution of N_2-fixers along the NO_3-N gradient in study streams. Only sites where N_2-fixers were recorded are shown, and only one sample per site (selected at random) is included. Open triangles correspond to the percent of total non-diatom algal biovolume comprised of heterocystous cyanobacteria (n = 51), asterisks mark sites where *Rivularia minutula* was recorded, Spearman rank ρ = -0.49, p = 0.001 (a); solid diamonds indicate the percent of total diatom valves comprised of *Epithemia* and *Rhopalodia* (n = 41), Spearman rank ρ = -0.29, p = 0.04 (b). The area to the left of the dashed line corresponds to N-limiting conditions in the data set (N:P <15:1), as well as the NO_3-N range within which N_2 fixation detected by separate molecular analyses. Note that the x-axis is log_{10}-scaled.

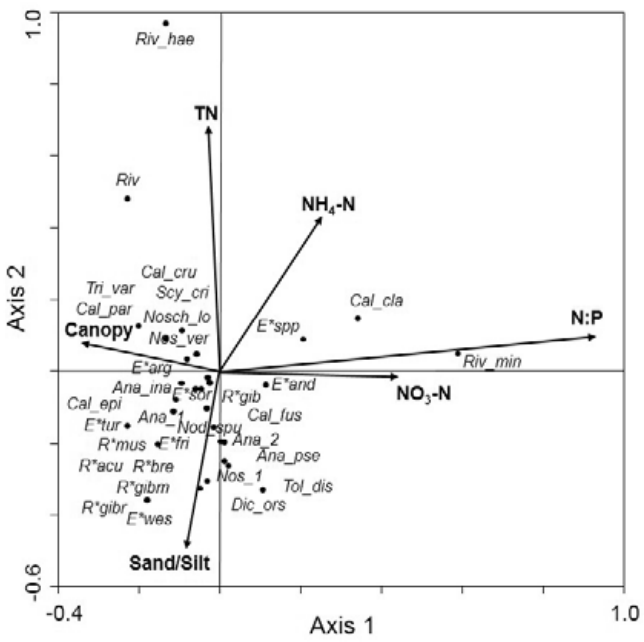

Figure 5. CCA ordination diagram showing the relation between N_2-fixer communities (heterocystous cyanobacteria and diatoms *Epithemia* and *Rhopalodia*) and significant environmental variables. Species name abbreviations are listed in Table 2. TN refers to filterable TN.

investigated two streams in northern California determined that the *Nostoc*, *Tolypothrix*, *Epithemia*, and *Rhopalodia* were widely distributed at low levels of N (<0.014 mg L^{-1}) and low N:P ratios. Similarly, Grimm and Petrone (1997) documented very high N_2 fixation rates for *Anabaena* and *Nostoc* mats, and epilithic *Calothrix* species in an Arizona desert stream with an ambient NO_3-N concentration of 0.06 mg L^{-1} and a low N:P ratio. Our field observations from a large stream data set corresponded well with the abovementioned regional studies on stream nutrient limitation. In addition, we provided several lines of evidence that stream nutrient levels can cause measurable changes in species composition and structure of algal community and hence enhances the suitability of using N_2 fixers as bioindicators for nutrient impairment in the study area. Molecular determination of conditions favoring N_2 fixation strongly validated our observations from the 104-site field stream survey.

We observed that both types of N_2-fixing cyanobacteria (i.e., free-living heterocystous and endosymbionts in *Epithemia* and *Rhopalodia*) showed similar threshold respond to an ambient nutrient conditions (threshold definition after Stevenson *et al.* 2012). Both were often recorded and very abundant in streams with NO_3-N <0.075 mg L^{-1}, NH_4-N <0.04 mg L^{-1}, and N:P ratios <15:1, above which their relative abundance and biodiversity sharply declined. N:P ratios <16:1 are considered to be N-limiting and promoting the growth of N_2-fixing cyanobacteria (Lohman *et al.* 1990, Pearl 1990). Nitrogenase gene expression detected herein suggested that

Table 3. Detected gene expression in N_2-fixing algae *Nostoc, Calothrix*, and *Rhopalodia gibba* cyanobacterial endosymbionts, and *Cocconeis, Cladophora*, *Ulva* from 16 streams sampled in September-October 2010 and May-June 2011. + = gene expression was detected; - = gene expression was not detected; N/A = gene expression was not tested (in the case of *Rhopalodia gibba*, reference is made to the genes of its cyanobacterial endosymbiont); BDT = below detection limit, which is 0.0002 mg L^{-1}; and LM = light microscopy.

