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Rapid measurements of periphytic responses to nutrients using PAM fluorimetry

Sarah Brooke Whorley

RAPID MEASUREMENTS OF PERIPHYTIC RESPONSES TO NUTRIENTS USING PAM FLUORIMETRY

by

Sarah Brooke Whorley

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Thesis Committee:

Steven N. Francoeur, PhD, Chair

Peggy Liggit, PhD

Kristin Judd, PhD

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APPROVED:

_____	_____
Steven N. Francoeur, PhD Thesis Chair	Date
_____	_____
Peggy Liggit, PhD Committee Member	Date
_____	_____
Kristin Judd, PhD Committee Member	Date
_____	_____
Marianne Laporte, PhD Department Head	Date
_____	_____
Deborah de Laski-Smith, PhD Dean of the Graduate School	Date

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ABSTRACT

Water quality monitoring has traditionally been done by measuring periphytic algal biomass that has grown on fertilized or unfertilized patches of habitat produced by nutrient-diffusing substrata (NDS). This method requires the destruction of the accumulated periphyton communities and thus does not allow for convenient monitoring through time. Optical fluorometric methods of estimating biomass and photosynthetic activity have been used in saline environments, but generally not over different nutrient treatments and not for a substantial duration. This study evaluated the use of a pulse amplitude modulated (PAM) fluorometer for measuring biomass and photosynthetic activity in conjunction with NDS over several weeks. The results of this study suggest that this is a comparable methodology not only for measuring periphytic responses to nutrients but also for evaluating the effect the nutrient changes have on overall photosynthetic efficiency.

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INTRODUCTION

The human population is growing exponentially, placing increased environmental stress placed on freshwater sources (Wetzel 2001). One type of anthropogenic stressor is nutrient enrichment in the form of municipal waste and agricultural (fertilizer) runoff, both of which are high in nutrients (Scrimegeour and Chambers 1997). Nutrient-rich water is flushed into the adjacent water bodies that the human population depends on, either directly or indirectly (Mitsch and Gosselink 2000). Algae are one of the first indicators of an excess of nutrients in natural waters (Wetzel 2001). When there is an abundance of nutrients, the algae quickly take advantage of the surplus (e.g. Borchardt 1996). Depending on the aquatic system characteristics, the types of algae present, and amount of nutrients present, they can rapidly grow and multiply. It is impractical to request that nutrient loading into aquatic systems from urban and suburban populations be ceased altogether. However, concerted conservation efforts can sharply decrease and limit the amount of anthropogenic nutrient deposition (Wetzel 2001). One of the ways to monitor the nutrient load of a particular water body, with respect to its natural state, is to observe its algae (Scrimgeour and Chambers 1997). Observing the growth response of algae under conditions of addition or subtraction of key nutrients can be used to determine 1) if algal growth in an aquatic system is limited by nutrient scarcity, 2) which nutrient is limiting algal growth and, 3) the extent of that limitation or excess (Francoeur et al. 1999).

Periphyton is the community of surface-associated microbes that grow attached to surfaces in aquatic ecosystems (Wetzel 2001). Periphyton communities typically include algae, bacteria, and protists. Periphytic algal growth responses to nutrient enrichment

have often been measured by biomass (quantitative chlorophyll extraction) or by community composition (microscopy and morphological taxonomy) responses in nutrient diffusing substrata (NDS) experiments (Borchardt 1996). In the standard NDS method (Scrimgeour & Chambers 1997), a mixture of agar and nutrients, such as nitrogen and/or phosphorus, are poured into a terra cotta pot. The porous surface provides a location for periphyton to grow upon and allows for the diffusion of nutrients out of the agar. This creates a localized zone of fertilization, which can be compared to unfertilized areas. Methods involving terra cotta or porcelain are not perfect. The pots are extremely cumbersome, contain varying amount of anomalies inherent in the materials, and lack a standardized pore size (Rugenski et al. 2008). Other popular methods use filters, such as nitrocellulose or glass fiber as a growth surface (Biggs and Lowe 1994, Francoeur et al. 1999), which alleviates some of the problems cited above. These NDS are then placed in selected areas for study and allowed to accumulate a community of algae for a set period of time (Fairchild et al. 1985). Samples of the periphytic community are then collected, and their biomass is measured. The amount of chlorophyll present can be used as an indicator of algal biomass (Winterbourn 1990), or area-specific algal cell densities (Fairchild et al. 1985) can be measured. Based on the results from these experiments, it can be determined whether an increased loading of nutrients will increase benthic algal growth in an ecosystem and which nutrient will cause that increase. One disadvantage to this type of experiment is that it is highly labor intensive and time-consuming. The average NDS experiment can take anywhere from 2-8 weeks to complete (Biggs and Kilroy 2000). The NDS themselves usually need to be in the field about a month to allow sufficient time for algal growth (Francoeur et al. 1999, Francoeur 2001). Additionally,

this method requires destructive sampling of benthic algal communities on the NDS analysis. This greatly complicates the measurement of the response of benthic algal communities to nutrient enrichment through time.

