Final Report

For the Project

Linking Observational and Experimental Approaches for the Development of Regional Nutrient Criteria for Wadeable Streams

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Study designs, choice of data analyses, presentation and interpretation of results, and conclusions and recommendations are those of the Principal Investigator and not those of the U.S. EPA, and no official endorsement from the U.S. EPA should be inferred.

EXECUTIVE SUMMARY

We employed a novel approach to developing a defensible, effects-based numerical target concentration of surface-water nutrients for wadeable streams in the Cross Timbers portion of Aggregate Nutrient Ecoregion IX of U. S. EPA Region 6. Our approach relied on an integration of collected field data from wadeable streams in Texas with experimental data generated from stream mesocosms at the Baylor Experimental Aquatic Research (BEAR) facility.

Observational Field Study

The observational field component of this study was conducted from August 2006 through June 2008. Data were collected quarterly (8 sampling events) from 26 wadeable streams in the Brazos River basin within the Cross Timbers Level III Ecoregion. The selected streams spanned a steep gradient of phosphorus enrichment (< 10 ug/L to >2000 ug/L TP). Sites with low, moderate, and high levels of P enrichment were widely distributed throughout the study area. The study design captured the full range of natural variability in geology, drainage networks, stream size, and other physiographic factors, while still achieving a wide spatial distribution of P enrichment and its likely sources (wastewater effluent discharges, pasture).

Field streams had similar hydrographs during the 2-year study. Differences in discharge were much greater among seasons and years rather than differences among the individual streams. August 2006-February 2007 was a drought; discharge was very low in all streams except those near effluent discharges. Heavy rains led to flooding in spring 2007, and streams maintained relatively high base-flow discharges through the remainder of the study.

Surface-water nutrients, particularly PO_4-P and TP, were more strongly influenced by differences among individual streams than by seasonal patterns of discharge. Streams with apparently few point or non-point sources of phosphorus exhibited consistently low TP. Streams with predominantly non-point sources of phosphorus (watersheds with pasture or other sources) showed sharp increases in TP during high discharge, reflecting transport of P from the land from surface-water runoff. Streams with point sources (effluent discharges) either had consistently high TP or had moderate declines in TP due to dilution by high surface-water runoff.

Periphyton, Filamentous Algae, and Macrophyte Responses to TP

We hypothesized excessive levels of enrichment of surface-water nutrients would be reflected in sharp changes in enrichment of the tissues of the periphyton. Indeed, sites with different levels of surface-water PO_4-P or TP differed markedly in their periphyton C:P (carbon-to-phosphorus) and N:P (nitrogen-to-phosphorus) molar ratios. TP consistently predicted nonlinear, threshold declines in periphyton C:P ratios. Sites with elevated surface-water TP had virtually constant periphyton C:P or N:P ratios over time (typically less than 300 and 20, respectively). Threshold declines in periphyton C:P, N:P, and C:N ratios were highly likely between 15 and 25 ug/L TP, with 19-20 ug/L representing the most consistent concentration yielding a threshold response. Moreover, despite the high variability of DIN (dissolved inorganic nitrogen) in surface waters

among sites over time, the periphyton N:P ratio was essentially identical to the C:P ratio, illustrating that P, not N, was driving differences in these ratios.

Significant threshold changes were detected in several of the periphyton, algal, and macrophyte biological response variables in response to TP. The thickness of microbial films (microalgae and other microbes; non-filamentous algae) growing on rocks showed consistently nonlinear declines in response to TP levels above 20 ug/L. This metric was computed as the percentage of observations in a reach (out of a maximum of 100 points along the reach-scale transect) in which biofilms exceeded 1 mm in thickness on rocks. Streams with TP < 20 typically had moderate to heavy growth of calcareous periphyton.

Streams above the threshold value (20 ug/L) usually had a thinner, darker layer of biofilms on the rocks but also tended to have more filamentous green algae. There were 2 distinct changepoints in filamentous algal cover: a consistent increase in cover among sites >20 ug/L TP, and a less consistent but more dramatic increase in cover at very high levels of TP (>200-1000 ug/L). Because filamentous algal cover depends highly on seasonal changes in light and water temperature, as well as sloughing from overgrowth and scouring from high flows, it is not surprising that many of the highly enriched sites had little filamentous algal cover on some dates. However, when high filamentous algal cover occurred, it was almost always associated with levels of P enrichment > 20 ug/L, and particularly at >200 ug/L.

The ratio of periphyton chlorphyll to AFDM revealed a nonlinear shift toward a greater fraction of chlorophyll-bearing organisms in the periphyton in response to TP > 20-30 ug/L. A secondary threshold appeared around 200 ug/L, likely related to the 2-tiered response of filamentous algae and chlorophyll-a at 20 and 200 ug/L TP.

One of the most important patterns that emerged from the field study was the sharp threshold declines in submersed macrophyte cover in response to TP. The two dominant submersed macrophyte taxa were *Najas* (a vascular plant) and *Chara* (a nonvascular charophyte); both virtually disappeared in streams with TP exceeding 25-54 ug/L TP.

Diel Dissolved Oxygen Response to TP

Interannual differences in stream discharge strongly influenced the daily (diel) variation in dissolved oxygen, water temperature, and pH. In 2006, a drought year, most of the field study streams had very low-to-no measureable discharge. This lack of flow limited the turbulent mixing of the water column. During the day, streams were supersaturated with dissolved oxygen (up 20 mg/L DO; > 250% saturation) which concomitantly caused pH to increase to relatively high levels (8.5-9.5). At night, DO was consumed and collapsed below 2 mg/L in some of the study streams. In September 2007, discharge was relatively high in all 26 study streams. Most of the streams were in flood stage for several of the preceding months and had recently subsided to wadeable flows. With higher flows and turblent mixing, minimum DO levels were rarely below 5 mg/L.

The harmful (0-2 mg/L) minimum DO values at several of the sites in 2006 were related to surface-water TP. All 6 streams with low flow and TP > 27.2 had minimum DO values < 2 mg/L, and two others with TP near 20 ug/L dropped below 3.5 mg/L DO. The four streams located immediately downstream of effluent discharges maintained relatively high minimum DO concentrations despite very high TP. This was related to reaeration associated with turbulent mixing and artificially high flows during a drought when most other streams were not flowing. However, these artificially high flows quickly declined within a few kilometers from the upstream effluent discharges due to evaporation and infiltration below the stream channel. Here the lack of reaeration by turbulent mixing coupled with high nutrient levels from the effluent caused DO to be almost completely consumed at night. Thus, effluent discharges were having dramatic effects on DO, but farther downstream than typically considered in monitoring studies.

Minimum DO in 2006 was also predicted significantly by mean biofilm thickness, filamentous algal cover, and the ratio of periphyton chlorophyll to AFDM. Thicker layers of calcareous periphyton were associated with low-P streams, and as TP increased, these biofilms succeeded to filamentous and colonial greens and other types of algae. Thus, these metrics appear to be sensitive to TP enrichment and may be linked quantitatively to aquatic life use standards that rely on dissolved oxygen as a measure of biological integrity.

Algal Species Composition Response to TP

Multivariate analyses of algal species composition during August 2006 and September 2007 continued to support the earlier conclusions that TP and related metrics had a strong influence on stream biota. In both years, most of the variance in algal species composition was related to TP amd related measures. Sites with the lowest TP and highest periphyton C:P ratios were clearly grouped away from sites with the highest TP and lowest C:P ratios, which were also distinctly grouped. Although algal species changed between years, the relative grouping of sites from low P to high P remained intact regardless of year. This was compelling evidence that algal species composition was altered by nutrient enrichment, and the magnitude of its effect was similar regardless of interannual variation in stream discharge.

Threshold Indicator Taxa Analysis (TITAN) continued to reveal stream biological thresholds at low levels of TP. Dozens of algal species essentially disappeared from streams between 12 and 30 ug/L TP, with the threshold of greatest overall decline at 19.2 (2006) and 21.6 (2007) ug/L TP, respectively. Simultaneously, numerous algal taxa showed sharp increases, either replacing taxa as they declined, or driving the declines via competitive exclusion (e.g., shading by *Cladophora,* a significant increasing taxon in both years).

Although most taxa declined or increased at relatively low levels of TP, a few taxa responded only to much higher levels of TP. These taxa were mostly associated with sites heavily influenced by effluent, and suggested a second tier of biological degradation at very high levels of TP (200-500 ug/L).

Macroinvertebrate Taxa Responses to TP

Results of ordinations and Threshold Indicator Taxa Analysis (TITAN) on macroinvertebrate taxonomic composition during November 2006 showed that nutrients, specifically phosphorus, corresponded to shifts in community structure. Because composition differed substantially between sites with and without flow, and because of established relationship between flow and other water chemistry variables (e.g., DO), we separated sites into groups by flow status prior to analysis.

Sites with no flow yielded more reliable relationships to TP than sites with flow, but this result should be interpreted with caution because there were only 9 sites in the group with flow. Ordinations clearly showed that in both sets of analyses, the sites with low TP and high periphyton C:P sorted together, whereas high TP and low C:P sites sorted away from the low TP sites. In both sets of TITAN analyses, more taxa were classified as negative threshold indicators (decline in response to TP) than as positive ones (increase).

Experimental Stream Study

The experimental stream study was conducted from 31 January to 7 April 2008 at the Baylor Experimental Aquartic Research (BEAR) facility. Twelve streams were used in the study. The experimental streams are approximately 0.6 m wide and 20 m in length. The streams are stratified into riffle, glide, and pool sections and are designed mimic natural habitat in central Texas streams. Water is pumped from the adjacent Lake Waco Wetland and delivered to the streams via a series of 12 valves, each adjusted to regulate flow equivalently among all 12 streams (180 L/min). Before entering the streams, water iss pumped into a mixing tank where chemicals representing experimental treatments can be dosed at a fixed rate using peristaltic pumps connected to a second, adjacent tank of chemical stock solution. Once dosed with the treatment levels of non-toxic chemicals, water is released into the streams and is discharged back into the Lake Waco Wetlands.

Clean river cobble and gravel were placed in streams in January 2008 to simulate erosional habitat in natural stream in our field study. Stream flow was initiated on 31 January 2008 and calibrated to 182 L/min (+/- 5 L/min) for each stream. Streams were seeded with organic matter, periphyton, and macroinvertebrates collected from two of the intensive field sites. These sites were chosen because their P concentrations were representative of the background (control) and high P treatments to be employed in the experiment. Streams were seeded twice: 1 February and 15 February 2008. Seeding was accomplished by transplanting organisms and organic matter from five 1-m^2 kick screen samples collected from into each of the 12 streams.

The streams were allowed to run without any nutrient additions from 31 January to 10 March 2008 in an effort to allow growth of periphyton at low nutrient concentrations. Established periphyton communities from riffle habitat from two intensive field sites, ROCK-01 (very low ambient phosphorus levels; 1-5 ug/L PO4-P, 4-12 ug/L TP) and NBOS-03 (moderately high P levels: 50-200 ug/L PO4-P, 75-250 ug/L TP) were also transplanted into the streams. Cobbles from ROCK-01 (300) and NBOS-03 (300) were placed in random cross sections throughout the

reach of each experimental stream on 7 and 8 March, respectively, a few days prior to initiation of the experiment.

Experimental dosing of phosphorus began on 11 March 2008. Background concentration of PO4-P during the colonization phase of the study was 6 and increased to 8 ug/L during the experiment. Four streams were randomly assigned no additional phosphorus, and thus a target concentration of **8 ug/L (Control)**. Because our field study had identified consistent nonlinear changes in numerous biological response variables between 10 and 20 ug/L PO4-P (15-25 ug/L TP), four streams were assigned a treatment of +15 ug/L PO4-P to achieve a target PO4-P concentration of **20 ug/L (Low P treatment)**. The remaining four streams received +95 ug/L PO4-P to achieve a target concentration of **100 ug/L (High P treatment).**

Periphyton and Filamentous Algae Response to P Dosing

Periphyton growing on the ceramic tiles responded rapidly to experimental P dosing. High P treatments had significantly more chlorophyll than Low-P and Controls on Day 14. By Day 28, Low P and High P treatments were not different, but had significantly more chlorophyll-a than Controls. The lack of difference between low and high P treatments is consistent with the threshold response observed in the field study.

Periphyton (biofilm) AFDM and chlorophyll-a from the bare rocks and transplanted rocks was highly variable among P treatments and did not differ significantly. However, this was largely due to the very strong response of *Cladophora* (filamentous green algae) in the low and high P treatments. *Cladophora* biomass exploded near the end of the study, and was significantly higher in the Low and High P streams than the Controls on the bare rocks and transplant rocks (ROCK-01, NBOS-03) on Day 28.

Periphyton C:P ratios from non-transplant bare rock samples differed significantly by treatment on Day 28. High P treated streams had the lowest C:P ratio (~150), Controls had the highest (~320) whereas Low-P streams were intermediately enriched (~230). Control C:P ratios were approaching levels deemed to be near or below a C:P threshold in the field study, suggesting that even the Control PO4-P (8 ug/L) and TP (19-20 ug/L) concentrations were high enough to cause sharp changes in periphyton nutrient content.

Periphyton C:P ratios from the ROCK-01 transplants (a field site with PO4-P < 5 ug/L and TP<10 ug/L) responded very strongly to all 3 experimental treatments. On Day 0, ROCK-01 periphyton had C:P ratios above 2,000. After transplanting these rocks into the BEAR streams for just 7 days, mean C:P ratios had dropped to 689, 346, and 215 among the Control, Low P and High P treatments, respectively. By Day 28, ROCK-01 Control C:P ratios had dropped to 250, whereas Low and High P values were near 150. This illustrated the remarkable affinity of stream periphyton for phosphorus, and provided additional evidence in support of for nonlinear uptake of P as the explanation for sharp declines in periphyton C:P with small increases in surface-water TP.

In contrast, the NBOS-03 transplants (a field site with PO4-P 50-100 ug/L and TP 75-200 ug/L) did not respond to any of the experimental P treatments on Day 7 or 28, supporting the hypothesis that recycling of P within the periphyton is an important mechanism for maintaining low C:P ratios over time in streams with highly variable surface-water TP. Moreover, it also showed that periphyton from NBOS-03 was already saturated with P and thus did not sequester more P per unit carbon in the low or high P treatments.

Algae and Macroinvertebrate Taxonomic Response to P Dosing

Transplanted algae from ROCK-01 were significantly different among P treatments on Day 28. ROCK-01 samples that were transplanted into Low and High P streams shifted significantly away from Control streams by day 28, and increasingly resembled algal communities from P-enriched field sites. However, consistent with a nonlinear threshold response, algal species composition in Low and High P treatments did not differ. In contrast, transplanted algae species composition from NBOS-03 did not differ among treatments after 28 days of exposure.

Indicator Species Analysis revealed that at least 5 diatom species were significantly less abundant (or absent) from ROCK-01 transplants in Low P and High P streams than Controls, whearas 2 species were significantly more abundant in the Low and High P treatments. Five of these 7 taxa also were identified as significant threshold indicators in response to TP gradients in the field study, validating the responses as caused by P. All five responded in the same direction (decline, increase) in the BEAR study as in the field study.

Macroinvertebrate taxonomic composition did not differ among treatments on Day 28. The relatively short dosing period, in contrast to the relatively long life cycle of these taxa, necessarily limited the potential response of this diverse group of organisms to P treatments. Future experiments focused on the effect of low flow and P interactions, or longer dosing periods, may produce more meaningful results in the context of animal responses to experimental P enrichment.

Conclusions and Recommendations

Shifts from periphyton communities comprised of sensitive diatoms, calcareous cyanobacteria, and other non-chlorophyll bearing microbes to communities with higher chlorophyll content and more filamentous green algae was repeatably demonstrated at concentrations of surface-water TP above 20 ug/L. Streams with TP > 200-1000 ug/L likely represent a second tier of degradation, and appear at greater risk for nuisance algal growth based on our threshold analyses. However, results from the P dosing experiment suggest that concentrations as low as 20 ug/L PO4-P can lead to high levels of *Cladophora* biomass in as little as 28 days. Adding more PO4-P (100 ug/L) did not result in more *Cladophora* in a 4-week period, but did result in more *Cladophora* after only 2 weeks. Thus, because faster growth rates were observed in the 100 ug/L treatment, field streams with very high levels of TP will have a greater probability of heavy coverage of filamentous algae because biomass accumulation between scouring or sloughing events will be more rapid.

Aquatic macrophyte cover consistently declined in streams with TP > 25-50 ug/L. These submersed plants serve as important refugia for juvenile fishes and macroinvertebrates, and provide a source of dissolved oxygen during low flows. Their decline likely represents key structural and functional degradation to these stream ecosystems.

Minimum dissolved oxygen levels are highly dependent upon an interaction between flow and nutrient enrichment. Our study suggests that TP levels > 20-30 ug/L, coupled with low flows, will cause detrimental declines in minimum dissolved oxygen levels. This is particularly important in the context of minimum flows, a contentious issue in the southwestern USA. It is unlikely that studies geared toward detecting effects of nutrients on DO will adequately characterize this relationship without sampling during periods of low flow when gas exchange with the atmosphere is very slow. Distance downstream and flow status are two very important considerations when evaluating the influence of WWTP discharges on wadeable streams in semi-arid regions. Future research is needed to better quantify the interaction between minimum flows and biological integrity in streams.

The weight of evidence from both the field stream study and experimental stream study demonstrates that streams of the study area are very sensitive to phosphorus enrichment. There is a very high probability that streams exposed to surface-water TP levels exceeding 20 ug/L, and possibly 15 ug/L, will experience a sharp decline in biological integrity, including loss of characteristic structure (periphyton and macrophytes), loss of numerous species (algae and macroinvertebrates), minimum dissolved oxygen levels unsuitable for supporting native fauna during low flows, and increase likelihood of nuisance algal growth that limits recreational use of strreams. Streams exceeding 200 ug/L may represent a second tier of degradation, with more consistent nuisance algal growth and additional losses of algal and macroinvertebrate species.

Table 1. Key to variable short names used throughout this document.

Variable	Description
SITE_ID	First 4 letters of site name followed by number (>1 for streams with more than one site)
NH3-N	Ammonia-nitrogen, surface water, ug/L
NO2NO3-N	Nitrite + nitrate-nitrogen, surface water, ug/L
PO4-P	Orthophosphate, surface water, ug/L
TN	Total nitrogen, surface water, ug/L
TP	Total phosphorus, surface water, ug/L
TURB_NTU	Turbidity, surface water, NTU
CHLA_UGL	Chlorophyll-a, surface water, ug/L
C_ALG	Total carbon, organic fraction of periphyton, %
C_BULK	Total carbon, bulk periphyton, %
C_SED	Total carbon, sediment fraction of periphyton, %
N_ALG	Total nitrogen, organic fraction of periphyton, %
N_BULK	Total nitrogen, bulk periphyton, %
N_SED	Total nitrogen, sediment fraction of periphyton, %
P_ALG	Total phosphorus, organic fraction of periphyton, %
P_BULK	Total phosphorus, bulk periphyton, %
P_SED	Total phosphorus, sediment fraction of periphyton, %
CN_ALG	Carbon:nitrogen ratio, OM fraction of periphyton
CN_BULK	Carbon:nitrogen ratio, bulk periphyton
CN_SED	Carbon:nitrogen ratio, sed fraction of periphyton
CP_ALG	Carbon:phosphorus ratio, OM fraction of periphyton
CP_BULK	Carbon:phosphorus ratio, bulk periphyton
CP_SED	Carbon:phosphorus ratio, sed fraction of periphyton
NP_ALG	Nitrogen:phosphorus ratio, OM fraction of periphyton
NP_BULK	Nitrogen:phosphorus ratio, bulk periphyton
NP_SED	Nitrogen:phosphorus ratio, sed fraction of periphyton
CHLA_M2	Chlorophyll a, periphyton, mg/m2 (rock surface area)
AFDM_M2	Ash-free dry mass, periphyton, g/m2
AFDM_PCT	Percent of dry mass as organic matter
CHL_AFDM	Chlorophyll-a:AFDM ratio, periphyton, mg/g
DISCHARG	Stream discharge, cubic meters/second
DO_MGL	Dissolved oxygen, mg/L
DO_PCT	Dissolved oxygen, %
PH	pH
SALINITY	Salinity, ppt
SPCOND	Specific conductance, uS/cm
TEMP	Water temperature
CANOPY	Tree canopy cover, mean %
VELOCITY	Stream velocity, mean m/s
THAL_DEP	Thalweg depth, mean cm
WETWIDTH	Stream wetted width, mean m
DEPTH	Water depth, mean (100 points)
FLOW_N	No flow velocity detected, % (100pts)
FLOW_M+	Flow velocity>0.2 m/s, % (100pts)
SED_CV	Sediment index, mean (100pts)
SED2+	Sediment index > 3, % (100pts)

SED3+	Sediment index > 2, % (100pts)
WET_PCT	% of reach with surface water (100pts)
BED_PCT	Bedrock, % (100pts)
BO_PCT	Boulder, % (100pts)
CO_PCT	Cobble, % (100pts)
GR_PCT	Gravel, % (100pts)
GRCO_PCT	Gravel+cobble, % (100pts)
SA_PCT	Sand, % (100pts)
SL_PCT	Silt-clay, % (100pts)
FINE_PCT	Silt-clay + sand, % (100pts)
MACALG_C	Filamentous algae cover score, mean (100pts)
MACALG3+	Filamentous algae cover > 25%, % (100pts)
MACALG4+	Filamentous algae cover > 50%, % (100pts)
MACALG5+	Filamentous algae cover > 75%, % (100pts)
MICALG_R	Biofilm thickness, mean (100pts)
MICALG2+	Biofilm thickness index > 0.5 mm, % (100pts)
MICALG3+	Biofilm thickness index > 1 mm, % (100pts)
PLNT_CV	Submersed macrophyte cover index, mean (100pts)
PLNT1+	Submersed macrophyte cover>0, % (100pts)
PLNT3+	Submersed macrophyte cover>25%, % (100pts)

PROBLEM DEFINITION AND BACKGROUND

Nutrient pollution is the most common cause of degraded water quality in lakes, streams, wetlands, and estuaries in the USA. Excessive inputs of nutrients, specifically phosphorus (P) and nitrogen (N), have many negative effects on aquatic ecosystems. One of the most noticeable consequences of nutrient pollution is the accelerated growth of aquatic vegetation. Termed "eutrophication," this increase in productivity caused by nutrient enrichment produces an undesirable disturbance to the balance of organisms present in the water. For instance, eutrophication is commonly associated with explosive growths of nuisance algae that can taint drinking water supplies, cause foul odors, and even harm humans. Eutrophication can also result in dissolved oxygen shortages that kill fish and other aquatic organisms and reduce aquatic biodiversity. Thus, nutrient pollution can significantly limit the ability of a water body to support its designated uses, such as fishing, swimming, or other recreational activities.

Nutrient criteria and total maximum daily load (TMDL) implementation plans are developed by States to improve water quality in streams and reservoirs. A TMDL determines how much of a particular pollutant can be assimilated by an aquatic ecosystem and still maintain its beneficial uses and ecological integrity and is integrally related to a numerical nutrient criterion. In Texas, phosphorus has been identified by the Texas Commission on Environmental Quality as the nutrient that would have the most effect in limiting algal and plant growth in many central Texas streams. TMDL development requires that numerical criteria for the parameters of interest be established in order to calculate necessary load reductions. However, to date, numerical nutrient criteria have largely been developed subjectively and without knowledge of the biological consequences, despite the directive issued by the U.S. EPA in its National Strategy for the Development of Regional Nutrient Criteria (1998). In this document, the U.S. EPA detailed a comprehensive plan for the development of scientifically defensible, numerical water-quality criteria. The plan emphasized the need for the inclusion of endpoints in criteria development that reflect physical, chemical, and *biological* integrity of aquatic ecosystems. However, quantitative linkages between critical levels of nutrients and stream biota have not been well developed in Region 6, largely because historical sampling protocols do not include relevant ecological indicators for streams.

Another major factor limiting the development of numerical nutrient criteria is the lack of experimental evidence to support field monitoring. Experimental studies allow for controlled nutrient treatments and, when coupled with field observations, substantially improve our ability to establish causation between water quality and biological integrity. Thus, coupling experimental evidence with observations from natural streams should allow States to more effectively develop defensible nutrient criteria for streams.

In this study, we employed a novel approach to developing a defensible, effects-based numerical target concentration of surface-water phosphorus for wadeable streams in the Cross Timbers portion of Aggregate Nutrient Ecoregion IX of Region 6. Our approach relied on an integration of collected field data from wadeable streams in Texas with experimental data generated from model streams, or stream mesocosms. We used attributes of species assemblages (macrophytes, filamentous macroalgae, periphyton and macroinvertebrates) as indicators of biological condition in response to observed and experimental phosphorus gradients that spanned multiple aquatic life

use designations. We evaluated a wide range of biological attributes (e.g., periphyton nutrient content and biomass, cover or abundance of nuisance algae, community diversity, ecosystem process metrics) that are predicted by the US EPA Tiered Aquatic Life Use (TALU) conceptual model and Texas' narrative aquatic life use categories to be degraded at different stressor concentrations along a human disturbance gradient (i.e., exhibit deflections from reference at different stressor levels corresponding to aquatic life use categories). Our approach explicitly associated numerical levels of phosphorus to threshold responses in ecological indicators of aquatic life uses as defined by narrative biological criteria in the TALU framework.

The approach we employed is consistent with the mandates of the Clean Water Act and the U.S. EPA's National Strategy for the Development of Regional Nutrient Criteria because we (a) explicitly address biological integrity and associated aquatic life uses, (b) developed cause-effect linkages between nutrient pollution and stream-ecosystem responses by reproducing field responses in an experimental setting, (c) identified critical levels of nutrient pollution that caused harm to biological integrity and aquatic life uses using a weight-of-evidence approach, and (d) estimated the probability of harmful effects associated with levels of nutrient pollution so that managers and stakeholders understand the likely ecological implications of selecting target nutrient concentrations for water quality standards and future TMDLs for streams.

The purpose of this document is to report the results of this two-year study. This report is structured into two main sections:

I. Field (observational) study. This section details the study area, methods, results and interpretation of 2 years of field data collected from 26 wadeable streams in the Cross Timbers Ecoregion of the Brazos River watershed in Texas. These data represent one of the most comprehensive evaluations of the effects of nutrient enrichment on wadeable streams in the USA.

II. Experimental stream study. Part 2 of the report details the results of a short-term phosphorus dosing study conducted in the Baylor Experimental Aquatic Research (BEAR) stream facility.

The Executive Summary and Conclusions and Recommendations provide a synthesis of findings, interpretation, conclusions, and recommendations.

SECTION I. FIELD STUDY

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STUDY AREA AND STREAM SAMPLING

Data were collected from 26 wadeable streams in the Brazos River basin within the Cross Timbers Level III Ecoregion (Figure 1; Griffin et al. 2004). The Cross Timbers ecoregion (Ecoregion 29) is a mosaic of forest, woodland, savanna, and prairie and is currently used mostly for rangeland and pastureland (Figure 2, Table 2).

Watershed variables describing physical characteristics and topography, land use, and distribution of disturbance points (outfalls and dams) were calculated for each site (Table 2). Watershed boundaries for each sample site were automatically digitized in ArcGIS 9.2 with the ArcHYDRO 9 extension using a 1:24,000 scale digital elevation model (DEM) expressed as a 30 m raster, available from the U. S. Geological Survey. Mean slope and elevation were calculated for each watershed using the digital elevation model. Mean annual precipitation was calculated for each watershed from a polygon coverage of average monthly and annual precipitation for the climatological period 1961-90. This dataset was obtained from USDA-NRCS. Number of wastewater outfalls and cumulative outfall (MGD) were calculated for each watershed based on the TCEQ municipal and industrial wastewater outfall shapefile available from http://www.tceq.state.tx.us/gis/sites.html. Landcover class percentages were calculated for each watershed using National Land Cover Database (NLCD 2001) available from http://www.mrlc.gov/nlcd_multizone_map.php. All watershed analyses were performed with ArcGIS 9.2 (ESRI, Redlands, CA.).

We collected physical, chemical, and biological data from each of the selected 26 stream sampling locations on a quarterly basis from August 2006 through June 2008. Locations were permanently marked reaches, defined as 40x the mean stream width, or a minimum of 150-m reach (TCEQ 2005). Selected reaches were required to include erosional (riffle/glide) habitats.

Surface-water nutrient samples (orthophosphate-P [SRP], total phosphorus-P [TP], ammonia-N [NH_3-N], nitrate-nitrite nitrogen [NO_2-NO_3-N], total nitrogen [TN]), chlorophyll-a, and turbidity were collected from each stream on a quarterly basis. Sampling periods were adjusted slightly to ensure sampling was conducted at least 2 weeks following storm flows. SRP, NH_3-N, and NO_2-NO_3-N samples were collected in triplicate and filtered immediately, whereas triplicate unfiltered TP and TN samples were only acidified. Single 1-L chlorophyll-a grab samples were collected and filtered in the laboratory within 24 h. Single 50 mL turbidity grab samples were collected and analyzed in the laboratory within 24 h. All samples were immediately placed on ice, stored in the dark, and transported back to the laboratory and stored at 4°C and analyzed in accordance with the approved project plan.

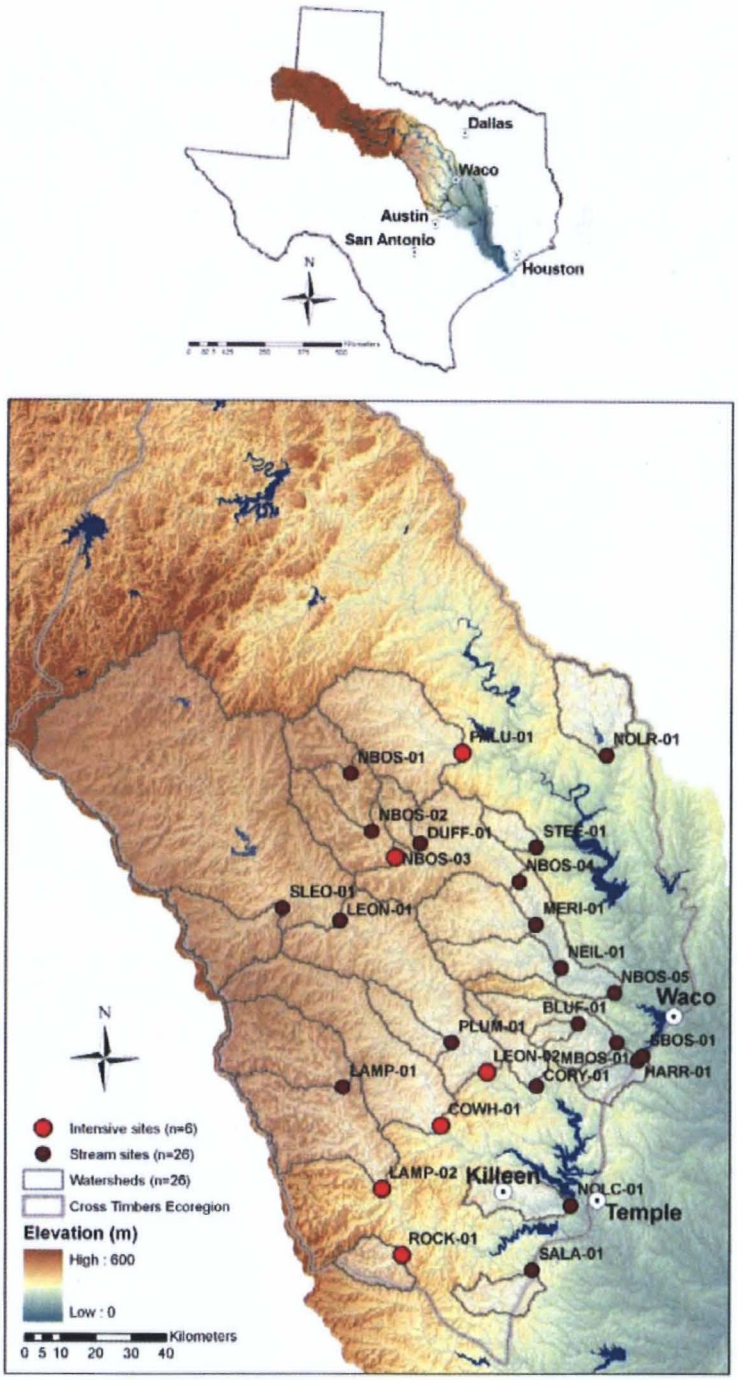

Figure 1. Map of Cross Timbers ecoregion and field study watersheds in relation to Texas and the Brazos River watershed. Twenty-six streams were selected for the field study, which entailed eight quarterly sampling events from August 2006 through June 2008. Six of the 26 streams were sampled for algal species composition during each of the 8 sampling events; these are shown as intensive sites. The remaining streams were sampled twice for algal species composition. All 26 sites were sampled during all 8 events for most other predictor and response variables (see Table 1).

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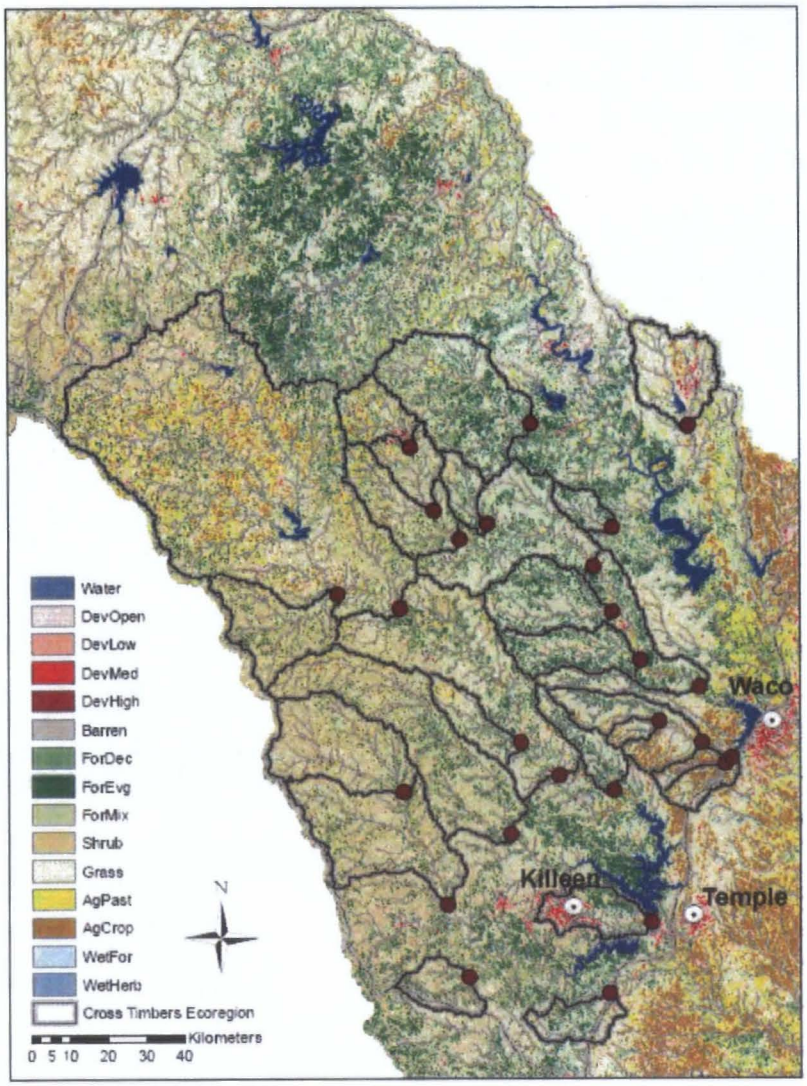

Figure 2. Spatial distribution of dominant land-cover classes among the 26 study watersheds (NLCD 2001). See Table 1 for percent cover of each land-cover class by watershed.

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Table 2. Physiographic and land cover characteristics among the 26 field-study watersheds.

SITE_ID	Precip. (in)	Elev (m)	Slope (%)	Area (km2)	Dams (n)	Outfalls (MGD)	Outfalls (n)	Water (%)	Dev. (%)	Forest (%)	Shrub (%)	Grass (%)	Pasture (%)	Crop (%)	Wetland (%)	Imp.. (%)
BLUF-01	33.4	216	2.83	68	0	0.00	0	0.1	0.6	8.8	0.0	69.1	1.4	17.2	2.9	0.0
CORY-01	33.0	201	4.78	220	0	0.00	0	0.0	1.5	26.9	5.3	59.2	1.5	3.5	2.0	0.2
COWH-01	31.4	232	4.53	1180	2	0.06	1	0.1	0.7	18.8	43.0	33.8	1.6	1.5	0.4	0.1
DUFF-01	31.6	297	2.98	157	0	0.00	0	0.2	0.4	22.4	13.1	57.1	1.5	4.1	1.1	0.2
HARR-01	34.4	153	1.48	77	0	0.00	0	0.1	12.0	1.6	0.0	42.6	3.0	38.6	2.1	2.0
LAMP-01	30.3	331	3.01	720	20	0.00	0	0.3	0.6	10.9	56.6	28.8	1.5	0.9	0.3	0.1
LAMP-02	31.1	271	3.08	1571	21	0.00	0	0.2	0.7	14.0	53.6	29.2	1.0	1.1	0.3	0.1
LEON-01	30.5	304	3.13	4705	141	3.00	10	0.8	3.1	12.2	33.1	33.3	8.4	8.5	0.8	0.3
LEON-02	32.2	226	3.31	6180	152	6.08	12	0.7	2.7	13.5	30.6	37.6	6.8	7.2	0.9	0.3
MBOS-01	33.9	161	2.92	478	0	0.09	1	0.1	1.6	10.5	0.0	63.2	2.0	19.9	2.7	0.1
MERI-01	32.6	198	5.14	480	4	0.04	1	0.4	0.3	32.3	0.4	63.7	0.5	1.0	1.4	0.2
NBOS-01	31.3	366	2.75	257	16	3.50	2	0.6	8.0	11.0	17.6	36.8	12.1	12.0	1.9	1.6
NBOS-02	31.0	324	2.94	489	30	3.50	2	0.6	6.5	10.7	20.1	43.9	8.5	7.8	1.9	1.3
NBOS-03	31.2	302	3.18	925	49	3.50	2	0.5	4.3	13.3	22.6	44.6	7.1	6.0	1.6	0.8
NBOS-04	32.1	220	3.60	1890	69	3.75	4	0.5	3.1	18.4	13.5	55.1	4.1	3.5	1.7	0.6
NBOS-05	33.8	154	4.20	3097	82	5.28	8	0.5	2.5	23.8	8.5	56.2	3.4	3.1	2.0	0.5
NEIL-01	32.9	177	5.20	357	1	0.00	0	0.2	0.2	35.6	1.4	57.7	0.7	2.2	1.8	0.2
NOLC-01	33.6	167	3.27	275	17	33.77	9	0.5	32.4	24.0	7.9	30.2	1.4	1.3	1.6	11.7
NOLR-01	33.6	200	2.35	451	2	6.73	5	1.6	12.3	3.2	0.1	65.7	8.8	5.9	2.3	2.5
PALU-01	32.0	214	6.06	933	43	0.00	0	0.4	1.1	32.9	11.0	49.8	3.1	0.8	1.0	0.2
PLUM-01	31.6	248	3.06	226	0	0.00	0	0.1	0.3	16.8	21.6	53.8	2.3	4.5	0.6	0.1
ROCK-01	31.4	258	2.75	221	0	0.00	0	0.1	1.3	24.3	39.9	34.0	0.1	0.1	0.3	0.2
SALA-01	33.0	198	1.94	215	0	0.25	1	0.1	2.1	27.9	5.2	59.7	0.6	3.1	0.9	0.4
SBOS-01	34.5	144	2.40	220	1	1.10	1	0.2	6.7	3.7	0.0	48.5	4.9	33.9	2.2	0.9
SLEO-01	30.1	335	4.75	518	4	0.08	1	0.4	0.5	14.7	52.4	26.8	2.6	2.1	0.5	0.1
STEE-01	32.2	210	4.19	127	4	0.00	0	0.8	5.1	30.9	0.0	59.6	0.6	1.5	1.6	0.5

Diel dissolved oxygen (DO) concentrations were measured from each stream once during TCEQ's (2005) critical index period in 2006 and 2007 using YSI 6600 or 600 XLM Datasondes. One datasonde was deployed in shallow glide habitats in each stream reach for at least 48 consecutive hours in order to characterize diel variability in DO, particularly daily minimums, that could be indicative of important functional changes caused by nutrients. Diel dissolved oxygen sampling corresponded closely in space and time with surface-water nutrient and biological sampling.

Periphyton, filamentous macroalgae, and aquatic macrophytes were sampled concurrently with quarterly nutrient sampling. Two approaches were utilized to assess aquatic vegetation growth. The first involved a direct sampling of periphyton occurring on rock (epilithic) substrate for the analysis and calculation quantitative measures of biomass per unit area (chlorophyll-*a* and AFDM) and algal species composition. Quantitative periphyton sampling followed the protocols of the US Geological Survey's National Water Quality Assessment (NAWQA) program (Moulton et al. 2002) for epilithic substrates using the targeted sampling approach (riffle or glide habitat with gravel, cobble, or bedrock). In brief, 5 separate rock samples of attached periphyton were collected from each of 5 erosional habitats throughout the reach (n=25 rocks). Surfaces of rocks were quantitatively delineated and scraped to remove periphyton. Material from the rocks was composited and quantitatively subsampled (aliquots) to determine chlorophyll-a, AFDM, and number of cells per species per unit area. Additional subsamples were taken for elemental analysis to estimate percent carbon, nitrogen, and phosphorus and corresponding ratios of these three elements as indicators of nutrient availability and enrichment. Methods are detailed in Back et al. (2008) and Scott et al. (2008).

The second approach utilized the modified version of Hawkins et al. (2001) vegetation cover protocols, which yielded a reach-wide assessment of aquatic macrophyte, macroalgae, and periphyton percentage cover and thickness of biofilm cover. The method entailed walking a zig-zag transect from one end of the reach to the other and estimating vegetation cover at each of 100 equally spaced points located along the transect. The protocol was modified to include an index of sediment film thickness on stream substrate (sediment cover index), dominant substrate (percent of different particle sizes in the reach), an estimate of flow velocity (flow index), and water depth.

Periphyton taxonomic composition was measured at each of the 26 streams during August 2006 and September 2007. Taxonomic data were obtained from 10-ml subsamples from the quantatitive periphyton composite sample. Samples were collected, homogenized, preserved, and identified in accordance with taxonomic methods for non-diatom and diatom algae described in TCEQ (2005). One non-diatom and one diatom taxonomic sample was identified per stream per year. At least 500 diatom and 300 non-diatom cells per respective sample were identified (TCEQ 2005). Dr. Barbara Winsborough, an expert periphyton taxonomist from central Texas, performed all of the species identifications in accordance with the approved project plan.

Six of the 26 streams were sampled for algal species composition on each of the 8 quarterly sampling events (see Figure 1, "intensive" sites). These six sites were chose because they were widely separated throughout the study region and spanned a steep gradient of nutrient enrichment.

Benthic macroinvertebrate densities and taxonomic composition were measured in November 2006. Macroinvertebrates were sampled quantitatively using a Hess sampler from the same 5 erosional habitats sampled quantitatively for periphyton within the reach. Four Hess samples were collected from each of the 5 locations within the reach (n=20). Samples were composited, homogenized, and preserved in 5% (v/v) buffered formalin stained with rose bengal.

A two-phase macroinvertebrate subsampling approach (King and Richardson 2002) was employed in the laboratory. The first phase was a comprehensive removal of all large-bodied taxa (organisms easily seen with the naked eye). The second phase was quantitative removal of a minimum of 500 individuals (fixed count method) using a gridded, numbered pan and random numbers table. All organisms will be removed from each randomly selected grid until enough grids have been sorted to achieve >500 individuals (Barbour et al. 1999), or the entire sample contents have been sorted, whichever came first. Total number of grids subsampled will allow estimation of densities (no/m^2; King and Richardson 2002).

Macroinvertebrates were identified in the laboratory using operational taxonomic units, usually genus or species for most taxa. A voucher collection of specimens is maintained in the Center for Reservoir and Aquatic Systems Research (CRASR).

Physical habitat, flow, substrate, riparian attributes, and basic water chemistry of each reach were characterized according to methods outlined in TCEQ (2005). Cross-sectional sampling transects were established as described in TCEQ (2005). Discharge (cfs) was measured each quarter along a representative transect within each reach using a portable electromagnetic flow meter (Marsh-McBirney Flo-Mate Model 2000).

DATA ANALYSES

We estimated potential threshold responses in the measurement endpoints to numerical levels of nutrients using nonparametric changepoint analysis (nCPA), a technique explicitly designed for detecting threshold responses using ecological data (King and Richardson 2003, Qian et al. 2003). This analysis is based on the fact that structural change in an ecosystem may result in a change in both the mean and the variance of an ecological response variable used to indicate a threshold. When observations are ordered along an environmental variable (gradient), a changepoint is a value that separates the data into the two groups that have the greatest difference in means and/or variances. This can also be thought of as the degree of within-group variance relative to the between group variance, or *deviance* (*D*). Analytically, the nCPA examines every point along the stressor gradient and seeks the point that maximizes the reduction in deviance.

There is one particular value of the predictor y (e.g, TP) that maximizes the reduction in deviance in the response data (in this case, the selected biological responses); however, there is uncertainty associated with that value. It is unlikely that any one value of the predictor (e.g., TP) is the only value that could represent a changepoint. In reality, depending on the acuteness of the biological change in response to TP, several observations of TP could represent the changepoint, each with varying probabilities. Thus, to assess the risk associated with particular levels of TP, nCPA

incorporates estimates of uncertainty in the changepoint (King and Richardson 2003). These estimates are calculated using a bootstrap simulation. This simulation resamples (with replacement) the original dataset and recalculates the changepoint with each simulation. Bootstrap simulations are repeated 1,000 times. The result is a distribution of changepoints that summarizes the uncertainty among multiple possible changepoints. This uncertainty is expressed as a cumulative threshold frequency based on the relative frequency of each changepoint value in the distribution.

Important gradients in algal and macroinvertebrate species compostion were identified using non-metric multidimensional scaling (nMDS). NMDS is a distance based procedure that ordinates study units based on rank dissimilarities (Minchin 1987, Clarke 1993, Legendre and Legendre 1998). We used Bray-Curtis dissimilarity (BCD) as the distance measure, a coefficient that has been repeatedly demonstrated to be robust for ecological community data (Faith and Norris 1989). A two-dimensional solution was used for all analyses as stress values (a measure of agreement between BCDs and the configuration of the ordination) were relatively low and did not substantially decrease when additional axes were included in ordinations. Before running ordinations on the data sets, algae or macroinvertebrate species occurring at only two sites within a data set were excluded, and abundances were log transformed. Variables from the watersheds and environmental measurements with high skewness (> 1) were also log transformed to improve linear relationships with the ordinations. Ordinations were performed in PC-Ord version 5.20 (MjM Software, Gleneden Beach, OR, U.S.A.).

We used rotational vector fitting to relate environmental and watershed variables to gradients in algal and macroinvertebrate community composition quantified by the NMS ordinations (Faith and Norris 1989). Vector fitting was used to find the direction of the maximum correlation for each environmental variable. Significance ($P \leq 0.05$) of each environmental vectors was estimated using 1,000 random permutations of the data. Vector fitting was performed using the ECODIST package in R version 2.5.1 (© 2007, The R Foundation for Statistical Computing).

For species abundance thresholds, we employed a new analytical approach, Threshold Indicator Taxa ANalysis (TITAN; Matthew E. Baker and Ryan S. King), with the goals of (1) exploring and identifying abrupt changes in both the occurence frequency and relative abundance of individual taxa along nutrient gradients, (2) quantifying uncertainty associated with both observed distributions of each taxon and the broader sample, and (3) estimating the relative synchrony of those changes as a non-parametric assessment of a community threshold. Current statistical methods used for grouping samples and detecting community ecological thresholds are not developed for distinguishing responses of individual taxa with low occurrence frequencies or highly variable abundances (Dufrêne and Legendre 1997, Brenden et al. 2008, Andersen et al. 2008). Some methods assume a linear, univariate response along all or part of an environmental gradient (e.g., Toms and Lesperance 2003), whereas others focus solely on aggregate, community-level dissimilarity (e.g., De'Ath 2002, King et al. 2005) or species turnover between samples (i.e., beta-diversity). Noisy, non-linear, and poorly distributed occurrences are typical properties of the vast majority of taxa in multivariate community data matrices (McCune and Grace 2002). Multivariate or multi-metric analysis can obscure distinct responses of taxa subsets in a community data set, especially if both predominant and rare species do not respond in a

similar fashion or focal species do not respond as expected. TITAN circumvents most of these problems.

TITAN represents a combination and extension of change-point and indicator species analysis. In TITAN, we use normalized indicator species taxa scores (z) to identify the value of a continuous variable, x, resulting in the optimal partitioning of sample units, such that the indicator score is maximized either for individual taxa or the additive response of all normalized indicator z -scores at the community level. Negatively responding taxa (z–) are distinguished from those responding positively (z+) to yield taxa-specific change-point distributions as well as cumulative responses of declining [sum(z–)] and increasing [sum(z+)] subsets of the community. Resampling procedures are used to measure both indicator reliability and purity, and to estimate uncertainty surrounding the existence of community change-points.

TITAN analysis was performed on the same species data sets as in the ordinations using log-transformed abundance data. Predictors included important nutrient variables identified from the environmental vector fitting analysis. TITAN was conducted in R version 2.5.1 (© 2007, The R Foundation for Statistical Computing) using the custom package TITAN written by M. E. Baker and R. S. King (Baker and King, accepted with revision; King and Baker, in review).

RESULTS AND INTERPRETATION

Temporal Patterns in Discharge and Nutrient Concentrations

The 26 selected streams spanned a steep gradient of phosphorus enrichment, which is critical for identifying numerical thresholds. Sites with low, moderate, and high levels of P enrichment were widely distributed throughout the study area. This latter point was important because our goal was to avoid spatial "clusters" of sites that had similar levels of enrichment. Spatial clustering in a predictor variable can lead to erroneous conclusions about the effect of the predictor (e.g., TP) on biological responses because other factors such as biogeography, geology, or other unmeasured variables may coincidently correspond to the predictor variable (e.g., King et al. 2005). Our study design avoided such spatial problems, capturing the full range of natural variability in geology, drainage networks, stream size, etc., while still achieving a heterogeneous arrangement of P enrichment and its likely sources (WWTP outfalls, pasture).

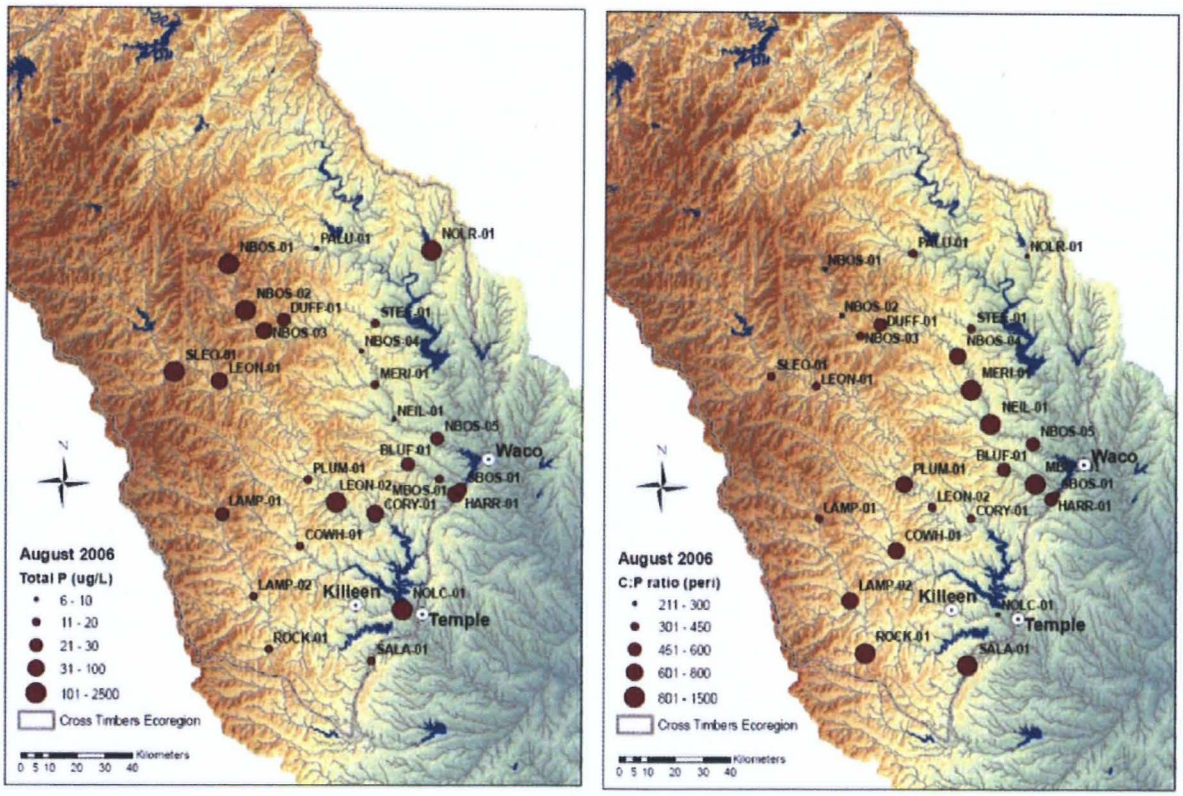

Figure 3. Spatial distribution of surface-water total phosphorus (TP, ug/L) and periphyton carbon-to-phosphorus (C:P, bulk) ratio during the first quarterly sampling event, August 2006.

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Streams throughout the study area had similar discharge patterns during the study period (Figure 4). Differences in discharge were much greater among seasons and years than among the individual streams (also see Figure 6).

Stream discharge was very low in summer and fall 2006 and early winter 2007. This was a period of near-record drought, causing flow to cease or nearly so in most of the 26 streams during November 2006. This provided an excellent opportunity to evaluate the effects of P enrichment on biological responses under low-flow conditions.

Heavy precipitation in spring and summer 2007 led to very high discharges and numerous scouring events. May 2007 (#4 in Figure 4) was completed during a brief period of stream-flow recession. During this event, 8 of the 26 streams were unwadeable so some variables were not sampled at those sites on that date. More rain and flooding continued through August.

Streams finally began to recede in September 2007 (#6 in Figure 4), allowing us to reinstate our quarterly sampling schedule. However, baseflows remained much higher than 2006.

February 2008 (#7 in Figure 4) corresponded to an extended period of normal to above-normal winter baseflows, without any major scouring events in the preceding few months. However, rains and flooding came back in March through May, thus the final sampling event (June 2008) followed heavy flows and scouring.

