An In Situ Substratum Fertilization Technique: Diatom Colonization on Nutrient-Enriched, Sand Substrata

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Using an in situ substratum enrichment method, we assessed the effects of nutrient release from sand substrata on the community structure of attached diatoms in a sand-bottomed, northern Michigan stream. Sand from the stream bottom was washed, sterilized, and then consolidated into small plastic petri dishes with agar solutions enriched with various concentrations of $NaNO_3$ and KH_2PO_4. Laboratory estimates of nutrient release rates were performed over a 144-h period with uncolonized substrata having different agar and nutrient concentrations. Release rates decreased 10-fold in an exponential fashion with no significant differences between replicates. Agar concentrations had no significant effect on release rates, although the rates were proportional to nutrient concentrations in the substratum. Racks of substrata were installed parallel to the stream current flow and retrieved after a 6-wk colonization period. Using multiple comparisons of treatment means for both nutrients and algal taxa, we found no differences between control and NO_3 enrichments; significant differences were found, however, between PO_4 and $NO_3 + PO_4$ (N:P = 25:1) treatment means relative to each other and the control. Diatom biovolume was two times as great on PO_4 treatments and four times greater on $NO_3 + PO_4$ treatments. Effects of PO_4 and NO_3 enrichment appear to be taxa specific, with PO_4 and $NO_3 + PO_4$ treatments favoring *Navicula* and *Nitzschia* spp. Control and NO_3 treatments were dominated by *Cocconeis placentula* Ehr. and *Achnanthes minutissima* Kutz.

En utilisant une méthode in situ d'enrichissement du substrat, les auteurs ont évalué l'incidence de la mobilisation des bioéléments du substrat sablonneux sur la structure de la communauté de diatomées fixées dans un cours d'eau à fond sablonneux du nord du Michigan. Le sable prélevé du fond a été lavé, stérilisé et mélangé dans de petits plats de pétri avec des solutions d'agar enrichies de diverses concentrations de $NaNO_3$ et de KH_2PO_4. En laboratoire, on a quantifié pendant 144 h les taux de mobilisation des bioéléments de substrats non colonisés contenant différentes concentrations de bioéléments et d'agar. Les taux de mobilisation ont baissé par un facteur de 10 de façon exponentielle, sans différences significatives entre les doubles. Les concentrations d'agar n'avaient aucune incidence significative sur les taux de mobilisation quoique les taux étaient proportionnels aux concentrations de bioéléments dans le substrat. Des grilles de substrat ont été installées parallèles au courant et récupérées après une période de colonisation de 6 sem. En utilisant des comparaisons multiples des moyennes de traitement obtenues pour les bioéléments et les diverses espèces d'algues, les auteurs n'ont découvert aucune différence entre le groupe témoin et le substrat enrichi de NO_3 ; par contre, ils en ont trouvé entre les moyennes des groupes enrichis de PO_4 et de $NO_3 + PO_4$ (N:P = 25:1) et entre ces groupes et le témoin. Le volume de diatomées était deux fois et quatre fois plus élevé en présence de PO_4 et de $NO_3 + PO_4$ respectivement. L'incidence du PO_4 et du NO_3 semble être spécifique ; ainsi, l'addition de PO_4 et de $NO_3 + PO_4$ semble favoriser *Navicula* et *Nitzschia* spp. *Cocconeis placentula* Ehr. et *Achnanthes minutissima* Kutz. peuplaient principalement les traitements témoins et les traitements au NO_3.