Active fluorimetry can be used to non-destructively measure photosynthetic performance and, under certain conditions, biomass (Consalvey et al. 2005; Honeywill et al. 2002). It has typically been used on macrophytes (Gilmore et al. 1995; Seaton and Walker 1992; Schreiber et al. 1986) though it has been in use on algae since the 1970s (e.g. Mauzerall 1972; Samuelsson and Öquist 1977). Various portable fluorescence methods are becoming increasingly popular as an alternative method for measuring algal biomass and productivity without disturbing these communities. The two main methods of fluorimetry currently used for *in situ* measurements are fast repetition rate fluorimetry (FRRF) (e.g. Sylvan et al. 2007) and pulse amplitude modulated (PAM) fluorimetry (e.g. Parkhill et al. 2001). Both methods work by first measuring the amount of photons being excited from PSII under non-saturating light conditions, typically complete darkness (dark-adapted F_o). Many studies have shown that dark-adapted F_o is well correlated to the amount of chlorophyll (chl) *a* in a given periphytic sample (Honeywill et al. 2002; Kiefer et al. 1989; Kolber and Falkowski 1993; Serôdio et al. 1997; Sylvan et al. 2007). These studies have predominantly looked at the application of fluorimetry in saline environments and for measuring biomass in a single event (Sylvan et al. 2007) or at varying depths within the periphyton (Honeywill et al. 2002). Only a few have explored the application of this method in inland freshwater systems (Ensminger et al. 2001; Vincent et al. 1984).

In the second phase of active fluorimetry, periphyton are subjected to a saturating amount of photosynthetically active radiation (PAR) (typically >6000 μmol $m^{-2}s^{-1}$, Consalvey et al. 2005) to measure the amount of photons being excited when all available photocenters are saturated (F_m). PAM uses a single pulse of light lasting typically between 300-1200 ms (Suggett et al. 2003), whereas FRRF employs a series of alternating flashes and pauses, typically 50-1000 ms (Kromkamp and Forster 2003) to achieve saturation (Kolber et al. 1998). The resulting F_m measurement gained through a repetitive flash can be approximately 50% higher than an F_m gained through a single pulse (Kromkamp and Forster 2003). Some investigators prefer using PAM (Parkhill et al. 2001; Schreiber et al. 1986) and others prefer to use FRRF (Sylvan et al. 2007; Kolber et al. 1998). There is no general consensus as to which method is superior. Few studies have directly compared these two methods, and Kromkamp & Forster (2003) concluded that both PAM and FRRF were well correlated with each other. Suggett et al. (2003) examined phytoplankton and concluded that although the two methods were comparable in results, FRRF methods were more suited to open ocean studies, and that PAM was more suited to inland freshwaters since it is not sensitive enough for use in open ocean conditions.

There are a few methodological concerns with using fluorimetry to assess periphytic biomass. Most notably is the issue of photoquenching (Gilmore et al. 1995; Schreiber et al. 1986; Schreiber et al. 1995; Oxborough and Baker 1997). Photoquenching is when chlorophyll becomes overstimulated by irradiance and dissipates excess electrons by closing down some PSII centers (Gilmore et al. 1995), which may result in artifactually low F_o readings. Although easily measureable, such light-adapted

F_o measurements do not reflect all active PSII centers, since some may be closed due to over-irradiance, and thus light-adapted F_o is not a good measure of biomass. In addition, since all reaction centers are not available for fluorescence, these centers cannot be measured as part of the F_m maximal fluorescence parameter. To relieve this, samples are typically dark-adapted for 10-15 min (Consalvey et al. 2005; Kromkamp & Forster 2003) to ensure that all reaction centers are at their lowest possible activity and thus are open and available for activity when finally exposed to PAR. The other issue encountered when using fluorimetry to assess periphyton communities is that these communities are not simply composed of algae with chlorophyll *a*-containing thylakoids. There are often many species of cyanobacteria, which do not posses chlorophyll *a*-containing organelles, have a greater ratio of PSI:PSII compared to eukaryotic algae (Campbell et al. 1998), and have varying amounts of other photosynthetically important pigments. These factors change the wavelength at which these organisms will fluoresce and have the potential to falsely lower F_o measurements made on a fluorometer that is calibrated to measure eukaryotic chlorophyll (Campbell et al. 1998).

