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Direct Comparison of Phosphate Uptake by Adnate and Loosely Attached Microalgae within an Intact Biofilm Matrix

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We report a direct comparison of phosphate uptake by adnate and loosely attached microalgae in an intact biofilm matrix, with resolution at the level of individual cells. Track scanning electron microscope autoradiography enabled assay of [^{33}P]phosphate uptake from the overlying water by adnate algae left undisturbed on mature leaves of the macrophyte *Potamogeton illinoensis* or on artificial plant mimics. The epiphyte communities developed in either phosphate-poor or moderately phosphate-enriched water, and they were assayed on both natural and artificial plants. All adnate taxa examined from both natural and artificial plants in both habitats took up significantly less radiolabel when assayed beneath the overlying matrix than when they were exposed to the water upon removal of the overstory material. Track scanning electron microscope autoradiography and track light microscope autoradiography were intercalibrated to enable comparison of [^{33}P]phosphate uptake by adnate and loosely attached components of the epiphyte matrix. Loosely attached cells on substrata from both habitats took up significantly more radiolabel than did underlying adnate cells, indicating that access to phosphate supplies from the water depended on the position of microbial cells in the matrix. In this short-term assay, the adnate microalgae were relatively isolated from the water column nutrient source.

Among the most significant contributors to total production in aquatic ecosystems are the benthic microorganisms that grow attached to sediment, rooted plants, and other surfaces (4, 27, 37). Benthic microflora mediate mineral cycling between nutrient-rich substrata and the overlying water (7, 32). Despite their importance, the metabolism of these organisms is poorly understood because available assay techniques have required either consideration of all the microorganisms and debris collectively or separation of the cells from the associated surface (34). The former approach does not isolate the response of the microbial components—bacteria, algae, fungi, and animals. Moreover, the metabolic response of the microbes in certain assays, such as phosphate uptake, is difficult to interpret because a major portion of the uptake may actually represent adsorption of the ions to debris (25). The latter procedure, physical separation of the microbes from the surface, results in complete disruption of the chemical microhabitat within the biofilm matrix and, often, damage to or death of the cells (6, 15).

Matrices of benthic microbes in aquatic systems are analogous to biofilm layers from many other habitats (8, 19). The cells exist within a 90 to 95% hydrated matrix of secreted-excreted glycoproteins and polysaccharides (8, 10, 12, 35) together with small animals and copious biotic-abiotic debris (1, 4). The matrix can be 2,000 μm or more in thickness and consists of an adnate component—microflora with the major cell axis in direct contact with the substratum—and a loosely attached component that grows away from the surface toward the water (5) (Fig. 1). The polysaccharides in the matrix provide resistance to diffusion, sites for phosphatases and other excreted enzymes, protection of these enzymes from proteolytic attack, and general capacity for ion adsorption (3, 18, 23). Dye studies indicate that aquatic communities of benthic microbes are relatively isolated from the water column by a thick surrounding boundary layer of quiescent water through which ions may pass only by slow diffusion (18, 31, 41). These isolating effects would be particularly acute for adnate microflora at the base of the matrix that are in contact with the substratum surface but at a substantial distance from the overlying water relative to the size of the cells.

We hypothesized that nutrient acquisition for microbes within a biofilm matrix depends on the position of the cells in the layer. The objective of this research was to test this hypothesis with techniques that would enable evaluation of phosphate uptake from the surrounding water by adnate versus loosely attached microalgae under conditions approaching those in situ. We used a novel technique, track scanning electron microscope (TSEM) autoradiography, to accomplish direct, cellular-level assays of [^{33}P]phosphate uptake by epiphytic adnate algae intact on leaves of aquatic plants. Further, calibration of TSEM autoradiography with the widely used track light microscope (TLM) autoradiography enabled comparison of [^{33}P]phosphate uptake by adnate and loosely attached epiphytes, respectively, from assays of the community as an intact, undisturbed matrix.

MATERIALS AND METHODS

Site description. Communities of epiphytic microalgae were sampled from Lawrence Lake, a phosphorus-limited hard-water lake of glacial origin located in Barry County, southwest Michigan (22, 33). Both phytoplankton and epi-