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In terrestrial ecosystems, the spatial distribution of many plant species has been closely correlated with the spatial distribution of soil nutrients (Pigott and Taylor 1964; Hanawalt and Whittaker 1976; Tilman 1982). Although the gross contribution of nutrients from marine and lake sediments to the primary production of overlying waters has received wide attention (Golterman et al. 1969; Fitzgerald 1970; Entsch et al. 1983), little has been published about the relationship between substratum fertility and species composition of attached periphyton. Observations that certain periphyton taxa reach high numbers on potentially nutrient-rich substrata have led several investigators to postulate a nutritional relationship between substratum and attached diatom communities (Cholnoky 1968; Linkins 1973; Lee et al. 1975). C. M. Pringle (University of Michigan, Ann Arbor, MI, unpubl. masters thesis, 1979) observed dense mats of *Navicula tripunctata* (O. Mull.) Borey and *Nitzschia linearis* (W. Sm.) covering larval chironomid tubes, similarly suggesting that a nutritional benefit was being derived by diatoms from the metabolic activities of the larval occupants.

Periphytic taxa locating themselves in nutrient-rich microenvironments have an adaptive advantage (Hellebust and Lewin 1977) that is especially significant in stream systems where spatially dependent nutrient reutilization is important (Ball and Hooper 1963; Short and Maslin 1977). If nutrients can be directly taken up by periphyton before becoming diluted in the water column and transported downstream, such tight coupling would represent a lower spatial limit within the framework of "nutrient spiralling" (Webster 1975; Newbold et al. 1981; Elwood et al. 1981a, 1981b).

Experimental field studies relating stream periphyton species composition and biomass to nutrient enrichment have been confined to direct nutrient additions to flowing water by whole-stream enrichment (Huntsman 1948; Warren et al. 1964; Elwood et al. 1981b) or through utilization of flow-through systems (Stockner and Shortreed 1978; Peterson et al. 1983). The substratum fertilization technique we describe here is introduced as a new in situ method to manipulate periphyton communities experimentally. To evaluate this technique we first examined NO_3 and PO_4 release rates from sand–agar substrata and the effects of agar concentration on release rates. Field studies then assessed effects of NO_3 and PO_4 singly and in combination on diatom biovolume and community composition in a northern Michigan stream.

Materials and Methods

Field portions of this study were conducted in Carp Creek, a second- to third-order stream located entirely on The University of Michigan Biological Station tract, Cheboygan County, MI. This sand-bottomed stream is spring-fed, exhibiting a constant discharge and relatively stable temperatures (10 ± 3°C in July). The stream is alkaline and nutrient poor, with nutrient levels during the study period averaging 4 $\mu g\ PO_4 \cdot L^{-1}$, 35 $\mu g\ NO_2 + NO_3N \cdot L^{-1}$, and 33 $\mu g\ NH_3N \cdot L^{-1}$. Carp Creek flows ~2.6 km to Burt Lake through an undisturbed wooded area composed mainly of conifers (*Thuja occidentalis*, *Pinus strobus*, and *Tsuga canadensis*).

Sand collected from the streambed was washed and autoclaved to kill attached microorganisms and then consolidated into plastic disposable petri dishes (5.2 cm diameter, 1.2 cm deep), with agar solutions enriched with various concentrations of $NaNO_3$ and KH_2PO_4. Each dish contained ~40 g of sand and 10 mL of agar solution. Agar and sand were thoroughly mixed and compacted such that liquid agar filled all interstitial spaces between sand grains. After solidification, the substratum was scraped flush with the lip of the petri dish, creating a hard, flat surface for periphyton colonization.

A laboratory experiment, in a three-level hierarchical design, was performed to characterize substratum nutrient release rates and to evaluate general effects of agar, NO_3, and PO_4 concentrations on rates of release. Twelve wide-mouth 4-L glass jars were acid rinsed, filled with 3 L of distilled water, and then placed in an incubator–shaker (Psychrotherm, New Brunswick) at 10°C. Artificial substrata placed within incubated jars were made with three different agar concentrations: 2.0, 2.5, and 3.0%. Within each of these treatments there were two different combinations of NO_3 and PO_4 concentrations (0.5 mol $NO_3 \cdot L^{-1}$:0.5 mol $PO_4 \cdot L^{-1}$ and 1.5 mol $NO_3 \cdot L^{-1}$:1.5 $PO_4 \cdot L^{-1}$), with two replicates per combination. Mixing of the water column above the substratum was achieved through the shaking action of the platform within the incubator. At time intervals of 0, 4, 8, 12, 24, 36, 49, 60, 72, 97, 120, and 144 h, the water in each jar was also vigorously stirred, after which a 20-mL water sample was removed with an acid-cleaned beaker. Samples were frozen and stored in acid-cleaned polyurethane bottles and analyzed for PO_4 and NO_3 using a Technicon II autoanalyzer (APHA 1975). Soluble reactive phosphate and nitrate were measured utilizing the molybdenum blue technique and the cadmium reduction technique, respectively.

