ASSESSMENT OF STREAM WATER QUALITY - EUTROPHICATION

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INTRODUCTION

Agricultural practices frequently disturb terrestrial ecosystems with concomitant effects on downstream aquatic ecosystems (Woodmansee, 1984). The impact of these disturbances on lakes and reservoirs is now reasonably predictable with respect to nutrients (Vollenweider, 1976). However, it is difficult to gauge the severity of these effects on rivers and streams. In running water (lotic) ecosystems, changes in discharge complicate an assessment of the effect of nutrients on the biota (Horner et al., 1983). For this reason, the study of nutrient limitation in streams has not progressed as rapidly as the study of nutrient limitation in lakes. There is a need for good methods to assess nutrient limitation in streams.

There are a number of methods for assessing nutrient limitation, and each has its strengths and weaknesses. They are as follows: nutrient concentrations in water, ratios of N:P nutrients in water, ratios of N:P in algae or periphyton, a substrate technique, and alkaline phosphatase activity. Whole stream fertilization and fertilization of stream-side troughs are other methods, but not applicable here because of cost.

Because it is easy to measure nutrient concentrations in water, it would be very useful if mere concentrations of a nutrient reflected nutrient limitation, i.e., nutrient limitation occurred at or below some threshold concentration. However, often this is not the case. The instantaneous standing quantity of a nutrient, particularly phosphate, in water does not reliably indicate how fast it is being used and recycled (Wetzel, 1983). If algae are P deficient, they will rapidly use P as fast as it is supplied.

Nutrients commonly measured are orthophosphate, nitrate, and ammonia. Orthophosphate is measured as soluble reactive phosphorus (SRP), and most of SRP is thought to be usable by algae as are both nitrate and ammonia.

Ratios of N:P as nutrients (supply ratios) could also be used to indicate potential for algal growth. Given the fact that N:P occur at a 20:1 ratio by atoms in nutrient replete algae, an increase

in the N:P ratio in water to higher values might indicate P-limitation (Cook, et al., 1986, Rhee and Gotham, 1980, and Redfield, 1958). Values less than 13:1 indicate N-limitation. However, these ratios are average ratios and individual species may have quite different ratios.

The substrate technique involves exposing artificial substrates that diffuse nutrients (Fairchild et al., 1985). After a suitable time, biovolume or biomass on substrates is determined, and treatments are compared to controls. A significant increase of biomass or biovolume on treatments indicates limitation by the treatment nutrient.

The alkaline phosphatase activity (APA) technique measures P-limitation only (Healey and Hendzel, 1979). The technique has its basis in that algae have the enzyme alkaline phosphatase when P limited, but not at other times. It is very rapid and potentially useful to screen many samples (sites).

Limitation of algae by N is relatively uncommon in freshwater (Wetzel, 1983), and thus techniques other than the substrate technique do not test for N- limitation. However, two facts could lead to a screening procedure for N- limitation in streams. Nitrogen fixation (NF) by autotrophs only occurs when N nutrients are very low, and NF in autotrophs occurs almost exclusively in one group of blue-green algae, those with heterocysts (Wetzel, 1983).

Naturally, techniques to measure nutrient limitation must account for time. While short-term limitation is interesting, the techniques should measure and integrate the effect of nutrients over the growth cycle of the organisms. Thus, time scales of weeks become appropriate. It is also important to have a measure of nutrient limitation that reflects and integrates the effects of exposure to stream water varying in velocity, nutrient concentration, and discharge.

This research was directed at assisting Oklahoma water managers in identifying when and where nutrient limitation occurs in streams, specifically in southeastern Oklahoma, where water quality problems are expected to result from projected expansion of the poultry industry. In McCurtain County alone, brooder houses will increase from 360 to 400 in one year. Relatively pristine streams are found in this area.

Poultry manure will be spread on agricultural land in the vicinity of the rivers. As a result, nitrogen (N) and phosphorus (P) nutrients could enter the streams. If this should occur, benthic aquatic plant growth will increase and could lower dissolved oxygen (DO) at low discharge at night.

OBJECTIVES

The purpose of the research was to compare techniques of measuring P-limitation in streams. The original objectives were as follows:

Objective 1. APA tests were compared to substrate tests and to biomass measures (N:P supply ratios and surplus P). The null hypothesis was that the results of APA do not agree with other measures of P-limitation.

Objective 2. Paul and Duthie (1989) showed that the epilithon recycled P more rapidly than seston, suggesting seston is more apt to be P limited. This idea will also be tested. The null hypothesis was that APA of seston is no different from the APA of the epilithon.

Objective 3. Conventional wisdom is that water movement over periphyton prevents local nutrient depletion and prevents nutrient limitation even when nutrients are very low. The null hypothesis was that riffle and pool results for substrata do not agree. Periphyton in riffles will reflect no P-limitation and lower APA.

Objective 4. Our data indicate that sometimes both P and N can be limiting. What effect does N- and P-limitation have on APA? The null hypothesis is that APA will be no different on P enriched as opposed to N+P enriched substrata.

The objectives spelled out in the proposal presupposed satisfactory precision of measurements in the first year of the project, but this was not the case when the data were finally analyzed. This was particularly the case for the enzymatic method (APA).

In addition, it proved difficult to find suitable sites in the river that were really pools or riffles and yet sufficiently deep to submerge the test substrates.

Thus, instead of pursuing objectives 3 and 4, I proposed to repeat experiments done previously with the objective of making technical changes in the methods and to focus our effort on P-limitation alone (memo to Dr. Norman Durham).

I also describe here research conducted in 1988-1990 to test for N- and P-limitation. These observations overlap an earlier OWRRI project, "Agricultural Impacts on Stream Water Quality" July 1, 1988 - June 30, 1990, experiments 1-10. The design of experiments is given in Table 1 on the following page.

Our 1991-1992 objective was to improve APA and biomass measurements, using clay pots as point sources of nutrients and repeat Objective 1. In particular, we attempted to find ways to improve the precision of chlorophyll measurements, using high and low concentrations of phosphorus in substrates acting as point sources of nutrients (experiments 11 and 12, Table 1).

Table 1. Design of nutrient limitation experiments in the Glover River, showing number of replicates. * = lost, ** also 1.0 M P.