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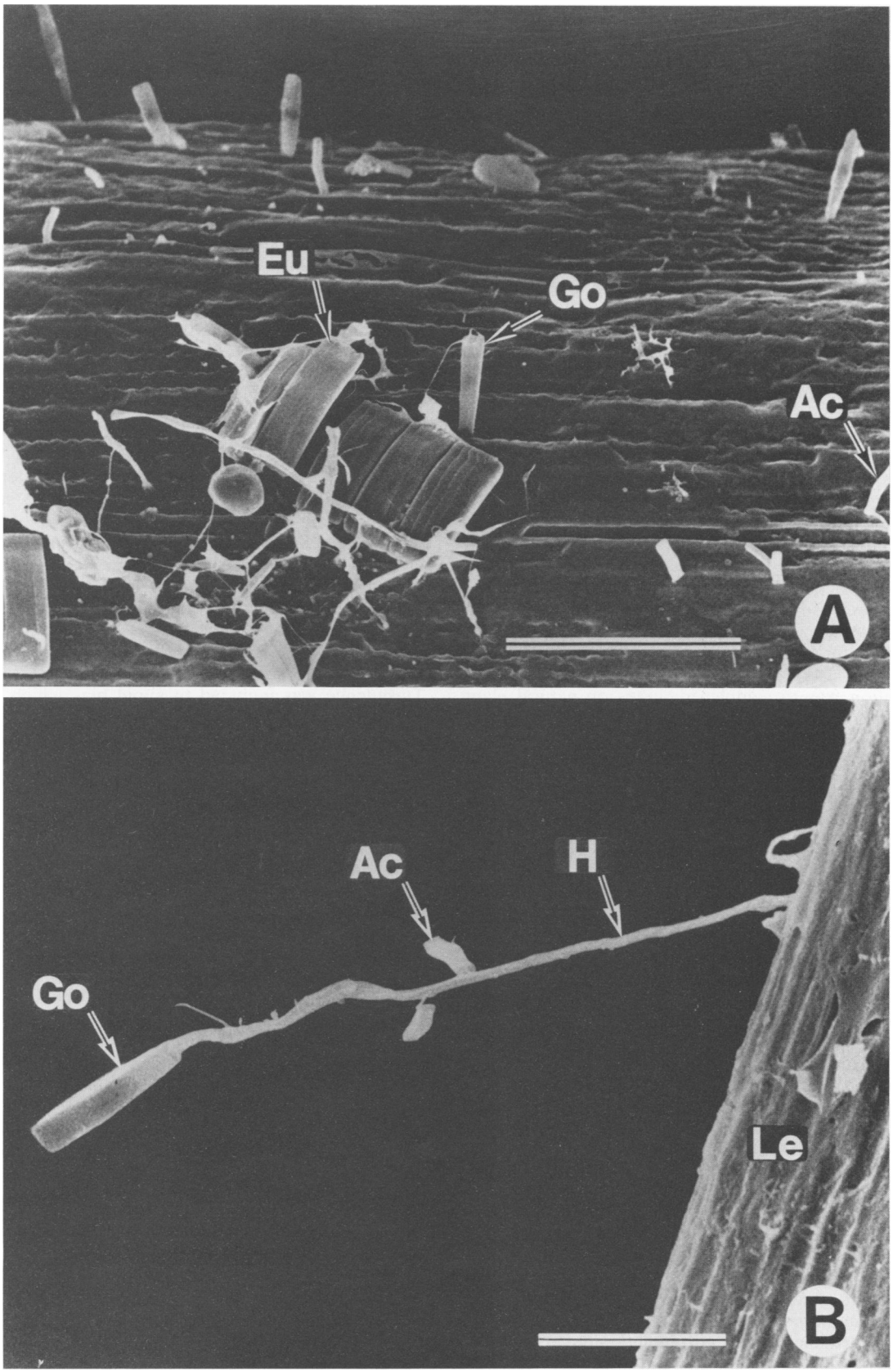

FIG. 1. Differences in the position of microalgal colonizers on a plant leaf. (A) Adnate epiphytes (major cell axis in direct contact with the leaf substratum) and pedunculate epiphytes (connected to the plant tissue by a short stalk) near the leaf surface, including diatoms *Eunotia* (Eu), *Gomphonema* (Go), and *Achnanthes* (Ac). Bar = 75 μm. (B) Algae extended out from the plant leaf (Le), including *Gomphonema* sp. (Go) on a long mucilaginous holdfast (H) and *Achnanthes* sp. (Ac). These algae are considered loosely attached because of their distance from the plant surface; they remained on this leaf surface after the overlying matrix (1,400 μm in thickness) had been removed. Bar = 25 μm.

phytic algae are P limited during the macrophyte growing season (June through September [6, 23]). The research area lay at a depth of 1.3 m within a sheltered region that was relatively protected from wind activity and extensive turbulence (4). The community structure of the epiphytes has been characterized in detail (4, 5). Approximately 98% of the littoral zone (depth, 0.5 to 5.0 m) contained abundant growth of submersed macrophytes, including the subdominant *Potamogeton illinoensis* Morong (33). New *Potamogeton* plants germinated from mid-April to early May. Growth was apical, and older leaves were maintained throughout the growing season.

Sampling design and assay procedure. Epiphyte communities which had developed for approximately 3 months during the macrophyte growing season were sampled from mid-August to mid-September. We compared two types of sites with similar macrophyte growth to test whether water column P enrichment increased phosphate uptake by the adnate or loosely attached epiphytes. In control sites the water column was unaltered, and total phosphorus was ≤ 4 μg liter^{-1} during the experiments. In a second group of sites approximately 20 m from the control areas, the water was enriched in phosphate to simulate mesotrophic conditions of 30 to 40 μg of total phosphorus liter^{-1}. The water column enrichment was accomplished by dissolution of small quantities of slow-release phosphorus pellets (Sierra Chemical Company, Milpitas, Calif.), added at a distance of 0.25 m from the plants for 16 to 24 days before the epiphytes were sampled.

Our intention was to sample epiphytes on natural plant substrata, approximating in situ conditions for epiphyte metabolism. The microalgae had 4- to 50-fold-higher surface area-to-volume ratios and high affinity for phosphate, relative to cells of the plant leaves (5, 6, 22). However, we recognized that the macrophyte might present a confounding factor by supplying phosphorus to the epiphytes (22, 26), particularly to adnate microalgae at the base of the matrix. Accordingly, in each sampling site we introduced artificial *Potamogeton* plants during emergence of the natural macrophytes in May, so that natural and artificial leaves were available to epiphyte colonization for a similar duration. This enabled us to compare phosphate uptake by epiphytes on substrata that potentially leached versus did not leach phosphorus to the matrix. Artificial structural plant leaves were obtained from plastic aquarium plants (Hygrophila; Living World Aquascapers, Metaframe Corporation, Compton, Calif. [5]). A small hole placed at the base of the artificial leaves facilitated alternate placement at suitable height along flexible acrylic rods. The leaves and rods were soaked for 24 h in a solution of dilute hydrochloric acid (1 N) to leach adsorbed phosphorus. The substrata were then rinsed 20 times in water which had been purified by successive filtrations (Millipore Super Q system) and were allowed to soak in ultrapure water for 12 h before being placed in the lake. The acrylic rods were anchored through small holes in lids of plastic freezer storage containers that had been filled with pure sand. We positioned the artificial substrata in the lake among abundant growth of natural macrophytes by burying the storage containers in the sediment.