In substratum construction for field experiments, 2.0% agar solutions were utilized. Agar solutions in three sets of plates were respectively enriched with 0.5 mol $NaNO_3 \cdot L^{-1}$, 0.5 mol $KH_2PO_4 \cdot L^{-1}$, and 0.5 mol $NaNO_3 \cdot L^{-1}$ + 0.02 mol $KH_2PO_4 \cdot L^{-1}$ (N:P = 25:1). A fourth set of control plates contained pure agar with no nutrients. Treatments consisted of three replicate substrata that were glued to pine boards (6.0 × 60.0 cm) and anchored with wire to the sand streambed of Carp Creek in an open sunny site during June–July of 1982 (Fig. 1). To minimize the variability of physical factors affecting substrata, we placed boards in a median range of current velocities (28–34 $cm \cdot s^{-1}$) and depths (10–15 cm below the water surface), such that the surfaces of the sand–agar plates were parallel with the current flow. Current velocities were measured with a standard Pygmy Gurley meter.

Previous studies in Carp Creek (C. M. Pringle, unpubl. data) indicated that periphyton biovolume reaches an asymptote after a colonization period of about 6 wk. Coring of our artificial sand–agar substrata has shown that diatoms are not located in sediment below the upper 3 mm of substratum during the day. Because of these observations, substrata were retrieved from the stream after a 6-wk colonization interval, and the top 3 mm of agar and sand was carefully cut out with a razor blade. This layer was placed in a 50-mL beaker and diluted with 25 mL of distilled water. The sample was then mixed for 10 min with a magnetic stirrer and decanted into a 100-mL beaker. The remaining sand grains were rinsed with 25 mL of fresh distilled water and the suspension again decanted into the 100-mL beaker. This procedure resulted in almost complete periphyton separation from sand grains, as evidenced by microscopic scanning of residual grains. Samples (0.4 mL) were then filtered (0.22-μm Millipore® filter) and subsequently cleared with immersion oil to allow algal cell enumeration under a compound microscope (1000×).

Diatoms comprised >90% of the periphyton biovolume on all treatments and counts were restricted to diatom taxa. At least 500 diatom frustules per slide were counted. The cell volume

