TEMPORAL CHANGES IN ALKALINE PHOSPHATASE ACTIVITY DURING DEVELOPMENT OF PHOSPHORUS LIMITED PERIPHYTON

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CHAPTER I

INTRODUCTION

The importance of factors limiting algal dynamics in freshwater ecosystems is recognized by many investigators engaging in research on eutrophicaton. Research and management efforts focus on the roles of nutrients, particularly phosphorus (P) and nitrogen (N) in the limitation of algal biomass (Stanley et al., 1990). Generally, phosphorus is the most frequently limiting nutrient in temperate fresh waters (Dillon and Rigler, 1974). Unlike the research on nutrient limitation of phytoplankton, research on nutrient limitation in periphyton was slow until workers began using nutrient-diffusing substrates or nutrient fertilization techniques (Aloi, 1990, Stanley et al., 1990). The periphyton community (periphyton) is the microflora living on stone, wood or sand substrates submerged in water (Wetzel 1983).

Identification of the limiting nutrients is essential for planning effective measures against eutrophication in management of water bodies (Rast et al., 1989). One experimental approach to determine the presence of and the type of nutrient limitation for periphyton is to measure physiological parameters as indicators.

Biomass accrual is frequently used as the indicator of phosphorus limitation (P-limitation) in fertilization experiments. Biomass is also used to normalize other indicators to a common base of reference (Mundie et al., 1991). Because of influences of current, grazing, and exposure time (Jones, 1978, Elwood et al., 1981, Steinman et al., 1991a, Borchardt, 1996), sometimes accrual rates of biomass on substrates may not reflect the nutrient status of periphyton.

It is also possible to use other physiological parameters as indicators of P-limitation. Because alkaline phosphatase activity (APA) is a direct, sensitive, and convenient indicator of P-limitation, it has been more frequently used in research on periphyton (e.g. Biggs, 1990, Mulholland et al., 1991b, Steinman et al., 1991b, Scholz and Boon, 1993, Lozona and Pratt, 1994, Invers et al., 1995, Mulholland et al., 1995, Johnson and Tuchman, 1997, McCormick and Stevenson, 1998). Surplus P, an indicator of P sufficiency, may be also useful in research on P-limitation but its use has been limited to filamentous algae and phytoplankton (Fitzgerald, 1972). In Oklahoma, APA and surplus P have been used to assess the effect of non-point sources of phosphorus on the eutrophication potential to the streams in the Illinois River basin (Toetz et al., In

Press).

Biggs (1988) stated that exposure (sampling) time is the most important factor to be considered when using artificial substrates to study periphyton. Periphyton development on artificial substrates follows a sigmoidal curve (Tilley and Haushild, 1975). Three phases occur for this process: a) the beginning of colonization; b) the logarithmic growth phase; c) the upper asymptote. The latter occurs when the instantaneous biomass accumulation rate approaches zero. Net production may be balanced by grazing and sloughing, the latter occurring when periphyton attachment to substrates is weakened by the death of underlying layers of cells and/or equilibrium shear stress of cells is exceeded by current (Biggs, 1988). Exposure time needs to be long enough for development of periphyton to reach a maximum biomass (Biggs, 1988). Practically, periphyton biomass usually fluctuates after the logarithmic growth phase, as demonstrated by the research of Biggs (1988). The fluctuation of biomass of a periphyton community may also cause different APA in samples taken at different times.

During development of periphyton under P-limitation, P demand increases (Bothwell, 1989). Increasing periphyton thickness decreases the rate of P diffusion into periphyton

(Stanley et al., 1990) because of the inverse relationship between thickness of the periphyton mat and P diffusion (Riber and Wetzel, 1987). Thus, the increase of P demand and decrease of P supply may cause a severe P-limitation and result in high APA levels. Stevenson (1996) suggested a positive effect of an increasing biomass on APA: APA increases as biomass increases. On the other hand, the increasing biomass also may have a negative effect on APA, because increasing periphyton thickness may increase periphyton self-shading, decrease photosynthesis and P demand of periphyton, thus decreasing APA. Furthermore P recycling in periphyton is greater when periphyton biomass increases (Paul and Duthie, 1989, Mulholland et al., 1991a, Mulholland, 1996). The increasing biomass may result in an increased rate of P recycling, a decreased P demand, and consequently low APA levels. So, a question arises as to whether high or low APA levels are related to increasing biomass. This question has not been answered conclusively.

In this paper the term diurnal means events during daylight (natural solar radiation) and also during the light-part of an artificial light-dark cycle as produced in the laboratory. Diel refers to an event that occurs once every 24 hours irrespective of the presence or absence of light.

It is not known whether a diurnal APA cycle occurs in periphyton so that an appropriate sampling time during the natural light-dark cycle might be selected. For phytoplankton, there have been a few studies on the diurnal APA cycle (Berman, 1970, Reichardt, 1971, Perry, 1976, Rivkin and Swift, 1979, Wynne, 1981, Huber and Kidby, 1984, Huber and Hamel, 1985, Klotz, 1985, Jamet et al., 1995). But, the length of time of observations in most of these studies just covers one light-dark cycle (24 hr). The results are often different. No common model exists for a diurnal APA cycle for phytoplankton, which could deductively be applied to periphyton.

Thus, when using APA for determination of P-limitation, we face the following questions. When during development should one determine APA as a measure of P-limitation? Does a diurnal cycle exist in periphytic APA? Does periphyton have an endogenous APA rhythm? To answer these questions, this study observed changes of APA during development of P-limited periphyton cultured in the laboratory with constant temperature and irradiance, minimum water movement, and absence of grazing. This study tested the following null hypotheses for P-limited periphyton:

I. No change in biomass, APA, and surplus P occurred during periphyton development.

II. No pattern of change in APA occurred during the diurnal period of light-dark cycles.

III. No significant difference occurred in APA in continuous darkness for periphyton that have been cultured in a light-dark cycle.

This research increases understanding of development of P-limited periphyton on artificial substrates. The research is a first step to establish criteria for optimum substrate exposure time and sampling time for APA assays, which can facilitate application of APA assays in stream eutrophication research.

CHAPTER II

LITERATURE REVIEW

Phosphorus Limitation

Nutrient limitation means growth limitation by a nutrient, i. e., adding the nutrient will increase growth of the alga (Darley, 1982). Nutrient limitation occurs when the supply of the nutrient can not meet the algal demand. Both P and N have been considered potentially limiting to periphyton growth, biomass accrual and production (Steinman et al. 1991b), but other candidates have also been identified.

An important concept underpinning algal nutrient kinetics is the idea that only a single nutrient can be limiting at a time. Two or more nutrients can be simultaneously near growth-limiting concentrations, but only one will actually be limiting (Borchardt, 1996).

Under P-limitation, the growth of an alga will follow the Monad model. The model is expressed as follows

$$\mu = \mu_m[S / (K_S+S)]$$

in which μ is the specific growth rate, μ_m is the maximum specific growth rate reached when nutrients are sufficient, S is the P concentration in the medium and K_S is the half-saturation constant (Darley, 1982). The latter is a P

concentration at which the growth rate is an half of the maximum growth rate (μ_m).

Phosphorus Availability

Orthophosphate is the only important inorganic phosphorus source for algae, although most of them can obtain the element from organic compounds through extracellular cleavage using the enzyme alkaline phosphatase (Darley, 1982, Lobban and Harrison, 1994). Algae also use surplus P (polyphosphate in their intracellular pool) as an additional supply when external P becomes limiting (Fitzgerald and Nelson, 1966, Reynolds, 1984). For periphyton, P availability is also controlled by the current velocity of water, mat thickness, internal P cycling.

A boundary-layer effect on the rate of periphyton nutrient supply has been suggested by Horner and Welch (1981), and Horner et al. (1983). It was quantitatively demonstrated by Riber and Wetzel (1987). The boundary layer is a stagnant layer adjacent to a permeable surface (Schetz, 1984). Nutrient mass transfer occurs by diffusion through the boundary layer into periphyton. The rate of diffusion can be described as a power function of the current of the water above the mat, and as a inverse power function of thickness of the mat. Thus, growth of periphyton is

directly controlled by nutrient concentration within the mat, and indirectly controlled by the mat thickness, ambient concentration, and current velocity.

A developing mat acquires a vertical structure, i. e., an overstory and an understory (Hudon and Bourget, 1983, Hamilton and Duthie, 1984, Paul and Duthie, 1989,). During mat development, overstory algae are better competitors for nutrient and light (Paul and Duthie, 1989), and the P supply to understory algae decreases.

Some of the nutrient demand can be met by animal excretion. Up to 12% of the external input of soluble reactive phosphorus (SRP) has been estimated from grazer excretion (Mulholland et al., 1991b). Grazers also facilitate nutrient diffusion from ambient water to periphyton understory by removing overstory algae (Grimm, 1988). Riber and Wetzel (1987) have found that the phosphorus turnover for periphyton suspended in a medium was within the range of the values for lake plankton.

Indicators of P-limitation

Parameters that are indicators of P-limitation of periphyton include ambient P concentration in the overlying water and the following properties of the mat: alkaline phosphatase activity (APA) (Perrin et al., 1987), surplus P

(Fitzgerald, 1972), N:P composition, and P uptake (Tate, 1990, Mulholland et al., 1991a). Other indicators are relative bicarbonate uptake (Paul et al., 1991), respiration (Fairchild and Everett 1988), biomass accrual and relative specific growth rate (Bothwell 1985), and biomass.

There are interactions among the effects of environmental factors, variations among species in response to low ambient concentrations (Cembella et al., 1984), and an inability of researchers to separate periphyton from bacteria, detritus, and invertebrates. In addition, one can not do controlled experiments on P-limitation in periphyton as one can do for unialgal cultures. Nevertheless, a set of indicators have been suggested for detecting P-limitation in algal cultures (Healey and Hendzel, 1979, Cembella et al., 1984), which may apply to periphyton. These indicators, ambient P concentration, APA, and surplus P are now described in detail.

Ambient P Concentration

Whereas many studies have identified P-limitation in periphyton, fewer studies have quantified the ambient P concentrations that are growth-limiting (Borchardt, 1996). Bothwell (1985) showed that growth rates of periphyton, which were dominated by diatoms, were near saturated at 3-4

μg P l^{-1} in the lower Thompson River at temperatures 1-4 °C. Also higher P concentrations, 25-50 μg P l^{-1} were needed to attain maximum periphyton biomass (Horner et al., 1983, Bothwell, 1988). In one study, 8 μg P l^{-1} was sufficient to achieve maximum biomass (Horner et al., 1990). Biggs (1990) reported a P-limited growth of periphyton with an ambient concentration of 6-10 μg P l^{-1}.

A number of objections occur to the use of ambient nutrient concentrations to indicate nutrient limitation. Cells may contain surplus P (Fitzgerald and Nelson, 1966, Reynolds, 1984) and nutrients may be easily recycled (Riber and Wetzel, 1987, Mulholland, 1996,). In both cases growth may continue even if nutrients are undetectable (Reynolds, 1984). But surplus P is used up quickly after the ambient P has been exhausted (Lobban and Harrison, 1994). When a high external P concentration occurs in the periphyton mat, diffusion could reverse P movement back to bulk water if the ambient water P concentration is low. Sooner or later diffusion out of the mat and/or use therein will reduce the internal concentration to a growth-limiting level. Overall, a deficiency of ambient P supply is always the basic cause of P-limitation. Thus, ambient P concentration is a potential indicator of P-limitation, especially if it is used with other indicators (e. g. Biggs et al., 1998.)

Alkaline phosphatase activity

Phosphatases are a group of enzymes which catalyze the hydrolysis of esters and anhydrides of phosphoric acid, including phosphomonoesterases, phosphodiesterases, phosphotriesterases. Phosphatases split anhydride bonds in phosphoryl-containing anhydrides, and P-N bonds (phosphoamidases) (Siuda, 1984). In aquatic ecology, studies on phosphatases have been focused on phosphomonoesterases, which are a group of enzymes catalyzing the hydrolysis of a variety of phosphomonoesters (Jansson et al., 1988). The term phosphatase has been applied to phosphomonoesterase (Jansson et al., 1988, Whitton, 1991). This definition of phosphatase is followed here for reasons of simplicity and consistency.

Phosphatases have maximum hydrolyzing capacity at different pH values; hence a division of them is made into alkaline and acid phosphatases (Jansson, et al., 1988). Although they have a broad specificity against different substrates, their activity is restricted only to the P-O bond on the phosphomonoesters (Cembella et al., 1984). Acid phosphatases generally are found intracellularly in the cytoplasm of cells. Alkaline phosphatases generally are located on the cell surface or are released into the surrounding water (Jansson, et al., 1988, Lobban and

Harrison, 1994). Both types of phosphatases may be found simultaneously in algae. For these organisms alkaline phosphatases are adaptive, hydrolyzing organic phosphorus to liberate orthophosphate as available P sources for algal uptake (Darley, 1982). Acid phosphatases playing a crucial role in cleavage and phosphate-transfer reactions in metabolic pathways (Lobban and Harrison, 1994).

The synthesis of enzymes is influenced most by the substrate supply or reaction products. Inducible enzymes are those whose synthesis starts in the presence of suitable substrates. Constitutive enzymes are produced independently of an activator, i.e., they are more or less constantly synthesized in the cell. Alkaline phosphatases are inducible and acid phosphatases constitutive (Jansson, et al., 1988, Lobban and Harrison, 1994).

Repression occurs when a compound, often the end product of the enzyme-catalyzed reaction, turns off enzyme synthesis. Alkaline phosphatases are phosphate-repressible. Acid phosphatases are mostly phosphate-irrepressible. A high external orthophosphate concentration or a high level of intracellular phosphate storage can repress synthesis of alkaline phosphatases (Jansson, et al., 1988, Lobban and Harrison, 1994).

Derepression causes the onset of enzyme production

after the depletion the repressors, i. e., the external orthophosphate and/or intracellular phosphorus storage (Jansson, et al., 1988).

Inhibition occurs when a compound reacts with the enzyme itself and stops its activity. Orthophosphate is a common inhibitor which competes with phosphate esters for the active sites on the phosphatases (Jansson, et al., 1988).

APA increases, after the external orthophosphate has been exhausted, and intracellular phosphorus storage has been used up quickly (Lundberg et al., 1989, Lobban and Harrison, 1994). The magnitude of the increase is species-specific and depends on the availability of organic phosphates and the degree of P-limitation experienced by the algae (Healey, 1973, Cembella et al., 1984, Lobban and Harrison, 1994).