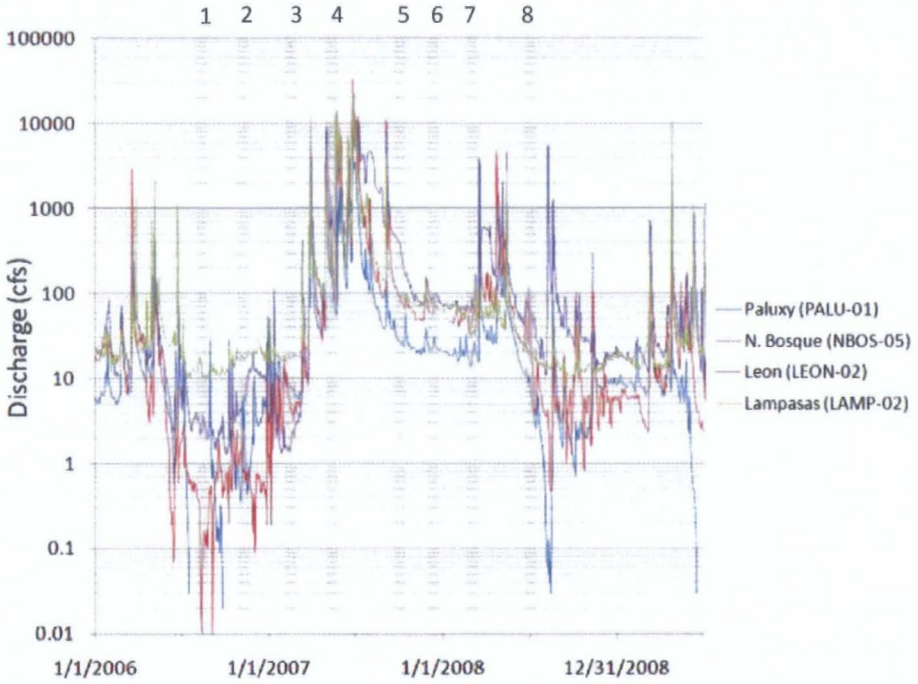

Figure 4. Hydrographs of four of the 26 study streams spanning a period before, during, and after the field study (data acquired from www.usgs.gov). The gray-shaded bars represent the sampling window corresponding to each of the 8 quarterly sampling events: 1=August 2006; 2=November 2006; 3=February 2007; 4=May 2007; 5=September 2007; 6=December 2007; 7=February 2008, and 8=June 2008.

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Figure 5. Photograph of Salado Creek (SALA-01) during August 2006, illustrating the typical channel and bank morphology of the region.

All of the study streams had streambeds comprised predominantly of calcareous gravel, cobble, boulders, and bedrock (e.g, Figure 5). Streams had short reaches of erosional (riffle) habitat comprised mostly of larger substrate (gravel and cobble). Erosional areas were interspersed by typically longer sections of either shallow, low gradient glides underlain by bedrock, or deeper, depositional pools that often were associated with limestone bluffs on the erosional side of the stream channel. This particular photo reveals a moderate amount of calcareous periphyton and patches of bright green filamentous algae, a common pattern seen in sites with low nutrient levels during extended periods of low flow. Nutrient-enriched sites typically had a thinner films of dark-colored periphyton as well as dark-green filamentous algae (mostly *Cladophora*).

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Instantaneous discharge measurements revealed that the hydrology of the 26 study streams was quite similar over time (Figure 6). All streams except for the 4 sites immediately downstream of significant effluent discharges (LEON-02, NOLC-01, NOLR-01, NBOS-01) exhibited a pattern of very low flow in August and November 2006, with a slight increase in February 2007 due to low evapotranspiration and increased groundwater discharge. However, the heavy rains and flooding of spring 2007 pushed discharge upward and maintained a relatively high baseflow through the end of the study in June 2008.

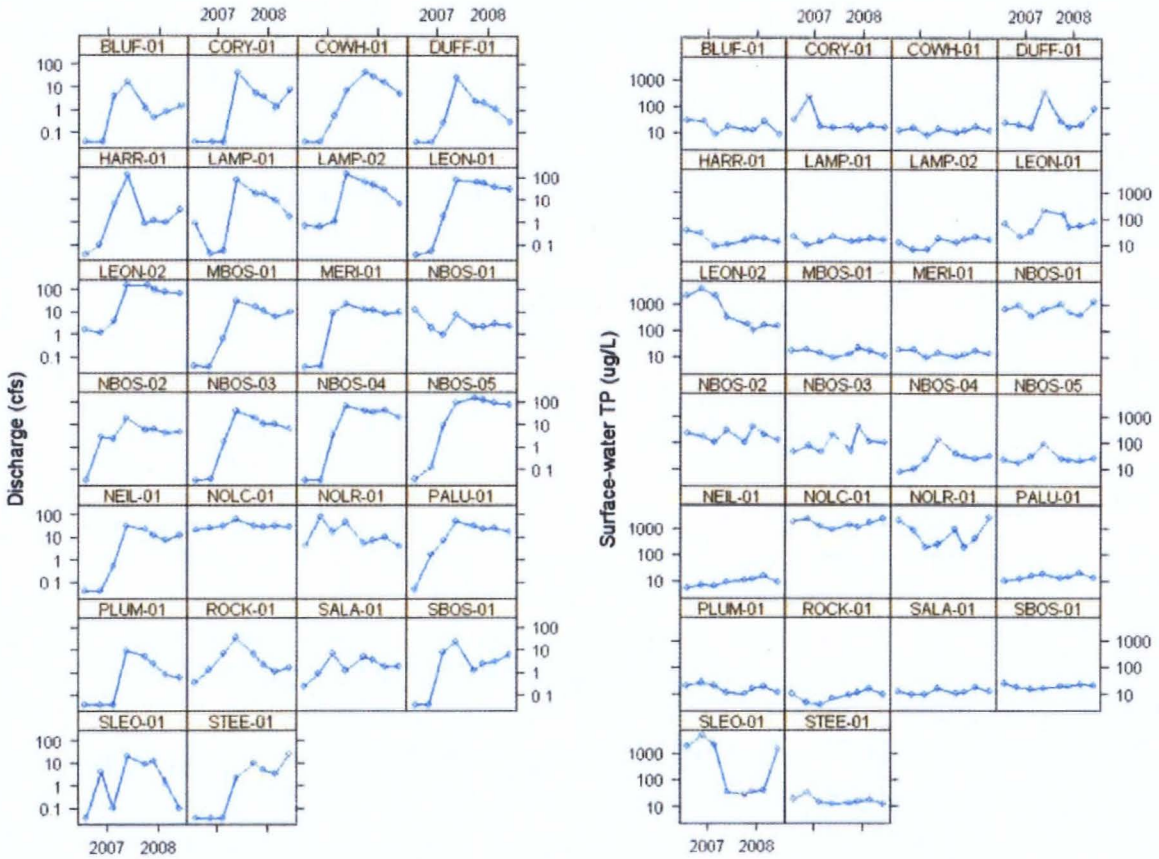

Figure 6. Temporal patterns of stream discharge (cfs) and surface water total phosphorus (TP, ug/L) among the 26 field sites, August 2006-June 2008. Both variables are plotted on a log-scale.

Surface-water TP, however, varied more by streams than by date of measurement. Streams with apparently few point or non-point sources of phosphorus (e.g., ROCK-01, NEIL-01, SALA-01, COWH-01, etc) exhibited consistently low TP, seen as relatively flat lines over time. Streams with predominantly non-point sources of phosphorus (watersheds with pasture or other sources) showed sharp increases in TP during high discharge, reflecting transport of P from the land from surface-water runoff. Streams with point sources (effluent discharges) either had consistently high TP (NOLC-01, NBOS-01) or were diluted by high surface-water runoff (LEON-02, NOLR-01, SLEO-01).

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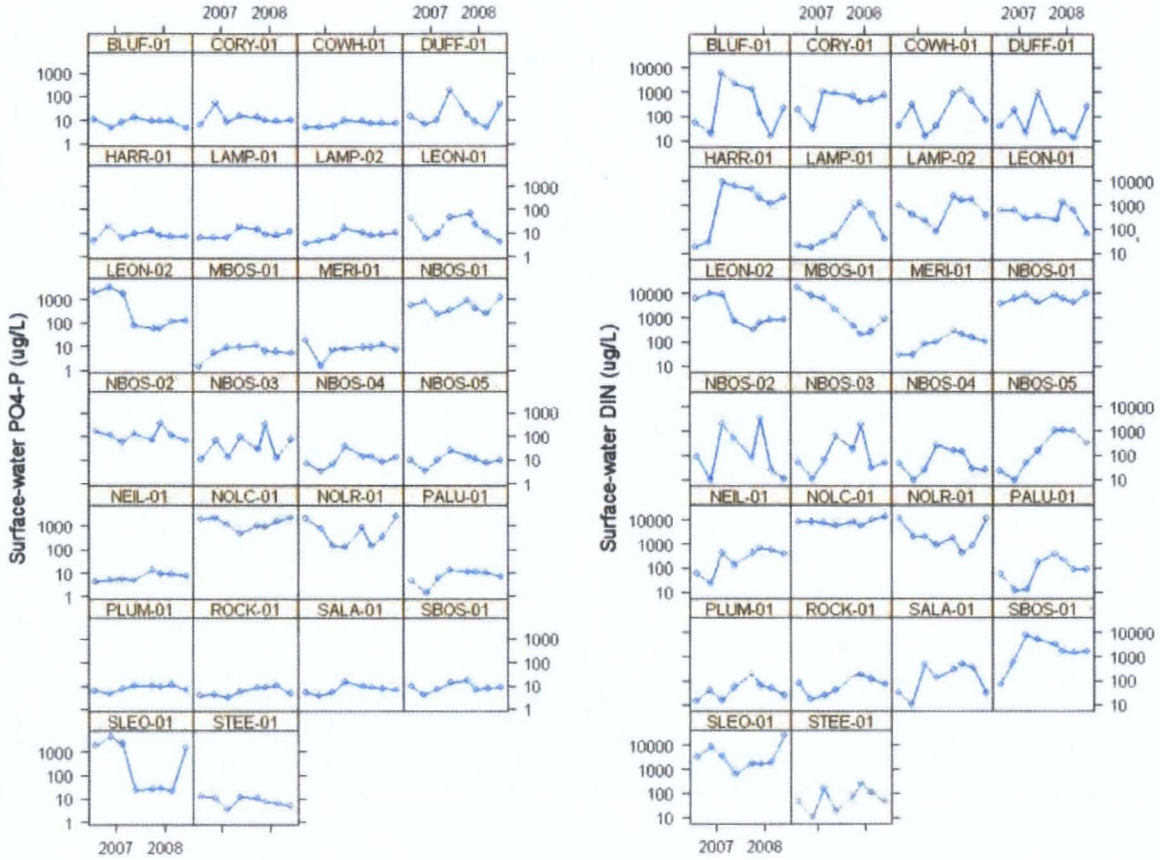

Figure 7. Temporal patterns of surface water orthophosphate (PO4-P, ug/L) and dissolved inorganic nitrogen (DIN) among the 26 field sites, August 2006-June 2008. Both variables are plotted on a log-scale.

Patterns in PO4-P among sites over time (Figure 7) were similar to that of TP, particularly for sites strongly influenced by point-source effluent discharges (NOLC-01, NOLR-01, NBOS-01, LEON-01, SLEO-01), as most of the TP at these sites was in the form of PO4-P. Orthophosphate comprised a smaller fraction (30-70%) of the TP at sites influence mostly by nonpoint sources (CORY-01, DUFF-01, PALU-01, LEON-01, etc). Orthophosphate represented a very small fraction of the TP at the sites with the fewest sources of phosphorus, and was often at or near the minimum detectable limit of 1-2 ug/L.

Dissolved inorganic nitrogen behaved much differently than PO4-P over time. During winter and spring of 2007, sites with rowcrop in their watersheds exhibited very high fluxes of DIN (mostly NO3-N). These sites tended to have consistently high DIN all year, but high concentrations were particularly evident as dissolved forms of N were transported from fields (mostly corn) via ground and surface water runoff to the streams (e.g., BLUF-01, CORY-01, HARR-01, MBOS-01, SBOS-01 NBOS-05). These high-DIN sites were important benchmarks because a few of them had low levels of TP (e.g., MBOS-01), revealing that biological variables did not respond to elevated DIN without a coincident subsidy of phosphorus (i.e., stream

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ecosystem processes were strongly limited by P, and additions of N did not have a detectable effect on the streams unless additional P was available).

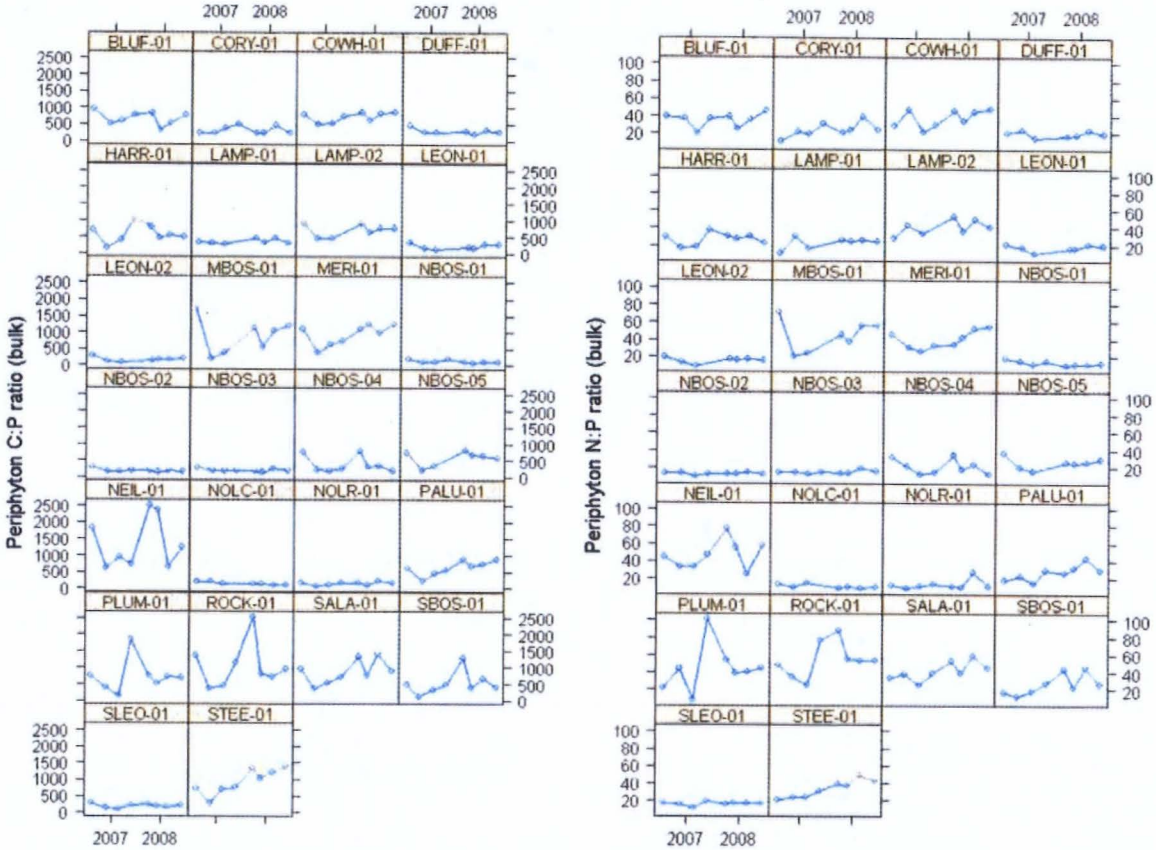

Figure 8. Temporal patterns of periphyton C:P ratio (bulk) and periphyton N:P ratio (bulk) among the 26 field sites, August 2006-June 2008.

We hypothesized that levels of enrichment of surface-water nutrients that were important would be reflected in enrichment of the tissues of the periphyton. Sites with elevated P, N or both should have more enriched periphyton P and/or N if either element was limiting its growth. Figure 8 illustrates that indeed sites differed markedly in their periphyton C:P and N:P ratios. Contrary to surface-water concentrations, *low values of C:P or N:P reflect greater enrichment* (i.e., more P per unit carbon or nitrogen in the periphyton = lower C:P or N:P ratio).

What is particularly compelling about these results is that sites with elevated surface-water TP had virtually constant C:P or N:P ratios in the periphyton. Moreover, despite the high variability of DIN in surface waters among sites over time, the periphyton N:P ratio was essentially identical to the C:P ratio, illustrating that P, not N, was driving differences in these ratios.

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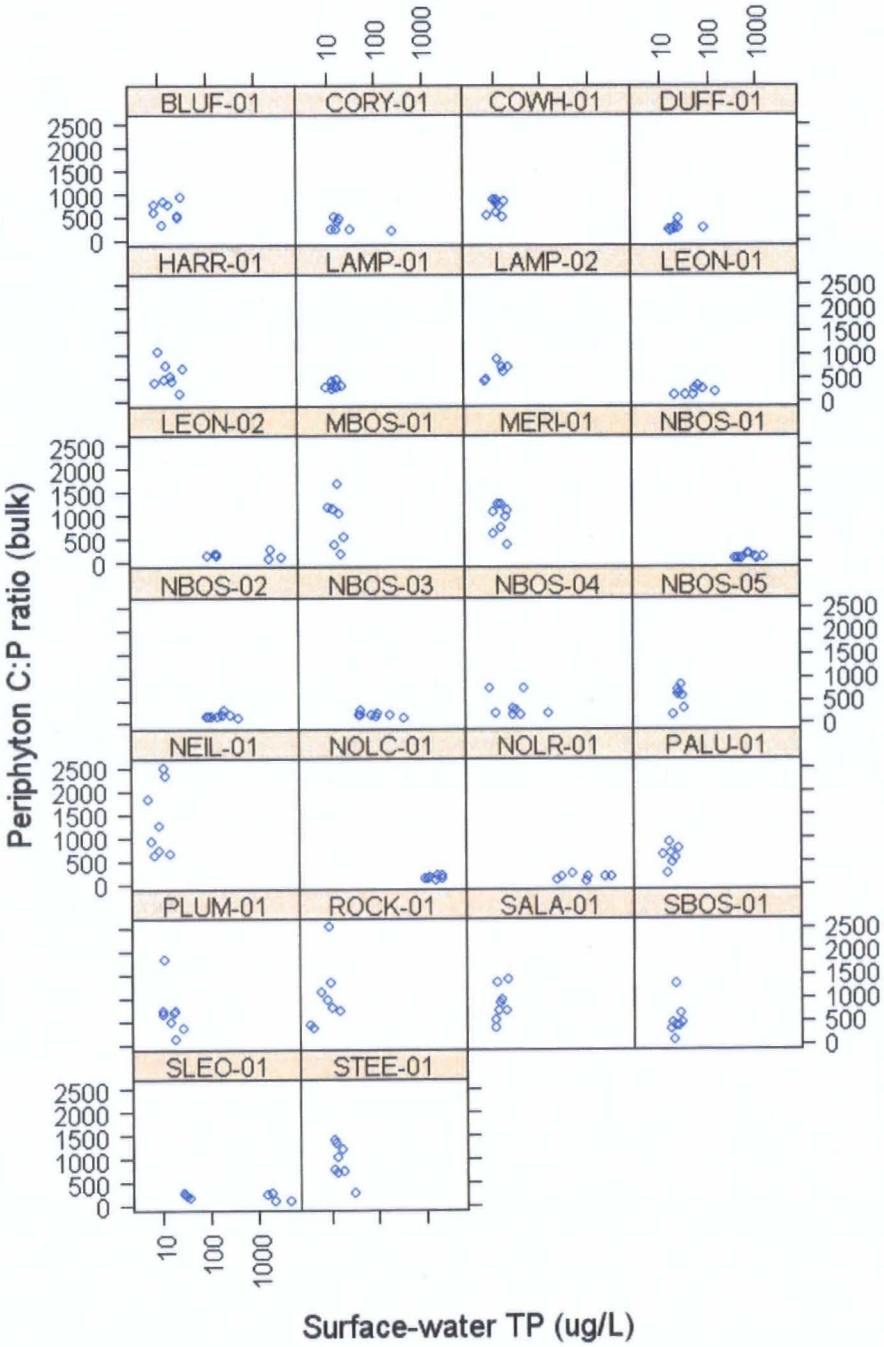

Figure 9. Scatterplot of surface-water TP (ug/L) versus periphyton C:P ratio (bulk) for each of the 8 sampling events, 2006-2008. Each point represents one of the eight dates of sampling. Surface-water TP is plotted on a log scale.

Figure 9 illustrates more directly the strong influence of surface-water TP on periphyton P content. There appear to be 3 types of relationships in this graph:

1. Sites with consistently low concentrations of surface-water TP (5-15 ug/L; e.g., NEIL-01, ROCK-01, SALA-01, STEE-01, etc) had highly variable periphyton C:P ratios. Importantly, these ratios almost always remained above 500. This showed that the periphyton was strongly limited by P, and variability in its P content was likely due to subtle seasonal differences in availability (either via recycling or pulses of low enrichment), taxonomic structure, and/or accumulation of biomass.

2. Sites with consistently moderate levels of TP (15-25 ug/L) had much less variability in the C:P ratios of the periphyton (e.g., BLUF-01, CORY-01, LAMP-01, HARR-01, SBOS-01) than sites with TP consistently < 15 ug/L. Moreover, these sites consisently had C:P ratios below 500, often below 200. This suggested that subtle increases in P availability resulted in a sharp, nonlinear response in the periphyton, with rapid uptake and storage of P.

3. Sites with consistently high levels of TP (e.g., effluent discharge sites such as NBOS-01, NOLC-01, NOLR-01), or with a wide range of levels of TP over time (e.g., SLEO-01, LEON-02, NBOS-02, NBOS-03) had consistently low (<200) periphyton C:P ratios, but these C:P ratios were not markedly lower than sites with consistently moderate levels of TP. This suggested that the periphyton was P-saturated (or nearly so) when exposed to low level P enrichment, and that temporal variability in surface-water TP did not correspond to variability in periphyton C:P because of its ability to store P during periods of high availability, and recycle P when it is less available.

In summary, these results imply that (1) periphyton was strongly limited by P, (2) it was very efficient at sequestering and recycling P, and (3) its C:P ratios were low and similar among sites ranging from subtle but consistent TP enrichment to consistently high levels of TP enrichment.

The next section of this report deals directly with quantification of levels of surface-water TP that result in thresholds, or nonlinear changes, in periphyton nutrient content and the implications of this enrichment on other biological response variables.

RESULTS AND INTERPRETATION, CONTINUED

Biological Responses to TP Gradients

The series of subfigures in Figure 10 effectively reveals the strikingly strong *nonlinear* relationship between surface-water TP and periphyton nutrient content. These figures use all of the data combined to show the remarkable consistency of the response, regardless of season or site. Threshold declines in periphyton C:P, N:P, and C:N ratios were highly likely between 15 and 25 ug/L TP, with 19-20 ug/L representing the most consistent concentration yielding a threshold response.

Note that C:N also declines with TP at the same threshold; this is important because it shows that as the periphyton is relaxed from its P limitation, it can also utilize the subsidy of nitrogen available in the streams. This pattern is not evident when C:P, N:P, and C:N are plotted against DIN or TN (see Figure 16).

Significant threshold changes were detected in biofilm thickness, filamentous macroalgal cover, and submersed macrophyte cover in response to TP, even when combining all of data from each site across the 8 sampling dates (Figure 11).

The thickness of microbial films (microalgae and other microbes; non-filamentous algae) growing on gravel, cobble, boulders, or bedrock showed consistently nonlinear declines in response to TP levels above 20 ug/L. This metric was computed as the percentage of observations in a reach (out of a maximum of 100 points along the reach-scale transect; see Methods) in which biofilms exceeded 1 mm in thickness on rocks. If no rocks were present at a point, or if the channel was too deep at a particular point to collect a rock, no measurement was recorded. Heavy accumulation of calcareous periphyton was a consistent trait of low-P streams (see Figure 12a). Streams above the threshold value of 20 ug/L usually had a thinner, darker layer of biofilms on the rocks (Figure 12b) but also tended to have more filamentous green algae (see next).

The percentage of points along the reach-scale transect that had filamentous macroalgae cover exceeding 75% of the substrate was also responsive to surface-water TP, but contrary to biofilm thickness, filamentous algae increased in response to TP (Figure 11). There were actually 2 distinct changepoints in filamentous algal cover: a consistent increase in cover among sites at about 20 ug/L TP, and a more diffuse but dramatic increase in cover at very high levels of TP (200-1000 ug/L). Because filamentous algal cover depends highly on seasonal changes in light and water temperature, as well as sloughing from overgrowth and scouring from high flows, it is not surprising that many of the highly enriched sites had little filamentous algal cover on some dates. However, when high filamentous algal cover occurred, it was almost always associated with levels of P enrichment > 20 ug/L, and particularly at >200 ug/L.

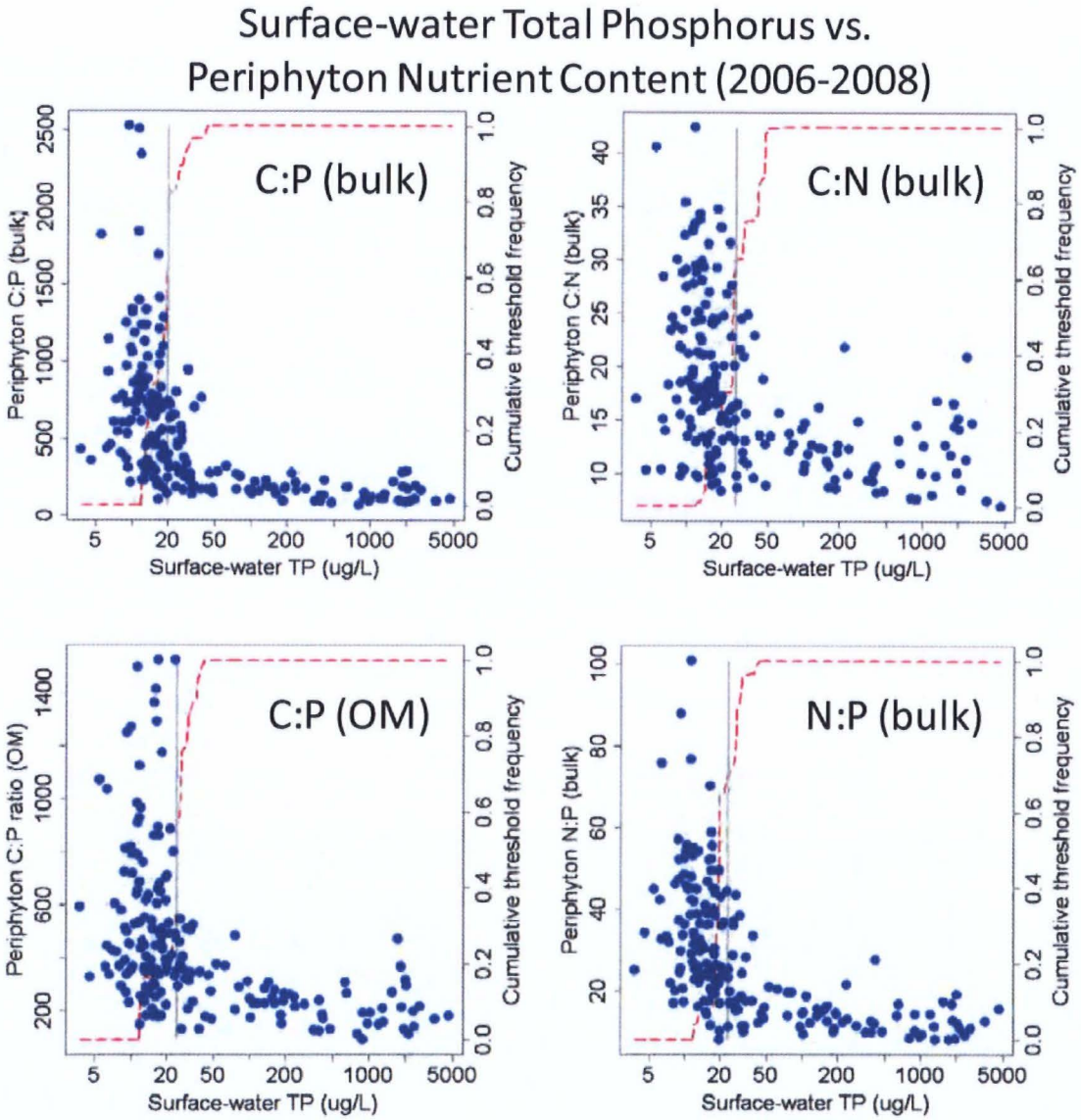

Figure 10. Results from nonparametric changepoint analysis using surface-water TP as a predictor of threshold changes in periphyton nutrient ratios across all 26 sites over the full study period. Bulk represents ratios of carbon, nitrogen, and phosphorus that were estimated using homogenized periphyton that was not subjected to the centrifugation separation. The OM (organic matter) ratio represents the fraction of homogenized periphyton that was separated from inorganic sediment using the centrifugation method described in Scott et al. (2008). Each blue dot represents one of the 26 sites, and each site is represented 8 times in the analysis, once for each sampling date. The gray vertical line is the observed TP threshold (the level of TP resulting in the greatest difference in the response variable to the left and right of that value). The dotted red line is the cumulative threshold frequency, an estimate of uncertainty based on 1,000 bootstrap samples of the data (see King and Richardson 2003). The cumulative threshold frequency illustrates the range of possible threshold values; different quantiles of this distribution can be interpreted as confidence intervals around the observed threshold. See Table 3 for summary of the corresponding statistical results.

0006302

One of the most important patterns that emerged from the field study was the sharp threshold declines in submersed macrophyte cover in response to TP >25-50 ug/L. The percentage of points along the reach-scale transect that had either some cover of macrophytes (top right panel, Figure 11) or cover exceeding 25% of the substrate (bottom right panel) both strongly declined in response to TP. The two dominant submersed macrophyte taxa were *Najas* (a vascular plant) and *Chara* (a nonvascular macroalga that is functionally equivalent to a vascular macrophyte). Other taxa less frequently encountered included *Potamogeton* and *Nitella*.

The mechanism for decline of these species may be related to filamentous algal growth on the plants themselves, which is a common problem in other systems experiencing eutrophication (e.g., Chesapeake Bay). *Chara* has also been shown to decline in response to TP levels above 30 ug/L in the Everglades (King et al. 2004, Richardson 2008), which is possibly related to changes in speciation of dissolved inorganic carbon with diel swings in pH associated with P-stimulated photosynthesis by algae. Regardless of mechanism, it is clear that P enrichment is associated with a significant negative change in an important structural component of these streams.

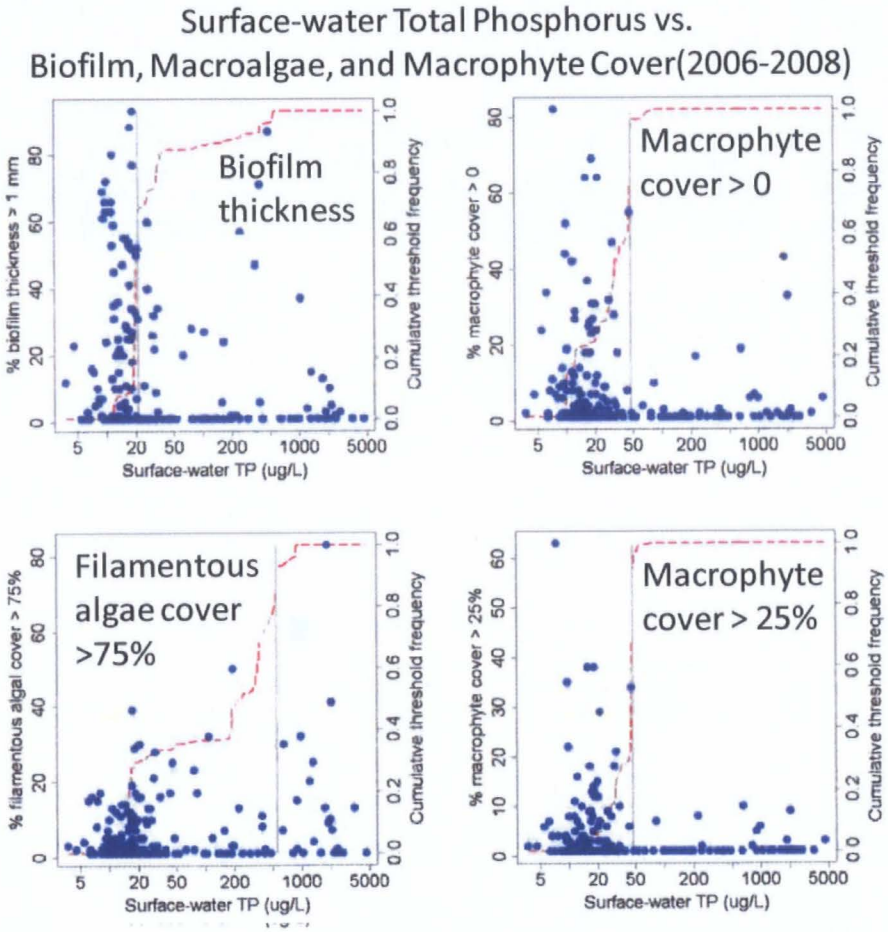

Figure 11. Results from nonparametric changepoint analysis using surface-water TP as a predictor of threshold changes in biofilm (non-filamentous periphyton) thickness on rocks, filamentous algae cover, and submersed macrophyte cover across all 26 sites over the full study period. See Figure 10 for other graphical details and Table 3 for statistical summaries corresponding to the figure.

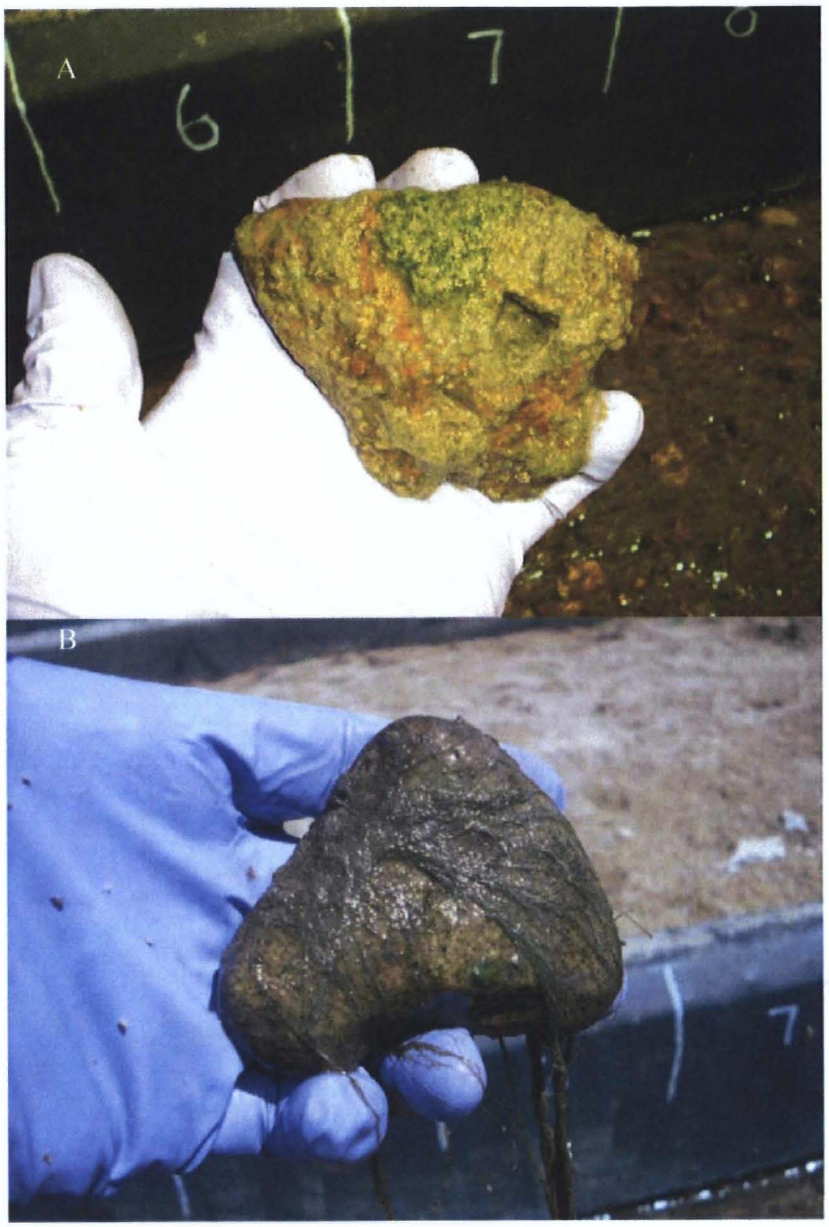

Figure 12. Photographs of typical periphyton (biofilm) color and thickness from sites below the TP threshold of <15-20 ug/L (panel A) and above the threshold (panel B). In panel A, the periphyton is mostly diatoms, calcareous cyanobacteria, and other microbes (bacteria and fungi), with little obvious filamentous green algae. In panel B, the periphyton is comprised of a thinner veneer of mostly diatoms and bacteria, but the overall biomass does not change markedly because of the increase in colonial and filamentous green algae. This shift in structure becomes evident in the next series of figures.

0006304

The preceding pair of photographs in Figure 12 places the results presented in Figure 13 into context and aids in their interpretation. Periphyton ash-free dry mass was highly variable in response to TP, and showed a weak decline in response to TP levels ranging from as low as 10 ug/L to about 50 ug/L. Because total ash-free dry mass combines all types of attached periphyton (diatoms, cyanobacteria, other microbes and filamentous/colonial green algae), it is intuitive AFDM might not respond as sharply as biofilm thickness (decline) or filamentous algal cover (increase), because AFDM combines both. Nevertheless, the pattern of decreasing biofilm thickness does result in a significant decline in AFDM, although the threshold level of TP is relatively uncertain.

Periphyton chlorophyll-a tended to track the pattern in % filamentous algal cover shown in Figure 11, with possibly 2 changepoints (20 and 200 ug/L), each leading to higher levels of chlorophyll-a. However, this result is also relatively uncertain and noisy because diatoms and cyanobacteria characteristic of low-P sites also contain chlorophyll.

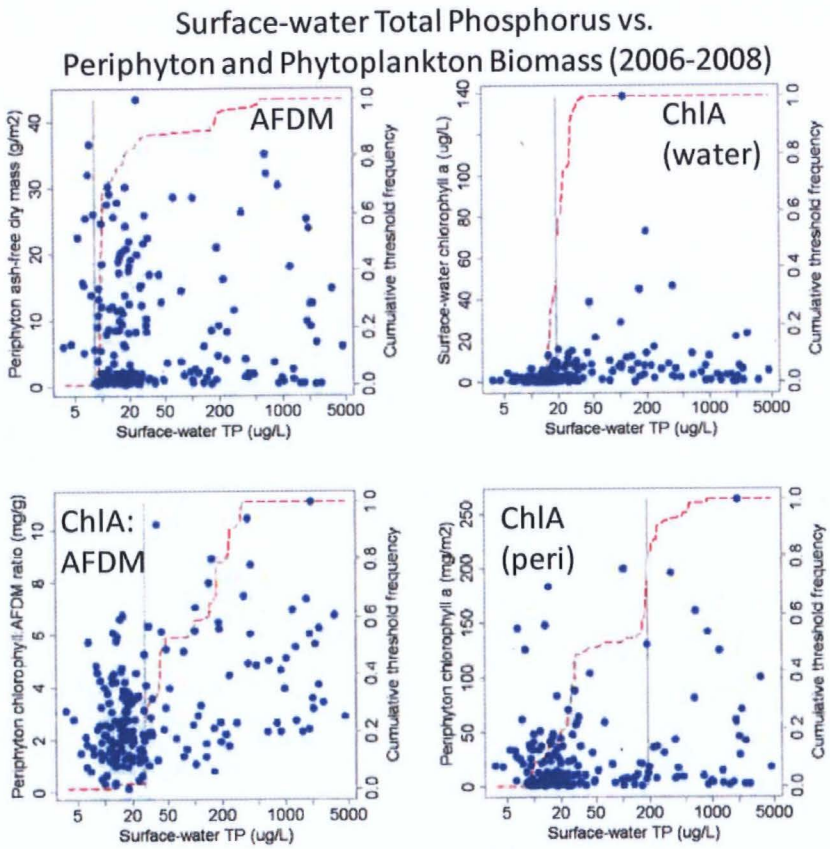

Figure 13. Results from nonparametric changepoint analysis using surface-water TP as a predictor of threshold changes in periphyton ash-free dry mass (AFDM, g/m^2), periphyton chlorophyll-a (ChlA (peri); mg/m^2), surface-water chlorophyll-a (ChlA (water); ug/L), and the ratio of periphyton ChlA to periphyton AFDM (ChlA:AFDM; mg/g) across all 26 sites over the full study period. See Figure 10 for other graphical details and Table 3 for statistical summaries corresponding to the figure.

0006305

The ratio of periphyton chlorphyll to AFDM did a much better job of revealing the shift in structure of the periphyton in response to P. This relationship (bottom left panel) reveals a clear, nonlinear shift toward a greater fraction of chlorophyll-bearing organisms in the periphyton in response to TP > 20-30 ug/L. A secondary threshold appears to be near 200 ug/L, coincident with the 2-tiered response of filamentous algae and chlorophyll-a.

Most primary production in wadeable streams is a result of periphyton and macroalgae rather than phytoplankton. However, wadeable streams in Texas typically have lower flows and thus longer residence times than wadeable streams in more mesic parts of North America. Longer residence times allow for some potential for phytoplankton blooms in response to TP. We observed a consistent nonlinear increase in surface-water chlorophyll-a at 20 ug/L TP. Streams with high TP did not consistently have high chlorophyll-a, but when high levels of chlorophyll were observed, TP was also elevated above 20 ug/L. This is somewhat circular, because TP is an unfiltered, digested sample that includes particulates such as phytoplankton. Thus, TP will necessarily increase if high chlorophyll is observed. It is also likely that much of the chlorophyll observed in the water column was from attached algal cells that had sloughed off of rocks.

Fine sediment runoff and deposition into streams may be an important contributor of non-point source phosphorus (Figure 14): C:P ratios in sediment that was separated from organic matter in the homogenized periphyton showed a strong nonlinear relationship to TP. It is likely that this relationship was at least partially caused by P-enriched organic matter (periphyton) that remained attached to sediment particles, but it also suggests that phosphorus bound to clay particles may be enriching the periphyton.

Streams with >20% of the channel dominated by silt or clay substrate were consistently associated with TP > 20 ug/L. Sediment-impacted streams often had high bank erosion, relatively high percentages of pasture in their watersheds, and usually had evidence of cattle activity in the stream channel and banks. However, many of the streams with TP>20 ug/L did not have obvious sedimentation problems, thus sedimentation alone was not the driving factor in these relationships. Rather, this suggests that sediment-bound P may have been an alternative source of enrichment that was driving the strong nonlinear biological responses.

The frequency of sediment cover classified as sufficiently heavy to obscure the color of periphyton (Sed>3) corresponded closely to the percentage of the reach dominated by fine silt-clay substrate. It also showed a sharp increase at 20 ug/L TP. This particular index was a direct measure of the potential effect of sediment on periphyton because it was a qualitative index of sediment film thickness on rock substrates. Again, not all streams with high TP had high sediment-index scores, suggesting that sediment alone was not the driver of threshold responses in biological endpoints.

Turbidity response to TP paralleled the responses of silt cover and the sediment index, sharply increasing at 20 ug/L TP. TP is partially dependent upon turbidity, however, because greater particulates in the water will necessarily lead to higher TP, assuming the particulates have P bound to them (clay) or the particulates are organic (detritus, phytoplankton).

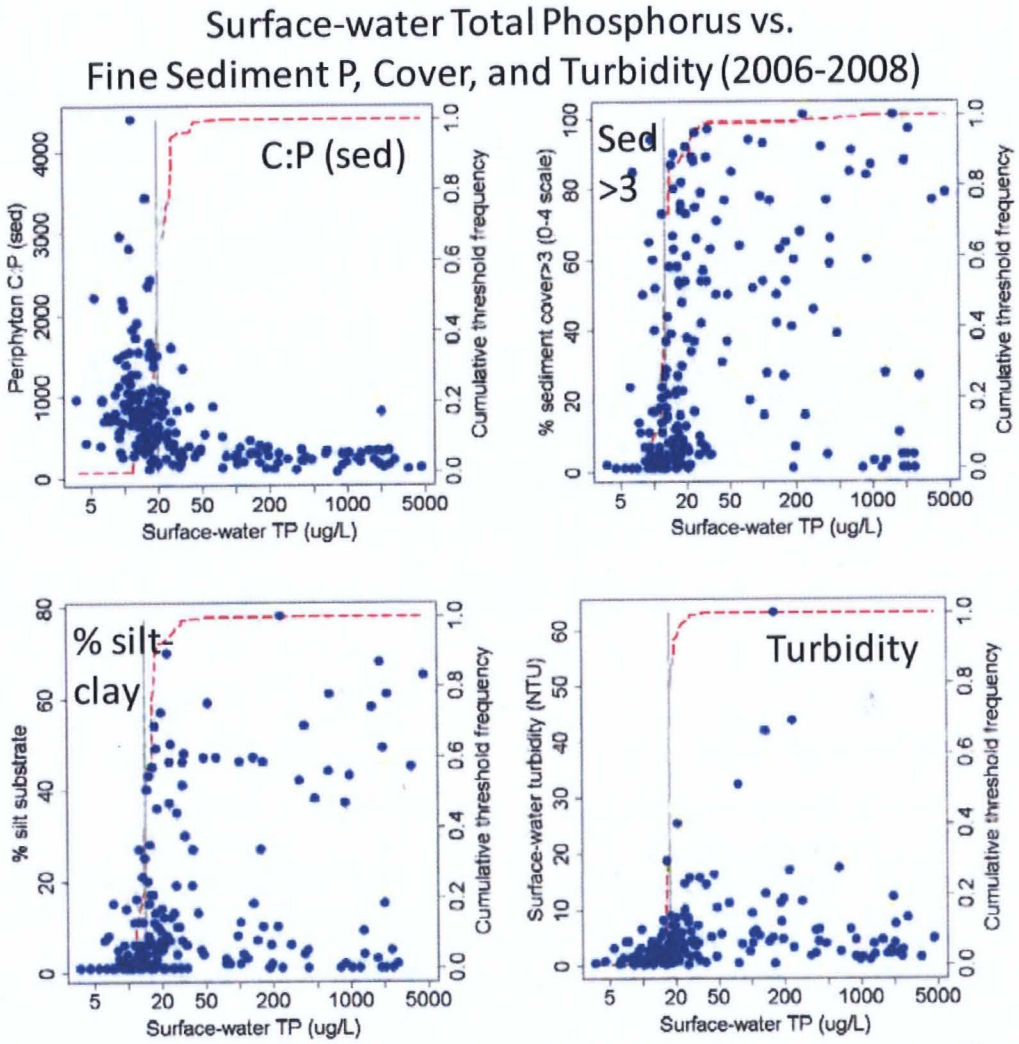

Figure 14. Results from nonparametric changepoint analysis using surface-water TP as a predictor of threshold changes in (1) periphyton C:P ratio (sediment fraction); (2) the percentage of points in the reach dominated by silt-clay substrate; (3) the percentage of points in the reach in which substrate had a heavy film of sediment (sediment index>3, where 3=sufficiently heavy to obscure color of periphyton); and (4) surface-water turbidity (NTU) across all 26 sites over the full study period. See Figure 10 for other graphical details and Table 3 for statistical summaries corresponding to the figure.

0006307

Numerical threshold responses of most of the measured water quality and biological endpoints from the the field study for all dates combined, 2006-2008 are presented in Table 3. The observed threshold value (shown as the gray vertical bar in Figures 10-14) is highlighted here in bold. The P-values represent the probabilities of Type I error (i.e., the probability of concluding that there is a threshold when in fact there is not) for each response to TP. Importantly, the cumulative threshold quantiles are narrow for most of the responses, with the 10% and 50% values corresponding closely to the observed value. The 90% quantile occasionally spiked up to near 200 ug/L for variables that were strongly influenced by filamentous algae, a response variable which likely had changepoints at ~20 and ~200 ug/L TP.

Table 3. Results of nonparametric changepoint analysis using **surface-water TP** as a predictor of threshold responses in selected biological and water quality response variables across 26 sites and 8 quarterly sampling events during 2006-2008. See Table 1 for full names and descriptions corresponding to variable IDs, and figures 10 through 14 for graphical display of most of these results.

				Cumulative Threshold Quantiles			Mean variable value	
Variable ID	**TP (ug/L)**	Response	P value	10%	50%	90%	Below	Above
AFDM_M2	**18.3**	Decline	0.0027	9.4	10.2	163.9	5.30	3.30
CHLA_UGL	**19.2**	Increase	<0.0001	15.5	19.2	28.3	0.81	3.86
CHL:AFDM	**27.6**	Increase	<0.0001	27.5	40.7	239.8	2.05	3.57
CHLA_M2	**186.0**	Increase	0.0042	12.0	57.6	239.8	7.53	19.88
CN_ALG	**20.1**	Decline	0.0001	12.0	20.1	46.5	14.98	12.18
CN_BULK	**26.2**	Decline	<0.0001	15.6	25.4	46.5	20.43	12.91
CP_ALG	**24.5**	Decline	<0.0001	12.1	24.5	32.9	564.95	259.96
CP_BULK	**19.8**	Decline	<0.0001	12.2	19.8	26.8	768.59	260.21
NP_ALG	**28.3**	Decline	<0.0001	22.7	32.9	174.5	34.59	19.60
NP_BULK	**27.5**	Decline	<0.0001	19.8	27.5	40.7	31.49	14.01
MACALG5+	**554.7**	Increase	0.0361	15.7	250.1	554.7	2.93	5.75
MICALG3+	**21.1**	Decline	0.0005	18.2	21.0	174.7	6.24	2.48
PLNT1+	**46.5**	Decline	0.0004	10.2	36.1	46.8	4.12	1.82
PLNT3+	**46.5**	Decline	0.0014	25.9	46.5	46.8	2.39	1.31
SED3+	**12.8**	Increase	<0.0001	10.8	12.8	18.4	5.78	20.85
TURB_NTU	**18.0**	Increase	<0.0001	15.5	17.8	19.8	1.08	4.24

Orthophosphate (PO4-P) was also a strong predictor of periphyton nutrient content. The relationships depicted in Figure 15 are very similar to the TP thresholds in Figure 10, but perhaps slightly more nonlinear. Virtually no sites with >18 ug/L PO4-P had bulk C:P ratios above 300 (upper left panel). This is further evidence for nonlinear uptake of P by periphyton, reaching a saturation point at the observed PO4-P threshold.

One disadvantage of PO4-P relative to TP as a predictor, however, is that it can be rapidly depleted during daylight when photosynthesis is active. Thus, some sites with low PO4-P are nevertheless highly enriched with P, shown by the low C:P ratios. TP is less variable and thus is a better indicator of P enrichment, but is still influenced by diel variability in photosynthesis and uptake. A major conclusion from this PO4-P result is that when there is a subsidy of bioavailable phosphorus (dissolved orthophosphate), it often indicates that the periphyton is near or beyond its point of saturation, thus excess dissolved P remains in the water column.

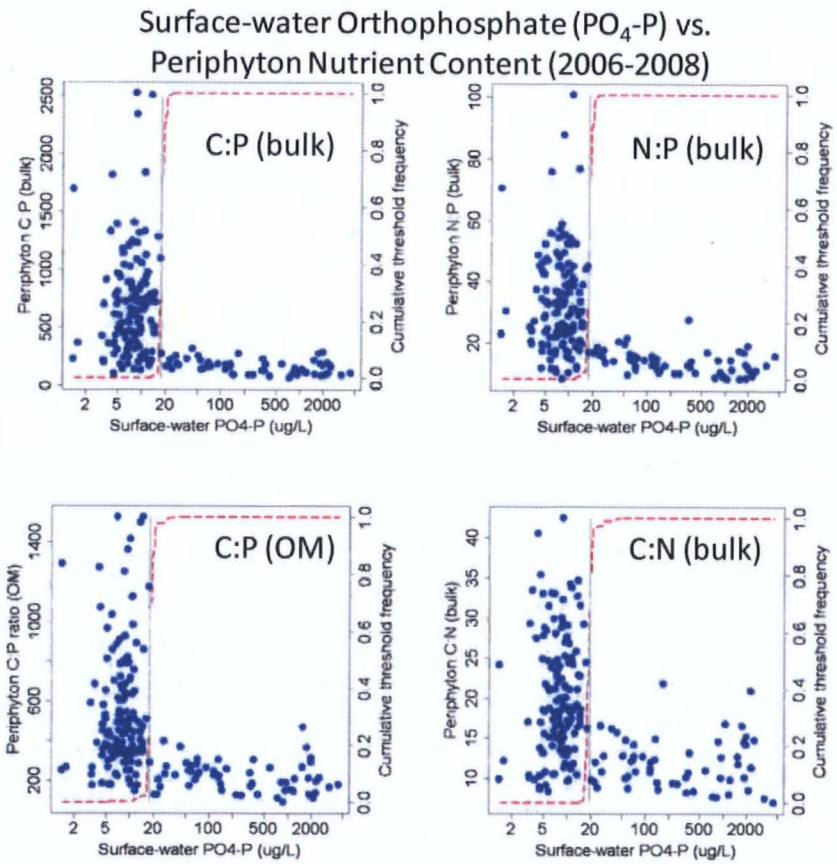

Figure 15. Results from nonparametric changepoint analysis using surface-water orthophosphate as a predictor of threshold changes in periphyton nutrient ratios across all 26 sites over the full study period. Bulk represents ratios of carbon, nitrogen, and phosphorus that were estimated using homogenized periphyton that was not subjected to the centrifugation separation. The OM (organic matter) ratio represents the fraction of homogenized periphyton that was separated from inorganic sediment using the previously described centrifugation method.

0006309

There were significant changes in periphyton C:P and N:P ratios in response to dissolved and total nitrogen, but the relationships were weak and noisy (Figure 16). Most of the signal in these relationships were driven by the covariation between P and N in effluent discharges (i.e., high P and N, but the periphyton was responding to the P), and the noise in the relationship is attributed to sites with high amounts of rowcrop in their watersheds but little pasture or effluent discharges (low P but high N, thus no response of the periphyton to the N because it was limited by P).