Experiment	Date	Site	Number of Substrates			
			Control	0.1 M P	0.1 M N	0.1 M P+0.1 M N
1	11/6-12/4/88	I	5	4	4	0
2	11/6-12/5/88	II	5	4	4	0
3	3/15-4/4/89	I	5	4	4	0
4	3/15-4/4/89	II	5	4	4	0
5	6/10-7/1/89	I	5	4	4*	0
6	7/29-8/19/89	I	8	8	8	0
7	9/29-10/19/89	I	8	8	8	0
8	6/16-6/28/90	I	8	8	8	8
9	9/9-9/26/90	I	8*	8	8	8
10	9/26-10/15/90	I	8	8	8	8
11	7/25-8/11/91	I	6	6	0	0
12	8/11-8/22/91	I	4	4**	0	0
13	2/18-3/8/92	I	4	4	0	0

STUDY SITE

The study site is in the Little River drainage basin (Figure 1). The study stream (Glover River) originates in the Quachita Mountains and flows southerly for about 90 km to its confluence with the Little River. The area of the drainage basin is 876 km^2 (Blazs, et al., 1992). The stream gradient varies from 19 m/km in the upper reaches to 1 m/km at the mouth. It is one of the remaining streams in Oklahoma that has not been impounded (US Army Corps of Engineers, 1975).

The watershed is largely forested, originally mixed pine and hardwoods. Current forestry practices include clear-cutting, forest fertilization, and replacement of mixed hardwoods and pine with monocultures. There are small farms in scattered clearings. Many farms contain chicken brooder houses, the basis of agriculture in the vicinity. Clear-cut regions can be found within 5 to 10 km of the study sites, but not in direct contact with a clear-cut.

At study site I (see Figure 1 on the following page), the Glover River is about 30 m wide; the substrate consists almost entirely of large smoothed rocks. Turbidity was 2-23 nephelometric units. Site II is upstream, similar to site I.

Discharge can be so low that stream water is virtually motionless, especially in summer. However, extremely high discharges can occur during flood events. At site I, flood debris was observed resting in streamside trees up to 2 m above the stream bank. Samples were frequently lost in such flood events.

Average discharge is extremely variable, with some monthly ranges exceeding 300 cubic feet per second (CFS), while others are as low as 14 CFS. August is the month of lowest discharge, and April is the month of peak flow. The river is not impounded and frequently floods in the spring and early summer.

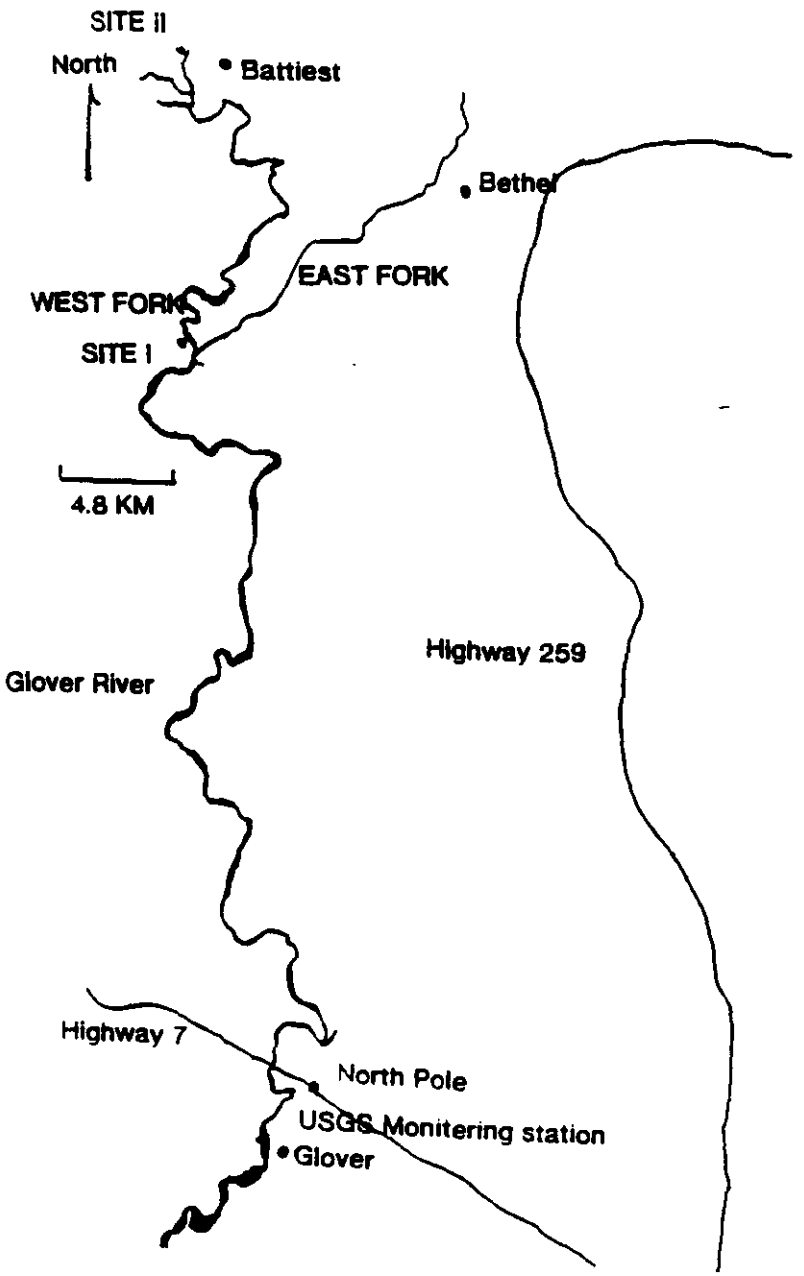

Figure 1. Location of the Glover River and the study sites for 1989 and 1990.

METHODS

Substrate Tests

I followed the technique of Fairchild et al. (1985), who used clay flower pots as substrates (substrata) diffusing nutrients. Clay flower pots were filled with 2% agar. Controls had no nutrients added (Table 1). Experimental units had 0.1 M nitrate, as $NaNO_3$ and/or 1.0 M phosphate as K_2PO_4 added to the agar, respectively. Nitrate treatments were omitted in 1991. Control substrata were set out upstream and those with nutrients downstream. After about 30 days periphyton was removed by scraping the pots with a razor blade. Known quantities of periphyton suspended in stream water were filtered through precombusted 0.7 µm GFF glass fiber filters (450 ° C) for analysis of particulate carbon (PC).. Chlorophyll a (chl. a) was also determined from known aliquots that had been removed from substrates by filtering through Millipore filters at 0.3 atm (pore size 0.45 µm).

A grab sample of stream water was obtained by submerging an acid-washed polypropylene bottle below the surface. The sample was stored on ice in the field. Temperature was measured in the field with a hand-held mercury thermometer.

The hydrogen ion concentration was determined in the laboratory with a Corning Model 7 pH meter. Turbidity was measured with a Hach Turbidometer (Model 16800). Immediately upon return to the laboratory, the sample was filtered through a 0.8 µm Millipore filter. The filtrate was split into three subsamples, which were stored in polypropylene bottles which had been cleaned with a solution of potassium dichromate-sulfuric acid. One subsample for ammonia analysis was stabilized to pH 2 with H_2SO_4 and frozen at -5 C. Samples for NO_3 and soluble reactive phosphate were frozen at -5 C.