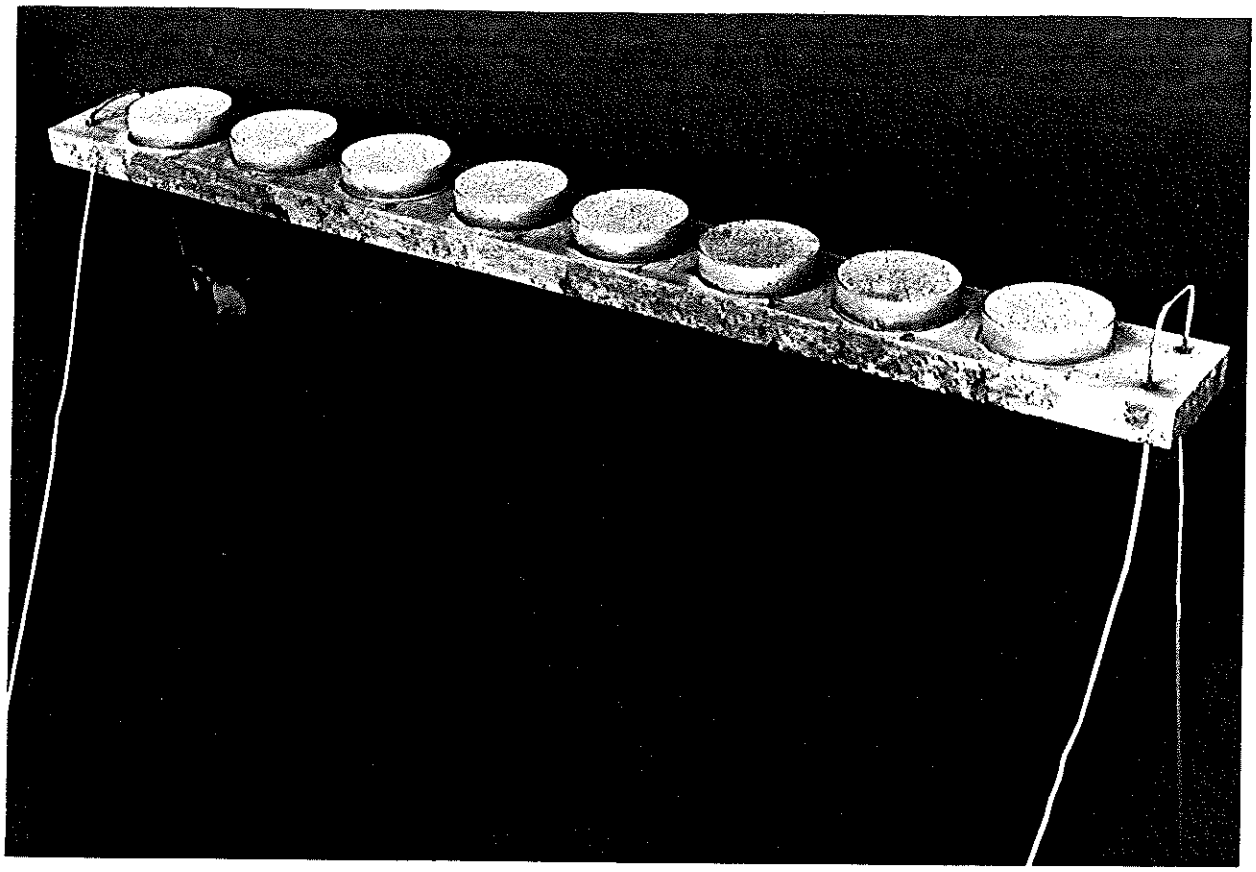

FIG. 1. Enriched sand–agar substrata consolidated into plastic petri dishes and mounted on a pine board.

of specific taxa was estimated by entering mean diatom measurements into geometric equations that best describe the three-dimensional shape of the frustule. Diatom cells were measured with an ocular micrometer and substage adjustment to determine length, width, and height. A minimum of 25 cells per diatom taxon has proven adequate in determining biovolumes (C. M. Pringle, unpubl. data).

Results

The laboratory nutrient release experiment indicated that release rates of NO_3 and PO_4 decreased exponentially through time. A 3 × 2 factorial ANOVA (three levels of agar, two levels of NO_3 and PO_4, two replicates per cell), based on the mean release rates through the 144-h time interval, indicated that agar concentrations of 2.0, 2.5, and 3.0% did not significantly affect NO_3 and PO_4 release rates ($P < 0.01$). However, those substrata having the 1.5 mol $NO_3 \cdot L^{-1}$ + 1.5 mol $PO_4 \cdot L^{-1}$ concentrations had release rates significantly higher than plates with the 0.5 mol $\cdot L^{-1}$ concentrations of these same nutrients ($P < 0.01$). NO_3 and PO_4 release rates representative of this experiment and those agar and nutrient concentrations used for the field experiments are illustrated in Fig. 2. N:P ratios of release rate were not significantly correlated ($r = -0.12$, $P < 0.01$) at N and P concentrations used, suggesting that N:P ratios during release either remain constant or vary in a random fashion.