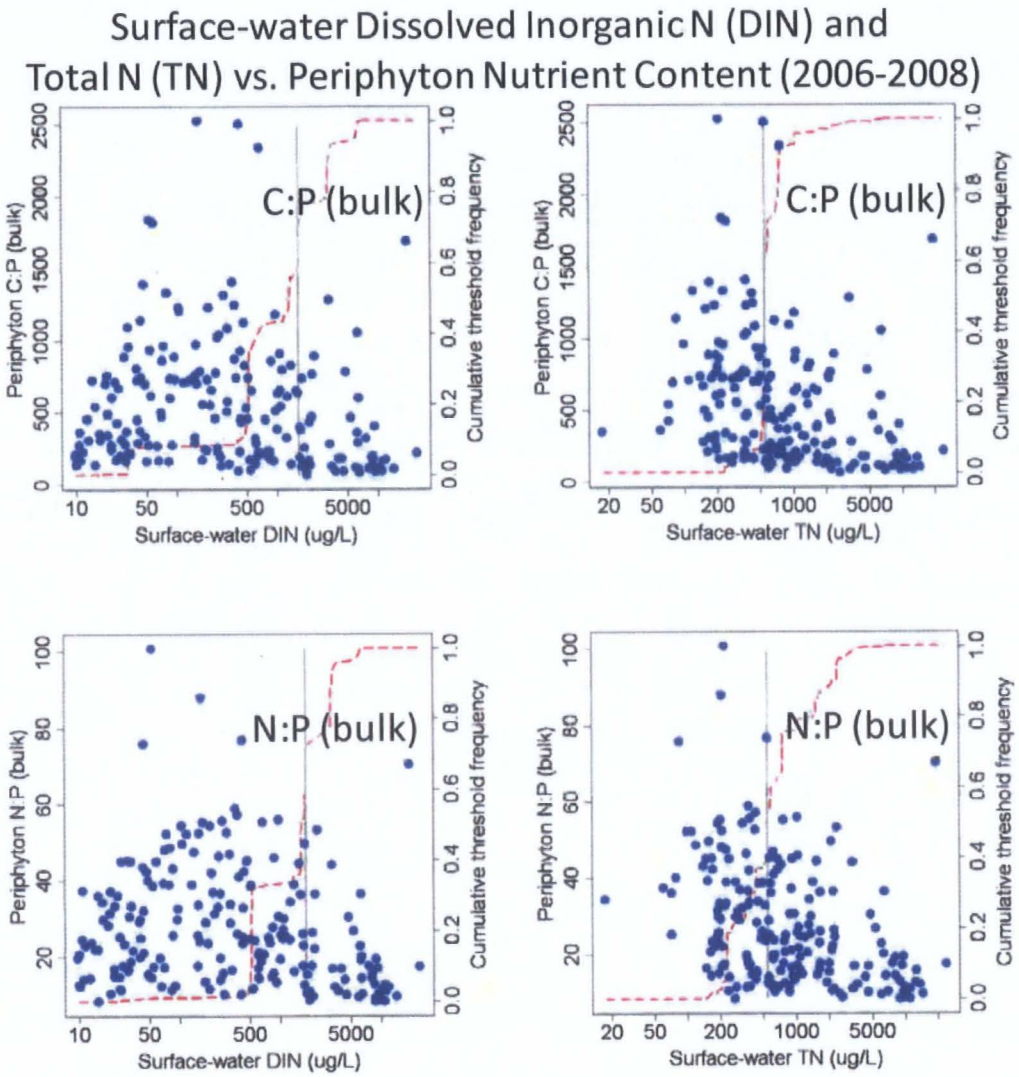

Figure 16. Results from nonparametric changepoint analysis using surface-water dissolved inorganic nitrogen (DIN, ug/L) and total N (TN, ug/L) as a predictor of threshold changes in periphyton nutrient ratios across all 26 sites over the full study period.

0006310

Table 4. Results of nonparametric changepoint analysis using **surface-water PO4-P, DIN, TN, and periphyton C:P ratio (bulk)** as a predictors of threshold responses in biological and water quality response variables from all 26 sites and 8 quarterly sampling events combined during 2006-2008. Significant thresholds are highlighted in bold. See Table 1 for full names and descriptions corresponding to variable IDs.

					Cumulative Threshold Quantiles		
Predictor	Variable	Response	Obs.	P value	10%	50%	90%
PO_4-P (ug/L)	**AFDM_M2**	**Decline**	6.8	<0.0001	6.8	7.4	7.9
	CHLA_UGL	**Increase**	28.9	0.0001	10.9	17.0	32.5
	CHL:AFDM	**Increase**	104.4	<0.0001	19.4	113.5	139.3
	CHLA_M2	**Increase**	84.3	<0.0001	6.8	7.7	145.2
	CN_ALG	**Decline**	7.0	<0.0001	5.5	8.7	13.8
	CN_BULK	**Decline**	19.4	<0.0001	17.3	19.2	20.7
	CP_ALG	**Decline**	17.9	<0.0001	17.3	17.9	20.4
	CP_BULK	**Decline**	17.9	<0.0001	16.5	17.9	19.9
	MACALG5+	**Increase**	509.0	0.0010	7.8	264.3	509.0
	MICALG3+	**Decline**	9.7	0.0015	5.7	10.0	235.1
	NP_ALG	**Decline**	17.9	<0.0001	19.2	49.8	141.9
	NP_BULK	**Decline**	17.9	<0.0001	17.3	17.9	21.0
	PLNT1+	**Decline**	6.8	<0.0001	5.5	8.1	9.4
	PLNT3+	**Decline**	8.2	<0.0001	5.1	7.7	11.7
	SED3+	**Increase**	9.8	<0.0001	5.6	9.6	145.2
	TURB_NTU	**Increase**	13.7	<0.0001	11.5	13.7	14.2
Periphyton C:P (bulk)	**AFDM_M2**	**Increase**	762.1	0.0159	431.6	736.2	963.6
	CHLA_UGL	**Decline**	496.1	<0.0001	181.1	416.4	739.4
	CHL:AFDM	**Decline**	717.3	<0.0001	141.6	442.3	869.1
	CHLA_M2	**Decline**	762.1	<0.0001	454.4	731.6	834.5
	CN_ALG	**Increase**	317.5	<0.0001	226.0	317.5	521.9
	CN_BULK	**Increase**	540.8	<0.0001	337.6	459.6	554.9
	MACALG5+	**Decline**	1050.7	<0.0001	129.3	272.6	1050.7
	MICALG3+	**Increase**	338.3	<0.0001	301.3	338.3	491.3
	NP_ALG	**Increase**	623.9	<0.0001	363.3	537.0	627.4
	NP_BULK	**Increase**	349.3	<0.0001	301.3	349.3	454.9
	PLNT1+	**Increase**	195.6	<0.0001	176.6	226.4	244.5
	PLNT3+	**Increase**	176.6	<0.0001	175.1	184.0	234.7
	SED3+	**Decline**	482.9	<0.0001	290.6	486.1	773.8
	TURB_NTU	**Decline**	403.5	<0.0001	301.2	403.5	736.3
TN (ug/L)	**AFDM_M2**	**Decline**	**2181.7**	**0.0234**	212.6	2181.7	3973.3
	CHLA_UGL	**Increase**	**351.8**	**<0.0001**	243.0	350.3	368.5

0006311

	CHL:AFDM	**Increase**	**1060.7**	**<0.0001**	987.6	1071.3	3965.0
	CHLA_M2	**Increase**	**2181.7**	**0.0002**	2075.0	2445.0	5083.4
	CN_ALG	**Decline**	**285.4**	**<0.0001**	261.4	285.4	580.8
	CN_BULK	**Decline**	**548.9**	**<0.0001**	524.9	574.4	1490.0
	CP_ALG	**Decline**	**593.5**	**<0.0001**	179.8	593.5	2445.0
	CP_BULK	**Decline**	**548.9**	**<0.0001**	511.2	548.9	771.7
	MACALG5+	**Increase**	**3335.0**	**<0.0001**	428.0	3071.3	7571.0
	MICALG3+	**Decline**	**243.0**	**0.0013**	204.4	240.5	587.4
	NP_ALG	**Decline**	**2116.7**	**0.0001**	1533.2	2116.7	2525.4
	NP_BULK	**Decline**	**2445.0**	**0.0003**	382.7	2145.0	3501.7
	PLNT1+	**Decline**	**786.4**	**0.0001**	258.1	786.4	939.0
	PLNT3+	**Decline**	**922.2**	**0.0001**	761.6	884.8	968.1
	SED3+	**Increase**	**279.9**	**<0.0001**	240.0	309.4	458.6
	TURB_NTU	**Increase**	**160.2**	**<0.0001**	160.2	208.2	587.4
DIN (ug/L)	**AFDM_M2**	**Decline**	**67.6**	**0.0001**	59.4	71.5	2092.4
	CHLA_UGL	Increase	9249.7	0.0754	64.0	69.1	3826.9
	CHL:AFDM	**Increase**	**609.4**	**<0.0001**	398.4	609.4	1939.6
	CHLA_M2	**Increase**	**3263.8**	**<0.0001**	1893.9	2912.5	3732.9
	CN_BULK	**Decline**	**1372.4**	**<0.0001**	323.6	1186.0	3525.3
	CP_ALG	**Decline**	**1627.1**	**<0.0001**	523.8	1660.1	3343.7
	CP_BULK	**Decline**	**1627.1**	**<0.0001**	436.2	1372.4	3343.7
	MACALG5+	Increase	2147.6	0.0591	61.6	1007.5	3900.0
	MICALG3+	**Decline**	**21.4**	**0.0147**	21.4	82.9	2060.6
	NP_ALG	Decline	1783.9	0.1290	72.6	1783.9	3358.2
	NP_BULK	**Decline**	**1783.9**	**<0.0001**	1609.3	1783.9	6138.7
	PLNT1+	Decline	71.5	0.0514	59.5	111.8	375.5
	PLNT3+	**Decline**	**71.5**	**0.0002**	61.6	101.4	492.4
	SED3+	**Increase**	**523.8**	**0.0008**	40.1	523.8	3202.8
	TURB_NTU	**Increase**	**73.2**	**<0.0001**	40.5	72.6	1372.4

TP and periphyton C:P were again used as predictors of threshold changes in each of the biological responses listed in Table 3 and 4, but for each of the 8 dates separately (Figures 17, 18; Tables 5, 6). This was done to evaluate seasonal changes and identify variables that responded consistently over the 2 year study.

The results of these date-specific analyses revealed several important conclusions:

- TP consistently predicted nonlinear declines in periphyton C:P ratios (Figure 17), and periphyton C:P ratios consistently predicted the same threshold responses in other variables that were predicted by TP, and even some that TP did not detect.

- The most consistent biological responses, aside from C:N:P ratios in the periphyton, were CHL_AFDM (ratio of periphyton chlorophyll to periphyton AFDM) and PLNT1 and/or PLNT3+ (percentage of the reach with > 0 or > 25% macrophyte cover). Both of these responses reflect fundamental changes to the structure and functioning of wadeable streams.

- Several biological variables were inconsistent responders to P enrichment, but usually this could be explained by seasonal patterns in light and/or discharge (scouring). The consistency of the direction of response (e.g., MACALG5+ always increasing, etc) supports these as reliable indices of excessive P enrichment, but also suggests that sites with TP levels above the reported thresholds will not always exhibit these responses (due to seasonal or hydrological limiting factors).

- Some metrics responded consistently among years within a particular season. In particular, MICALG3+ (% of reach with biofilm thickness > 1 mm) significantly declined only in the summer months (Aug 2006, Sep 2007, and June 2008), but this is perhaps the most critical time of the year in terms of primary production and other ecosystem processes. The consistent increase of CHLA:AFDM ratio also implies that biofilm structure changed with TP enrichment even when there was no effect on biofilm thickness (MICALG3+).

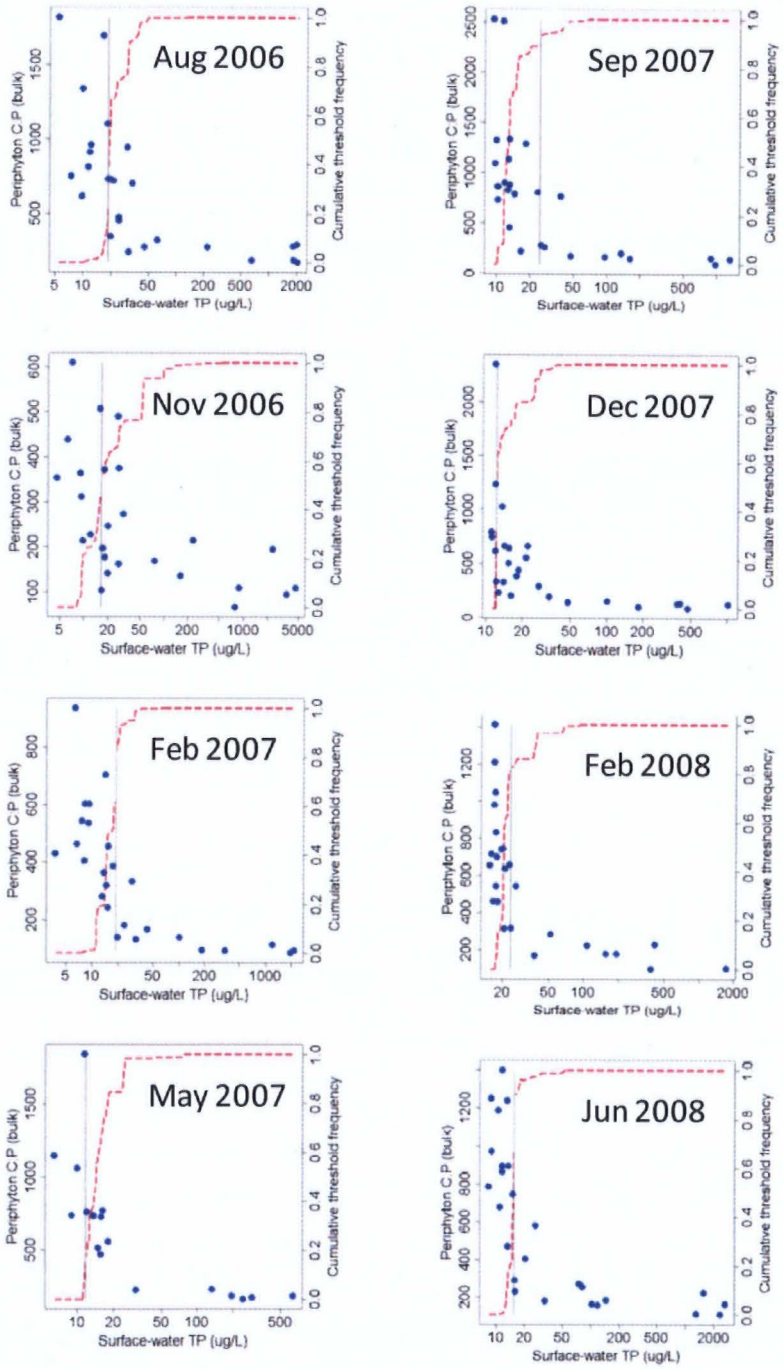

Figure 17. Results from nonparametric changepoint analysis using **surface-water TP (ug/L)** as a predictor of threshold changes in **periphyton C:P ratios (bulk)** across all 26 sites for each of the 8 quarterly sampling events separately, illustrating the consistency of the threshold decline of C:P (bulk), regardless of year, season, or discharge. All TP thresholds were significant, and ranged from a minimum of 11.7 ug/L (May 2007) and a maximum of 24.6 ug/L TP (September 2007). See Table 5 for detailed summaries of the statistical results.

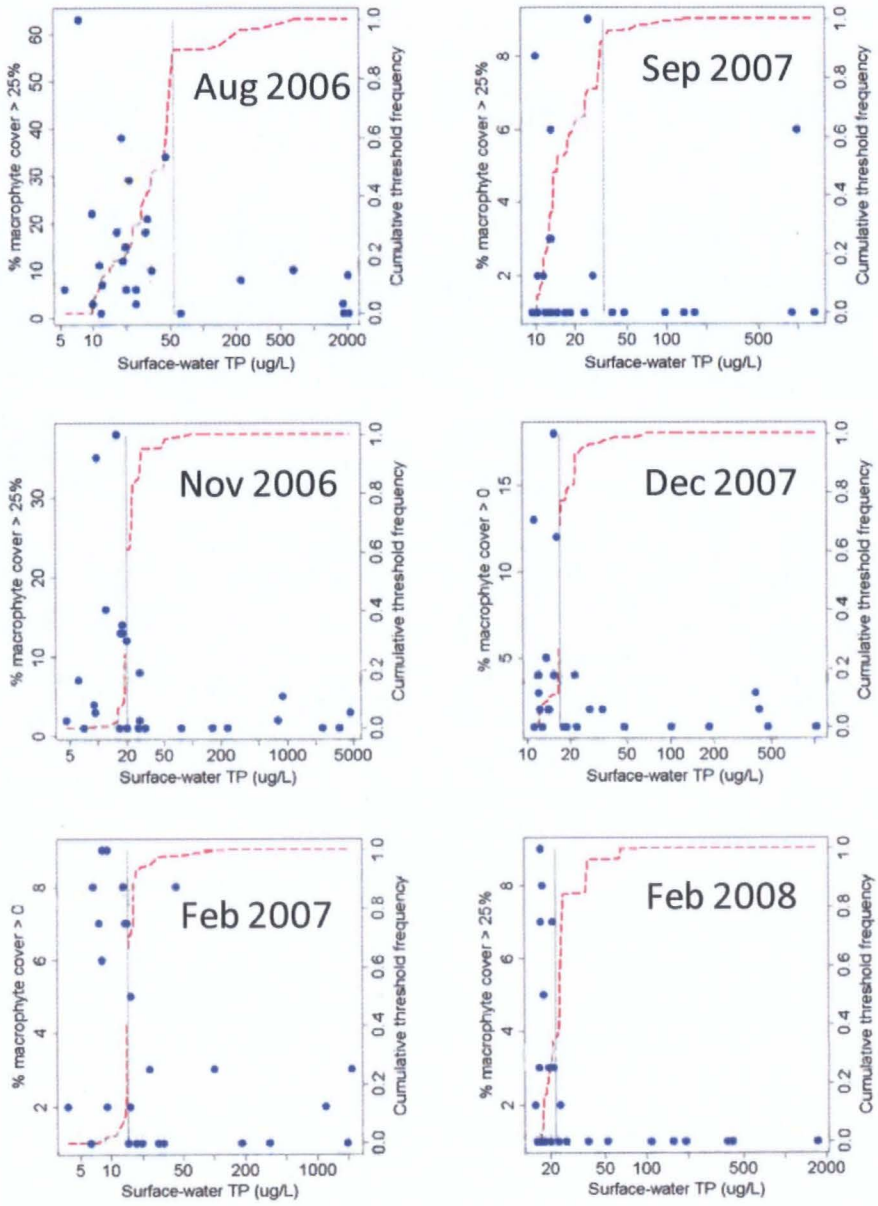

Figure 18. Results from nonparametric changepoint analysis using **surface-water TP (ug/L)** as a predictor of threshold changes in **macrophyte cover** across all 26 sites for 6 of the 8 quarterly sampling events separately, illustrating the consistency of the threshold decline of macrophyte cover regardless of year, season, or discharge. All TP thresholds were significant, and ranged from a minimum of 14.6 ug/L to a maximum of 54.2 ug/L TP (September 2007). See Table 5 for detailed summaries of the statistical results.

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Table 5. Results from nonparamateric changepoint analysis using **surface-water TP (ug/L)** as a predictor of threshold responses in biological and water quality variables among each of the 8 quarterly sampling events from the 26 field study streams during 2006-2008. TP thresholds (Obs) and bootstrap quantiles are shown for each response variable and date combination. Direction (increase or decrease) of the response with increasing TP is shown for thresholds deemed significant (**bold**).

				Cumulative Threshold Quantiles			
Date	Variable	Response	Obs.	10%	50%	90%	P value
Aug-06	**AFDM_M2**	**Decline**	**30.5**	**14.4**	**27.8**	**48.3**	**0.0357**
Nov-06	AFDM_M2		120.1	12.4	31.1	120.1	0.1596
Feb-07	AFDM_M2		26.4	11.5	24.4	28.0	0.1046
May-07	AFDM_M2		14.3	11.9	14.3	18.1	0.1593
Sep-07	AFDM_M2		115.8	10.9	18.4	54.7	0.3967
Dec-07	AFDM_M2		15.5	13.4	15.5	22.0	0.1587
Feb-08	AFDM_M2		45.3	19.1	20.9	39.5	0.1532
Jun-08	AFDM_M2		29.4	10.6	26.6	54.1	0.3157
Aug-06	CHLA_UGL		653.7	14.4	32.8	220.7	0.1326
Nov-06	CHLA_UGL		14.0	12.8	14.5	47.2	0.1156
Feb-07	**CHLA_UGL**	**Increase**	**15.5**	**14.6**	**15.5**	**17.3**	**0.0016**
May-07	**CHLA_UGL**	**Increase**	**82.2**	**11.4**	**15.6**	**30.7**	**0.0503**
Sep-07	**CHLA_UGL**	**Increase**	**21.1**	**18.4**	**20.2**	**26.5**	**0.0050**
Dec-07	**CHLA_UGL**	**Increase**	**22.0**	**15.0**	**22.0**	**40.8**	**0.0105**
Feb-08	**CHLA_UGL**	**Increase**	**45.3**	**20.8**	**36.8**	**65.9**	**0.0037**
Jun-08	**CHLA_UGL**	**Increase**	**18.2**	**13.4**	**18.2**	**21.0**	**0.0087**
Aug-06	**CHL_AFDM**	**Increase**	**437.2**	**27.2**	**45.6**	**233.0**	**0.0028**
Nov-06	**CHL_AFDM**	**Increase**	**801.0**	**23.7**	**76.2**	**236.3**	**0.0074**
Feb-07	**CHL_AFDM**	**Increase**	**37.6**	**21.8**	**32.2**	**65.7**	**0.0044**
May-07	CHL_AFDM		16.0	12.9	15.6	18.1	0.1849
Sep-07	CHL_AFDM		10.2	10.2	12.8	36.4	0.3732
Dec-07	**CHL_AFDM**	**Increase**	**40.8**	**18.9**	**24.7**	**40.8**	**0.0170**
Feb-08	**CHL_AFDM**	**Increase**	**32.2**	**21.0**	**52.5**	**108.0**	**0.0082**
Jun-08	CHL_AFDM		131.2	14.2	32.7	102.7	0.1321
Aug-06	CHLA_M2		54.2	20.5	35.0	141.7	0.1179
Nov-06	CHLA_M2		801.0	14.0	27.4	236.3	0.3263
Feb-07	**CHLA_M2**	**Increase**	**26.4**	**16.5**	**26.4**	**37.6**	**0.0188**
May-07	**CHLA_M2**	**Increase**	**14.3**	**12.9**	**14.3**	**16.5**	**0.0438**
Sep-07	CHLA_M2		10.2	10.2	13.0	42.9	0.4436
Dec-07	**CHLA_M2**	**Increase**	**22.0**	**14.2**	**22.0**	**24.7**	**0.0375**
Feb-08	**CHLA_M2**	**Increase**	**32.2**	**19.7**	**23.4**	**39.5**	**0.0178**
Jun-08	CHLA_M2		29.4	13.4	48.0	82.2	0.1926

Aug-06	**CP_ALG**	**Decline**	**18.5**	**17.6**	**18.5**	**20.5**	**0.0034**
Nov-06	**CP_ALG**	**Decline**	**9.6**	**9.6**	**16.3**	**97.6**	**0.0211**
Feb-07	**CP_ALG**	**Decline**	**37.6**	**8.5**	**26.4**	**37.6**	**0.0075**
May-07	**CP_ALG**	**Decline**	**11.7**	**10.7**	**12.5**	**17.0**	**0.0435**
Sep-07	**CP_ALG**	**Decline**	**12.3**	**12.3**	**13.0**	**31.1**	**0.0202**
Dec-07	**CP_ALG**	**Decline**	**30.6**	**12.2**	**22.0**	**35.1**	**0.0061**
Feb-08	**CP_ALG**	**Decline**	**17.5**	**16.9**	**17.5**	**23.6**	**0.0058**
Jun-08	**CP_ALG**	**Decline**	**13.3**	**12.5**	**13.3**	**45.7**	**0.0051**
Aug-06	**CP_BULK**	**Decline**	**18.5**	**17.6**	**19.3**	**30.5**	**0.0058**
Nov-06	**CP_BULK**	**Decline**	**16.3**	**9.4**	**16.6**	**53.7**	**0.0317**
Feb-07	**CP_BULK**	**Decline**	**18.7**	**11.3**	**17.1**	**20.7**	**0.0036**
May-07	**CP_BULK**	**Decline**	**11.7**	**11.7**	**14.3**	**23.5**	**0.0331**
Sep-07	**CP_BULK**	**Decline**	**24.6**	**11.6**	**13.0**	**24.6**	**0.0141**
Dec-07	**CP_BULK**	**Decline**	**12.2**	**12.0**	**12.3**	**24.9**	**0.0125**
Feb-08	**CP_BULK**	**Decline**	**23.5**	**18.2**	**20.6**	**39.5**	**0.0052**
Jun-08	**CP_BULK**	**Decline**	**15.4**	**13.3**	**15.4**	**15.5**	**0.0011**
Aug-06	**MACALG5+**	**Increase**	**141.7**	**18.5**	**29.2**	**141.7**	**0.0388**
Nov-06	MACALG5+		8.5	11.0	19.1	120.1	0.5798
Feb-07	**MACALG5+**	**Increase**	**8.3**	**8.3**	**8.7**	**26.4**	**0.0420**
May-07	MACALG5+		15.7	11.7	15.6	17.2	0.4341
Sep-07	MACALG5+		11.6	11.6	13.9	42.9	0.6199
Dec-07	MACALG5+		30.6	13.6	22.9	35.1	0.3143
Feb-08	MACALG5+		17.3	17.3	19.1	36.7	0.2957
Jun-08	MACALG5+		10.8	10.8	15.9	75.5	0.6003
Aug-06	**MICALG3+**	**Decline**	**20.6**	**12.1**	**20.6**	**22.3**	**0.0252**
Nov-06	MICALG3+		200.2	14.0	26.8	200.2	0.2573
Feb-07	MICALG3+		9.3	9.3	13.5	72.6	0.5553
May-07	MICALG3+		11.7	11.7	13.8	22.8	0.4841
Sep-07	**MICALG3+**	**Decline**	**10.2**	**10.2**	**10.3**	**13.9**	**0.0013**
Dec-07	MICALG3+		11.9	11.9	15.4	58.4	0.1905
Feb-08	MICALG3+		32.2	17.5	20.3	39.5	0.1097
Jun-08	**MICALG3+**	**Decline**	**13.4**	**13.4**	**14.2**	**14.8**	**0.0368**
Aug-06	**PLNT1+**	**Decline**	**54.2**	**15.8**	**30.1**	**54.2**	**0.0484**
Nov-06	**PLNT1+**	**Decline**	**20.2**	**16.3**	**20.2**	**29.3**	**0.0153**
Feb-07	**PLNT1+**	**Decline**	**14.6**	**14.3**	**14.6**	**17.7**	**0.0267**
May-07	**PLNT1+**	**Decline**	**15.3**	**13.2**	**15.3**	**15.4**	**0.0860**
Sep-07	PLNT1+		13.9	10.3	13.9	31.9	0.3884
Dec-07	**PLNT1+**	**Decline**	**17.1**	**14.3**	**17.1**	**22.0**	**0.0400**

Feb-08	**PLNT1+**	**Decline**	**25.1**	**19.6**	**23.8**	**38.2**	**0.0269**
Jun-08		NA					
Aug-06	**PLNT3+**	**Decline**	**54.2**	**12.1**	**45.6**	**115.4**	**0.0643**
Nov-06	**PLNT3+**	**Decline**	**20.2**	**19.2**	**20.2**	**29.3**	**0.0227**
Feb-07	PLNT3+		72.6	9.3	14.9	42.9	0.1456
May-07	PLNT3+	NA					
Sep-07	PLNT3+		33.0	11.4	14.9	31.9	0.5600
Dec-07	**PLNT3+**	**Decline**	**17.1**	**12.0**	**17.1**	**19.0**	**0.0528**
Feb-08	**PLNT3+**	**Decline**	**22.1**	**18.2**	**23.8**	**38.2**	**0.0460**
Jun-08	PLNT3+	NA					
Aug-06	SED3+		22.8	14.4	22.2	41.0	0.1015
Nov-06	**SED3+**	**Increase**	**9.6**	**9.4**	**11.4**	**17.1**	**0.0407**
Feb-07	**SED3+**	**Increase**	**8.0**	**7.6**	**9.3**	**42.9**	**0.0472**
May-07	SED3+		20.4	11.8	14.7	23.2	0.0699
Sep-07	**SED3+**	**Increase**	**12.8**	**10.2**	**12.8**	**13.0**	**0.0128**
Dec-07	**SED3+**	**Increase**	**12.0**	**12.0**	**14.2**	**22.0**	**0.0429**
Feb-08	**SED3+**	**Increase**	**22.1**	**17.5**	**20.7**	**39.5**	**0.0279**
Jun-08	**SED3+**	**Increase**	**15.9**	**9.9**	**15.9**	**54.1**	**0.0183**
Aug-06	**TURB_NTU**	**Increase**	**17.4**	**15.3**	**17.4**	**19.9**	**0.0267**
Nov-06	TURB_NTU		9.6	9.5	16.3	86.9	0.1315
Feb-07	**TURB_NTU**	**Increase**	**9.3**	**8.9**	**9.3**	**13.5**	**0.0040**
May-07	**TURB_NTU**	**Increase**	**17.2**	**12.5**	**15.9**	**24.4**	**0.0360**
Sep-07	**TURB_NTU**	**Increase**	**17.6**	**13.9**	**20.2**	**31.9**	**0.0077**
Dec-07	**TURB_NTU**	**Increase**	**15.9**	**15.8**	**16.8**	**24.9**	**0.0066**
Feb-08	**TURB_NTU**	**Increase**	**45.3**	**19.4**	**22.4**	**39.5**	**0.0054**
Jun-08	**TURB_NTU**	**Increase**	**18.2**	**13.2**	**18.1**	**21.0**	**0.0027**

Table 6. Results from nonparamateric changepoint analysis using **periphyton C:P ratio (bulk)** as a predictor of threshold responses in biological and water quality variables among each of the 8 quarterly sampling events from the 26 field study streams during 2006-2008. Periphyton C:P thresholds (*Obs.*) and bootstrap quantiles are shown for each response variable and date combination. Direction (increase or decrease) of the response with increasing periphyton C:P is shown for thresholds deemed significant (**bold**). **Note that an increasing value of C:P corresponds to a decline in P enrichment in the periphyton.*

				Cumulative Threshold Quantiles			
Date	Variable	Response*	Obs.	10%	50%	Date	Variable
Aug-06	**AFDM_M2**	**Increase**	**275.1**	**274.7**	**306.4**	**597.9**	**0.0386**
Nov-06	AFDM_M2		373.6	122.5	195.4	368.0	0.2509
Feb-07	AFDM_M2		373.6	135.7	373.6	461.2	0.1407
May-07	AFDM_M2		182.5	182.7	368.3	726.0	0.1484
Sep-07	AFDM_M2		155.0	155.0	534.4	886.0	0.6122
Dec-07	AFDM_M2		328.9	212.9	334.6	624.7	0.3682
Feb-08	AFDM_M2		297.5	175.8	297.5	499.0	0.2741
Jun-08	AFDM_M2		278.7	160.6	278.7	709.1	0.4895
Aug-06	CHLA_UGL		618.3	275.1	387.7	706.6	0.4483
Nov-06	CHLA_UGL		368.0	149.2	213.9	364.1	0.4072
Feb-07	**CHLA_UGL**	**Decline**	**394.4**	**205.6**	**347.4**	**457.9**	**0.0191**
May-07	CHLA_UGL		731.0	204.0	477.2	731.0	0.0520
Sep-07	**CHLA_UGL**	**Decline**	**814.8**	**204.9**	**814.8**	**859.0**	**0.0086**
Dec-07	**CHLA_UGL**	**Decline**	**328.9**	**169.9**	**408.5**	**659.8**	**0.0158**
Feb-08	**CHLA_UGL**	**Decline**	**297.5**	**266.2**	**297.5**	**369.3**	**0.0068**
Jun-08	**CHLA_UGL**	**Decline**	**762.4**	**180.5**	**743.1**	**803.1**	**0.0108**
Aug-06	**CHL_AFDM**	**Decline**	**306.4**	**212.3**	**306.4**	**344.2**	**0.0052**
Nov-06	CHL_AFDM		205.1	140.5	205.1	236.6	0.0514
Feb-07	**CHL_AFDM**	**Decline**	**123.2**	**116.9**	**164.7**	**429.3**	**0.0280**
May-07	CHL_AFDM		226.4	226.4	391.5	639.2	0.1566
Sep-07	**CHL_AFDM**	**Decline**	**1207.6**	**501.2**	**867.0**	**1130.4**	**0.0220**
Dec-07	**CHL_AFDM**	**Decline**	**659.8**	**169.9**	**354.9**	**659.8**	**0.0225**
Feb-08	**CHL_AFDM**	**Decline**	**171.2**	**175.2**	**225.3**	**271.0**	**0.0086**
Jun-08	**CHL_AFDM**	**Decline**	**523.3**	**236.7**	**523.3**	**863.2**	**0.0294**
Aug-06	CHLA_M2		212.3	212.3	317.5	740.0	0.1763
Nov-06	CHLA_M2		205.1	109.4	204.0	212.1	0.1650
Feb-07	**CHLA_M2**	**Decline**	**123.2**	**123.2**	**300.3**	**429.3**	**0.0480**
May-07	CHLA_M2		226.4	204.0	345.9	616.1	0.0958
Sep-07	**CHLA_M2**	**Decline**	**1207.6**	**510.8**	**851.5**	**993.1**	**0.0342**
Dec-07	**CHLA_M2**	**Decline**	**328.9**	**194.6**	**328.9**	**658.9**	**0.0460**

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Feb-08	**CHLA_M2**	**Decline**	**297.5**	**269.2**	**297.5**	**385.4**	**0.0178**
Jun-08	CHLA_M2		278.7	170.5	345.9	877.6	0.1525
Aug-06	MACALG5+		306.4	299.4	306.4	658.6	0.1331
Nov-06	MACALG5+		173.5	109.4	173.5	242.2	0.6289
Feb-07	MACALG5+		537.6	123.2	262.2	457.9	0.7244
May-07	MACALG5+		470.2	204.0	455.6	726.0	0.5601
Sep-07	MACALG5+		143.5	143.5	352.5	867.0	0.1605
Dec-07	MACALG5+		213.3	162.3	213.3	606.6	0.2077
Feb-08	**MACALG5+**	**Decline**	**642.1**	**252.0**	**642.1**	**705.8**	**0.0481**
Jun-08	MACALG5+		158.4	158.4	241.6	709.1	0.3257
Aug-06	**MICALG3+**	**Increase**	**306.4**	**299.4**	**317.5**	**724.5**	**0.0497**
Nov-06	MICALG3+		324.0	122.6	259.1	333.2	0.2077
Feb-07	MICALG3+		354.6	114.4	277.7	441.6	0.6632
May-07	MICALG3+		470.2	204.0	474.1	726.0	0.8096
Sep-07	**MICALG3+**	**Increase**	**993.1**	**758.6**	**840.7**	**993.1**	**0.0208**
Dec-07	MICALG3+		328.9	127.5	328.9	528.8	0.2828
Feb-08	MICALG3+		540.2	297.5	526.2	686.3	0.1434
Jun-08	**MICALG3+**	**Increase**	**762.4**	**335.8**	**679.7**	**877.6**	**0.0264**
Aug-06	**PLNT1+**	**Increase**	**212.3**	**212.3**	**363.4**	**711.2**	**0.0273**
Nov-06	**PLNT1+**	**Increase**	**195.6**	**169.9**	**195.6**	**221.0**	**0.0234**
Feb-07	**PLNT1+**	**Increase**	**139.8**	**136.1**	**262.2**	**427.0**	**0.0563**
May-07	PLNT1+		486.6	368.3	486.6	727.9	0.2028
Sep-07	PLNT1+		1207.6	233.5	843.5	1109.9	0.2403
Dec-07	**PLNT1+**	**Increase**	**470.7**	**169.9**	**470.7**	**524.4**	**0.0591**
Feb-08	**PLNT1+**	**Increase**	**676.6**	**269.2**	**596.2**	**713.4**	**0.0331**
Jun-08	PLNT1+	NA					
Aug-06	PLNT3+		212.3	229.3	524.4	598.9	0.1627
Nov-06	**PLNT3+**	**Increase**	**173.5**	**169.9**	**188.0**	**230.1**	**0.0546**
Feb-07	**PLNT3+**	**Increase**	**154.0**	**153.7**	**154.0**	**416.4**	**0.0436**
May-07	PLNT3+						
Sep-07	PLNT3+		843.5	217.0	821.7	1013.6	0.3490
Dec-07	PLNT3+		659.8	169.9	418.7	659.8	0.2043
Feb-08	**PLNT3+**	**Increase**	**297.5**	**269.2**	**456.7**	**725.8**	**0.0572**
Jun-08	PLNT3+						
Aug-06	**SED3+**	**Decline**	**779.8**	**484.6**	**749.6**	**908.4**	**0.0558**
Nov-06	**SED3+**	**Decline**	**292.3**	**177.2**	**292.3**	**313.4**	**0.0691**
Feb-07	SED3+		93.6	93.6	160.2	444.9	0.3262
May-07	SED3+		486.6	205.1	486.6	726.0	0.1023

Sep-07	**SED3+**	**Decline**	**793.0**	**767.2**	**806.2**	**993.1**	**0.0337**
Dec-07	SED3+		701.3	137.9	308.5	635.6	0.3543
Feb-08	**SED3+**	**Decline**	**297.5**	**269.2**	**386.0**	**676.6**	**0.0692**
Jun-08	**SED3+**	**Decline**	**523.3**	**270.7**	**523.3**	**803.1**	**0.0187**
Aug-06	**TURB_NTU**	**Decline**	**779.8**	**724.5**	**770.2**	**829.0**	**0.0181**
Nov-06	TURB_NTU		259.1	122.6	207.7	278.9	0.3126
Feb-07	**TURB_NTU**	**Decline**	**373.6**	**352.2**	**394.4**	**457.9**	**0.0326**
May-07	**TURB_NTU**	**Decline**	**486.6**	**368.3**	**486.6**	**731.0**	**0.0307**
Sep-07	TURB_NTU		814.8	188.3	754.5	830.3	0.0822
Dec-07	**TURB_NTU**	**Decline**	**169.9**	**166.0**	**173.9**	**401.4**	**0.0197**
Feb-08	**TURB_NTU**	**Decline**	**385.4**	**297.5**	**385.4**	**642.1**	**0.0081**
Jun-08	**TURB_NTU**	**Decline**	**626.4**	**278.7**	**626.4**	**660.4**	**0.0054**

RESULTS AND INTERPRETATION, CONTINUED

Effects of TP on Diel Dissolved Oxygen

Interannual differences in stream discharge strongly influenced the daily (diel) variation in dissolved oxygen, water temperature, and pH (Figure 19).

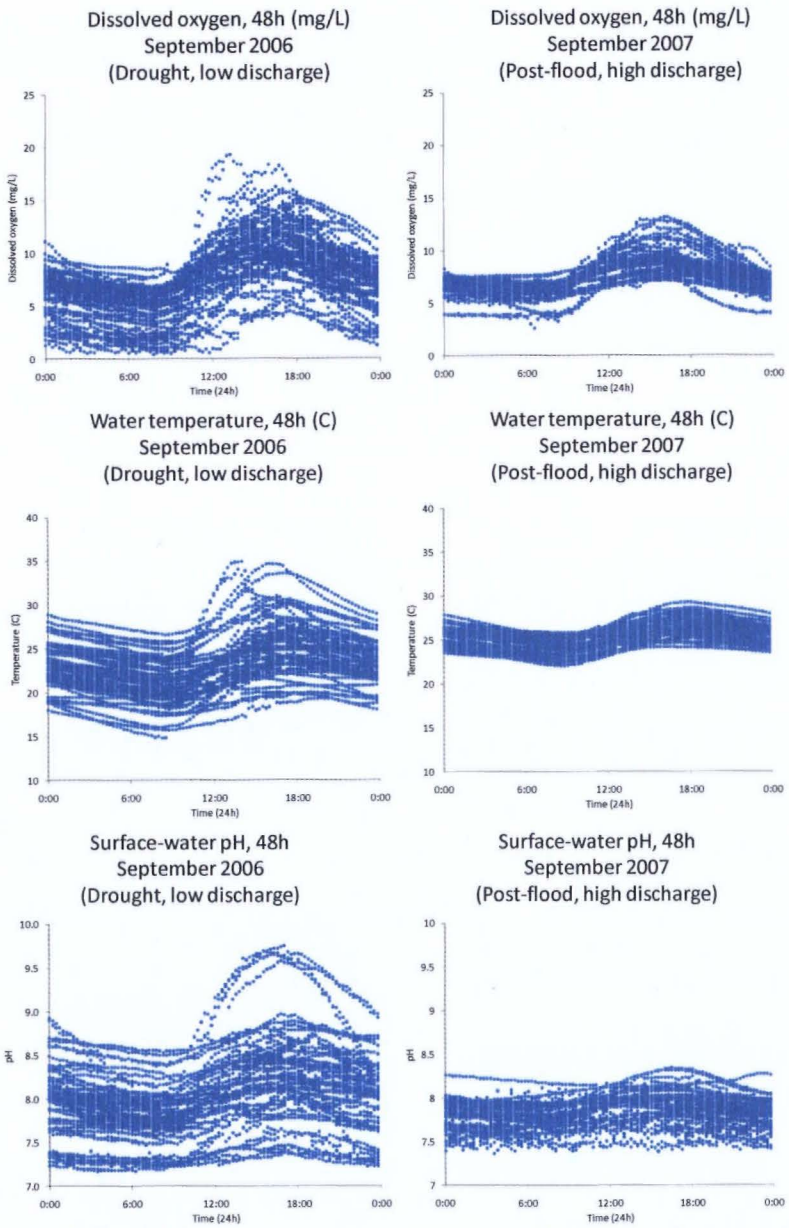

Figure 19. Patterns of dissolved oxygen, water temperature, and pH across 2-consecutive 24-hour periods at each of the 26 field study sites during a period of very low discharge (September 2006) and during a period of high flows following significant flooding (September 2007).

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Figure 19 clearly illustrates the important influence of stream flow on diel water chemistry. In 2006, a drought year, most of the field-study streams had very low-to-no measureable discharge. This lack of flow limited the turbulent mixing of the water column. Without turbulence, gas exchange between air and water was limited to passive diffusion. During the day, this caused many of the streams to become supersaturated with dissolved oxygen (up to 20 mg/L DO; > 250% saturation) which concomitantly caused pH to increase to relatively high levels (8.5-9.5). At night, DO was rapidly consumed. Several streams had minimum DO levels near 0 mg/L, often for several hours (typically between 00:00-09:00, but in some cases until 12:00). Water temperature also varied widely at different hours of the day, reaching temperatures up to 30-35 C during late afternoon (Figure 19).

In September 2007, discharge was relatively high in all 26 study streams. Most of the streams were in flood stage for several of the preceding months, and had just recently subsided to wadeable flows. Even under these higher flows and turbulent mixing conditions, dissolved oxygen showed clear diel variation, peaking around 16:00 at concentrations up to 12 mg/L (120-150% saturation), and declining during the night to typically 6-8 mg/L DO. However, minimum DO levels were rarely below 5 mg/L, and no stream had minimum DO levels that would be considered detrimental to native fishes or macroinvertebrates in this region, especially in moving water.

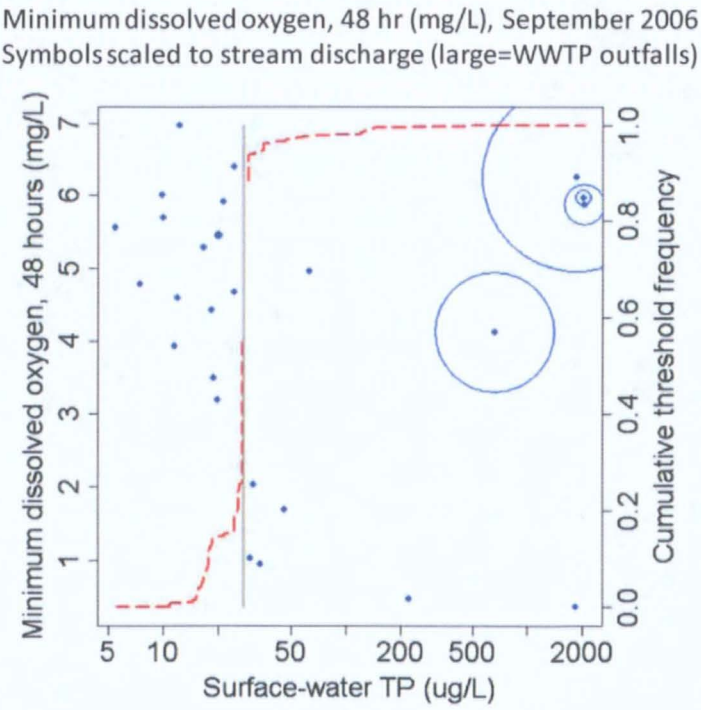

Figure 20. Results of nonparametric changepoint analysis using surface water TP (ug/L) as a predictor of threshold declines in minimum dissolved oxygen (DO, mg/L) during September 2006. The four points in the upper right corner of the plot correspond to streams immediately downstream of WWTP discharges; these were the only flowing streams during this sampling event.

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The very low minimum DO values at several of the sites in 2006 were strongly related to surface-water TP, but also stream discharge (Figure 20). All 6 streams with no detectable flow and TP > 27.2 had minimum DO values < 2 mg/L, and two others with TP near 20 ug/L dropped below 3.5 mg/L DO. The four streams located immediately downstream of effluent discharges maintained relatively high minimum DO concentrations despite very high TP. This was related to reaeration associated with turbulent mixing. Despite these outliers, TP was nevertheless a significant predictor of threshold declines in minimum DO (Table 7).

The diel pattern of DO at NBOS-02 (North Bosque River - 02) is further evidence that WWTP discharge sites maintained relatively high minimum DO because of turbulent mixing associated with flow. NBOS-02 is located a few kilometers downstream of NBOS-01, one of the 4 WWTP discharge sites depicted in Figure 20. NBOS-02 had DO levels fall to near 0 mg/L the same night that NBOS-01 maintained DO above 5 mg/L. Because of the drought, the water table had dropped below the stream channel and discharge from NBOS-01 subsided to a slow trickle by the time it reached NBOS-02. Here, P enrichment stimulated photosynthesis during the day whereas microbial respiration, also fueled by P-rich organic matter, was even higher at night. Thus, the stream was essentially anoxic for several hours at night and early morning.

Minimum DO also corresponded to mean biofilm thickness, filamentous algal cover, and the ratio of periphyton chlorophyll to AFDM (Figure 21). As previously shown, thicker layers of calcareous periphyton comprised mostly of diatoms, cyanobacteria, and other microbes were associated with low-P streams, and as TP increased, these biofilms succeeded to filamentous and colonial greens and other types of algae. The results in Figure 21 explicitly link TP to sharp nonlinear responses of primary producers, which, in turn, cause sharp nonlinear declines in minimum DO. Thus, these metrics appear to not only be sensitive to TP enrichment, but also can be linked quantitatively to aquatic life use standards that rely on dissolved oxygen as a measure of biological integrity.

These flow-dependent results imply that studies on the effects of nutrients on DO will not adequately characterize risk to biota and associated aquatic life use designations without sampling during periods of low flow.

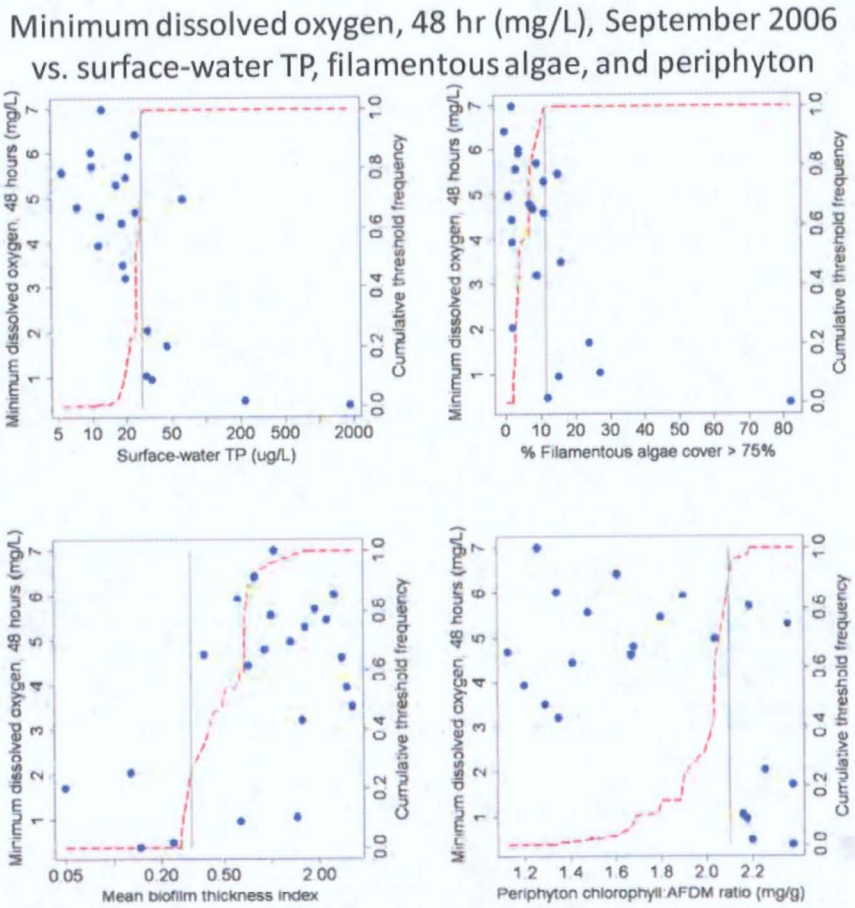

Figure 21. Results of nonparametric changepoint analysis using surface water TP (ug/L), biofilm thickness, filamentous algal cover, and periphyton chlorophyll-to-AFDM ratio as predictors of threshold declines in minimum dissolved oxygen (DO, mg/L) during September 2006. The four streams immediately downstream of WWTP discharges were excluded from the analysis because these were the only flowing streams during this sampling event.

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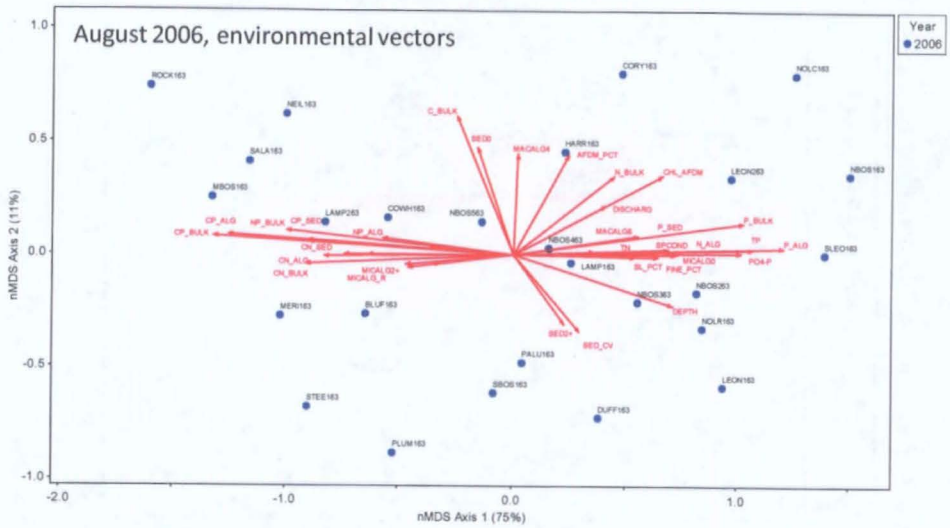
Table 7. Results from nonparametric changepoint analysis using surface-water TP, periphyton C:P bulk, filamentous macroalgal cover > 75% (MACALG5+) and mean biofilm thickness (MICALG_R) as threshold predictors of **minimum dissolved oxygen** during 48 h among 26 stream sites in September 2006 and 2007. Significant thresholds in DO are highlighted in bold. *All 26 sites. **Four WWTP sites with high discharge and turbulent mixing removed from analysis. See Figures 20 and 21 for graphical display of results.

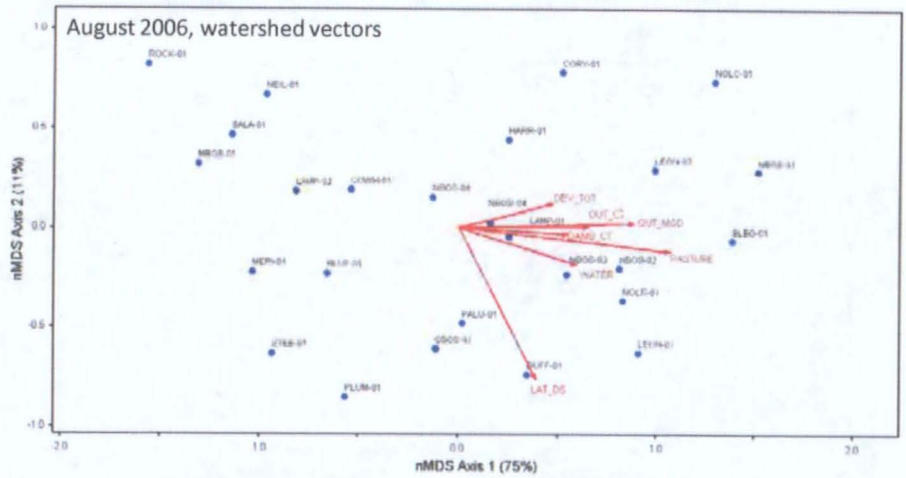
			Cumulative Threshold Quantiles				Mean DO 48 h min (mg/L)	
	Predictor	Obs.	10%	50%	90%	*P* value	Below	Above
September 2006 (drought, low flow)								
	TP*	**27.2**	**17.6**	**27.2**	**29.2**	**0.0499**	**5.09**	**3.06**
	TP**	**27.2**	**19.4**	**24.5**	**27.2**	**0.0068**	**5.09**	**1.64**
	CP_BULK**	**299.4**	**299.4**	**321.9**	**698.8**	**0.0237**	**1.14**	**4.63**
	MACALG5+**	**11.5**	**2.5**	**4.0**	**11.0**	**0.0170**	**4.96**	**1.92**
	MICALG_R**	**0.31**	**0.31**	**0.51**	**0.775**	**0.0237**	**1.14**	**4.63**
September 2007 (post-flood, high flow)								
	TP	12.9	11.6	12.9	47.3	0.5489	5.59	6.13
	CP_BULK	143.4	143.4	517.8	885.9	0.1694	5.25	6.05

RESULTS AND INTERPRETATION, CONTINUED

Periphyton Taxonomic Responses to TP: Ordinations



Increasing P enrichment, sedimentation, chlorophyll, filamentous macroalgae
Decreasing periphyton C:P, C:N ratios, biofilm thickness



Increasing WWTP outfall discharge, pasture, reservoirs, dams, and urban land

Figure 22. Nonmetric multidimensional scaling ordination of algal species composition among the 26 field study sites in August 2006. Abundance data (no. of cells/cm^2) was $\log_{10}(x+1)$ transformed prior to analysis. Bray-Curtis distance was used as the dissimilarity metric. Distances between sites in the ordination space are proportional to taxonomic dissimilarity (near=similar, far=dissimilar). In each figure, the red arrows (vectors) represent the direction and magnitude of significant ($p<0.05$) correlations between environmental variables and algal species composition. The upper panel shows the significant environmental predictors of algal species: nutrients (e.g., TP, periphyton C:P), sediment, biofilm thickness, and filamentous algae. The lower panel shows the significant watershed predictors of algae species: pasture, outfalls, and urban development in watersheds. See Table 1 and 2 for full variable names.