Natural and artificial leaves with intact epiphyte matrices were carefully collected underwater and were transported to the laboratory for immediate assay. Collection jars had been acid cleaned and filled with filtered (0.22-μm-pore-size Millipore GS filters) pelagic water (containing <2 μg of total phosphorus liter^{-1}) 12 h before use. Each leaf sample was carefully checked for signs of epiphyte matrix disruption, which was especially important to avoid for intact treatments. The sample was discarded if any matrix cracks, other disruption, or sloughed epiphytes were apparent. Epiphyte communities from both natural and artificial leaves were contained within cohesive matrices and were dominated by various diatoms (*Chrysophyta*) and blue-green algae (cyanobacteria), also with abundant filamentous green algae (e.g., *Mougeotia* sp.; *Chlorophyta*) in the P-enriched sites.

Four replicates were included for each experimental condition consisting of ultrafiltered water (0.22-μm pore size, Millipore Super Q system) and (i) natural leaves with epiphytes from control sites, (ii) natural leaves with epiphytes from water column P-enriched sites, (iii) artificial leaves with epiphytes from control sites, or (iv) artificial leaves with epiphytes from water column P-enriched sites. Trace concentrations of [^{33}P]phosphate (New England Nuclear Corp., Billerica, Mass.) were injected into the ultrafiltered water. We used an isotope concentration to potentially label algal cells at an average decay rate of approximately 1 disintegration per day per cell (17), based on separate determinations of algal cell density per unit of macrophyte surface area. Each replicate of artificial leaves included epiphytic material which was quantitatively comparable in surface area to that included from two leaves of the natural plant-epiphyte systems. Controls consisted of similar samples from which [^{33}P]phosphate had been omitted. We completed short-term 1-h incubations at 21 to 23°C and 50 microeinsteins of light intensity m^{-2} s^{-1} at 75 rpm on a Gyrotory shaker (Model G-2; New Brunswick Scientific Co., Inc., Edison, N.J.). Turbulence was included to avoid bias which would have been imposed if the samples had been maintained perfectly stationary. The rotation effected turbulence that was about 10- to 100-fold greater than that for calm conditions in the lake research area (20). This turbulence would have resulted in an underestimate of the potential isolation of adnate algae from the water by overlying loosely attached material, since it would have reduced the thickness of the boundary layer immediately adjacent to the epiphyte matrices (38).

Incubations were terminated by transferring each plant leaf with epiphytes to a solution that consisted of filtered pelagic water enriched with 50 μg of unlabeled phosphate (as K_2HPO_4) liter^{-1} to remove the epiphyte-leaf complex from the source of radiolabel and to exchange adsorbed isotope with unlabeled phosphate. Samples in the solution of unlabeled phosphate were maintained for 45 to 60 min under similar conditions of light, temperature, and mixing. Comparison of TLM autoradiographs for epiphytes exposed to the unlabeled-P rinse for 15 and 30 min with those exposed for ≥ 45 min indicated that about 10% less ^{33}P was associated with algae maintained in the unlabeled rinse for at least 45 min. This difference was assumed to have resulted from exchange of adsorbed labeled phosphate for adsorbed unlabeled phosphate. Only after assays were terminated was the epiphyte matrix disrupted to separate loosely attached from adnate components, while still maintaining the adnate microbes intact on the leaf surface. Loosely attached epiphytes were displaced from leaves by using gentle abrasion with a soft, sterile Teflon stopper. Both cohesive layers and matrices with filamentous algae were easily sloughed from the leaves after tapping the material 1 to 3 times (5). We gently dispersed the epiphyte slurry with a loosely fitting glass tissue grinder, diluted the slurry with filtered lake water to attain suitable cell density for autoradiography, and quantitatively placed subaliquots on glass slides. The slides had previously been cleaned, coated with a sterile-filtered (0.22-μm-pore-size Millipore-type GS filters) 5% gelatin–chrom-

alum solution (34, 36), dried in a laminar flow hood, and stored at room temperature with desiccant. The epiphyte slurry was allowed to settle on the slide surface for 10 to 15 min. The slides were then placed on a stainless steel surface that had been chilled to −70°C with liquid nitrogen (24).

Adnate epiphytes and associated leaves were quick-killed by freezing over liquid nitrogen. The leaf-adnate epiphyte samples were cored into subsections and held at −70°C until they were lyophilized. Samples were mounted on round aluminum electron microscope specimen mounts (15-mm height by 15-mm diameter). Both LM and SEM samples were held under desiccation in final preparation for autoradiography.

The experimental design also included analysis of [^{33}P]phosphate uptake by adnate microalgae on natural leaves from control areas, in which the adnate epiphytes were exposed directly to the water by gently dislodging and removing the loosely attached matrix immediately prior to assay. Results were compared with the data for ^{33}P uptake by adnate algae assayed beneath the intact, loosely attached matrix to determine whether the adnate cells were capable of taking up additional label once the overlying material was removed.

Autoradiographic techniques. TLM autoradiography has been used extensively in marine and freshwater research (11, 13, 22, 28). The technique for TLM autoradiography selected in this research was modified from Pip and Robinson (28). Kodak NTB3 nuclear track emulsion (melted at 33 to 34°C; layer thickness, 26 to 30 μm; Eastman Kodak Company, Rochester, N.Y.) was applied to the slides in complete darkness, except for periodic use of a safe light when the dipping chamber was filled (15-W incandescent bulb screened by a Kodak no. 2 safe filter and mounted at least 1.3 m from the samples and emulsion [36]). Slides were dried over desiccant at 22°C for at least 1 h. The samples were exposed to the emulsion for 9 days at 4°C in complete darkness with desiccant. Samples were then developed in complete darkness by using Kodak D-19 developer (7 min), followed by a 1% acetic acid stop bath (5 min), 30% fixer (30 min), 10% fixer (30 min), and two final rinses in bacteria-free deionized water (10 to 15 min for each rinse). Solutions with samples were not agitated in any way to minimize background grain development. The developed TLM autoradiographs were dried in a laminar-flow hood and stored at room temperature with desiccant to minimize fungal attack. Immediately prior to viewing, the autoradiographs were rehydrated with fresh 30% glycerin solution. The autoradiographs were viewed for track production at ×600 magnification under phase contrast with a Zeiss Standard RA light microscope.