It is with these limitations in mind that this study has several goals: To 1) modify the standard NDS methodology by which benthic algae can be monitored, 2) use PAM fluorimetry to include rapid, repeated measurements of biomass and photosynthetic activity *in situ*, and 3) combine the above objectives into a protocol that can be used for the purposes of aquatic system restoration, conservation, and management.

MATERIALS AND METHODS

Study Site

This study was conducted at Eastern Michigan University's Loesell Wetland in Ypsilanti, MI (N42.25563^{o}, W083.66071^{o}). This 1.62ha freshwater wetland has no surface inflow or outflow and is surrounded by residential development enclosing a region of deciduous forest that borders a wetland pool of standing water over deep, flocculent sediment. On 29 June, 6 July, 12 July, and 22 July of 2007, water samples were filtered (0.7 μm GFF) for NO_3, NH_4, soluble reactive phosphorous (SRP) analysis. Analysis was conducted on a Seal AQ-2 discrete analyzer according to EPA-approved manufacturer's protocols for NO_3 and NH_4 (Seal Analytical 2005). PO_4 was analyzed using the molybdate blue method (Lind 1985). A YSI 63 meter (YSI Incorporated, Yellow Springs, Ohio, USA) was used to measure *in situ* conductivity, temperature, and pH.

NDS Construction and Calibration

Four different nutrient treatments were employed for this study. A 2% agar solution (modified from Biggs & Kilroy 2000) had either 0.5M nitrogen (N) as $NaNO_3$, 0.05M phosphorous (P) as $NaHPO_4$, or both 0.5M nitrogen and 0.05M phosphorous added (B), or a control of no additional nutrients (C). Agar solutions were poured into 75mL acid-washed (10% HCl) polypropylene jars. After the agar had solidified, it was covered with nitrocellulose filters (pore size = 0.8μm). Jars were capped with lids through which had been bored a 38.1mm diameter hole. The rate of diffusion of nutrients from the agar was determined by placing 3 replicates of the B treatment in 1L of distilled

water. Acid-washed beakers containing water and jars of agar were placed on a shaker table oscillating at 60rpm for 33 days at room temperature. Water samples were collected every 3 days for the first 15 days and then every 6 days thereafter. During sample collection, each jar was wiped to remove any bacterial growth. The jars were then replaced in a clean beaker with fresh distilled water. Water samples were filtered through a 0.7μm glass fiber filter and frozen in acid washed polypropylene bottles. At the end of the collection, samples were thawed and nutrient concentrations were determined (see field samples above for method). Nutrient diffusion rates (mg of NO_3 or PO_4/L/d) were calculated, and modeled using logarithmic regression.

Field Experiment

For the *in situ* experiment, each nutrient treatment was replicated 5 times. One jar of each type was attached to a piece of angle-iron 1m in length. These were suspended in the wetland pool at a depth of 10-15 cm, just above the sediment, from a boardwalk running through the wetland. The NDS were deployed on June 29, 2007, and remained in the wetland for 24 days. The jars were continuously submerged, except when removed for measurement. Measurements were made on 6, 12, and 22 July 2007 using a Walz Diving PAM Fluorimeter (Walz 1998). Light-adapted measurements were conducted in ambient sunlight between noon and 3pm. Dark-adapted measurements were conducted immediately following the light-adapted measurements by covering jars with aluminum cylinders for 15 minutes. A black plastic hood was then used to cover each jar, the PAM fluorimeter, and the investigator before the dark-adapted measurement was made. Measurements in each light treatment consisted of recording the base fluorescence (F_o),

the maximum fluorescence (F_m), and the yield (Y) (Kromkamp and Forster 2003). Fluorescence yield is calculated with the Gentry parameter F_m-F_o/F_m (Gentry 1989). One-way ANOVA and Spearman's correlations were conducted using SYSTAT 11™ to determine statistical significance of differences among nutrient treatments and relationships among response variables.

Chlorophyll Analysis

After the final PAM measurements, 18mm diameter sample disks were cut from the filter of each NDS jar and frozen for later spectrophotometric chlorophyll analysis using a hot 90% ethanol extraction and acidification to correct for phaeopigments (Biggs and Kilroy 2000).