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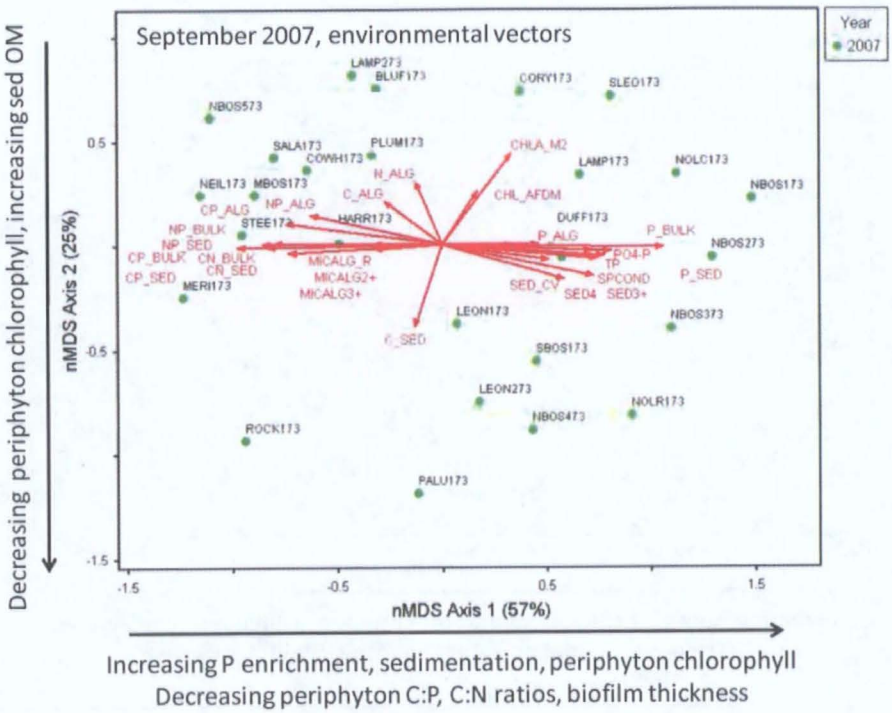

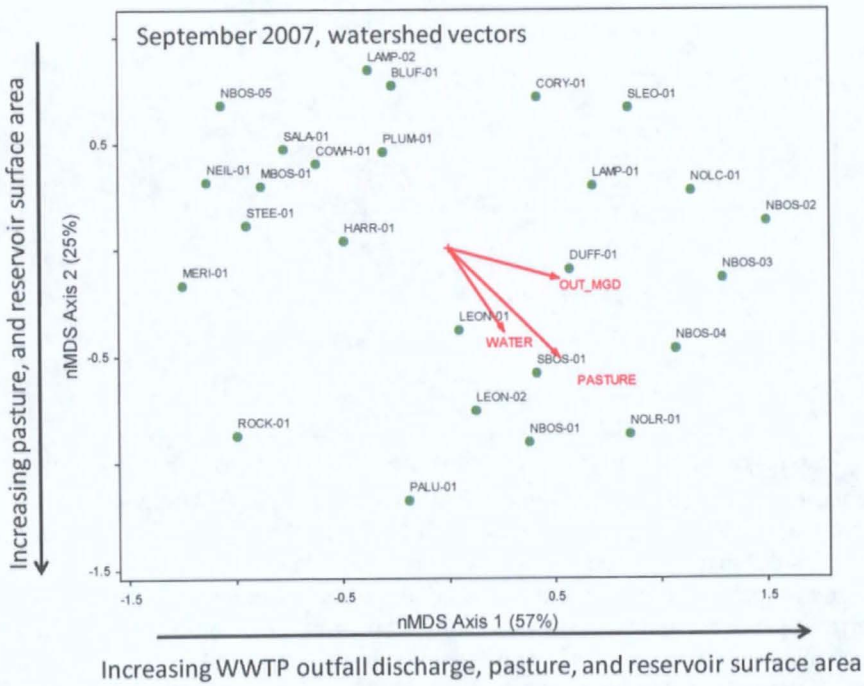

Figure 23. Nonmetric multidimensional scaling ordination of algal species composition among the 26 field study sites in September 2007. Most of the environmental and watershed variables that were strong predictors of algal species composition in 2006 were also significant predictors in these ordinations. See Figure 22 for details.

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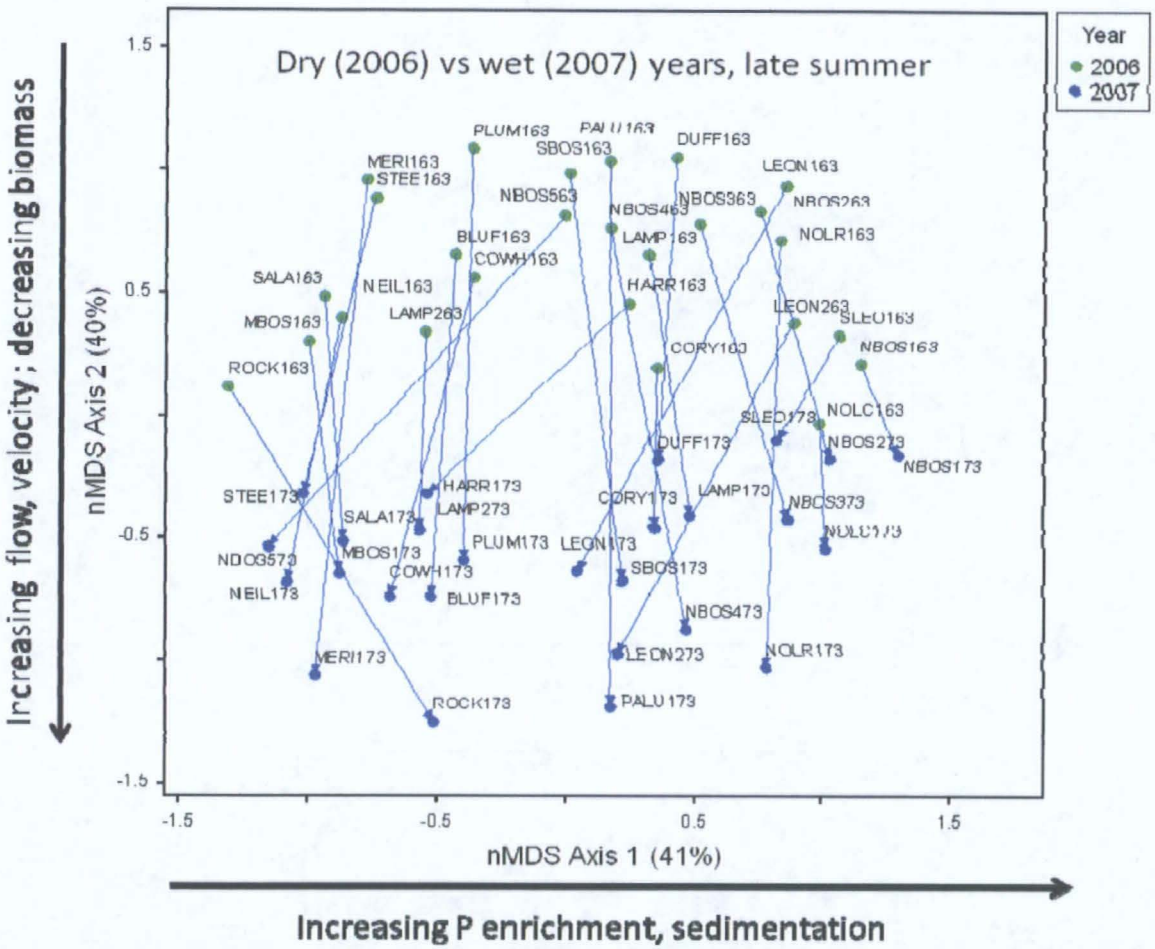

Figure 24. Nonmetric multidimensional scaling ordination of algal species composition among the 26 field study sites in August 2006 and September 2007. The arrows indicate directions and magnitude of change in algal species composition between years at each of the 26 sites.

The multivariate analyses of algal species composition during August 2006 and September 2007 continued to support the earlier conclusions that TP and related metrics had a strong influence on stream biota (Figures 22-24). In both years, most of the variance in algal species composition was related to TP. Sites with the lowest TP and highest periphyton C:P ratios were placed at the low end of axis 1 whereas sites with the highest TP and lowest C:P ratios were at the opposite end of axis 1. Figure 24 is particularly interesting because it showed that although algal species changed between years (illustrated by the shift in sites from 2006 to 2007 along axis 2), the relative order of sites from low P to high P remained intact on axis 1. This is compelling evidence that algal species composition was altered by nutrient enrichment, and the magnitude of its effect was similar regardless of interannual variation in stream discharge.

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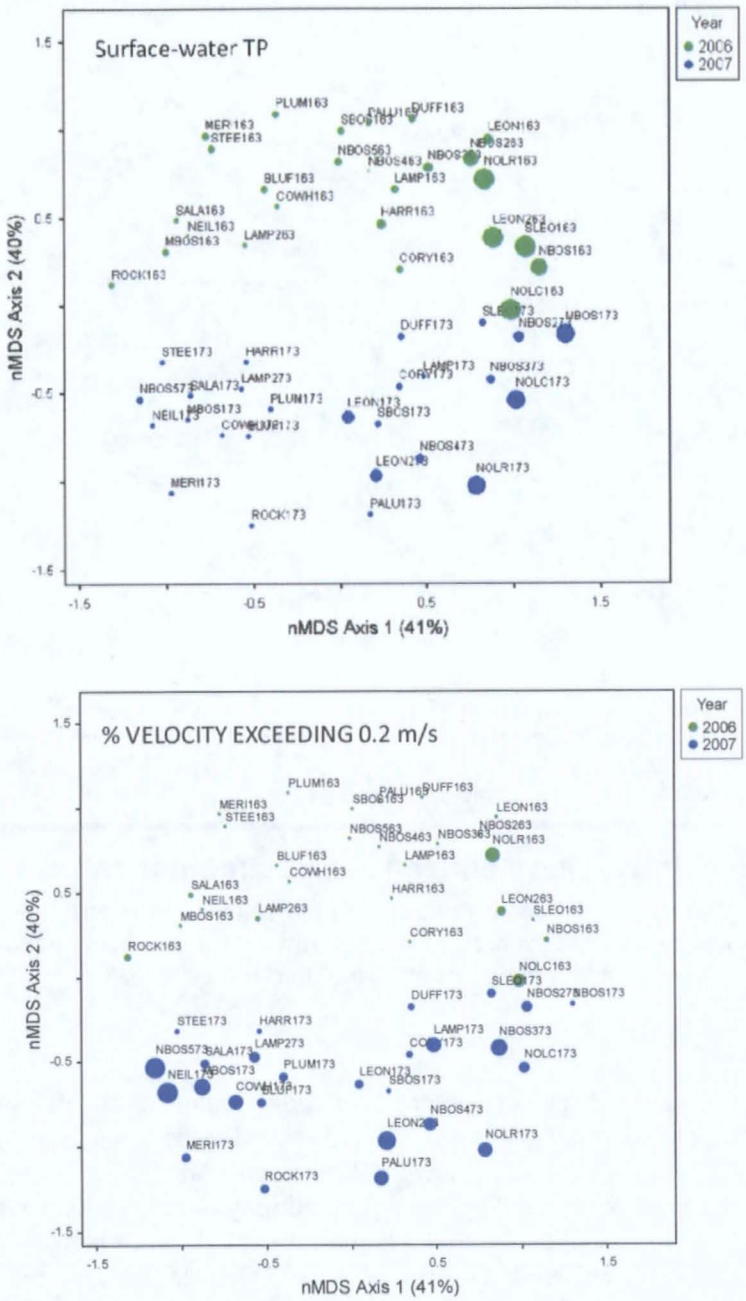

Figure 25. Nonmetric multidimensional scaling ordination of algal species composition among the 26 field study sites in August 2006 and September 2007. The ordination diagram is identical to Figure 24, except that site symbols are scaled in proportion to surface-water TP (upper panel) and the proportion of the reach with velocity > 0.2 m/s (lower panel).

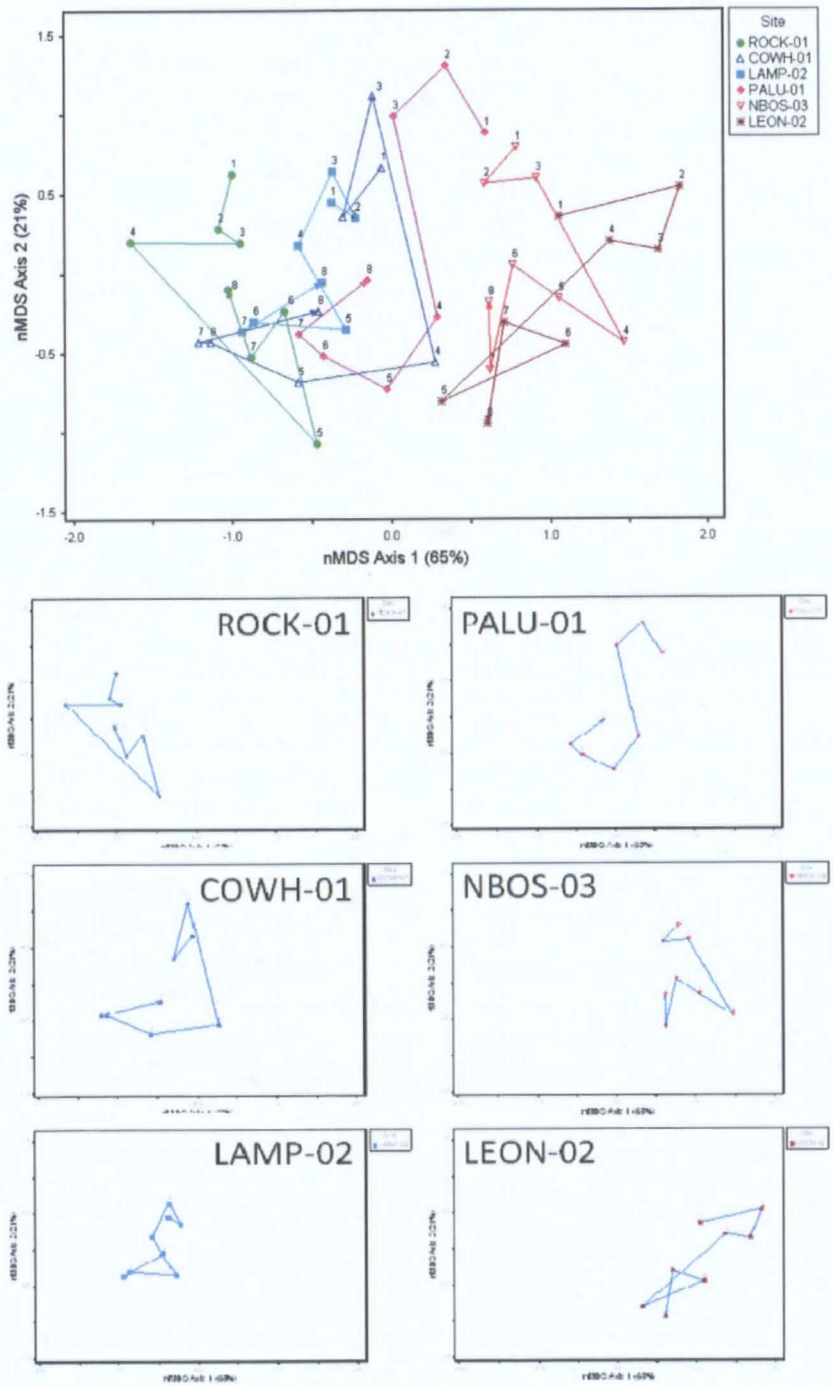

Figure 29. Nonmetric multidimensional scaling ordination of algal species composition over the 8 quarterly sampling events at the 6 intensive sampling sites (see Figure 1). Lines connect dates within each site in chronological order, starting with August 2006 (1) and ending with June 2008 (8). For clarity, patterns of community succession are shown for each site separately in the lower 6 panels.

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As shown previously in the ordination of sites between years (2006 vs. 2007), algal species composition varied significantly over time (Figure 29). The 6 sites selected for species analysis on all 8 quarterly sampling events ("intensive sites") followed the same general trajectory of change from along nMDS Axis 2, shifting down axis 2 during the high flow periods of May and September 2007, but turning around and moving back up axis 2 toward their original positions in August 2006.

Importantly, each site stayed in the same approximate position along axis 1 (the nutrient axis) over time. ROCK-01, COWH-01, and LAMP-02 overlapped somewhat in their trajectories, but each of these sites was consistently below the observed TP thresholds reported in the preceding results. Thus, their similarity is to be expected.

PALU-01 remained consistently intermediate along Axis 1, distinctly separated from the low-P sites during the first few sampling events, but overlapping with COWH-01 near the end of the study. NBOS-03 and LEON-02, both highly enriched sites, consistently stayed on the right end of axis 1. NBOS-03 had high TP levels for most of the study (~100-200 ug/L). In 2006, LEON-02 had very high levels of TP (> 2 mg/L), but TP declined to ~100 ug/L in the latter half of the study (Figure 6). The decline in TP was also coincident with a slight shift to the left in algal species composition, possibly indicating a decline in the few taxa that were shown to proliferate mostly at the levels of TP > 200 ug/L (Tables 10-13).

RESULTS AND INTERPRETATION, CONTINUED

Periphyton Taxonomic Responses to TP: Threshold Indicator Taxa Analysis (TITAN)

TITAN analyses on algal species composition in 2006 and 2007 continued to add evidence to an already substantial case for a threshold at low levels of TP. Dozens of algal species essentially disappeared from streams between 12 and 30 ug/L TP, with the threshold of greatest overall decline (sum(z-)) at 19.2 and 21.6 ug/L in 2006 and 2007, respectively (Figure 26, Table 8). Simultaneously, numerous algal taxa showed sharp increases, either replacing taxa as they declined, or driving the declines via competitive exclusion (e.g., shading by *Cladophora*=CLAglome, a significant increasing taxon in both years; Table 10).

Although most taxa declined or increased at relatively low levels of TP, a few taxa responded only to much higher levels of TP (Figure 26, Table 10). These taxa were mostly associated with sites heavily influenced by effluent.

Fewer taxa declined in response to TP in 2007 than 2006, but as many or more increased in response to TP. Because of the protracted period of flooding prior to September 2007, many of the streams were in a period of rapid recolonization (community succession). Scouring of rocks reduced periphyton biomass substantially compared to 2006. It is likely that part of the explanation was that fast-responding "weedy" algal taxa proliferated with elevated TP, whereas slower responding, low-P indicator taxa had not become as consistently established across all streams as in 2006.

Another explanation for differences between 2006 and 2007 was that surface-water TP was not as strong of an indicator of P-enrichment status during higher and more variable flows. There is some support for this hypothesis given that more threshold indicator taxa were deemed significant in 2007 when using periphyton C:P ratios (bulk or OM) than TP (Table 9, 11; Figure 27, 28). This implies that exposure and availability (via recycling, sediment-bound P, etc) at these sites was better indicated by the P in the periphyton than in an instantaneous measurement from the water.

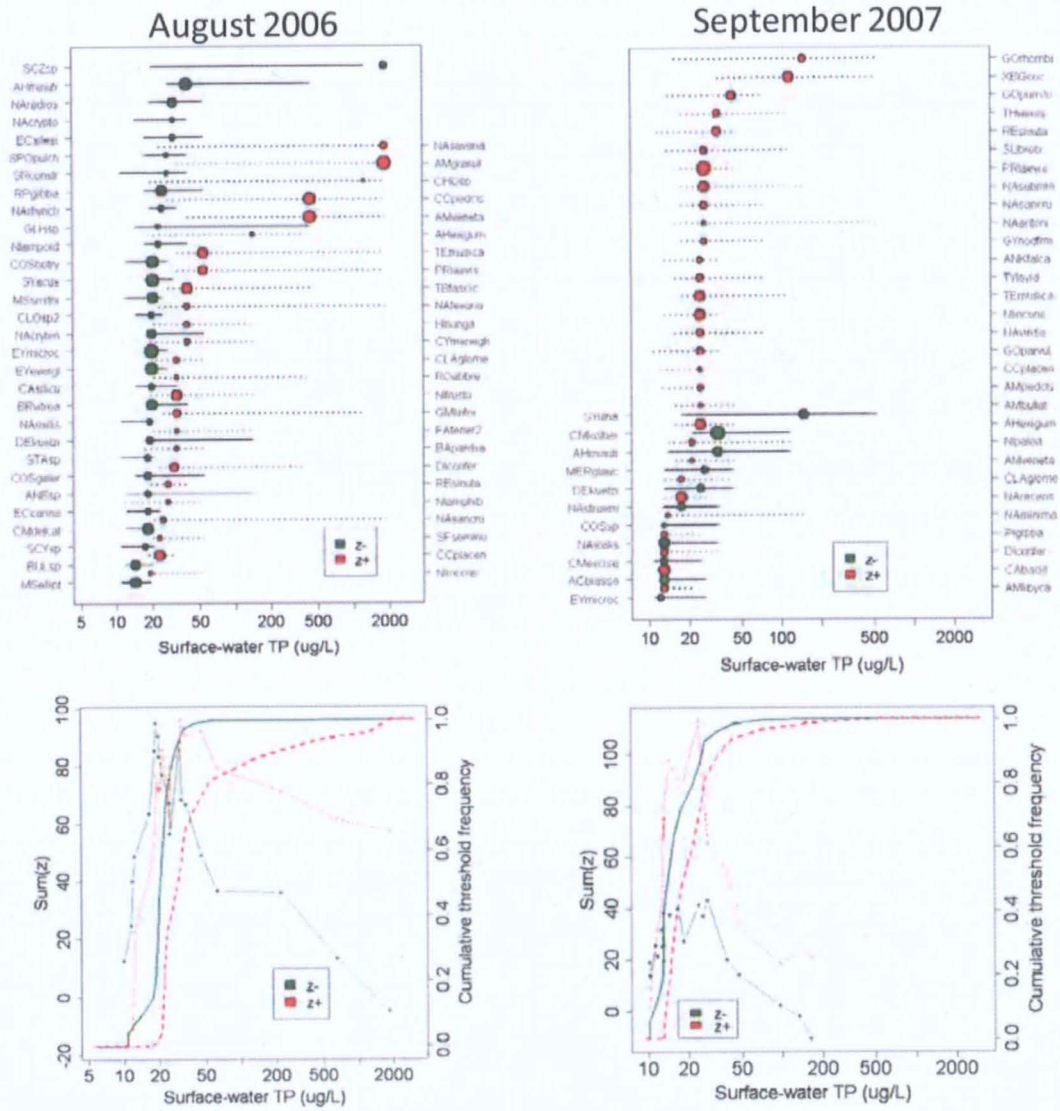

Figure 26. Results of Threshold Indicator Taxa ANalysis (TITAN) using surface-water TP a a predictor of threshold changes in individual algal species during August 2006 and September 2007. Taxa are classified as either negative (z-) or positive (z+) threshold indicators based on the direction of response to TP. The upper panels show the observed TP threshold value (colored symbols) for each taxon deemed to change significantly. Taxon IDs (see Appendix 1) are shown on the left (negative indicators) and right (positive indicators) y-axes, in rank order of their TP thresholds. Line segments around each symbol are 90% confidence intervals around the TP threshold. The lower two panels show the aggregate response of negative (sum(z-)) and positive (sum(z+)) threshold indicator taxa. The TP value resulting in the highest sum(z) value is the point in which the greatest cumulative negative (z-) or positive (z+) occurs. Bootstrapping is used to estimate the cumulative threshold frequency for negative (green) and positive (red) responses, respectively. See Tables 8 and 9 for community level (sum(z)) thresholds, and Tables 10 and 12 for taxa-specific thresholds.

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Table 8. Community-level results from Threshold Indicator Taxa Analysis (TITAN) on algal species composition in response to water and periphyton nutrient concentrations during **August 2006** (drought, n=26 sites). Thresholds (*Obs.*) are based on the value of the predictor resulting in the greatest aggregate decrease (sum(z-)) or increase (sum(z+)) in the frequency and abundance of taxa in the community. Taxa responses associated with lower nutrient conditions are shown in **bold.** The lower (*5%, 10%*), middle (*50%*), and upper (*90%, 95%*) quantiles of 1,000 bootstraps represent measures of uncertainty around the observed threshold. **Note that lower C:P values = higher P enrichment relative to organic carbon in the periphyton, thus taxa that "decrease" sharply in response to increasing C:P are associated with higher levels of P-enrichment,, whereas "increaser" taxa are associated with lower levels of P enrichment..* Figures 26-28 for details.

August 2006 (n=26 streams)			Bootstrap Cumulative Distribution of Thresholds				
Predictor	Obs.	Response > Obs Threshold Value	5%	10%	50%	90%	95%
Surface-water TP (ug/L)	**19.2**	**Decline (z-)**	**11.7**	**17.1**	**20.6**	**30.4**	**32.4**
	32.4	Increase (z+)	22.8	22.8	30.4	437.2	1250.2
Surface-water PO4-P (ug/L)	**4.4**	**Decline (z-)**	**4.4**	**4.4**	**5.1**	**14.3**	**16.2**
	30.8	Increase (z+)	9.6	9.6	14.3	1181.8	1808.3
Periphyton C:P ratio (OM)	369	Decline *(z-)	313	338	461	500	515
	628	**Increase* (z+)**	**445**	**461**	**563**	**833**	**1017**
Periphyton C:P ratio (bulk)	465	Decline* (z-)	284	306	546	711	725
	780	**Increase* (z+)**	**659**	**659**	**729**	**925**	**926**
Surface-water DIN (ug/L)	**56.9**	**Decline (z-)**	**28.1**	**28.1**	**54.4**	**64.9**	**85.3**
	85.3	Increase (z+)	56.9	56.9	834.3	7411.5	7411.5
Surface-water TN (ug/L)	**280.6**	**Decline (z-)**	**222.6**	**222.6**	**280.6**	**663.0**	**1162.5**
	1162.5	Increase (z+)	613.0	663.0	1852.5	8510.0	8510.0

Table 9. Community-level results from Threshold Indicator Taxa Analysis (TITAN) on algal species composition in response to water and periphyton nutrient concentrations during **September 2007** (post-flood, n=26 sites). Thresholds (*Obs.*) are based on the value of the predictor resulting in the greatest aggregate decrease (sum(z-)) or increase (sum(z+)) in the frequency and abundance of taxa in the community. Taxa responses associated with lower nutrient conditions are shown in **bold.** See Table 8 and Figures 26-28 for details.

September 2007 (n=26 streams)			Bootstrap Cumulative Distribution of Thresholds				
Predictor	Obs.	Response > Obs. Threshold Value	5%	10%	50%	90%	95%
Surface-water TP (ug/L)	**21.6**	**Decline (z-)**	**10.3**	**11.6**	**15.6**	**26.5**	**33.0**
	24.5	Increase (z+)	13.1	13.8	21.1	42.8	71.9
Surface-water PO4-P (ug/L)	**13.5**	**Decline (z-)**	**10.0**	**10.9**	**13.5**	**14.8**	**17.3**
	13.5	Increase (z+)	12.1	13.2	14.3	28.4	45.1
Periphyton C:P ratio (OM)	421	Decline *(z-)	279	283	379	457	485
	737	**Increase* (z+)**	**356**	**379**	**612**	**973**	**1187**
Periphyton C:P ratio (bulk)	775	Decline* (z-)	182	235	749	815	844
	775	**Increase* (z+)**	**362**	**749**	**844**	**1328**	**1328**
Surface-water DIN (ug/L)	**189.4**	**Decline (z-)**	**159.3**	**159.3**	**189.4**	**446.5**	**446.5**
	1827.1	Increase (z+)	446.5	446.5	1827.1	3981.5	3981.5
Surface-water TN (ug/L)	**435.5**	**Decline (z-)**	**362.0**	**362.0**	**423.0**	**753.0**	**753.0**
	1720.0	Increase (z+)	753.0	753.0	2080.0	4080.0	4080.0

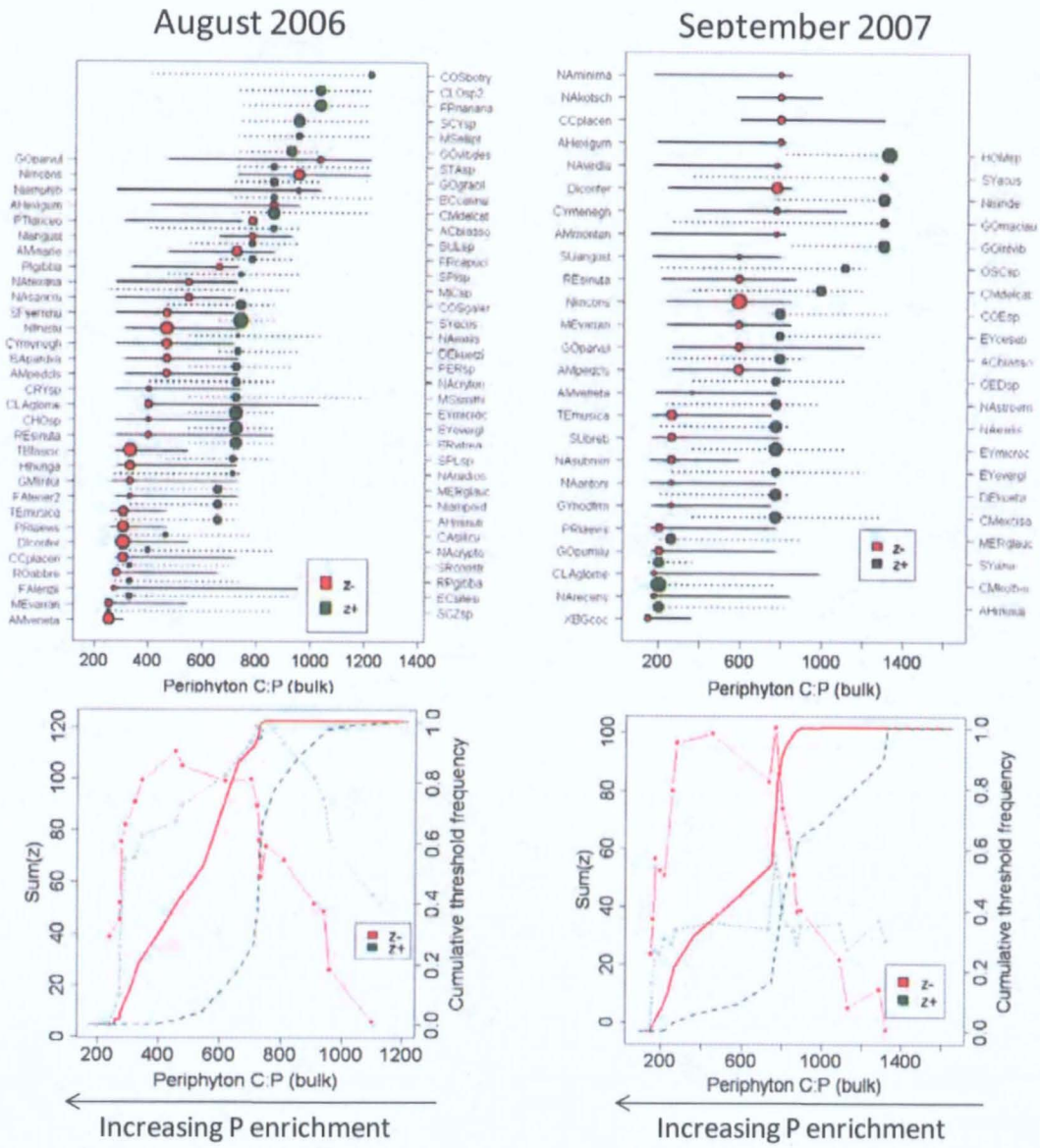

Figure 27. Results of Threshold Indicator Taxa ANalysis (TITAN) using periphyton C:P ratio (bulk) as a predictor of threshold changes in individual algal species during August 2006 and September 2007. Taxa are classified as either negative (z-) or positive (z+) threshold indicators based on the direction of response to TP. *Note that lower C:P values = higher P enrichment relative to organic carbon in the periphyton, thus taxa that are negative indicators (z-) in these results are associated with higher levels of P-enrichment,, whereas positive threshold taxa are associated with lower levels of P enrichment (high C:P ratios).* See Figure 26 for other graphical details. See Tables 8 and 9 for community level (sum(z)) thresholds, and Tables 11 and 13 for taxa-specific thresholds.

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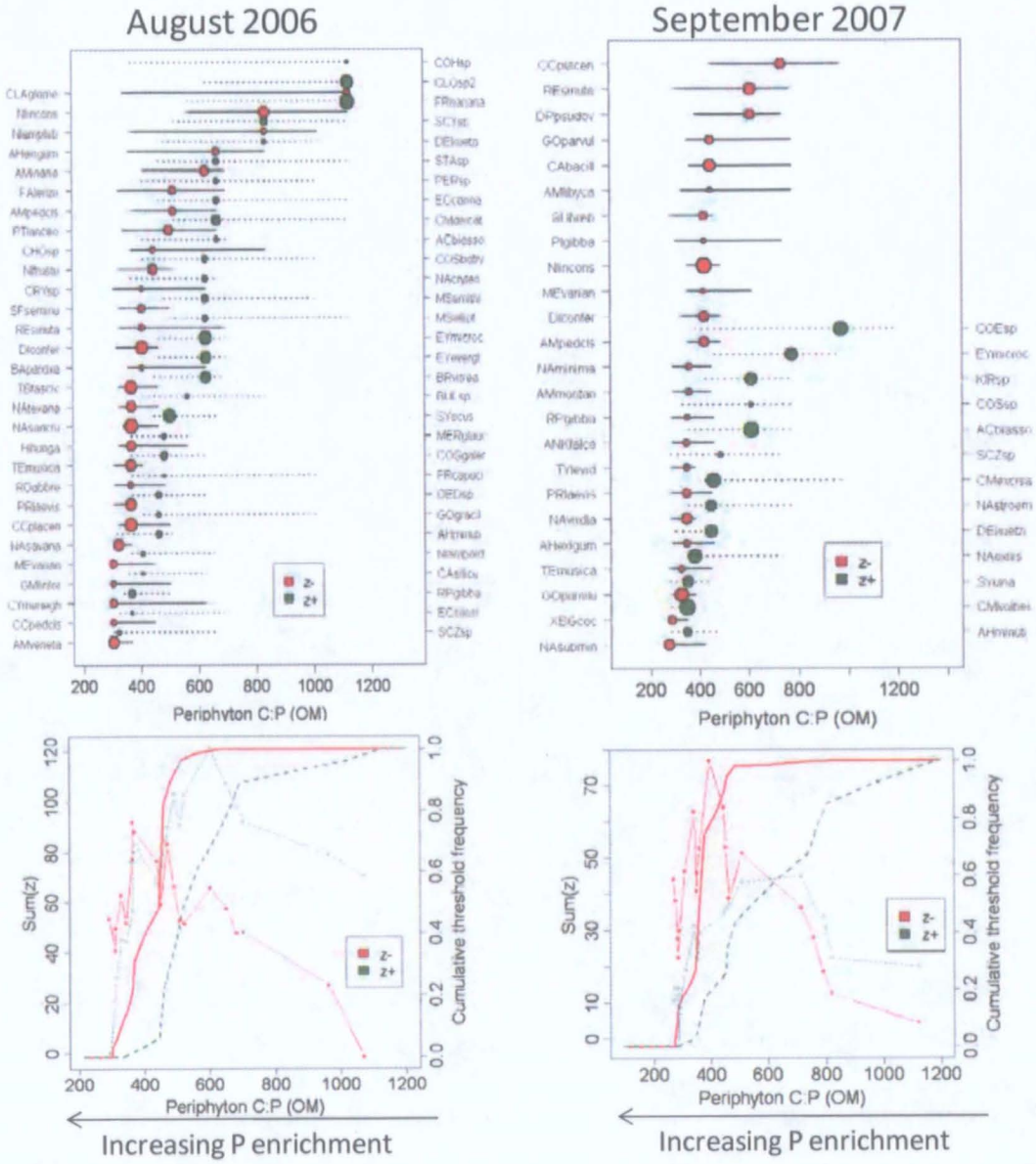

Figure 28. Results of Threshold Indicator Taxa ANalysis (TITAN) using periphyton C:P ratio (organic fraction, sediment removed) as a predictor of threshold changes in individual algal species during August 2006 and September 2007. Taxa are classified as either negative (z-) or positive (z+) threshold indicators based on the direction of response to TP. *Note that lower C:P values = higher P enrichment relative to organic carbon in the periphyton, thus taxa that are negative indicators (z-) in these results are associated with higher levels of P-enrichment,, whereas positive threshold taxa are associated with lower levels of P enrichment (high C:P ratios).* See Figure 26 for other graphical details. See Tables 8 and 9 for community level (sum(z)) thresholds, and Tables 11 and 13 for taxa-specific thresholds.

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Table 10. Taxa-specific results from Threshold Indicator Taxa Analysis (TITAN) on algal species composition in response to **surface-water total phosphorus (TP, ug/L)** in **August 2006** (n=26 sites). Only species that showed significant threshold declines or increases to **surface-water (TP)** are included in this table. The observed (*Obs*) threshold value of TP for each taxon is shown in bold, whereas lower (*10%*), middle (*50%*), and upper (*90%*) quantiles of 1,000 bootstraps represent measures of uncertainty around the observed threshold. *Z* represents the standardized indicator score from TITAN (larger numbers = stronger threshold response), *IndVal* is the unstandardized indicator score (scaled from 0-100%, with 100=perfect indicator). *Purity* is the relative consistency of the response direction among the 1,000 bootstraps (*purity* > 0.95 is significant). *P-value* is the likelihood of getting an equal or larger IndVal if the score were computed with random shuffling of the observed data (*P*<0.05 is significant). See Appendix 1 for full species names corresponding to *Taxon IDs*.

							Cumulative Threshold Quantiles		
Taxon ID	**Obs.**	Response > obs. value	*z*	IndVal	Purity	*P* value	10%	50%	90%
AHminuti	**39.7**	Decline	5.97	87.76	0.994	<0.004	27.2	39.7	437.2
BRvitrea	**19.9**	Decline	5.37	70.39	0.998	<0.004	18.5	22.8	39.7
CAsilicu	**19.9**	Decline	2.93	39.70	0.960	0.044	14.4	20.6	32.4
CMdelcat	**18.5**	Decline	5.41	55.56	0.998	0.008	11.7	17.4	19.9
DEkuetzi	**19.2**	Decline	3.95	68.49	0.998	0.012	17.4	20.6	141.7
ECcarina	**18.5**	Decline	3.01	33.33	0.958	0.040	12.1	18.5	22.8
ECsilesi	**30.4**	Decline	3.40	60.53	0.982	0.012	17.4	27.2	54.2
EYevergl	**19.9**	Decline	5.94	75.95	1.000	<0.004	17.4	20.6	27.2
EYmicroc	**19.9**	Decline	6.22	77.91	1.000	<0.004	17.4	20.6	27.2
MSellipt	**14.4**	Decline	5.02	57.14	0.982	<0.004	10.8	14.4	19.2
MSsmithi	**20.6**	Decline	5.09	50.00	0.998	<0.004	11.7	19.9	24.4
NAcrypto	**30.4**	Decline	2.69	43.75	0.982	0.028	14.4	22.8	39.7
NAcryten	**19.9**	Decline	4.35	65.68	1.000	0.008	19.2	27.2	54.2
NAexilis	**19.2**	Decline	3.05	30.00	0.962	0.024	10.8	14.4	20.6
NAradios	**30.4**	Decline	4.54	67.68	0.984	<0.004	19.2	27.2	54.2
NArhynch	**24.4**	Decline	2.92	42.86	0.962	0.012	19.2	24.4	32.4
NIampoid	**22.8**	Decline	3.42	48.77	0.990	0.016	17.4	22.8	39.7
RPgibba	**24.4**	Decline	4.97	71.87	1.000	<0.004	17.4	27.2	54.2
SRconstr	**27.2**	Decline	2.87	47.38	0.994	0.028	10.8	19.9	39.7
SYacus	**20.6**	Decline	5.60	75.66	1.000	<0.004	17.4	20.6	30.4
ANBsp	**18.5**	Decline	3.13	57.14	0.984	0.012	12.1	19.2	141.7
BULsp	**14.4**	Decline	4.30	62.60	0.978	<0.004	11.7	17.4	20.6
CLOsp2	**19.9**	Decline	3.03	36.36	0.974	0.040	14.4	19.9	24.4
COSbotry	**20.6**	Decline	5.73	66.67	1.000	<0.004	12.1	19.2	27.2
COSgaler	**18.5**	Decline	4.01	65.53	0.988	0.008	14.4	19.2	54.2
GLHsp	**22.8**	Decline	2.38	57.28	0.964	0.024	14.4	22.8	437.2
SCYsp	**17.4**	Decline	3.24	37.50	0.954	0.036	10.8	17.4	20.6
SCZsp	**1853.3**	Decline	3.71	53.86	0.996	<0.004	19.9	32.4	1250.2

SPOpulch	**27.2**	Decline	2.77	40.00	0.956	0.020	17.4	24.4	39.7
STAsp	**18.5**	Decline	3.30	33.33	0.962	0.024	10.8	17.4	20.6
AHexigum	**141.7**	Increase	3.40	72.59	0.994	0.012	18.5	32.4	437.2
AMgranul	**1853.3**	Increase	8.83	92.88	0.964	<0.004	32.4	1250.2	1853.3
AMveneta	**437.2**	Increase	8.45	80.00	0.992	<0.004	39.7	437.2	1853.3
BApardxa	**32.4**	Increase	4.08	58.27	0.998	0.012	19.2	27.2	54.2
CCpedcls	**437.2**	Increase	8.34	60.00	0.954	<0.004	27.2	437.2	1853.3
CCplacen	**22.8**	Increase	7.53	83.85	1.000	<0.004	18.5	22.8	30.4
CYmenegh	**39.7**	Increase	4.80	73.39	1.000	<0.004	19.2	30.4	141.7
DIconfer	**30.4**	Increase	6.32	82.53	1.000	<0.004	22.8	32.4	437.2
FAtener2	**32.4**	Increase	4.01	49.79	0.992	<0.004	19.9	32.4	141.7
GMlinfor	**32.4**	Increase	5.21	62.98	0.974	<0.004	24.4	39.7	1250.2
HIhunga	**39.7**	Increase	4.83	71.10	0.982	<0.004	20.6	30.4	141.7
NAsancru	**24.4**	Increase	4.32	50.00	1.000	<0.004	20.6	32.4	1853.3
NAsavana	**1853.3**	Increase	5.80	62.68	0.958	0.008	22.8	1250.2	1853.3
NAtexana	**39.7**	Increase	4.23	44.83	0.998	0.008	22.8	36.1	1853.3
NIamphib	**27.2**	Increase	4.05	54.51	1.000	<0.004	11.7	19.2	54.2
NIfrustu	**32.4**	Increase	7.08	87.20	1.000	<0.004	19.2	30.4	141.7
NIincons	**19.2**	Increase	3.55	68.12	0.998	<0.004	18.5	24.4	54.2
PRlaevis	**54.2**	Increase	6.75	71.43	0.998	<0.004	32.4	141.7	1853.3
REsinuta	**27.2**	Increase	4.63	66.23	0.966	<0.004	19.2	27.2	41.2
ROabbre	**32.4**	Increase	3.73	40.42	0.990	0.008	20.6	32.4	437.2
SFseminu	**22.8**	Increase	3.72	46.15	0.994	0.020	19.9	27.2	54.2
TBfascic	**39.7**	Increase	7.63	82.70	0.992	<0.004	27.2	39.7	437.2
TEmusica	**54.2**	Increase	6.98	71.43	0.994	<0.004	32.4	141.7	1853.3
CHOsp	**1250.2**	Increase	2.57	53.57	0.976	0.016	19.2	32.4	1853.3
CLAglome	**32.4**	Increase	4.34	67.04	0.986	<0.004	19.2	30.4	54.2

Table 11. Taxa-specific results from Threshold Indicator Taxa Analysis (TITAN) on algal species composition in response to **C:P ratio in the periphyton** (bulk, no separation from sediment) in **August 2006** (drought, n=26 sites). Only species that showed significant threshold declines or increases to **periphyton C:P (bulk)** are included in this table. The observed (*Obs*) threshold value of **C:P** for each taxon is shown in bold. **Note that lower C:P values = higher P enrichment relative to organic carbon in the periphyton, thus taxa that "decrease" sharply in response to increasing C:P are associated with higher levels of P-enrichment,, whereas "increaser" taxa are associated with lower levels of P enrichment.*

							Cumulative Threshold Quantiles		
Taxon ID	**Obs.**	Response > obs. value	*z*	IndVal	Purity	*P* value	10%	50%	90%
ACbiasso	**859.2**	Increase*	3.94	66.74	0.972	<0.004	398.8	724.5	950.8
AHminuti	**658.6**	Increase*	5.35	76.65	1.000	<0.004	275.1	398.8	724.5
BRvitrea	**724.5**	Increase*	7.25	85.66	1.000	<0.004	546.4	729.1	859.2
BULsp	**779.8**	Increase*	3.90	53.41	0.992	0.008	546.4	740.0	950.8
CAsilicu	**465.1**	Increase*	3.18	40.00	0.974	0.028	333.1	658.6	779.8
CLOsp2	**1030.0**	Increase*	6.01	70.87	0.990	<0.004	729.1	950.8	1218.3
CMdelcat	**859.2**	Increase*	6.73	71.43	1.000	<0.004	740.0	925.2	1218.3
COSbotry	**1218.3**	Increase*	3.98	83.30	0.998	<0.004	398.8	779.8	1218.3
COSgaler	**740.0**	Increase*	5.86	79.48	1.000	<0.004	465.1	729.1	859.2
DEkuetzi	**729.1**	Increase*	3.95	68.49	0.990	0.008	658.6	740.0	950.8
ECcarina	**859.2**	Increase*	4.07	42.86	0.966	0.028	711.2	925.2	1218.3
ECsilesi	**333.1**	Increase*	3.92	66.93	0.996	<0.004	283.8	546.4	925.2
EYevergl	**724.5**	Increase*	8.11	90.91	1.000	<0.004	546.4	729.1	859.2
EYmicroc	**724.5**	Increase*	8.18	90.91	1.000	<0.004	546.4	729.1	859.2
FRcapuci	**779.8**	Increase*	4.40	64.66	0.978	<0.004	658.6	740.0	925.2
FRnanana	**1030.0**	Increase*	6.70	70.64	0.994	<0.004	740.0	950.8	1218.3
GOgracil	**859.2**	Increase*	4.96	74.19	0.992	<0.004	711.2	859.2	1030.0
GOvibdes	**925.2**	Increase*	6.33	50.00	0.960	<0.004	729.1	925.2	1030.0
MERglauc	**658.6**	Increase*	4.79	71.00	0.980	<0.004	333.1	658.6	729.1
MICsp	**740.0**	Increase*	1.69	57.43	0.960	0.044	255.8	658.6	925.2
MSellipt	**950.8**	Increase*	4.58	55.96	0.986	0.008	724.5	925.2	1218.3
MSsmithi	**724.5**	Increase*	4.86	54.55	0.998	0.012	546.4	729.1	1218.3
NAcrypto	**398.8**	Increase*	3.07	43.75	0.974	0.016	330.4	658.6	859.2
NAcryten	**724.5**	Increase*	4.86	68.32	1.000	<0.004	398.8	724.5	859.2
NAexilis	**729.1**	Increase*	2.75	30.00	0.950	0.048	546.4	740.0	1030.0
NAradios	**711.2**	Increase*	3.37	61.58	0.994	0.008	273.0	465.1	740.0
NIampoid	**658.6**	Increase*	4.84	61.54	0.994	0.008	333.1	658.6	740.0
PERsp	**724.5**	Increase*	5.52	54.55	0.998	<0.004	546.4	729.1	925.2
RPgibba	**333.1**	Increase*	3.88	66.94	0.994	<0.004	275.1	465.1	740.0
SCYsp	**950.8**	Increase*	6.80	60.00	0.956	<0.004	740.0	990.4	1218.3
SCZsp	**255.8**	Increase*	2.80	52.84	0.978	0.012	255.8	398.8	859.2

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SPIsp	**740.0**	Increase*	3.85	60.21	0.980	<0.004	465.1	779.8	950.8
SPLsp	**711.2**	Increase*	3.91	56.36	0.956	0.008	465.1	724.5	859.2
SRconstr	**333.1**	Increase*	3.30	52.94	0.958	0.016	283.8	398.8	711.2
STAsp	**859.2**	Increase*	4.68	42.86	0.958	0.012	724.5	950.8	1218.3
SYacus	**740.0**	Increase*	8.28	92.42	1.000	<0.004	658.6	729.1	859.2
AHexigum	**859.2**	Decrease*	4.62	78.95	1.000	<0.004	398.8	779.8	950.8
AMinarie	**724.5**	Decrease*	5.18	75.36	0.980	<0.004	465.1	724.5	859.2
AMpedcls	**465.1**	Decrease*	4.23	60.72	0.988	<0.004	306.4	546.4	729.1
AMveneta	**255.8**	Decrease*	5.86	70.99	0.984	0.008	255.8	273.0	306.4
BApardxa	**465.1**	Decrease*	4.45	57.57	0.998	<0.004	306.4	546.4	729.1
CCplacen	**306.4**	Decrease*	5.22	81.46	0.998	<0.004	275.1	333.1	724.5
CHOsp	**398.8**	Decrease*	3.05	52.60	0.976	<0.004	275.1	398.8	729.1
CLAglome	**398.8**	Decrease*	4.31	67.96	1.000	<0.004	398.8	740.0	1030.0
CRYsp	**398.8**	Decrease*	3.30	49.39	0.958	0.016	274.9	398.8	740.0
CYmenegh	**465.1**	Decrease*	5.55	73.04	0.998	<0.004	275.1	398.8	711.2
DIconfer	**306.4**	Decrease*	7.31	91.22	1.000	<0.004	275.1	333.1	546.4
FAlenzii	**275.1**	Decrease*	3.40	71.86	0.986	0.008	273.0	333.1	950.8
FAtener2	**333.1**	Decrease*	3.29	49.79	0.992	0.008	275.1	398.8	729.1
GMlinfor	**333.1**	Decrease*	3.86	48.26	0.986	<0.004	255.8	333.1	729.1
GOparvul	**1030.0**	Decrease*	3.65	77.92	0.950	<0.004	465.1	950.8	1218.3
HIhunga	**333.1**	Decrease*	5.60	74.27	0.998	<0.004	283.8	398.8	724.5
MEvarian	**255.8**	Decrease*	4.69	46.93	0.952	0.012	255.8	273.0	546.4
NAsancru	**546.4**	Decrease*	4.42	50.00	0.998	<0.004	275.1	398.8	711.2
NAtexana	**546.4**	Decrease*	3.57	41.67	0.992	<0.004	275.1	465.1	724.5
NIamphib	**950.8**	Decrease*	3.01	54.67	0.996	0.012	275.1	724.5	1030.0
NIangust	**779.8**	Decrease*	3.97	66.67	0.962	0.008	658.6	779.8	925.2
NIfrustu	**465.1**	Decrease*	6.62	83.23	1.000	<0.004	306.4	546.4	729.1
NIincons	**950.8**	Decrease*	5.95	90.48	0.996	<0.004	724.5	925.2	1218.3
PIgibba	**658.6**	Decrease*	3.73	38.46	0.954	0.028	333.1	658.6	729.1
PRlaevis	**306.4**	Decrease*	5.83	62.50	0.994	<0.004	255.8	306.4	465.1
PTlanceo	**779.8**	Decrease*	4.47	66.67	0.998	0.008	304.1	658.6	740.0
REsinuta	**398.8**	Decrease*	3.90	63.69	0.996	0.008	275.1	465.1	859.2
ROabbre	**283.8**	Decrease*	4.62	53.44	0.996	0.008	273.0	306.4	658.6
SFseminu	**465.1**	Decrease*	4.43	54.55	0.998	0.008	275.1	398.8	711.2
TBfascic	**333.1**	Decrease*	6.56	72.75	0.998	<0.004	275.1	333.1	546.4
TEmusica	**306.4**	Decrease*	5.23	62.50	0.990	<0.004	255.8	283.8	465.1

Table 12. Taxa-specific results from Threshold Indicator Taxa Analysis (TITAN) on algal species composition in response to **surface-water total phosphorus (TP, ug/L)** in **September 2007** (post-flood, n=26 sites). Only species that showed significant threshold declines or increases to **surface-water (TP)** are included in this table. The observed (*Obs*) threshold value of TP for each taxon is shown in bold.

							Cumulative Threshold Quantiles		
Taxon ID	**Obs.**	Response > obs. value	Z	IndVal	Purity	Pr. (Type I)	10%	50%	90%
ACbiasso	**12.9**	Decrease	4.45	63.21	1.000	<0.004	12.3	15.6	26.5
AHminuti	**32.9**	Decrease	4.56	76.14	1.000	<0.004	13.8	26.5	115.8
CMexcisa	**12.9**	Decrease	3.57	55.34	0.982	0.008	10.9	12.9	24.5
CMkolbei	**32.9**	Decrease	6.15	88.52	0.998	<0.004	21.0	32.9	115.8
DEkuetzi	**24.5**	Decrease	4.08	71.96	1.000	<0.004	12.9	21.0	42.8
EYmicroc	**12.3**	Decrease	3.12	56.18	0.976	0.020	10.9	12.9	26.5
NAexilis	**12.9**	Decrease	5.02	72.15	1.000	<0.004	12.8	13.8	32.9
NAstroem	**17.6**	Decrease	4.12	64.13	0.988	0.008	12.8	15.6	32.9
SYulna	**149.3**	Decrease	4.17	66.36	0.978	<0.004	17.6	42.8	529.3
COSsp	**13.1**	Decrease	2.60	61.08	0.984	0.016	12.3	13.1	32.9
MERglauc	**26.5**	Decrease	3.83	58.82	0.968	0.016	13.1	21.0	42.8
AHexigum	**24.5**	Increase	5.56	60.00	0.996	<0.004	15.6	24.5	42.8
AMbullat	**24.5**	Increase	3.08	30.00	0.956	0.028	15.6	26.5	45.7
AMlibyca	**12.9**	Increase	4.00	58.82	0.984	0.012	12.3	13.1	21.0
AMpedcls	**24.5**	Increase	3.63	54.59	0.998	0.008	12.8	13.8	26.5
AMveneta	**21.0**	Increase	2.73	27.27	0.960	<0.004	15.6	24.5	42.8
CAbacill	**12.9**	Increase	6.03	82.35	1.000	<0.004	11.6	12.9	13.8
CCplacen	**24.5**	Increase	3.23	64.12	0.992	0.012	12.3	13.8	26.5
DIconfer	**13.1**	Increase	4.14	56.25	0.998	0.008	12.9	15.6	32.9
GOparvul	**24.5**	Increase	4.05	68.06	0.994	0.012	10.9	17.6	32.9
GOpumilu	**42.8**	Increase	4.74	74.47	0.978	<0.004	13.8	24.5	71.9
GOrhombi	**149.3**	Increase	4.34	45.34	0.954	0.048	15.6	42.8	529.3
GYnodfrm	**26.5**	Increase	3.46	33.33	0.964	0.020	17.6	26.5	71.9
NAantoni	**26.5**	Increase	3.08	33.33	0.952	0.032	15.6	26.5	529.3
NAminima	**13.8**	Increase	2.87	35.71	0.966	0.008	13.1	17.6	149.3
NArecens	**17.6**	Increase	6.05	80.36	0.992	<0.004	13.1	17.6	26.5
NAsancru	**26.5**	Increase	4.35	44.44	0.992	0.012	17.6	26.5	71.9
NAsubmin	**26.5**	Increase	5.50	55.56	0.998	<0.004	17.6	32.9	149.3
NAvirdla	**24.5**	Increase	4.19	50.00	0.994	0.008	15.6	24.5	76.3
NIincons	**24.5**	Increase	5.62	76.31	1.000	<0.004	12.9	17.6	32.9
NIpalea	**21.0**	Increase	3.72	45.45	0.994	0.016	13.8	24.5	115.8
PIgibba	**13.1**	Increase	3.67	53.33	0.988	<0.004	12.8	13.1	24.5
PRlaevis	**26.5**	Increase	6.96	85.44	1.000	<0.004	15.6	24.5	42.8

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REsinuta	**32.9**	Increase	4.76	72.10	0.998	<0.004	11.6	13.8	42.8
SUbreb	**26.5**	Increase	4.61	60.28	1.000	<0.004	13.8	24.5	115.8
TEmusica	**24.5**	Increase	5.66	60.00	1.000	<0.004	17.6	26.5	115.8
THweiss	**32.9**	Increase	4.19	37.50	0.966	0.012	17.6	32.9	115.8
TYlevid	**24.5**	Increase	4.03	40.00	0.984	0.012	15.6	24.5	71.9
ANKfalca	**24.5**	Increase	3.71	59.42	0.980	0.016	12.9	17.6	32.9
CLAglome	**17.6**	Increase	3.79	64.41	0.980	<0.004	12.9	17.6	42.8
XBGcoc	**115.8**	Increase	5.64	76.12	0.978	<0.004	32.9	115.8	529.3

Table 13. Taxa-specific results from Threshold Indicator Taxa Analysis (TITAN) on algal species composition in response to **C:P ratio in the periphyton** (bulk, no separation from sediment) in **September 2007** (drought, *n*=26 sites). Only species that showed significant threshold declines or increases to **periphyton C:P (bulk)** are included in this table. The observed (*Obs*) threshold value of **C:P** for each taxon is shown in bold. Note that lower C:P values = higher P enrichment relative to organic carbon in the periphyton, thus taxa that "decrease" sharply in response to increasing C:P are associated with higher levels of P-enrichment,, whereas "increaser" taxa are associated with lower levels of P enrichment.