Nutrients

Soluble reactive P (SRP) was measured spectrophotometrically on filtered samples using the molydate blue/ascorbic acid for color development (EPA, 1979). Total P was measured on

unfiltered samples following persulfate digestion and development of color as for SRP as above (EPA, 1979).

Ammonia (hereafter called NH_4 - N) was measured spectrophotometrically on unfiltered samples, using phenol-sodium citrate method of Solorzano (Wetzel and Likens, 1979). Nitrate plus nitrite (hereafter called NO_3 - N) was measured spectrophotometrically after passage of filtered water through a cadmium column and color development with sulfanilamide (Wetzel and Likens, 1979).

Biomass

Chlorophyll and samples were stored in aluminum foil in paper envelopes in the dark at -5° C until analysis. Chlorophyll a samples were analyzed using the monochromatic method in 1991-1992 and corrected for phaepigments (Wetzel and Likens, 1979). Chlorophyll a was determined by fluorescence in 1988-1990 with a Turner Model 111 fluorometer, which had been calibrated using known concentrations of chl. a (Sigma Chemical Co.).

Surplus Phosphorus

Surplus phosphorus analyses were performed on periphyton that had accumulated on the clay pot nutrient-diffusing substrates. Duplicate samples of periphyton were obtained from subsamples of biofilm taken from substrata (above) for analyses of surplus P. Biomass calculations and APA analysis were also concurrently performed, in duplicate on each substrate.

The sample used for surplus phosphorus analysis was rinsed with 40.0 ml of distilled, deionized water and gently boiled for one hour. The sample was cooled to room temperature and filtered through a pre-rinsed 1.2 µm Whatman 4.25 cm glass fiber filter. A 2.7 ml volume of supernatant was reacted in a 5 cm cuvette with 0.3 ml of mixed reagent, following Strickland and Parsons (1972), for ten minutes and then absorbed and measured at 885 nm on a Shimadzu model TB-85 spectrophotometer.

Surplus P was normalized to biomass in 1988-1989 using ash weight as a measure of biomass. Duplicate samples were filtered through a pre-muffled 1.2 µM Whatman 4.25 cm glass fiber filter and dried at 250 ° C for 24 hours. The dried filter was then weighed. The filter was then muffled for 2 hours at 450° C. The filter was re-weighed, and the difference between the dried and muffled filter was determined. This difference provided a measurement of ash-free weight of the sample. Surplus P was calculated in µm P/grams ash weight.

In 1990, the subsamples were filtered through a 0.7 µm type AA Millipore filter to obtain surplus P samples. Surplus P was normalized to chlorophyll a concentration, following Wynne and Berman (1980).

Alkaline Phosphatase Activity (APA)

Periphyton was removed from nutrient-diffusing substrates and placed into a polyethylene bottle for transport to Stillwater, and frozen, following Perrin et al. (1987). Whole water and filtered water was analyzed for total APA. Total APA includes both a dissolved fraction and a fraction associated with particulate matter or sestonic APA.

APA was measured by the hydrolysis of 100 µm 3-0-methylfluorescein phosphate (o-MFP). A 4.5 ml volume of water of concentrated cells was placed in a fluorometer tube. An addition of 100 µmoles of p-MFP in 10.0 micromolar Tris buffer was added to the sample to begin the reaction. The fluorometer tubes were sealed with parafilm, inverted, and the fluorescence was read.

APA samples were measured against Tris controls. APA was measured as the average increase in fluorescence and converted to absolute units using a standard average increase in fluorescence and converted to absolute units using a standard curve of fluorescence verses o-MFP concentration. Distilled, deionized water was used as a blank. Activities were expressed in micromoles MFP hydrolyzed per unit of biomass. Parallel fluorometric analysis of chl. a concentration was used to normalize APA values to biomass units (chl. a).

Measurement Precision

Because chl.a biomass measurements were so essential to this study, an experiment was conducted in the late winter of 1992 to learn if chl.a measurements could be made more precisely. The experimental approach was to use smaller clay flower pots (area 67 cm^2) and to immerse the entire pot in 90% acetone upon removal from the stream.

On February 18, 1992, 9 control and 9 treatment (0.1 M P) flower pots (substrates) were set into the Glover River tied to steel bars and in linear order, respectively. On March 8, 1992, substrates were removed from the river. Four substrates were selected at random from each linear sequence of controls and treatments, respectively, (two control substrates were lost) and put immediately into 90% acetone in sealed jars and transported on ice in the dark to the laboratory. The next day, each jar was sonicated for 5 minutes. Then three replicates of each sample were centrifuged and analyzed spectrophotometrically.

Periphyton was scraped off the remaining substrates and stored on ice in glass jars until the next day when ash-free dry weight (AFDW) was determined. Samples were added to preweighed crucibles and allowed to evaporate to dryness at 60 ° C. After weighing to obtain the dry weight, the crucibles were muffled at 50 ° C for 2 hours, cooled, and then reweighed to obtain the ash weight. AFDW was the difference between dry weight and ash weight.

Samples of periphyton on rocks and on the plexiglass bases of substrates and river water were also analyzed for chl.a, APA, and surplus-P. Physical-chemical parameters were also determined.

Statistical Tests

A rank transformation was performed on the biomass data in experiments 1-10 before analysis (Conover and Iman, 1981). Following transformation, a one-way ANOVA was performed to determine differences in biomass accumulation among treatments. A Tukey analysis was then used to make pairwise comparisons of treatments ($P < 0.005$).

ANOVA was performed using log transformed 1991-1992 data using SAS (1988). Then a Student-Newman Keuls multiple range test was used to determine differences between treatments. Data were not log transformed in experiments and a T-test was used to test for differences in experiments 11 and 12. In all cases the P was <0.05.

RESULTS

Substrata Tests 1988-1990

During 1988-1990, nutrient limitation was determined ten times, using the substrata technique. No nutrient limitation was found in experiments 1 and 2 using chlorophyll as a measure of biomass (Figure 2). But, P was limiting in experiment 1, when PC was used as a measure of biomass.

During March and April, 1989 (experiments 3 and 4), nitrate and phosphate treatments, respectively, had higher particulate carbon (PC) than controls at sites I and II, respectively (Figure 3). However, when biomass was measured as chlorophyll, P treatments were no different than controls, but N treatments were greater than controls at both sites.

