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Microscopic examination of photoautotrophic and phosphatase-producing organisms in phosphorus-limited Everglades periphyton mats

Abstract—Using a fluorescent-labeled enzyme substrate, we examined the location of in situ phosphatase activity in a periphyton mat and explored the potential associations of phosphatase-producing organisms (PPO) and cyanobacteria within these mats. Our results indicate that most PPOs are concentrated in the lower section of the mat, and the phosphatase activity appears to be associated with heterotrophic organisms that are in close proximity to chlorophyll-containing cyanobacteria. The lack of observed phosphatase by larger photosynthetic cells and the close association of these cells with PPOs indicate a possible interaction whereby PPOs obtain photosynthetically fixed carbon from cyanobacteria and, in turn, provide inorganic phosphorus (P) and other compounds to the cyanobacteria. We believe these results may represent additional evidence for algal–bacterial symbiosis in aquatic systems and, in particular, the P-limited cyanobacterial mat communities.

By supporting various endangered and threatened species and maintaining high genetic and ecological diversity, the Florida Everglades is a unique wetland ecosystem of global importance (Maltby and Dugan 1994). The Everglades ecosystem also supports high levels of productivity despite its phosphorus (P)-limited nature. Much of this high productivity is attributed to the growth and dominance of periphytic communities (McCormick and Stevenson 1998), which can cover much of the open water regions and serve as a base of the Everglades food web (Browder et al. 1994). Through its biotic activities (e.g., photosynthesis and nitrogen fixation) the Everglades periphyton community has a pronounced effect on the biogeochemistry of the water column and the ecosystem as a whole. For this reason, periphyton communities and their associated functions are critical to the health and stability of the Everglades ecosystem.

The Everglades periphyton are complex microbial assemblages based on cyanobacterial filaments of *Schizothrix* sp. and *Scytonema* sp. (Gleason and Spackman 1974). These periphytic forms can occur in association with the benthos (benthic) or in association with submersed and emergent macrophytes (epiphytic) such as *Typha*, *Cladium*, and *Utricularia purpurea*. They may be either thin films (ca. 1–2 mm) or well-developed, thick (ca. 1–4-cm) growths referred to as floating and benthic periphyton 'mats' or epiphytic 'sweaters.' Both epiphytic and benthic mats can detach from the substrata via buoyancy from trapped gases and form floating periphyton mats at the water surface. Photosynthetic activity within the mats influences local pH and in hardwater Everglades regions can lead to precipitation of calcium car-

bonate within the mat (Browder et al. 1994). In this regard, the Everglades periphyton mats are similar to other calcifying cyanobacterial communities (Rejmankova and Komarkova 2000).

In thick cyanobacterial mats, vertical gradients of light, oxygen, pH, nutrients, and microbial metabolic products may exist (Jorgensen 1983; Stal et al. 1985). Mat organisms structure themselves in response to these physico-chemical gradients, leading to the formation of a biogeochemically distinct layer. With this structure, a cyanobacterial mat can simultaneously support diverse groups of microorganisms and their associated biogeochemical activities. This diversity of organisms and functions is a key factor in the ability of these mats to exist and thrive in extreme environments such as the extremely P-limited Florida Everglades (Cohen and Rosenberg 1989).

Under conditions of P limitation, the availability of P is often regulated by activity of the enzyme phosphatase which hydrolyzes organic P (P_o) compounds to bioavailable inorganic phosphate (P_i) (Chrost 1991). Studies have shown increased phosphatase activity of bacterioplankton (Campbella et al. 1984) and cyanobacterial mats (Rejmankova and Komarkova 2000) in P-limited environments. High phosphatase activity has also been observed in periphyton mats of the Everglades (Newman et al. 2003) and is likely a major factor contributing to the dominance of periphyton in P-limited systems such as the Everglades.

In aquatic systems, it is often presumed that phosphatase activity of periphyton consortia is the simple summation of the activities of the component organisms, with the response of the community being linked to the overall conditions of P limitation in the system. Many aquatic organisms, including cyanobacteria (Grainger et al. 1989), diatoms, and green algae (Gonzalez-Gil et al. 1998), and eubacteria (Jansson et al. 1996) are known to produce phosphatase in pure cultures; however, several studies have also shown that the physiological and biochemical properties of bacteria in isolation do not reflect those of organisms growing in natural consortia (Deretic et al. 1994; Caldwell et al. 1997). For this reason, production of phosphatase in periphyton consortia may be the result of one or more specific groups of organisms. Identifying active phosphatase-producing organisms (PPO) and investigating the potential associations with other mat organisms serve as the initial steps to understanding phosphatase expression in a periphyton community.