							Cumulative Threshold Quantiles		
Taxon ID	**Obs.**	Response > obs. value	Z	IndVal	Purity	*P* value	10%	50%	90%
ACbiasso	**793.0**	Increase*	3.70	54.99	0.994	0.016	234.9	775.2	886.0
AHminuti	**204.9**	Increase*	3.51	69.35	0.972	0.004	165.7	234.9	817.7
CMdelcat	**993.1**	Increase*	3.47	65.32	0.972	0.012	362.3	843.5	1207.6
CMexcisa	**775.2**	Increase*	4.36	57.94	0.990	0.004	362.3	793.0	1303.4
CMkolbei	**204.9**	Increase*	4.94	81.92	1.000	0.004	165.7	204.9	775.2
DEkuetzi	**775.2**	Increase*	4.26	71.73	1.000	0.008	204.9	749.4	843.5
EYcesati	**793.0**	Increase*	2.96	33.33	0.984	0.020	772.6	886.0	1303.4
EYevergl	**775.2**	Increase*	3.05	50.34	0.952	0.016	263.5	793.0	1207.6
EYmicroc	**775.2**	Increase*	4.92	58.10	0.996	0.004	362.3	793.0	1109.9
GOintvib	**1303.4**	Increase*	4.16	45.86	0.964	0.024	843.5	1303.4	1327.6
GOmaclau	**1303.4**	Increase*	3.36	61.24	0.956	0.024	263.5	1109.9	1327.6
NAexilis	**775.2**	Increase*	4.35	64.18	1.000	0.004	204.9	592.0	843.5
NAstroem	**775.2**	Increase*	3.66	59.11	0.950	0.012	234.9	775.2	993.1
NIsinde	**1303.4**	Increase*	4.26	67.35	0.984	0.008	775.2	1109.9	1327.6
SYacus	**1303.4**	Increase*	2.54	60.60	0.950	0.020	362.3	886.0	1327.6
SYulna	**204.9**	Increase*	3.58	61.58	0.954	0.008	155.0	182.3	362.3
COEsp	**793.0**	Increase*	3.75	41.67	0.994	0.004	749.4	843.5	1327.6

HOMsp	**1327.6**	Increase*	4.98	63.39	0.958	0.012	814.8	1255.5	1327.6
MERglauc	**263.5**	Increase*	3.82	58.82	1.000	0.016	204.9	775.2	896.7
OEDsp	**775.2**	Increase*	3.08	38.46	0.980	0.048	362.3	814.8	1109.9
OSCsp	**1109.9**	Increase*	3.24	68.82	0.990	0.004	204.9	886.0	1207.6
AHexigum	**793.0**	Decrease*	4.06	42.86	0.996	0.008	182.3	592.0	814.8
AMmontan	**775.2**	Decrease*	3.05	38.46	0.974	0.040	155.0	592.0	814.8
AMpedcls	**592.0**	Decrease*	5.57	70.16	1.000	0.004	263.5	749.4	843.5
AMveneta	**362.3**	Decrease*	2.78	30.00	0.958	0.048	182.3	362.3	775.2
CCplacen	**793.0**	Decrease*	5.14	72.90	0.998	0.004	592.0	814.8	1303.4
CYmenegh	**775.2**	Decrease*	4.04	65.60	0.992	0.008	362.3	814.8	1109.9
DIconfer	**775.2**	Decrease*	6.82	69.23	1.000	0.004	234.9	749.4	843.5
GOparvul	**592.0**	Decrease*	5.11	72.24	1.000	0.004	263.5	793.0	1207.6
GOpumilu	**204.9**	Decrease*	5.12	74.47	0.990	0.004	165.7	234.9	775.2
GYnodfrm	**263.5**	Decrease*	3.11	33.33	0.972	0.036	165.7	234.9	749.4
MEvarian	**592.0**	Decrease*	5.33	58.63	0.990	0.004	234.9	749.4	843.5
NAantoni	**263.5**	Decrease*	3.00	33.33	0.962	0.036	155.0	234.9	775.2
NAkotsch	**793.0**	Decrease*	4.08	62.24	0.956	0.004	569.0	793.0	993.1
NAminima	**793.0**	Decrease*	3.00	35.71	0.978	0.008	165.7	592.0	843.5
NArecens	**182.3**	Decrease*	3.38	69.06	0.982	0.012	165.7	263.5	843.5
NAsubmin	**263.5**	Decrease*	4.84	55.56	0.998	0.004	155.0	234.9	592.0
NAvirdla	**775.2**	Decrease*	3.17	38.46	0.994	0.032	165.7	263.5	793.0
NIincons	**592.0**	Decrease*	8.00	86.56	1.000	0.004	234.9	749.4	843.5
PRlaevis	**204.9**	Decrease*	4.98	72.21	0.998	0.004	165.7	263.5	775.2
REsinuta	**592.0**	Decrease*	5.25	71.84	0.998	0.004	204.9	362.3	867.0
SUangust	**592.0**	Decrease*	3.57	36.36	0.986	0.020	165.7	362.3	793.0
SUbreb	**263.5**	Decrease*	5.20	64.36	1.000	0.004	165.7	263.5	793.0
TEmusica	**263.5**	Decrease*	5.66	66.67	1.000	0.004	165.7	263.5	749.4
CLAglome	**182.3**	Decrease*	2.94	68.42	0.988	0.016	165.7	592.0	993.1
XBGcoc	**155.0**	Decrease*	4.55	67.72	0.958	0.004	155.0	182.3	362.3

RESULTS AND INTERPRETATION, CONTINUED

Macroinvertebrate Taxonomic Responses to TP

Results of ordinations and Threshold Indicator Taxa Analysis (TITAN) on macroinvertebrate taxonomic composition during November 2006 showed that nutrients, specifically phosphorus, corresponded to shifts in community structure (Figure 30, 31). Because composition differed substantially between sites with and without flow, and because of established relationship between flow and other water chemistry variables (e.g., DO), we separated sites into groups by flow status prior to analysis.

Sites with no flow yielded more reliable relationships to TP than sites with flow, but this result should be interpreted with the caveat that there were only 9 sites in the group with flow. Ordinations clearly showed that in both sets of analyses, the sites with low TP and high periphyton C:P sorted on the left side of axis 1, whereas high TP and low C:P sites fell out on the right side of axis 1 (Figure 30).

In both sets of TITAN analyses, more taxa were classified as negative threshold indicators (decline in response to TP) than as positive ones (increase). A few of the negative indicators of note included the stoneflies *Zealeuctra* and *Perlesta*, the caddisflies *Chimarra,* and *Hydroptila*, the mayfly *Neochoroterpes nanita*, and the dryopid beetle *Postelichus*. Notable increasers included the filter-feeding caddisfly *Cheumatopsyche*, the amphipod *Hyallela azteca*, the fingernail clam Sphaeriidae, and aquatic worms (Oligochaeta).

Because macroinvertebrates are so highly tied to flow, these results likely represent a weak representation of the potential influence of nutrient enrichment on macroinvertebrates. Many of the sites had gone dry or nearly so between August and October 2006, so some of the sites with flow during this event had been reduced to tiny disconnected pools in weeks prior to sampling. We anticipate producing a larger macroinvertebrate data set (J. A. Back, Ph. D dissertation) spanning more flow conditions, and therein more completely evaluate macroinvertebrate responses to TP enrichment.

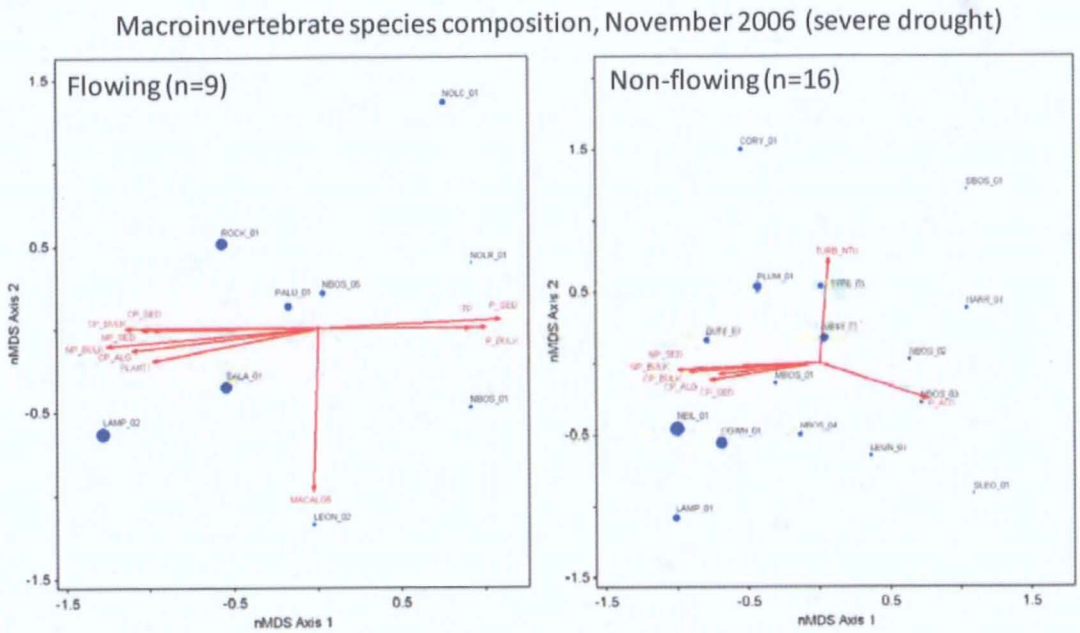

Figure 30. Nonmetric multidimensional scaling ordination of macroinvertebrate species composition during a period of drought in November 2006. Dissimilarity (Bray-Curtis) was computed using log-transformed densities (no./m2) per taxon. Ordinations were conducted separately for sites with detectable flow (n=9) and those without flow (n=16; one site had insufficient surface water for macroinvertebrate sampling and was removed). Red arrows (vectors) correspond to significant predictors of taxonomic composition.

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Table 14. Community-level results from Threshold Indicator Taxa Analysis (TITAN) on **macroinvertebrate species composition** in response to **surface-water TP and periphyton C:P ratio (bulk)** during November 2006 (severe drought, n=26 sites). Analyses were conducted separately for sites that had measureable flow preceding and during the sampling (flow, n=9), and those that were not flowing (no flow, n=16). Thresholds (*Obs.*) are based on the value of the predictor resulting in the greatest aggregate decrease (sum(z-)) or increase (sum(z+)) in the frequency and abundance of taxa in the community. See Figure 31 for details.

				Bootstrap Cumulative Distribution of Thresholds				
Data set	Predictor	Method	**Obs.**	5%	10%	50%	90%	95%
No flow (n=16)	Surface-water TP (ug/L)	Decline (sumz-)	**16.3**	16.3	16.3	18.3	20.2	20.2
		Increase (sumz+)	**17.5**	18.3	19.3	27.3	120.1	120.1
No flow (n=16)	Periphyton C:P ratio (bulk)	Decline (sumz-)	**230.1**	152.1	152.1	195.4	230.1	259.1
		Increase (sumz+)	**373.6**	173.5	195.4	230.1	373.6	373.6
Flow (n=9)	Surface-water TP (ug/L)	Decline (sumz-)	**10.7**	10.7	10.7	14.8	850.4	1636.5
		Increase (sumz+)	**409.2**	10.7	14.8	850.4	1636.5	1636.5
Flow (n=9)	Periphyton C:P ratio (bulk)	Decline (sumz-)	**195.6**	151.8	151.8	195.6	290.9	359.2
		Increase (sumz+)	**359.2**	195.6	195.6	290.9	359.2	359.2

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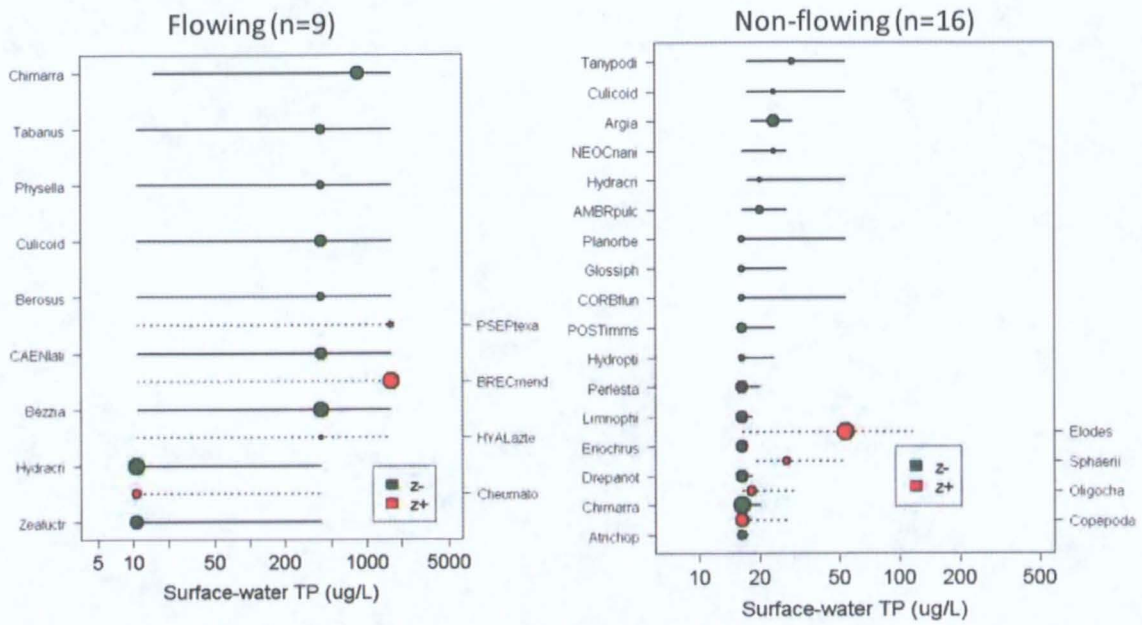

Figure 31. Results of TITAN using surface-water TP as a predictor of threshold changes in macroinvertebrate taxa abundances from sites with flow (n=9) and those without flow (n=16) in November 2006.

Table 15. Taxa-specific results from Threshold Indicator Taxa Analysis (TITAN) on **macroinvertebrate species composition** in response to **surface-water TP** during November 2006 (severe drought, n=26 sites). Analyses were conducted separately for sites that had measureable flow preceding and during the sampling (flow, n=9), and those that were not flowing (no flow, n=16). Only species that showed significant threshold declines or increases to **surface-water (TP)** are included in this table. The observed (*Obs*) threshold value of TP for each taxon is shown in bold. See Table 14 and Figure 31 for details.

							Cumulative Threshold Quantiles		
Taxon ID	**Obs.**	Response > Obs. Value	z	IndVal	Purity	P-value	10%	50%	90%
Non-flowing sites (n=16)									
AMBRpulc	**20.2**	Decline	3.82	75.00	0.990	0.020	16.3	20.2	27.3
Argia	**23.7**	Decline	5.63	95.20	1.000	<0.004	18.3	23.7	29.3
Atrichop	**16.3**	Decline	4.60	50.00	0.872	0.048	16.3	16.3	17.5
Chimarra	**16.3**	Decline	7.29	50.00	0.878	0.016	16.3	16.3	20.2
CORBflun	**16.3**	Decline	3.19	80.09	0.966	0.012	16.3	19.3	53.7
Culicoid	**23.7**	Decline	2.64	66.48	0.942	0.024	17.5	23.7	53.7
Drepanot	**16.3**	Decline	5.09	50.00	0.880	0.040	16.3	16.3	18.3
Enochrus	**16.3**	Decline	5.16	50.00	0.872	0.036	16.3	16.3	17.5
Glossiph	**16.3**	Decline	3.40	81.85	0.980	<0.004	16.3	19.3	27.3
Hydracri	**20.2**	Decline	2.63	63.98	0.872	0.024	17.5	20.2	53.7
Hydropti	**16.3**	Decline	3.17	70.72	0.950	0.012	16.3	17.5	23.7
Limnophi	**16.3**	Decline	4.98	50.00	0.880	0.040	16.3	16.3	18.3
NEOCnani	**23.7**	Decline	2.61	55.56	0.984	0.044	16.3	19.3	27.3
Perlesta	**16.3**	Decline	5.29	50.00	0.872	0.036	16.3	16.3	20.2
Planorbe	**16.3**	Decline	2.75	74.33	0.938	0.032	16.3	19.3	53.7
POSTimms	**16.3**	Decline	4.83	89.02	0.988	<0.004	16.3	17.5	23.7
Tanypodi	**29.3**	Decline	3.13	62.64	0.982	0.008	17.5	23.7	53.7
Copepoda	**16.3**	Increase	4.05	56.40	0.884	<0.004	16.3	17.5	27.3
Elodes	**53.7**	Increase	4.98	50.00	0.884	0.040	16.3	53.7	120.1
Oligocha	**18.3**	Increase	2.96	53.86	0.982	0.024	16.3	19.3	29.3
Sphaerii	**27.3**	Increase	2.60	67.64	0.876	0.024	19.3	27.3	53.7
Flowing sites (n=9)									
Berosus	**409.2**	Decline	1.82	75.40	0.904	0.032	10.7	409.2	1636.5
Bezzia	**409.2**	Decline	3.14	91.83	0.994	0.020	10.7	409.2	1636.5
CAENlati	**409.2**	Decline	2.57	82.76	0.982	0.040	10.7	409.2	1636.5
Chimarra	**850.4**	Decline	2.76	76.34	0.894	<0.004	14.8	850.4	1636.5
Culicoid	**409.2**	Decline	2.62	86.52	0.988	0.020	10.7	409.2	1636.5
Hydracri	**10.7**	Decline	3.48	100.00	0.964	0.024	10.7	10.7	409.2

Physella	**409.2**	Decline	1.97	75.05	0.930	0.028	10.7	409.2	1636.5
Tabanus	**409.2**	Decline	2.26	82.85	0.972	0.040	10.7	409.2	1636.5
Zealuctr	**10.7**	Decline	3.03	66.67	0.880	0.084	10.7	10.7	409.2
BRECmend	**1636.5**	Increase	4.82	100.00	0.894	<0.004	10.7	1636.5	1636.5
Cheumato	**10.7**	Increase	3.08	100.00	0.960	<0.004	10.7	10.7	409.2
HYALazte	**409.2**	Increase	1.71	72.75	0.894	0.100	10.7	409.2	1636.5
PSEPtexa	**1636.5**	Increase	2.05	82.98	0.910	0.100	10.7	850.4	1636.5

SECTION 2: EXPERIMENTAL STREAM STUDY

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SITE DESCRIPTION

Experimental evidence derived from controlled studies is important for the development of scientifically defensible water quality standards, yet most studies are limited only to field observations from natural lakes, wetlands, and streams because these habitats are too large and complex to manipulate experimentally. The intention of the Baylor Experimental Aquatic Research (BEAR) facility in Waco, TX, is to bridge the gap between field observations, which represent the habitat of interest but may be influenced by many interacting chemicals or other aquatic stressors simultaneously, and laboratory or small field experiments, which allow for control of environmental variables yet are too small and unrepresentative of natural conditions to be realistic. Because of its size (over 30,000 square feet), outdoor location, and close proximity to natural aquatic habitats the BEAR facility is a unique, state-of-the-art resource for conducting controlled yet realistic water research studies (Figure 32).

The BEAR facility supports 12 experimental stream mesocosms. The facility is located at the Lake Waco Wetlands, an 80-hectare constructed wetland. Water is pumped from the outflow of cell 2 of the 5-cell wetland to the BEAR facility. Here, the water has relatively low levels of dissolved N and P and thus provides high-quality source water for a nutrient addition experiment.

Water from the wetland is delivered to the BEAR facility via a 2500 L/min pump. Water is pumped into a 160,000 L water tower and delivered in controlled volumes to the streams via a series of 12 valves, each adjusted to regulate flow equivalently among all 12 streams (180 L/min). Before entering the streams, water is pumped into a mixing tank where chemicals representing experimental treatments can be dosed at a fixed rate using peristaltic pumps connected to a second, adjacent tank of chemical stock solution. Once dosed with the treatment levels of non-toxic chemicals, water is released into the streams and is discharged back into the Lake Waco Wetlands.

The experimental streams are approximately 0.6 m wide and 20 m in length. The streams are stratified into riffle, glide, and pool sections and are designed mimic natural habitat in central Texas streams. A riffle is a high-gradient habitat that is usually fast-flowing, quite shallow, and usually composed of gravel or cobble substrate. Glides are also shallow but usually much slower in water velocity. Pools are deep, slow areas in streams. Each of these habitats tends to support different species with specific adaptations to those particular environments.

The BEAR stream facility was covered by a canopy that excluded 60% of incoming solar radiation to approximate riparian canopies of a typical stream in the field study (800-1,000 μE, RSK, unpublished data), and reduce variability of sunlight across experimental units.

Figure 32. Aerial view of the Lake Waco Wetland and the location of the Baylor Experimental Aquatic Research (BEAR) stream facility (red arrow, upper panel). The lower panel is an aerial view the BEAR experimental stream facility (80' x 100', covered in 60% shade cloth), adjacent pond mesocosms, and a 40K gallon water tower used to store water pumped from the Lake Waco Wetlands en route to the experimental streams. See http://www.baylor.edu/aquaticlab/index.php?id=45868 for a video tour of the BEAR streams.

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EXPERIMENTAL DESIGN

Clean river cobble and gravel were placed in streams in January 2008 to simulate erosional habitats found in natural streams in our field study. Stream flow was initiated on 31 January 2008 and calibrated to 182 L/min (+/- 2 L/min) for each stream.

Streams were seeded with organic matter, periphyton, and macroinvertebrates collected from ROCK-01 and NBOS-03, two of the intensive field sites. These sites were chosen because their P concentrations were representative of the background (control) and high P treatments to be employed in the experiment. Inoculating the streams with organisms only from the low-P streams would bias the study because of the reduced probability of introducing taxa that proliferate under high P conditions.

Streams were seeded twice: 1 February and 15 February 2008. The first event was to primarily introduce algae, bacteria, fungi, and detritus, as we anticipated high mortality of macroinvertebrates due to the lack of food in the streams at the beginning of the colonization period. The second seeding was done to facilitate establishment of macroinvertebrates found in both low and high P streams in the region.

Seeding was accomplished by collecting five 1-m^2 kick screen samples from ROCK-01 and NBOS-03 for each of the 12 experimental streams. Samples were taken from 5 different locations in the field study sites, with one sample collected for each of the 12 streams at each of the 5 different locations. A composite of the five samples was placed in one 20-L bucket per experimental stream. Each bucket was aerated during transport from the field back to the BEAR facility. One bucket per stream was mixed and gently dumped into the top of the riffle section to permit drifting organisms to colonize downstream areas in the reach.

The streams were allowed to run without any nutrient additions from 31 January to 10 March 2008 in an effort to allow growth of periphyton at low nutrient concentrations. However, we were also interested the potential effect of nutrients on established periphyton communities from real streams. To this end, we transplanted 600 cobbles with established periphyton communities from riffle habitat in ROCK-01 and NBOS-03. Cobbles from ROCK-01 and NBOS-03 were placed in random cross sections throughout the reach of each experimental stream on 7 and 8 March 2008, respectively, a few days prior to initiation of the experiment.

Experimental dosing of phosphorus began on 11 March 2008. Background concentration of PO4-P during the colonization phase of the study was approximately 6 ug/L, slightly higher than our lowest field sites, but nevertheless relatively low. Four streams were randomly assigned no additional phosphorus, and thus a target concentration of **6 ug/L (Control)**. Because our field study had identified consistent nonlinear changes in numerous biological response variables between 10 and 20 ug/L PO4-P (usually corresponding to 15-25 ug/L TP), we selected a target PO4-P concentration of 20 ug/L for our lower nominal P treatment. Four streams were assigned a treatment of +15 ug/L PO4-P to achieve a target PO4-P concentration of **20 ug/L (Low P treatment)**. The remaining four streams received +95 ug/L PO4-P to achieve a target concentration of **100 ug/L (High P treatment).** The higher P concentration was selected

because it was clearly above the level of P enrichment that was correlated with significant biological changes in the field study.

Streams were dosed with NaH_2PO_4 using peristaltic pumps. Pumps were calibrated to deliver phosphorus stock solutions from 50-L carboys to mixing tanks located at the head of each experimental stream.

Figure 33. Collage of BEAR stream photographs illustrating the layout of the stream facility (top left), a view from within a glide section of a stream channel (top right), the substrate of a typical riffle section during the colonization phase of the study (bottom left), and a lateral view of the streams from the far left side of the facility, looking toward the adjacent Lake Waco Wetlands.

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SAMPLING AND DATA ANALYSES

Dissolved nutrients (PO4-P, NH3-N, and NO2NO3-N) were sampled in triplicate biweekly from the mixing tank and pool (terminal outlet) of the streams. Because of the relatively short length of the streams and insufficient amount of time for phytoplankton growth before water is emptied from the streams, turbidity and chlorophyll-a in surface water were not monitored. Because of the effect of turbulent mixing on dissolved oxygen in streams and lack of response of DO to TP in field streams during periods of high flow, diel dissolved oxygen was not monitored in this study.

Biotic sampling was stratified into riffle, and glide habitats. Each section was partitioned into approximately forty 20-cm wide cross-sections. Each cross section was assigned a number for random sampling location for biota sampling.

Quantitative periphyton samples from rocks that were exclusively colonized in the experimental streams ("bare rock", non-transplant periphyton) were collected on Day 0 (day before dosing commenced) and Day 28 (4 weeks of dosing) of the study. On each date, four random cross-sections from the riffle sections were sampled. Within each of the four cross sections, 5 cobbles were removed quantitatively sampled in accordance with the field study protocol. The composite of 20 rocks was subsampled for chlorophyll-a, AFDM, and CNP chemical analysis following the QAPP (Appendix B). On Day 28, filamentous algae (*Cladophora*) was gently removed from the rocks (most of it was loosely attached) and quantified separately for AFDM, chlorophyll-a and CNP chemical analysis. The remaining attached periphyton was processed as previously described.

Quantitative periphyton samples from transplanted cobbles (ROCK-01 and NBOS-03) were processed on Day -2 and -1 (two and one days prior to dosing, the same day rocks were collected from the field streams) to characterize the baseline AFDM, chlorphyll a, and CNP content prior to transplanting. Four separate composite samples were collected from each site (four sets of 5 rocks). Five transplanted cobbles per field stream were collected from each experimental stream on Day 7 (17 March 2008) and 28 (7 April 2008). Each set of five rocks represented a single composite sample (following Day -1 and -2) from each of the 2 field streams. AFDM, chlorophyll-a, and CNP content of the periphyton was quantified for each composite sample. Additionally, algal species composition was measured from samples from Day -2/-1 (hereafter, Day 0) and Day 28.

An additional set of quantitative periphyton samples was collected from 5 x 5 cm ceramic tiles. Clean tiles were placed in the glide section of streams on Day -14. A set of 10 tiles was randomly removed from each stream on Day 0, 14, and 28 for analysis of chlorophyll-a and AFDM.

Quantitative macroinvertebrate sampling was conducted on Day 0 and 28 and was coordinated with periphyton sampling so that the same cross-sections were sampled on the same dates. Samples were collected with a custom-made quantitative sampler developed specifically for the BEAR facility. The sampler, called a BEAR Trap, is functionally similar in design and function to a Hess sampler, but has dimensions that fit within the 20-cm cross-sections of the BEAR

streams (52 x 20 cm; area=0.104 m^2). Four quantitative samples were collected per stream per riffle and run sections per date and were processed as described in the QAPP (Appendix B).

Depending upon the response variable, most data were analyzed as a one-way ANOVA with P treatment as a 3-level main effect (Control, Low P, High P). Individual streams were replicates (n=4 per treatment). Appropriate transformations were applied when necessary. Multiple comparison tests were done when ANOVA deemed the main effect significant ($p<0.05$).

Algal and macroinvertebrate species composition were analyzed using nonmetric multidimensional scaling ordination (see Section I) and analysis of successional vectors. Tests for diffrerences in treatments were done using Multi-Response Permutation Procedure (MRPP). Indicator Species Analysis was conducted to identify taxa that changed significantly in response to P treatments if the overall MRPP test of community differences was deemed significant. Multivariate analyses were conducted in PC-ORD v.5.20.

RESULTS AND INTERPRETATION

Nutrient Concentrations among the Experimental Streams

Experimental PO4-P additions yielded concentrations among treatments that were very close to target nominal concentrations of 6, 20, and 100 ug/L PO4-P (Figure 34, Table 16). The 4-week PO4-P average from the control streams ranged between 7.7 and 8.1 ug/L PO4-P, slightly higher than anticipated due to slight increases in nutrients in the wetland during the study period. The average PO4-P for the Low P treatment ranged from 17.7 to 19.3 among streams, slightly lower than the target of 20 ug/L. High P treatments ranged from 102 to 112 ug/L PO4-P, slightly higher than the 100 ug/L target concentration.

DIN and total N were very similar among all 12 study streams (Table 16). DIN and TN were within the range of the least impacted field study streams. TP was higher than expected, largely due to wind re-suspension of fine sediment in the wetland. Mean TP ranged from 19.9-20.5 among the Control streams during the 4 week dosing period. This TP level was close to the threshold observed from the field study.

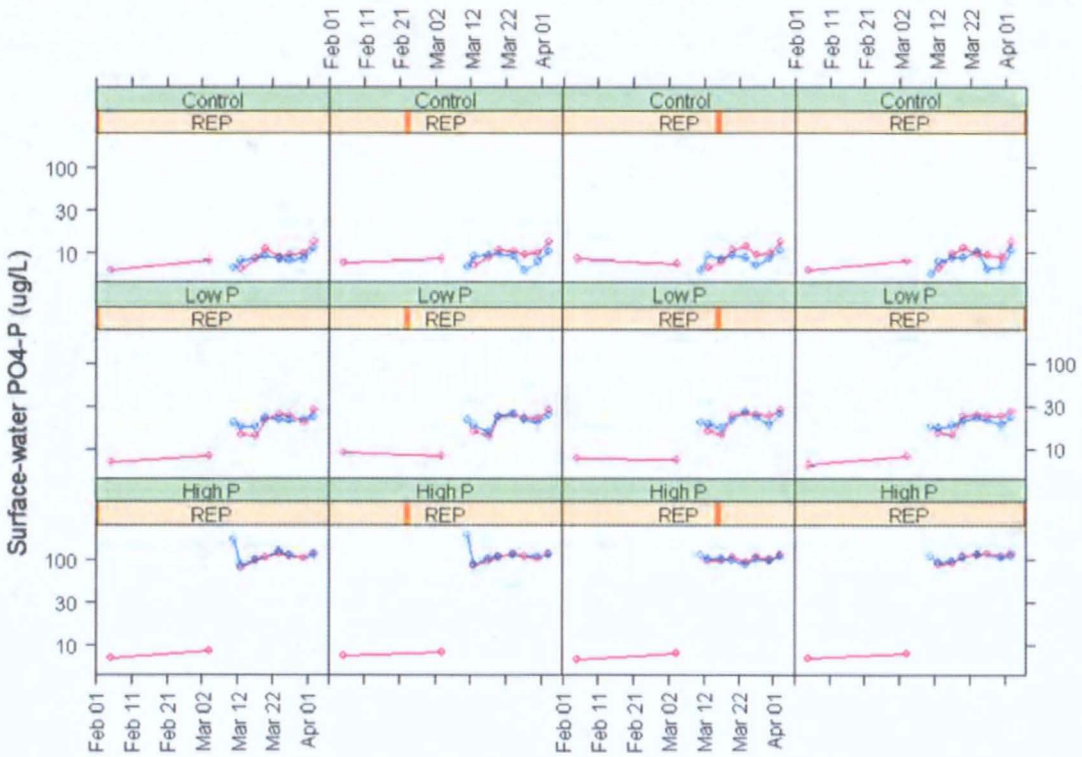

Figure 34. Time series multi-panel plot showing concentrations of PO4-P among the 12 experimental streams during the colonization (pre-dosing; 1 February – 10 March 2008) and dosing (11 March – 7 April 2008) phases of the experiment. The magenta points correspond to the samples collected from the outlet of each stream, whereas the blue points are samples from the mixing tank. No mixing tank samples were collected prior to dosing. Control = no P addition (6 ug/L), Low P = 20 ug/L and high P = 100 ug/L. REP = stream 1 -4 per treatment.

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Table 16. Mean dissolved and total P and N concentrations during the 28-days of PO4-P dosing in the experimental streams. Dissolved nutrients (PO4-P, DIN) were sampled in triplicate per location per stream, twice weekly during the study period (11 March – 7 April 2008; n=9 sampling events). Total N and P were sampled once per week during the dosing period (n=4 dates). See figure 34 for temporal patterns in PO4-P prior to and during dosing.

Treatment	Stream rep.	Location	**PO_4-P**	DIN	NH_3-N	NO_2NO_3-N	TN	TP
Control	1	Mixing tank	**8.1**	264.9	15.7	249.1	419.3	19.9
Control	2	Mixing tank	**7.9**	272.1	15.6	256.6	468.5	20.2
Control	3	Mixing tank	**7.8**	288.1	15.7	272.4	472.5	20.5
Control	4	Mixing tank	**7.7**	267.2	15.0	252.3	501.3	20.5
Low	1	Mixing tank	**18.3**	258.5	15.4	243.1	452.0	37.8
Low	2	Mixing tank	**19.3**	273.2	14.9	258.2	449.3	39.9
Low	3	Mixing tank	**19.3**	273.2	14.7	258.6	389.8	40.3
Low	4	Mixing tank	**17.7**	250.9	14.6	236.4	432.5	37.8
High	1	Mixing tank	**109.8**	267.2	15.6	251.6	469.5	136.2
High	2	Mixing tank	**112.7**	273.3	16.2	257.2	464.3	137.2
High	3	Mixing tank	**102.7**	265.4	14.7	250.8	447.0	127.4
High	4	Mixing tank	**109.5**	277.6	14.2	263.4	421.9	136.0
Control	1	Outlet	8.5	259.2	18.0	241.2	463.1	19.4
Control	2	Outlet	8.8	259.7	16.2	243.5	459.3	19.1
Control	3	Outlet	9.1	272.3	16.6	255.6	521.0	19.5
Control	4	Outlet	8.9	251.5	14.6	236.9	488.3	19.2
Low	1	Outlet	17.8	226.8	15.5	211.3	411.0	37.7
Low	2	Outlet	19.5	262.8	15.0	247.8	396.5	39.3
Low	3	Outlet	18.4	245.4	15.5	229.9	426.5	40.3
Low	4	Outlet	17.4	224.2	15.5	208.7	457.3	38.5
High	1	Outlet	96.6	216.6	16.1	200.5	431.4	128.7
High	2	Outlet	98.7	214.9	15.6	199.3	441.8	134.4
High	3	Outlet	102.8	250.9	15.7	235.2	466.8	127.2
High	4	Outlet	103.1	225.9	18.0	207.9	420.8	134.5

RESULTS AND INTERPRETATION, CONTINUED

Periphyton and Filamentous Algal Biomass Response to P Dosing

Periphyton growing on the ceramic tiles responded rapidly to experimental P dosing (Figure 35). There were no differences in treatments on Day 0 (Figure 36). High P treatments had significantly more chlorophyll than Low-P and Controls on Day 14, although Low-P chlorophyll-a was trending higher than the Control. By Day 28, Low P and High P treatments were not different, but had significantly more chlorophyll-a than Controls (Figure 35, 36). The lack of difference between low and high P treatments is consistent with the threshold response observed in the field study.

Figure 35. Comparison of periphyton growing on ceramic tiles among the 3 P treatments on Day 14 (left) and Day 28 (right). Note the obvious difference in color between Conrol and Low/High P treatments, even on Day 14. By Day 28, Low/High P tiles were covered in green algae.

0006361

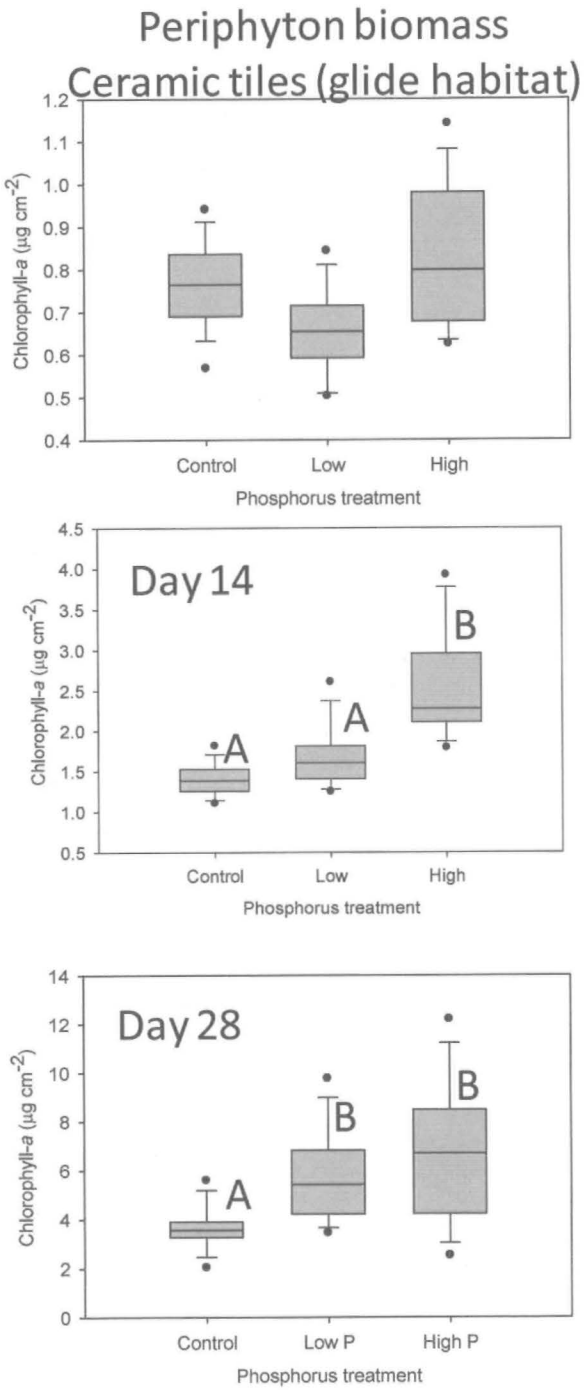

Figure 36. Results of ANOVA comparing mean chlorophyll-a (ug/cm2) among P treatments on Day 0, 14, and 28. Means significantly differed on Day 14 and 28, but not Day 0. Means with the same letter are not different (p>0.05).

0006362

Periphyton AFDM and chlorophyll-a from the bare rocks and transplanted rocks was highly variable among P treatments and did not differ significantly. However, this was largely due to the very strong response of *Cladophora* (filamentous green algae) in the low and high P treatments. *Cladophora* biomass exploded near the end of the study, and was significantly higher in the Low and High P streams than the Controls on the bare rocks and transplant rocks (ROCK-01, NBOS-03) on Day 28 (Figures 37-40).

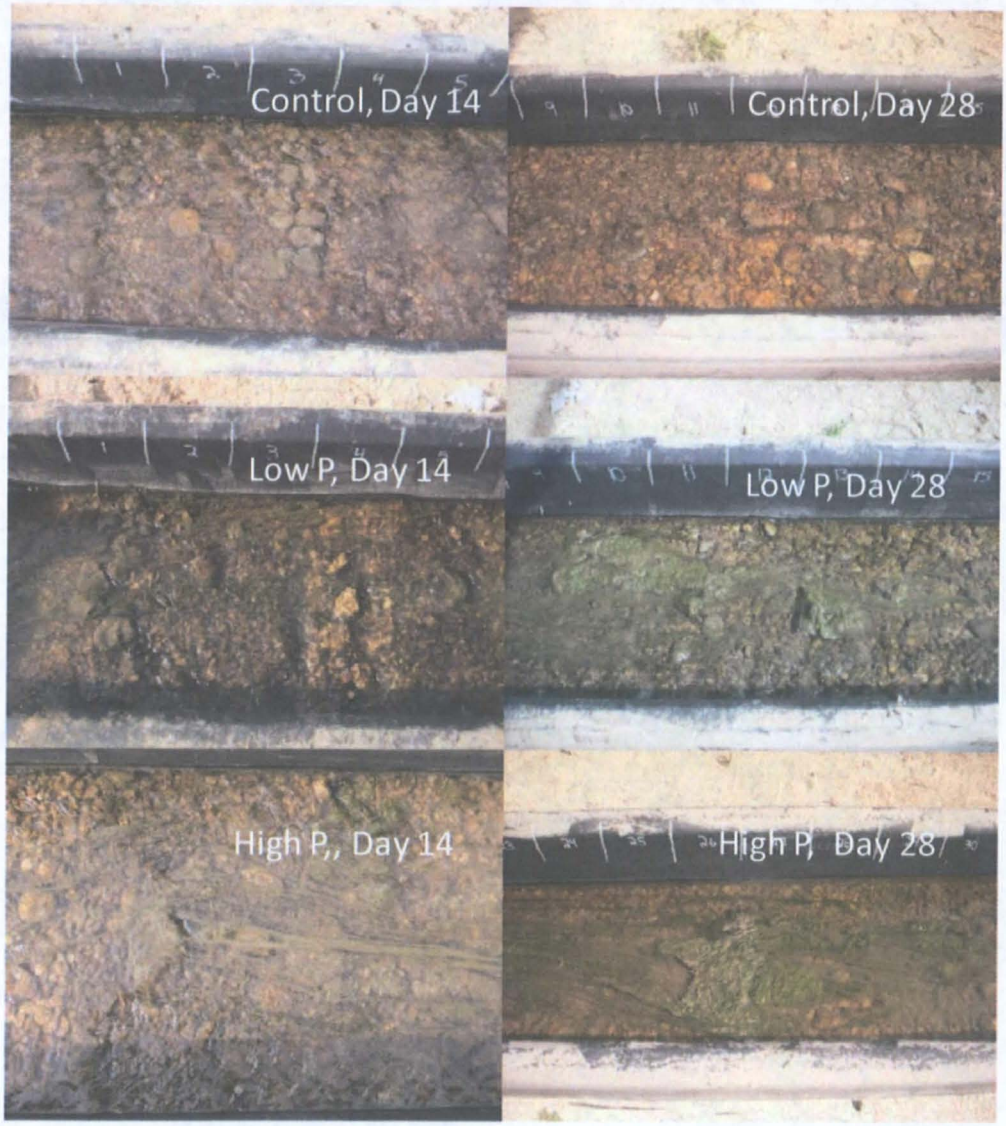

Figure 37. Photographs of riffle sections of Control, Low P, and High P treated experimental streams on Day 14 (left) and Day 28 (right). On Day 14, patches of *Cladophora* had begun to appear in Low and High P streams, but was more visually abundant in the High P streams. By Day 28, Low and High P streams were virtually indistinguishable, both supporting high biomass of *Cladophora*. Controls maintained a golden-brown coating of diatoms on rocks thorughout the study, although some *Cladophora* had begun to appear by Day 28.

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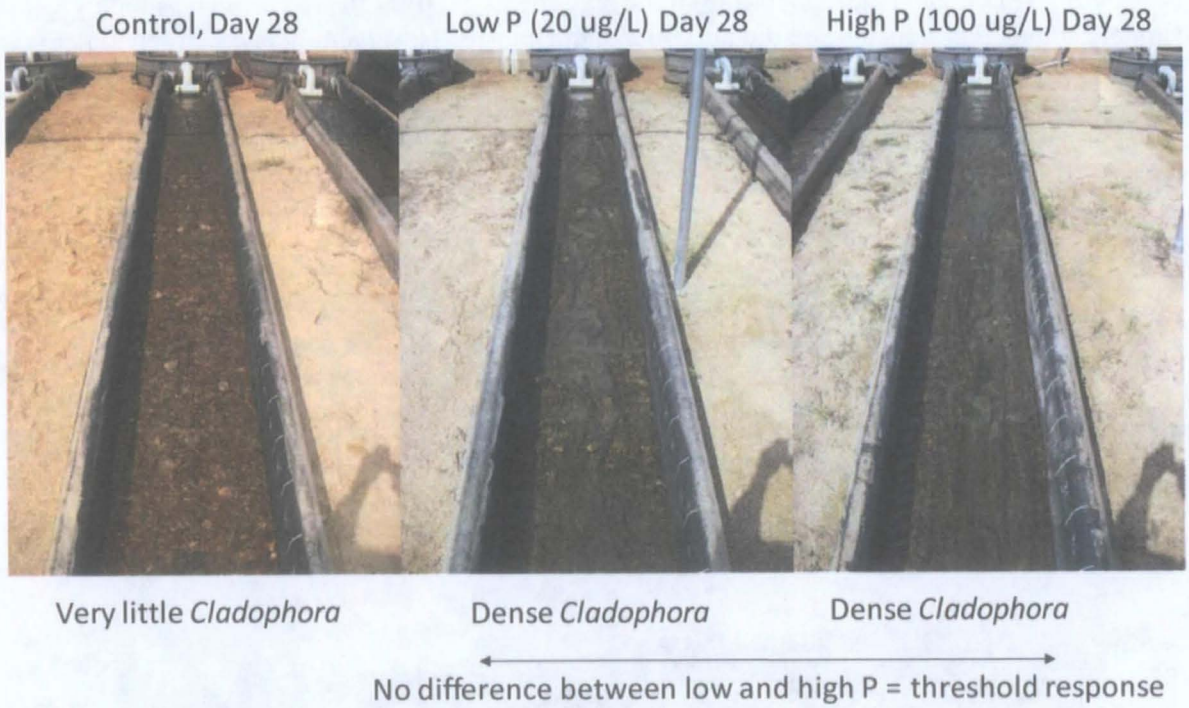

Figure 38. A longitudinal view of Control, Low-P, and High-P streams on Day 28.

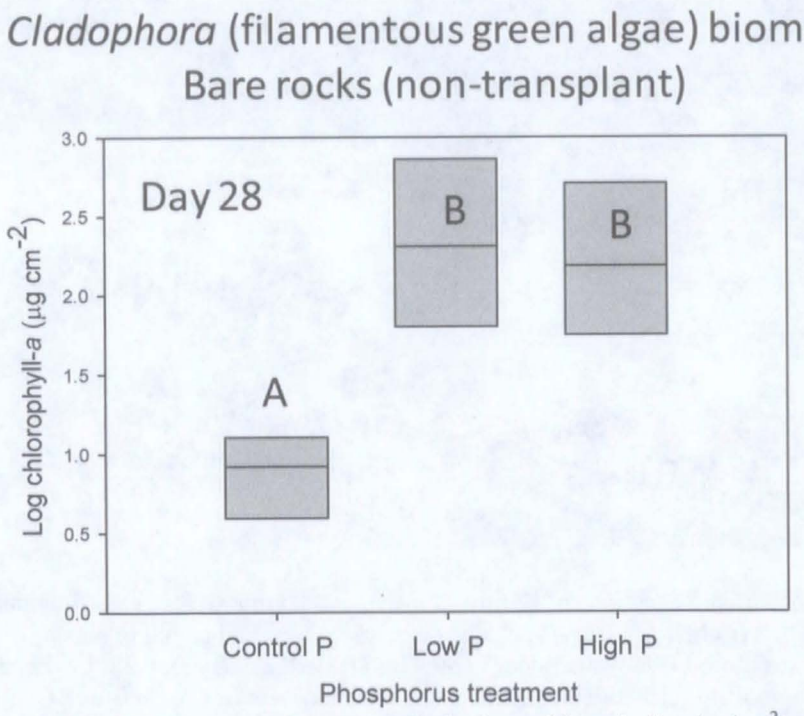

Figure 39. Results of ANOVA comparing mean *Cladophora* chlorophyll-a (ug/cm^2) among P treatments on Day 28. Means with the same letter are not different (p>0.05).

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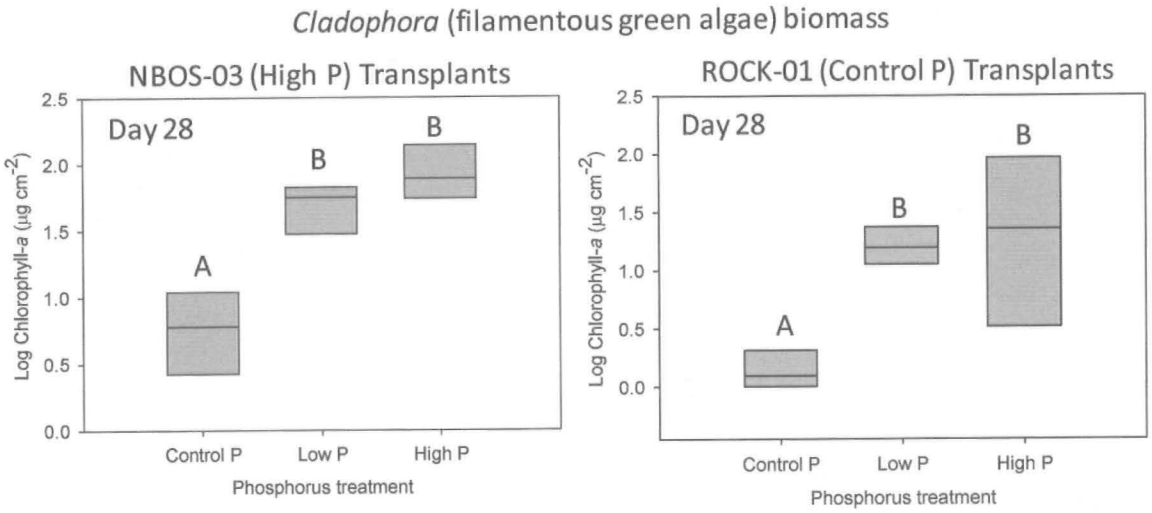

Figure 40. Results of ANOVA comparing mean *Cladophora* chlorophyll-a (ug/cm2) on transplanted rocks (ROCK-01=very low nutrient site, NBOS-03=high nutrient site) among P treatments on Day 28. Means with the same letter are not different (p>0.05).

0006365

RESULTS AND INTERPRETATION, CONTINUED

Periphyton Nutrient Content Response to Experimental P Dosing

Periphyton C:P, C:N, and N:P ratios from non-transplant bare rock samples did not differ among streams on Day 0, but differed significantly by treatment on Day 28 (Figure 41). High-P treated streams had the lowest C:P ratio (~150), Control streams had the highest (~320), whereas Low-P streams were intermediate (~230). Even the control C:P ratios were approaching levels deemed to be near or below a C:P threshold in the field study, suggesting that the background PO4-P concentrations were high enough to allow periphyton to sequester much higher amounts of P than typical of the lowest nutrient field sites.

Periphyton C:P ratios from the ROCK-01 transplant rocks responded very strongly to all 3 experimental treatments (Figure 42). On Day 0, ROCK-01 periphyton had C:P ratios above 2,000. After transplanting these rocks into the BEAR streams for just 7 days, mean C:P ratios had dropped to 689, 346, and 215 among the Control, Low P and High P treatments, respectively. By Day 28, ROCK-01-Control C:P ratios had dropped to 250, whereas Low and High P values were near 150 (Figure 43). This illustrated the remarkable affinity of stream periphyton for phosphorus, and provided additional evidence in support of nonlinear uptake of P as the explanation for sharp declines in periphyton C:P with small increases in surface-water TP.

In contrast to the ROCK-01 transplants, the NBOS-03 transplants did not respond to any of the experimental P treatments on Day 7 or 28 (Figure 42, 43), supporting the hypothesis that recycling of P within the periphyton is an important mechanism for maintaining low C:P ratios over time in streams with highly variable surface-water TP. Moreover, it also showed that periphyton from NBOS-03 was already saturated with P and thus did not sequester more P per unit carbon in the low or high P treatments.