Community Composition

Another representative sample was cut (18mm diameter) for community composition, preserved in 5% gluteraldehyde, and refrigerated. Preserved periphyton was removed from the filter substrate and rinsed in distilled water several times to remove gluteraldehyde. Relative abundance of algal taxa was determined by identification and enumeration of a minimum of 100 algal cells per sample with an Olympus BH-2 at 400x. All fields viewed were randomly selected and counted to completion. Cells were identified to genus, when possible, using the taxonomy of Wehr and Sheath (2003) for diatoms and Prescott (1973) for all other algae. Using CANOCO 4™, community data were subjected to a Principle Components Analysis (PCA). All taxa were weighted equally, and any taxon that failed to achieve 5% relative abundance in at least one sample

was omitted prior to PCA. A PCA biplot of the relative abundance of alga taxa was created to illustrate any relationships between nutrient treatment and specific algal taxa. Divisional relative abundance by was also analyzed in SYSTAT 11^{TM} with a Kruskal-Wallis test and subsequent Bonferroni comparison (Zar 1999) to determine broad-scale taxonomic differences between nutrient treatments.

RESULTS

Environmental Measurements and NDS Diffusion Gradient

All measured environmental parameters remained relatively constant during the field experiment (Table 1). 6 July measurements indicated a small increase in water temperature as well as an increase in pH. While levels of ammonia were below the detection limits of the methods used, levels of nitrate/nitrite decreased from 0.238mg/L through the course of the trial. Also, levels of SRP ranged from a low of 14.9 μg/L on June 29 to a high of 59.8 μg/L on July 12.

At the end of 33 days in submersion and agitation, the final release of NO_3 and PO_4 from the NDS was recorded at 0.556 mg NO_3/L/d and 1.087 mg PO_4/L/d. Concentrations of NO_3 decreased quickly in the first half of trial before the release rate slowed. Concentrations of PO_4 decreased similarly though not as quickly as NO_3 (Fig. 1). From an initial concentration of 0.5M of NO_3, the pattern of diffusion decreased in a log slope, where the amount of NO_3 diffusing across the filter at any given day can be predicted by the equation mg NO_3/L/d = $103.32e^{-0.151(days)}$. A similar situation was observed for PO_4, where the equation mg PO_4/L/d = $7.1265e^{-0.055(days)}$ could predict the amount of PO_4 diffusing across the filter at any given day.

PAM Measurements & Correlations

Within one week, PAM fluorimetry detected significant ($p=0.003$) dark-adapted base fluorescence (F_o) responses to simultaneous addition of NO_3 and PO_4 (Fig. 2c). A continuing increase in this response was also observed on July 12 ($p=0.031$) and on July 22 ($p<0.001$). This biomass response was confirmed by a traditional spectrophotometric

biomass assay (Fig. 2c) where the concentration of chl *a* mg/m^2 present on July 22 for treatments with both NO_3 and PO_4 was significantly greater than the control treatment ($p<0.001$). Final F_o measurements were highly correlated ($r_s=0.846$, $p<0.001$) with the spectrophotometric measurements of chl *a* mg/m^2 (Fig. 3a). Similar effects of nutrient addition were observed for both dark-adapted ($p<0.001$) and light-adapted ($p<0.001$) yield measurements (Fig. 2b, 2a). Dark-adapted yield ($r_s=0.703$, $p<0.001$) and light-adapted yield ($r_s=0.742$, $p<0.001$) were also correlated with algal biomass for July 22 (Fig. 3b, 3c). Correlations of dark- and light-adapted yield measurements increased through time (Fig. 4).

Community Composition

Communities across all treatments were dominated by chlorophytes, and chlorophyte relative abundance was significantly greater in the B treatment. Cyanobacterial taxa also comprised a large portion of community relative abundance, and appeared to decline in the B treatment although this decline was not statistically significant (Table 2). Table 3 lists the genera encountered.

The PCA biplot captured 33.2% of the variance in the composition dataset with the first axis, and the second axis included an additional 20.8%. The four major taxa (all of which were chlorophytes) were plotted to aid in interpretation of possible relationships between nutrient treatment and dominant community type (Fig. 5). There was a tendency for the B treatments to have a larger proportion of the chlorophyte *Scenedesmus* and an unidentified coccoid chlorophyte than other nutrient treatments. A weaker relationship

existed between the N treatment and the chlorophyte *Gleocystis*; however, no other relationships emerge between any other taxa and nutrient treatments.