Forms of grain density SEM autoradiography have been used since the 1970s in medical sciences, metallurgy, and biological sciences (9, 16, 25). However, we developed TSEM autoradiography and used this new technique in the present research. We applied a thin coat of Kodak NTB3 emulsion (40 to 42°C) to samples with the aid of a safe light used at a distance of at least 1.3 m from the samples. Emulsion was slowly drawn into a syringe base (without needle), and excess emulsion coating the syringe base was removed with a Kimwipe. A 30-gauge needle was attached, and 5 to 8 drops of emulsion were applied to each sample. The silhouette of each emulsion droplet was viewed by working over a small square of smooth black Plexiglas placed on a sheet of white waterproof paper. The EM specimen mount with coated sample was immediately tipped on its side and rolled through several turns to remove excess emulsion. The sample was then placed upright into an appropriate holder, avoiding contact with the perimeter of the upper surface adjacent to the sample.

The adnate samples were dried over desiccant at 22°C in complete darkness for at least 1 h. They were exposed to the emulsion for 9 days and then were developed under conditions identical to those for the TLM autoradiographs. The samples were stored with desiccant until they were carbon coated with a vacuum evaporator, after which they were stored under vacuum to prevent recharging. The TSEM autoradiographs were viewed for track production with a JEOL JSM-35C scanning EM equipped with a dual annular photolithographic disc backscatter electron detector. Energy from β-decay of [^{33}P]phosphate incorporated into formerly live algal cells produced patterns of silver grain formation in the emulsion. A track was designated as four or more silver grains in consecutive sequence which developed within 5 μm of a pigmented cell in TLM (17). Diatoms under TSEM were considered to have been alive during assay if the outer organic membrane was intact, obscuring the cell wall ornamentation.

Track quantification. Tracks of silver grain development, marking uptake of labeled [^{33}P]phosphate ions, were quantified on the basis of algal biovolume for diatom taxa which were common to all sample types (i.e., on natural and artificial leaves from both control and P-enriched areas). Size classes for diatoms were arbitrarily designated in order to determine the mean cell biovolume of each taxon (4). For each taxon, 40 to 50 cells from each included size class were measured if sufficient cell numbers were available. Biovolumes were calculated using formulas for solid geometric shapes which most closely matched the cell shape. Mean biovolumes within each size class were then used to calculate the total biovolume contributed by the taxon to its respective sample.

In TLM autoradiography, four replicates including at least 100 cells of each abundant diatom taxon were analyzed for track production of epiphytes from natural and artificial leaves. However, in TSEM autoradiography the emulsion tended to separate easily from artificial leaf sections. Hence, only 40 to 50 cells per taxon from two replicates were analyzed for epiphytes from artificial plants in control sites; and it was possible to analyze only two replicates (with at least 100 cells per taxon) for epiphytes from artificial plants in P-enriched sampling sites. *Achnanthes minutissima* Kütz. was the most common adnate diatom encountered on both natural and artificial plants. At least 400 cells of this taxon per replicate from all natural macrophyte samples and from one of two replicates for artificial plants from the P-enriched site were analyzed by TSEM autoradiography.

Comparison of track counts for adnate and loosely attached algae required calibration of the two autoradiographic procedures. A slurry of benthic microalgae from Lawrence Lake which consisted mainly of *Amphora ovalis* (Kütz.) Kütz. (98% by biovolume, 70% by cell number) was assayed for [^{33}P]phosphate uptake in filtered pelagic lake water (0.22-μm-pore-size Millipore-type GS filters). Sufficient [^{33}P]phosphate was added to the water to potentially label the algae at an average radioactive decay of approximately 1 disintegration day^{-1} cell^{-1}. Short-term 1-h assays were completed for four replicate samples under the conditions described previously. Assays were terminated by gently centrifuging samples for 1 min at approximately 2,500 rpm (International Centrifuge Model HN), followed by removal of the supernatant which contained [^{33}P]phosphate, and suspension of the algae in an unlabeled phosphate solution