NO_3-N (mg L^{-1})	PO_4-P (mg L^{-1})	Stream ID	Year	*Nostoc nifK*	*Nostoc* 16S rRNA	*Calothrix nifK*	*Calothrix* 16S rRNA	*Rhopalodia nifK*	*Rhopalodia* 16S rRNA	*Cocconeis* 18S rRNA	*Cladophora* 18S rRNA	*Ulva rbcL*	*LM identification of Nostoc, Calothrix, Rhopalodia, Epithemia*
BDT	0.0073	IC	11	+	+	+	+	-	+	N/A	N/A	N/A	*N. verrucosum, Calothrix* sp.
BDT	0.0044	SJTC2	11	+	+	+	+	-	-	N/A	N/A	N/A	*N. verrucosum, Calothrix* sp.
0.0002	0.0116	SJTC3	10	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, Calothrix sp., R. gibba, E. turgida, E. sorex*
0.0009	0.0108	TJKT2	11	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, Calothrix sp., R. gibba, E. turgida, E. sorex*
0.0014	0.019	SMAS1	11	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, C. epiphytica, R. gibba, R. gibberula, E. turgida*
0.0018	0.0233	SLFY1	11	+	+	+	+	-	+	N/A	N/A	N/A	*Nostoc* sp.
0.0025	0.0251	PC	11	+	+	+	+	-	+	N/A	N/A	N/A	*Nostoc* sp.
0.0034	0.0137	SASA4	10	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, N. parmelioides, R. gibba*
0.0042	0.006	TJKT2	10	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, Calothrix sp., R. gibba, E. turgida, E. sorex*
0.008	0.0304	DGSY1	10	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, Calothrix sp., R. gibba, E. adnata, E. turgida, E. sorex*
0.0096	0.0099	TJPC2	11	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, Calothrix sp., R. gibba, R. gibberula, E. turgida, E. sorex*
0.0105	0.0079	SJBL1	10	+	+	+	+	+	+	N/A	N/A	N/A	*N. verrucosum, Calothrix sp., R. gibba, E. turgida, E. sorex*
0.0735	0.0223	DGSY1	11	+	+	+	+	-	+	N/A	N/A	N/A	*N. verrucosum, Calothrix* spp.
0.1477	0.0011	SAMI1	10	-	-	-	-	-	-	+	-	-	*Epithemia sp.*
0.2468	0.0437	CBLA1	10	-	-	-	-	-	-	+	+	+	Not observed
0.3224	0.0057	SGCT1	11	-	+	-	+	-	-	+	+	-	*Calothrix* cf. *parietina*
6.2735	0.2299	SDSD3	10	-	-	-	-	-	-	+	+	-	Not observed
11.207	0.1025	SMRC1	10	-	-	-	-	-	-	+	+	-	Not observed

heterocystous cyanobacteria *Nostoc* and *Calothrix*, and inhabitants in diatoms (*Rhopalodia* and presumably *Epithemia*) fixed atmospheric N_2 at ambient NO_3-N <0.074 mg L^{-1} and <0.01 mg L^{-1}, respectively, which corresponded very closely with NO_3-N threshold, as revealed by the field study. Furthermore, our morphometric study on cyanobacterial endosymbions in *Epithemia* and *Rhopalodia* suggested that their biovolume is a function of NO_3-N availability under low ambient NO_3-N concentrations (<0.02 mg L^{-1}). Although N_2-fixing algae were sometimes recorded in higher ambient NO_3-N concentrations (>0.075 mg L^{-1}, but not exceeding 1.36 mg L^{-1}), their abundance was very low and we did not detect nitrogenase gene activity in samples where NO_3-N exceeded 0.14 mg L^{-1}. This result is in very good accordance with Reir and Stevenson (2006) who reported that algal growth rates in stream mesocosms were N limited below 0.086 mg L^{-1} fiterable inorganic N and molar N:P ratios of 12:1.