Traditional methods of measuring phosphatase activity of natural periphyton primarily consist of assaying bulk samples without attempting to separate the relative contribution

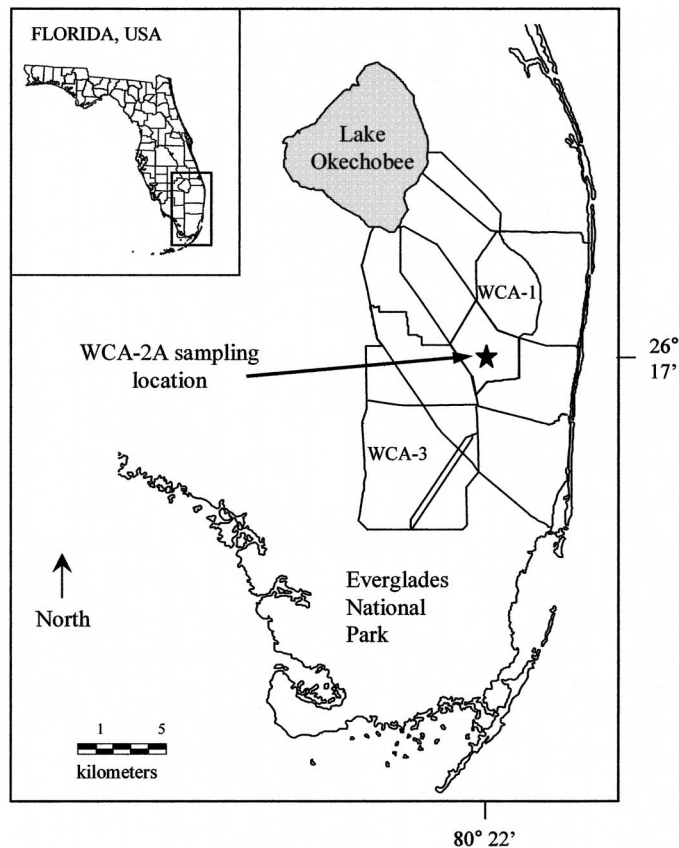


Fig. 1. Location of the site used for sampling P-limited floating periphyton mats in WCA-2A of the Florida Everglades.

of individual mat organisms. For this reason, we conducted the following study to investigate phosphatase production by specific groups of organisms (i.e., algae/bacteria, phototrophs/heterotrophs) as they occur within an intact, calcareous periphyton mat of the Florida Everglades. Our goals in this study were to determine the spatial distribution of phosphatase activity within the mat and to examine the associations between photoautotrophic and phosphatase-producing bacterial groups in a periphyton mat. To demonstrate the location of phosphatase activity in situ, we employed a microscopic technique based on the fluorescent substrate ELF[®] 97 (Molecular Probes). Studies such as this have important implications for understanding microbial cycling of P within these and similar periphyton mat communities.

Study site and sampling—Periphyton mats used in this study were obtained from a site in the interior of Water Conservation Area 2A (WCA-2A) of the Florida Everglades (Fig. 1). This area is typical of the P-limited regions (soil total phosphorus (TP), 0.6 g kg⁻¹; pore water P, 0.1 mg L⁻¹; and periphyton TP, 75 mg kg⁻¹) of the Northern Everglades and is characterized by ridges and open slough areas. Typical biochemical parameters of this site are discussed by Inglett et al. (2004). Vegetation on the ridges is dominated by *Cladium* sp., while periphyton mats occur predominantly in open slough areas dominated by *Nymphaea*, *Eleocharis*, and *Utricularia* spp. Floating periphyton mats were sampled in No-

vember 2002. Several intact mats exhibiting well-developed layers (Fig. 2a) were collected in site-water-filled polyethylene containers and stored on ice while they were transported to the Wetland Biogeochemistry Laboratory at the University of Florida in Gainesville, Florida.