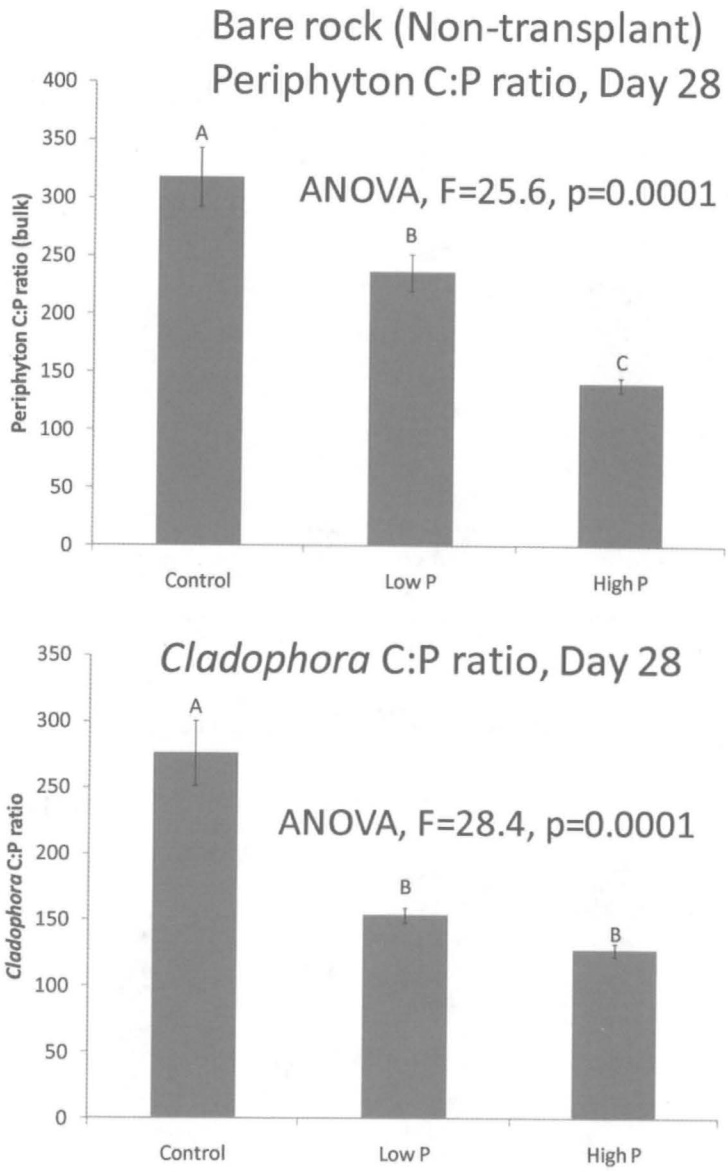

Figure 41. Results of ANOVA comparing mean periphyton C:P ratio (upper panel) and *Cladophora* C:P ratio (lower panel) among P treatments. Means with the same letter are not different (P>0.05).

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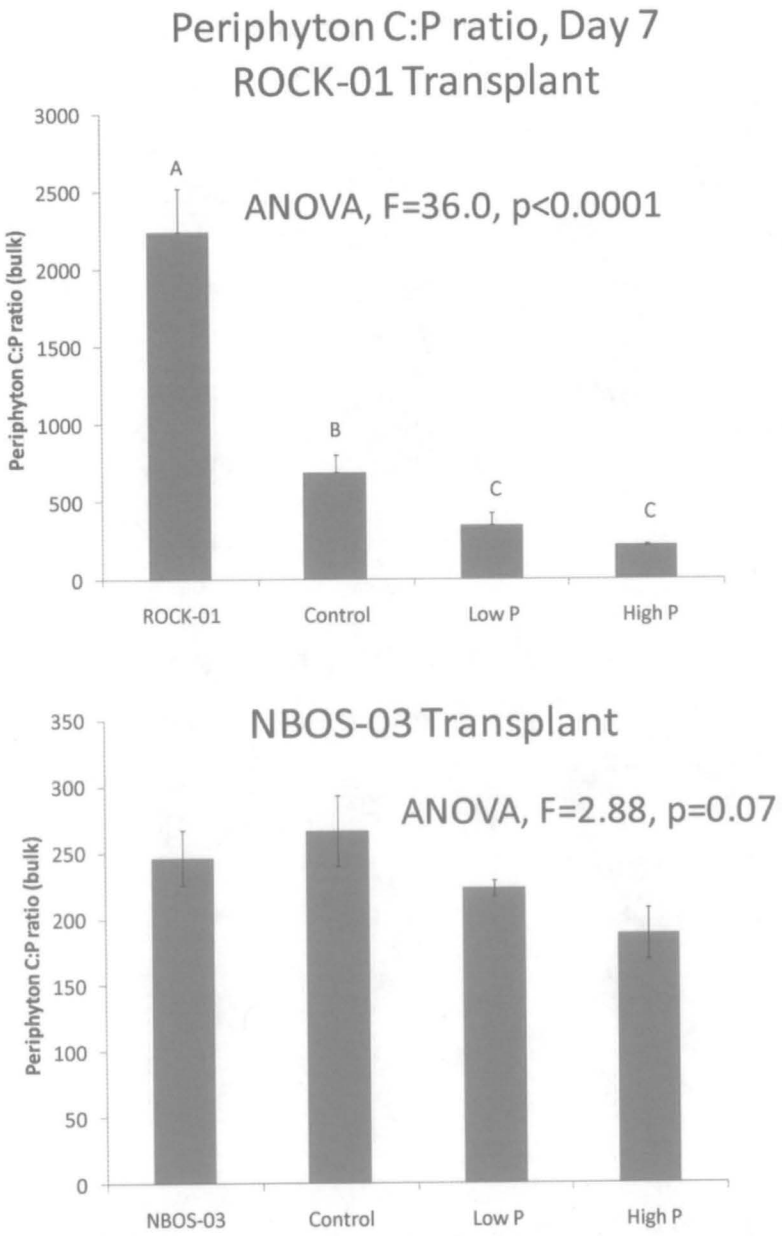

Figure 42. Results from ANOVA comparing periphyton C:P ratios from transplant rocks from ROCK-01 (top panel) and NBOS-03 (lower panel) after 7 days of exposure in the experimental streams. The ROCK-01 (top panel) and NBOS-03 (bottom panel) column corresponds to the mean from 4 composite samples collected on Day 0 of the study (prior to transplanting into the streams). Means with the same letter are not significantly different.

0006368

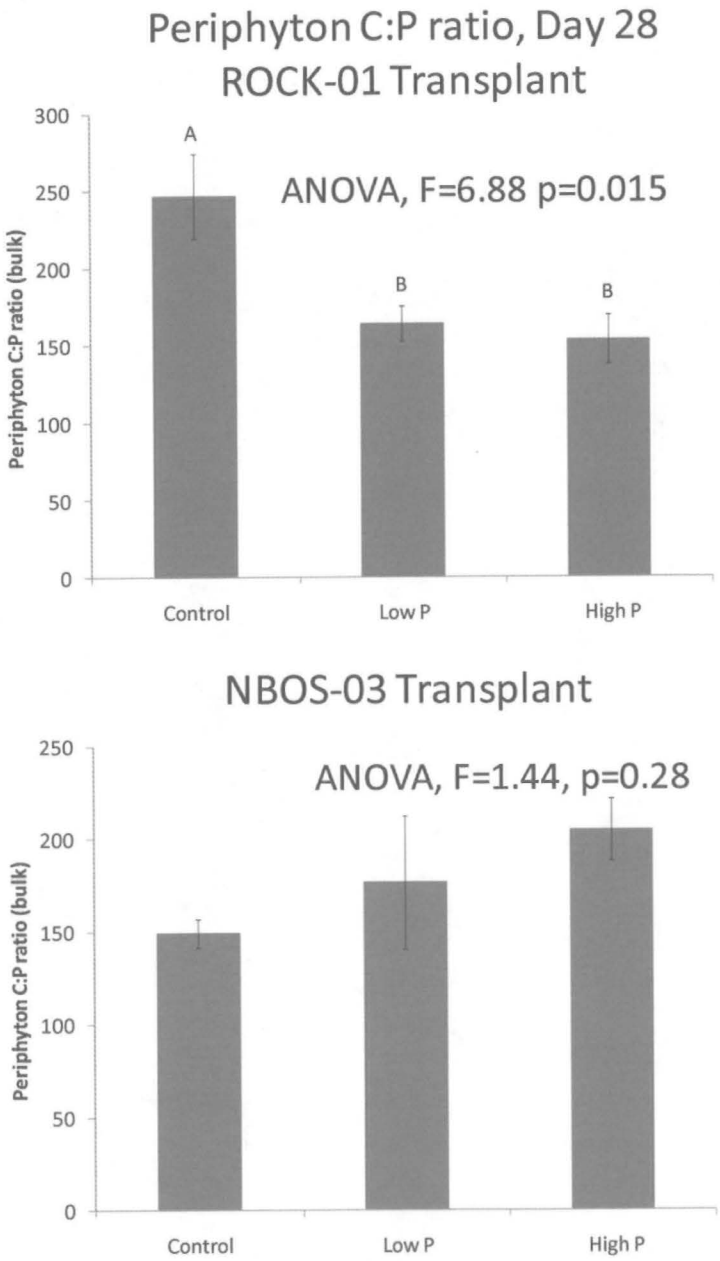

Figure 43. Results from ANOVA comparing periphyton C:P ratios from transplant rocks from ROCK-01 (top panel) and NBOS-03 (lower panel) after 28 days of exposure in the experimental streams. Means with the same letter are not significantly different.

0006369

RESULTS AND INTERPRETATION, CONTINUED

Algae Species Responses to Experimental P Dosing

Nonmetric multidimensional scaling ordination of algal species composition from the 6 intensive field sites in February 2008 and June 2008, and the ROCK-01 and NBOS-03 transplant rocks on Day 0 and Day 28 revealed several interesting responses (Figure 44). ROCK-01 samples from Day 0 were significantly different than the Control, Low P or High P samples on Day 28 (MRPP, $p<0.0001$). Algal species composition at ROCK-01 in February 2008 (prior to the BEAR study) and in June 2008 (after the BEAR study) remained clearly separated from any of the ROCK-01 samples that were transplanted in the BEAR streams.

ROCK-01 Control species composition shifted significantly to the right along nMDS Axis 1 by Day 28, moving in the direction of the NBOS-03 samples and toward field sites with species indicative of moderate to high TP (PALU-01, LEON-02). However, ROCK-01 Low and High P samples shifted significantly farther along this axis (MRPP, $P<0.0032$), indicating changes in species composition in Low and High P treatments were significantly greater than Controls. Consistent with a threshold response, algal species composition in Low and High P treatments did not differ (Figure 44).

In contrast, NBOS-03 rocks shifted in the same direction as the June 2008 NBOS-03 sample, remaining on the high-P end of Axis 1, regardless of P treatment. Algal species composition did not differ among treatments after 28 days of exposure (Figure 44).

Indicator Species Analysis of ROCK-01 transplants from Day 28 revealed that at least 5 diatom species were significantly less abundant (or absent) from Low P and High P samples than Controls, and 2 diatoms were significant indicators of the Low/High P treatments (Table 17). Five of these 7 taxa were identified as significant threshold indicators in response to TP gradients in the field study. All five responded in the same direction (decline, increase) in the BEAR study as in the field study.

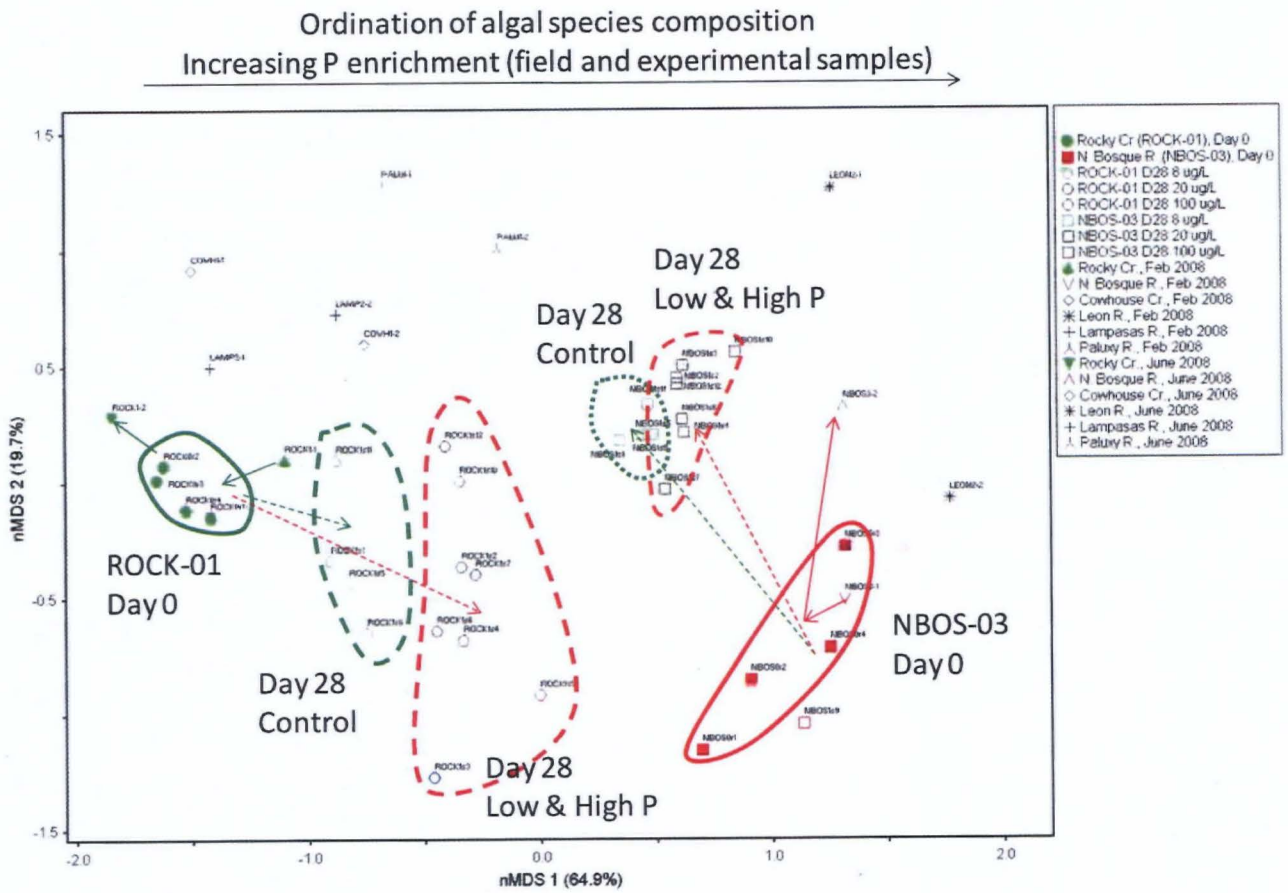

Figure 44. Nonmetric multidimensional scaling ordination of algal species composition from ROCK-01 and NBOS-03 transplant rocks on Day 0 and Day 28. Algal species composition from the 6 intensive field sites during February (before) and June 2008 (after) are also ordinated with the BEAR samples for a frame of reference. Sites sorted from low P sites (left end of Axis 1) to high P sites (right end of Axis 1). All ROCK-01 samples shifted toward NBOS-03 samples after 28 days of exposure in the BEAR facility, but Low and High P treated rocks shifted farther away from the natural stream and were significantly different than Controls. In contrast, NBOS-03 rocks shifted in the same direction as the June 2008 NBOS-03 sample, and algal species composition did not differ among treatments after 28 days of exposure.

Table 17. Results of Indicator Species Analysis (IndVal) using **Control vs. Low/High P** treatments as a predictor of algal species composition growing on ROCK-01 transplanted cobbles at Day 28 of the BEAR experimental stream study. Only taxa with significant IndVal scores are shown below. Taxa that showed significant declines or increases in response to P in both the experimental dosing study and in the field are highlighted in **bold.**

Taxon ID	Indicator group	Response to experimental PO4-P enrichment	Response to field study TP gradients	IndVal	p
ACbiasso	Control	**Decline**	**Decline**	51.8	0.0408
AHminuti	Control	**Decline**	**Decline**	77.6	0.0034
CMaffins	Control	Decline		88.2	0.0022
EYevergl	Control	**Decline**	**Decline**	100	0.0022
MDcircul	Control	Decline		100	0.0022
CCpedcls	Low/High P	**Increase**	**Increase**	58.9	0.0022
REsinuta	Low/High P	**Increase**	**Increase**	87.5	0.0102

RESULTS AND INTERPRETATION, CONTINUED

Macroinvertebrate Community Response to Experimental P Dosing

Macroinvertebrate taxonomic composition did not differ among treatments on Day 0 or Day 28, although composition shifted significantly between dates (Figure 45). Forty-five taxa were identified from the experimental streams, including 17 genera from the orders Ephemeroptera, Plecoptera, and Trichoptera (Appendix A). The relatively short dosing period, in contrast to the relatively long life cycle of these taxa, necessarily limited the potential response of this diverse group of organisms to P treatments. Future experiments spanning a longer dosing period or focused on the effect of low flow and P interactions may produce more meaningful results in the context of animal responses to experimental P enrichment.

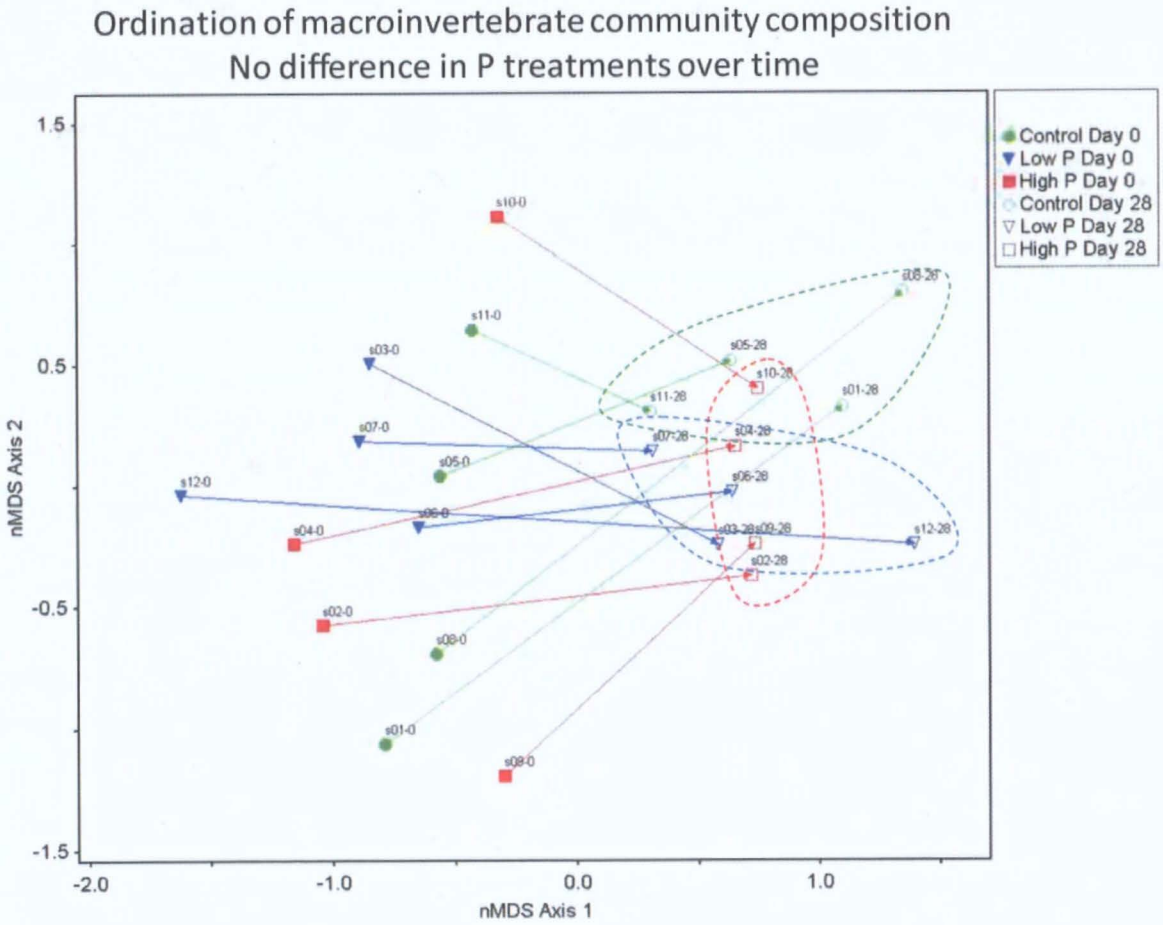

Figure 45. Nonmetric multidimensional scaling ordination of macroinvertebrate community responses to experimental P additions at the BEAR facility, Day 0 and Day 28. Arrows indicate the direction and magnitude of community change within each stream between Day 0 and 28. Ellipses are drawn to envelop all 4 streams by treatment on Day 28. There was extensive overlap among treatments, and community composition did not differ (MRPP, p=0.13).

0006373

SECTION III.
CONCLUSIONS AND RECOMMENDATIONS

Shifts from periphyton communities comprised of sensitive diatoms, calcareous cyanobacteria, and other non-chlorophyll bearing microbes to communities with higher chlorophyll content and more filamentous green algae is highly likely at concentrations of surface-water TP above 20 ug/L. Streams with TP > 200-1000 ug/L likely represent a second tier of degradation, and appear at greater risk for nuisance algal growth based on our threshold analyses. However, results from the P dosing experiment suggest that concentrations as low as 20 ug/L PO4-P can lead to high levels of *Cladophora* biomass in as little as 28 days. Adding more PO4-P (100 ug/L) did not result in more *Cladophora* in a 4-week period. However, faster growth rates were observed in the 100 ug/L treatment, which may explain why field streams with very high levels of TP (>200 ug/L) had greater frequency of heavy growth of filamentous algae.

Aquatic macrophyte cover consistently declined in streams with TP > 25-50 ug/L. These submersed plants serve as important refugia for juvenile fishes and macroinvertebrates, and provide a source of dissolved oxygen during low flows. Their decline likely represents a key structural and functional change in these stream ecosystems.

Minimum dissolved oxygen levels are highly dependent upon an interaction between flow and nutrient enrichment. Our study suggests that TP levels > 20-30 ug/L, coupled with low flows, will cause detrimental declines in minimum dissolved oxygen levels. This is particularly important in the context of minimum flows, a contentious issue in the southwestern USA. It is unlikely that studies geared toward detecting effects of nutrients on DO will adequately characterize this relationship without sampling during periods of low flow when gas exchange is with the atmosphere is very slow. Distance downstream and flow status are two very important considerations when evaluating the influence of WWTP discharges on wadeable streams in semi-arid regions. Future research is needed to better quantify the interaction between minimum flows and biological integrity in streams.

Based on the weight of evidence from the coupling of the field stream study and experimental stream study suggests that streams of the study area are very sensitive to phosphorus enrichment. There is a very high probability that streams exposed to surface-water TP levels exceeding 20 ug/L, and possibly 15 ug/L, will experience a sharp decline in biological integrity, including loss of characteristic structure (periphyton and macrophytes), loss of numerous species (algae and macroinvertebrates), minimum dissolved oxygen levels unsuitable for supporting native fauna during low flows, and increase likelihood of nuisance algal growth that limits recreational use of strreams. Streams exceeding 200 ug/L may represent a second tier of degradation, with more consistent nuisance algal growth and additional losses of algal and macroinvertebrate species.

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Appendix A. List of algal taxa identified in the field and experimental dosing studies. Only taxa that occurred >2 times are included in the table (n=295). Taxonomic identifications were conducted exclusively by Dr. Barbara Winsborough, Winsborough Consulting, Leander, TX.

TYPE	SPECIES	TAXON_ID
Diatom	*Achnanthes biassolettiana*	ACbiasso
Diatom	*Achnanthes brevipes*	ACbrevip
Diatom	*Achnanthes hungarica*	AChungar
Diatom	*Adlafia bryophila*	ADbryoph
Diatom	*Achnanthidium exiguum*	AHexigum
Diatom	*Achnanthidium minutissimum*	AHminuti
Diatom	*Amphipleura pellucida*	ALpelluc
Diatom	*Amphora bullatoides*	AMbullat
Diatom	*Amphora coffeaeformis*	AMcoffea
Diatom	*Amphora granulata*	AMgranul
Diatom	*Amphora inariensis*	AMinarie
Diatom	*Amphora libyca*	AMlibyca
Diatom	*Amphora montana*	AMmontan
Diatom	*Amphora ovalis*	AMovalis
Diatom	*Amphora pediculus*	AMpedcls
Diatom	*Amphora veneta*	AMveneta
Diatom	*Anomoeoneis sphaerophora*	ANsphaer
Diatom	*Aulacoseira ambigua*	AUambig
Diatom	*Aulacoseira granulata*	AUgranlt
Diatom	*Aulacoseira granulata var. angustissima*	AUgrnang
Diatom	*Bacillaria paradoxa*	BApardxa
Diatom	*Brachyseira vitrea*	BRvitrea
Diatom	*Caloneis bacillum*	CAbacill
Diatom	*Campylodiscus clypeus*	CAclypeu
Diatom	*Caloneis molaris*	CAmolars
Diatom	*Caloneis schumanniana*	CAschuma
Diatom	*Caloneis silicula*	CAsilicu
Diatom	*Cocconeis pediculus*	CCpedcls
Diatom	*Cocconeis placentula*	CCplacen
Diatom	*Cymbella cistula*	CMcistul
Diatom	*Cymbella cymbiformis*	CMcymbis
Diatom	*Encyonema delicatula*	CMdelcat
Diatom	*Encyonema elginensis*	CMelgine
Diatom	*Cymbella excisa*	CMexcisa
Diatom	*Cymbella falaisensis*	CMfalais
Diatom	*Cymbella hustedtii*	CMhusted
Diatom	*Cymbella kolbei*	CMkolbei
Diatom	*Cymbella laevis*	CMlaevis
Diatom	*Cymbella tumida*	CMtumida
Diatom	*Cyclostephanos tholiformis*	CStholif

Diatom	*Cymatopleura elliptica*	CTellipt
Diatom	*Cymatopleura solea*	CTsolea
Diatom	*Cyclotella cf. stelligera*	CYcfstel
Diatom	*Cyclotella meneghiniana*	CYmenegh
Diatom	*Denticula kuetzingii*	DEkuetzi
Diatom	*Denticula subtilis*	DEsubtil
Diatom	*Diadesmis (Navicula) confervacea*	DIconfer
Diatom	*Diploneis ovalis*	DPovalis
Diatom	*Diploneis pseudovalis*	DPpsudov
Diatom	*Diploneis puella*	DPpuella
Diatom	*Encyonema carina*	ECcarina
Diatom	*Encyonema minutum*	ECminutu
Diatom	*Encyonema silesiacum*	ECsilesi
Diatom	*Encyonema triangulum*	ECtriang
Diatom	*Epithemia adnata*	EPadnata
Diatom	*Epithemia sorex*	EPsorex
Diatom	*Epithemia turgida*	EPturgid
Diatom	*Eucocconeis (Achnanthes) flexella*	ESflexel
Diatom	*Eunotia pectinalis*	EUpectin
Diatom	*Eunotia arcus*	EUarcus
Diatom	*Encyonopsis cesatii*	EYcesati
Diatom	*Eunotia bilunaris*	EUbilun
Diatom	*Encyonema (Encyonopsis) evergladianum*	EYevergl
Diatom	*Encyonema (Encyonopsis) microcephala*	EYmicroc
Diatom	*Navicula (Fallatia) lenzii*	FAlenzii
Diatom	*Fallacia litoricola*	FAlitori
Diatom	*Fallacia monoculata*	FAmonoc
Diatom	*Fallacia pygmaea*	FApygmae
Diatom	*Fallatia tenera*	FAtener2
Diatom	*Fragilaria capucina*	FRcapuci
Diatom	*Fragilaria elliptica*	FRellptc
Diatom	*Fragilaria famelica*	FRfameli
Diatom	*Fragilaria nanana*	FRnanana
Diatom	*Fragilaria pinnata var. lancettula*	FRpinlan
Diatom	*Fragilaria tenera*	FRtenera
Diatom	*Geissleria decussis*	GEdecu
Diatom	*Gomphosphenia (Gomphonema) lingulatiformis*	GMlinfor
Diatom	*Gomphosphenia reicheltii*	GMreicht
Diatom	*Gomphonema acuminatum*	GOacumin
Diatom	*Gomphonema affine*	GOaffine
Diatom	*Gomphonema angustatum (micropus)*	GOangstt
Diatom	*Gomphonema angustum*	GOangust
Diatom	*Gomphonema clavatum*	GOclavat
Diatom	*Gomphonema gracile*	GOgracil
Diatom	*Gomphonema insigne*	GOinsign

Diatom	*Gomphonema intricatum*	GOintric
Diatom	*Gomphonema intricatum var vibrio*	GOintvib
Diatom	*Gomphonema mclaughlinii*	GOmaclau
Diatom	*Gomphonema parvulum*	GOparvul
Diatom	*Gomphonema pumilum*	GOpumilu
Diatom	*Gomphonema rhombicum*	GOrhombi
Diatom	*Gomphonema truncatum*	GOtrunca
Diatom	*Gomphonema vibrioides*	GOvibdes
Diatom	*Gyrosigma nodiferum*	GYnodfrm
Diatom	*Gyrosigma obscurum*	GYobscur
Diatom	*Gyrosigma spencerii*	GYspence
Diatom	*Hantzschia amphioxys*	HAamphio
Diatom	*Hippodonta (Navicula) hungarica*	HIhunga
Diatom	*Craticula cuspidata*	KCcuspid
Diatom	*Craticula (Navicula) halophila*	KChaloph
Diatom	*Achnanthes (Lemnicola) hungarica*	LEhungar
Diatom	*Luticola goeppertiana*	LUgoept2
Diatom	*Luticola mutica*	LUmutica
Diatom	*Meridion circulare*	MDcircul
Diatom	*Melosira varians*	MEvarian
Diatom	*Mastogloia elliptica*	MSellipt
Diatom	*Mastogloia smithii*	MSsmithi
Diatom	*Navicula (Mayamaea) atomus*	MYatomus
Diatom	*Navicula antonii*	NAantoni
Diatom	*Navicula cf. pseudanglica*	NAcfpsan
Diatom	*Navicula circumtexta*	NAcirctx
Diatom	*Navicula constans*	NAconstn
Diatom	*Navicula cryptocephala*	NAcrypto
Diatom	*Navicula cryptotenella*	NAcryten
Diatom	*Navicula erifuga*	NAerifga
Diatom	*Brachyseira neoexilis (Navicula exilis)*	NAexilis
Diatom	*Navicula gregaria*	NAgregar
Diatom	*Navicula ingenua*	NAingua
Diatom	*Navicula integra*	NAintgra
Diatom	*Navicula kotschii (texana)*	NAkotsch
Diatom	*Navicula leptostriata*	NAleptos
Diatom	*Navicula libonensis*	NAlibone
Diatom	*Navicula menisculus*	NAmenscl
Diatom	*Navicula (Eolima) minima*	NAminima
Diatom	*Navicula oblonga*	NAoblong
Diatom	*Navicula perminuta*	NApermnt
Diatom	*Navicula phyllepta*	NAphylpt
Diatom	*Navicula radiosa*	NAradios
Diatom	*Navicula recens*	NArecens
Diatom	*Navicula rhynchocephala*	NArhynch

Diatom	*Navicula sanctaecrucis*	NAsancru
Diatom	*Navicula savannahiana*	NAsavana
Diatom	*Navicula schroeteri var escambia*	NAschesc
Diatom	*Sellaphora (Navicula) stroemii*	NAstroem
Diatom	*Fallatia (Navicula) subminuscula*	NAsubmin
Diatom	*Navicula symmetrica*	NAsymtrc
Diatom	*Navicula texana (Grimmei)*	NAtexana
Diatom	*Navicula tridentula*	NAtriden
Diatom	*Navicula trivialis*	NAtrivis
Diatom	*Navicula veneta*	NAveneta
Diatom	*Navicula viridula var. rostellata*	NAvirdla
Diatom	*Neidium iridis*	NEiridis
Diatom	*Nitzschia acuminata*	NIacuata
Diatom	*Nitzschia amphibia*	NIamphib
Diatom	*Nitzschia amphibioides*	NIampoid
Diatom	*Nitzschia angustatula*	NIangtu
Diatom	*Nitzschia angustata*	NIangust
Diatom	*Nitzschia brevissima*	NIbrevis
Diatom	*Nitzschia calida*	NIcalida
Diatom	*Nitzschia compressa var. balatonis*	NIcombal
Diatom	*Nitzschia constricta*	NIcstric
Diatom	*Nitzschia debilis*	NIdibili
Diatom	*Nitzschia dissipata*	NIdissip
Diatom	*Nitzschia filiformis*	NIfilifr
Diatom	*Nitzschia fonticola*	NIfontic
Diatom	*Nitzschia frustulum*	NIfrustu
Diatom	*Nitzschia inconspicua*	NIincons
Diatom	*Nitzschia linearis*	NIlinear
Diatom	*Nitzschia obtusa*	NIobtusa
Diatom	*Nitzschia palea*	NIpalea
Diatom	*Nitzschia serpentiraphe*	NIserpen
Diatom	*Nitzschia sigmoidea*	NIsigdea
Diatom	*Nitzschia sigma*	NIsigma
Diatom	*Nitzschia sinuata v delognii*	NIsinde
Diatom	*Nitzschia solita*	NIsolita
Diatom	*Nitzschia subacicularis*	NIsubaci
Diatom	*Nitzschia tropica*	NItropic
Diatom	*Nitzschia tryblionella*	NItrybla
Diatom	*Nitzschia vitrea*	NIvitrea
Diatom	*Pseudostaurosira brevistriata*	PDbrevis
Diatom	*Pseudostaurosira parasitica var. subconstricta*	PDparsub
Diatom	*Plagiotropis lepidoptera*	PGlepidp
Diatom	*Pinnularia acrosphaeria*	PIacro
Diatom	*Pinnularia gibba*	PIgibba
Diatom	*Pinnularia microstauron*	PImicros

Diatom	*Pinnularia streptoraphe*	PIstrept
Diatom	*Pleurosira (Ceratulina) laevis*	PRlaevis
Diatom	*Achnanthes (Planothidium) lanceolata*	PTlanceo
Diatom	*Reimeria sinuata*	REsinuta
Diatom	*Rhoicosphenia abbreviata*	ROabbre
Diatom	*Rhopalodia brebissonii*	RPbrebsn
Diatom	*Rhopalodia gibba*	RPgibba
Diatom	*Rhopalodia musculus*	RPmuscul
Diatom	*Sellaphora laevissima*	SFlaevis
Diatom	*Sellaphora pupula*	SFpupula
Diatom	*Sellaphora seminulum*	SFseminu
Diatom	*Fragilaria (Staurosira) construens*	SRconstr
Diatom	*Fragilaria construens var. venter*	SRconven
Diatom	*Stauroneis phoenicentron*	SSphoeni
Diatom	*Stephanodiscus sp1*	ST1
Diatom	*Surirella angusta*	SUangust
Diatom	*Surirella bifrons*	SUbifron
Diatom	*Surirella brebissonii*	SUbreb
Diatom	*Surirella elegans*	SUelegan
Diatom	*Surirella minuta*	SUminuta
Diatom	*Surirella ovalis*	SUovalis
Diatom	*Surirella spiralis*	SUspiral
Diatom	*Surirella splendida*	SUsplen
Diatom	*Synedra (Fragilaria) acus*	SYacus
Diatom	*Synedra goulardi*	SYgoular
Diatom	*Synedra (Fragilaria) ulna*	SYulna
Diatom	*Fragilaria (Tabularia) fasciculata*	TBfascic
Diatom	*Terpsinoe musica*	TEmusica
Diatom	*Thalassiosira weissflogii*	THweiss
Diatom	*Tryblionella apiculata*	TYapicul
Diatom	*Tryblionella (Nitzschia) calida*	TYcaldid
Diatom	*Nitzschia (Tryblionella) levidensis*	TYlevid
Soft	*Aphanothece sp.*	AFCsp
Soft	*Aphanizomenon sp.*	AFNsp
Soft	*Aphanocapsa sp.*	AFPsp
Soft	*Aphanochaete sp.*	AFTsp
Soft	*Anabaena sp.*	ANBsp
Soft	*Ankistrodesmus falcatus*	ANKfalca
Soft	*Audouinella hermannii*	AUDhernn
Soft	*Blennothrix sp.*	BLNsp
Soft	*Bulbochaete sp.*	BULsp
Soft	*Calothrix sp.*	CALsp
Soft	*Chlorococcum sp.*	CHCOAUL
Soft	*Chlamydomonas sp.*	CHLsp
Soft	*Chlorophytan zoospores*	CHLzoo

Soft	*Chroococcus sp.*	CHOsp
Soft	*Characium sp.*	CHRsp
Soft	*Chaetophora sp.*	CHTsp
Soft	*Cladophora glomerata*	CLAglomer
Soft	*Chlorobotrys simplex*	CLBsimp
Soft	*Closterium sp*	CLOsp2
Soft	*Coelastrum sp.*	COEsp
Soft	*Coelosphaerium sp.*	COHsp
Soft	*Cosmarium botrytis*	COSbotry
Soft	*Cosmarium galeritum*	COSgaler
Soft	*Cosmarium sp.*	COSsp
Soft	*Crucigenia sp.*	CRUsp
Soft	*Cryptomonas sp.*	CRYsp
Soft	*Dictyosphaerium sp.*	DICsp
Soft	Unidentified dinoflagellates	DINuid
Soft	*Euastrum binale*	EUAbinal
Soft	*Euglena sp.*	EUGsp
Soft	*Gloeocystis sp.*	GLCsp
Soft	*Gloeothece sp.*	GLHsp
Soft	*Gloeoskene turfosa*	GLKturf
Soft	*Gloeocapsa sp.*	GLOsp
Soft	*Gongrosira sp.*	GOGsp
Soft	*Golenkinia sp.*	GOLsp
Soft	*Gomphosphaeria sp.*	GOMsp
Soft	*Homoeothrix sp.*	HOMsp
Soft	*Hormidium sp.*	HORsp
Soft	*Kirchneriella sp.*	KIRsp
Soft	*Kirchneriella obesa*	KIRobesa
Soft	*Merismopedia glauca*	MERglauc
Soft	*Micractinium sp.*	MIAsp
Soft	*Microcystis sp*	MICsp
Soft	*Mougeotia sp.*	MOUsp
Soft	*Oedogonium sp.*	OEDsp
Soft	*Oocystis sp.*	OOCsp
Soft	*Ophiocytium capitatum*	OPHcapit
Soft	*Oscillatoria sp.*	OSCsp
Soft	*Pandorina morum*	PANmorum
Soft	*Pediastrum boryanum*	PEDboryn
Soft	*Pediastrum duplex*	PEDduplx
Soft	*Pediastrum simplex*	PEDsimpl
Soft	*Peridinium sp.*	PERsp
Soft	*Phacus sp.*	PHAsp
Soft	*Phormidium sp*	PHOsp
Soft	*Raphidiopsis curvata*	RAPcurvt
Soft	*Scenedesmus abundans*	SCEabund

Soft	*Scenedesmus acuminatus*	SCEacumn
Soft	*Scenedesmus acutiformis*	SCEacutf
Soft	*Scenedesmus armatus*	SCEarmat
Soft	*Scenedesmus bijuga var. alternans*	SCEbialt
Soft	*Scenedesmus bijuga*	SCEbijug
Soft	*Scenedesmus dimorphus*	SCEdimor
Soft	*Scenedesmus quadricauda*	SCEquadr
Soft	*Scenedesmus sp.*	SCEsp
Soft	*Schroderia setigera*	SCRsetig
Soft	*Scytonema sp.*	SCYsp
Soft	*Schizothrix sp.*	SCZsp
Soft	*Sphaerocystis schroeteri*	SPHschro
Soft	*Sphaerocystis sp.*	SPHsp
Soft	*Spirogyra sp.*	SPIsp
Soft	*Spirulina sp.*	SPLsp
Soft	*Spondylosium pulchrum*	SPOpulch
Soft	*Staurastrum sp.*	STAsp
Soft	*Synechococcus sp.*	SYCsp
Soft	*Tetraedron caudatum*	TETcaudt
Soft	*Tetraedron minimum*	TETminum
Soft	*Tetraedron trigonum*	TETtrign
Soft	*Trachelomonas sp.*	TRAsp
Soft	Centric diatoms	Uncent
Soft	Pennate diatoms	Unpennt
Soft	Unknown Cyanophyte coccoid	XBGcoc
Soft	Unknown Cyanophyte filament	XBGfilam
Soft	Unidentified dinoflagellates	XDFalga
Soft	Unknown alga sp.	XXAsp
Soft	*Zygnema sp.*	ZYGsp

Appendix A,continued List of dominant macroinvertebrate taxa identified in the BEAR experimental streams, Day 0 and 28, including several **EPT (Ephemeroptera, Trichoptera, Plecoptera) taxa (bold).**

Argia
Baetis
Baetodes
Berosus
Brechmorhoga
Caenis
Camelobaetidius
Cheumatopsyche
Chimarra
Chironomidae
Chironomus
Coptotmus
Erpetogomphus
Erpobdellidae
Gammarus
Gyrinus
Haematopota
Helicopsyche
Hetaerina
Hydracarina
Hydrocanthus
Hydroperla
Hydropsyche
Isonychia
Lutrochus
Macaffertium
Micromenetus
Naididae
Neochoroterpes
Oecetis
Perlesta
Petrophila
Physella
Simulium
Sphaeriidae
Stenelmis
Stenonema
Thraulodes
Trichocorixa
Tricorythodes
Turbellaria
Veliidae

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APPENDIX B. PUBLICATIONS SUPPORTED IN PART OR FULL BY #CP-966137-01

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Scott, J. T.*, J. A. Back*, J. M. Taylor*, and R. S. King. 2008. Does nutrient enrichment decouple algal-bacterial production in periphyton? Journal of the North American Benthological Society 27:332-334.

Scott, J. T.*, D. A. Lang*, R. S. King, and R. D. Doyle. 2009. Nitrogen fixation and phosphatase activity in periphyton growing on nutrient diffusing substrata: Evidence for differential nutrient limitation in stream benthos. Journal of the North American Benthological Society 28:57-68.

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Ontogenic differences in mayfly stoichiometry influence growth rates in response to phosphorus enrichment

Jeffrey A. Back[1]*, Jason M. Taylor[1], Ryan S. King[1], Kari L. Fallert[2] and Emily H. Hintzen[2]

With 2 figures and 4 tables

Abstract: We contrasted the carbon, nitrogen, and phosphorus (C : N : P) stoichiometry of *Caenis* spp. (Ephemeroptera:Caenidae) nymphs from 2 stream reaches differing in P enrichment. We also estimated growth rates of nymphs reared on algae of different P content across four development classes in a laboratory experiment. C : N ratios of field-collected nymphs exhibited variable responses across development classes between sites whereas C : P and N : P ratios showed a clear unimodal response, increasing from classes II through IV but then declining sharply in class V (nymphs nearing eclosion) at both sites. C : P was lower at the highly enriched site for all but the last development class. Growth rates increased in response to P enrichment at the earliest development class, but this growth response diminished in later development classes resulting in a significant interaction between P treatments and development classes. Trends in field data imply that later stages of development have higher P requirements than earlier classes and nutrient enrichment may affect sequestration of P by nymphs. Laboratory data suggest that early development classes are more P limited but in light of field results, nymphs may shift P allocation from somatic growth to reproductive development as organisms mature.

Key words: C : N : P ratios, phosporus content, development classes, growth rates, Ephemeroptera, Caenidae.

Introduction

Elemental stoichiometry is gaining favor in ecology as a way to study ecosystems, especially the interactions between organisms at all trophic levels and their environment. Stoichiometry examines the ratios of key elements (nutrients), particularly carbon (C), nitrogen (N) and phosphorus (P), in organisms and their food, and how differing elemental ratios affect organisms and ecosystems (Elser et al. 1996). Nutrient ratios are an important aspect of stream ecology because they influence organism growth, reproduction and life history traits and are therefore the basis of trophic interactions. Studies on the stoichiometry of benthic invertebrates and their food sources should improve overall understanding of trophic interactions, as they are an essential part of the food web. However, current knowledge of organism stoichiometry is lacking in terms of species examined, ontogenic changes, and spatial and temporal variation in elemental composition (Frost et al. 2002).

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The importance of phosphorus in affecting growth rates has recently been demonstrated (Weider et al. 2005). Phosphorus-rich ribosomal (r)RNA is necessary for protein synthesis which directly influences growth rates. Thus, rapid growth rates are associated with increased P requirements because organisms must disproportionately increase their allocation to P-rich rRNA to meet the protein synthesis demands of rapid growth. This is the basic tenet of the Growth Rate Hypothesis (GRH) which suggests that organisms with rapid growth rates must build P-rich biomass which makes them more susceptible to P-limitation (Elser et al. 1996, 2006, Vrede et al. 2002).

Invertebrates at a given life stage are more homeostatic with respect to their body nutrient content than autotrophs despite variation in the chemical makeup of their food, although the degree of elemental homeostasis varies between species (Sterner & Elser 2002, Peck & Walton 2006). In a field study examining consumer-resource stoichiometry, Cross et al. (2003) found that P content of invertebrates exhibited much greater variability than C or N, and that some taxa might not be stoichiometrically homeostatic across life stages. Although data from this study did not find a significant correlation between body size and %P for the 40 invertebrate taxa examined, the P content of certain functional feeding groups did decrease with increasing body size. Frost & Elser (2002) found a negative linear relationship between P content and body size in *Ephemerella* spp. indicating that P content in this taxon is not necessarily fixed for its lifespan but shifts ontogenetically during development. Similar ontogenetic shifts in P content have been observed in aquatic crustaceans (DeMott 2003, Faerøvig & Hesson 2003).

Mayflies (Ephemeroptera) are a widespread and important component of stream ecosystems. Previous studies have examined the effects of C: N and C: P ratios on mayfly species growth in both field and laboratory settings. Data demonstrate that high quality food (high P and or N content) increases growth and fecundity of several species of mayfly (Soderstrom 1988, Frost & Elser 2002). Mayflies are a good organism for studying possible stoichiometric difference across their ontogeny because development classes have been described (e.g. Taylor & Kennedy 2006) or can be easily determined for most species. Whether or not the growth rate is constant across all development classes is unknown. Moreover, in organisms which undergo metamorphosis (e.g. insects), growth and the loss of juvenile structures and development of adult structures follow a time sequence which may require different nutrient levels. Because adult mayflies do not feed, all the chemical requirements of the adults must be met by the nymphs. Thus nymphal nutrition must play a large role in adult reproductive success. Since egg elemental composition is key to early nymph growth and survivorship (Tessier et al. 1983, DeMott 2003), the availability of nutrients to actively feeding nymphs is of paramount importance. Because all egg production is realized in mayfly nymphs, there should be a direct linkage between nymphal food quality, fecundity, and egg nutrient content in mayflies. This relationship has been shown in *Daphnia* (Sterner 1993, Urabe & Sterner 2001). On the other hand it is possible that this linkage is relaxed because males may contribute phosphorus in their sperm packet that is available for incorporation into eggs. This has been demonstrated in *Drosophila* spp. (Markow et al. 2001).

The objective of this study was to determine growth rates for *Caenis* spp. across a range of development classes spanning several levels of nutrient enrichment. We hypothesize that (1) nutrient stoichiometry will vary through the development cycle of *Caenis* spp., with highest levels of P at early and late development stages due to relatively high levels of somatic and reproductive growth, respectively, (2) that nutrient stoichiometry within a development class from a site will differ depending on nutrient content of food, and (3) growth rates will increase with increasing P content of food resources and decrease with increasing development class.

Methods

Study area

The North Bosque River is a 4th-order (Straehler system, 1:250,000 scale) perennial tributary of the Brazos River located in central Texas, USA. The North Bosque flows predominantly through the Cross Timbers Level III ecoregion (Griffith et al. 2004), an area characterized by semi-arid climate (annual precipitation 40–60 cm/y), shallow alkaline clay soils overlaying heavily fractured limestone bedrock, and flashy stream flow. The longitudinal profile of the North Bosque River exhibits a strong nutrient gradient caused by municipal waste water inputs and runoff associated with concentrated animal feeding operations. These inputs are highest in the upper reaches of the watershed. Consequently, concentrations of dissolved and total phosphorus and nitrogen decrease with downstream direction (Back 2003).

Two sites along the longitudinal P gradient were selected for the field study of *Caenis* spp. to contrast C : N : P ratios among nymphs of differing development classes and from habitats differing in nutrient content of food resources. We chose NBOS-03 (31.97692° N, 98.03974° W), a 4th-order reach near Hico, TX as a relatively high phosphorus site. We selected NBOS-05 (31.63760° N, 97.36640° W), a 4th-order reach

Table 1. Nutrient concentrations for the North Bosque River sample locations corresponding to the field collection of *Caenis* nymphs.

	Site	
Nutrient (μg/L)	NBOS-03	NBOS-05
TP	104	46.1
PO_4-P	10.9	9.3
TN	2058	610
NO_2-N+NO_3-N	2.1	6.6
NH_3-N	50.2	71.3

near Del Mar Ranch at Valley Mills, TX as a contrasting site of low-to-moderate levels of phosphorus enrichment (Table 1). Both stream reaches exhibited similar physical characteristics, typified by short limestone cobble riffles interspersed with long, shallow bedrock glides and pools with moderate deposits of fine sediment. Historical stream flows (United States Geological Survey, http://tx.usgs.gov/basins.html) at each site were also similar, although surface discharge was undetectable at the time of sampling and streams were reduced to a series of long glides and pools interconnected by subsurface flow.

Field study: stoichiometry of *Caenis* development classes

Caenis spp. nymphs were collected from both study reaches between 28 September and 7 October 2006. *Caenis* spp. nymphs were collected using a 250 μm Hess sampler from shallow gravel substrates at pool margins. Samples (15–25) from each site were sieved through soil sieves (2-mm, 1-mm, 0.5-mm, and 0.25-mm). The retained material in the three smallest sieves was stored on ice for transport to the laboratory. We removed in the field all large *Caenis* spp. nymphs retained in the 2 mm sieve. In the laboratory we sorted nymphs into one of five development classes following Taylor & Kennedy (2006) using a Nikon SMZ 1500 dissecting microscope equipped with a Nikon DXM 1200f digital imaging system. Development classes corresponded to external wing pad morphology and pigmentation. Development classes I through V are determined as: I = no wing-pads present, II = clear wing-pads present in thoracic region, III = wing-pads with veins present in thoracic region, IV = wing-pads with veins present in abdominal region or with veins and mottling present in thoracic region, V = wing pads enlarged, with veins and dark mottling reaching abdominal region (Taylor & Kennedy 2006). These external morphological classes are also related to internal changes associated with adult development. The use of development classes is superior to arbitrarily making groups based upon size alone because equal size among individuals does not necessarily mean they are at an equivalent development stage. Because only mature development class V nymphs can be reliably identified to species we could only contrast development classes at the genus level. We dried all development class samples to a constant mass at 50 °C, pulverized and homogenized with a mini-beadbeater-8 (Biospec Products) and stored the powder in a desiccator until determination of nutrient content was completed.

Periphyton (defined here as a composite mixture of algae, fine particulate organic matter, and sediment) was collected from each site for determination of C : N : P stoichiometry to evaluate whether food resources at the two sites differed in terms of nutrient content. Periphyton was removed from rocks by scrubbing with hard-bristle brushes. The entire slurry was dried at 50 °C for 24 hours and then pulverized and homogenized the dried periphyton with a mini-beadbeater-8 (Biospec Products) and stored the powder in a desiccator until determination of nutrient content was completed.

Surface-water samples were collected from each site for determination of dissolved (0.45 μm filter) and total (unfiltered) N and P. Samples were collected in triplicate for NH_4-N, NO_2-NO_3-N, TN, PO_4-P, and TP and stored and analyzed samples according to standard methods (APHA 1998). All surface-water nutrients were analyzed on a Lachat Quik-Chem 8500 flow-injection autoanalyzer.

We estimated %C and N content of periphyton and *Caenis* spp. nymphs from the field sites using a ThermoQuest Flash EA™ 1112 elemental analyzer. Percent P content was estimated using a Lachat QuikChem 8500 flow-injection autoanalyzer using the molybdate colorimetric method following digestion in 5-mL 32M sulfuric acid at 350 °C on a digestion block for 2-h. Soil (Thermo Finnigan 1.99 %C) and peach leaf (SRM 1547, 0.137 %P, 2.98 %N) standards were used for QA/QC to determine C, N, and P recoveries, which were all quite high (89–107 %) and consistent among replicates (n = 5 per standard).

Because of the small size of *Caenis* nymphs (development classes I–V mean mass per individual were 0.002, 0.01, 0.071, 0.289, and 0.461 mg, respectively) relative to the mass required to achieve detectable concentrations of P in our digests (10–20 mg), we analyzed composite samples of several to hundreds of nymphs from each site (Table 2). In those cases that we had less than the required mass, we decreased the acid volume and dilution volume proportionately (3–9 mg material would be digested in 2.5 ml H_2SO_4 and then diluted to 37.5 mls). We were unable to collect sufficient biomass of development class I nymphs for C : N : P determination.

Polynomial least-squares regression was used to fit continuous relationships between increasing development stages and C : P and N : P ratios of nymph composites. Regression equations were fitted to data for each site and patterns were qualitatively contrasted between sites to assess whether the higher-P site (NBOS-03) tended to have lower C : P and N : P ratios than the lower-P site (NBOS-05).

Laboratory growth experiment

Green algae (*Cladophora* and *Spirogyra* spp.) mats were collected from Neils Creek, a low-nutrient tributary of the North Bosque River, near Valley Mills, TX, to culture for food treatments in the growth experiment. Algal mats were collected on 24 September 2006 and split this material into four equal masses of approximately 20 g wet mass. We placed split fractions of algae in 1 L beakers of filtered site water enriched with Na_2HPO_4 ranging from +0 (no enrichment, background TP 10 μg/L), + 30, + 90 and + 270 μg/L P, respectively. Algal cultures were held in control and enriched stream water at 20°C with continuous light for 48 hours to allow uptake of P into algal tissues. We did not determine the ingestion rates of *Caenis* spp. across its development classes and potentially could have not provided enough food at higher development classes. However, our lowest supply (0.15 mg C/cm^2) was 3-times higher than that used by Frost & Elser (2002) to represent low food quantity for similarly sized larvae (our development class I). Algal samples were

removed from beakers and allowed to air dry at 20 °C for several days. Samples were ground to a fine powder using a mortar and pestle and stored in airtight containers. Nine subsamples were analyzed from each algal enrichment treatment for C, N and P content using laboratory methods previously described in the field study.

We collected *Caenis* spp. nymphs for the growth study from a perennial pool in shallow gravel/sand substrates from the lower-P site (NBOS-05) on the North Bosque River. Twenty-five Hess samples (250 μm mesh) were collected as described in the field study in order to obtain enough nymphs for the experiment. Live nymphs were separated into the five development classes and measured each individual for head capsule width (HCW) using a Nikon SMZ 1500 stereomicroscope equipped with a Nikon DXM 1200f digital imaging system. We initially considered all five development classes, but preliminary results suggested that development class V nymphs were too close to maturation and would likely emerge during the experiment. Thus, we focused only on nymphs of development classes I–IV for the growth experiment.

Nymphs were placed individually into 20 mL glass scintillation vials filled with 15 mL of filtered stream water. Ten nymphs were reared from each of the four development classes, on each of the four algal treatments in incubators set at 20 °C with 12 hour light cycles for ten days. Nymphs were fed in order of increasing development class 0.15, 0.3, 0.6, and 1.2 mg C cm^{-2}, respectively. We replaced filtered stream water and algae every 3–4 days. Post incubation HCW measurements were made for each individual and used these measurements to calculate growth rate. Growth rate was calculated as:

$$\mu = [\ln(B_2) - \ln(B_1)] / time$$

where μ = growth rate, B_1 = estimated initial mass and B_2 = estimated final mass. We estimated initial and final masses (mg) by inserting HCW measurements (μm) into a published HCW-weight regression (dry mass = $23.09548\, e^{HCW + 3.19737}$, $r^2 = 0.97$) developed for *Caenis latipennis* by Taylor & Kennedy (2006).