DISCUSSION

Nutrient Release by NDS

The rates and negative logarithmic pattern of nutrient diffusion from the NDS were in line with results experienced by other similar systems (Fairchild et al. 1985, Gibeau and Miller 1989, Matlock et al. 1998). Nutrient release continued for at least 33 days, indicating that nutrient treatments were maintained throughout the 24-day field study.

Nutrient Effects on Algal Growth

Benthic algae in the Loesell wetland displayed co-limitation by N and P, as biomass accrual (measured by spectrophotometric chlorophyll *a* assay) was stimulated by simultaneous addition of N and P, but not in either the N or P treatments. Nutrient limitation, and even co-limitation, can occur over a wide range of environmental N:P ratios (Francoeur et al. 1999) depending on the composition of the algal communities as well as the characteristics of the water body. It is not surprising, therefore, that co-limitation was observed at these low but detectable nutrient levels where the atomic N:P ratio in Loesell was 8:1. Lind (1985) suggests that algae typically require 7 N atoms for every P atom, and others have suggested N:P demand ratios are even higher (e.g. 16:1 from Redfield 1958). N-limitation has a significant effect on photosynthetic efficiency due to its requirement for chlorophyll production as well as other chloroplast proteins (Berges et al. 1996), where as P-limitation does not effect photosynthesis as much as it hinders the rate at which carbon is processed through phosphorylated intermediates (Wykoff et al. 1998). These metabolic consequences of nutrient limitation have an effect

not only on the amount of chlorophyll present but also on the ability of the chlorophyll present to produce a fluorescence signal.

Nutrient Effect on Fluorescence

The co-limitation that was observed through spectrophotometric chlorophyll analysis was also reflected in the fluorescence measurements. These results support the theoretical and empirical arguments that dark-adapted minimum fluorescence is an accurate indicator of algal biomass (e.g., Serôdio et al. 1997, Honeywill et al. 2002, Kromkamp & Forster 2003) and indicate that *in situ* optical measurements of biomass and activity can be used in conjunction with NDS to determine algal responses to nutrient addition in aquatic ecosystems. The occurrence of this relationship most probably exists due to N and P's significant role in the photosynthetic capabilities of benthic algae. N-limitation would cause a decrease in the amount and quality of chlorophyll (Berges et al. 1996), which would reflect as a lower F_o, since there would be fewer photocenters. Also, P-limitation would be observed as a lower F_o since without proper carbon processing (Wykoff et al. 1998) a lower level of algal growth and reproduction would take place, resulting in smaller and less numerous algal cells.

This study also suggests that both traditional (i.e., spectrophotometric measurement of biomass accrual) and PAM fluorimetry-based (i.e., optical measurement of biomass accrual and PSII activity) assessments are equivalent in their ability to detect responses to nutrient addition for the reasons given above. Spectrophotometric chlorophyll analysis could not be conducted for every date of the study due to the destructive nature of the assay. The number of replicates required would be so numerous

as to make this method of analysis impractical. Because fluorescent analysis is non-destructive, a manageable number of replicates could be used throughout the entire study for optical estimation of biomass. This allowed a relatively quick detection of algal biomass responses to nutrient enrichment (one week). In contrast, use of traditional, destructive chl *a* analysis encourages use of a longer incubation period (3-4 weeks) to ensure that any potential growth response to nutrient enrichment will have had sufficient time to occur and reach a measurable level.

In addition to the strong correlation of dark-adapted F_o to algal biomass, both light-adapted ($p,0.001$, $r_s=0.742$) and dark-adapted ($p,0.001$, $r_s=0.703$) yield also showed a strong correlation to biomass. The yield measurement represents the algae's capacity for efficient light use for photosynthesis, either as actual efficiency under current illumination (light-adapted yield) or as maximum potential efficiency (dark-adapted yield) (Consalvey et al. 2005). Should the yield decrease, this would be an indicator of stress (Consalvey et al. 2005) as a result of lack of nutrients, grazing, or other environmental stressors. Similarly, an increase would indicate favorable conditions for algal growth. It is important, therefore, to not only take into account dark-adapted F_o as an optical measure of biomass but to also keep record of the yield, since it is this parameter that measures overall health and efficiency of the algae present.