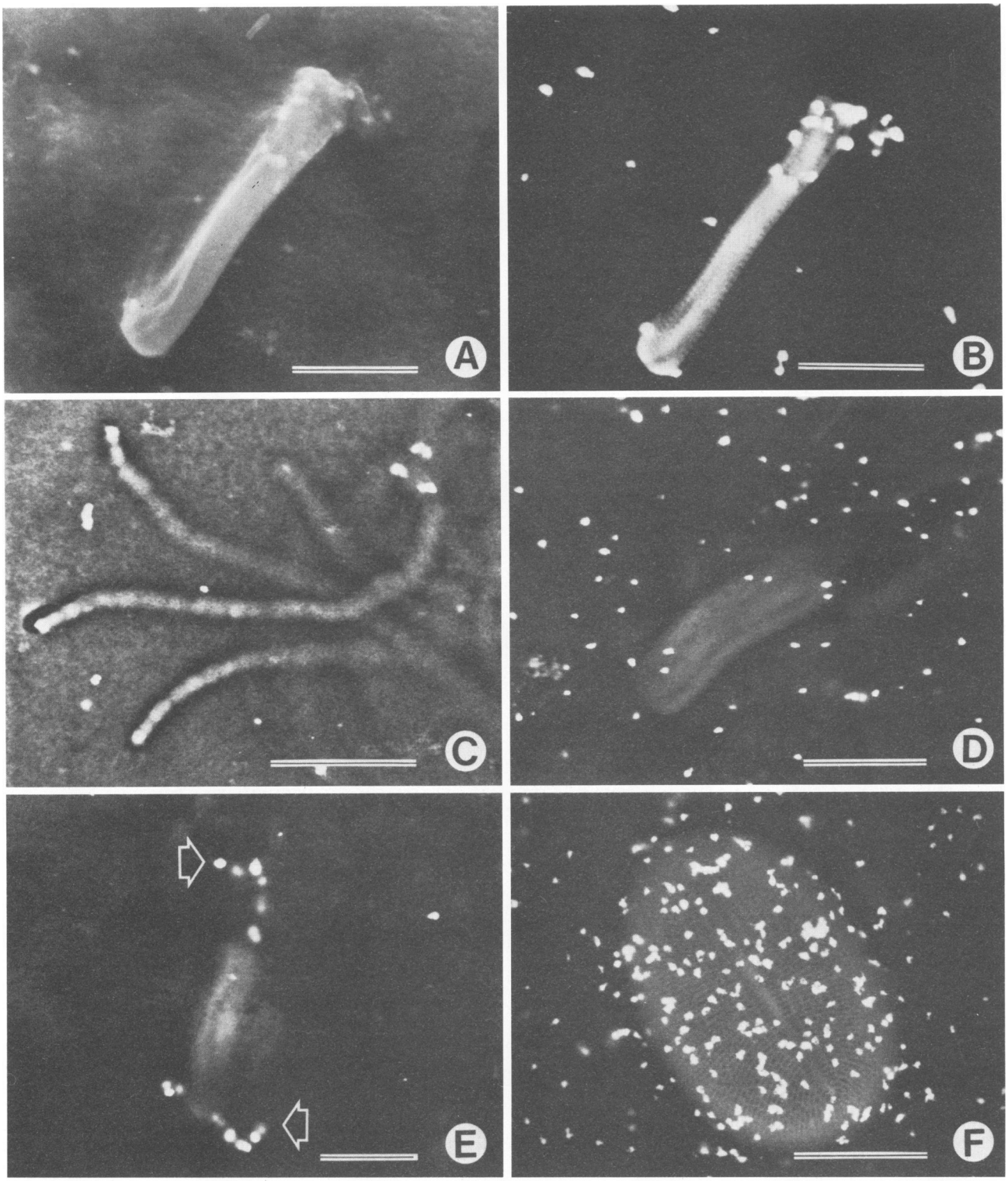

FIG. 2. SEM produced with secondary electron image (A) compared with TSEM autoradiographs produced with backscatter electron imagery (B through E) for adnate microalgae on *Potamogeton* leaves. The algae were assayed for [^{33}P]phosphate uptake from the overlying water, either when maintained beneath a thick, intact overlying matrix (A through E) or when exposed directly to the water column ^{33}P source with the loosely attached matrix removed (F). (A) SEM of *A. minutissima* L. coated with Kodak NTB3 emulsion and developed for autoradiography (bar = 5 μm). (B) TSEM with backscatter imagery enabling resolution of areas with distinct elemental composition (e.g., silver). Development of silver grains above background occurred at the upper edge of the diatom cell, indicating a region of [^{33}P]phosphate uptake (bar = 5 μm). (C) Unlabeled blue-green algal (*Anabaena*) filaments against low background silver grain development (bar = 20 μm); (D) unlabeled *Achnanthes* cell against high background development (bar = 10 μm); (E) *Achnanthes* cell that was labeled with two tracks (arrows) (bar = 5 μm); (F) *Cocconeis* cell with numerous tracks (bar = 15 μm).

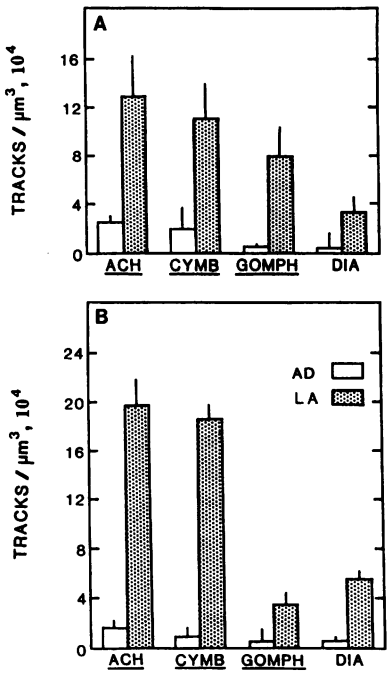

FIG. 3. [^{33}P]phosphate uptake from the water by adnate (AD) compared with loosely attached (LA) algae on natural *Potamogeton* leaves from (A) control sites and (B) phosphate-enriched sites. Abundant taxa included in the analysis were *A. minutissima* (ACH), *C. minuta*. (CYMB), and *G. parvulum* (GOMPH), as well as other diatom cells considered collectively (DIA). Tracks per unit of biovolume are represented; bars represent mean ± standard error for 100 to 400 cells for each taxon, from four replicates.

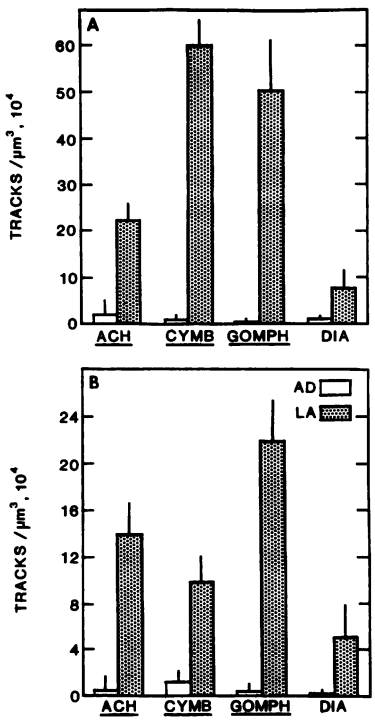

FIG. 4. [^{33}P]phosphate uptake from the water by adnate compared with loosely attached algae on artificial *Potamogeton* leaves from (A) control sites and (B) phosphate-enriched sites. Data presentation is similar to that in Fig. 3, with tracks per unit of biovolume represented for the mean of two replicates and 40 to 50 cells analyzed for each taxon (except 400 *Achnanthes* cells were analyzed in one replicate from the enriched sites). See text for further explanation. Abbreviations are as in Fig. 3 legend.

(50 μg of phosphate liter^{-1} in filtered lake water). This second slurry was maintained with gentle turbulence for 45 to 60 min and then was centrifuged. The supernatant was removed, and the algae were suspended in filtered lake water at a suitable dilution for autoradiography.