Diatom analysis of substratum plates after a 6-wk colonization period in Carp Creek indicated that the NO_3 + PO_4 treatment (N:P = 25:1) supported almost four times the biovolume (57.43×10^7 $\mu m^3 \cdot cm^{-2}$) found on control substrata (14.68×10^7 $\mu m^3 \cdot cm^{-2}$; Fig. 3). The 0.5 mol $PO_4 \cdot L^{-1}$ treatment supported a biovolume only two times greater (28.92×10^7 $\mu m^3 \cdot cm^{-2}$) than the control whereas the 0.5 mol $NO_3 \cdot L^{-1}$ treatment alone supported only a slightly higher biovolume.

To assess results of pilot field studies, we used two-way ANOVA, and both treatment effects and the interaction term were highly significant ($P < 0.001$). Multiple comparisons (Student–Newman–Keuls test, $P = 0.05$) of treatment biovolume means for both nutrients and algal taxa indicated no difference between the control and NO_3 enrichments. However, the PO_4 and NO_3 + PO_4 additions were significantly different from each other and the first two treatments (control and 0.5 mol $NO_3 \cdot L^{-1}$).

The control and NO_3 treatments were dominated by *Cocconeis placentula* Ehr. and *Achnanthes minutissima* Kutz., whereas the PO_4 and NO_3 + PO_4 treatments were characterized by a predominance of motile pennate diatoms (e.g. *Navicula* and *Nitzschia* spp.; Fig. 3). Taxa biovolume means fell into three different groups: (1) *A. minutissima* and *C. placentula*, (2) *Navicula* spp., and (3) *Nitzschia* spp. and the "other" category. The significant interaction term indicates that enrichment with PO_4 alone and NO_3 + PO_4 produced taxa-specific effects. Addition of PO_4 triggered a fourfold increase in *Navicula* and a threefold increase in *Nitzschia* whereas *A. minutissima* and *C. placentula* biovolumes were not significantly different from the control. The NO_3 + PO_4 treatment resulted in a greater than 10-fold increase in *Navicula* and *Nitzschia*, with *A. minutissima* and *C. placentula* displaying significantly lower biovolumes relative to the control.