After April, 1989, the research focus shifted entirely to site I. No nutrient limitation by P was detected by the substrata tests in June or October, 1989 (experiments 5 - 7) (Figure 4), except for N treatments in experiment 7 when chl. a was used as a measure of biomass. However, N limitation could also be demonstrated in August in experiment 6 when nitrate treatments had significantly higher PC and chl. a than controls.

Figure 5 shows results of the 1990 substrata experiments. In experiment 8 nitrate treatments were significantly greater than controls when chlorophyll a was used as a measure of biomass, but not carbon. In experiment 9, controls were lost. In experiment 10, no N treatments were significantly different than controls using either measure of biomass. Limitation by P could not be shown in 1990.

The interaction of N and P was also determined in 1990 (Figure 5). In experiment 8, the N + P treatment was significantly greater than controls for chl. a but not PC. The N + P treatments in experiment 9 were greater than either N or P alone using both measures of biomass. The control chl. a was higher than the N + P treatment in experiment 10, but control PC was not significantly different from N + P treatments.

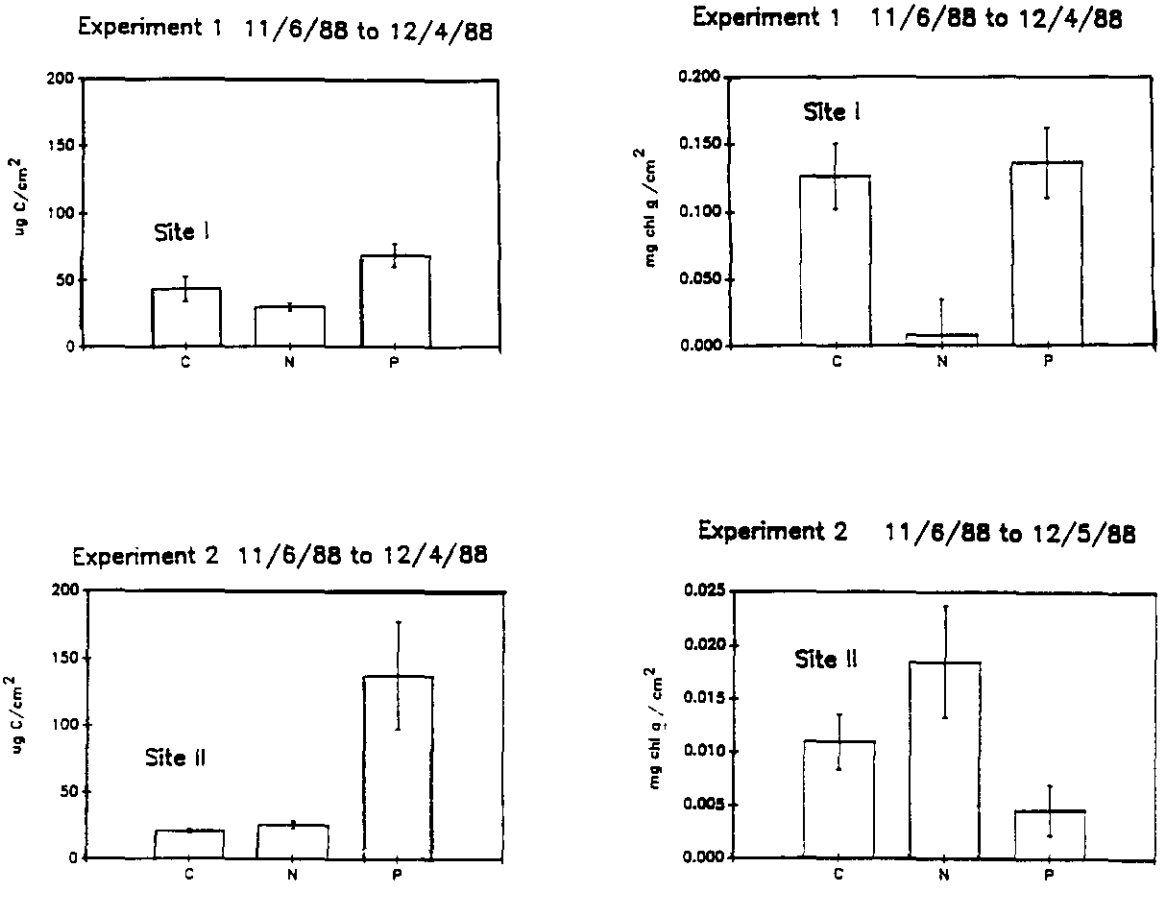

Figure 2. Biomass measured as particulate carbon and chlorophyll a 1998 in substrate experiments. C = control; N and P treatments. Error bars are Standard Error. Values for Chlorophyll a are micrograms not milligrams.