Two-way ANOVA was performed on growth-rate data using development class (I-III) and food quality (four C : P treatments) as main effects and a development class × food quality interaction term. High mortality of nymphs in class IV treatments reduced our degrees of freedom below an acceptable level for detecting statistical interactions so we only analyzed growth-experiment data from development classes I–III . One-way ANOVA was run *a posteriori* to test the influence of food quality on growth rate within each development class, including development class IV. Tukey's studentized range test was used to assess the relationships of means between groups for ANOVA results. Because not all data was distributed normally, all analyses were conducted on ranked data. Effects were considered significant when $p \leq 0.05$. ANOVA was performed in SAS 9.1 (SAS Institute, Cary, NC, USA).

Results

Field study: stoichiometry of *Caenis* development classes

Surface-water and periphyton had higher N and P levels at NBOS-03 when compared to the downstream site (NBOS-05). This suggests that nutrient availability to periphyton indeed was higher at NBOS-03 and nutrient content of an important food resource to *Caenis* reflected these differences in its elemental composition (Table 2).

C : P ratio of *Caenis* spp. nymphs exhibited a unimodal relationship with increasing development class ($r^2 = 0.993$, $F_{2,1} = 74.7$, $p = 0.081$ and $r^2 = 0.995$, $F_{2,1} = 107.8$, $p = 0.048$ for NBOS-03 and NBOS-05, respectively; Fig. 1a). C : P ratio increased from development class II to III, but decreased slightly (NBOS-03) to markedly (NBOS-05) from class III to V. C : P ratio at NBOS-05 was greater than the more P-enriched NBOS-03 for all development classes except class V (Fig. 1a). We observed a similar hump-shaped N : P relationship across development classes and between sites ($r^2 = 0.971$, $F_{2,1} = 17.32$, $p = 0.168$ and $r^2 = 0.998$, $F_{2,1} = 207.8$, $p = 0.041$ for NBOS-03 and NBOS-05, respectively). This pattern in N : P ratio was largely due to less dramatic shifts in %N content across development classes than those observed for %P (Table 2, Fig. 1b). Neither C : N nor % C showed a consistent trend with increasing development class between sites, increasing from II–V at NBOS-03, but no clear pattern at NBOS-05 (Table 2).

Table 2. Elemental content of periphyton and *Caenis* spp. nymphs across developmental classes at each stream location.

Periphyton or Developmental Class	NBOS-03				NBOS-05			
	n[1]	% C	% N	% P	n	% C	% N	% P
Periphyton	–	10.7	1.12	0.116	–	9.70	0.96	0.085
II	504	48.2	11.7	0.93	609	47.0	11.7	0.71
III	231	48.9	12.0	0.81	352	47.2	10.4	0.59
IV	40	51.4	12.0	0.83	40	50.7	10.8	0.74
V	18	53.1	11.5	0.89	9	48.1	10.6	1.17

[1] number of individual nymphs analyzed in each composite sample

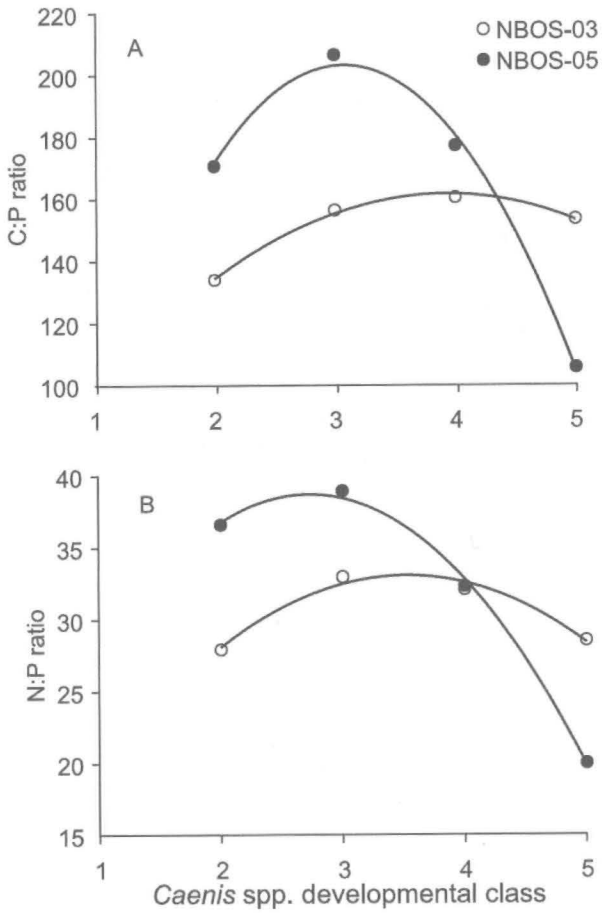

Fig. 1. Regressions of molar C : P **(A)**, and N : P **(B)** ratios across *Caenis* spp. development classes at NBOS-03 and NBOS-05 collection sites.

Table 3. Elemental composition and molar ratios for the control and three P-enriched food treatments used in the growth experiments.

Treatment	%C	%N	%P	C : N	C : P	N : P
+ 0 (Control)	26.4	2.09	0.07	14.7	960	65
+ 30 µg/L	25.6	2.02	0.26	14.8	254	17
+ 90 µg/L	25.5	2.09	0.70	14.3	95	6.6
+ 270 µg/L	25.2	2.06	1.04	14.3	62	4.4

Table 4. One-way ANOVA and Tukey's Studentized Range Test results for the effects of food P content on ranked growth rates for each development class of *Caenis* spp.

Development class	df	*F*	*p*
I	3, 22	8.36	0.0007
II	3, 24	0.74	0.5411
III	3, 21	1.12	0.3650
IV	3, 15	0.18	0.9062

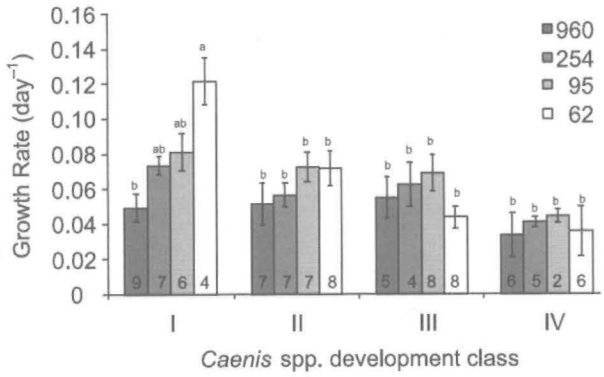

Fig. 2. Mean ± 1 SE growth rates of individually reared *Caenis* spp. nymphs in four different development classes across four different food quality treatments. All food treatments are presented as molar C : P ratios. Sample sizes are indicated within bars. Lower case letters above bars represent significant differences between rank sums of food quality treatments within each development class determined by a Tukey's Stundentized Range Test.

Laboratory growth experiment

Artificially enriched algal samples produced a strong P gradient across the four algal treatments with P increasing substantially while little change was observed in C and N. This resulted in a shift in molar C : P ratios from 960 for the control to 62 for the highest P treatment (Table 3).

Caenis spp. growth rates increased in response to P enrichment in the earliest development class but this growth response diminished in later development classes (Fig 2). Growth rate of *Caenis* spp. was significantly influenced by development class ($F_{2, 67}$ = 3.41, p = 0.0388) and the interaction between development class and P treatment ($F_{6, 67}$ = 2.36, p = 0.0397); however, P treatment ($F_{3, 67}$ = 2.51, p = 0.0661) had no effect on growth rate (two-way ANOVA on ranked data). Tukey's studentized range test (α = 0.05) could not separate the rank sums of the three development classes into statistically different groups. A post hoc analysis on data analyzed separately for development classes I through IV showed that P treatment had a highly significant effect on growth rate in development class I but not development classes II, III and IV (One-way ANOVA on ranked data; Table 4). Only the rank sums of P treatments 62 and 960 were significantly different for development class I (Tukey's studentized range test on ranked data, α = 0.05; Fig. 2).

Discussion

Ontogenetic differences in *Caenis* stoichiometry

The C : P ratios for *Caenis* were all in the lower range of values reported 100–800 (mean 263 ± 113) for mayflies in Evans-White et al. (2005). Although we have no measurement of the amount of variation within each development class, C : P and N : P ratios showed a clear pattern of increasing and then decreasing values across development classes at both sites. Admittedly, having no measure of variation in C : P and N : P ratios decreases the certainty of a unimodal pattern. However Frost & Elser (2002) showed that the smallest mayflies had the highest P content. Extending this trend to Fig. 1 would further support a unimodal pattern. If individual species could be identified for all development classes, their stoichiometry could result in patterns different than those we observed. However, *Caenis* are collector-gathering detritivores and variability in food type and quality among species is probably low. Therefore, the level of elemental imbalance between different *Caenis* species and their food should be similar. This could lead to species with unique elemental content but not necessarily differing patterns in stoichiometry across their ontogeny.

Why would *Caenis* spp. nymphs differ in their C : N : P stoichiometry across development classes? We believe that it is a result of differing N and P demands needed for the development of somatic and reproductive structures across the ontogeny of *Caenis* spp. When eggs hatch and nymphs grow and metamorphosis progresses through development classes, the early development classes first produce somatic tissue, and later (development classes IV and especially V) produce reproductive structures and gametes. Thus the decline in C : P ratios in development class IV and V represents a high P investment in gametes, which is probably most evident in females. This idea is supported by Markow et al. (1999, 2001) who demonstrated that adult *Drosophila melanogaster* and *D. nigrospiracula* females are 3-times more phosphorus rich than males and that eggs and male ejaculate are P rich. Furthermore, males of *D. nigrospiracula*, which feed on P-poor cacti, had a longer time lag in mating after eclosion than those of *D. melanogaster*, which feed on P-rich fruit. This time lag for *D. nigrospiracula* was thought to represent a longer P acquisition time for males because of the low P content of its food (Markow et al. 2001). Follicular development in *D. nigrospiracula* was much slower than that in *D. melanogaster* at eclosion. Markow et al. (2001) suggest that *D. nigrospiracula* may have to allocate a higher proportion of P to somatic growth in the larval stages due to low P food. Thus more egg maturation takes place in the adult stage in this species. Elser et al. (2006) showed that P content of larvae in five *Drosophila* spp. decreased as larvae grew. This is not the pattern we observed for *Caenis* spp. However, mayflies have a major difference in their life history: adults do not feed. Thus, larvae must acquire all materials necessary for adult survival and reproduction. Based on the high P content of adult *Drosophila* reproductive structures (Markow et al. 2001), it makes sense that organisms with non-feeding reproductive stages would necessarily have late development stage larvae that are richer in P than earlier stages because gametes develop and mature in the larvae. The onset of morphological sexual differentiation and development of reproductive structures coincides with decreasing C : P ratios in *Caenis* spp. nymphs in our study. Examinations of mature female mayfly larvae (i.e. darkened wing pads) of *Caenis* spp. and two other mayflies at our sites, *Neochoroterpes nanita* and *Stenonema femoratum*, revealed their entire abdomens were full of eggs and no digestive tract was evident. Frost & Elser (2002) showed a steady decline in %P content in *Ephemerella* sp. mayflies. Although they did not report development classes in their study, mature larvae of *Ephemerella* sp. are 9–15 mm in total length and they complete their life cycle in 9–12 months (Edmunds et al. 1976). Since Frost & Elser (2002) collected newly hatched larvae and their experiment duration was six days, they could not have included late development class nymphs in their experiment. Thus the declining body %P demonstrated in their study supports what we demonstrated in early development class larvae of *Caenis* spp.

Trends in *Caenis* spp. nutrient stoichiometry between sites of differing nutrient status are consistent with those shown for aquatic insects by Cross et al. (2003). They showed insects from enriched sites had higher nutrient content than those from less enriched sites. Mayfly development classes showed higher percent nutrient content at the enriched (NBOS-03) site relative to the less enriched site (NBOS-05). Percent C increased across development classes. This probably represents an increase in exoskeleton and other C rich structures associated with growth (i.e. larger body size). The %N and P at NBOS-03 was equivalent or higher than NBOS-05 in every case except development class V. Again, this probably reflects the nutrient content of the dominant food source which was higher at NBOS-03 and those mayflies had the higher nutrient content.

The decrease in C : P ratio of development class V (Fig. 1a, b) at NBOS-05 when compared to NBOS-03 may be the result of the majority of individuals being female, and contributing a disproportional amount of P to the sample. The latter is supported by Markow et al. (1999, 2001) findings on the high P content of female *Drosophila* spp. Consideration of life history strategies and adult feeding status needs to be included in future studies of ontogenetic changes of elemental composition. Villar-Argaiz & Sterner (2002) demonstrated in a freshwater copepod that P deficiency in late stages of development prevented larval *Diaptomus clavipes* from developing into adults, yet younger stages grew just as well as individuals fed a P replete diet. Thus timing of increased P acquisition is probably important. Even in insects that have feeding adults, the quality of food used by adults needs to be investigated in light of larval food quality. Many holometabolous insects have larvae and adults that do not use the same food resources and each stage may face differing elemental imbalances. Future work is needed to determine the variability of C : N : P ratios within *Caenis* spp. development classes and between sexes to better understand the degree of plasticity in body chemistry among development classes and sexes.

Laboratory growth experiment

A high potential for rapid growth provides many potential advantages to species because growth and development rates can affect many life history traits (e.g. age and size at first reproduction) and ecological features (e.g. predation risk) (Elser et al. 2006). Our findings suggest that increased P content of food increases growth rates of smaller development classes of *Caenis* mayfly nymphs (Fig. 2). The GRH suggests that growth of organisms with higher potential for rapid growth will be more P limited. Our data supports this hypothesis within the context of the life history of a single taxon. As nymphs mature, growth rates and associated P requirements decrease resulting in less influence of food P content on somatic growth rate. Stream insects typically exhibit higher mortality during early stages of development (Benke & Huryn 2007). While many factors such as density affect survivorship, it is plausible that increased growth rates during early stages of development have the potential to decrease mortality and increase overall production of populations when P is not limited.

Our data showed a significant interaction between P content of food and development class on growth rates of *Caenis* spp. This interaction appears to be driven by P content of food having an effect on growth rates in development class I but not development class II through IV (Fig. 2, Table 4). However, in light of our field results it is plausible that later development classes shift P resources from somatic growth to reproductive development. Nymphs within higher P content treatments could have potentially begun developing reproductive structures earlier than nymphs in lower treatments resulting in diminishing growth rates based on HCW measurements earlier than in lower treatments. Thus linear dimensions may not increase even though biomass is accumulated via reproductive development. This supports the hypothesis that not only growth but development may be P limited. Increased development rates have the potential to limit fecundity as it is usually correlated with organism size.

Although P limitation of growth has previously been demonstrated for other mayflies including *Caenis* spp. (Frost & Elser 2002) we provide the first observation of ontogenetic shifts in P limitation of growth related to size and development class within the life cycle of a benthic consumer. Our results agree with studies on terrestrial insects done by Elser et al. (2006) that showed similar shifts in P limitation across the short life cycles of several species of *Drosophila*. However, our experimental design was limited by higher mortality rates than expected in development class IV and the short time period of the experiment. We could not determine whether these shifts were related to size specific growth potential, shifts in P allocation from somatic growth to reproductive development or a combination of the two. Nonetheless, our study demonstrates that P requirements for growth and development can vary across a species life cycle and that P availability has the potential to limit many life history factors within aquatic benthic consumers. Future stoichiometric studies of benthic consumers should consider ontogenetic shifts in P limitation of growth, the mechanisms that control these shifts and the cumulative consequences that varying P limitation across life cycles may have on populations of aquatic organisms.

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Does nutrient enrichment decouple algal–bacterial production in periphyton?

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Abstract. Coupled production between algae and bacteria in stream epilithon was assessed along a nutrient-enrichment gradient in 8 Texas streams with open canopies. Photosynthesis (PS) and bacterial biomass production (BBP) were measured simultaneously using a dual-label radioassay (^{14}C-HCO_3^- uptake and ^3H-L-leucine incorporation into protein) on multiple samples within a stream reach. PS and BBP were measured after light (1200–1500 μmol m^{-2} s^{-1}) and dark incubations. The degree of coupled production between algae and bacteria within a stream was estimated as the covariation (i.e., correlation or covariance) between PS and BBP derived from unshaded replicates in each stream. Streamwater nutrients ranged from 0.18 to 8.1 mg/L total N and 0.009 to 2.0 mg/L total P. Epilithon N and P content (as % dry mass) and C:N:P ratios varied widely among streams and were positively correlated with streamwater nutrient concentrations. Mean BBP measured in light incubations (BBP_L) was greater than mean BBP measured in dark incubations (BBP_D), and the difference between the 2 means ($BBP_L - BBP_D$) was positively correlated with mean PS among streams ($R^2 = 0.53$). Covariance between PS and BBP_L within streams (COV_{PS-BBP}) decreased as epilithon nutrient content increased. COV_{PS-BBP} was positively correlated with both epilithon C:N ($R^2 = 0.78$) and C:P ($R^2 = 0.77$) among streams. These results suggest that algal and bacterial production are decoupled by nutrient enrichment, and that algae might rely more heavily on bacterial-regenerated nutrients than on streamwater nutrients to support production in nutrient-poor streams.

Key words: algal–bacterial interaction, bacterial production, ecological stoichiometry, eutrophication, microbial interactions, nutrient criteria, nutrient ratios, nutrient regeneration, periphyton, photosynthesis, streams, water quality.

The inherent link between autotrophic and heterotrophic microbial production has been a recurring topic in aquatic ecology. In planktonic systems, the covariation between phytoplankton and bacterioplankton abundance and production often is a function of bacterial metabolism of phytoplankton-derived extracellular organic C (EOC) (Cole et al. 1982, Coveney 1982, Coveney and Wetzel 1989). However, bacterioplankton growth also can be limited by P (Toolan et al. 1991), and inorganic P is the preferred form of P for both phytoplankton and bacterioplankton (Cotner and Wetzel 1992). Therefore, 2 competing models of covariation have been adopted for pelagic systems. The 1st model suggests that phytoplankton production varies with P concentration, and bacterioplankton vary in response to phytoplankton. The 2nd model suggests that both phytoplankton and bacterioplankton respond simultaneously to P. However, patterns in P dynamics and phytoplankton and bacterioplankton production observed at multiple scales have not conformed consistently to either model. As a result, some researchers have speculated that the observed covariation depends on interactions involving multiple resources (i.e., P and C) used by, or pressures (i.e., grazing) experienced by, both algae and bacteria (Currie 1990, Coveney and Wetzel 1995).

Most microbial production in low- to mid-order stream ecosystems generally occurs in benthic biofilms (Vannote et al. 1980). As in planktonic systems, bacterial production in stream biofilms (namely epilithon) often is coupled to algal production and has been attributed to bacterial metabolism of algal EOC (Haack and McFeters 1982, Murray et al. 1987). However, streams with significant canopy cover, such

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as forested systems, often have heterotrophic biofilms that rely on allochthonous organic matter rather than algal-derived EOC (Findlay et al. 1993). Nevertheless, bacterial production usually is coupled with algal production in epilithon when light intensities are sufficient to stimulate benthic photosynthesis (Kaplan and Bott 1989, Jones and Lock 1993).

The involvement of nutrients in the interplay between autotrophic and heterotrophic production in stream epilithon has been debated, but it has been studied less than in planktonic systems. Rier and Stevenson (2001) found that algal biomass was the best predictor of bacterial cell densities and surmised that nutrients indirectly influenced bacteria by controlling algal biomass. They also found no quantitative relationship between algal biomass and bacterial density when algal biomass was <5 μg chlorophyll a/cm^2. Low algal biomass occurred primarily in nutrient-poor streams, suggesting a potential competitive interaction between algae and bacteria for nutrients. In a subsequent experimental study, Rier and Stevenson (2002) demonstrated that epilithic bacteria were colimited by organic C and inorganic nutrients, but they also found that competition for nutrients did not decrease algal and bacterial covariation. Carr et al. (2005) also found no evidence of competition between algae and bacteria for nutrients. Rier and Stevenson (2002) and Carr et al. (2005) suggested that the coupling between algae and bacteria in stream biofilms is related to the construction of the polysaccharide matrix, which provides ample buffering to EOC supply (Freeman and Lock 1995) and is an area of intense nutrient retention and recycling (Wetzel 1993, Scott et al. 2007). Rier and Stevenson (2002) went one step farther to suggest that nutrient-poor biofilms might exhibit stronger algal–bacterial coupling than nutrient-rich biofilms.

Based on the results of these studies, we present 2 hypotheses: 1) Algae and bacteria do not compete for nutrients in stream biofilms, but algal and bacterial production are tightly coupled through simultaneous cycling of C, N, and P. Increased nutrient availability decouples algal and bacterial production as algae become less reliant on bacterial remineralization of nutrients and bacteria become less stressed for photosynthetically derived EOC (Fig. 1A). 2) Algal–bacterial coupling decreases with increasing nutrient availability (as in hypothesis 1), but decoupling also occurs in very-low-nutrient environments because of algal–bacterial competition for nutrients (Fig. 1B). To test these hypotheses, we measured algal and bacterial production along a nutrient-enrichment gradient in several streams in Texas. We quantified the covariation between algal and bacterial production within each stream and assessed whether this covariation was correlated across streams with several metrics of nutrient availability to periphyton. Based on evidence from other systems (i.e., King and Richardson 2007), we suspected that epilithon nutrient content would track the gradient of nutrient enrichment in surface water. In particular, we were interested in relating the degree of coupling between epilithic algal and bacterial production to the nutrient content of the epilithon itself.

Methods

Study area

The study was conducted in the Cross Timbers Ecoregion within the Brazos River Basin in central Texas (Fig. 2). Eight wadeable and historically perennial streams were selected as candidates based on surface-water soluble reactive P (SRP) and total P (TP) concentrations as measured by the Texas Commission on Environmental Quality within the previous 5 y (TCEQ 2007). The candidate streams were intended to represent a continuum of nutrient enrichment. Reconnaissance sampling in June 2006 revealed that these streams were distributed along a steep nutrient gradient, spanning <10 μg/L to >1 mg/L TP. In a 3-wk period from July to August 2006, one 300- to 500-m study site was sampled in each stream to assess coupling between algal photosynthesis (PS) and heterotrophic bacterial biomass production (BBP), and C, N, and P content of epilithon. Surface-water total N (TN) and TP concentrations and discharge were measured in each site.

Field sampling

In each site, 5 rocks (~50 cm^2) were collected for simultaneous measurements of PS and BBP under high-light (1400–1700 μmol m^{-2} s^{-1}) conditions, 5 rocks were collected for PS and BBP measurements in dark incubations, and 2 rocks were collected to serve as killed (4% buffered formalin) controls. Rocks were collected from shallow pools (<0.3 m) and from areas of open canopy with high light exposure. Epilithon growing in unshaded environments does not generally experience photoinhibition (Boston and Hill 1991). The rocks in all streams were colonized by thin biofilms. Some small patches of macroalgae occurred sporadically in all streams, and these growth forms were intentionally avoided.

Twenty-five rocks were collected from each site (5 rocks × 5 locations/site) for measurement of epilithon ash-free dry mass (AFDM) and C, N, and P content. Epilithon was removed from rocks in the field. Rocks

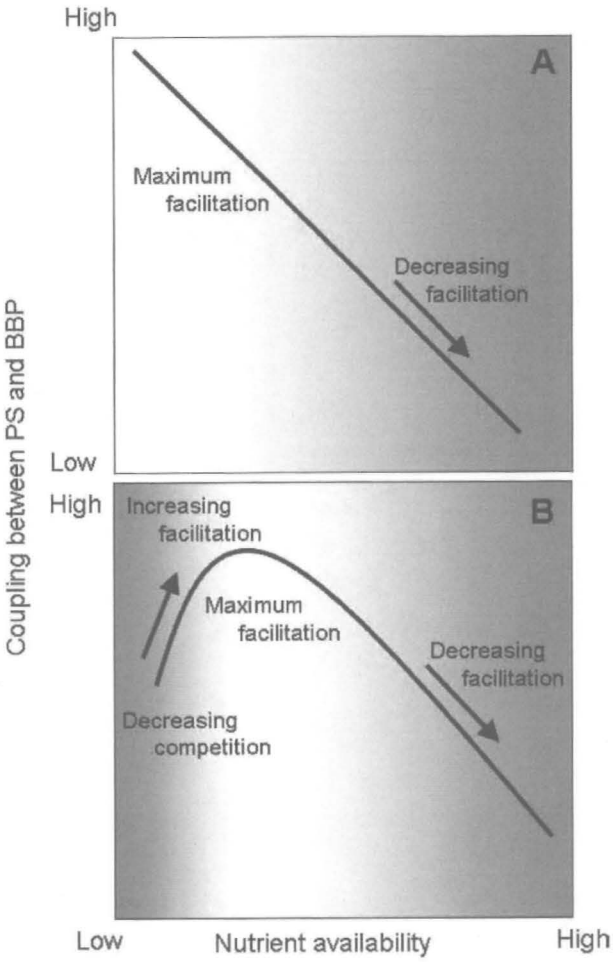

FIG. 1. Two alternative models (hypotheses) of photosynthesis (PS) and bacterial biomass production (BBP) coupling in stream epilithon along a nutrient-enrichment gradient. Areas of no shading within each graph indicate the zone of maximum facilitation (greatest coupling) along a nutrient gradient. A.—Coupling is highest in nutrient-poor streams and diminishes as nutrient availability increases. B.—Algae and bacteria are slightly decoupled in lowest-nutrient streams because of competition for nutrients, coupled under moderate-nutrient availability, and decreasingly coupled as nutrient availability increases.

were scraped with a knife, scrubbed with a toothbrush, and rinsed into a pan with stream water. Epilithon from the 25 rocks was pooled into a single sample, diluted to a known volume in a 1-L dark bottle, and returned to the laboratory. Rock surface area was measured by pressing aluminum foil over each rock and cutting the foil margin to match the size of the scraped area. We weighed the cleaned and dried foil in the laboratory and estimated rock surface area based on a foil mass–area relationship.

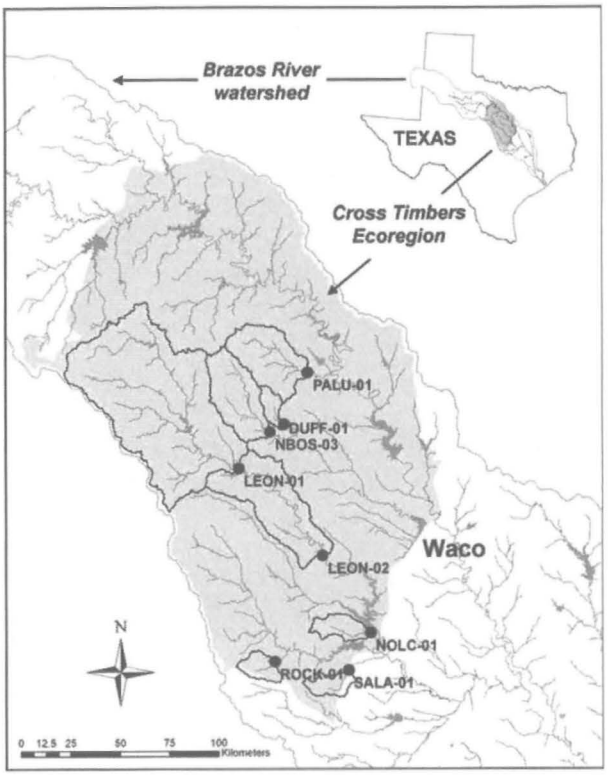

FIG. 2. Study sites in 8 wadeable streams in the Cross Timbers Ecoregion within the Brazos River watershed in central Texas. Watershed boundaries for each stream sampled are indicated with dark outline.

PS and BBP measurements

PS and BBP were estimated simultaneously on individual rocks using $^{14}C\text{-}HCO_3^-$ uptake and ^3H-L-leucine incorporation into protein, respectively. Rocks were put top-down into 60-mL wide-mouth jars. Jars were filled with filtered (0.2-μm pore size) stream water until no air space remained, and they were sealed with open-top caps lined with silicon septa. Rocks were inverted so that the jars could be placed upside down in streams for incubation. Five jars were left unwrapped for light incubation, and the other 5 were wrapped in aluminum foil for dark incubation. Killed controls were incubated in light.

Each jar was injected with 50 μL of ^{14}C-labeled $NaHCO_3$ solution (54 μCi/mL) and placed in the stream at a depth of 7 to 10 cm for 1.5 h. After that time, 50 μL of ^3H-labeled L-leucine were injected into each jar, and the incubation was continued for 30 min. Concentrations of leucine ranged from 500 to 580 nmol/L (specific activity = 0.166 μCi/nmol leucine, assuming negligible background leucine concentration), depending on the volume of rock being

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incubated. Leucine concentrations >400 to 500 nmol/L were necessary to saturate leucine incorporation into protein in similar microbial communities (Thomaz and Wetzel 1995, Gillies et al. 2006, JTS, unpublished data). Incubations were stopped by adding buffered formalin to a final concentration of 4%. Samples were returned to the laboratory and stored at 4°C until processing.

Proteins in intact epilithon were precipitated with trichloroacetic acid (TCA). A previous study on metaphyton showed that TCA protein precipitation was necessary to maximize ^{3}H-L-leucine recovery and had little effect on ^{14}C retention by photoautotrophs (Scott and Doyle 2006). Incubation water was removed from each jar and replaced with 5% TCA. Jars were placed on ice for 1 h, after which samples were removed from TCA. Epilithon was scraped from rocks with a toothbrush, rinsed into a slurry with 5% TCA, and diluted to a known volume (30–50 mL) with 5% TCA. Samples were homogenized with a vortex mixer. A subsample was filtered onto a precombusted and weighed glass-fiber filter (GF/F; pore size = 0.7 μm) for determination of AFDM. A 2nd subsample was filtered onto a 0.2-μm-pore-size polycarbonate filter and washed twice with 5% TCA, once with 80% ethanol, and once with deionized (DI) water. These filters were placed in scintillation vials with 5 mL of alkaline solution (0.5 mol/L NaOH, 25 mmol/L ethylenediamine-tetra-acetic acid [EDTA], and 0.1% sodium dodecylsulfate) and agitated for 1 h at 85°C. Material attached to polycarbonate filters dissolved in the alkaline solution (Buesing and Gessner 2003). A 2.5-mL aliquot of this solution was radioassayed for ^{14}C and ^{3}H activity on a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Fullerton, California). Activities of both isotopes were corrected for quench with external standards and then converted from radioactivity estimates to HCO_3^- and leucine uptake rates based on the specific activity of each isotope used in incubations.

The specific activity of inorganic C in each sample was determined by combining the quantity of ^{14}C-labeled HCO_3^- with the quantity of unlabeled dissolved inorganic C (DIC) in incubation. DIC concentration in filtered incubation water was measured with a Shimadzu TOC-Vcsh C analyzer (Shimadzu Scientific Instruments, Columbia, Maryland). DIC concentration was multiplied by the volume of water in incubation jars to estimate the total quantity of inorganic C available. The volume of each individual rock was measured via liquid displacement and subtracted from 60 mL to arrive at the final water volume in incubation. The measured ^{14}C radioactivity of samples was converted into PS using the specific activity of inorganic C for all incubations. PS was normalized to the surface area of the rock from which the epilithon originated (see *Field sampling* section) or to measured AFDM and expressed as μg C cm^{-2} h^{-1} and μg C mg^{-1} AFDM h^{-1}, respectively (Wetzel and Likens 2000).

Background leucine concentration in incubation water was assumed to be negligible. Therefore, the specific activity of leucine was constant, and measured ^{3}H radioactivity was converted directly to leucine uptake. BBP was calculated from leucine uptake (nmol leucine/h) by assuming that the fraction of leucine in protein = 7.3%, cellular C/protein = 86%, and isotope dilution was negligible (Kirchman 2001). BBP was normalized to the surface area of the rock from which the epilithon originated (see *Field sampling* section) or to measured AFDM and expressed as μg C cm^{-2} h^{-1} or μg C mg^{-1} AFDM h^{-1}, respectively.

AFDM and nutrients

Epilithon from samples used in PS and BBP incubations was processed for AFDM only. Epilithon from the 25-rock composite sample collected from each stream was processed for determination of AFDM and C, N, and P content. The epilithon–streamwater slurry was homogenized using a blender and diluted to a known volume with DI water, and 3 subsamples were removed. While the epilithon slurry was mixed vigorously, a 2- to 5-mL subsample was transferred onto a precombusted (500°C, 4 h) and weighed GF/F for AFDM measurement. A 2nd 100- to 160-mL subsample was transferred into a large weighing boat. The water was allowed to evaporate, and the sample was dried at 60°C. The bulk sample was then homogenized into a powder for C, N, and P analysis. A 3rd subsample was transferred into a 50-mL centrifuge tube. Epilithon in these tubes was separated from inorganic material by centrifugation in colloidal Si using the method of Hamilton et al. (2005). Following separation and several rinsing steps to remove Si, organic matter (OM) samples were dried and homogenized for C, N, and P determination. C and N content in bulk epilithon and epilithon OM were measured simultaneously using a Thermo Finnigan FlashEA 1112 elemental analyzer (Thermo Fisher Scientific, Waltham, Massachusetts). P content was measured colorimetrically on a Lachat Quickchem 8500 (Hach, Loveland, Colorado) following a 3-h digestion in concentrated H_2SO_4 at 350°C (APHA 1998). Total N and P content in water samples collected from each stream was measured colorimetrically on a Lachat Quickchem 8500 following either alkaline persulfate (N) or acid persulfate (P) digestions (APHA 1998).

TABLE 1. Water chemistry and flow at sampling locations. DIC = dissolved inorganic C.

Stream	Site code	DIC (mg/L)	Total N (μg/L)	Total P (μg/L)	Discharge(m^3/s)
Paluxy River	PALU-01	26.6	215.0	9.89	0.001
Rocky Creek	ROCK-01	19.9	176.3	10.0	0.009
Salado Creek	SALA-01	17.2	296.0	12.3	0.005
Duffau Creek	DUFF-01	16.7	507.3	24.4	0.0
North Bosque River	NBOS-03	8.92	1686	45.6	0.0
Leon River upstream	LEON-01	22.2	1197	62.7	0.0
Nolan Creek	NOLC-01	23.4	6925	1860	0.556
Leon River downstream	LEON-02	25.9	8110	2030	0.046

Data analyses

The amount of BBP stimulated by PS in each stream was calculated by subtracting the mean BBP rate measured in dark incubations (BBP_D) from the mean BBP rate measured in light incubations (BBP_L) (mean BBP_L − mean BBP_D). The respective errors were propagated additively. Model II major-axis regression was used to assess the relationship between (mean BBP_L − mean BBP_D) and mean PS because our predictor (mean PS) and response (mean BBP_L − mean BBP_D) variables were random estimates with error (Legendre and Legendre 1998). Statistical tests on slope and y-intercept were conducted using 999 random permutations of our data. A FORTRAN program available online (Casgrain and Legendre 2007) was used for this analysis because model II regression is not available in most standard statistical software packages. The relationship between PS and BBP_L across all streams was assessed using ordinary least squares (OLS) regression (Proc GLM, SAS version 9.1; SAS Institute, Cary, North Carolina). The covariance value between PS and BBP_L within each stream reach was calculated as

$$COV_{PS-BBP} = \sum_{i=1}^{n} \frac{(PS_i - PS)(BBP_{Li} - BBP_L)}{n},$$

where i is the incubation replicate. The correlation coefficient ($CORR_{PS-BBP}$) was the covariance divided by the product of individual standard deviations (SD) of PS and BBP_L. OLS regression was used to determine how strongly each metric of PS–BBP_L coupling (i.e., correlation and covariance) was related to streamwater nutrient concentrations and epilithon (bulk and OM) nutrient content. Negative relationships were expected between PS–BBP_L coupling metrics and streamwater and epilithon nutrient concentrations. Positive relationships, indicative of decreasing covariance between PS and BBP_L with increasing nutrient content in epilithon, were expected between PS–BBP_L coupling and epilithon C:N or C:P.

Results

Habitat and epilithon elemental composition

Streamwater nutrient concentrations confirmed that a strong nutrient-enrichment gradient existed among sites. TP levels ranged from <10 μg/L to >2 mg/L, and TN levels ranged from <200 μg/L to >8 mg/L among sites (Table 1). DIC concentrations varied little among sites, with the exception of NBOS-03, where DIC was 2× to 3× lower than in other sites. Three sites, DUFF-01, LEON-01, and NBOS-03, had substantial standing water but no detectable surface flow during sampling events. Surface flow was only slightly above detection levels at PALU-01, ROCK-01, and SALA-01, but it was relatively high in sites below wastewater discharges (LEON-02 and NOLC-01) (Table 1).

Epilithon elemental composition also confirmed the existence of a nutrient-enrichment gradient. Epilithon bulk P content ranged from 0.028% at ROCK-01 to 0.224% at NOLC-01, and bulk N content ranged from 0.28% at PALU-01 to 1.4% at NOLC-01 (Table 2). Epilithon OM P content ranged from 0.030% at ROCK-01 to 0.235% at NOLC-01, and OM N content ranged from 0.76% at ROCK-01 to 1.98% at LEON-02. Both bulk and OM C content were highly variable among sites but did not appear to be related to the nutrient-enrichment gradient (Table 2). Epilithon OM nutrient content was more similar among streams than were streamwater nutrient concentrations. For instance, sites with very similar streamwater P (PALU-01, ROCK-01, and SALA-01; Table 1) had decidedly different OM P contents (Table 2). Epilithon AFDM was variable among sites, but this variation was not explicitly related to differences in nutrient levels among sites (Table 2).

Epilithon PS and BBP_L

PS and BBP_L were highly variable among sites (Table 3). BBP_L rates were always greater than BBP_D rates, and mean BBP_L − mean BBP_D was positively correlated with mean PS across all sites (Fig. 3).

TABLE 2. C, N, and P content (% of dry mass) measured from bulk epilithon and epilithon organic matter (OM) separated by centrifugation. Values represent a single composite sample from 25 rocks sampled in each stream reach. Mean (±1 SD) ash-free dry mass (AFDM) is of epilithon from 12 individual rocks used in ^{14}C and ^{3}H assays (5 replicate light incubation, 5 replicate dark incubation, 2 killed controls). IS = insufficient amount of sample available for analysis.

Site code	% bulk C	% bulk N	% bulk P	% OM C	% OM N	% OM P	AFDM (mg/cm^2)
PALU-01	8.61	0.28	0.036	IS	IS	0.080	0.99 ± 0.32
ROCK-01	13.57	0.55	0.028	14.99	0.76	0.030	0.67 ± 0.19
SALA-01	18.53	0.75	0.030	18.11	0.98	0.050	1.39 ± 0.27
DUFF-01	7.83	0.45	0.050	12.38	0.82	0.073	1.39 ± 0.40
NBOS-03	12.42	0.77	0.117	18.19	1.45	0.142	1.87 ± 0.65
LEON-01	8.42	0.62	0.068	12.71	1.10	0.090	1.35 ± 0.59
NOLC-01	16.14	1.38	0.224	19.19	1.68	0.235	0.85 ± 0.21
LEON-02	11.25	0.86	0.100	33.00	1.98	0.189	1.21 ± 0.33

Epilithon PS and BBP_L values in individual replicates were positively correlated across streams sites regardless of whether surface area (Fig. 4A) or AFDM (Fig. 4B) was used to normalize PS and BBP_L. However, the relatively large amount of epilithon biomass at NBOS-03 (Table 2) exerted disproportional influence on areal production rates. When this site was excluded from the regression analysis of area-normalized rates, the slope of the linear fit decreased from 0.4 to 0.16, and the amount of BBP_L variation explained by PS increased by 23% (Fig. 4A). In a similar analysis using the AFDM-normalized rates, slope did not change, but the amount of explained variation increased by 20% (Fig. 4B).

Replicate data within sites indicated that PS and BBP_L were positively correlated in all streams, with the exception of area-normalized rates at DUFF-01 (Table 3). Mean correlation coefficients for PS and BBP_L among sites were similar for area-normalized ($r = 0.78 \pm 0.42$ SD) and AFDM-normalized ($r = 0.79 \pm 0.26$ SD) rates. PS–BBP_L covariance was much more variable, and covariance of area- and AFDM-normalized rates ranged across 4 orders of magnitude (Table 3). Average area-normalized PS–BBP_L covariance was $1.6 \times 10^{-3} \pm 1.8 \times 10^{-3}$, whereas average AFDM-normalized PS–BBP_L covariance was $2.3 \times 10^{-3} \pm 4.7 \times 10^{-3}$.

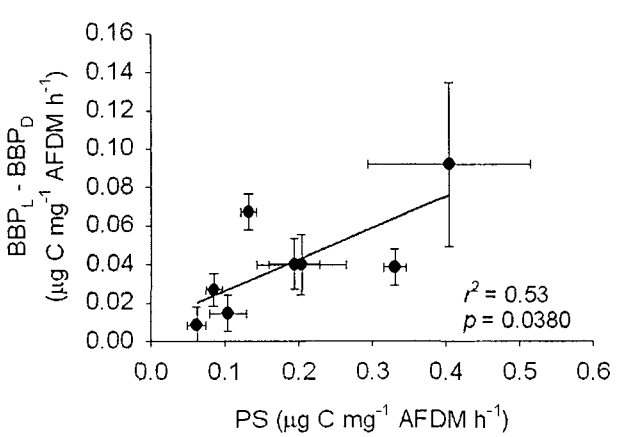

FIG. 3. Major-axis regression of mean (±1 SE) difference between bacterial biomass production in light (BBP_L) and in dark (BBP_D) incubations ($BBP_L - BBP_D$) versus photosynthesis (PS) of epilithon at sites in 8 Texas streams along a nutrient-enrichment gradient. Data were normalized relative to AFDM of the epilithon; $n = 5$ in each 8 streams.

PS and BBP_L covariation along nutrient-enrichment gradient

Correlation coefficients ($CORR_{PS-BBP}$) and covariance values (COV_{PS-BBP}) for area-normalized PS and BBP_L rates were not correlated with any measure of nutrient concentration. $CORR_{PS-BBP}$ for AFDM-normalized PS and BBP_L rates also were not correlated with any measure of nutrient concentration. However, COV_{PS-BBP} values for AFDM-normalized PS and BBP_L ($COV_{PS-BBP,AFDM}$) were correlated with multiple metrics of nutrient concentration across sites.

$COV_{PS-BBP,AFDM}$ was not significantly related to streamwater TN (Fig. 5A) or TP (Fig. 5B), epilithon bulk % N (Fig. 5C) or bulk % P (Fig. 5D), or to epilithon bulk C:N (Fig. 5E). In 2 streams, study sites were directly downstream of wastewater inputs. Data from these 2 sites were particularly influential in several of these regressions (white circles; Fig. 5A–F). Exclusion of these streams from the regression analyses improved the relationship between $COV_{PS-BBP,AFDM}$ and streamwater TN and TP (Fig. 5A, B), and epilithon bulk % P and bulk C:P (Fig. 5D, E, respectively), but only the relationship between $COV_{PS-BBP,AFDM}$ and bulk % P was statistically significant after excluding these streams (Fig. 5D). The relationship between $COV_{PS-BBP,AFDM}$ and bulk C:P was statistically significant regardless of whether wastewater-influenced streams were includ-

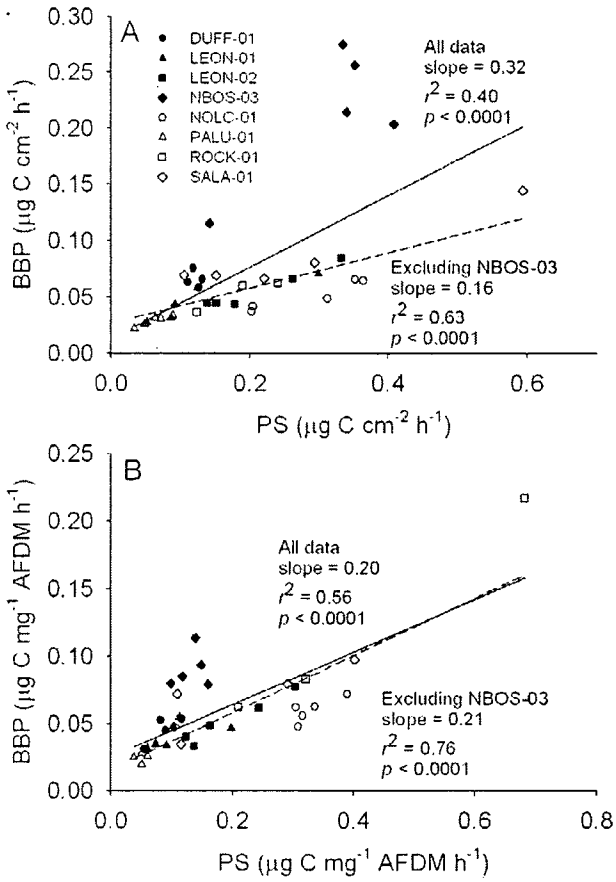

FIG. 4. Linear regression of bacterial biomass production in the light (BBP_L) versus photosynthesis (PS) normalized relative to surface area (A) and ash-free dry mass (AFDM) (B) of epilithon on 5 individual rocks at sites in 8 Texas streams along a nutrient-enrichment gradient. Solid lines are regressions based on data from all streams. Dashed lines are regressions based on data that excluded site NBOS-03. Site codes are given in Table 1.

ed, but the relationship was much stronger when wastewater-influenced streams were excluded (Fig. 5F).

$COV_{PS-BBP,AFDM}$ was not significantly related to OM % N regardless of whether the wastewater-influenced streams were included (Fig. 6A). $COV_{PS-BBP,AFDM}$ was not significantly related to OM % P when all sites were included, but it was significantly negatively related to OM % P when wastewater-impacted streams were excluded (Fig. 6B). $COV_{PS-BBP,AFDM}$ was significantly positively related to OM C:N and C:P (Fig. 6C, D). Exclusion of wastewater-influenced sites had little effect on the relationship between $COV_{PS-BBP,AFDM}$ and OM C:N (Fig. 6C) but strengthened the relationship between $COV_{PS-BBP,AFDM}$ and OM C:P (Fig. 6D).

Discussion

The objective of our study was to assess how nutrient availability was related to coupling between algal and bacterial production in stream epilithon. We hypothesized that: 1) nutrient enrichment would decrease the covariation between algal and bacterial production, and 2) covariation between algal and bacterial production would diminish in nutrient-poor epilithon because of competition for nutrients. Our results support hypothesis 1 but not hypothesis 2. $COV_{PS-BBP,AFDM}$ decreased with increasing nutrient concentration, and the relationship was particularly strong when comparing $COV_{PS-BBP,AFDM}$ with epilithon OM C:N and C:P (Fig. 6C, D). This result suggests that algae (and perhaps both algae and bacteria) rely on regenerated nutrients to support production in nutrient-poor streams, but they rely less on regenerated nutrients for production as nutrient availability increases. This paradigm has been well established in oceanic plankton communities (sensu Dugdale and Goering 1967), but it has not been well

TABLE 3. Mean (±1 SD) photosynthesis (PS) and bacterial biomass production in the light (BBP_L) for all streams and multiple metrics for estimating the relationship between PS and BBP_L at each site. $CORR_{PS-BBP}$ is the correlation coefficient between PS and BBP_L in light incubations in each stream. COV_{PS-BBP} is the covariance between these variables in each stream. Metrics were calculated for rates normalized to surface area of rocks and epilithon ash-free dry mass (AFDM).

Site code	PS (µg C cm^{-2} h^{-1})	BBP_L (µg C cm^{-2} h^{-1})	$CORR_{PS-BBP,area}$	$COV_{PS-BBP,area}$	$CORR_{PS-BBP,AFDM}$	$COV_{PS-BBP,AFDM}$
PALU-01	0.061 ± 0.021	0.029 ± 0.005	0.96	7.45×10^{-5}	0.96	3.03×10^{-4}
ROCK-01	0.184 ± 0.059	0.053 ± 0.014	1.0	5.24×10^{-4}	1.0	1.37×10^{-2}
SALA-01	0.272 ± 0.193	0.086 ± 0.033	0.97	4.92×10^{-3}	0.88	2.30×10^{-3}
DUFF-01	0.121 ± 0.009	0.066 ± 0.007	–0.24	-7.52×10^{-6}	0.86	1.64×10^{-4}
NBOS-03	0.315 ± 0.101	0.213 ± 0.062	0.76	3.77×10^{-3}	0.21	6.70×10^{-5}
LEON-01	0.130 ± 0.010	0.046 ± 0.018	0.86	1.25×10^{-3}	0.64	2.44×10^{-4}
NOLC-01	0.287 ± 0.079	0.052 ± 0.013	0.92	7.81×10^{-4}	0.79	2.02×10^{-4}
LEON-02	0.212 ± 0.082	0.057 ± 0.018	0.99	1.19×10^{-3}	0.99	1.06×10^{-3}

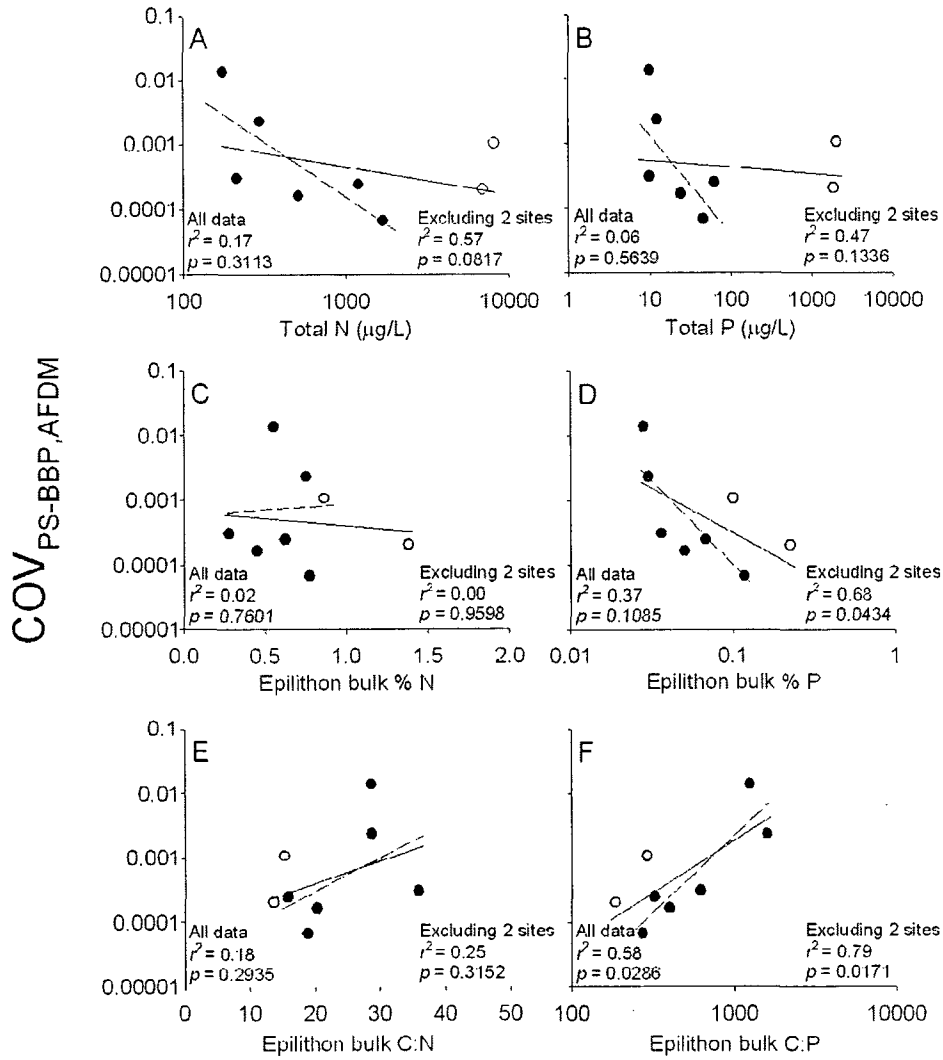

FIG. 5. Linear regression of the covariance of photosynthesis (PS) and bacterial biomass production in the light (BBP_L) normalized to epilithon ash-free dry mass (AFDM) ($COV_{PS-BBP,AFDM}$) vs streamwater total N (TN) (A) and total P (TP) (B), epilithon bulk % N (C) and % P (D) content, and epilithon bulk C:N (E) and C:P (F) in 8 Texas streams along a nutrient-enrichment gradient. Solid lines are regressions based on data from all streams. Dashed lines are regressions based on data excluding 2 streams from which sites were directly below wastewater discharges (open circles; NOLC-01 and LEON-02). Site codes are given in Table 1; $n = 5$ rocks/stream for $COV_{PS-BBP,AFDM}$.

demonstrated in attached microbial communities, such as epilithon.

We found no evidence that competitive interactions decoupled algal and bacterial production in nutrient-poor streams. In fact, $COV_{PS-BBP,AFDM}$ was greatest in the most nutrient-poor streams, and this result is similar to a model proposed for planktonic microbial communities (Cotner and Biddanda 2002). The lack of competitive decoupling between epiphytic algae and bacteria also is consistent with results of other studies on epilithic stream biofilms (Rier and Stevenson 2002, Carr et al. 2005), which provide support for the existence of mutually beneficial interactions between microbial autotrophs and heterotrophs. Efficient recycling of C, N, and P within biofilm matrices has long been suspected as an important driver of community function in biofilms (Wetzel 1993). A recent stable-isotope study of metaphyton N cycling provides support for this idea (Scott et al. 2007). Larned et al. (2004) demonstrated that mass transfer of nutrients into epilithon is the most likely rate-limiting step in nutrient transfer from stream water into epilithic algal cells. Therefore, nutrient regeneration in biofilms might be the most significant process affecting nutrient

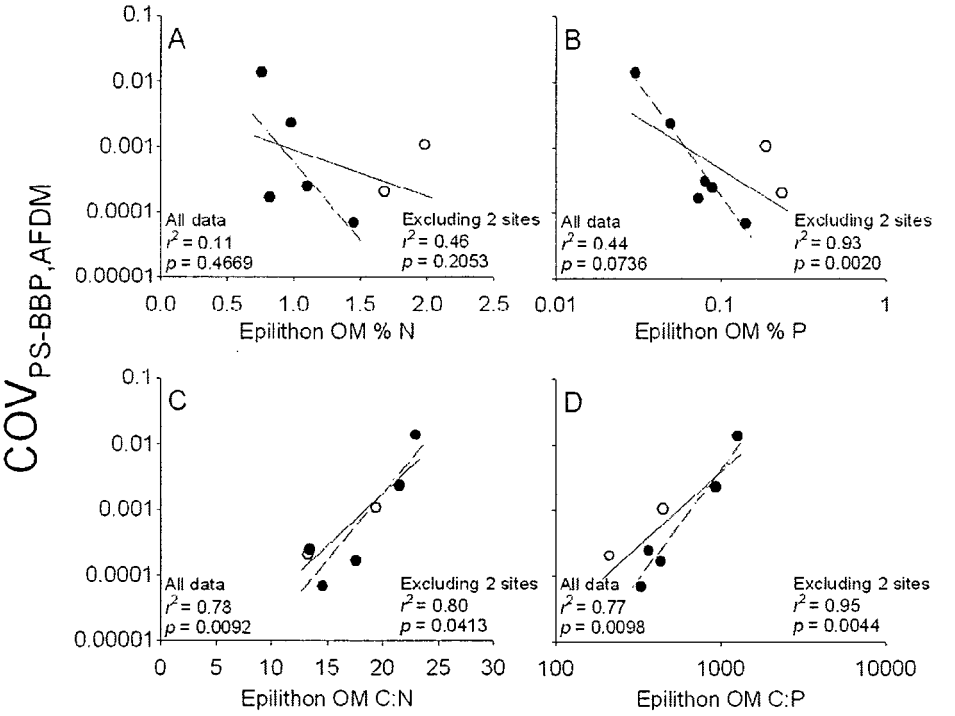

FIG. 6. Linear regression of the covariance of photosynthesis (PS) and bacterial biomass production in the light (BBP_L) normalized to epilithon ash-free dry mass (AFDM) ($COV_{PS-BBP,AFDM}$) vs epilithon organic matter (OM) % N content (A), % P content (B), C:N (C), and C:P (D) in 8 Texas streams along a nutrient-enrichment gradient. Solid lines are regressions based on data from all streams. Dashed lines are regressions based on data excluding 2 streams from which sites were directly below wastewater discharges (open circles; NOLC-01 and LEON-02). Site codes are given in Table 1; $n = 5$ rocks/stream for $COV_{PS-BBP,AFDM}$.

availability to cells growing within its polysaccharide matrix.