Implications of Community Composition

Some differences in the relative abundances of algal divisions related to nutrient treatment were observed. In particular, chlorophyte abundance increased significantly on B treatments, while cyanobacterial taxa appeared to decrease. The PCA biplot also

indicated that nutrient treatments did not support consistently different algal communities, with the exception that B treatments tended to have increased relative abundance of coccoid chlorophytes and the chlorophyte *Scenedesmus*. PAM fluorometers are typically spectrally optimized for use with either eukaryotes or cyanophytes, not both (Walz 1998); thus it is then possible that some of the observed PAM signal could have been due to shifts in divisional abundance. Measuring cyanobacterial fluorescence with a PAM fluormeter calibrated for eukaryotic samples has the capacity to result in artifactually low F_o reading due to the different ratio of PSII:PSI as well as the presence of other photosynthetic pigments (Campbell et al. 1998). Thus, it is possible that some of the increased F_o response in the B nutrient treatment was due to a community shift away from cyanobacteria in this treatment. However, the close agreement of PAM and spectrophotometric analyses suggest that any potential influence was small. Further studies are needed to determine the degree of uncertainty caused by analysis of mixed cyanobacterial/eukaryote algal communities.

Conclusions

A co-limitation of algae by N and P in Loesell was observed, using both a traditional spectrophotometric and a novel optical measurement of benthic algal biomass. This confirmed that dark-adapted F_o is a suitable measurement for *in situ* algal biomass, that those measurements are reflective of nutrient conditions, and that *in situ* optical measurements can be used to monitor the community's changes in biomass and photosynthetic activity in response to changing nutrient conditions through time. The potential effect that cyanobacteria could have on dark-adapted F_o required further

investigation, but this method is likely currently suitable for use as an ecological monitoring procedure in impacted or protected aquatic systems.

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Table 1: Auqatic environmental data from Loesell wetland. DIN:SRP (the sum of amount present of NH_4 and NO_3 compared the amount of PO_4) ratio approximately 8:1. Asterisk indicates data is from July 20. nd = not determined.

	June 29	July 6	July 12	July 22
Water Temp (^oC)	26.9	32	27	23.2
pH	6.81	7.2	6.95	6.93
Conductivity (μs)	638	646	701	769
NO3/NO2	0.238mg/L	0.173mg/L	0.128mg/L	0.078mg/L*
NH4	nd	nd	nd	nd*
SRP	14.96μg/L	44.89μg/L	59.86μg/L	49.88μg/L*

Table 2. Relative abundance (proportion of cells) of algal divisions by nutrient treatment. Asterisk indicates a significant difference from C, determined with a Bonferroni pairwise comparison.

	C	P	N	B
Chlorophytes	0.383	0.535	0.672	0.882*
Cyanophytes	0.380	0.402	0.346	0.059
Bascilliariophytes	0.168	0.210	0.098	0.052
Euglenophytes	0.048	0.041	0.025	0.007
Dinophytes	0.020	0.009	0.000	0.000

Table 3. Counts (# of cells) of algal taxa by treatment.

Genera	**C**	**P**	**N**	**B**
Chlorophytes				
Botryococcus	0	26	0	0
Cosmarium	5	9	9	2
Eudorina	0	0	7	0
Gleocystis	55	1	167	82
Oedogonium	0	94	149	0
Palmella	8	0	0	20
Pediastrum	16	25	0	4
Scenedesmus	54	4	48	337
Selenastrum	2	2	16	3
Sphaerocystis	0	0	4	0
Staurastrum	0	0	1	0
Stigeoclonium	0	0	0	29
Tetraedon	1	4	1	6
Unident. 1	9	27	7	235
Unident. 2	0	0	9	0
Unident. 3	15	0	0	0
Cyanophytes				
Anabaena	72	48	24	5
Chroococcus	3	0	0	8
Merismopedia	20	19	0	0
Oscillatoria	87	18	111	39
Bascilliariophytes				
Cocconeis	2	1	0	0
Eunotia	0	1	0	0
Navicula-like	67	50	54	32
Nitschzia	5	2	1	3
Ophiocytium	1	0	0	0
Rhopalodia	0	1	0	0
Synedra-like	4	2	0	0
Tabellaria-like	0	0	4	0
Euglenophytes				
Euglena	1	0	0	1
Phacus	1	2	3	0
Trachelemonas	19	12	13	4
Dinophytes				
Gymnodinium	2	1	0	0
Peridinium	8	2	0	0

Figure 1a. NO_3 diffusion pattern for NDS. Final mean value = 0.556 mg NO_3/L/d. Error bars ±1SD.