While all species of *Epithemia* and *Rhopalodia* were uniform indicators of stream N-limiting conditions, and their abundance declined under increased availability of fiterable inorganic N, a second peak in the relative biovolume of heterocystous cyanobacteria was observed under elevated N:P ratios (>30:1), NO_3-N (0.1-0.25 mg L^{-1}), and NH_4-N (0.01 to 0.02 mg L^{-1}). It was attributable to an increase in a members of Rivulariaceae, known for their phosphatase-producing capability, which may confer a competitive advantage in an inorganic-phosphate-poor environment (Mateo *et al.* 2010). Conditions of phosphate deficiency influence the trichome morphology of Rivulariaceae by inducing the formation of long colorless multicellular hairs, which are the sites of phosphomonoesterase activity (Whitton and Mateo 2012). We observed long hairs (up to 250 µm; Figure 1b) in all populations of *Rivularia minutula* in the field data set (n = 6 out of 104 sites) and in another member of Rivulariaceae: *Calothrix* cf. *parietina*, collected for the molecular assay from *R. minutula'* locality. Nitrogenase gene expression, however, was not detected for *Calothrix* in that sample. This finding may be indicative of P-limitation, instead of N-limitation in some of the studied streams, because cyanobacterial N_2 fixation is likely repressed when preferred inorganic N sources (such as NO_3-N or NH_4-N) are bioavailable. According to Rhee (1974), algae respond to nutrient limitation with sharp transitions between limitation by one nutrient vs. another. While two or more nutrients can be simultaneously near growth-limiting concentrations, only one will actually be limiting (Borchardt 1996). In addition, the CCA showed that *R. minutula, R. haematites*, unidentified Rivulariaceae, and *Calothrix clavata* were far removed from the rest of N_2-fixing cyanobacteria due to their association with elevated N:P ratios and fiterable inorganic N concentrations. On the other hand, *Rivularia* is characteristic of environments where nutrient ratios and concentrations vary during the year (Yelloly and Whitton 1996), so the difference between the nutrient environment of *R. minutula* and the other cyanobacterial taxa may reflect seasonality, because all our samples of this species were collected during the summer. However, we documented species-specific response to nutrient limitation by heterocystous cyanobacteria in studied streams (albeit, always at the low end of the NO_3-N concentration gradient) and therefore provided data for their nutrient ranges and optima at species level.

In our data set, free-living heterocystous cyanobacteria and endosymbiont-containing diatoms were recorded together, and in significant quantities, only in streams under N-limiting conditions (based on N:P ratios). Conversely, N_2 fixers from either diatom or non-diatom algal assemblages could be found in the absence of the other in some streams with higher N:P ratios. We found that when the two algal assemblages were used separately for inferring nutrient conditions, over 20% of N-limited sites remained unidentified as such, based on algal community data. Thus our results suggest that the use of diatoms in conjunction with non-diatom algae provides a more robust assessment (i.e., "weight-of-evidence") of nitrogen limitation than monitoring with either algal group alone.

The differential effects of various environmental factors, aside from nutrients, on the distribution of N_2 fixers, free-living and endosymbionts in *Epithemia/ Rhopalodia*, provides further motivation for utilizing both diatom and non-diatom algae in assessments of stream nutrient status. Light and temperature are well established as variables that control algal growth and N_2-fixation rates and in streams (Hill and Knight 1988, Grimm and Petrone 1997, Marcarelli *et al.* 2008), but our results showed that microhabitat characteristics and silicon deficiency may be important as well (Martin-Jézéquel *et al.* 2000). Substratum type may play a role in determining whether N_2-fixing free-living cyanobacteria or in diatoms dominate in certain sites, and we found diatoms *Epithemia* and *Rhopalodia* were more likely

than heterocystous cyanobacteria to inhabit sites with fine substrata, in accordance with Potapova and Charles (2005). Differences in light response by major taxonomic categories of algae (i.e., diatoms and cyanobacteria) have been suggested, and indeed, taxonomic categories are defined in part by differences in light-capturing pigments and membranes (Hill 1996). Moreover, the cyanobacterial endosymbionts in *Rhopalodia gibba* do not seem to be photosynthetically active and probably import energy-rich molecules from the host for the nitrogen-fixation process (Prechtl *et al*. 2004). This phenomenon may explain our findings that *Epithemia* and *Rhopalodia* were relatively more abundant in more shaded sites, while the heterocystous filamentous cyanobacteria were more abundant in sites with less shading.

Finally, our study contributed to a better understanding of quantitative relationships between stream benthic algae and nutrients, and can inform regional refinement of algal indicators in order to enhance their utility in stream bioassessment. This work represents one of the first efforts to integrate host-associated quantitative molecular methods with algal species composition and environmental variables from a large stream data set. The interdisciplinary strategy we used not only demonstrates increased resolution in assessing the status and impact of nutrients on California streams, but also suggests several other avenues of research that will help to improve this approach. Much research is needed to relate the response, acclimation, and seasonal patterns of algal species composition to biogeochemical processes.

Taken together, our results provide weight-of-evidence for a threshold of response of stream heterocystous cyanobacteria and endosymbionts in *Epithemia* and *Rhopalodia* to ambient nutrient concentrations and limitation, based on data collected at different scales, and using different types of measurements. Our findings indicate that abundance of heterocyst-containing cyanobacteria and endosymbiont-containing diatom cells could be assessed for rapid nutrient biomonitoring. Furthermore, because heterocystous cyanobacteria and *Epithemia/Rhopalodia* were not always recorded together at N-limited sites, we suggest that both cyanobacterial and diatom assemblages be used jointly for assessment of stream nutrient limitation based on algal community composition, as this approach provides a more comprehensive assessment than examining either assemblage alone.

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Supplemental Information

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