Kuenzler and Perras (1965) and Fitzgerald and Nelson (1966) have reported high APA in P-limited algae. Their work formulated the basis for use of APA as an indicator for phosphorus deficiency of algae in culture and in mixed populations in the field. Derepression of APA is often associated with a depletion of intracellular phosphorus storage (Olsen et al., 1983, Petterson, 1980, 1985, Jansson et al., 1988), so high APA reflects algal P deficiency. On

the other hand, high APA also reflects the limiting external orthophosphate supply, because high APA can be detected only when the concentrations of orthophosphate are so low they do not inhibit alkaline phosphatases (Doonan and Jensen, 1980, Jansson et al., 1988). APA above a critical level indicates phosphorus deficiency in external and intracellular supply of phosphorus. Critical APA levels, where switching between P-sufficiency and P-limitation occurs, have been suggested (Table 1).

As an indicator of P-limitation, APA has its shortcomings. In lake water alkaline phosphatases are also produced by bacteria, and zooplankton not just phytoplankton (Cembella et al., 1984, Jansson et al., 1988). Thus, APA measured in periphyton is probably not all from algal origins. Also, APA is inhibited by polyphenols which may be at high concentrations in streams (Stewart and Wetzel, 1982, Freeman et al., 1990, Serrano and Boone, 1991). Furthermore, APA assays are usually conducted with only one substrate. Multiple phosphatases with different substrate affinities have been reported for some algae (e.g., *Euglena*), the phosphatase activity assayed with a substrate may substantially underestimate or overestimate the actual phosphatase potential of the algae (Cembella et al., 1984). So, cautious use of APA as a indicator of P-limitation has

Table 1. Threshold values of APA when switching occurs from sufficiency to P-limitation

Author	Temperature °C	APA			Units
		Ac	Aw	As	
Gage and Gorham, 1985	15	24	48	125	nM P mg^{-1}POM hr^{-1}
Healey and Hendzel, 1979	35	20	between 20-100	100	nM MFP mg^{-1}POM hr^{-1}
		3	between 3-5	5	nM MFP μg^{-1}chl *a* hr^{-1}

Ac critical level when no P limitation occurs

Aw warning level for incipient P starvation

As critical level for severe P starvation

POM particulate organic matter

MFP o-methylfluorescein phosphate

been suggested (Healey and Hendzel, 1979, Cembella et al., 1984, Jansson et al., 1988).

Nevertheless, APA has been used to demonstrate P-limitation of periphyton (Bothwell, 1985, 1989, Perrin et al., 1987, Biggs, 1990, Mulholland et al., 1991b, Steinman et al., 1991b, Lozona and Pratt, 1994, Invers et al., 1995, Mulholland et al., 1995, Johnson and Tuchman, 1997, McCormick and Stevenson, 1998). Moreover, the response of APA to P-limitation usually occurs coincident with that of other indicators (e. g. Bothwell, 1985, Mulholland and

Rosemond, 1992).

Surplus P

Algae accumulate P as polyphosphates by "luxury uptake", when P supply is sufficient. This P storage is called surplus P. Algae use stored P for growth when the external supply is not sufficient. When the growth of algae is P-limited, little or no surplus P occurs (Fitzgerald and Nelson, 1966, Reynolds, 1984). Thus, a low surplus P may indicate that algae are replete with P for phytoplankton (e.g., Fitzgerald and Nelson, 1966, Petterson, 1980) and filamentous algae, *Cladophora* (Fitzgerald, 1972).

Threshold values for surplus P are shown in Table 2. Because certain cellular P fractions, probably polyphosphates, control repression of APA, surplus P and APA are not independent from each other (Cembella et al., 1984). An inverse relation should occur between them (Jansson, et al., 1988). Cellular polyphosphates also act as a noncompetitive inhibitor of phosphate uptake in P-limited culture of *Scenedesmus* sp. (Rhee, 1974).

Table 2. The critical threshold values for surplus P

Author	Surplus P			Units
	Pa	Pw	Ps	
Gage and Gorham, 1985	10	8	4.3	μg particulate P mg^{-1}POM
Chrost and Overbeck, 1987	15%		10%	surplus P/particulate P
Fitzgerald and Nelson, 1966		0.8		μg surplus P mg^{-1}DW
Healey, (1978) in Gage and Gorham, 1985			5	μg particulate P mg^{-1}DW
Petterson, 1980		0.2		μg surplus P mg^{-1}C
Whitton et al., 1990	0.76%		0.3%	cellular P/DW

no data in blank cells

Pa critical level to begin surplus P accumulation

Pw warning level for P limitation

Ps critical level for severe P starvation

POM particulate organic matter

DW dry weight

Diel Rhythms of Algal Physiological Parameters

Many algal physiological parameters are known to fluctuate over the diel cycle (Darley, 1982). By timing the maximum rates of various activities for different times during the diel cycle, algae could reduce interspecific

competition (Chisholm et al., 1978), and could be allocating resources to take maximum advantage of the oscillating levels of environmental resources (Darley, 1982).

The diel rhythms of activities of phytoplankton have been summarized by Sournia (1974). The rhythms of cell division, photosynthetic capacity, and P-uptake are closely related to the diel rhythm of APA (Cembella et al., 1984). The cell division of many species is phased (partially synchronized) in that a large percentage of the cells divide at a certain time of the day or night. Nocturnal division apparently is favored by many species. Short-term photosynthetic measurements at constant, saturating light intensities show maximum rates in the morning and minimum rates in the early evening. Diel rhythms in P_m (light-saturated photosynthetic rate in photosynthesis-irradiance curve) are found in many species (Darley, 1982). The time at which P_m is maximal varies but is most often in the morning or midday (Henley, 1993). Usually rates of P-uptake are higher during the day than at night, a trend that reflects light stimulation of uptake rates (Darley, 1982).

Few studies on these three rhythms and the diel rhythm of APA have focused at P-limited algae. Some of these studies will be described in the Discussion.

Periphyton Culture

Because this study was done in the laboratory, a brief review of periphyton culture is presented next. Most laboratory cultures have been performed with flow-through systems and river water. Little research has been reported on methods to grow periphyton in static growth media. Some media have been developed to culture the filamentous alga *Cladophora* (Gerloff and Fitzgerald, 1976, Rosemarin, 1982). Hansson (1988) used Chu-10 growth medium to seed and culture periphyton on a nylon net placed in an aquarium. Otten and Willemse (1988) have developed a new medium to culture periphyton in Petri dishes to observe modes of attachment. Lozano and Pratt (1994) have used synthetic, moderately hard dilution water to culture natural periphyton to study the effect of nutrients on toxicant stress (EPA, 1989).

Studies of periphyton in the laboratory suffer at present because observations are not entirely reproducible, because inocula are not composed of exactly the same algae species and/or mix of detritus and bacteria. To address this issue we replicated mesocosms and repeated the same observations in time.

CHAPTER III

MATERIALS AND METHODS

Introduction

Periphyton communities, which were collected from littoral rock substrates, were used to colonize a Styrofoam sheet in an aquarium in a growth chamber. The control factors for each experiment were temperature, light intensity, photoperiod, depth of medium, nutrient concentrations of medium, and length of the period of culture. The following parameters were measured: chlorophyll a (chl a), APA, surplus P, soluble reactive P (SRP). The diurnal sampling intervals were four hours, five days elapsed between sampling days for most experiments.

Except for two pilot experiments, twelve experiments were conducted. An experiment consisted of monitoring colonization of a biofilm in one aquarium over time. A pair of experiments was conducted simultaneously. The design of these experiments is shown in Table 3. Experiments 1-8 were conducted with almost the same protocols to test hypotheses I and II. To test hypothesis I that no change in biomass, APA, and surplus P occurred during periphyton development, samples of these three parameters were taken at six sampling days in five-day intervals throughout 37 days. Linear

Table 3. The protocols for experiments

Experiment	Hypothesis tested	P concentration in added medium µg P l^{-1}		Temperature °C	Medium change	Light-dark cycle	Sampling days in culture	Sampling time
		1-12 days	after 12 days					
1, 2	I, II	5.0	1.0	12.0	daily	11.00 : 13.00	12, 17, 22, 27, 32, 37	four hour intervals in light period
3, 4	I, II	5.0	1.0	13.5	daily	11.50 : 12.50	12, 17, 22, 27, 32, 37	four hour intervals in light period
5, 6, 7, 8	I, II	5.0	5.0	16.6	daily	13.00 : 11.00	12, 17, 22, 27, 32, 37	four hour intervals in light period
9, 10	III	5.0	5.0	16.6	daily until day 19	13.00:11.00 for days 1-18, in the dark for days 19-21	19, 20, 21	four hour intervals continuously for 3 days in the dark
11, 12	II, III	5	5	16.6	daily until day 19	13.00:11.00 for days 1-18, in the dark for days 19-21	18, 19, 20, 21	four hour intervals continuously in light period of day 18, and the following 3 days in the dark

regressions were utilized to determine if there were significant correlations for these parameters with the days of periphyton in culture. To test hypothesis II that no pattern of change in APA occurred during the diurnal period of light-dark cycles, APA was sampled at the interval of four hours during the light period of each sampling day. Then ANOVA was utilized to determine if there was a significant effect of sampling time of the day on APA. Experiments 9-12 were different in sampling design, however in that observations began after 18 days, not 12 days. The observations on APA at the interval of four hours continued then for three days in the dark. To test hypothesis III that no significant difference occurred in APA in continuous darkness for periphyton that have been cultured in a light-dark cycle, data on APA in experiments 9-12 were analyzed with ANOVA to determine if there was a significant difference among APA at different sampling times of the day.

Periphyton were colonized in replete media with 5 µg P/l for 12 days. As shown in Table 3, the culture temperatures were below 15 °C for experiments 1-4, and above 15 °C for experiments 5-12. After 12 days of colonization, the P concentration in the added media for experiments 1-4 was 1 µg/l, which is below the critical levels (3-4 µg/l) for P-limitation of growth at temperatures below 10 °C as

reported by Bothwell (1985). For experiments 5-12, the P concentration in the added media was 5 µg/l, which is also below the critical value (25 µg/l) for P-limitation of growth at temperatures above 15 °C as reported by Horner et al.(1983). Below are details of the methods.

Collection of Periphyton for Seeding

Periphyton, growing on light exposed rock substrates (25 -50 cm^2), were collected at 1630 hr (CDT) from Arcadia Lake (Oklahoma County, OK) for pilot experiments and Sooner Lake (Noble County, OK) for experiments 1-12. Immediately after removing the rocks from water, visible invertebrates and sand were removed with forceps. Periphyton were scraped with a razor blade into a clear acid-washed high-density polyethylene (HDPE) 500 ml bottle. Then the bottle was half-filled with water from the collection site and immediately transported to the laboratory in the dark on ice. On each sampling day, a grab sample of water was collected for analyses of nutrients (total P, soluble reactive P (SRP), nitrate, ammonia) in a clear acid-washed HDPE bottle, and transported in the dark on ice to the laboratory. The water samples were preserved in the laboratory according to the techniques recommended by EPA (1979). Water temperature was measured in the field with a

hand-held thermometer; pH was measured in the laboratory with a Corning Model 17 pH meter.

Setting Up Aquaria for Experiments

For each experiment, an aquarium (31.2 cm x 26.2 cm x 51.8 cm), an undergravel filter (1.6 cm x 25.4 cm x 50.8.8 cm, Lee's Aquarium & Pet Products), and an open-cell Styrofoam sheet (3.0 cm x 23.6 cm x 49.8 cm, Flora Craft) were used. The undergravel filter is a molded plastic plate with 1" water lift tubes. The filter is perforated with many slits to let the water flow through. The aquarium and the filter were washed with phosphorus-free detergent, rinsed three times with tap water and deionized water, then air dried. The Styrofoam sheet was soaked three times sequentially with tap water and deionized water for up to 12 hours and air dried.

The dry Styrofoam sheet was tied to the filter with seven plastic ties, and then was fixed to the bottom of the aquarium (sheet up) with True Value contact cement and Dow Corning general purpose sealant. After the setup dried in the air for three days, two water lift tubes with connectors were fixed to the ready-made holes at the two nonadjacent corners of the filter. Also, two holes with the diameter of 1.025 cm^2 were made with a brass cork borer (size number 8)

through the center of the Styrofoam sheet. In addition to facilitating the circulation of medium for the ongoing experiment, these holes released the tension caused by the buoyant force of the styrofoam sheet in the medium.

To reduce the possible effect of toxicants from the styrofoam sheet and the glue used, the setup was soaked once for one day, and then twice for one hour or more with deionized water. Then the soaking procedure was repeated with deionized distilled water (DD water). The aquarium was air-dried in the culture chamber, and covered with aluminum foil until used.

At the start of each experiment, culture medium was added to the aquarium. Two air stones were put into each of the two lift tubes and remained at the bottom. An Aquaculture Model ULMK-503 double outlet aquarium air pump pumped air through the two air stones to aerate and gently disturb the medium in the aquarium.

Colonization and Culture

The periphyton collected from Sooner Lake were harvested by filtration with 1.2 mm Whatman 4.24 cm GF/C glass fiber filters after blending for about 1 minute with a blender (Model No. 400.829002, Sears Roebuck Co.). Visible invertebrates and sand were removed from the filters with

forceps. The periphyton were then rinsed into a clear acid-washed 1000 ml cylinder with the culture medium. The cylinder was filled with the culture medium, inverted several times to evenly distribute the periphyton, and divided into 500 ml aliquots. Each of these aliquots was then inoculated into one aquarium. Each aquarium was placed in a Percival E-514U growth chamber for an experiment. On each collection day for periphyton, two experiments started at 1900 hr. I allowed periphyton to colonize for 37 days for experiments 1-8, and 21 days for experiments 9-12.

The open-cell Styrofoam sheet at the aquarium bottom served as substrate for periphyton colonization. The volume of the culture medium in the aquarium was 6.78 $\pm$ 0.2 l. The upper surface of the substrate was 4.4 cm below the surface of culture media.

The photon flux density (PFD), which was measured with a LI-COR Model LI 189 photometer, was 200.3 $\mu E\ m^{-2}\ s^{-1}$ at the medium surface. This PFD level is within the range (135-400 $\mu E\ ^{-2}\ s^{-1}$) of saturation intensity for photosynthesis of periphyton reported by Hill and Boston (1991).

As shown in Table 3 and Table 4, the culture temperatures for experiments 3-6 were constant and close to that measured on the day of collection. The temperature measured at the time of periphyton collection for

experiments 1-2 was 8.7 °C (Table 3), and could not be reached in the growth chamber because of facility limitation. The culture temperature for experiments 1-2 was set at 12 °C, which is the lowest of the range of maintainable constant temperatures for the growth chamber. For experiments 1-6, the diel photoperiods were also adjusted to close to that in the season when collection was made (Table 3).