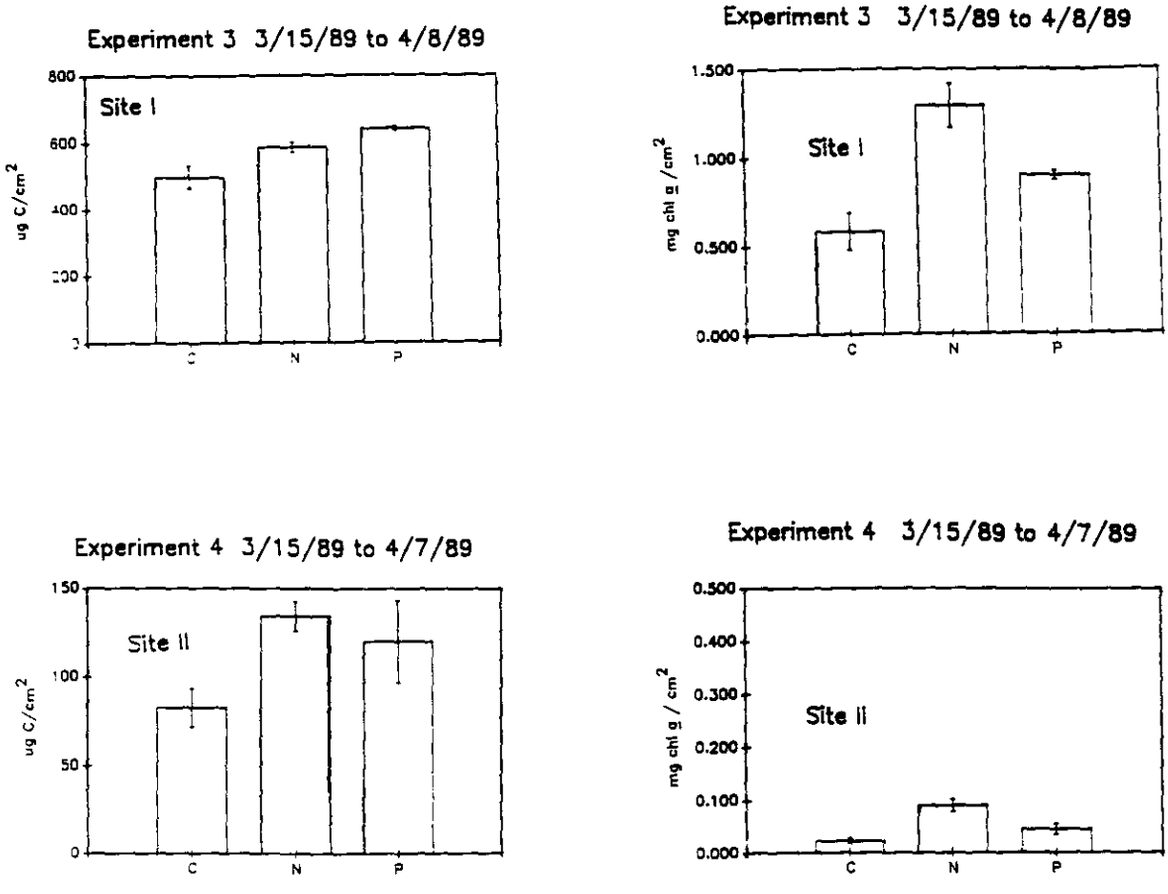

Figure 3. Biomass measured as particulate carbon and chlorophyll a 1989 in substrate experiments. C = control; N and P treatments. Error bars are one Standard Error. Values for chlorophyll a are micrograms not milligrams.

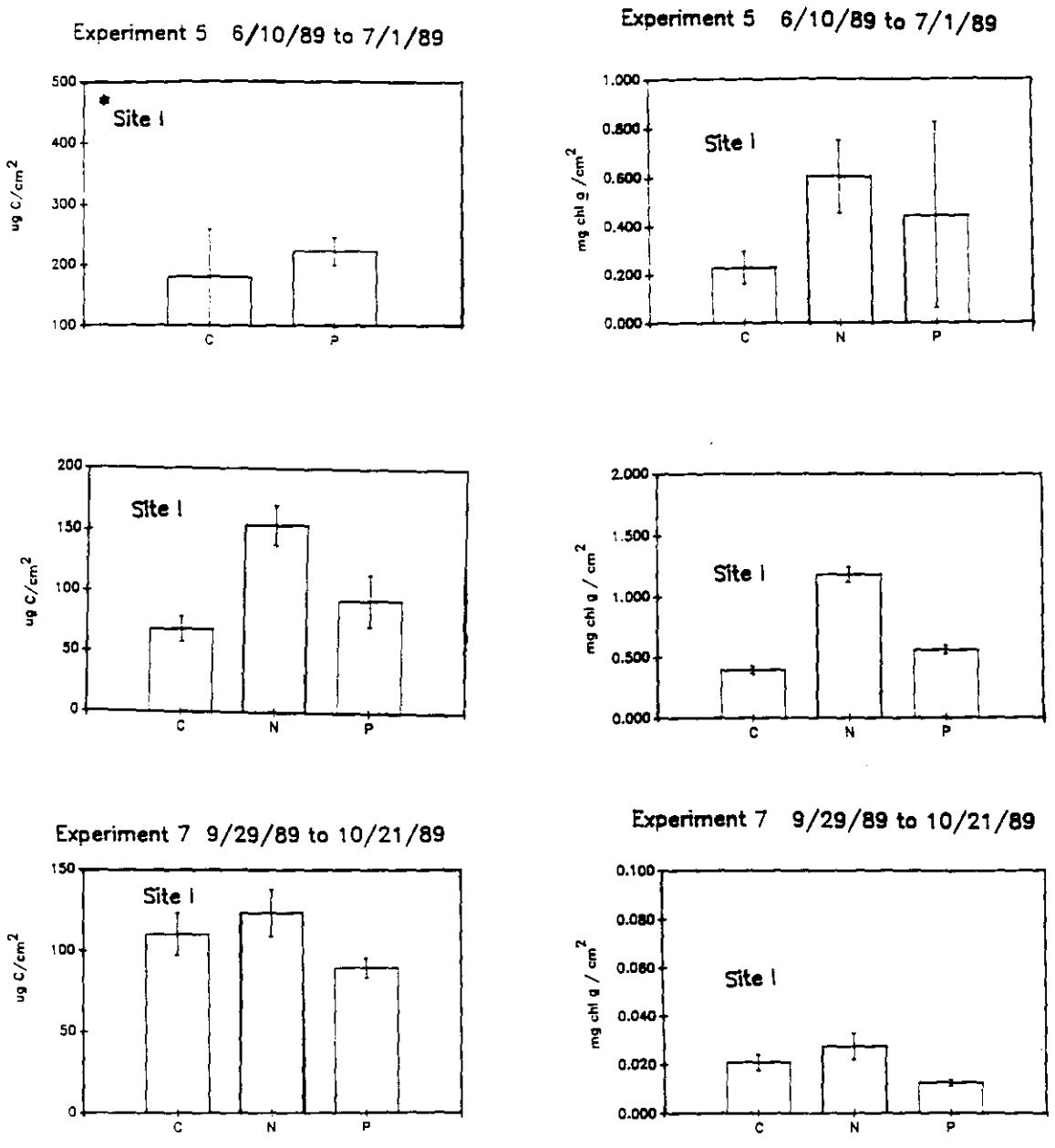

Figure 4. Biomass measured as particulate carbon and chlorophyll a 1989 in substrate experiments. *Nitrogen treatments were lost in Experiment 5. C = control; N and P treatments. Error bars are one Standard Error. Values for chlorophyll a are micrograms not milligrams.

