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A Micro-Bioassay for Epilithon using Nutrient-Diffusing Artificial Substrata

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ABSTRACT

A modification of the nutrient-diffusing substrata method was used to evaluate nutrient and invertebrate grazer effects on attached epilithic algae in an arctic river. An acid-cleaned porcelain or fused silica disk was melted into the opening of a small plastic, agar-filled vial, supplemented with water soluble materials. These materials were such that they would have an impact on the biomass and/or productivity of the autotrophic and/or heterotrophic communities colonizing hard surfaces in rivers and lakes. The vials were independent replicates (3-6/treatment), providing many more treatments per unit cost and unit effort than other methods currently employed. After 3 week incubation periods in the river, we not only showed that this method works very well, we also were able to demonstrate that the river is limited by phosphorus and nitrogen, thereby confirming the results of previous assays. This method would also allow for the use of, nutrients, xenobiotics, antibiotics, and/or organic substrates to be used to enhance or minimize the effects on autotrophs or heterotrophs in complex communities.

INTRODUCTION

In this paper, we describe an in situ nutrient-diffusible artificial substrata method that has been miniaturized. This system allows many replicates of numerous treatments to be used with a minimum of time and cost as compared to other methods in the literature (Fairchild and Lowe 1984, Fairchild et al. 1985, Pringle and Bowers 1984, Lowe et al. 1986). Other studies addressing the factors controlling or modifying the biomass, growth, and species composition of attached epilithic algal/bacterial communities in rivers and lakes have examined the role of nutrient concentration, nutrient supply rates and ratios (Tilman et al. 1982), nutrient storage (Stevenson and Stoermer 1982) and grazers (Steinman et al. 1987, Lamberti et al. 1987). In the field, researchers determining these interactions have used methods of enclosing a substratum and its associated biota in a chamber, then modifying that environment and examining the effect. Significant contributions to the study of periphyton nutrient bioassays have been made with the development of the diffusible-substrata flower pot technique (Fairchild and Lowe 1984, Fairchild et al. 1985). This method is inexpensive, in both labor and costs, in comparison to other studies, as well as allowing more replicates at more stations. As an in situ method, it allows colonization at ambient environmental parameters (light intensities, etc.). While sample surface area is not limiting, the samples have to be removed physically by abrasion before responses can be measured. Because of the heterogeneity in stream current within the microhabitats, when the flower pots are placed on boards or spikes, the variance between/among pots within a given treatment can be high. On a single flower pot, the location of the subsample represents a wide gradient in both current velocity and light, as a function of its height above the substratum and its position relative to the direction of the current or the sun. The mounting method and size of the pots also mean that the large surface area of the chambers were exposed to current. In one experimental study, 23 of 96 (24%) flower pots were lost due to storm events (Lowe et al. 1986). Although samples are incubated under natural sunlight, surface irradiance may vary due to position and size of pots. This heterogeneity may simulate a natural rock, but could also allow greater variance in composition than a uniformly flat surface (Stevenson 1983, Stevenson 1984).

METHODS

All field studies were conducted on the Kuparuk River, a meandering fourth order tundra stream, located on the north slope of the Brooks Range, Alaska (68°38' N, 149°36' W) in the Toolik Lake/Kuparuk River Arctic Long Term Ecological Research (LTER) site. The river contains a population of arctic grayling with an average length of 33 cm (Peterson et al. 1983, Peterson et al. 1985), and is low in soluble phosphate all season. In addition, nitrification at the stream margin increases the soluble nitrate concentration as the summer progresses. The river bottom is characterized largely by cobble and boulder coverage originating from the glacial till that covers this Pleistocene landscape (Miller et al. 1986). These experiments were carried out in three 3-week periods during the ice free season (June-Aug.) of 1986 in which river conditions varied from low to high flow (Table 1).