Cryoembedding and cryosectioning—Periphyton mats with intact layers in their vertical profiles were chosen and sectioned within 24 h of collection (Fig. 2). The samples were cryoembedded in Tissue-Tek[®] OCT compound (Miles; Yu et al. 1994). Embedded samples were sectioned (5 μm thick) with a cryostat (Leica), and orientation of the mats was adjusted such that the sections would contain all the layers in the vertical profile of mats. Sections were mounted on glass slides and treated with formaldehyde (0.01%) before storing them at 4°C until further staining analysis.

Fluorescent staining—Sections of periphyton mats were stained with enzyme-labeled fluorescence substrate, ELF[®] 97 [(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone] and/or 4', 6-diamidino-2-phenyl-indole (DAPI). Enzymatic hydrolysis of water-soluble ELF[®]97 phosphatase substrate (ELF-P) yields a water-insoluble, yellow-green ELF-alcohol (ELF-A) precipitate that is extremely photostable. The sites of phosphatase production were visualized by epifluorescence microscopy. Fluorescent stain DAPI binds to double-stranded DNA, and the stained cells fluoresce blue. Immediately prior to use in this study, ELF-P was diluted at ratio of 1:20 in ELF Detection Buffer and filtered through 0.2-μm spin filters to remove substrate precipitates. Each prefixed mat section was incubated with 30 μL of ELF-P for 30 min in the dark at room temperature. Stained samples were washed with 10 mmol L⁻¹ phosphate-buffered saline to stop the reaction. Negative controls were prepared by treating the sections of mats as described above except that they were incubated with ELF detection buffer without ELF-P substrate. Some randomly chosen ELF-P-stained cryosections were also stained with DAPI for 5 min in dark.

Microscopy and image analysis—A fluorescent morphometric microscope was used to examine prepared sections. The excitation spectrum of chlorophyll (Chl) *a* and *b* and ELF-A are different; therefore, the yellow-green signals of ELF-A and the red fluorescence of chlorophyll were visualized sequentially with separate filter sets. ELF-P-treated samples were visualized with an Olympus type U filter. Filters used for ELF-A detection were 360 ± 40 nm for excitation and 530 ± 25 nm for emission. The Texas red filter was used for images of Chl *a* autofluorescence (CHL images). DAPI-stained samples were observed under a fluorescent microscope equipped with long-pass filter set (excitation 365 ± 8 nm; emission > 420 nm). Images captured at the same spot by the different filters were digitized with a cooled color charge-coupled device camera. ELF-A, DAPI, and Chl images of the same field were stored in a single file and later overlaid to show the location of the chlorophyll-containing organisms and the zones of phosphatase production.