The culture medium Rodhe 8 (pH 7.0-7.5) was used, which had a pH (about 7.5) close to that in the collection site of Sooner Lake (Nichols 1973). Stock solutions for the medium constituents were made with fresh deionized glass-distilled water. Then, these constituent stocks were immediately sterilized for 30 minutes at 15 psi. Each time after using the constituent stocks to make the medium stock, the remains of the constituent stocks were pasteurized for 30 minutes at a temperature higher than 73 °C (James, 1978). The stock medium was stored in the dark. Just before using, medium was made with freshly deionized water.

To replace the medium, one clear alkali-washed rubber tube was used to gently siphon medium out from the aquarium. Particles on the Styrofoam sheet, especially the periphyton which had died or were not attached to the Styrofoam sheet, were also siphoned out the aquarium after 10-12 days of

colonization.

During 12 days of colonization, the medium was replaced by siphon daily at the rate of 20% total volume, and the medium was aerated to attain uniform colonization. After 12 days, medium was replaced daily at the rate of 96.8 ± 0.2% (6.56 ± 0.2 l). Replacement of medium occurred for experiments 1-8 through the whole experiment, and for experiments 9-12 until 19 days.

Sampling Periphyton of Culture

In the experiments 1-8, the cultured periphyton and medium were sampled at 12, 17, 22, 27, 32 and 37 days (Table 3). To sample randomly, the central part (20.3 cm x 45.7 cm) of Styrofoam sheet was divided to 144 squares with 18 columns and 8 rows. Except for the two corner squares with the water lift tubes and one central square with the above-mentioned two holes, each of the remaining 142 squares was assigned a sampling sequence number, which was among 1-142 and was obtained from a Random Number Table (Steel and Torrie, 1980). On each sampling day, periphyton were sampled with a brass cork borer at 4 hour intervals during the light cycle to determine chlorophyll *a* (chl *a*). Three replicates for each sample of chl *a* were taken in three squares. At each sampled square for chl *a*, the samples for

alkaline phosphatase activity (APA) were taken (duplicates for experiments 3-12). In experiments 9 and 10, sampling was conducted at 4 hour intervals for the 72 hours in the dark after day 18. During the light period of day 18 and the following 72 hours in the dark, sampling was conducted at 4 hour intervals in experiments 11 and 12.

The periphyton samples for surplus P (n=3), and medium samples for SRP analysis were taken only for experiments 1-8 at 1100 hr of each sampling day. No samples for surplus P and medium were taken for experiments 9-12.

A brass cork borer with an area of 0.258 cm^2 was used to sample periphyton for APA; the borer had an area of 1.025 cm^2 for chl _a_ and surplus P. After sampling, the contents were removed from borers with a glass rod. The attached periphyton and about 1 mm Styrofoam were sliced from the remainder of the styrofoam. The chl _a_ samples were placed into screw-cap centrifuge tubes with 12.5 ml of 90% acetone for 24 hour extraction at 4 °C in the dark. The sample for APA was put into a fluorometer tube with 0.5 ml of medium, which was made with newly boiled and cooled DD water. Then the tube was sealed with parafilm and immediately frozen at -84 °C. Samples for surplus P were harvested onto a Whatman GF/C glass fiber filter, and rinsed twice with 50 ml DD water. Then the filter with sample was folded, wrapped with

aluminum foil, and immediately frozen at -20 °C. The samples for SRP in the medium were filtered through Whatman GF/C glass fiber filters, stored in acid-washed HDPE bottles, and immediately frozen at -20 °C.

Laboratory Analyses

All the chemicals used in the analyses were of analytical grade or equivalent. All the standard stocks, standards, blanks, and reagents were made with fresh distilled deionized water except the blanks and standards for the analysis of medium soluble reactive P, which were made with phosphorus-free medium. Triplicate measurements were made for chl *a* and APA; duplicates for all other samples. Standard curves were prepared daily for each series of measurements. The phosphorus standards made from the prepared stock were compared with the standards made from Hach standard stock solutions. A Shimadzu TB-85 spectrophotometer was used to measure surplus P, chl *a* and nutrients; a Model 10 Turner fluorometer was used to measure APA.

Analyses of Lake Water and Medium in Culture Aquaria

Nutrient Analyses of Lake Water

Ammonia analysis followed the phenol / nitroprusside

method after water samples were adjusted to pH 7 (Solorzano, 1969). Nitrate analyses followed the cadmium column method of Strickland and Parsons (1968). Soluble reactive P (SRP) analysis for water samples followed the ascorbic acid method of EPA (1979). The measurement of soluble reactive P includes orthophosphate which is readily useable by algae plus a variable amount of organic P, which may not be useable. Thus, SRP is an approximation of useable P in the water (Lind, 1985). Total phosphorus analysis followed the ammonium persulfate digestion method of EPA (1979).

Analysis of Soluble Reactive P in Culture Medium

The malachite green method was followed without cartridge concentration and automation (Camarero,1994). Three parts of 0.45 % (wt/vol) malachite green oxalate solution and one part of 4.2 % (wt/vol) ammonium heptamolybdate in 8 N sulfuric acid solution were mixed as the reagent. Then 150 μl of detergent Tergitol NP-10 (Sigma Chemical Co.) and 100 ml reagent was mixed. The mixed reagent was stirred for 20 minutes and then passed through a Waterman GF/C glass fiber filter. The detergent caused flocculation of the possible contaminating P contained in the reagent, which was retained on the filter.

Because of the nature of medium samples, phosphorus-

free Rodhe 8 medium was used to make blanks and standards instead of distilled deionized water. Blanks, standards, and samples were filtered through Waterman GF/C glass fiber filters.

Analysis of Chlorophyll a

Chlorophyll a analysis followed the method described by APHA (1992). The sample was extracted with 90% acetone at 4 °C in the dark for 24 hours. The absorbances of the extraction at 664 nm and 750 nm before, and at 665 nm and 750 nm after adding one drop of 1 N HCl were measured. Chl a was calculated as follows:

$$\mu g \text{ chl } a \text{ per sample} = 26.7 \ (OD_b - OD_a) \ v \ / \ l$$

Where OD_b = absorbance at 664 nm corrected for turbidity at 750 nm; OD_a = absorbance at 665 nm corrected for turbidity at 750 nm; v = volume (ml) of solvent for extraction; l = length (cm).

A 90% acetone blank with about same amount of Styrofoam as that attached by periphyton sampled was also measured each time. There was no influence of Styrofoam on the measurement. When a small difference of 0.001 to 0.002 occurred, a correction for the difference was made.

Analysis of Surplus P

Analysis of surplus P followed the method of Wynne and Berman (1980). A sample in 50 ml DD water was extracted for 60 minutes at 100°C in a boiling water bath. After cooling to room temperature, the sample was filtered through a Whatman GF/C glass fiber filter. The filtrate was analyzed for SRP as above.

APA Analysis

Analysis of particulate APA followed Bothwell (1988). Exactly 0.6 ml of 100 μM 3-0-methylfluorescein phosphate (MFP) and 10 μm tris buffer was added to a fluorometer tube with 5 ml medium, which had been prepared with newly boiled and cooled DD water, and sample. The tube was sealed with parafilm and inverted several times. Then, the fluorescence of the sample was measured immediately and after 1 hour with a Model 10 Turner fluorometer. About 30 minutes after the first measure, the tubes were mixed by inversion again.

APA samples were compared to a control of medium made with newly boiled and cooled DD water. APA was measured as the increase of fluorescence, which was related to a standard curve prepared with different concentrations of 3-0-methylfluorescein (MF) and blank. The measurements of chl *a* of the samples were used to normalize APA measurements

to biomass.

The correction of non-enzymatic hydrolysis of substrate in APA measurement has been made by some researchers(e.g. Boon, 1989, Vrba et.al., 1995). The method for the correction is to process autoclaved samples alongside the treatment samples (Scholz and Boon, 1993). Because of the interference caused by autoclaving, the correction of non-enzymatic hydrolysis of substrate was not used (e.g. Scholz and Boon, 1993). Because phosphate precipitation occurs when the medium is autoclaved, the correction for the non-enzymatic hydrolysis of MFP could not be made in this study.

Statistical Analysis

To test hypothesis I in experiments 1-8, the linear regressions of chl a, APA, surplus P with the days of periphyton in culture were utilized to determine if there were significant correlations among these parameters with the days of periphyton in culture. If there were such a significant correlation, hypothesis I would be rejected.

To test hypothesis II, analysis of variance (ANOVA) techniques were utilized. The data were in a split-split-plot arrangement in a completely random design with subsampling. This design was utilized for APA in paired

experiments 1-2 and 3-4, and pooled experiments 5-8. A three-way ANOVA was used to examine the effect of month of seeding (Month), time after seeding (Day), and sampling time during the day (Time) on APA for pooled experiments 5-8. A two-way ANOVA was used to examine effects of above-mentioned factors, with exception of the effect of Month, on APA for the paired experiments 1-2 and 3-4. For the observations during the light period of day 18 and the following dark period in paired experiments 11-12, a two-way ANOVA was used to test the effects of sampling time and time after seeding. The above-mentioned three-way ANOVA procedures were also used to test hypothesis III in pooled experiments 9-12. If there were a significant effect of TIME on APA in paired experiments 1-2, 3-4, and pooled experiments 5-8, hypothesis II would be rejected. And hypothesis III would be rejected if there were a significant effect of Time on APA in pooled experiments 9-12.

Also the interactions (two-way and three-way) among Time and other factors were tested. When the effect of Time and its interaction with one other factor was significant, the data were sorted by the other factor. ANOVA for data in a split-plot arrangement in a completely random design was used to separately test the effect of Time for the sorted data. When ANOVA for the sorted data exhibited a

significant effect of Time, multiple comparisons with Duncan's procedure were used to learn how Time affected APA in the sorted data. For example, if an interaction occurred between Day and Time, data were sorted by Day. ANOVA was used to separately test the effect of Time on each sampling day. For the sampling days, in which a significant effect of Time was shown, multiple comparisons were applied to APA. SAS software was used for all above-mentioned statistical analyses (SAS Institute Inc.).

CHAPTER IV

RESULTS

Physical and Chemical Conditions of Water at the Collection Site

The physical and chemical conditions of water at the sampling site of Sooner Lake are summarized in Table 4. SRP was lower than the detection limit (5 µg Pl^{-1}) except on May, 1993, when SRP and TP were 31 and 33 µg Pl^{-1}, respectively. TP during the summer ranged from 10 to 18 µg Pl^{-1}. Sooner Lake was meso-eutrophic, because meso-eutrophic lakes have a range of TP 10-30 µg Pl^{-1} (Wetzel 1983). The water was also slightly alkaline (7.4-7.7), similar to the culture media used for experiments.

The N:P rate of supply was estimated as the molar ratio of NH_4^+-N + NO_3^{--}-N + NO_2^--N^-/SRP. Values of 5 µg Pl^{-1} were used when SRP was below the detection limit. The N:P supply ratios ranged from 16:1 to 565:1 (Table 5). Because an N:P supply ratio of 20:1 or more indicates P limitation, the algae obtained from Sooner Lake were probably already under moderate to severe P stress.

Table 4. The date of periphyton collection, related experiments (Exp.), temperature (T, °C), pH, total phosphorus (TP, µg P l^{-1}), soluble reactive phosphorous (SRP, µg l^{-1}), ammonia (NH_4^+, µg N l^{-1}), nitrate and nitrite ($NO_3^-+NO_2^-$, µg N l^{-1}) and standard deviations at the Sooner Lake sampling site

Date	Exp.	T	pH	TP	SRP	NH_4^+	$NO_3^-+NO_2^-$
Dec.12 1992	1, 2	8.7	7.4	31.0 ±3.6	bd	13.7 ±0.1	1214.9 ±2.0
Feb.19 1993	3, 4	13.5	7.5	22.4 ±0.4	bd	bd	471.7 ±6.0
Apr.11 1993	5, 6	16.6	7.6	14.8 ±0.9	bd	87.7 ±0.9	55.7 ±2.4
May 24 1993	7, 8	22.0	7.6	33.6 ±1.3	30.8 ±0.7	58.4 ±0.9	24.0 ±3.3
Jul.6 1993	9, 10	25.0	7.7	10.0 ±0.2	bd	43.5 ±1.5	23.0 ±2.6
Aug.22 1993	11, 12	30.3	7.6	18.14 ±0.92	bd	20.7 ±0.9	14.1 ±0.1

bd: below detection limits for SRP and NH_4^+

Table 5. Molar N:P supply ratio in Sooner Lake

Date	Dec.12	Feb.19	Apr.11	May 20	July 6	Aug.22
N:P	565:1	217:1	66:1	38:1	30:1	16:1

Development of Cultured Periphyton

Changes of Chlorophyll a, APA, Surplus P and SRP in Experiments 1-8

A consistently synchronous change occurred in chlorophyll a (chl a) during paired experiments (Fig. 1). During experiments 3 and 4 , and 5 and 6, chl a decreased relative to the days of periphyton in culture. After day 22 in culture in all the experiments with exception of experiments 3 and 4, chl a was relatively stable or rose slightly. Daily means of chl a were less than 1 μg cm^{-2} in all these experiments, and were less than 0.7 μg cm^{-2} after 22 days of culture.

A tendency occurred for APA/area (APA normalized by area) and APA/chl a (APA normalized by chl a) to increase relative to the days in culture in all experiments (Figures 2 and 3). APA/area increased more than 50% in experiments 1 and 2, but not as much in the other experiments (Figure 2). APA/chl a increased more than 50% in experiments 1, 3, 4, 5, and 6, but only slightly in the rest (Figure 3).