Influences of streamwater nutrients vs epilithon nutrients on algal–bacterial coupling

$COV_{PS-BBP,AFDM}$ was more strongly related to epilithon OM nutrient content than to epilithon bulk nutrient content or streamwater nutrient concentrations. Previous studies exploring algal–bacterial covariation along nutrient gradients have used primarily streamwater nutrient concentrations as metrics of nutrient availability (Carr et al. 2005). However, epilithon nutrient content, including extracellular nutrients within the biofilm matrix, integrate short-term temporal changes in streamwater nutrient availability. Therefore, epilithon nutrient content might be a more representative indicator of average stream conditions than a single water sample. In our study, epilithon nutrient content, and OM nutrient content in particular, differed more among streams than did streamwater nutrient concentrations. This contrast was particularly evident in streams with low streamwater nutrient concentrations. Thus, our study indicates that epilithon nutrient content is a better indicator of nutrient availability to biofilm microorganisms than are streamwater nutrient concentrations.

The strongest correlations with $COV_{PS-BBP,AFDM}$ were revealed when epilithon OM was explicitly isolated and elemental ratios of this organic fraction (e.g., C:N and C:P) were used. Epilithon from wastewater-impacted streams had the highest % N and % P, but also the highest % C. Therefore, C:N and C:P values in epilithon of wastewater-impacted streams were higher than in other streams, even though wastewater-impacted streams had the most nutrient-rich epilithon (as a percentage of dry mass). This result suggests that epilithon C content in wastewater-impacted streams might be influenced by the dissolved organic C available in effluent. For example, $COV_{PS-BBP,AFDM}$ was higher than expected for wastewater-impacted streams when evaluated using streamwater nutrient (Fig. 5A, B), bulk epilithon nutrient (Fig. 5C, D), or epilithon OM nutrient (Fig. 6A, B) models. However, elemental ratios accurately predicted $COV_{PS-BBP,AFDM}$ across all streams, particularly when derived from epilithon OM (Fig. 6C, D). Thus, a stoichiometrically explicit approach (sensu

Sterner and Elser 2002) might be particularly useful for understanding trophic dynamics in stream microbial communities (Frost et al. 2002, 2005).

Nutrient regeneration as a basis for trophic coupling

Based on evidence gathered in this and other studies (Cotner and Biddanda 2002, Rier and Stevenson 2001, 2002, Carr et al. 2005), we suggest that nutrient deficiency strengthens trophic coupling between algae and bacteria in the epilithon of oligotrophic streams through the interdependence of C, N, and P cycles within the biofilm matrix. For example, bacterial metabolism of algae-derived EOC is supported by studies that show increased bacterial extracellular enzyme activity associated with algal photosynthesis (Espeland et al. 2001, Francoeur and Wetzel 2003). Furthermore, the decomposition of P-containing compounds at night, or during periods of low photosynthetic activity, is supported by observations of concomitant increases in bacterial phosphatase activity (Espeland and Wetzel 2001). Rier et al. (2007) found that bulk phosphatase (derived from algae, bacteria, and fungi) activity remained high at night in stream periphyton growing on inert substrata and on leaf detritus in a high-light environment. Therefore, sustained bulk phosphatase (Rier et al. 2007) or increased bacterial phosphatase (Espeland and Wetzel 2001) activity coupled with decreased algal uptake of P at night (Reshkin and Knauer 1979) would result in substantially higher bacterial P uptake or P accumulation in the biofilm matrix. P accumulation at night could fuel P-limited photosynthesis and bacterial EOC decomposition in the subsequent daylight hours. Other studies have suggested that bacteria specifically control P recycling within biofilm communities. Sharma et al. (2005) found that phosphatase activity was spatially segregated from chlorophyll-containing microorganisms in metaphyton from the strongly P-limited Florida Everglades. They hypothesized that bacteria provided the primary mechanism for P remineralization, which directly increased algal photosynthesis and subsequently EOC production within metaphyton.

Recent studies have indicated that dissolved biochemicals such as deoxyribonucleic acid (DNA) can be an important source of P in marine sediments (Dell'Anno and Danavaro 2005) and that bacteria might use extracellular DNA specifically as a nutritional supplement (Palchevskiy and Finkel 2006). Furthermore, decomposition of biochemical aggregates, which are likely to include P-rich lipids, might provide another important source of P recycling in biofilms (Wotton 2007).

The same argument might be presented for N cycling. However, fewer studies on N recycling in periphyton exist, and results of those studies are inconsistent. For instance, Francoeur and Wetzel (2003) found that experimental periphyton communities exhibited higher leucine-aminopeptidase (LAMP) activity when grown in dark conditions with low dissolved inorganic N (DIN) availability. However, the addition of glucose resulted in higher LAMP activity in DIN-deficient periphyton grown under lighted conditions. Furthermore, LAMP activity in natural periphyton communities was affected inconsistently by light, glucose, and DIN additions (Francoeur and Wetzel 2003). However, Scott and Doyle (2006) demonstrated that bacterial production and algal production were strongly coupled in nutrient-deficient metaphyton, but they became decoupled following N enrichment.

Interactions between microbial autotrophs and heterotrophs no doubt exist, and growing evidence from photosynthesis, microbial enzyme linkages (Romaní and Sabater 1999, Espeland et al. 2001, Francoeur and Wetzel 2003, Francoeur et al. 2006, Rier et al. 2007), and coupled algal–bacterial production studies (Scott and Doyle 2006, our study) suggest that mutually beneficial interactions might be common. In mutualistic interactions, bacteria facilitate increased algal production via nutrient regeneration, and algae in turn, facilitate increased bacterial production via increased EOC generation. Mutualism in this form might increase production efficiencies, support the relatively high and sustained production observed in nutrient-poor systems (Wetzel 1993), and exert substantial influence on the energy budgets of oligotrophic ecosystems (Vadeboncoeur et al. 2002).

Spatial heterogeneity within streams and sources of variation among streams

Our results support the idea that epilithon functions as microbial landscapes (Battin et al. 2007). Both PS and BBP_L were highly variable within and among sites. In our study, the standard deviation of epilithic biomass on rocks within the same stream (determined from samples used in radioassays) was usually 30% to 50% of mean biomass value (Table 2). Furthermore, biomass substantially influenced both PS and BBP_L on individual rocks (Fig. 4A). However, AFDM-normalized PS and BBP_L still showed considerable variation within sites (possible sources of variation discussed next). We exploited this spatial variation to better understand the coupling between PS and BBP along the nutrient gradient. However, many studies overlook microscale variability and struggle to control the

substantial noise generated by spatial heterogeneity in ecological data. We echo the sentiments of Battin et al. (2007) and urge benthic microbial ecologists to exploit microscale heterogeneity in biofilm communities, which is undoubtedly important to ecological processes.

Our study was designed to identify only correlative relationships across a stream nutrient-enrichment gradient. Therefore, we could not control other factors that might have contributed to differences in epilithon metabolic activity and coupling between algal and bacterial production. We attempted to minimize differences in stream habitat conditions, but some variables exhibited substantial variation among streams. For instance, our intention was to sample open-canopy stream habitats with similar flow conditions, but an extended drought in Texas during the summer of 2006 resulted in diminished stream flow in most of our study streams and no measurable flow in some (Table 1). The 2 streams with greatest current velocity were those in which wastewater effluent provided most of the flow. Current velocity and discharge were not correlated directly with any measurements of algal and bacterial metabolism or epilithon nutrient content in our study. However, indirect effects of reduced flow, which might have contributed to measured variations between streams, such as boundary-layer conditions, periphyton thickness, and periphyton community composition (Stevenson 1996), were not explicitly examined.

The intentional focus of our study was on stream epilithon growing in open-canopy, high-light conditions. Similar patterns seem unlikely for periphyton growing in shaded habitats. Findlay et al. (1993) found that algae and bacteria were not strongly coupled in heterotrophic biofilms of forested streams. Nutrient cycling might be governed by similar mechanisms in shaded and unshaded periphyton communities, but the links between autotrophs and heterotrophs probably are damped by the reliance of heterotrophs on allochthonous organic matter.

Concluding remarks

Evidence from our study suggests that the degree of coupling between autotrophs and heterotrophs in periphyton decreases along a nutrient-enrichment gradient (Fig. 1A). However, decoupling does not appear to occur as a result of competition for nutrients in nutrient-poor streams (Fig. 1B). Because our study was correlative by design, these results should be confirmed with reach-scale or stream-mesocosm manipulative experiments. Our results support the idea of mutual facilitation between photoautotrophs and heterotrophic bacteria in aquatic microbial communities. Microbial mutualisms of this sort might support high sustained production in attached communities and heavily influence the energetic budgets of oligotrophic ecosystems. Nutrient enrichment of surface waters and accelerated eutrophication of aquatic ecosystems undoubtedly alter ecosystem functions. Our study provides another example of the effect of anthropogenic nutrient enrichment on aquatic ecosystem function by demonstrating that stream-nutrient enrichment can decouple algal and bacterial production in stream epilithon.

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Nitrogen fixation and phosphatase activity in periphyton growing on nutrient diffusing substrata: evidence for differential nutrient limitation in stream periphyton

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Abstract. We explored N_2 fixation and alkaline phosphatase activity (APA) in periphyton from a N-limited stream ecosystem by coupling measurements of these processes with nutrient diffusion substrata (NDS) experiments. We measured periphyton biomass accumulation (as ash-free dry mass [AFDM] and chlorophyll *a* [CHLA]), N_2 fixation, and APA to evaluate the relative importance of N_2 fixation as an N source to the periphyton community and APA as an indicator of P deficiency in a seemingly N-limited system. We used fritted-glass-disc NDS and estimated AFDM, CHLA, N_2 fixation, and APA on days 6, 18, and 29 after deployment. Periphyton AFDM steadily increased on NDS over time, but was not influenced by nutrients. CHLA was elevated in the N treatment on days 18 and 29, indicating autotrophic N limitation. Consistent with N limitation, N_2 fixation was high but not different in the control and P treatments and was virtually undetectable on the N treatment. N_2 fixation in control and P treatments was detectable in both light and dark incubations, and dark rates were 4 to 73% of the light rates on days 18 and 29. The average contribution of total N_2 fixation to periphyton in control and P treatments was 0.93 mg N/m^2 on day 18 and 1.0 mg N/m^2 on day 29. APA was significantly elevated on the control and was highest in the N treatment despite no apparent P limitation of periphyton biomass accumulation. P enrichment always decreased APA. Measurable N_2 fixation and the change in CHLA suggest that autotrophs were primarily N limited. However, APA observed in controls demonstrated that some portion of the periphyton community was experiencing P deficiency. This result suggests that periphyton metabolism was related to both N and P availability, but that biomass accumulation might have been limited primarily by N. One explanation for these findings is that different organisms, perhaps occupying different trophic positions within the community, might have been limited by different elements.

Key words: algae, epilithon, NDS, nitrogenase activity, nitrogen fixation, phosphatase activity.

A common paradigm in aquatic ecology has been that N usually limits primary production in coastal marine environments, whereas P usually limits primary production in freshwater environments. However, this paradigm is not withstanding challenges, particularly those directed from meta-analyses on large data sets (Francoeur 2001, Elser et al. 2007). As a result, the efficacy by which freshwater systems were thought to overcome N limitation and come into equilibrium with P limitation has come into question. Schindler (1977) suggested that P generally would limit primary production in freshwater lakes over long time scales because N_2 fixation could supply sufficient N to counterbalance ecosystem N deficiency. This paradigm has been generally accepted by stream ecologists, but few studies have explored the role of instream N_2 fixation as a source of N to streams (Marcarelli et al. 2008), and the

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importance of N_2 fixation in alleviating N limitation to benthic microbial communities has been assessed only rarely (Scott et al. 2007). What is certain is that N limits primary production in streams over relatively short time scales as frequently as P does (Francoeur 2001) and that populations within the periphyton community might experience differential nutrient limitation (i.e., some limited by N, some limited by P).

Nitrogenase catalyzes the fixation of gaseous N_2 into biologically available NH_4^+ (Voet and Voet 2004). In aquatic environments, N_2 fixation is carried out by certain cyanobacteria, heterotrophic bacteria, and some diatoms with cyanobacterial endosymbionts (Howarth et al. 1988). Periphyton N_2 fixation and its relationship with dissolved inorganic N (DIN) availability has been studied extensively, but most of these studies have focused on lentic habitats (Doyle and Fisher 1994, Rejmánková and Komárková 2000, Inglett et al. 2004, Scott et al. 2007). Even modest levels of DIN will signal organisms to suspend nitrogenase synthesis and subsequently stop N_2 fixation because N_2 fixation is energetically expensive. However, N_2 fixation can provide a significant amount of N to the entire periphyton community when DIN concentrations are low. For instance, Grimm and Petrone (1997) demonstrated that fixed N_2 provided up to 85% of the net N flux into the benthos of a desert stream.

Marcarelli and Wurtsbaugh (2006) recently showed that P and temperature interacted to control N_2 fixation in periphyton from oligotrophic streams. P enrichment increased periphyton N_2 fixation rates by increasing the density of N_2-fixing organisms within the periphyton community. Higher temperature also increased N_2 fixation by increasing the density of N_2-fixing organisms and increasing the rate of cell-specific N_2 fixation. P enrichment also increased periphyton biomass (as chlorophyll *a* [CHLA]), but N enrichment had no effect on periphyton biomass. Therefore, the study by Marcarelli and Wurtsbaugh (2006) provided a good example of how N_2 fixation can supplement the N supply to periphyton communities that are strongly P limited. What remains unclear is how much N can be provided by N_2 fixation in streams experiencing stronger N limitation. Eutrophic streams might experience stronger and more frequent N limitation than oligotrophic streams because anthropogenic nutrient sources usually have a lower N:P ratio than do natural nutrient sources (Downing and McCauley 1992). However, N_2 fixation in eutrophic streams is an area of research that has been neglected (Marcarelli et al. 2008).

Enzyme activities are an important source of information on the physiological state of microbial communities. In particular, enzymes associated with nutrient cycling are informative indicators of nutrient status (Hill et al. 2006). For instance, phosphatases catalyze the hydrolysis of dissolved organic phosphate esters and are generated by a number of aquatic microbes, including bacteria, algae, and protozoans (Chróst 1991). Studies on natural systems and in laboratory cultures have demonstrated that microbial phosphatase activity increases when P limits growth (Healey and Hendzel 1980, Chróst and Overbeck 1987). Alkaline phosphatase activity (APA) has been widely studied in planktonic systems (Rose and Axler 1998), but has received less attention in benthic environments. Patterns of increasing periphyton APA are related to decreasing inorganic P availability and decreasing periphyton P content in lotic (Klotz 1992, Mulholland and Rosemond 1992) and lentic (Kahlert et al. 2002, Scott et al. 2007) benthic habitats. However, most periphyton APA measurements, particularly for streams, were done in studies with the goal of relating microbial enzyme activities to microbial biomass distributions (Sinsabaugh et al. 1991, Romaní and Sabater 2000, 2001). Scott et al. (2007) used APA as an indicator of P deficiency in N_2-fixing microbial communities in a freshwater wetland, but similar studies have not been done in streams.

Nutrient-diffusing substrata (NDS) are useful tools for identifying nutrient limitation in periphyton communities over short time scales (15–60 d). NDS have been used in lakes (Fairchild and Lowe 1984, Pringle and Bowers 1984, Fairchild et al. 1985), wetlands (Scott et al. 2005), streams (Lowe et al. 1986, Winterbourn and Fegley 1989, Tate 1990, Marcarelli and Wurtsbaugh 2006, Tank et al. 2006), and large rivers (Corkum 1996, Scrimgeour and Chambers 1997). Periphyton accumulates on diffusion surfaces when NDS are deployed in natural waters, and differences in accumulation rates between nutrient treatments and controls are interpreted as evidence of periphyton nutrient limitation. Periphyton accumulation is most often measured as ash-free dry mass (AFDM) or CHLA. AFDM represents the combined organic matter of algae and heterotrophs (e.g., bacteria, fungi) in the periphyton, whereas CHLA represents the biomass of algae only. Many studies also have used taxonomic assessments to explore changes in algal community composition after nutrient enrichment with NDS. However, few studies have coupled NDS experiments with more-complex measurements of periphyton community function such as N_2 fixation and APA.

We measured periphyton N_2 fixation, APA, and biomass accumulation (as AFDM and CHLA) to understand the importance of N_2 fixation in alleviating N limitation to stream periphyton. We worked in a stream-like outflow of a constructed freshwater marsh. N_2 fixation and APA must be measured in enclosed containers. Therefore, we used the micro-NDS design

developed by Gibeau and Miller (1989) and modified by Marcarelli and Wurtsbaugh (2006), wherein fritted glass discs (FGDs) are used as diffusion surfaces at the end of plastic tubes containing enrichment media. FGDs provide a solid surface that can be removed from NDS and placed inside closed syringes for N_2 fixation and APA assays. We estimated nutrient diffusion rates from NDS by measuring the rate of nutrient depletion from enriched agar because our NDS design differed slightly from those previously described in the literature.

Based on ambient nutrient levels and previous periphyton work in our study area (Scott et al. 2005), we expected that surface-water N would be sufficiently low and P would be sufficiently high to induce N limitation in periphyton. Thus, we hypothesized that biomass accumulation rates would be enhanced by N enrichment but not by P enrichment. We also hypothesized that N_2 fixation would be elevated on unenriched control NDS and highest on P-enriched NDS, where the N:P ratio of nutrient supply was lowest. We also hypothesized that N_2 fixation would be deactivated by N enrichment, whereas APA would be elevated on the N treatment only if P became limiting in response to N enrichment. Last, we hypothesized that N_2 fixation and APA would be more sensitive than AFDM and CHLA to changes in nutrient availability. Our goals were to evaluate N_2 fixation as a source of N to the periphyton community and to assess the importance of P deficiency in a seemingly N-limited system.

Methods

Study site

We deployed NDS in the stream-like outflow of the Lake Waco Wetland (LWW), near Waco, Texas, USA, from 23 May 2006 to 21 June 2006. Previous research on metaphyton in the study area indicated that N limits periphyton production primarily in the downstream areas of this created wetland (Scott et al. 2005, 2007). The outflowing stream connecting LWW with Lake Waco is ~100 m long, has an average width of 3 m, and has primarily sandy substrate with *Typha latifolia* growth along its margins. Thalweg depth at the deployment location was 0.4 m. Velocity and discharge during the study were ~0.15 m/s and ~0.1 m^3/s, respectively. The deployment location was an open site with low marginal *Typha* growth and lacked canopy cover.

NDS and experimental design

We constructed NDS based on the designs of Gibeau and Miller (1989) and Marcarelli and Wurtsbaugh (2006). Both of these designs used a FGD as a growth substrate that enabled nutrients to diffuse from an agar medium through the porous surface of the FGD. Our design differed from previous designs in that larger vessels (50-mL centrifuge tubes) were used to store agar in an effort to minimize the ratio of FGD surface area to agar volume. We cut 22-mm-diameter holes in the caps of 50-mL centrifuge tubes (Carolina Biological Supply, Burlington, North Carolina), and inserted FGDs (25-mm diameter, 4–5.5-μm porosity; Wilmad LabGlass, Buena, New Jersey) inside the cap, exposing most of the FGD surface through the top of the cap. We inserted a 3-mm-wide rubber gasket inside the cap to seal the bottom of the FGD to the top rim of the centrifuge tube.

We prepared NDS with 20 g/L agar and either no nutrient enrichment (control), N enrichment (0.51 M N from $NaNO_3$), or P enrichment (0.065 M P from $Na_3PO_4 \cdot 12H_2O$). We randomly placed NDS in a rack (60 × 90 cm) made from wire netting framed with plastic-coated plant stakes. We used a 2nd set of stakes to anchor the rack in the substrate at its deployment location in the wetland outflow. We deployed 51 NDS of each treatment (control, N, and P) to determine rates of biomass accumulation (AFDM and CHLA), N_2 fixation, APA, and N and P diffusion from NDS for a 29-d study period. We retrieved 3 randomly chosen NDS from each treatment on days 1, 2, 3, 12, and 24 to determine agar N and P concentrations and periphyton AFDM. We retrieved 12 NDS from each treatment on days 6, 18, and 29 to determine agar N and P concentrations and periphyton AFDM, CHLA, N_2 fixation rates, and APA.

Water chemistry and temperature

We collected 50-mL surface-water grab samples upstream and immediately downstream of the NDS rack on days 3, 6, 12, 18, and 24 after deployment. We estimated total N (TN), total P (TP), $NO_2 + NO_3$-N (NO_x-N), and PO_4-P levels using colorimetric methods (APHA 1992) on a Lachat® Quik-Chem 8500 flow-injection autoanalyzer (Hach, Loveland, Colorado). We analyzed TN and TP following alkaline and acid persulfate digestions, respectively. We monitored water temperature throughout the study period with a HOBO® Water Temp Pro temperature logger (Onset Computer Corporation, Bourne, Massachusetts) attached to the NDS rack.

N and P diffusion rates

We estimated nutrient diffusion rates by monitoring how rapidly nutrients disappeared from NDS agar. We sampled 3 randomly chosen NDS from each treatment

(control, N, and P) on days 1, 2, 3, 6, 12, 18, 24, and 29 after deployment. We extracted agar from the centrifuge tubes and melted it in a beaker with ~900 mL deionized water. We cooled the solution to room temperature, diluted it to 1 L, and collected 7-mL duplicate subsamples that we held frozen for <28 d until analysis for dissolved NO_x-N and PO_4-P concentrations.

Estimation of N_2 fixation, APA, AFDM, and CHLA

We kept periphyton intact on FGDs from NDS for measurement of N_2 fixation and APA before removal for biomass estimation (AFDM, CHLA). We measured N_2 fixation on 5 FGDs and APA on another 5 FGDs from each treatment × sampling day combination. We used the 2 remaining NDS as dark incubation controls in N_2-fixation assays. We conducted N_2 fixation and APA assays in 50-mL Popper micromate syringes with 3-way stopcocks (Popper and Sons, New Hyde Park, New York). We transferred periphyton on FGDs into syringes with either 30 mL of stream water for N_2 fixation assays or 30 mL of 1.2% Tris buffer for APA assays.

N_2 fixation.—We estimated N_2 fixation using acetylene reduction (Flett et al. 1976). We injected 5 mL of acetylene gas, generated from the dissolution of calcium carbide, into syringes containing samples in 30 mL stream water. We gently agitated samples to dissolve the acetylene and then incubated them under artificial lighting (250 μmol m^{-2} s^{-1}) for 2 to 4 h. We incubated 2 samples from each treatment in the dark to estimate the importance of N_2 fixation by heterotrophs. In addition, we incubated streamwater samples under lights to account for any background N_2 fixation in seston. We removed samples from incubation and analyzed them one at a time and recorded the length of incubation to the nearest minute. We drew 15 mL of air into each syringe and agitated the samples to establish equilibrium between water and vapor phases. We withdrew 1 μL of air from each syringe and injected it into a Carle AGC series gas chromatograph (Hach Carle, Loveland, Colorado) equipped with a flame ionization detector and a 1.8-m column packed with 80% Porapack N and 20% Porapack Q. The column temperature was 70°C. We used 10-ppm ethylene standards to calibrate the instrument daily. To estimate N_2 fixation, we assumed that the production of 3 μmol ethylene was equivalent to the fixation of 1 μmol N_2 (Flett et al. 1976).

APA.—We estimated APA using the rate by which phosphate was cleaved from 4-methylumbelliferyl phosphate (MUFP) resulting in the production of methylumbelliferone (MUF), which fluoresces (emission wavelength 455 nm) when irradiated at 365 nm wavelength (Healey and Hendzel 1979). We injected 4 mL of 0.2-mM MUFP into syringes containing samples in 1.2% Tris buffer. We gently agitated syringes, incubated samples at room temperature, and measured fluorescence after 5, 20, and 45 min. We removed subsamples from incubation and analyzed samples one at a time and recorded the length of incubation to the nearest minute. We measured fluorescence on a Turner 10 AU fluorometer (Turner Designs, Sunnyvale, California) calibrated with 50-, 100-, 250-, 500-, and 1000-μg/L MUF standards. We used the slope of a linear regression of time (min) vs MUF concentration to calculate APA activity. Instances in which the MUFP concentration was not saturating were rare. However, we derived APA activity from the exponential increase in MUF concentration vs time using regression when we observed this phenomenon.

Periphyton biomass.—After N_2 fixation and APA assays, we removed FGDs from syringes and scraped and rinsed periphyton to form a 50-mL slurry. We collected two 25-mL aliquots onto separate glass fiber filters (GFFs) for AFDM and CHLA analyses, respectively. We estimated AFDM on prewashed, precombusted, and preweighed GFFs. We dried AFDM samples at 100°C for 1 h, weighed them to estimate dry mass, combusted them for 1 h at 500°C, and weighed them again to estimate ash content. We determined phaeophytin-corrected CHLA using the method described by Biggs and Kilroy (2000). We extracted CHLA overnight in 90% ethanol (heated) and measured pigments by absorbance on a Beckman DU series spectrophotometer (Beckman Coulter, Fullerton, California).

Data analysis

We used paired *t*-tests (SAS 9.1.3; SAS Institute, Cary, North Carolina) to compare water-column TN, TP, NO_x-N, and PO_4-P concentrations upstream and downstream of the NDS rack. We used nonlinear regression models of nutrient diffusion vs day of retrieval (SigmaPlot 9.0; SYSTAT, Point Richmond, California) to estimate nutrient diffusion rates from agar.

We used 2-factor analysis of variance (ANOVA; SAS) with day of retrieval (date) and nutrient treatment (nutrient) as main effects and a date × nutrient interaction term on AFDM, CHLA, N_2 fixation, and APA. We used standard 2-way factorial ANOVA rather than repeated-measures ANOVA because we did not take repeated measurements from the same samples over time, but rather sampled a unique set of NDS during each collection; thus,

TABLE 1. Mean (±1 SE) surface-water N and P concentrations upstream and downstream of the nutrient diffusing substrate rack. TN = total N, TP = total P, NO_x-N = NO_2 + NO_3-N. $n = 5$.

	Concentration (μg/L)			
Analyte	Upstream	Downstream	Paired t	1-tailed p
TN	556.2 ± 27.3	592.8 ± 35.2	1.58	0.09
TP	105.5 ± 5.3	104.3 ± 6.1	–0.64	0.28
NO_x-N	49.6 ± 3.2	62.8 ± 4.5	2.46	0.03
PO_4-P	24.5 ± 0.4	29.9 ± 3.1	1.70	0.08

adjustments for serial autocorrelation were inappropriate. Post hoc comparisons of significant main effects or interaction terms were done with LSMEANS with Tukey adjustments (SAS). The significance level for all tests was $p < 0.05$.

We applied $\log_{10}(x)$ transformations to most data sets before analyses because group variances tended to increase with mean response magnitude. Before transformation, we anchored each data set by setting the minimum data value to 1.0 by adding or subtracting a constant to the values in the data set. This technique standardizes the effect of transformations (Sokal and Rohlf 1995, Osborne 2002) and keeps the resultant log-transformed values positive.

Results

Water chemistry and N and P diffusion rates from NDS

Mean NO_x-N concentrations were higher downstream than upstream of the NDS rack during the 29-d study period ($t = 2.46$, $p = 0.03$). TN, TP, and PO_4-P concentrations did not differ between upstream and downstream samples (Table 1). Mean water temperature was 25.5°C (range: 23–28°C) during the study period.

Mean agar concentrations of NO_x-N and PO_4-P declined exponentially during the study period. Day-29 NO_x-N and PO_4-P remained within ~40% and 75% of day-0 concentrations, respectively. NO_x-N concentrations of the control and P treatment were ~0. PO_4-P concentrations of the control and N treatment also were low, but the PO_4-P concentrations tended to be slightly higher in the N treatment than in the control. The 1st derivatives of exponential-loss equations predicted the release rates for NO_x-N and PO_4-P of the N and P treatments, respectively (Fig. 1A, B). The predicted release rate of NO_x-N from the N treatment decreased by >50% by day 2, but the release rate decreased more gradually after day 3 (Fig. 1A). NDS released 2.9 mg NO_x-N cm^{-2} d^{-1} on day 6, 1.2 mg NO_x-N cm^{-2} d^{-1} on day 18, and 0.57 mg NO_x-N cm^{-2} d^{-1} on day 29. The release rate of PO_4-P from the P treatment did not exhibit a rapid initial decline (Fig. 1B). NDS released 0.41, 0.26, and 0.18 mg PO_4-P cm^{-2} d^{-1} on days 6, 18, and 29, respectively.

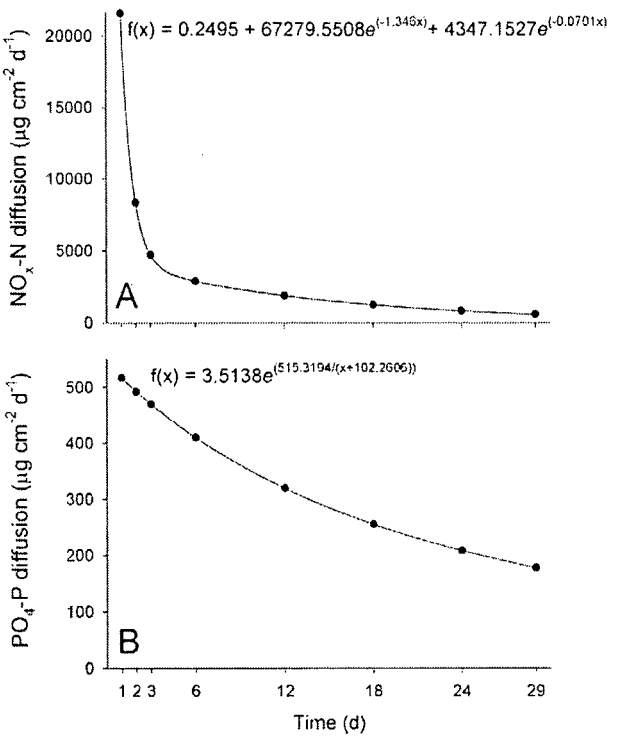

FIG. 1. N (A) and P (B) release rates from nutrient diffusing substrates.

Effects of N and P enrichment on periphyton accrual

Periphyton AFDM increased significantly during the study period ($F_{7,129} = 92.88$, $p < 0.0001$; Fig. 2). However, area-normalized AFDM did not differ significantly among nutrient treatments ($F_{2,129} = 1.30$, $p = 0.2751$), nor was the nutrient × date interaction significant ($F_{14,129} = 0.54$, $p = 0.9056$). AFDM accrual appeared linear and was similar among nutrient treatments between days 1 and 6. AFDM accrual rate was 0.1197 mg cm^{-2} d^{-1} between days 1 and 6 and was 0.2197 mg cm^{-2} d^{-1} between days 24 and 29. Area-normalized CHLA varied significantly among nutrient treatments over the study period ($F_{4,97} = 5.12$, $p = 0.0009$; Fig. 3). CHLA was significantly greater in the N treatment than in control or P treatments on days 18 and 29.

N_2 fixation and APA following nutrient enrichment

N_2 fixation varied significantly among nutrient treatments during the study period, regardless of

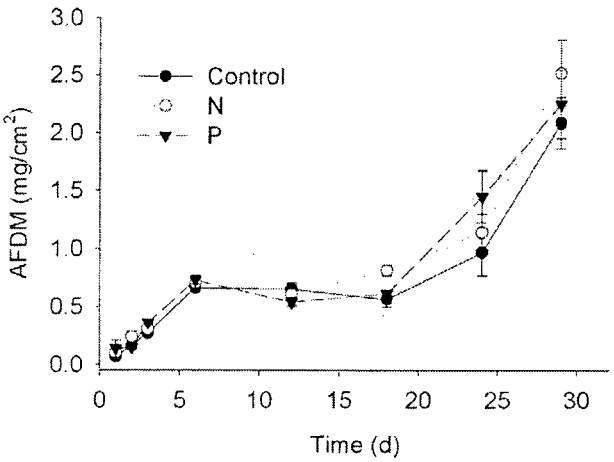

FIG. 2. Mean (±1 SE) periphyton biomass measured as area-normalized ash-free dry mass (AFDM) on control, N, and P nutrient diffusing substrate treatments over 29 d.

normalization method ($p < 0.001$; Fig. 4A–C). N_2 fixation did not differ significantly among nutrient treatments on day 6. However, N_2 fixation was significantly greater in control and P than in N treatments on days 18 and 29. N_2 fixation did not differ significantly between control and P treatments (Fig. 4A–C). Biomass (CHLA)-normalized N_2 fixation in control and P treatments was significantly lower on day 29 than on day 18 (Fig. 4C). A similar pattern was apparent for biomass (AFDM)-normalized N_2 fixation, but the difference between days was not significant

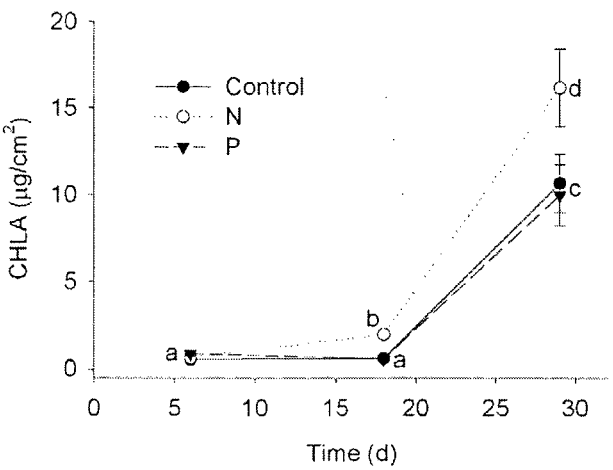

FIG. 3. Mean (±1 SE) periphyton biomass measured as chlorophyll *a* (CHLA) on control, N, and P nutrient diffusing substrate treatments over 29 d. Values with the same letter are not significantly different (Tukey–Kramer adjusted posthoc comparisons).

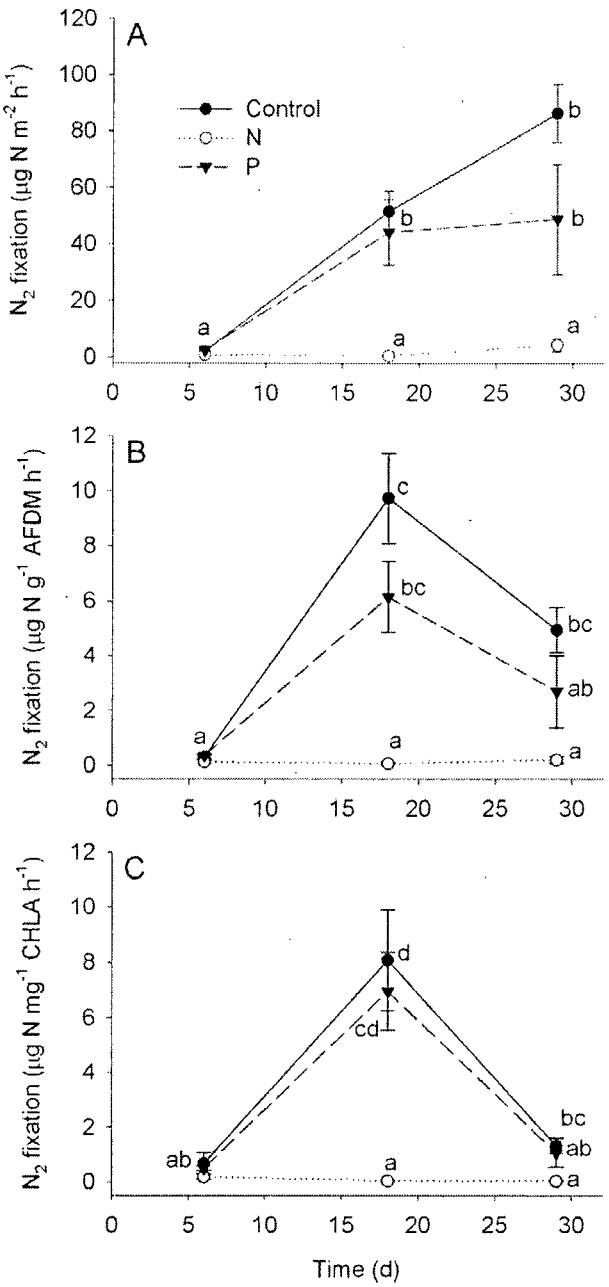

FIG. 4. Mean (±1 SE) N_2 fixation rates normalized by area (A), periphyton ash-free dry mass (AFDM) (B), and periphyton chlorophyll *a* (CHLA) (C) on control, N, and P nutrient diffusing substrate treatments over 29 d. Values with the same letter are not significantly different (Tukey–Kramer adjusted post hoc comparisons).

(Fig. 4B). N_2 fixation in dark incubations was consistently >0 in control and P treatments (Table 2). N_2 fixation in dark incubations accounted for 4.0 to 73.3% of N_2 fixation in light incubations, except in the

TABLE 2. Mean periphyton N_2 fixation rates (±1 SE) in dark incubations. $n = 2$.

Treatment	Day	Dark N_2 fixation ($\mu g\ N\ m^{-2}\ h^{-1}$)	% of mean light N_2 fixation
Control	6	15.0 ± 9.17	727.0
	18	19.1 ± 3.28	37.0
	29	10.0 ± 6.14	11.6
N	6	0.00 ± 0.00	0.0
	18	0.00 ± 0.00	0.0
	29	0.00 ± 0.00	0.0
P	6	0.81 ± 0.81	33.3
	18	32.3 ± 16.18	73.3
	29	1.96 ± 0.67	4.0

control treatment on day 6 when N_2 fixation was 7× higher in dark than in light incubations (Table 2).

APA always was significantly lower in P than in control or N treatments, regardless of normalization method ($F_{2,36} = 57.17$–91.67, $p < 0.0001$; Fig. 5A–C). APA varied significantly among nutrient treatments over the study period ($F_{4,36} = 2.92$–5.19, $p = 0.0022$–0.0343; Fig. 5A–C). On day 18, area-normalized APA was greater in the N than in the control treatments (Fig. 5A). In the control treatment, area-normalized APA did not differ significantly between days 6 and 18, but was significantly greater on day 29 than on days 6 and 18. In the N treatment, area-normalized APA increased significantly between days 6 and 18, but did not change between days 18 and 29. In control and N treatments, biomass-normalized APA was significantly lower on day 29 than on day 18 (Fig. 5B, C).

Discussion

NDS diffusion rates and surface-water chemistry

Initial rapid loss of nutrients from NDS limits their usefulness in long-term studies (Corkum 1996, Rugenski et al. 2008). Most NDS studies are limited to the period of measurable nutrient diffusion from treatments. Deployment periods for NDS designs similar to ours range from 2 wk (Bernhardt and Likens 2004) to 2 mo (Von Schiller et al. 2007), but typically are 3 to 4 wk so that diffusion rates remain relatively constant (Tank and Dodds 2003). Bernhardt and Likens (2004) reported that day-14 N and P diffusion rates were only 10% and 25%, respectively, of day-2 rates. In our study, depletion of N was initially rapid and exponential, but resulted in only 60% loss during the 29-d study period. Depletion of P was more gradual, and resulted in only 25% loss during the study period. Our results also suggest that N and P diffusion remained high enough on day 29 (0.57 mg NO_x-N $cm^{-2}\ d^{-1}$, 0.18

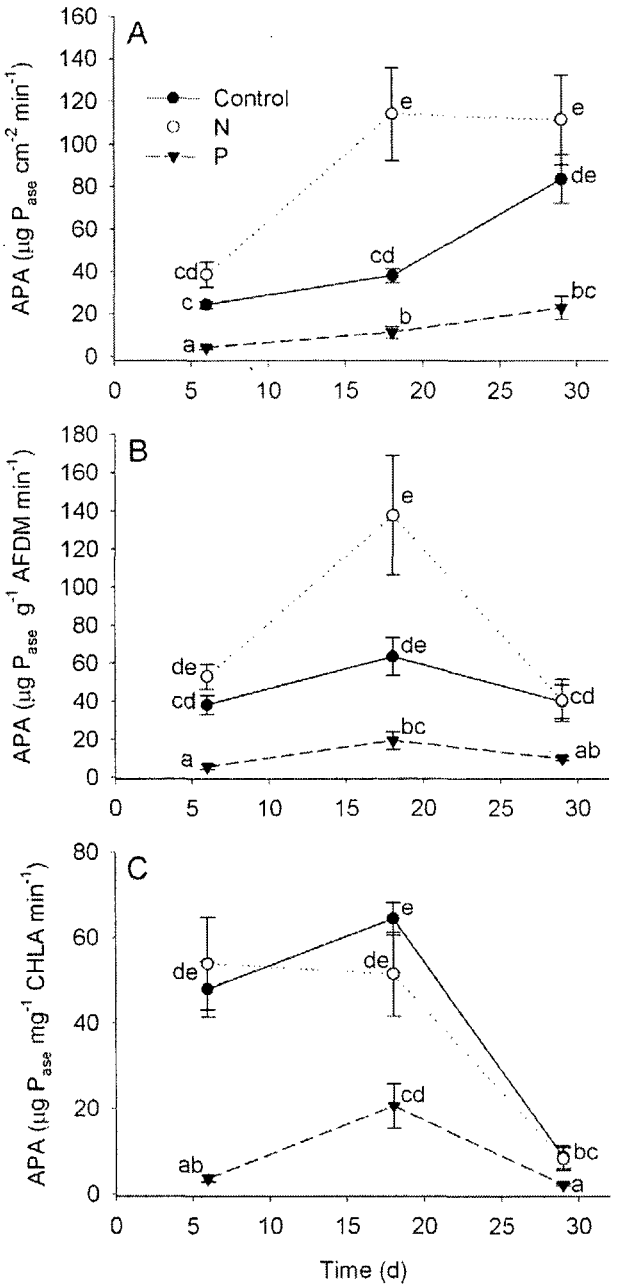

FIG. 5. Mean (±1 SE) alkaline phosphatase activity (APA), expressed as mass phosphatase (P_{ase}) per minute and normalized to area (A), periphyton ash-free dry mass (AFDM) (B), and periphyton chlorophyll *a* (CHLA) (C) on control, N, and P nutrient diffusing substrate treatments over 29 d. Values with the same letter are not significantly different (Tukey–Kramer adjusted posthoc comparisons).

mg PO_4-P $cm^{-2}\ d^{-1}$) to inhibit periphyton N_2 fixation and APA. Furthermore, N and P diffusing from NDS at the end of the study period was high relative to N and P requirements of periphyton growing on them. We

did not measure periphyton N and P content, but N and P content of stream epilithon (Stelzer and Lamberti 2001) and metaphyton from the wetland that provides the water for our study stream (Scott et al. 2007) range from 1.0 to 2.0% N and 0.1 to 0.2% P. Mean periphyton dry mass on day 29 of our study was ~3 mg/cm^2. If we apply N and P contents from Stelzer and Lamberti (2001) and Scott et al. (2007) to periphyton in our system, periphyton N content probably ranged from 30 to 60 μg/cm^2 and periphyton P probably ranged from 3 to 6 μg/cm^2. Thus, the N and P diffusion rates were sufficient to replace periphyton N 10 to 20× and periphyton P 30 to 60× on day 29. Sufficient N and P were provided by NDS by the end of our study period to maintain the integrity of our nutrient-enrichment treatments.

We attempted to minimize the effect of confounding experimental factors by randomizing NDS positions in the stream. Thus, concentrations of dissolved N might have been slightly higher at downstream than at upstream NDS. NO_x-N concentrations were slightly higher in water collected 0.5 m downstream than in water collected immediately upstream of the rack. The slightly higher NO_x-N concentrations supplied to downstream NDS might have dampened periphyton response to nutrient treatments in downstream samples, but the concentrations of NO_x-N in downstream water were certainly lower than the exposure concentrations of NO_x-N on the NDS. Moreover, we did not detect any spatial pattern in biomass or enzyme activity as a function of the position of samples in the rack. Thus, we conclude that upstream–downstream differences in streamwater nutrients had minimal influence on our results, but the relative position of treatments should be considered carefully in the design of future NDS studies (Tate 1990).

N_2 fixation, APA, and biomass on NDS

N_2 fixation.—Periphyton N_2 fixation ranged from 44 to 86 μg N m^{-2} h^{-1} in control and P treatments on days 18 and 29. This range is 4 to 8× higher than the median of 22 values reported by Marcarelli et al. (2008). However, mean rates in some of the studies reviewed by Marcarelli et al. (2008) were as high as 410 to 8400 μg N m^{-2} h^{-1} (Buckley and Triska 1978, Howard-Williams et al. 1989, Grimm and Petrone 1997, Marcarelli and Wurtsbaugh 2006). The highest rates were reported from Sycamore Creek (Grimm and Petrone 1997), a strongly N-limited stream in the Sonoran Desert (Grimm and Fisher 1986). Furthermore, the highest rates occurred during mid-summer in all studies, consistent with summer N_2-fixation maxima in lentic systems (Howarth et al. 1988). Our study was done in late May through mid-June, and rates might be substantially higher during July and August.

We hypothesized that N_2 fixation would be highest in control and P treatments and that N enrichment would suppress N_2 fixation by periphyton. N_2 fixation rates were lower in N than in control treatments, but P enrichment did not stimulate N_2 fixation in our system. Mean N_2 fixation was always lower on P treatments than on controls, although this difference was never statistically significant. That P enrichment did not stimulate N_2 fixation was unexpected. We see no obvious explanation for this result, but offer the following possibilities. 1) The composition of the microbial community might have changed in the P treatment (sensu Fairchild et al. 1985), with the consequence that a different community of algae and bacteria had slightly lower N_2 fixation potential. However, we lack taxonomic data to support this hypothesis. Marcarelli and Wurtsbaugh (2006) found that P enrichment increased the number of N_2-fixing taxa in an oligotrophic stream, but other studies have reported that P enrichment decreased the rate of N_2 fixation in some stream periphyton communities (Marcarelli and Wurtsbaugh 2007). 2) Increased N remineralization might have occurred in response to P enrichment. Several studies have demonstrated the importance of N-mineralizing extracellular enzymes in periphyton (Francoeur and Wetzel 2003, Francoeur et al. 2006, Rier et al. 2007), but to our knowledge, no studies have explored how P enrichment affects N remineralization within the periphyton matrix. 3) Heterotrophic N_2 fixation might have decreased in response to P enrichment. Sundareshwar et al. (2003) reported reduced heterotrophic N_2 fixation in estuarine sediments after P enrichment, but found that glucose alone and glucose + P additions stimulated N_2 fixation. We did not replicate dark N_2 fixation measurements sufficiently for statistical comparisons, but N_2 fixation in the dark was 4 to 73% of N_2 fixation in the light on days 18 and 29. Thus, heterotrophic N_2 fixation could have influenced our results and might have been affected by availability of dissolved organic C in the periphyton matrix. More work is needed to elucidate these mechanisms.

Daily N_2 fixation was 0.93 mg N m^{-2} d^{-1} on day 18 and 1.0 mg N m^{-2} d^{-1} on day 29 (mean of control and P treatment N_2 fixation rates integrated over a 14:10 h day:night cycle). The rates of N diffusion in the N treatment suppressed N_2 fixation on days 18 (12.0 g N m^{-2} d^{-1}) and 29 (5.7 g N m^{-2} d^{-1}) and supplied 6×10^3 to 12×10^3 times more N to the N-enriched periphyton than was supplied by N_2 fixation to periphyton in control and P treatments. We cannot say what minimum rate of N supply from NDS might have

inhibited N_2 fixation. However, agar N concentrations can be modified to manipulate diffusion rates (Rugenski et al. 2008), and future studies might be able to clarify the relationships between diffusion rates of NO_3^- or NH_4^+ from NDS and up- or down-regulation of N_2 fixation by periphyton.

N_2 fixation rates in streams generally are much lower than instream denitrification and stream DIN uptake rates (Marcarelli et al. 2008). This conclusion suggests that N_2 fixation might seldom be an important source of N in stream ecosystems. However, the rates used in these comparisons were derived for the whole-reach scale and were expressed on an areal basis. Therefore, they do not consider N standing stocks within stream reaches. A more appropriate rate to compare might be the N-specific uptake rate (or N turnover rate of the periphyton N pool) expressed in units of time^{-1}. If we assume that periphyton N in our study probably ranged from 30 to 60 μg N/cm^2 on NDS, then N-specific uptake from N_2 fixation on control and P treatments in our study was 1.5×10^{-3}/d to 3.1×10^{-3}/d on day 18 and 1.7×10^{-3}/d to 3.4×10^{-3}/d on day 29. We did not measure total N uptake, but Dodds et al. (2004) reported that N-specific total N uptake by primary producers across several streams in North America ranged from 10^{-4}/d to 10^{-1}/d, a range that brackets our N_2 fixation estimates. Therefore, the importance of N_2 fixation as a source of N to primary producers and, ultimately, to consumers within the food web might be more obvious when considered in terms of stream biomass N.

APA.—We hypothesized that P enrichment would inactivate APA and that N enrichment would stimulate APA. As in other studies (e.g., Klotz 1992), P enrichment always decreased APA. N enrichment stimulated area-normalized APA on day 18 but not on day 29. N enrichment stimulates APA in phytoplankton (e.g., Rose and Axler 1998) and in stream epilithon (Klotz 1992). Stimulation of APA by N enrichment suggests that organisms originally limited by N become P limited after N enrichment or that N enrichment enables P-limited organisms to up-regulate APA. Thus, P deficiency appears to be an important limitation on periphyton biomass production and metabolism. The concentration of inorganic P in our study stream (24.4–29.9 μg P/L) approached concentrations that saturate periphyton biomass accumulation (Bothwell 1989). However, mass transfer of nutrients from stream water into the periphyton matrix is the primary rate-limiting step for nutrient uptake by periphytic algal cells (Larned et al. 2004). Thus, internal nutrient regeneration within the periphyton matrix could be important in both oligotrophic and eutrophic streams because the rate at which nutrients enter the biofilm matrix might not keep up with demand by cells. This idea is supported by our data, which suggest that P deficiency existed in control and N treatments even though streamwater inorganic P concentration was consistently high.

Periphyton APA values approached 0 only on P-enriched NDS. We cannot say what minimum rate of P supply from NDS was necessary to inhibit periphyton APA. However, as with N, agar P concentrations can be modified to manipulate diffusion rates (Rugenski et al. 2008), and future studies might be able to clarify the relationships between diffusion rates of P from NDS up- or down-regulation of APA by periphyton.

Biomass.—We hypothesized that N enrichment, but not P enrichment, would increase periphyton biomass. CHLA, a measure of autotrophic biomass (Steinman et al. 2006), was higher in N than in control or P treatments on days 18 and 29, but N enrichment had no effect on AFDM. P enrichment had no effect on CHLA or AFDM. However, both N_2 fixation and APA were greatly influenced by periphyton biomass. AFDM and CHLA increased substantially between days 18 and 29. Area-normalized N_2 fixation and APA were highest (or equal to the highest observed value) on day 29 when CHLA and AFDM were highest, but AFDM- and CHLA-normalized N_2 fixation and APA were highest on day 18 when CHLA and AFDM were relatively low. This pattern suggests that periphyton was more metabolically active (with regard to N_2 fixation and APA) on day 18 than on day 29 and that the higher area-normalized rates on day 29 were driven by high periphyton biomass.

We hypothesized that N_2 fixation and APA would be more sensitive than periphyton biomass to differences in nutrient enrichment. N_2 fixation and APA were strongly influenced by nutrient treatments. CHLA accrual was stimulated by N enrichment, but not P enrichment, on days 18 and 29, but AFDM accrual was not sensitive to N or P enrichment. These results indicate that autrotrophs must have been limited by N availability throughout the study. However, the high rates of APA on control and N treatments suggest that some part of the periphyton community (autotrophs, heterotrophs, or both) was P limited. This conclusion is supported by the lower APA in P than in control or N treatments. Thus, our results suggest the potential for differential nutrient limitation among individual autotrophic and, perhaps, heterotrophic taxa in this periphyton community.

Differential nutrient limitation in periphyton

Our study design did not enable us to distinguish whether APA was derived from autotrophs or hetero-

trophs. We do not know whether only a subset of autotrophs, mostly heterotrophs, or a combination of both were experiencing P deficiency. Different autotrophic taxa within the same community can be limited by different elements (Dodds 1991, Armitage et al. 2006), as can autotrophs and heterotrophs within the same community (Sundareshwar et al. 2003, Zohary et al. 2005). Differential nutrient limitation of autotrophs and heterotrophs might maximize resource use within an ecosystem if groups facilitate resource delivery to each other, rather than compete for the same resource (Sundareshwar et al. 2003). The strength of algal–bacterial coupling in periphyton decreases as nutrient availability increases (Scott and Doyle 2006, Scott et al. 2008). This result suggests facilitation of N and P exchange between trophic levels. Thus, periphyton communities experiencing differential nutrient limitation between trophic levels might be more productive than predicted under a single-limiting-nutrient scenario. Exchange of resources between trophic levels in microbial benthos experiencing differential nutrient limitation could explain how benthic communities increase ecosystem production efficiency in nutrient-poor systems and could provide a mechanism to explain why benthic production is of primary importance in oligotrophic ecosystems (Vadeboncoeur et al. 2002).

Results of our study suggest that N_2 fixation can provide an important source of N to periphyton communities, but that P deficiency might still play a critical role in periphyton community metabolism and production. Both N_2 fixation and APA are sensitive indicators of microbial nutrient limitation in periphyton. N enrichment increased autotrophic biomass in our periphyton community. Moreover, although changes in total periphyton biomass did not generally reflect a response to added nutrients, N enrichment inhibited N_2 fixation and stimulated APA, and P enrichment inhibited APA. Therefore, microbial metabolism was related to both N and P availability. Furthermore, the simultaneous activity of both N_2 fixation and APA and their respective responses to nutrient enrichment suggests the importance of differential nutrient limitation and supports the need for a multielement approach to understanding nutrient constraints on ecological communities and the systems in which they live (sensu Sterner and Elser 2002). However, more work is needed to determine if differential nutrient limitation is a general characteristic of aquatic microbial communities and if patterns observed in nature might be explained by differences in elemental constraints among microorganisms of the same or of different trophic positions.

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