Subsamples for technique intercalibration were taken by following the autoradiographic procedures described previously, except that subsamples for TSEM were placed on carbon planchettes which had been attached to the EM specimen mounts. As expected, the scanning electron microscopy enabled resolution of tracks formed by grains sufficiently small to be overlooked under light microscopy. A mean of 2.82-fold more tracks per unit of algal biovolume were counted with TSEM than with TLM autoradiography (mean ± standard error of 79 ± 6 and 28 ± 4 tracks per unit of biovolume, respectively, considering three replicates [J. M. Burkholder, R. G. Wetzel, and K. L. Klomparens, *in* R. G. Wetzel, ed., *Periphyton Methods Manual*, in press]). Track counts per unit of biovolume for adnate algae were divided by 2.82 to compare [^{33}P]phosphate uptake by the adnate and loosely attached epiphytes.

RESULTS AND DISCUSSION

TSEM autoradiographs produced by using our technique were easily interpreted and indicated regions of ^{33}P uptake by adnate epiphytes intact on *Potamogeton* leaves (Fig. 2). TSEM and TLM autoradiography in combination enabled direct comparison of [^{33}P]phosphate uptake from the water by adnate and loosely attached microalgae, respectively, that were assayed intact on macrophyte leaves. In these short-term assays, the loosely attached matrix served as an effective barrier for restricting the quantity of phosphate which penetrated to the adnate algae. For every taxon analyzed, cells located in the loosely attached matrix took up significantly more label than did adnate cells at the base of the layer (Wilcoxon's signed-ranks test [40]; $P < 0.05$ [Fig. 3, 4]). This trend was evident for epiphytes on natural and artificial plants from both control and P-enriched sampling sites. In control sites, loosely attached *Cymbella minuta* Hilse ex Rabh. and *Gomphonema parvulum* Kütz. from artificial leaves were labeled with significantly more tracks than cells from natural leaves ($P < 0.01$), indicating that these taxa may have been more P limited on the artificial plants. By contrast, in P-enriched sites, loosely attached *C. minuta* and *G. parvulum* on both natural and artificial leaves were fairly comparable in [^{33}P]phosphate uptake, and uptake of [^{33}P]phosphate by adnate algae generally was comparable under all experimental conditions.

Sufficient cell numbers were analyzed for *A. minutissima* to justify in-depth comparison of [^{33}P]phosphate uptake by this taxon on natural versus artificial leaves. Uptake of radiolabel from the water was comparable among loosely attached *A. minutissima* from natural and artificial leaves and comparable among adnate *A. minutissima* from the two leaf types (Table 1). Adnate *A. minutissima* from natural and artificial plants obtained less labeled phosphate from the water in fertilized than in control areas, although the difference was not significant. This trend could have been observed because phosphate enrichment stimulated growth of

TABLE 1. [^{33}P]phosphate uptake by *A. minutissima* on natural and artificial leaves from control and moderately phosphate-enriched sampling sites[a]

Epiphyte sample site	Uptake by:		Ratio[b]
	Loosely attached cells	Adnate cells	
Natural plants			
Control	1,983 ± 401	301 ± 99	3.7
Enriched	1,963 ± 390	153 ± 56	12.8
Artificial plants			
Control	1,111 ± 367	186 ± 64	6.0
Enriched	1,853 ± 369	73 ± 33	25.4

[a] Data are given as tracks per unit of algal biovolume, 10^4 (mean ± standard error). Four replicates were taken from natural plants, and 400 cells were analyzed per replicate. Two replicates were taken from artificial plants; 50 cells were analyzed per replicate from control areas and for one replicate taken from the enriched sites. Four hundred cells from the other replicate were analyzed. See text for further explanation.

[b] Ratio of uptake by loosely attached cells to uptake by adnate cells.

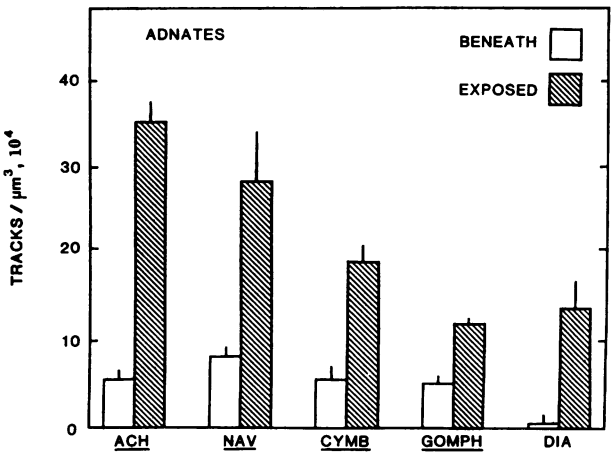

FIG. 5. [^{33}P]phosphate uptake from the water by adnate algae assayed beneath an intact, loosely attached matrix compared with uptake by adnate algae exposed directly to the water prior to assay, with the overlying matrix removed. Data presentation is similar to that in Fig. 3 for *A. minutissima* (*ACH*), *Navicula microcephala* Grunow (*NAV*), *C. minuta* (*CYMB*), *G. parvulum* (*GOMPH*), and other diatom cells considered collectively (DIA).

loosely attached algae and resulted in a thicker overlying matrix, so that isolation of adnate algae from the water column nutrient source was exacerbated, at least over the short-term assay periods. Moreover, the ratio of tracks from loosely attached cells to tracks from adnate cells was significantly greater for fertilized areas than for control areas ($P < 0.01$), considering epiphytes from natural or artificial plants (Fig. 3 and 4). The data suggest that loosely attached *A. minutissima* which had been acclimated to a relatively phosphate-rich environment may have been better adapted physiologically to take up phosphate from the P-poor water used in assays than were extremely P-limited *A. minutissima*, at least for short periods immediately following long-term exposure to phosphate-rich conditions.