To measure algal responses to nutrient supplements, small agar-filled substrata supplemented with nutrients were placed on the river bottom. Each chamber was composed of a 10 dram plastic vial (Dynalab Corporation #2636-0010) used as a reservoir, filled with various nutrient-supplemented agar treatments, and sealed with a coarse, porous porcelain or fused silica (2.6 cm diameter disc) crucible cover (Leco Corporation #528-042). Since they were relatively chemically inert, they were cleaned by soaking in a 10%

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HCl solution for 48 hr, with a final copious rinsing with distilled water. Each vial was filled with 37 ml of a 2% (w/v) Difco Ultrapure Agar solution augmented with one each of the following treatments: 1) control (CON) (plain agar); 2) humic acid extract plus phosphorus (H&P) (2 gm humics/L and 0.5ml conc. HCl plus 0.005 moles K_2HPO_4/L); 3) phosphorus (P) (0.005 moles K_2HPO_4/L); 4) ammonia (N) (0.05 moles NH_4Cl/L); 5) phosphorus plus ammonia (N&P) (0.005 moles K_2HPO_4/L and 0.05 moles NH_4Cl/L); 6) vitamins (VIT) (B1 0.1 mg/L, plus Biotin 5 mg/L); and 7) a trace metal mixture (T.M.) (Woods Hole formula plus 0.0999g NTA/500ml as a chelator, Stein 1973). All agar/nutrient mixtures were autoclaved to ensure sterility. Individual discs were heated on a hot plate and sealed into the top of a filled plastic vial by melting the plastic and molding it around the disc. The vial was then turned upside down, allowing the agar mixture to solidify in contact with the porous disc. Finished vials were capped and color coded according to the treatment they contained.

TABLE 1. Physical/chemical/nutrient characteristics of the Kuparuk River during experimental batches, 1986.

		BATCH 1 Jun 27-Jul 18	BATCH 2 Jul 11-Aug 1	BATCH 3 Jul 21-Aug 8
SRP	mMoles/L	0.09	0.17	0.08
NH_4^+	mMoles/L	1.03	0.90	0.79
NO_3^-	mMoles/L	4.29	0.83	0.99
CONDUCTIVITY	mMhos/cm @ 25°C	22	23	23
ALKALINITY	meq/L	0.296	0.16	0.252
pH		6.83	6.96	7.14
TEMPERATURE	Degrees C	12.07	9.52	8.57
OXYGEN	mG/L	10.2	9.46	10.4
STAGE @ WEIR	cm	17.13	53.21	68.14
DISCHARGE	m^3/sec	1.11	5.93	7.79
DEPTH @ STATION	cm	30.48	35	53
CURRENT	cm/sec	13	25	31

Six replicates of each of the 7 treatments were used per batch. Wooden holders comprised of 61 cm lengths of 5 cm x 10 cm lumber with 3 cm diameter holes drilled through, and attached to a piece of plywood 1.2 M x 31 cm, served as the means of anchoring the vials to the river bottom. The vial assemblies were attached to these holders with a small spot of silicon sealant on the bottom of the vials. A very small amount was used to minimize any possible effects from acetic acid leaching from the sealant. The wooden holders were placed on the river bottom using two restraints, a rope running between opposite shores and looped through a hole on the upstream side of the wooden base; and two metal spikes at each end of the board, driven through the base and into the rocky bottom, with flat rocks placed over the stakes at each end. This ensured that even during periods of high flow, the boards remained stationary on the river bottom. The boards containing the agar vials were left in place, undisturbed, on the river bottom for a period of 3 weeks. Three such batches were incubated over the short arctic summer (June 27-Aug. 5) with an overlap of 1 week between batches (Batch 1 = June 27-July 18, Batch 2 = July 11-Aug. 1, and Batch 3 = July 21-Aug. 8). At the end of the requisite time period, the entire board was removed from the river bottom, kept submerged, and carefully maneuvered near the shore. The vials were removed one at a time by color code, and the disc removed into individual pre-labeled specimen containers (plastic 125 ml urine cups) by gently squeezing the mouth of the vial with pliers. Samples to be assayed for chlorophyll a were stored with no additional water, while samples to be analyzed for primary productivity had approximately 50 ml of river water added to the container in order to completely submerse the disc. Samples were then returned to the field lab for subsequent analysis.