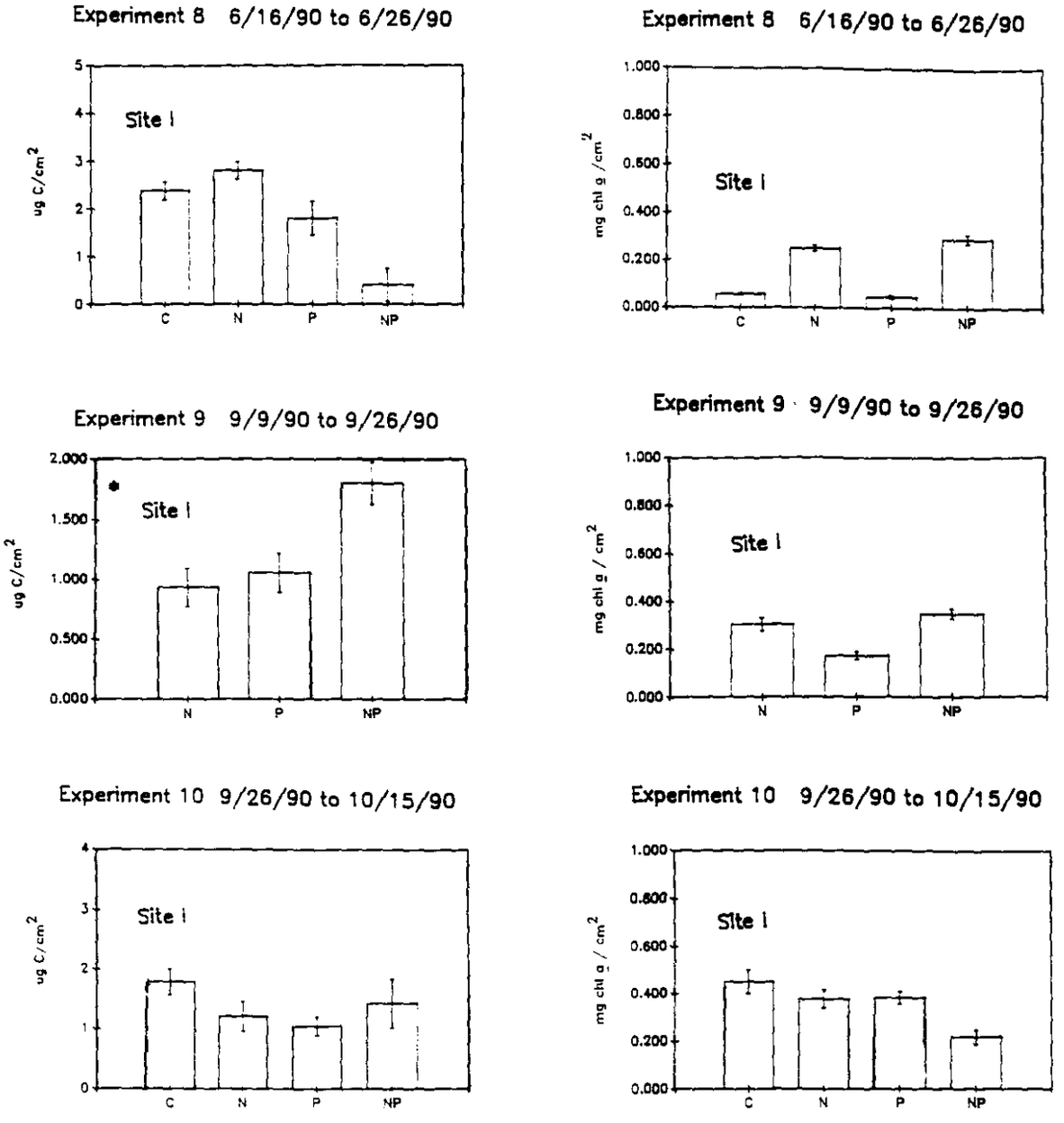

Figure 5. Biomass measured as particulate carbon and chlorophyll a 1990 in substrate experiments. *Controls were lost in Experiment 9. C = control; N and P treatments. Error bars are one Standard Error. Values for chlorophyll a are micrograms not milligrams.

In summary, substrata tests showed little evidence for P-limitation and some evidence of N-limitation.

Surplus P 1988-1990

Following the value originally established by Fitzgerald and Nelson (1966), the surplus P concentration will be considered indicative of P-limitation if the value falls below 0.08 mg P/100 mg dry weight. To apply this threshold to the Glover River, conversion of the biomass term (denominator) needs to be made.

Since chl. a is 0.5 - 2 % dry weight of algae (Reynolds, 1984), the threshold is 40-160 µg P/ mg chl. a. Ash weight as a % of dry weight in algae is 10 - 40% (Reynolds, 1984). Thus, the Fitzgerald and Nelson (1966) threshold is 2-8 µg P/mg ash weight. Surplus phosphorus concentration of periphyton accumulated on nutrient-diffusing substrates was analyzed in experiments 5-10. In experiments 5-7 all substrata (controls and treatments) had surplus P in excess of threshold values, thus indicating no limitation by P (Table 2). The expression of surplus P in terms of ash weigh was done because the large volume of sediment in subsamples made dry weight measurements impossible. The resulting data are suspect, however. Surplus phosphorus of periphyton sampled during 1990 (experiments 8-10) were below levels indicative of P-limitation, suggesting that the periphyton was P deficient (Table 2). Values of surplus P for controls in 1990 were also below the critical value in experiments 8 and 10 (Table 2).

APA 1988-1990

APA testing was performed on whole and filtered water samples, following the technique outlined by Perry (1972). No increase in fluorescence was detected for water sampled February 2, or April 4, 1990. The levels of fluorescence were so low that APA was probably not significant. APA of biofilms of control substrata in 1990 were 0.235 and 0.057 nm P/µg chl. a.min^{-1}, respectively, in experiments 8 and 10. These values are above threshold values of Healey and Hendzel (1979), thus indicating P limitation. Control substrata were not recovered in experiment 9 due to a flood.

Table 2. Surplus-P of periphyton from nutrient diffusing substrata in the Glover River. Standard deviation in parenthesis; ND = no data.

Experiment	Treatment			
	Control	N	P	N+P
		(µg P /ng ash weight)		
5	498 (156)	165 (56)	1584 (619)	ND
6	77 (15)	62 (6)	255 (181)	ND
7	18 (5)	49 (14)	205 (21)	ND
		µg P /mg chl.a		
8	2.48 (1.3)	1.0 (0.01)	2.9 (0.17)	2.67 (1.30)
9	ND	0.05 (0.01)	0.07 (0.01)	0.097 (0.02)
10	Not Detected	6.4 (4.3)	8.3 (3.7)	6.7 (4.43)

Thresholds: 0.08 mg P /100 mg dry weight;

2-8 µg P /mg ash weight;

40-160 µg P /mg chl. a

Nutrients

The concentration of soluble reactive phosphate (SRP) was extraordinarily high at all stations in late winter, 1988, possibly due to forest fertilization. Consequently, failure to observe P-limitation is not surprising. Similarly, the concentrations of SRP in March - April 1989 should have precluded P-limitation, although nitrate concentrations may have been low enough to cause the N-limitation that was observed. The N:P supply ratio (nitrate and ammonia: SRP) indicated a switch from potential N limitation to P limitation between October, 1989 and January, 1990 when it increseased for 2:1 to 640:1 (Table 3).

A summary of the 1988-1990 experiments is given in Tables 4 and 5. There was good agreement among some methods in 1989 for P-limitation. Surplus P, APA and N:P ratios indicated P-limitation in 1989-1990, but substrata tests did not. Low precision in biomass measurements on substrata was the apparent cause.