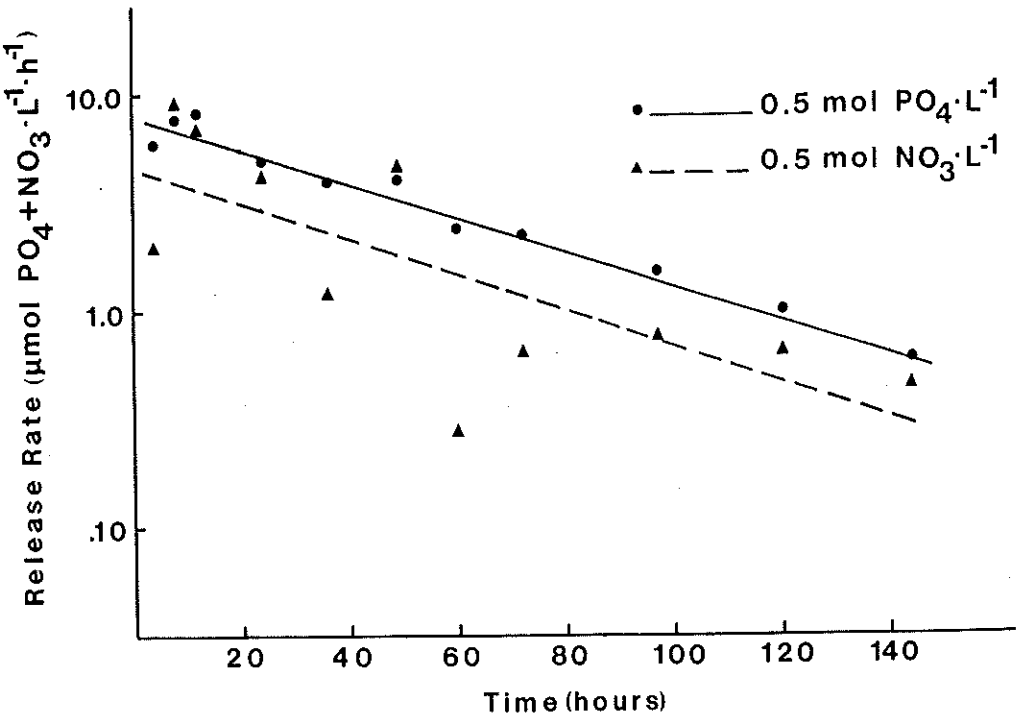

FIG. 2. Mean phosphate (PR) and nitrate (NR) release rates from enriched, 2.0% agar substrata into distilled water at 10°C. Linear regressions of release rates versus time on a semi-logarithmic scale indicated an exponential rate decrease. For PO_4, ln PR = $-0.02T + 2.09$ where $r^2 = 0.97$, $s_b = 0.01$, and $n = 11$. For NO_3, ln NR = $-0.02T + 1.48$ where $r^2 = 0.58$, $s_b = 0.01$, and $n = 11$.

Discussion

Our technique detected taxa-specific effects that were similar to previously observed distribution patterns on potentially nutrient-rich natural substrata in Carp Creek (C. M. Pringle, unpubl. data). *Navicula* and *Nitzschia* spp. dominated the diatom assemblages covering clumps of decomposing mayfly (*Baetis vagans*) exuviae and the larval retreats of the chironomid *Pseudodiamesa* cf. *pertinax* (Garrett) in Carp Creek. The tubes of *P. pertinax* are composed of consolidated sand grains and support high diatom biovolumes (112.9×10^7 $\mu m^3 \cdot cm^{-2}$). *Achnanthes minutissima* and *C. placentula* were the dominant diatom taxa on wood substrata unmodified by chironomid tubes and were likewise predominant on unenriched control and NO_3 treatments.

Alteration of N:P ratios also resulted in differences in species composition that were proportionally similar to results obtained in nutrient enrichment studies of other oligotrophic streams (Stockner and Shortreed 1978; Peterson et al. 1983). In these studies, increases in NO_3 alone had no appreciable effects on algal growth. Likewise, preliminary results from Carp Creek indicate no significant differences between diatom biovolume on control and NO_3 treatments. Additions of NO_3 + PO_4 (N:P = 25:1) resulted in almost four times the biovolume of the control whereas addition of PO_4 alone resulted in a twofold increase. Similarly, Stockner and Shortreed (1978) found that trebling concentrations of both NO_3 and PO_4 (N:P = 35–40:1), over ambient concentrations, resulted in greatest algal growth. They likewise observed that when PO_4 alone was trebled, thereby reducing the N:P ratio to 10–13:1, maximum growth was only a little greater than half that seen when both N and P were trebled. Peterson et al. (1983) found that PO_4 addition alone and PO_4 + NH_4NO_3-N enrichment resulted in significantly higher levels of chlorophyll *a* and CO_2 uptake (from six to eight times that of control).

In contrast with previous studies in which nutrients were added at a constant rate into flowing water, release rates from our artificial substrata decrease exponentially through time. This pattern of exponential decay is similar to nutrient release rate curves observed for naturally decomposing substrata such as leaves (Hynes and Kaushik 1969; Howarth and Fisher 1976; Elwood et al. 1981b). In the laboratory, release rate curves reached an asymptote within 6 d, with an estimated 78.5% P and 82.3% N left in substrata. Greater diatom biovolume observed on nutrient-enriched substrata after a 42-d colonization period indicated significant long-term effects of in situ enrichment.