Of the six replicates for each treatment, three were used for biomass analysis. In order to obtain biomass estimates, the disks were first scrubbed and the resultant material, as well as the entire disk, was used for the extraction procedures. Biomass, estimated as chlorophyll a, was monitored after a 24 hr extraction in 20 ml of 90% acetone in darkness at 4° C. The amount of chlorophyll a was quantified using a calibrated fluorometer (Turner 111) with a 1 cm light path and the method of Strickland and Parsons (1968). Phaeopigments were estimated by the reduction in fluorescence with mild acid (1 drop 10% HCl).

Primary productivity was assayed on the remaining three discs of each treatment by a modified $s^{14}C$ technique. The discs were left in the specimen containers, and the volume of each brought up to 100 ml with river water. The orientation of the disc (top vs bottom) was then corrected. It should be noted that we experienced no loss of attached epilithic mat by our handling techniques. Each sample was then injected with 0.2 ml ^{14}C-bicarbonate (18 µCi/ml), and incubated at constant temperature (10°-12° C) and light (500 footcandles, 51.7 µEinsteins $cm^{-2}*sec^{-1}$), for approximately four hr, which is comparable to a normal sunny day at the river location). At the end of the incubation period the discs were scrubbed (with an electric screwdriver to which a toothbrush head was attached) to remove all attached algae, and a well-mixed 20 ml aliquot was filtered through a Gelman A/E 25 mm glass filter. The filter was placed into a 10 ml scintillation vial and allowed to dry before sealing. Samples were later counted on a Packard Tri-Carb 460 C liquid scintillation system, utilizing channel ratio quench correction. From the dpm's obtained, primary productivity was calculated based on the equations of Wetzel and Likens (1979).

The vials were also checked for diffusion rates of PO_4^{-3} and NH_4^+ into distilled water, utilizing both new and used vials. Samples were taken every 36 hr from continuously stirred beakers of distilled water in which the top of the vial (containing the porous disc) was immersed. These samples were analyzed for NH_4^+ and PO_4^{-3} with standard spectrophotometric techniques.

RESULTS

The suitability of these porcelain discs as a substratum for algal growth is equivalent to the results obtained in a comparative study of the areal colonization of Teflon, glass beads, nylon mesh, and scrubbed rocks (Hullar and Vestal, U. Cincinnati, personal communication). The rate of colonization after 14 days was proportional to surface roughness, declining from, porcelain, rock, beads, mesh, and Teflon, in that order. The colonization rate on porcelain discs was linear with time for 24 days. Smoother surfaces, like rock and beads, were colonized almost exponentially during the second 10 days (Hullar and Vestal, U. Cincinnati, personal communication). Thus, for the purposes of this method, the time of harvest was not as critical since colonization of rough surfaces has less of a lag time than smooth surfaces, which may cause difficulties during the formation of a microbial glycocalyx required for algal colonization (Stevenson 1984, and M. Lock, U. North Wales, personal communication).

TABLE 2: Two way ANOVA of chlorophyll biomass by treatment and seasonal batch in Kuparuk River. Student-Newman-Keuls multiple range test shows the significant treatments and seasonal variations (batch) ($p<.05$).

2-WAY ANALYSIS OF VARIANCE DEPENDENT VARIABLE: CHLOROPHYLL

SOURCE	DF	ANOVA SS	F VALUE	PR > F
BATCH	2	1.159	5.91	0.0055
TREATMENT	6	6.865	11.65	0.0001
BATCH*TREATMENT	12	6.989	5.93	0.0001
ERROR	42	4.123		

STUDENT-NEWMAN-KEULS TEST FOR VARIABLE: CHLOROPHYLL. MEANS WITH IDENTICAL UNDERLINE ARE NOT SIGNIFICANTLY DIFFERENT. UNITS ARE - ug Chlor/cm^2.

TREATMENT n=9

1.232	0.702	0.555	0.332	0.297	0.26	0.257
N&P	HUM&P	P	CONTROL	VITAMINS	METALS	N

BATCH (SEASONALITY) n=21

0.693	0.502	0.362
1 (EARLY)	2 (MID)	3 (LATE)

TABLE 3: Two way ANOVA of primary productivity assay by treatment effects and seasonal batch in the Kuparuk River. Student-Newman-Keuls multiple range test shows the significant treatments and seasonal variations (batch) ($p<0.5$).