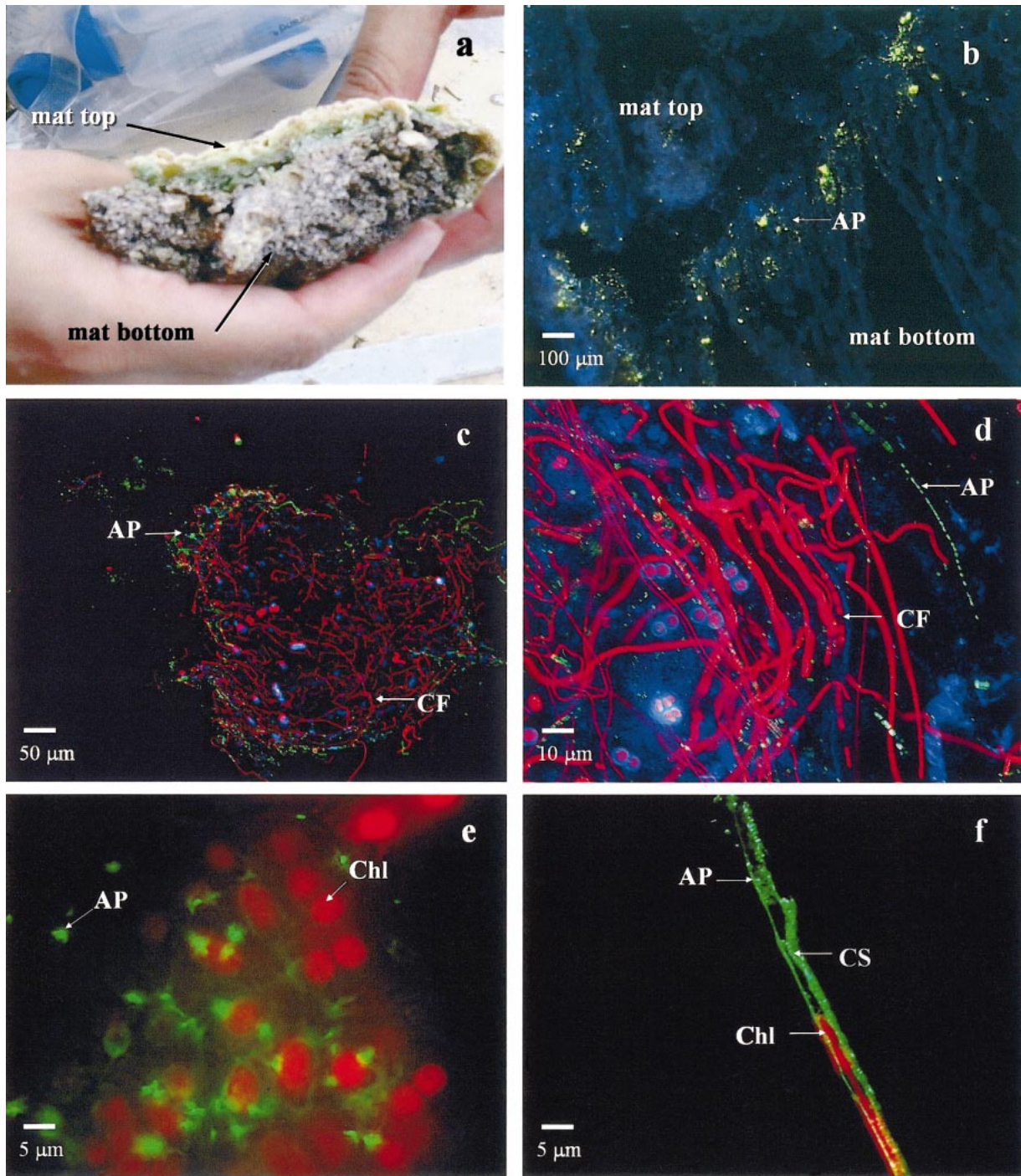


Fig. 2. Photo depicting the three layers in a typical P-limited Everglades periphyton mat similar to that used in this study. (a) Photomicrographs of vertical cryosections (5- μm thickness) of the mat stained with fluorescent phosphate substrate. (b–f) Sites of alkaline phosphatase (yellow–green fluorescence) and chlorophyll (red fluorescence) activity are evident in (b) vertical mat cross section; (c, d) localized aggregate of filamentous cyanobacteria; (e) isolated colony of coccoid cyanobacteria; and (f) along remnant cyanobacterial sheaths/slime trails. AP, alkaline phosphatase; Chl, chlorophyll; CF, cyanobacterial filaments; CS, cyanobacterial sheath.

Results—Well-formed periphyton mats in P-limited areas were between 2 and 2.5 cm thick, with three clearly defined layers (Fig. 2a). The top mat layers were pale yellow to white in color, probably the result of photobleaching at the

water surface and/or the presence of high concentrations of the pigment scytonemin (Dillon et al. 2003). Deeper mat layers were not exposed to high intensities of solar radiation, and as a result, they appear green from the dominance of

photosynthetic cyanobacteria and green algae. Bottom mat layers were gray/black and likely contained remnants of soil from the benthic surface.

The presence of photoautotrophic organisms in the mat sections was confirmed by high autofluorescence when examined with the Texas red filter (Fig. 2c–f). Chlorophyll-containing filamentous and coccoidal cells were distributed throughout the mat as expected. The most conspicuous photosynthetic structures were numerous filaments ranging in size from 3 to 5 μm in diameter and $>100 \mu\text{m}$ in length. This size is also in agreement with the reported dominance of filamentous cyanobacteria (e.g., *Schizothrix*) in the Everglades periphyton (Gleason and Spackman 1974).

Attempts to stain bacterial cells using DAPI were largely unsuccessful in the mat sections, as observed in Fig. 2c–e. Isolated DAPI-stained cells were observed in some slides; however, application of DAPI predominantly resulted in staining large portions of observed field areas with blue fluorescence. This staining pattern cannot be attributed to suspected patterns of nucleic material and likely represents non-specific binding of DAPI with polysaccharide materials present in the mat sections. Alternately, it is also possible that extremely intense signal of ELF-A (which fluoresces at the same wavelength as DAPI) may have overwhelmed the DAPI signal of the cells, making it difficult to separate the two signals. We did note in many cases that ELF-A precipitation coincided with strong DAPI signals.