During 1991, I attempted to relate results of substrata tests to results from enzyme assays (APA) and surplus P. The hypothesis was that if these measures are useful indicators of P nutrient limitation, then an interrelationship between them should be predictable as follows:

1. If P is limiting, biomass on treatments (where P is supplied by diffusion) should exceed biomass on controls.
2. If P is limiting, APA of controls should be higher than APA of treatments. Also, surplus P of controls should be lower than surplus P of treatments.
3. APA and surplus P treatments should be inversely related to one another.

In experiment 11, a significant difference in chlorophyll biomass could not be demonstrated between controls and the 0.1 M P treatment (Table 6). However, surplus P was significantly higher and APA was significantly lower on the P treatment, confirming predictions 2 and 3 above.

Table 3. Nitrogen (NH_3+NH_4): phosphorus (SRP) ratios by atoms at Site I. + = Limitation; - = No limitation; E = Exponent, base 10.

Date	Ratio	Limitation	
		N	P
11-5-88	1: 4	+	-
12-4-88	1: 1	+	-
3-15-89	1: 24	+	-
4-7-89	1: 7	+	-
6-10-89	1: 34	+	-
7-1-89	1.68E18: 0	-	+
7-29-89	1: 3	+	-
8-19-89	2: 1	+	-
9-29-89	2: 1	+	-
10-21-89	2: 1	+	-
1-27-90	640: 1	-	+
2-23-90	80: 1	-	+
6-16-90	184: 1	-	+
6-29-90	448: 1	-	+
7-19-90	323: 1	-	+
9-9-90	9.5E17: 0	-	+
9-26-90	92: 1	-	+
10-15-90	2.31E18: 0	-	+

Table 4. Evidence for P-Limitation.

Year	Exp.	Difference from Control				
		N:P Ratios	Chl.a Biomass	PC Biomass	Surplus P	APA
1988	1	no	no	yes		
	2	no	no	no		
1989	3	no	no	yes		
	4	no	no	no		
	5	yes	no	no		
	6	no	no	no		
	7	no	no	no		
1990	8	yes	no	no	yes	yes
	9	yes	lost	lost	no data	no data
	10	yes	no	no	yes	yes

Table 5. Evidence For Nitrogen Limitation.

Year	Exp.	Difference from Control		
		N:P Ratios	Chl.a Biomass	PC Biomass
1988	1	yes	no	no
	2	yes	no	no
1989	3	yes	yes	no
	4	yes	yes	yes
	5	no	no data	no data
	6	yes	yes	yes
	7	yes	yes	no
1990	8	no	yes	no
	9	no	lost	lost
	10	no	no	no

Table 6. Summary of two 1991 experiments in the Glover River, Oklahoma, to test relationships among parameters indicating phosphorus limitation. Units are biomass as ng chlorophyll a per cm^2, alkaline phosphatase activity (APA) as nM P/µg chl. a x min. x 10^{-3} and surplus P as µg P/ mg chl. a. Values in parentheses below means are 95% confidence intervals.

	Chlorophyll a	Surplus P	APA
Experiment 11			
Control	190.7	2.5 limited	24.9
	(151.6-229.8)	(0.2-5.1)	(20.5-29.3)
0.1 M P	342.0	210.9 *	10.3 *
	(-2.3-686.4)	(-19.3-441.2)	(-1.28 to 21.9)
Experiment 12			
Control	674.3	12.9	9.4
	(492.0-856.7)	(10.1-15.6)	(7.22-11.51)
0.1 M P	675.7	52.0 *	3.07 *
	(426.9 to 924.6)	(35.5-68.4)	(1.68 to 4.4)
1.0 M P	1033.4	138.0 *	0.602 *
	(869.4 to 1197.5)	(18.8-275.2)	(-0.35 to 1.559)
Threshold		> 40-160	> 50

Notes: Experiment 11, July 25 - August 11; Experiment 12, August 11 - 29.

* = significant difference

In experiment 12, biomass was not significantly higher on either treatment compared to the control (Table 6). As in experiment 1, surplus P was higher and APA lower on both the 0.1 and 1.0 M P treatments, compared to the controls, respectively. Thus, surplus P and APA results are consistent with the hypothesis that supplying P should increase the former and decrease the latter. Moreover, as expected, surplus P of all treatments exceeded literature threshold values above which algae are thought to be P-replete (storing P) (Table 6). When algae were P replete on treatments, they would not be expected to have high APA. Indeed, this was the case, since the APA of treatments was always lower than APA threshold values for P-limitation (Table 6). This shows the treatments were not P limited, again, as expected.

The APA and surplus P of controls should reflect potential nutrient limitation by naturally occurring river periphyton. The surplus P of controls was always less than the surplus P threshold, implying control periphyton were P limited (Table 6). The APA of controls was less than the APA threshold, suggesting control periphyton were not P limited. The thresholds used here were not developed for biofilms but for phytoplankton, so their use may be suspect.

An additional experiment (13) in the winter of 1992 attempted to learn if some of the variability in biomass measurements could be reduced by submerging substrata in acetone. The chl.a data revealed a difference between the P treatment and controls ($F = 7.06$, $Pr > F = 0.0377$). However, within both controls and treatments, there were also significant differences between individual substrata (Table 7). In particular, control substratum A was significantly different from controls B, C, and D. Likewise, treatments A and B were significantly different from treatments C and D.

Table 7. Student-Newman Keuls test for differences within controls and treatments, 1992 data. Underlined substrata were not significantly different.

Control ANOVA

Source	df.	Sum of Squares	F	Pr > F
Model	3	0.15381209	4.96	0.031
Error	8	0.08264096		
Total	8	0.23645305		

log chl.a/substrate	2.0253	1.817	1.799	1.734	
	A.	C.	B.	D.	Control substrata

Treatment ANOVA

Source	df.	Sum of Squares	F	Pr > F
Model	3	0.1578059	25.88	0.0002
Error	8	0.00162611		
Total	8	0.01740671		

log chl.a/substrate	2.0556	2.0554	1.98671	.9823	
	C.	D.	B.	A.	Treatment substrata

Table 8 shows the coefficients of variation (CV) of replicated analyses of individual substrates. For controls, the CV was as high as 39% and as low as 1.9%. Excluding the high CV for control 2, the CV was generally less than 7.5%. These data indicate that when using the best spectrophotometric techniques a precision of less than 5% is obtained when subsampling a homogeneous periphyton sample. Thus, if whole substrates are subjected to 90% acetone treatment and sonication, reasonable precision can be expected.

Table 8. Results of 1992 substrata tests for biomass, n=3. Units are micrograms chl. a/substrate. Mean is in parentheses.