Surplus P/area (surplus P normalized by area) increased in experiments 1 and 2, decreased in experiment 3 and 4, and slightly decreased in the other experiments relative to the days in culture (Figure 4). Surplus P/chl a (surplus P

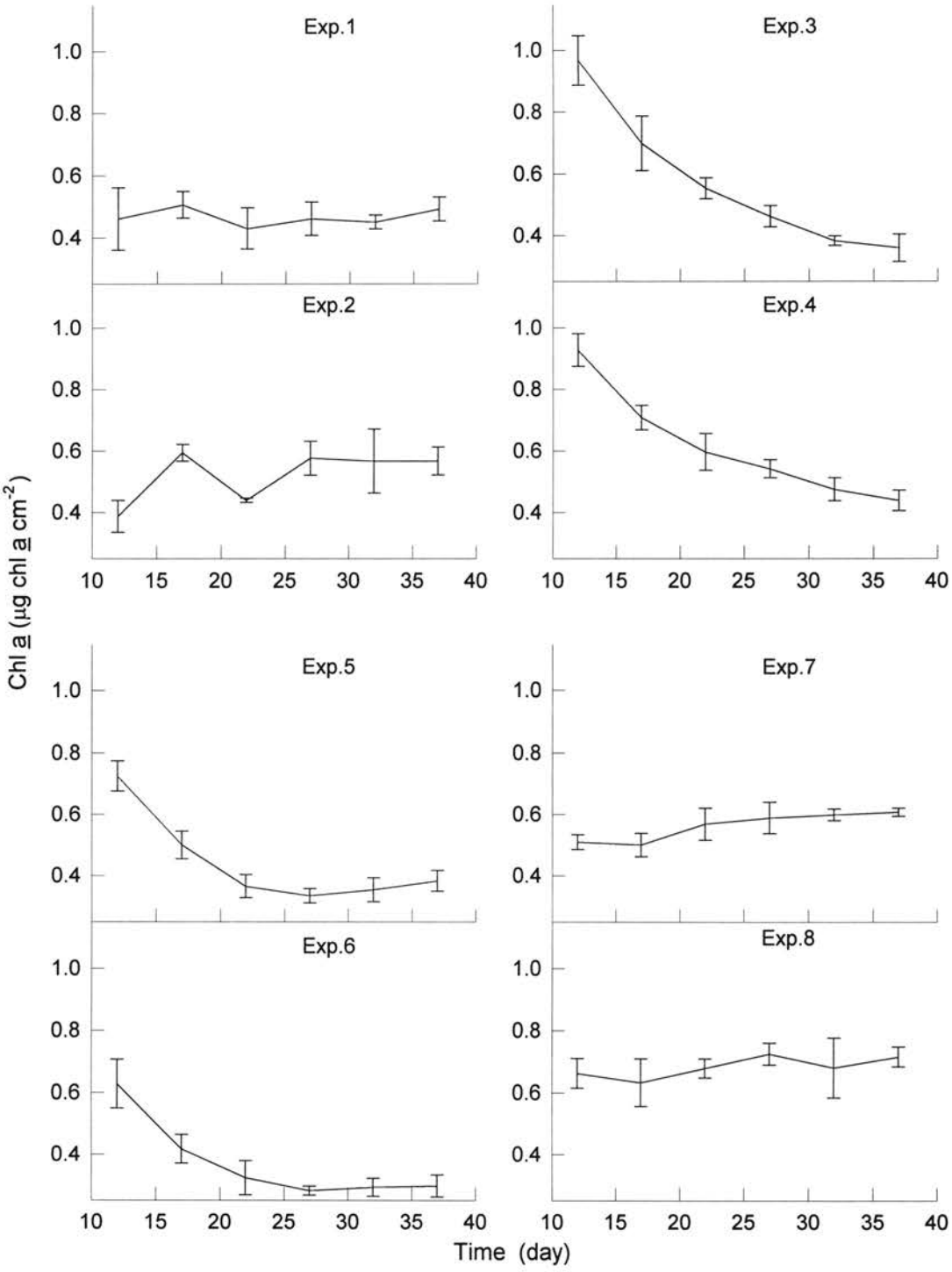

Figure 1. Changes of Chl a during periphyton development.
Values plotted are daily means ± 1SD; n=12.

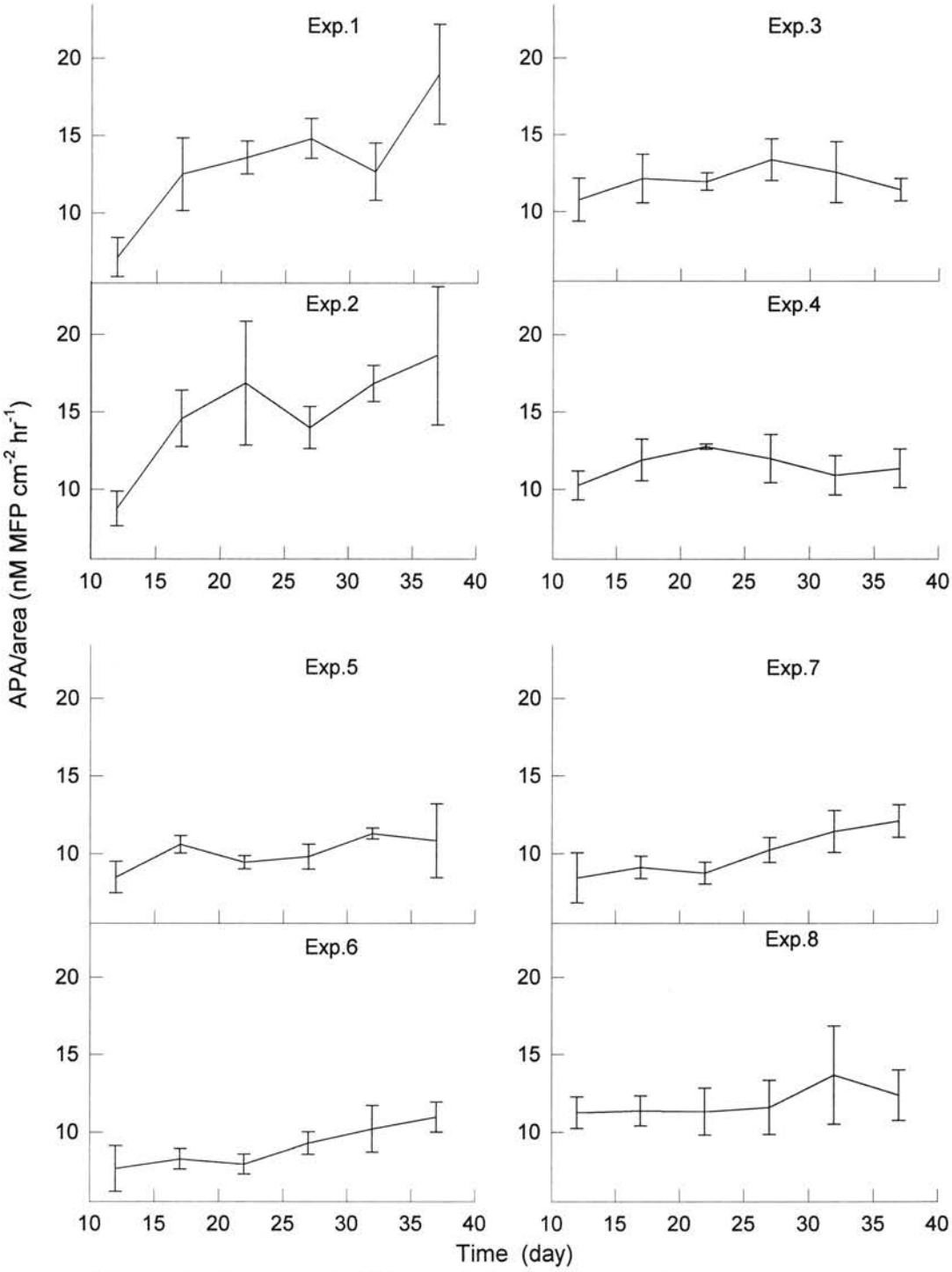

Figure 2. Changes of APA/area during periphyton development. Values plotted are daily means ± 1SD; n=24.

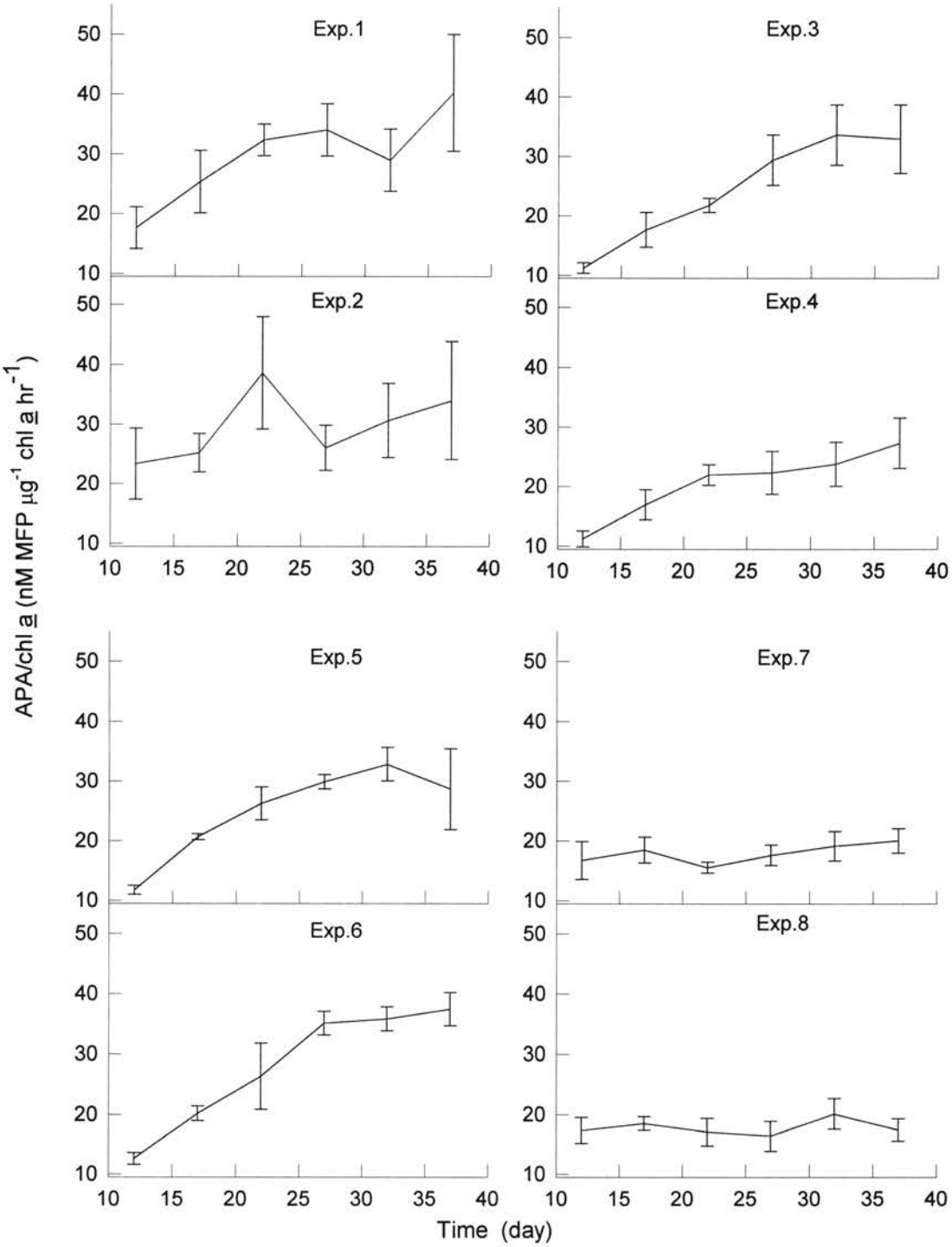

Figure 3. Changes of APA /chl a during periphyton development.
Values plotted are daily means ± 1SD; n=24.

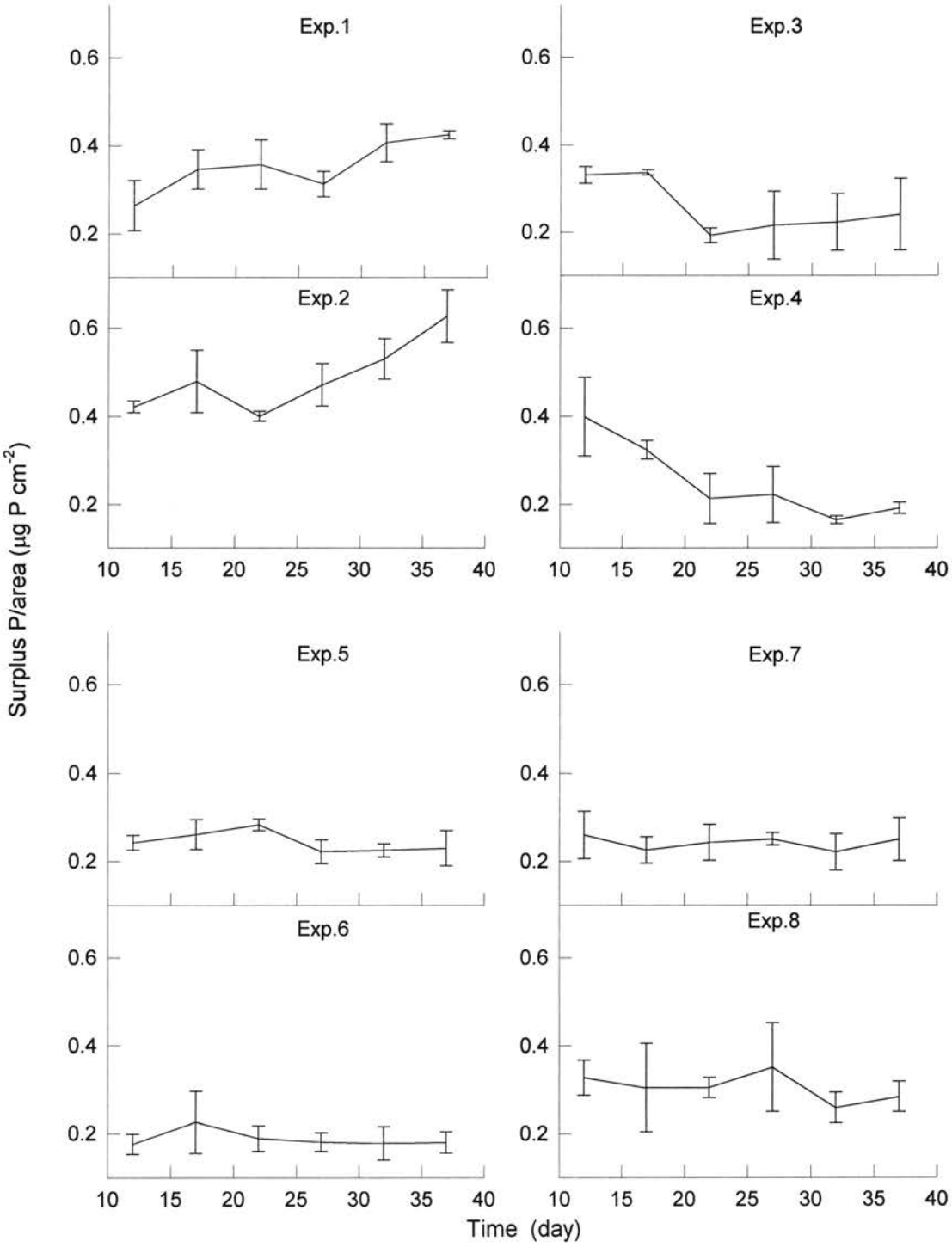

Figure 4. Changes of surplus P/area during periphyton development. Values plotted are daily means ± 1SD; n=3.

normalized by chl a) had wide variation in experiments 1, 2, 3, and 6. It increased in experiments 1 and 3 (Figure 5). In experiments 7 and 8, it tended to decline toward the end of the experiments. In experiments 5 and 6, it rose and then declined. No consistent trend occurred in the other experiments.