Sites of phosphatase activity were determined by ELF-A deposition on mat sections. Within the mat profiles, phosphatase activity was mainly present in the middle and the lower mat sections, while ELF-A precipitation was largely absent in the topmost mat layers of the mat that were exposed to air at the water surface (Fig. 2b). Within the middle and lower mat sections, ELF-A fluorescence was concentrated in dense clusters. Closer examination of some of these ELF-A concentrations revealed higher phosphatase activity along the edges of aggregates of chlorophyll-containing filaments and cells (Fig. 2c). When the same field images of DAPI long-pass and Texas red filters were overlaid, dense aggregations of ELF-A precipitates were observed in close association with chlorophyll-containing cells (Fig. 2e,f). In some cases, ELF-A precipitation appeared with filamentous, sheathlike structures of the cyanobacteria. No red fluorescence was observed with some of these structures, indicating that they may have been remnant sheaths of dead cyanobacteria or mucilaginous slime trails (Fig. 2f).

Discussion—It is unclear whether the phosphatase activity observed in this study using ELF was a result of surface-bound or free dissolved enzyme. The appearance of ELF fluorescence at localized, concentrated sites, however, indicates a dominance of surface-bound enzymes rather than free dissolved phosphatase, which would likely be randomly distributed throughout the mat. Because phosphatase activity generates the bioavailable P required for basic cell growth and functions, the presence and activity of PPO (and sites of phosphatase activity) are indicative of the location of P transformations within the mat. Because of the highly P-limited nature of the Everglades system, we expected to observe a wide distribution of phosphatase activity in the pe-

riphyton mats. Contrary to this hypothesis, however, the microscopic examination of ELF-stained periphyton mat sections revealed an uneven distribution of phosphatase activity, with the majority of activity localized in the middle and lower sections of the mat (Fig. 2b). Appearance of enzyme activity at specific sites within a periphyton mat indicates that not all mat-forming organisms are producing phosphatase and/or that this function may be limited to specific sites of high growth/metabolism, where P demand is presumably greatest.

One explanation for spatial segregation of PPO may be attributable to the influence of various biochemical factors that are known to determine the distribution of the groups of bacteria in microbial mats (Jorgensen et al. 1983; Stal 1994). Seasonal changes in light and temperature have been shown to affect periphyton growth rates (McCormick et al. 1998). These effects may also be observed through altered spatial distribution of organismal groups in a mat. Ultraviolet radiation at the mat surface may also contribute to structuring of mat PPO by causing the migration of diatoms and cyanobacteria to the lower mat layers (Janssen et al. 1996). In this manner, the lack of phosphatase activity in the upper regions of the periphyton mat of this study may be due to the absence of these and other PPO from exposed layers. Whether or not the localization of phosphatase activity within the mat structure is advantageous for the functioning of P-limited cyanobacterial mat is presently unclear. One advantage may be that the localization of phosphatase maximizes internal recycling, leading to increased P turnover within the mat structure.

The absence of any phosphatase activity in the interior of aggregated filaments indicates that the aggregate interior was P sufficient relative to the exterior (Fig. 2c), which exhibited high ELF-A fluorescence. The fact that the organisms on the aggregate exterior were nonphotosynthetic (i.e., lacking chlorophyll) indicates that heterotrophic bacteria may be the dominant producers of phosphatase in the mat. Another important observation supporting this hypothesis is the presence of phosphatase activity on the outer sheath of intact cyanobacterial cells, as well as the remnant sheaths of dead filaments (Fig. 2f). Even though there is no quantitative evidence in this study to demonstrate that heterotrophic bacteria are the dominant producers of phosphatase, it is surprising that in the low-P conditions of the Everglades periphyton, only bacteria should actively produce phosphatase. Such an occurrence would likely indicate a significant P limitation of the periphyton bacterial populations and would be contrary to the general observation that bacteria have a higher uptake affinity for P relative to larger algae (Smith and Kalff 1981). By this reasoning, larger algal cells should be P limited (and exhibit higher phosphatase activity), whereas adjacent bacteria should remain limited by another nutrient (e.g., nitrogen [N]) or by availability of carbon (C) substrates (Wynne and Rhee 1988).