Adnate algae which were exposed directly to trace concentrations of [^{33}P]phosphate in the water for short periods took up significantly more labeled phosphate than did adnate algae beneath the intact, loosely attached matrix ($P < 0.01$ [Fig. 5]). Phosphate uptake was comparable for exposed adnate and loosely attached algae. Hence, the adnate and loosely attached algae were similar in affinity for the phosphate source, but the presence of an overlying matrix significantly reduced [^{33}P]phosphate uptake from the water by the adnate component.

These experiments demonstrated that on a short-term basis and after acclimating to either P-poor or moderately P-enriched conditions, loosely attached epiphytes could significantly restrict the quantity of phosphorus which penetrated from the water through the biofilm layer to adnate cells. The response of epiphytes to phosphate supplies in the water column depended on their position in the matrix. The data support the premise that there is a physiological assimilation gradient among microalgae coinciding with the position of the cells within the matrix. The TLM technique considered all loosely attached algae collectively, and technique modifications to identify the exact location of the cells would strengthen data interpretations. Possibilities include TLM or TSEM autoradiography after matrix freeze-fracture or cryomicrotoming and nuclear magnetic resonance techniques such as high-field spectroscopy (2, 14). Nonetheless, loosely attached algae consistently took up significantly more labeled phosphate from the water than did underlying adnate algae. The boundary layer adjacent to the macrophyte-epiphyte complex can form a significant barrier to nutrient entry from the water or nutrient passage in the opposite direction—from the matrix to the water—even in moderately turbulent aquatic habitats (30, 31, 39). Increasing turbulence reduces the thickness of the boundary layer (38) and would facilitate nutrient passage from the water to the matrix interior (31).

In this study, the trend of significantly less uptake by adnate than by loosely attached cells was observed despite the relatively high turbulence imposed during the assays, suggesting that adnate algae in mature benthic microbial communities from P-poor or moderately P-enriched waters may, to a great extent, be isolated from the water column nutrient source. The study indicates that the isolating effect for adnate microalgae would be likely in the case of small concentrations of newly released, localized phosphate supplies in the medium, such as those contributed from excretion by small animals or lysis of algal cells suspended in the overlying water. Our data may represent selective diffusive entrance of [^{33}P]phosphate to the adnate component under short-term conditions, and verification of isotopic equilibrium is needed before conclusions can be made about long-term utilization of the total P_i pool. The response of loosely attached epiphytes under long-term conditions (months) imposed by Moeller et al. (22) supports our hypothesis that the nutrient regimen for epiphytic algae differs depending on the location of cells within the layer. In that study, *A. minutissima* (generally on short stalks near the base of the matrix [5]) relied on the macrophyte as the major supply of phosphorus, whereas *G. parvulum* (often on long stalks, growing toward the outer fringe of the matrix [5]) obtained most of its phosphorus from the overlying water. Moreover, from long-term (days) research on periphyton in a nutrient-poor stream, Pringle (29) observed that sessile (adnate) taxa without an overstory of (loosely attached or long pedunculate) microalgae increased in number when the water was enriched with inorganic N and P. These data, based on changes in species abundances, also support our hypothesis that adnate algae in thick biofilm matrices are relatively isolated from nutrient supplies in the overlying medium.

This research represents the first direct measurement of phosphate uptake by adnate algae in their unaltered microenvironment. An hypothesis which follows for future testing is that adnate epiphytes obtain the major proportion of their phosphorus requirement from the adjacent macrophyte tissue during macrophyte growth and senescence. Application of TSEM with TLM autoradiography will enable examination of both carbon and phosphorus metabolism (e.g., growth rates as well as phosphorus uptake) among adnate versus loosely attached microflora. TSEM autoradiography may also help determine the role of adnate epiphytes in phosphorus cycling among lakes of varying trophic status, with resolution at the level of individual cells.