The sample means of SRP measured in all the experiments were from 2.1 to 6.2 μg Pl^{-1}. In all cases, SRP declined synchronously in paired experiments with the days in culture (Figure 6). In experiments 5 and 6, SRP rose slightly at the end of the experiments. In experiments 7 and 8, SRP increased at day 27 then declined toward the end of the experiments. The tendency for SRP to decline during the experiments suggests that P demand was increasing and the periphyton were experiencing greater P-limitation. To learn if these trends were statistically significant, linear correlations between the variables above-mentioned and culture time were calculated.

Linear Correlations of Chl a, APA, Surplus P and SRP with Culture Time

Linear correlations of APA/area and APA/chl a with the days in culture were positive and significant in most experiments (Table 6). When they were not significant

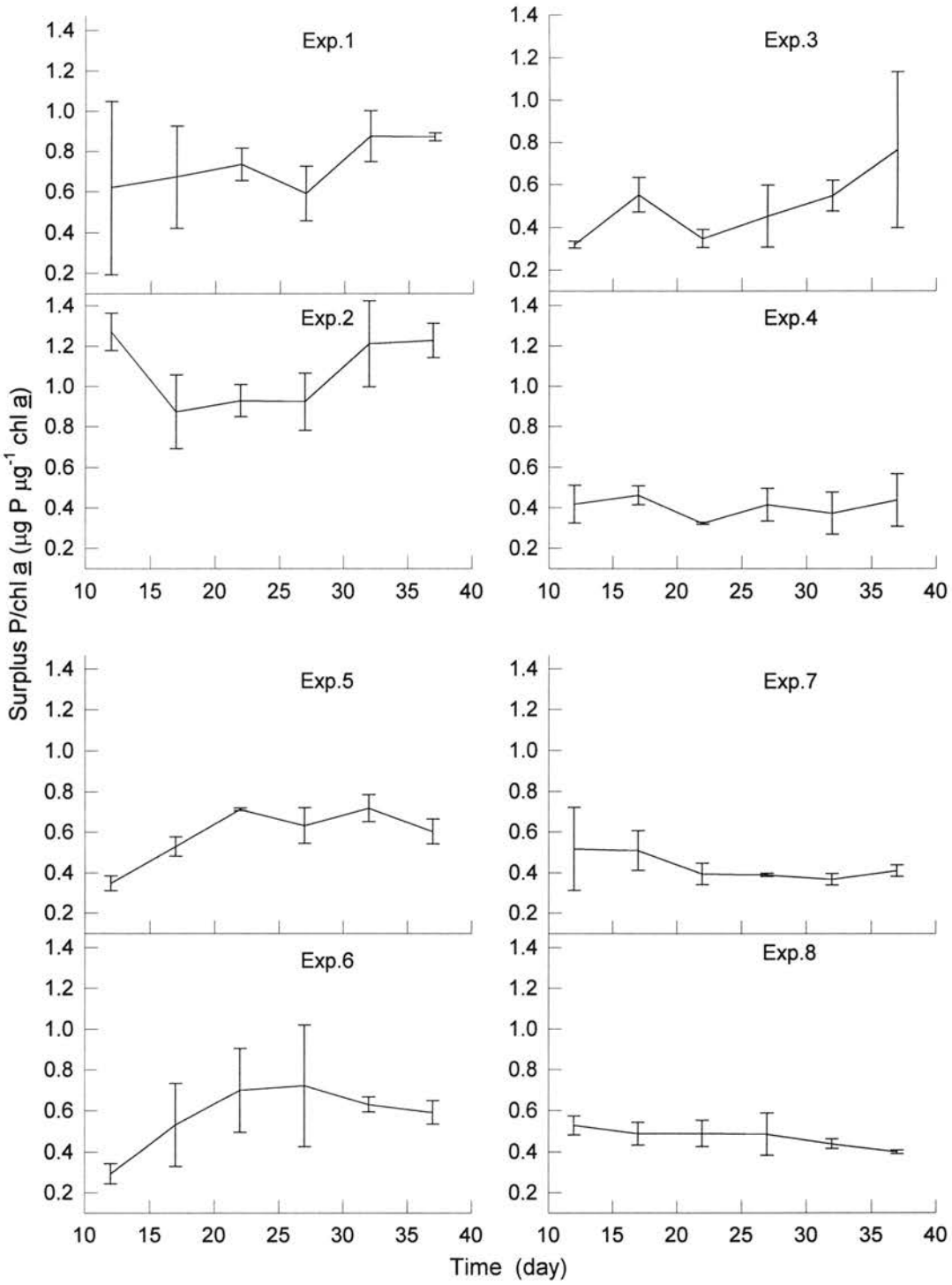

Figure 5. Changes of surplus P/chl a during periphyton development. Values plotted are daily means ± 1SD; n=3.

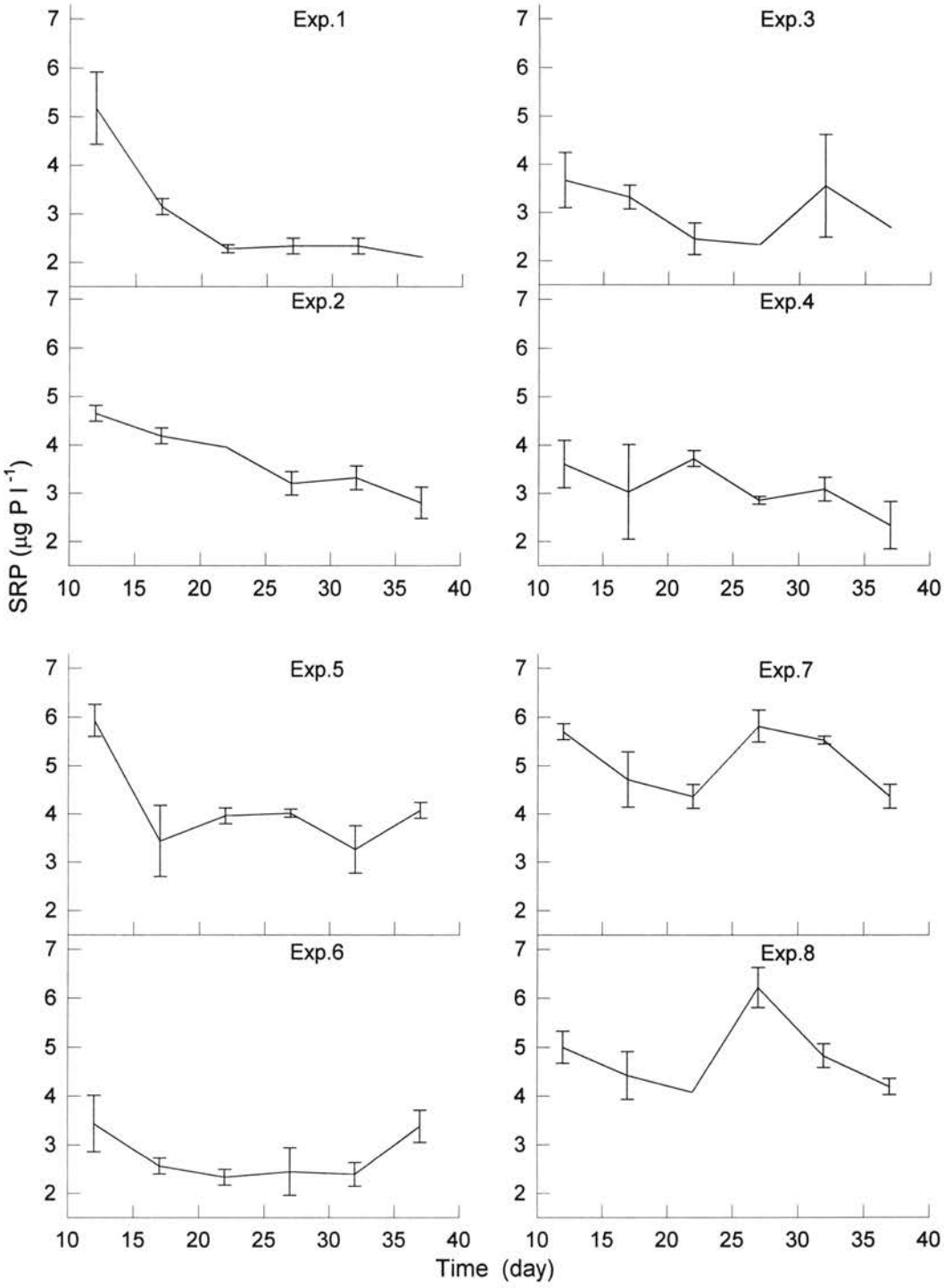

Figure 6. Changes of SRP during periphyton development.
Values plotted are daily means ± 1SD; n=2.

Table 6. Correlation coefficients (r), observed significance levels (P), and sample size (n) for correlations of measured variables with the days in culture.

Experiment		Chl *a*	APA/area	APA/chl *a*	Surplus P/area	Surplus P/chl *a*	SRP
1	r	+0.01	+0.66[h]	+0.54	+0.70	+0.39	-0.79
	P	0.925	<0.001	<0.001	<0.001	0.017	0.002
	n	72	72	72	36	36	12
2	r	+0.38	+0.57	+0.29	+0.73	+0.20	-0.94
	P	<0.001	<0.001	0.015	<0.001	0.248	<0.001
	n	72	71	71	36	36	12
3	r	-0.89	+0.13	+0.78	-0.52	+0.56	-0.328
	P	<0.001	0.115	<0.001	0.001	<0.001	0.299
	n	72	144	144	36	36	12
4	r	-0.84	+0.036	+0.67	-0.80	-0.04	-0.59
	P	<0.001	0.668	<0.001	<0.001	0.813	0.050
	n	72	144	144	36	36	12
5	r	-0.72	+0.30	+0.65	-0.37	+0.64	-0.52
	P	<0.001	<0.001	<0.001	0.028	<0.001	0.082
	n	72	143	143	36	36	12
6	r	-0.63	+0.46	+0.76	-0.18	+0.44	-0.06
	P	<0.001	<0.001	<0.001	0.304	0.007	0.843
	n	72	144	144	36	36	12
7	r	+0.51	+0.45	+0.21	-0.08	-0.48	-0.21
	P	<0.001	<0.001	0.011	0.645	0.003	0.518
	n	72	144	144	36	36	12
8	r	+0.19	+0.20	+0.05	-0.24	-0.62	-0.05
	P	0.101	0.014	0.565	0.154	<0.001	0.890
	n	72	144	144	36	36	12

normalized one way, e. g. as APA/chl a, they were significant another way, e. g. as APA/area. Thus, APA increased with culture time.

Linear correlations of chl a with the days in culture were negative and significant in experiments 3 and 4 and 5 and 6. In paired experiments 1-2 and 7-8 correlations were significant and positive in one experiment of the two, but not significant in the other. Thus, the weight of these analyses shows chl a declined during most experiments and/or rose slightly during some experiments.

The correlation coefficients of surplus P/area with the days in culture were relatively high (greater than 0.70), positive in experiments 1 and 2, and negative in experiment 3, 4, and 5. The linear correlations of surplus P/chl a with the days in culture were positive in experiments 1, 3, 5, and 6, and negative in experiments 7 and 8. Surplus P therefore did not change in any predictable manner, given these results.

Linear correlations of SRP with the days in culture were negative and significant only in experiments 1, 2, 4, and 5. Thus, SRP tended not to change, but when it did, it decreased relative to time.

Linear Correlations of Chl a, APA, Surplus P with SRP

Linear correlations between chl a. APA, and Surplus P with SRP for individual experiments and pooled experiments 1-8 are given in Table 7. It was found that a significant correlation existed between chl a and SRP, and APA and SRP only in some individual experiments. When the data for experiments 1-8 were pooled, however, the positive correlation between chl a and SRP and the negative correlation between APA and SRP were consistent with the proposition that higher concentrations of SRP may cause higher chl a and lower APA. The latter is more reasonable than the former, however.

Linear correlations of surplus P/area with SRP were positive in experiments 3, 4, and 8 as expected, but negative in experiments 1 and 2. Surplus P/chl a was negatively correlated with SRP in experiments 4, 5, and 6. Also, surplus P/chl a was poorly and negatively correlated with SRP using pooled data ($r=-0.23$).

We can conclude that surplus P decreased or remained constant as SRP increased in some experiments, which is inconsistent with the idea that more P is stored as SRP increases, at least in the intracellular pool that was measured. But the overall negative relationship between APA and SRP suggests that APA was induced by low levels of SRP

as expected.

Table 7. Correlation coefficients (r), observed significance levels (P), and sample size (n) for correlations of measured variables with SRP, and expected correlations based upon assumed relationships to SRP (all samples were taken at 1100 hr of each sampling day).

Experiment		Chl *a*	APA/area	APA/chl *a*	Surplus P/area	Surplus P/chl *a*
1	r	+0.22	-0.82	-0.72	-0.63	-0.27
	P	0.376	<0.001	<0.001	<0.001	0.112
	n	18	18	18	36	36
2	r	-0.38	-0.72	-0.54	-0.70	-0.09
	P	0.116	<0.001	0.020	<0.001	0.620
	n	18	18	18	36	36
3	r	+0.51	-0.22	-0.39	+0.54	-0.07
	P	0.002	0.201	0.016	<0.001	0.678
	n	18	36	36	36	36
4	r	+0.57	+0.10	-0.41	+0.39	-0.32
	P	<0.001	0.572	0.012	0.019	0.056
	n	18	36	36	36	36
5	r	+0.81	-0.30	-0.58	-0.02	-0.76
	P	<0.001	0.080	<0.001	0.913	<0.001
	n	18	35	35	36	36
6	r	+0.44	-0.01	-0.28	-0.15	-0.52
	P	0.007	0.946	0.102	0.374	0.001
	n	18	36	36	36	36
7	r	+0.07	-0.18	-0.28	+0.05	+0.02
	P	0.664	0.297	0.098	0.752	0.908
	n	18	36	36	36	36

Table 7 (continued). Correlation coefficients (r), observed significance levels (P), and sample size (n) for correlations of measured variables with SRP, and expected correlation coefficients based upon assumed relationships to SRP (all samples were taken at 1100 hr of each sampling day).

Experiment		Chl *a*	APA/area	APA/chl *a*	Surplus P/area	Surplus P/chl *a*
8	r	+0.14	+0.08	-0.06	+0.32	+0.20
	P	0.415	0.649	0.731	0.059	0.245
	n	18	36	36	36	36
1-8	r	+0.34	-0.16	-0.42	+0.08	-0.23
	P	<0.001	0.013	<0.001	0.204	<0.001
	n	144	251	251	252	252
Expected correlation		+	-	-	+	+

Linear Correlations of APA/area with Chl *a*

The linear correlations of APA/area with chl *a* were poor ($r<0.41$), positive in experiments 2, 7, and 8, and negative in experiments 3, 4 and 5 (Table 8). Having established the conditions of the culture and periphyton, the issue of the diurnal fluctuation of APA will now be addressed.