2-WAY OF ANALYSIS OF VARIANCE DEPENDENT VARIABLE: PRIMARY PRODUCTIVITY

SOURCE	DF	ANOVA SS	F VALUE	PR > F
BATCH	2	3.587	1.48	0.2389
TREATMENT	6	61.329	8.44	0.0001
BATCH*TREATMENT	12	7.605	0.52	0.8873
ERROR	42	50.842		

STUDENT-NEWMAN-KEULS TEST FOR VARIABLE: PRIMARY PRODUCTIVITY. MEANS WITH IDENTICAL UNDERLINE ARE NOT SIGNIFICANTLY DIFFERENT. UNITS ARE - ug Chlor/cm^2.

TREATMENT n=9

3.682	2.7	2.066	1.34	1.06	0.965	0.833
N&P	HUM&P	P	CONTROL	VITAMINS	METALS	N

BATCH (SEASONALITY) n=21

2.07	1.858	1.492
1 (EARLY)	2 (MID)	3 (LATE)

The results of the sample diffusion experiments indicated that in general, nutrient diffusion decreased with time, and decayed as a negative exponential as found by Pringle and Bowers (1984) and Fairchild et al. (1985). Rate of release of ammonia decayed as a function of time calculated by the equation: $\log(\mu m\ NH_4^+ * cm^{-2} * day^{-1}) = -.0845 * Days + 0.725$ ($r^2 = .915$, df = 6). After 21 days, the calculated rate of release of NH_4^+ was 2% of the initial rate. However, only a calculated 7% of the NH_4^+ initially contained in each vial was released from the agar in that period. Rate of release of phosphate decayed similarly, as predicted by the equation: $\log(\mu m\ PO_4^{-3} * cm^{-2} * day^{-1}) = -.058 * Days + 0.608$ ($r^2 = .792$, df = 6). After a 21 day incubation period the calculated rate of release was 7% of the initial rate, however, 77% of the amount initially in the vial had been released.

In river experiments, Phosphate, and PO_4^{-3} + humic acid developed the highest biomass as estimated by chlorophyll a in the early July batch. As the inorganic nitrogen became more limiting in mid and late July, however, the $NH_4^+ + PO_4^{-3}$ treatment developed the highest biomass. Trace metals, vitamins, and NH_4^+ alone exhibited no significant differences when compared with the control vials.

A 2-way ANOVA of total chlorophyll a extracted was significantly different between treatments, by seasonal batch and by treatment*batch interactions (Table 2). Student-Newman-Keuls multiple range test showed that $NH_4^+ + PO_4^{-3}$ additions were statistically significant from other treatments. The late July batch (#3) showed N+P stimulation and was statistically more significant than in the early July batch (#1) (Table 2).

The assay of primary production showed significant differences between both the $NH_4^+ + PO_4^{-3}$ and the Humic + PO_4^{-3} treatments when compared with the remaining treatments (Table 3). However, the seasonal batches were not different statistically, although the mean of all treatments was greatest for the late July batch.

In our static bioassay for primary productivity, $NH_4^+ + PO_4^{-3}$ and Humic + PO_4^{-3} treatments exhibited the greatest productivities (Figure 1). At our site, primary production appeared to be more variable than the biomass measurements. Thus, assimilation ratios ranged more widely, from 2 to 15 ($\mu g\ C * cm^{-2} * h^{-1}$)/($\mu g$ chl a $* cm^{-2}$) in control samples. In general, the higher the chlorophyll a, the lower the assimilation ratio.