The hydrolysis reaction of phosphatase enzymes liberating inorganic P has also been shown to result in the release of labile C compounds (Heath and Hanson pers. comm.). For this reason, bacterial expression of phosphatase is now being considered as a possible mechanism to overcome C limitation (Benitez-Nelson and Buesseler 1999). Carbon limitation

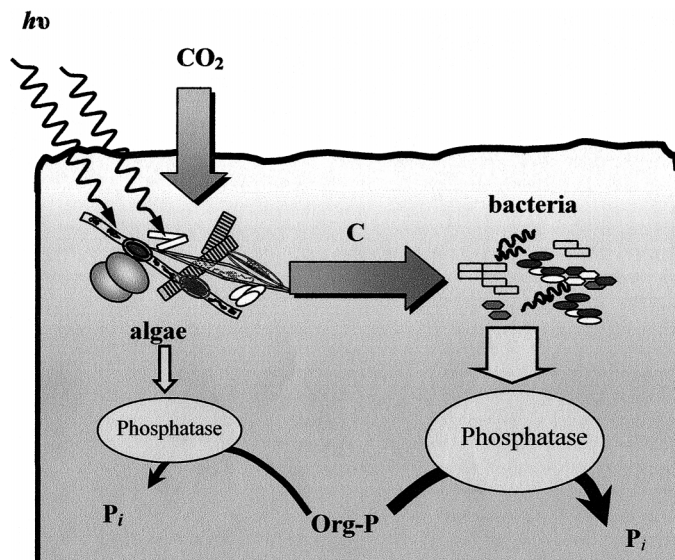


Fig. 3. Schematic diagram of proposed association between phosphatase-producing organisms and cyanobacteria in periphyton mats. Phosphatase-producing organisms live in close association with the eukaryotic algae filaments and the cyanobacterial cells and filaments, perhaps providing them with inorganic P through activity of cell-bound phosphatase.

may explain the dominance of bacterial phosphatase expression in the current microscopic study. Conversely, the lack of observed algal phosphatase production in this study may indicate there is a sufficient supply of P to the algal component of the Everglades periphyton. As there was a general lack of phosphatase expression by algal cells in the mats we examined, it is possible that the P source to algal cells in these mats was primarily derived from the bacterial phosphatase activity. For this reason, we propose that there is some type of cooperative interaction between the algae and bacteria within the Everglades periphyton mat complex (summarized in Fig. 3).

Cooperative interactions between cyanobacteria and bacteria have been discussed in the past, and they primarily revolve around the exchange of one or more nutrients or substrates (Marshall 1989). Chlorophyll-containing cyanobacteria have the ability to photosynthesize and fix atmospheric N_2 . They are also known to maintain their colonial structure by exudation of exopolysaccharides such as mucilage and/or firm sheaths (Browder et al. 1994). These active secretions, combined with products produced during cell death and senescence, become an important source of C and N for the heterotrophic bacteria. Close proximity of bacteria may be advantageous to algae because PPO generate bioavailable P that is perhaps used by the algal cells. The high uptake affinity of bacteria for P would dictate that most available P would enter the mat through the bacterial component. Once the bacterial stoichiometric needs of P are satisfied, additional P would become available for algal uptake. This available P would then support additional algal photosynthesis to complete the exchange.

In conclusion, this study attempted to better establish the roles of various organismal groups in the production of phos-

phatase within a P-limited Everglades periphyton mat. In our proposed model, algae may provide photosynthetically fixed C, while bacteria may increase levels of bioavailable P. The combined activities of these groups may thus facilitate the existence of a periphyton mat community under conditions of extreme P limitation. Because of the qualitative nature of the microscopic techniques in this work, however, we can only speculate regarding this association.

Studies in the past have attributed the associations of autotrophic and heterotrophic organisms to C and N exchange. We believe these current results may represent additional support for algal-bacterial symbiosis involving P in aquatic systems and, in particular, the P-limited cyanobacterial mat communities. More information is required to definitively document the role of the heterotrophs in cyanobacterial mat phosphatase production. For this reason, the eventual fate and ecological importance of phosphatase produced by heterotrophic bacteria within such mat communities represent an exciting and potentially important area of new research.

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