Table 8. Correlation coefficients (r) of APA/area with chl *a*, related significance levels(P) , and sample size (n)

Experiment	1	2	3	4	5	6	7	8
r	+0.049	+0.21	-0.18	-0.14	-0.15	+0.02	+0.40	+0.31
P	0.683	0.085	0.035	0.093	0.068	0.784	<0.001	<0.001
n	72	71	144	144	143	143	144	144

Diurnal Changes of APA during the Light-dark Cycle

Diurnal APA changes were observed in experiments 1-8. For each individual experiment, the daily highest and the lowest APA/area or APA/chl *a* did not appear consistently at a certain sampling time. A representative example is given for APA/area in experiment 7 (Figure 7). Also, after the same number of days of culture in all individual experiments the highest and lowest APA did not consistently occur at a certain sampling time.

Experiments 1-2, 3-4, and 5-8 were analyzed separately, because culture conditions were markedly different in respect to temperature, SRP added to the culture, and light-dark cycle duration (Table 3).

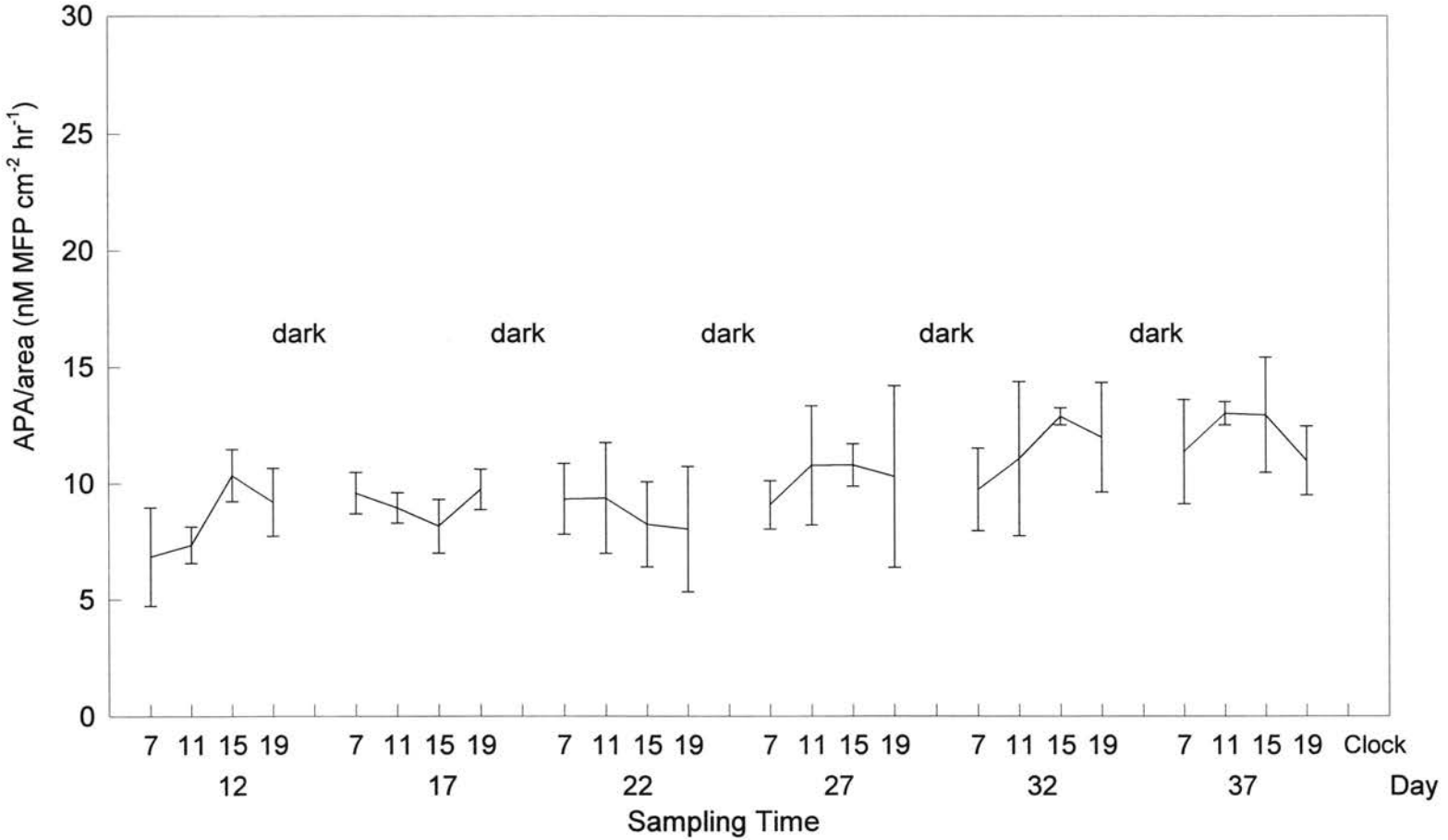

Figure 7. Changes of APA/area during light period for Experiment 7. Values plotted are sample means ± 1SD; n=6. Zeros were omitted on legend for time of day on abscissa.

A two-way ANOVA was used to determine the effect of days in culture (Day), and sampling time during the day (Time) on APA for pooled experiments 1-2 and 3-4. Because experiments 5, 6, 7, and 8 were cultured under the same conditions, the pooled data of these four experiments (experiments 5-8) were used for ANOVA. A three-way ANOVA was used to determine the effects of above-mentioned variables and the variable of month of seeding (Month) on APA for pooled experiments 5-8. The interactions among Month, Day, and Time were also tested by ANOVA. Table 9 contains a summary of the results. Results of a similar ANOVA are presented below for pooled experiments 9-12 where endogenous changes in APA were examined.

For paired experiments 1-2 and 3-4, ANOVA did not reveal any significant effect of sampling time (Time) on both APA/area and APA/chl _a_ and interaction between the effects of Day and Time (Table 9). ANOVA revealed that days in culture (Day) apparently affected APA in paired experiments 1-2 and pooled experiments 5-8. It was previously established that there was a positive correlation between APA and the days in culture in individual experiments (Table 6), so this analysis confirms the trend noted earlier.

Table 9. Results of ANOVA for APA in paired experiments 1-2 and 3-4, and pooled experiments 5-8 and 9-12. Observed significance levels of effects of month of seeding periphyton (Month), days in culture (Day), and sampling time during the day (Time) on APA/area and APA/chl *a*, and the two-way interactions between Month and Day (Month*Day), Month and Time (Month*Time), Day and Time (Day*Time), and the three-way interactions among Month, Day, and Time (Month*Day*Time).

APA	Experiment	Month	Day	Time	Month*Day	Month*Time	Day*Time	Month*Day*Time
APA/area	1-2	na	0.071	0.628	na	na	0.828	na
	3-4	na	0.475	0.887	na	na	0.159	na
	5-8	0.319	<0.001	0.004	0.913	0.986	0.006	0.560
	9-12	0.469	0.272	0.095	0.326	0.462	0.921	0.340
APA/chl *a*	1-2	na	0.007	0.247	na	na	0.923	na
	3-4	na	0.387	0.686	na	na	0.160	na
	5-8	0.028	<0.001	0.147	<0.001	0.935	0.761	0.968
	9-12	0.134	0.059	0.116	0.770	0.055	0.944	0.996

na: not applicable because of seeding on the same month

Next the results of ANOVA for APA/chl *a* and then the results for APA/area in pooled experiments 5-8 will be discussed. In these pooled experiments, the three-way interactions of Month*Day*Time were not significant (Table 9). The effect of Time on APA/chl *a* was not significant,

but the effect of Day and the interactions between Month and Day were highly significant in pooled experiments 5-8 (Table 9).

Because of the interaction between effects of Month and Day on APA/chl a in pooled experiments 5-8, data were sorted by Month and then the effect of Day on APA/chl a in the different months was tested by ANOVA. The effect of Day on APA/chl a was highly significant ($P<0.01$) in April for paired experiments 5-6, and marginally significant ($P<0.10$) in May for paired experiments 7-8 (Table 10a). Data were also sorted by Day to test the effect of Month on APA/chl a on the different days of culture. The effect of Month was significant on days 12, 17, 22, 27, and 32 for APA/chl a but not later (Table 10b). In other words, APA in periphyton, seeded in different months, was different on the same day of culture, with exception of day 37.

Table 10. Results of ANOVA for APA/chl a using sorted data in pooled experiments 5-8

10a: Significance levels for effects of days in culture on APA/chl a for data sorted by month of seeding

Experiments	5-6	7-8
Month of seeding periphyton	April	May
Significance of effects of Day in different months	<0.001	0.077

Table 10 (continued). Results of ANOVA for APA/chl a using sorted data in pooled experiments 5-8

10b: Significance levels for effects of month of seeding on APA/chl a for data sorted by days of culture

Days in culture	12	17	22	27	32	37
Significance of effects of Month in different sampling days	0.011[s]	0.011[s]	0.006[h]	0.029[s]	0.011[s]	0.089[m]

The effect of Time on APA/area, and the interaction between effects of Day and Time on APA/area were highly significant for pooled experiments 5-8 (Table 9). When data were sorted by Time, the effect of Day was significant at 1100, 1500, 1900 hr, and marginally significant (p=0.068) at 0700 hr (Table 11). When data were sorted by Day, the effect of Time was significant on days 12, 22, 37 (Table 11). Thus, our experiments revealed the detectable light-period changes of APA only on days 12,22, and 37 respectively, for pooled experiments 5-8.

APA/area measured on each of these three days in pooled experiments 5-8 was compared with Duncan's test of multiple range (Table 12). APA/area measured during light-period was highest at 1500 hr on day 12, at 0700 hr on day 22. Two peaks were observed on day 37 at 1100 and 1500 hr. And APA was essentially the same at 0700, 1100 and 1900 hr on day

Table 11. Results of ANOVA for APA/area using sorted data in pooled experiments 5-8. Significance levels of effects of days in culture for data sorted by sampling time (Time), and significance levels of effects of sampling time (Time) for data sorted by days in culture (Day)

Sampling Time (hr)	0700		1100	1500		1900
Significance of effects of Day at different sampling time	0.068		<0.001	0.014		0.003
Days in culture	12	17	22	27	32	37
Significance of effects of Time in different sampling days	0.009	0.622	0.038	0.268	0.269	0.018

Table 12. Comparison of means of APA/area at different sampling times on days 12, 22, 37 in pooled experiments 5-8 using Duncan's test of multiple range. The means are arranged from the highest on the left to the lowest on the right. The means accompanied by the same letter are not significantly different (P>0.05).

Sampling Day	Mean of APA/area (nM MFP cm^{-2} hr^{-1})/ Sampling Time (hr)				Time with highest or higher APA (hr)	Time with lowest or lower APA (hr)
12	10.5/1500 a	9.0/1900 b	8.4/1100 b	7.8/0700 b	1500	0700,1100, 1900
22	10.0/0700 a	9.8/1100 ab	9.2/1500 ab	8.3/1900 b	0700	1900
37	13.2/1100 a	12.3/1500 a	10.6/1900 b	10.3/0700 b	1100, 1500	1900, 0700

12. APA was significantly lower at 1900 hr on day 22. Two low values were observed at 0700 and 1900 hr on day 37. The common features of these data are that the higher or highest APA generally occurred around noon with one exception; the lower or lowest APA occurred late or early during light-period (1900 or 0700 hr) with one exception. Therefore, APA was highest at least several hours before the end of light period as expected.

Diel Changes of APA after Periphyton Cultured in Light-dark Cycle Was Put in Continuous Darkness

In experiments 9-12, periphyton were cultured in light-dark cycles as reported above for experiments 1-8. Up to day 18, lights were turned on at 0700 hr and turned off at 2000 hr. From 2000 hr on day 18, the cultures were subjected to continuous darkness to learn if rhythms of APA were endogenous. Thus, observations for APA were made during the light-period of day 18 and the following 72 hours in continuous darkness in experiments 11-12. In experiments 9-10, observations were made only in continuous darkness. A representative example of changes of APA is given for APA/area in experiment 11 (Figure 8).

The 24-hour period beginning from 0700 hr of day 18 to

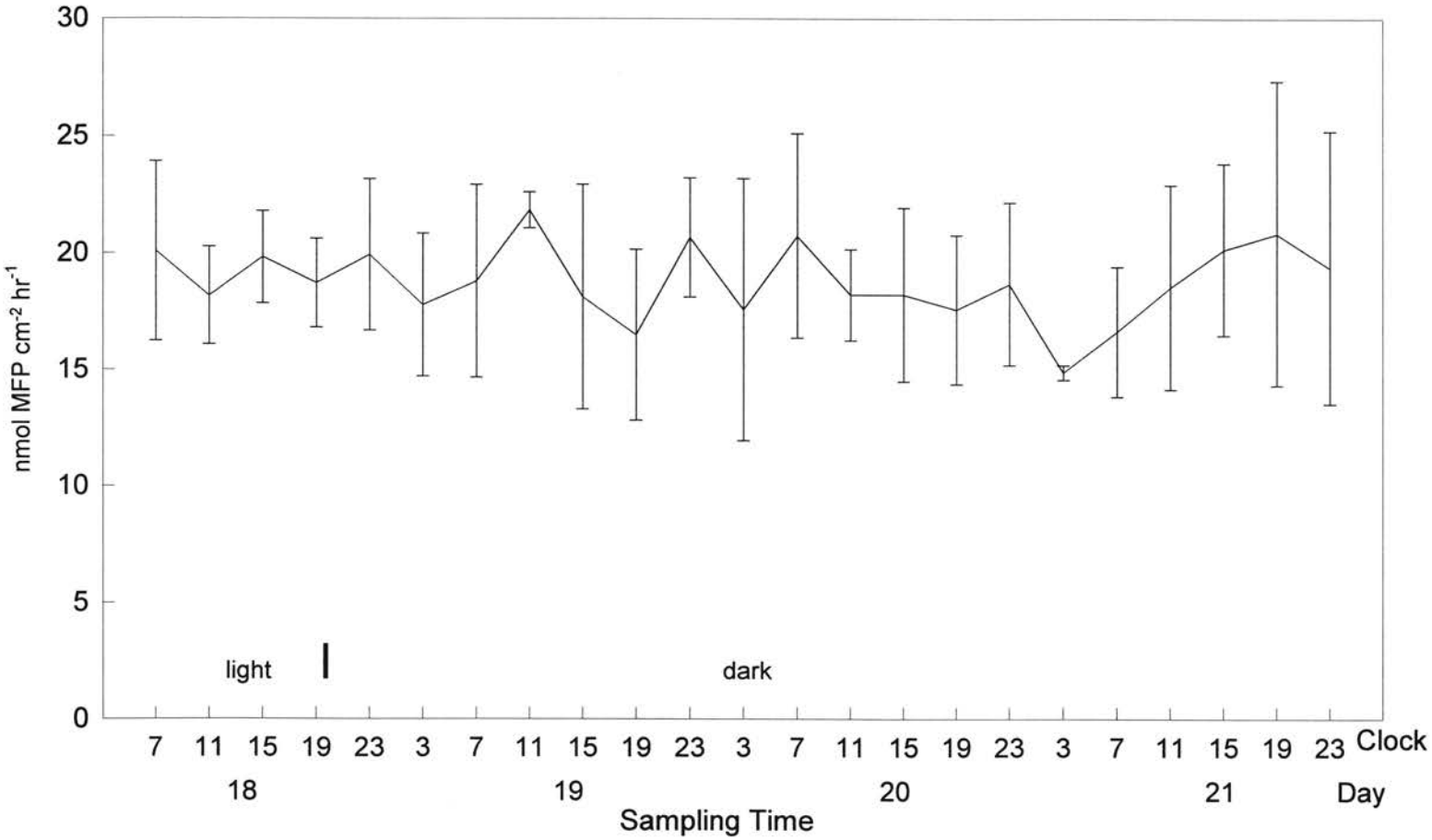

Figure 8. Changes of APA/area measured in Experiment 11. Values plotted are sample means ± 1SD; n=6. Light was off at 20:00 of day 18 (the end of light period of day 18). Zeros were omitted on legend for time of day on abscissa. Cultured periphyton were continuously in the dark for the following three days.