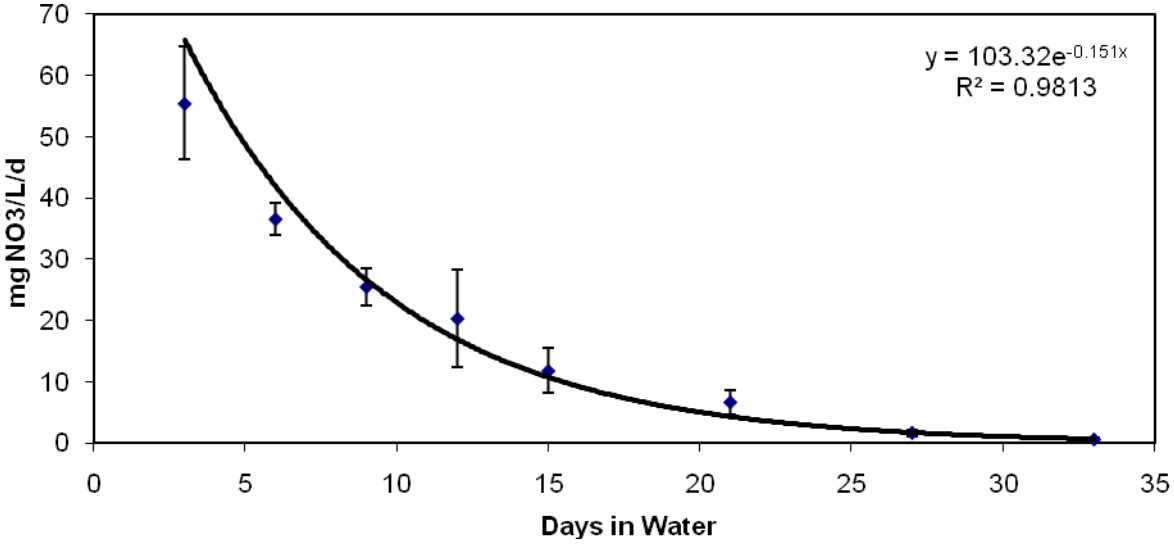

Figure 1b. PO_4 diffusion pattern for NDS. Final mean value = 1.087 mg PO_4/L/d. Error bars $\pm$1SD.

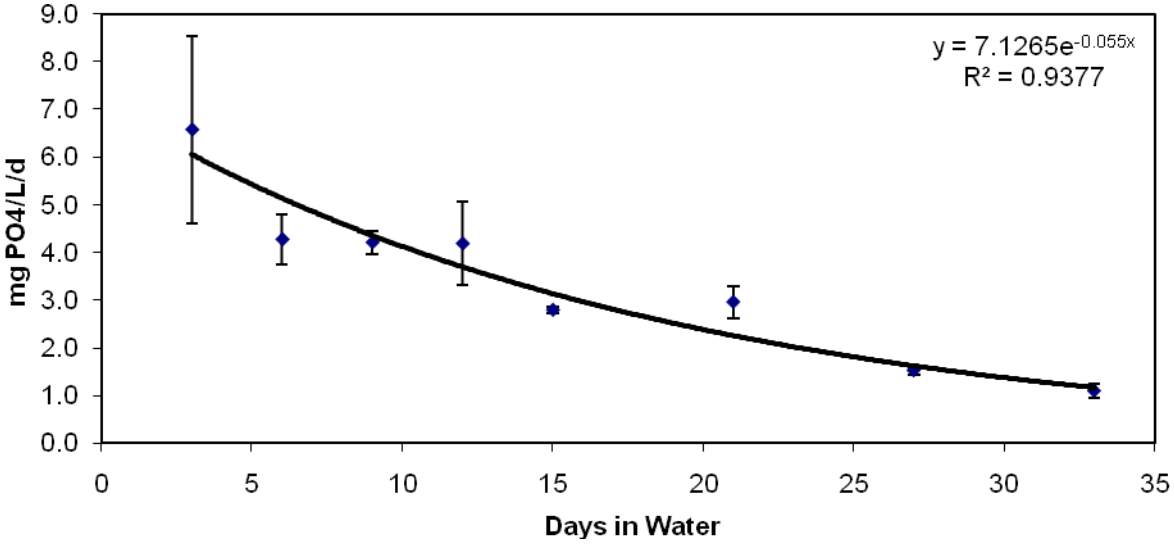

Figure 2. The change in photosynthetic activity and biomass on a per weekly basis, with the final graph showing both dark-adapted minimum fluorescence with final chlorophyll concentrations. Asterisks indicate where B is significantly different from C, Tukey HSD p values given. N and P treatments were never significantly different from C.