Control			Treatment		
Sample	chl.a	CV(%)	Sample	chl.a	CV (%)
A	105		A	95	
	109			97	
	105			95	
	(106)	1.9		(96)	1.0
B	35		B	100	
	81			94	
	73			96	
	(63)	39.0		(97)	3.2
C	60		C	117	
	68			107	
	69			116	
	(66)	7.5		(114)	4.8
D	53		D	111	
	57			113	
	53			117	
	(54.2)	4.9		(114)	2.7

The problem with the substrate technique is that substrate-to-substrate differences within a treatment are highly variable. For example, control A had a CV of only 1.9%, but was significantly different than other controls. Actually control A was twice as high as control D. Differences of this sort cannot be attributed to any one cause, but they impose a practical limitation to use of the nutrient diffusing substrate technique in that many replicates are required to account for substrate-to-substrate variability.

The mean ash-free dry weight (AFDW) for controls was 1.755 mg/substrata (SE = 0.509, CV = 29%) and treatments 8.382 mg/substrata (SE = 1.192, CV = 14%). A t-test showed that treatment AFDW was greater than controls (t = 4.656, df = 7, P = 0.002).

Sestonic APA

Table 9 shows a partitioning of APA between the dissolved fraction (that passing a 0.45 µm membrane filter) and whole water fraction (unfiltered). All of the APA was in the dissolved fraction on August 29, 1991, and February 18, 1992. But, only 33% was dissolved on August 11, and 67% was dissolved on July 25, 1991.

Nutrients and physical/chemical data for experiments 11 - 13 are given in Tables 10 and 11.

Table 9. APA and chlorophyll a in grab samples of water from the Glover River 1991-1992. APA as nM P/min/μg chl.a. All other values are μg/1. Standard deviations are in parentheses.

	APA			chl.a
Experiment 1	Whole Water	Dissolved	% dissolved	
July 25, 1991	0.324	0.216	67	1.883
	(0.002)	(0.023)		
Aug 11, 1991	5.9397	1.9793	33	0.338
Experiment 2				
Aug 29, 1991	3.7013	3.7489	100	0.231
				(0.038)
Experiment 3				
Feb 19, 1992	0.0695	0.0658	100	5.9
	(0.0005)	(0.004)		(2.58)

Table 10.Concentration of nutrients in the Glover River, July and August 1990. Nutrients are in units of micrograms, l^{-1}. Standard deviations are in parentheses.

Date	pH	Turbidity NTU	SRP	TP	Nitrate-N	Ammonia-N
July 25	6.4	15.2	1.505	134	16	30
			(0.116)	(32)	(1.0)	(9.2)
Aug. 11	6.7	9.7	0.048	517	34	30
			(0.034)	(301)	(0.8)	(5.1)
Aug. 14	6.8	12.5	0.017	25	42	42
			(0.000)	(0)	(20.0)	(3.2)
Aug. 29	6.5	14.9	0.011	no	9.23	104
			(0.000)	data	(0.7)	(1.4)

Table 11. Physical-chemical characteristics of Glover River on February 18 and March 8, 1992. Standard deviations in parenthesis. All values are μg liter $^{-1}$ except turbidity, ND= not detected.

Date	Temp. °C	chl.a	SRP	TP	NO_3-N	NH_4-N	pH	Turbidity NTU
Feb. 18	14	No data	ND	ND	39 (0.3)	407 (28)	6.32	2.31
March 8	15	5.9 (2.58)	0.3 (0.6)	ND	40 (0.3)	205 (34)	6.45	6.57

DISCUSSION

The substrata (clay pot) technique was not always useful in identifying almost certain limitation by P in the Glover River. However, other measures of P limitation (surplus P, N: P supply ratios, and APA) did indicate P stress. Serious problems of measuring biomass may be one reason that the substrata technique was apparently insensitive in 1988-1990. When chl. a was measured for whole substrates submerged in 90% acetone, a significant difference was detected between controls and treatments, and the precision of replicates were usually reduced to less than 7.5% (Table 8). This still does not solve the problem of large variation between substrata that sometimes occurs. The source of the latter variation is not known, but may be due to effects of positioning of substrata in the current.

Aside from problems with measuring biomass, other explanations are possible to explain why substrata tests failed to demonstrate P limitation. Low light in this turbid river may have been the major factor affecting growth. This fact combined with short incubations could have resulted in biomasses so low that difference between controls and treatments were not detected. Still,

differences were observed between N treatments and controls, so perhaps the results of substrata tests accurately reflect the P status of periphyton. If so, external P concentrations and/or rapid recycling in the biofilm were adequate to meet the needs of periphyton in this river.

Biofilms should be viewed as communities of algae, bacteria, and fungi. Organisms in biofilms are limited in their ability to use compounds outside their biofilm boundary by diffusion kinetics (Riber and Wetzel, 1987). Biofilms are thought to have rapid internal recycling of nutrients, which may be another reason they do not respond to external concentration changes. Further, algae in biofilms are thought to be P limited, while bacteria are not because bacteria are better competitors for P. Thus, while the use of these techniques together revealed P-limitation in experiments 11 and 12, much more must be known before any one of them can be used alone to predict the potential for P-limitation or before thresholds can be developed for use with biofilms.

Part of the difficulty in interpreting data such as these is that the history of nutrients in the stream is limited to grab samples taken 14 days apart (before and after the experiment). Second, the growth history of the biofilm on these substrates is unknown. Different growth rates could result in quite different biofilm thickness and species composition even in the same stream, so standardization of exposure times for nutrient diffusing substrata may not be possible.

Still, the use of several techniques has an advantage. If all measures indicate nutrient limitation, the case for that limitation can be more sure than if only one is used. Some measure of biomass must be measured. Since APA and surplus P must be normalized to some measure of biomass, the easiest method for most monitoring might be to measure chl. a.

The substrata technique has some shortcomings aside from obvious problems of high variability of biomass on substrata. In particular, readers are cautioned on its use in strongly disturbed streams such as Glover River. In these systems researchers should test for light limitation and the ability of substrata to continue to supply nutrients during high discharge (Lowe, et al., 1986).

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Figure 3. Biomass measured as particulate carbon and chlorophyll a 1989 in substrate experiments. C = control; N and P treatments. Error bars are one Standard Error. Values for chlorophyll a are micrograms not milligrams.

Figure 4. Biomass measured as particulate carbon and chlorophyll a 1989 in substrate experiments. *Nitrogen treatments were lost in Experiment 5. C = control; N and P treatments. Error bars are one Standard Error. Values for chlorophyll a are micrograms not milligrams.

Figure 5. Biomass measured as particulate carbon and chlorophyll a 1990 in substrate experiments. *Controls were lost in Experiment 9. C = control; N and P treatments. Error bars are one Standard Error. Values for chlorophyll a are micrograms not milligrams.

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