0700 hr of day 19 was a complete light-dark cycle in experiments 11 and 12. During this time, ANOVA did not reveal a significant effect of sampling time on APA/area and APA/chl _a_, i. e., detectable diel changes of APA.

For the observations made during the next 72 hours in continuous darkness in experiments 9-12, ANOVA also did not reveal a significant effect of sampling time on APA/area and APA/chl _a_ in individual experiments. For pooled experiments 9-12, the results of ANOVA showed only a marginally significant effect of Time on APA/area, and also a marginally significant effect of Day on APA/chl _a_. A marginally significant interaction of effects of Month and Time also occurred for APA/chl _a_. The three-way interactions of Month*Day*Time were not significant for APA/area and APA/chl _a_ (Table 9). No significant diel change of APA/area and APA/chl _a_ for periphyton existed in continuous darkness in individual and pooled experiments. Thus, little evidence from these analyses support the view that APA cycled in a predictable way in either a light-dark cycle or in continuous darkness.

CHAPTER V

DISCUSSION

Validity of P-limitation in Periphyton Culture

The change in chlorophyll a biomass is a surrogate for growth which was not measured. After day 22 in culture, biomass slightly fluctuated or decreased in experiments 1-8 (Figure 1). The daily means of chl a were always less than 1.0 $\mu g\ cm^{-2}$.

Periphyton communities in this study were P-limited when observations on diurnal APA cycles were made. Evidence for this conclusion comes from the magnitude of APA observed. The daily means of APA measured in individual experiments 1-8 were 7.7-19.0 nM MFP $cm^{-2}\ hr^{-1}$, and 11.7-40.2 nM MFP μg^{-1}chl a hr^{-1} as expressed as an areal and biomass basis, respectively. When these values are compared to the deficiency criteria for APA as proposed by Healey and Hendzel (1979) for phytoplankton, and cited by Biggs (1990) for periphyton, a case can be made that the periphyton in this study were severely P-limited. The daily means of APA expressed on a biomass basis in the present experiments were always higher than the critical value of 5 nM MFP μg^{-1}chl a hr^{-1} for severe P-limitation. This also agreed with the APA range (19 to 29 nM MFP μg^{-1}chl a hr^{-1}) reported by Bothwell

(1985) for P-limited periphyton.

Biomass accrual was probably not limited by irradiance. In a review article, Hill (1996) reported that in 16 studies saturation irradiance for periphyton photosynthesis was 100-400 μmol photons m^{-2} s^{-1}; in two thirds of the studies light saturation began at 200 μmol photons m^{-2} s^{-1} or lower. The reported compensation irradiance (when P = R) was 10-40 μmol photons m^{-2} s^{-1}. In my experiments, the irradiance was 200.3 μmol photons m^{-2} s^{-1}, which is within the range of saturation irradiance and above the compensation irradiance reported in the literature. Also, P-limited growth of periphyton was supported at an irradiance of 125 μmol photons m^{-2} s^{-1} (Hepinstall and Fuller, 1994). Thus, light was not the factor limiting growth of periphyton in this study.

The temperatures maintained in this study were constant and above 10 °C, temperatures unlikely to limit growth. Thus, P-limited periphyton mats were produced in the laboratory under controlled conditions. The mats in each paired experiments seemed to behave synchronously during development and early senescence and were P-limited (Figure 1-8).

Rhythmic Changes of APA

Diel changes of APA in algae have not been frequently studied, and most of the research on this topic has focused on phytoplankton (Table 13). Other limitations exist in comparing my data to other studies. With the exception of the study of Rivkin and Swift (1979), none of these studies employed statistical tests. Four studies (Reichardt, 1971, Wynne, 1981, Klotz, 1985) presented the precision of APA measurements only as error bars. Furthermore, the length of time of observations in research to date was usually 24 hr, occasionally 48 hours (Jamet et al., 1995).

To avoid confusion about cycles, a number of operational definitions were adopted. The term pattern describes any type of changes of APA (field or laboratory) during a period equal to or less than 24 hr but including one light-period. And the term "consistent pattern " is used to mean that a pattern of significant APA change that occurs consistently for at least two light-periods.

Because diel changes of APA in algae are the result of endogenous circadian rhythms and exogenous influence of light-dark cycles, two time-scales are potentially operative for any observed changes for the two rhythms: a time-scale for endogenous rhythms, and the time-scale for exogenous changes, which are related to the light-dark cycle.

Table 13. Summary of methods and materials of previous research on diel changes of APA, and comparison to the present work

Reference	Algae measured	Numbers of all experiments or surveys	Length of time for experiments or surveys	SRP in water or culture media (μg P/l)	APA normalized by	Statistics
Berman, 1970	Lake phytoplankton	2	24 hr	1-4	water sample volume	none
Reichardt, 1971	lake phytoplankton	one for each of three lakes	about 26 hr	6.2-11.7	water sample volume protein	standard error
Perry, 1976	chemostat culture of marine diatom *Thalassiosira pseudonana*	at least two	24 hr	0.6	water sample volume	none
Rivkin and Swift,1979	marine phytoplankton dominated by *Pyrocystis noctiluca*	2	16.5-30 hr	1.5-4.4	cell	none
	culture of *Pyrocystis noctiluca*	2	28-32 hr	<2.4	cell	Student's *t*-test
Wynne, 1981	lake dinoflagellate *Peridinium*	2	24 hr	not specified	protein	none
	culture of *Peridinium*	2	30 hr	6.0-6.3	cell	standard error
Huber and Kindby, 1984	estuarine phytoplankton	3	24-72 hr	not specified	water sample volume	none
Huber and Hamel, 1985	estuarine algal blooms of *Nodularia spumigena*	2	24 hr	25-200	water sample volume	none

Table 13. Summary of methods and materials of previous research on diel changes of APA, and comparison to the present work (continued)

Reference	Algae measured	Numbers of all experiments or surveys	Length of time for experiments or surveys	SRP in water or culture media (μg P/l)	APA normalized by	Statistics
Klotz, 1985	culture of phytoplankton *Selenastrum capricornutum*	two for each of two culture regimes	24 hr	0 or 35.6	cell	standard deviation
Jamet et al., 1995	reservoir phytoplankton	2	48 hr.	270-300	water volume	none
					chl a	none
					protein	none
This study	Lake periphyton (*Cladofora*, diatoms, unicellular green algae, and blue green algae)	8 for APA changes during light-period, 2 for APA changes during light-dark cycle	six light periods during 37-day cultures for APA changes during light periods, 24 hours for APA changes during light-dark cycle	2.1-6.2 for 8 experiments aimed at APA changes during light-period	sampling area and chl a	ANOVA

Moreover, diel changes in algal APA may be different between field and laboratory culture, because artificial light-dark cycles may not exactly simulate the natural diel cycles. It was assumed that the on-off artificial light-dark cycle used here reproduced natural cycles.

In experiments 11-12, I measured APA over one diel cycle, and while these observations were not extensive, I begin this part of the discussion by comparing my results to those of others. The intent is to set the stage for a comparison of my diurnal results to those of others. The diurnal data of other workers is nested in their reports of diel cycling.

Diel Change of APA

The changes of APA during the diel cycle reported in the literature are summarized in Table 14 with a comparison to the results of my experiments on day 18-19. On day 18-19 of pooled experiments 11- 12, no significant differences occurred in APA among sampling times. This is similar to the results of Perry (1976) and Klotz (1985). In Perry's study with chemostat cultured marine diatom *Thalassiosira pseudonana*, the APA measured between times was essentially the same. In the Klotz's study, where cultures of the freshwater phytoplankton *Selenastrum capricornutum* were

Table 14. Summary of results of previous research on diel changes of APA, and comparison to the present work

Reference	Diel changes of APA	Type of diel changes of APA	Compared to this study
Perry, 1976	no change	no detectable change	the same as that on day 18 of experiments 11-12
Klotz, 1985	no change	no detectable change	the same as that on day 18 of experiments 11-12
Huber and Kidby, 1984	dissimilar results for surveys	no consistent pattern	different
Jamet et al., 1995	dissimilar results for 48-hour monitoring	no consistent pattern	different
Berman, 1970	the highest before midnight, the lowest at the morning or early afternoon	not light-induced	different
Reichardt, 1971	the highest before sunrise, the lowest at early or late night	not light-induced	different
Wynne, 1981	the highest around midnight, the lowest at the morning for lake *Peridinium*	not light-induced	different
Rivkin and Swift, 1979	the highest at the morning, the lowest at late night for marine phytoplankton dominated by *Pyrocystis noctiluca*	light-induced	different
	the highest at the morning, the lowest before dawn for the culture of *Pyrocystis noctiluca*	light-induced	different
Huber and Hamel, 1985	the highest at morning or noon, the lowest at night	light-induced	different
This study	no change	no detectable change	

used, the ranges of APA samples overlapped one another between sampling times. The overlapped portion was more than 50% of the smallest one of the two overlapped ranges. The changes of APA in these studies probably were not significant. Both authors concluded that the algae studied showed no detectable diel cycle of APA (Perry, 1976, Klotz, 1985).

Some researchers have found detectable diel changes of APA, but these changes did not show consistent patterns. Huber and Kidby (1985) stated that distinct patterns of diel changes of APA were always found, but the patterns were not consistent between either sampling stations or sampling dates. In one continuous two-day study, the highest APA on the first day and the lowest APA on the next day occurred around noon. So it could be concluded that no consistent pattern of diel changes of APA was observed in their study. Jamet et al.(1995) monitored freshwater phytoplankton at intervals of 1-2 hr for 48 hr. They found that APA normalized to either water volume, chl a, and protein content showed no consistent patterns of diel changes.

On the other hand, some patterns of diel change of APA have been reported. Berman (1970) and Reichardt (1971) measured APA of lake phytoplankton and normalized their measurements to water volume. APA was highest before

midnight and lowest in the morning or early afternoon in Bermans' survey; APA was highest before sunrise and lowest before sunset or at early night in Reichardt's survey. Wynne (1981) found that the APA was highest around midnight and lowest in the morning for the dinoflagellate, *Peridinium* spp., in lake water, and highest before sunrise and lowest around noon for *Peridinium* spp. in culture. In these three studies, the lowest APA appeared between morning and early evening, and the highest APA at night. This type of diel change is apparently not light-induced.

Some authors have reported lowest APA at night and highest APA during the day. The latter was interpreted as light induced, a practice that I will continue. Rivkin and Swift (1979) monitored the diel changes of APA normalized to cell numbers in marine phytoplankton dominated by *Pyrocystis noctiluca* and also of this flagellate in culture. Their results showed the lowest APA occurred at late night and the highest APA in the morning. Huber and Hamel (1985) also found that the APA was highest from the morning to noon and lowest at night for a bloom of *Nodularia spunigena,* a cyanobacterium.

Therefore, three types of the diel change of APA have been reported. Type I: changes with the highest APA during the day, which is described here as light-induced without

exclusion of other causes for this type of change. Type II: not light-induced changes of APA with the highest APA at the night. Type III: no detectable change. The diel changes of APA in this study may be consistently type III. As mentioned above, changes of APA during day 18 of pooled experiments 11-12 were not detectable. Changes of APA only during the daytime for the pooled experiments 5-8 will next be compared to the results of previous studies.

Diurnal Changes of APA

These experiments revealed detectable diurnal changes of APA only on days 12, 22, and 37 respectively, for pooled experiments 5-8 (Table 11). The common features of these changes are that the APA was highest at least several hours before the end of light period.

The diurnal changes of APA in previous studies are compared with my results in Table 15. The same studies cited above for diel changes had diurnal changes as well and were used below in Table 15. The diel changes of APA were light-induced in the studies of Rivkin and Swift (1979) and Huber and Hamel (1985). The diurnal changes of APA can be summarized as follows. APA reached its highest level at some time other than just before sunset, which is similar to the detectable diurnal changes of APA in pooled experiments

5-8.