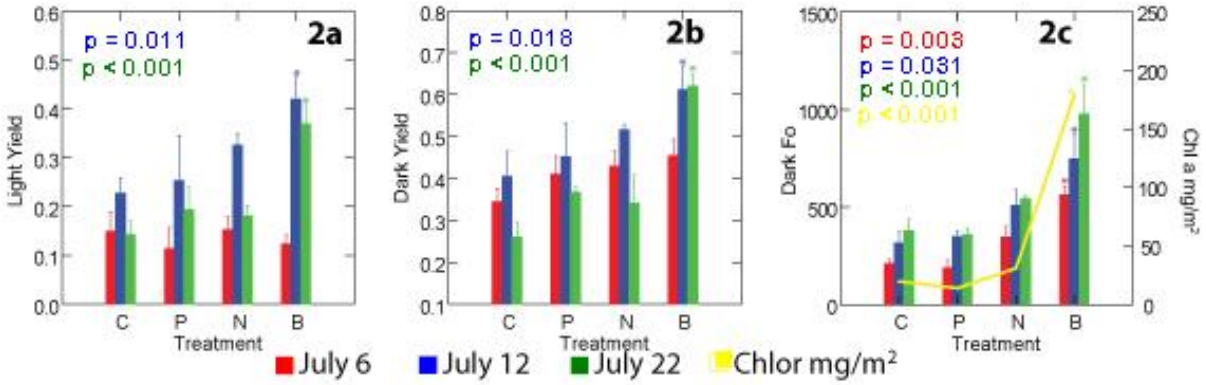

Figure 3. Spearman correlations for July 22 spectrophotometric chlorophyll content with dark-adapted minimum fluorescence, dark yield, and light yield.

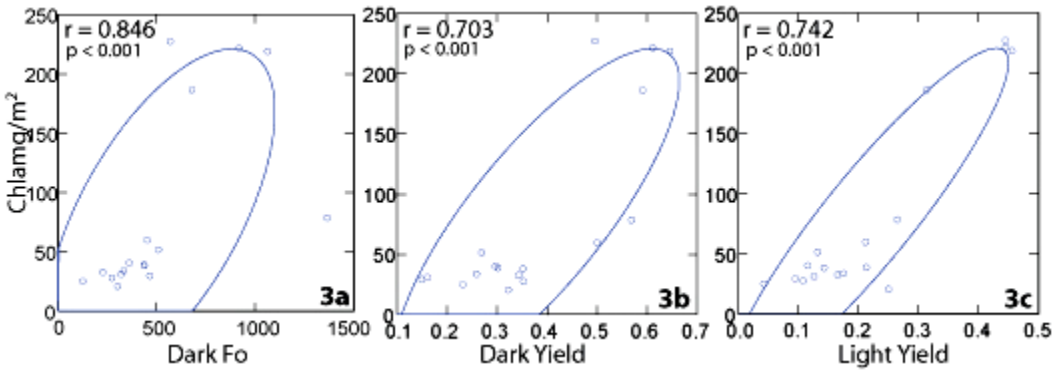

Figure 4. Spearman correlations of light yield with dark yield on a per week basis, showing increasing correlation as periphytic communities become established.

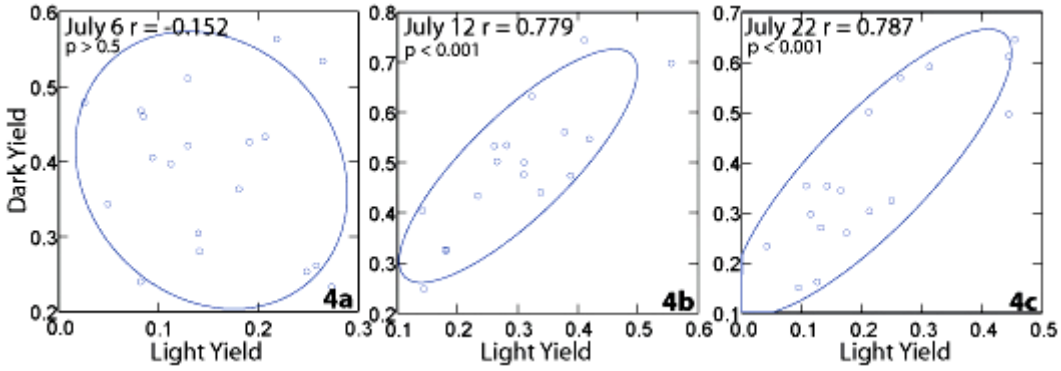

Figure 5. PCA biplot of selected algal genera in relation to nutrient treatment.