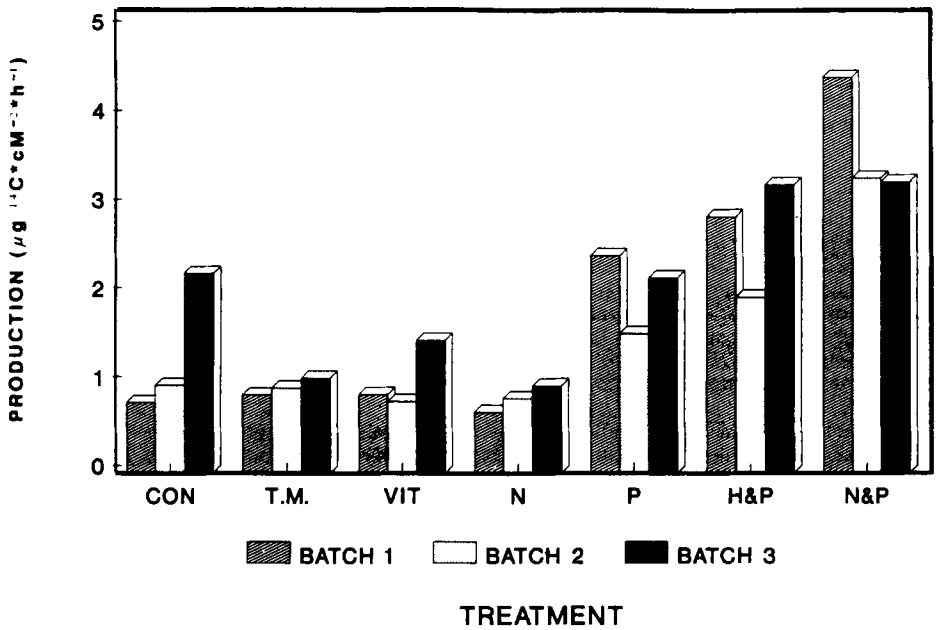

FIGURE 1: Primary production assay (mean n=3) to added nutrients, for 3 batches, over the 1986 ice free season in the Kuparuk River.

DISCUSSION

In the Kuparuk River, the nutrient-diffusing, artificial substrata method confirmed the observations of earlier model stream assays (Peterson et al. 1983, Hullar 1986) showing acute phosphorus and nitrogen limitation. River discharge did not reduce accumulated epilithic algal biomass, even in the fastest current during near record mid-season discharge. The algal biomass developed as a function of thaw days, inversely

correlated with current velocity, and directly correlated with temperature of the water (Table 1). The limitation found was related to trace metals and phosphate in the early summer, and to both phosphate and ammonia in mid and late summer. The concentration of phosphate in the river declined seasonally as discharge increased (Table 1).

The method described offers several improvements over other methods using artificial substrata. First, the physical handling of the substratum is greatly reduced since the disc is removed from the vial directly into the container in which analysis will be performed. Second, the discs are all the same size, thereby eliminating any experimental error brought about by the non-uniform scrubbing of smaller sub-areas of a larger substrata. Third, the vials are small enough to allow many replicates and treatments to be used simultaneously. Only 2 out of 504 vials (0.4%) were lost due to river flow and experimental handling in a larger 1986 study, even though they were exposed to a very high discharge event in mid-July. Finally, all the replicates used in the same area are statistically independent so that the potential pseudoreplication found in bioassay tubes, or in subsampled flower pots, was not possible (Peterson et al. 1983).

Recent work of Pringle (1987) has compared the diffusible sand substratum in a flowing-water bioassay similar to that of Peterson et al. (1983). Her study demonstrated that the response of attached algae was to both, nutrients from overlying water, and from nutrient-diffusing substrata. Differences in colonization were based on the changes of nutrient concentration in water over time.

The in situ methods normally carried out, which include whole stream and/or river treatments, are upstream/downstream comparisons that lack statistical independence (Hurlbert 1984). Fertilizing a whole river with nutrients or organic substrates is costly, and perhaps inappropriate for assessing algal responses (Miller et al. 1986, Warren et al. 1964, Elwood et al. 1981).

With this method, numerous possibilities exist as to the experimental manipulations that can be carried out. Many nutrient types can be studied without the expense and time involved with trying to manipulate an entire river. In addition, invertebrate grazer effects on algal production can be studied by utilizing insecticides to inhibit their colonization of the substratum. This has already been successfully accomplished by the authors.

Thus, this inexpensive bioassay system is a significant first step in evaluating the probable influence of many factors on the attached algal and microbial community in rivers and streams.

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