Table 15. Summary of results of previous research on diurnal changes of APA, and comparison to the present work

Reference	diurnal changes of APA	Type of diel change of APA	Compared to the present work
Perry, 1976	no change	no change	the same as that on some days of some experiments
Klotz, 1985	no change	no change	the same as that on some days of some experiments
Huber and Kidby, 1984	dissimilar patterns	no consistent pattern	similar to or different from that on days 12, 22, 37 for pooled experiments 5-8
Jamet et al., 1995	dissimilar results for 48-hour monitoring	no consistent pattern	similar to or different from that on days 12, 22, 37 for pooled experiments 5-8
Berman, 1970	the highest before sunset, the lowest at the morning or early afternoon	not light-induced	different
Reichardt, 1971	the highest at the morning, the lowest at early morning or before sunset	not light-induced	the same as that on days 12, 22, 37 for pooled experiments 5-8
Wynne, 1981	the highest before sunset, the lowest at the morning or afternoon for lake phytoplankton	not light-induced	different
Rivkin and Swift, 1979	the highest at the morning, the lowest at afternoon for marine phytoplankton	light-induced	the same as that on days 12, 22, 37 for pooled experiments 5-8
	the highest after sunrise or late afternoon, the lowest before sunset for phytoplankton culture	light-induced	the same as that on days 12, 22, 37 for pooled experiments 5-8

Table 15 (continued). Summary of results of previous research on diurnal changes of APA, and comparison to the present work

Huber and Hamel, 1985	the highest at morning or noon, the lowest before sunset or lower at afternoon	light-induced	the same as that on days 12, 22, 37 for pooled data of experiments 5-8
The present work	peaked before or at mid-afternoon then decreased on days 12, 22, 37 for pooled experiments 5-8	light-induced	
	no changes sometimes	no change	

On the other hand, when diel changes of APA were not light-induced (APA is highest at night), the highest diurnal APA appeared before sunset (Berman, 1970, and Wynne, 1981). This is different from the results in that the highest APA did not appear before the end of light-period on days 12, 22, 37 of the pooled experiments 5-8. But in Reichardts' study (1971), the highest diurnal APA occurred in the morning, although the diel APA change was not light-induced. This is similar to the situation for days 12, 22, 37. On the other days of the pooled experiments 5-8, no detectable diurnal changes of APA occurred, similar to that reported by Perry (1976) and Klotz (1985). Furthermore, other studies have shown no consistent pattern for diurnal and diel changes of APA (Huber and Kidby, 1984, Jamet et al., 1995).

Controlling Factors of Diel Changes of APA

Explanations for Type I and Type II diel change of APA

Many factors control APA of algae (Invers et al., 1995). Logically, any factor that has a direct or indirect effect on the algal supply and demand of phosphorus also has effect on algal APA. The periodicity of these factors also affects diel changes of APA. Few studies on the controlling mechanisms of diel changes of APA in algae in the field. This may be partially attributed to the precise nature of the control of APA which has not yet been established (Cembella et al., 1984, Invers et al., 1995).

In some studies diel changes of light, photosynthesis, and cell division have been used to explain Type I diel APA change. Light can affect P demand indirectly through photosynthesis, which then affects APA. In Type I diel APA change (where APA is the highest in the light-period), it is implies that light may control APA. Rivkin and Swift (1979) reported a rapid increase of APA for *Pyrocystis noctiluca* in culture during the transition between the light and dark period, which implies that light stimulated APA. Wynne and Rhee (1988) measured APA for six species of phytoplankton grown under P limitation and different light intensities and wavelengths. They found that under the same degree of P

limitation both light intensity and spectral quality affected APA in a species specific manner.

Few studies have been completed on the mechanism of the influence of light on APA. Rivikin and Swift (1979) exposed *Pyrocystis noctiluca* in culture to photosynthetic inhibitors (DCMU and CCCP), two respiratory inhibitors (CN and DNP), and prolonged darkness (about 68 hours). In all these treatments, APA was markedly reduced. That implies that the effect of light on APA is mediated at least partially though photosynthesis. The data of Rivikin and Swift suggest that a reduction of oxidative metabolism was responsible for the decrease of APA for algae when inhibitors were used. This decrease in APA may result from the interference of the synthesis of alkaline phosphatases (Cembella et al., 1984). Because the energy required for enzyme synthesis comes from photosynthesis and respiration, the inhibition of these two metabolic processes will reduce or stop the energy supply to alkaline phosphatase synthesis. On the other hand, reduced metabolism caused by inhibited photosynthesis or respiration will accompany reduced P demand and P uptake. Consequentially, the accumulation of orthophosphate at external sites of P uptake will repress APA. These two explanations also could be used to explain the appearance of the low APA under low irradiancies. Besides light and

photosynthesis, Rivkin and Swift (1979) demonstrated an endogenous control of APA for Type I pattern of APA diel change.

For the Type II pattern of APA diel change, rate of external P supply and cell division have been considered as controlling factors. Enzyme-hydrolyzable organic phosphorus has been suggested to affect APA by Reichardt (1971). He correlated diel changes of APA of phytoplankton to enzyme-hydrolyzable organic phosphorus in lake water. If autolysis and zooplankton excretion are chief sources of organic matter, the rhythm of supply of the substrate for APA may explain the diel changes of APA. Berman (1970) attributed the increase of APA in the late afternoon and evening to local exhaustion of available phosphorus as a result of intense photosynthesis. Based on the coincidence of time of the appearances of maximum rate of division and highest APA values of *Peridinium* spp. in Lake Kinneret, Wynne (1981) suggested a connection between APA and the rate of cell division.

Enzyme-hydrolyzable organic phosphorus may have affected periphyton in my experiments much more than algae in the field. In the field dead phytoplankton sink and unhealthy periphyton are more easily scoured by water currents. But in the experiments described in this paper

dead periphyton accumulated in aquaria as a potential source of enzyme-hydrolyzable organic phosphorus. In my experiments, the P concentration in the media in aquaria fluctuated and was sometimes even higher than the concentration in added medium. This may be caused by increases in enzyme-hydrolyzable organic phosphorus from autolysis of dead periphyton cells.

Endogenous Control of APA

Few studies exist on endogenous diel changes of APA for algae. In the study of Rivkin and Swift (1979), the culture of *Pyrocystis noctiluca* exposed to a diel light-dark cycle showed Type I of diel APA change. And when the alga was in continuous darkness, it still showed a diel change of APA with the highest value during daytime, which suggested endogenous control of APA. But the highest value of APA for the alga in continuous darkness was much lower than that in the control exposed to a diel light-dark cycle. And the APA for the control was higher or equal to that in continuous darkness at night and much higher during the day. This demonstrates the dominant role of light in controlling APA diel changes as opposed to endogenous control. Therefore, the Type I pattern of APA diel change for this alga still could be considered as light-induced.

In the study of Wynne (1981), highest APA values for *Peridinium* spp. occurred at night in Lake Kinneret and in the dark-period in culture synchronous with a natural day-night cycle. This was Type II of APA diel change. But under continuous light, the highest value of APA occurred during the day. This switch of a nocturnal peak under a light-dark cycle to a daytime peak under a continuous light indicated that the nocturnal peak occurred because of lack of light. So, non-endogenous factors control the appearance of the highest values of APA for *Peridinium* spp. at night in Lake Kinneret and during the dark-period in the culture synchronous with the natural day-night cycle (Wynne, 1981). Hence, Type I and Type II of APA diel change in these two studies were demonstrated to be dominantly controlled by non-endogenous factors. Among them, light control may be the most important one.

In contrast to these studies, experiments 9-12 did not show detectable diel changes of APA when periphyton were in continuous darkness. The absence of detectable changes of APA may result from the lack of light, because in pooled experiments 5-8, detectable diurnal changes of APA were shown when periphyton were exposed to light-dark cycles. But, detectable changes did not appear on some days in pooled experiments 5-8. Therefore, sometimes the detectable

changes of APA may not appear both in continuous darkness and light-dark cycles. Thus, the absence of detectable changes of APA in continuous darkness may be not related to the lack of light.

Furthermore, endogenous diel changes were weaker than light controlled diel changes in the study of Rivkin and Swift (1979). The diurnal changes of APA in experiments 5-8 were small. Thus, if the diel changes in continuous darkness in pooled experiments 9-12 were weaker than that in pooled experiments 5-8, these changes may have been masked by some environmental interference. The results suggest that no endogenous control was observed.

Control of APA Diurnal Cycles

In these experiments, diurnal APA changes were small and strong cycles were not observed. One reason for this observation might be the relatively large variability in APA measurements, i. e., large error bars in Figures 7 and 8. No doubt some of this error could occur because of the composition and density of algae species in a given sample. It is well understood that species differ widely in physiological states and these same states change with age, time of cell division, cell quota N:P requirements, etc. (Darley, 1982, Lobban and Harrison, 1994). Also each sample

included bacteria, which could also influence the magnitude of periphytic APA and possibly mask the APA changes of algae.

In pooled experiments 5-8, significant diel changes APA occurred on days 12, 22, and 37. The sudden appearance and disappearance of significant diurnal changes in APA, may be related to the succession of the cultured periphyton community, in which different species were dominant at different stages of the development process. Also, the significant effect of Month for seeding periphyton, and effect of Day of culture on APA as revealed by ANOVA in these experiments may be related to the successional stage of the community.

One clue to mechanisms involved in the weak APA diel cycles is that light induced changes of physiological parameters are weaker in P-limitation (reviewed by Cembella et al., 1984). In monospecific cultures, diel variation of ratios of chl a to cellular C and ATP to cellular C had a smaller range during nutrient limitation than during light-limitation, which occurs with replete nutrient supply.

Several physiological characteristics exhibit diel periodicity: cell division, photosynthetic capacity, respiration and nutrient uptakes characteristics (Sournia, 1974). These characteristics are mutually interdependent.

Cembella et al. (1984) suggested that the breakdown of P uptake periodicity occurs during P-limitation. This may be also true for APA.

Also, species of algae in periphyton may have different physiological periodicities. By timing the maximum rates of various activities for different times during the diel cycle, algae could reduce interspecific competition (Chisholm et al., 1978), and could be allocating resources to take maximum advantage of the oscillating levels of environmental resources (Darley, 1982). This divergence of periodicity among species may result in weak periodicity for the whole community.

Furthermore, alkaline phosphatase cleaves some organic compounds, enzymatically hydrolyzable phosphate (EHP), which is one part of pool of dissolved organic phosphate (DOP), and releases orthophosphate for uptake. Algae also secrete more DOP during P-limitation (Cembella et al., 1984). Dead algae in periphyton also accumulate and serve as potential source of DOP. The external DOP pool was not measured in this research, but the SRP that was measured contains orthophosphate and some DOP. Conceivably, the size of pools of SRP and DOP could affect APA, because each would change depending upon uptake and excretion of algae. It appears however that APA is largely under internal control of the

cell (Cembella et al., 1984) and, thus, the internal control mechanisms governing diel APA described above are probably more directly involved in regulation than are the external concentrations of P.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

The growth of periphyton was severely limited by phosphorus, because the daily means of APA in individual experiments 1-8 were 11.7-40.2 nM MFP μg^{-1}chl *a* hr^{-1}, which were higher than the critical value of 5 nM MFP μg^{-1}chl *a* hr^{-} for severe P-limitation found in the literature. This P-limitation was also confirmed by a significant correlation ($P<0.001$, $n=144$) between chl *a* and SRP in pooled experiments 1-8. Three null hypotheses were tested for such P-limited periphyton.

Hypothesis I was that no change in biomass, APA, and surplus P occurred during periphyton development. A consistently synchronous change occurred in chl *a* during paired experiments. Relative to the days of periphyton in culture, chl *a* decreased in experiments 3 and 4 , and 5 and 6, increased in experiments 2 and 7, but did not show any trend in experiments 1 and 8. After 22 days of culture chl *a* was relatively stable or rose slightly in all experiments with exceptions of experiments 3 and 4, where it decreased. The reason for these differences is not known. Daily means of chl *a* were less than 1 $\mu g\ cm^{-2}$ in all these experiments, and were less than 0.7 $\mu g\ cm^{-2}$ after 22 days of culture.

Further research is needed to determine why chl a concentrations declined in some, but not all cases, and to modify the cultural procedure to obtain reproducible changes in the biomass of P-limited periphyton seeded at different times. APA significantly increased with time in all experiments with three exceptions, twice when APA was expressed on an areal basis, and once when it was expressed as chl a. In these exceptions, the correlations of APA with culture time were not significant. The reason for these results may be large variances of APA, which are discussed below. No consistent trend existed between surplus P normalized by area or by biomass with culture time in individual experiments. Surplus P was not a useful indicator of P-limitation in this study, because surplus P measured could not be differentiated between living or dead algae.

Hypothesis II was that no pattern of change in APA occurred during the diurnal period of light-dark cycles. Diurnal values of APA normalized by area were significantly different during the light period of three of six sampling days for pooled experiments 5-8. On the other three sampling days no significant changes of diurnal APA occurred. Again, perhaps the reason resulted from the large APA variance as mentioned above. Large variance is common

in periphyton research. New and innovative approaches are needed to deal with this problem, because increasing sample size to reduce variance is usually impractical. It is recommended to further workers that they determine APA in pilot experiments and use a power analysis to test for the sample size needed to establish statistical differences between APA means for Type I and Type II error, respectively. This approach would establish the feasibility of measuring diurnal APA changes and the general magnitude of significant changes to be expected.

During the three days when significant changes of diurnal APA/area occurred, APA/area was higher or highest at least several hours before the end of light period. In addition to the highly significant effect of sampling time on APA/area for pooled experiments 5-8 ($P=0.004$), the effect of days in culture ($P<0.001$) and interaction between sampling time and days in culture ($P=0.006$) were highly significant. For APA normalized by chl *a*, the effect of sampling time ($P=0.147$) was not significant, but the effect of days in the culture ($P<0.001$) and the interaction between days in culture and month of seeding (0.001) were highly significant. And no significant three-way interaction was observed among the effects of sampling time, days in culture, and month of seeding on APA/area and APA/chl *a*.

Hypothesis III was that no significant difference occurred in APA in continuous darkness for periphyton that have been cultured in a light-dark cycle. ANOVA showed that no significant diel changes occurred in APA normalized by area and chl _a_ in continuous darkness; endogenous control of APA did not occur. Also, no significant effects were observed of the days in culture and the month of seeding, and two-way and three-way interactions among the effects of sampling time, the days in culture, and the month for seeding on APA/area and APA/ chl _a_.

The normalization of APA with chl _a_ is potentially misleading, because the correlations between APA/area and chl _a_ expressed on an areal basis in this study were poor ($r<0.41$) and showed different trends in individual experiments. The normalization of APA with area is recommended, when comparing APA between periphyton samples that have large differences in biomass.

Accurate measurement of APA in the field requires that the researcher account for changes in APA during colonization and during the day. Because APA increased during colonization in this study, it is recommended that artificial substrates set out at the same time be used to insure the same age of the periphyton mat when comparing APA between sites (streams). The results of this study suggest

that APA samples would be highest between 0700 and 1500 hrs, but not at 1900 hrs. Thus, when comparing the APA at different sites on the same day, samples should be taken between roughly 0700-1500 hrs to avoid low values.

Field verification of this and other suggestions is strongly recommended, because the significant effect of sampling time on APA/area occurred only on some sampling days for pooled experiments 5-8. Moreover, diurnal changes in APA for periphyton cultured under laboratory conditions may be different from that for periphyton growing under field conditions.

The objective of this research was to determine when to sample APA during the light period. The results suggest a 50% probability that APA does not change during the day and a 50% probability that it does change with high or highest values occurring from 0700-1500 hr. This information will greatly assist people who need to sample APA during a normal working day. Such information increases the feasibility that APA will become a routine monitoring tool.

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