The method we describe here displays reproducible results and its compact design facilitates implementation in the field. Also, it more realistically reproduces the texture of natural substrata as compared with artificially barren substrata such as glass slides. This latter, widely used substratum has been found to select against filamentous and loosely attached metaphytonic species with a higher stature than tightly adhering forms (Brown 1976). Since release rates from artificial sand–agar substrata were found to be independent of agar concentration, physical manipulation of the substratum is possible. Agar concentrations can be manipulated to affect the firmness of the substratum surface and hence its resistance to biogenic influences such as burrowing by chironomid larvae. Artificial substrata can also be enriched with a variety of substances, and substance-specific release rates ascertained. This may allow distinctions to be made between periphyton community response along a resource ratio gradient to specific elements within the gross array of materials that are found in and often added to natural systems.

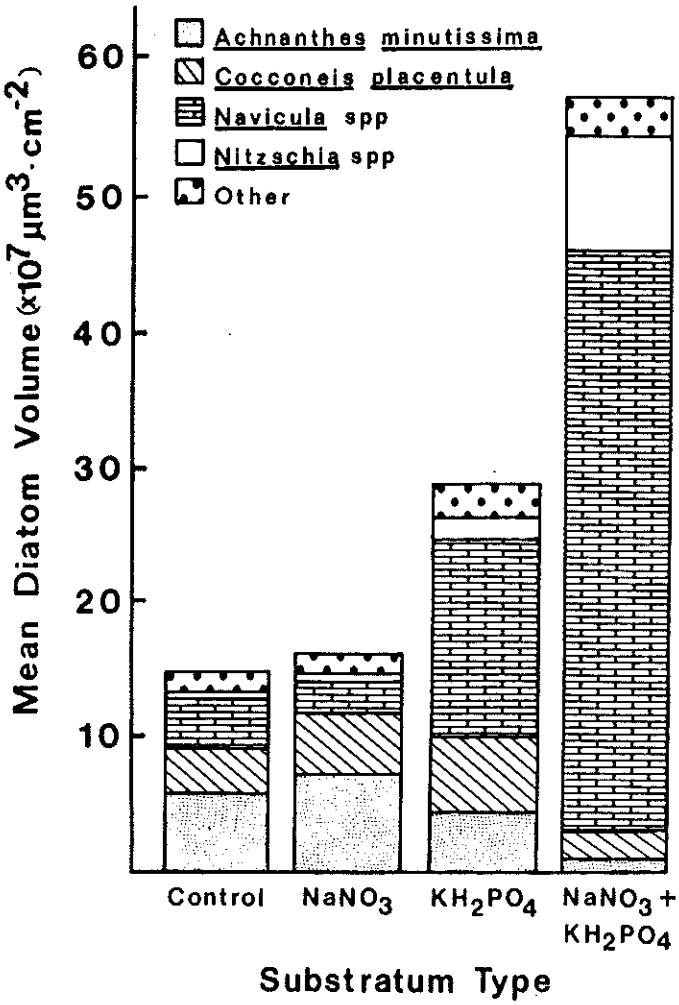

FIG. 3. Relative biovolume abundance of diatoms colonizing sand–agar substrata in Carp Creek. $NaNO_3$ substratum type = 0.5 mol $NaNO_3 \cdot L^{-1}$, KH_2PO_4 substratum type = 0.5 mol $KH_2PO_4 \cdot L^{-1}$, and $NaNO_3$ + KH_2PO_4 substratum type = 0.5 mol $NaNO_3 \cdot L^{-1}$ + 0.02 mol $KH_2PO_4 \cdot L^{-1}$ (N:P = 25:1).

For example, many vitamins, amino acids, and other organic materials influence the growth and metabolism of periphyton, but species and community effects have yet to be elucidated. Lastly, when used in conjunction with methods of direct fertilization of the water column (Stockner and Shortreed 1978; Peterson et al. 1983), this substratum enrichment technique will enable closer examination of the relative contributions of nutrients from substrata and the water column to periphyton community structure.

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