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LITERATURE CITED

1. **Allanson, B. R.** 1973. The fine structure of the periphyton of *Chara* sp. and *Potamogeton natans* from Wytham Pond, Oxford, and its significance to the macrophyte-periphyton metabolic model of R. G. Wetzel and H. L. Allen. Freshwater Biol. **3**:535–542.
2. **Alleman, J. E., E. K. Russell, W. L. Lantz, Jr., and R. F. Wukasch.** 1985. Biofilm cryo-preparation for SEM. Water Res. **19**:1073–1078.
3. **Armstrong, S. M., and F. Barlocher.** 1989. Adsorption and release of amino acids from epilithic biofilms in streams. Freshwater Biol. **22**:153–159.
4. **Burkholder, J. M., and R. G. Wetzel.** 1989. Epiphytic microalgae on a natural substratum in a phosphorus-limited hardwater lake: seasonal dynamics of community structure, biomass and ATP content. Arch. Hydrobiol. **83**(Suppl.):1–56.
5. **Burkholder, J. M., and R. G. Wetzel.** 1989. Microbial colonization on natural and artificial macrophytes in a phosphorus-limited, hardwater lake. J. Phycol. **25**:55–65.
6. **Burkholder, J. M., and R. G. Wetzel.** 1990. Epiphytic alkaline phosphatase on natural and artificial plants in an oligotrophic lake: re-evaluation of the role of macrophytes as a phosphorus source for epiphytes. Limnol. Oceanogr. **35**:736–746.
7. **Carlton, R. G., and R. G. Wetzel.** 1988. Phosphorus flux from lake sediments: effect of epipelic algal oxygen production. Limnol. Oceanogr. **33**:562–570.
8. **Costerton, J. W., G. G. Geesey, and K. J. Cheng.** 1978. How bacteria stick. Sci. Am. **238**:86–95.
9. **Darley, P. J., and B. J. McFarlane.** 1977. Scanning electron microscope autoradiography: a technique for the location and study of microscopic-active particles. Health Phys. **32**:259–270.
10. **Fletcher, M.** 1980. Adherence of marine micro-organisms to smooth surfaces, p. 345–374. *In* E. H. Beachey (ed.), Bacterial adherence. Chapman & Hall, Ltd., London.
11. **Fuhs, G. W., and E. Canelli.** 1970. Phosphorus-33 autoradiography used to measure phosphate uptake by individual algae. Limnol. Oceanogr. **15**:962–967.
12. **Geesey, G. G., R. Mutch, J. W. Costerton, and R. B. Green.** 1978. Sessile bacteria: an important component of the microbial population in small streams. Limnol. Oceanogr. **23**:1214–1223.
13. **Hudon, C., H. C. Duthie, and B. Paul.** 1987. Physiological modifications related to density increase in periphytic assemblages. J. Phycol. **23**:393–399.
14. **James, T. L., and A. R. Margulis (eds.).** 1984. Biological magnetic resonance. Radiology Research and Education Foundation, San Francisco.
15. **Karl, D. M.** 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbiol. Rev. **44**:739–796.
16. **Klomparens, K. L., M. A. Petersen, D. C. Ramsdell, and W. G. Chaney.** 1986. The use of scanning electron microscope autoradiography to localize the blueberry shoestring virus in its aphid vector. J. Electron Microsc. Technique **21**:590–596.
17. **Knoechel, R., and J. Kalff.** 1976. Track autoradiography: a method for the determination of phytoplankton species productivity. Limnol. Oceanogr. **21**:590–596.
18. **LaMotta, E. J.** 1976. Internal diffusion and reaction in biological films. Environ. Sci. Technol. **10**:765–769.
19. **Lock, M. A., R. R. Wallace, J. W. Costerton, R. M. Ventullo, and S. E. Charlton.** 1984. River epilithon: toward a structural-functional model. Oikos **42**:10–22.
20. **Losee, R. F., and R. G. Wetzel.** 1987. Water movement within submersed vegetation. Verh. Int. Verein. Limnol. **23**:62–66.
21. **Madsen, B. L.** 1972. Detritus on stones in small streams. Mem. Ist Ital. Idrobiol. Dott Marco Marchi. **29**(Suppl.):385–403.
22. **Moeller, R. E., J. M. Burkholder, and R. G. Wetzel.** 1988. Significance of sedimentary phosphorus to a rooted submersed macrophyte (*Najas flexilis* (Willd.) Rostk. and Schmidt) and its algal epiphytes. Aquat. Bot. **32**:261–281.
23. **Moeller, R. E., and R. G. Wetzel.** 1988. Littoral vs. profundal components of sediment accumulation: contrasting roles as phosphorus sinks. Verh. Int. Verein. Limnol. **23**:386–393.
24. **Paerl, H. W.** 1984. An evaluation of freeze fixation as a phytoplankton preservation method for microautoradiography. Limnol. Oceanogr. **29**:417–426.
25. **Paul, D., A. Grobe, and F. Zimmer.** 1970. Autoradiography in the scanning microscope. Nature (London) **227**:488–489.
26. **Penhale, P., and G. W. Thayer.** 1980. Uptake and transfer of carbon and phosphorus by eelgrass (*Zostera marina* L.) and its epiphytes. J. Exp. Mar. Biol. Ecol. **42**:113–123.
27. **Penhale, P. A.** 1977. Macrophyte-epiphyte biomass and productivity in an eelgrass (*Zostera marina* L.) community. J. Exp. Mar. Biol. Ecol. **26**:211–224.
28. **Pip, E., and G. G. C. Robinson.** 1982. A study of the seasonal dynamics of three phycoperiphytic communities, using nuclear track autoradiography. I. Inorganic carbon uptake. Arch. Hydrobiol. **94**:341–371.
29. **Pringle, C. M.** 1990. Nutrient spatial heterogeneity: effects on community structure, physiognomy, and diversity of stream algae. Ecology **71**:905–920.
30. **Raven, J. A.** 1970. Exogenous inorganic carbon sources in plant photosynthesis. Biol. Rev. Camb. Philos. Soc. **45**:167–221.
31. **Riber, H. H., and R. G. Wetzel.** 1987. Boundary layer and internal diffusion effects on phosphorus fluxes in lake periphyton. Limnol. Oceanogr. **32**:1181–1194.
32. **Riber, H. H., J. P. Sorensen, and A. Kowalczewski.** 1983. Exchange of phosphorus between water, macrophytes and epiphytic periphyton in the littoral of Mikolajskie Lake, Poland, p. 235–243. *In* R. G. Wetzel (ed.), Periphyton of freshwater ecosystems. Dr. W. Junk Publishers, Boston.
33. **Rich, P. H., R. G. Wetzel, and N. V. Thuy.** 1971. Distribution, production and role of aquatic macrophytes in a southern Michigan marl lake. Freshwater Biol. **1**:3–21.
34. **Robinson, G. G. C.** 1983. Methodology: the key to understanding periphyton, p. 245–252. *In* R. G. Wetzel (ed.), Periphyton of freshwater ecosystems. Dr. W. Junk Publishers, Boston.
35. **Roemer, S. C., K. D. Hoagland, and J. R. Rosowski.** 1984. Development of a freshwater periphyton community as influenced by diatom mucilages. Can. J. Bot. **62**:1799–1813.
36. **Rogers, A. W.** 1979. Techniques of autoradiography, revised ed. Elsevier/North-Holland, Amsterdam.
37. **Round, F. E.** 1981. The ecology of algae. Cambridge University

Press, New York.
38. **Schlichting, H.** 1968. Boundary layer theory, 6th ed. McGraw-Hill, New York.
39. **Smith, F. A., and N. A. Walker.** 1980. Photosynthesis by aquatic plants: effects of unstirred layers in relation to assimilation of CO_2 and HCO_3^- and to carbon isotope discrimination. New Phytol. **86**:245–259.
40. **Sokal, R. R., and F. J. Rohlf.** 1981. Biometry, 2nd ed. W. H. Freeman and Co., San Francisco.
41. **Williamson, J., and P. L. McCartey.** 1976. Verification studies of the biofilm model for bacterial substrate utilization. J. Water Pollut. Control Fed. **48**:281–296.