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Active fluorometry improves nutrient-diffusing substrata bioassay

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Abstract. Benthic algal nutrient bioassays traditionally have 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 destruction of the accumulated periphyton communities and, thus, does not allow for convenient monitoring through time. Variable fluorescence methods of estimating algal biomass and photosynthetic activity have been used in aquatic environments, but generally not over different nutrient treatments and not for a substantial duration. We evaluated the use of a pulse amplitude modulated (PAM) fluorometer for measuring algal biomass and photosynthetic activity in conjunction with NDS over several weeks in a wetland under several nutrient-addition treatments. We were able to detect a significant fluorometric response as early as 1 wk into the study with addition of both N and P. Wetland periphyton was co-limited by N and P. Dark-acclimated minimal fluorescence was highly correlated with chlorophyll *a* in different nutrient treatments. Our results suggest that active fluorometry is a useful method for measuring periphytic responses to nutrients and for evaluating the effect of nutrient additions on overall photosynthetic efficiency.

Key words: fluorometry, periphyton, wetlands, NDS, PAM, algae.

Benthic algal responses to nutrient enrichment often have been measured by observing biomass (quantitative chlorophyll *a* extraction) or community composition (microscopy and morphological taxonomy) responses in nutrient-diffusing substrata (NDS) experiments (Fairchild et al. 1985, Borchardt 1996). Comparison of algal responses under ambient and nutrient-enriched conditions can be used to detect whether algal growth in an aquatic system is nutrient-limited, which nutrient is limiting algal growth, and the extent of that limitation (Francoeur et al. 1999).

In typical NDS experiments, researchers deploy growth surfaces in a water body. Some surfaces release nutrients to create local nutrient-enriched zones, whereas other surfaces do not release nutrients and serve as controls. Benthic algae are allowed to colonize and to accumulate on the surfaces and are collected later for analysis. Such experiments can be time consuming and labor intensive. The average NDS experiment requires 2 to 8 wk to complete (Biggs and Kilroy 2000), and 3 to 4 wk of exposure typically are needed to allow sufficient time for algal growth (Francoeur et al. 1999, Francoeur 2001). In addition, the biomass, community structure, or physiological assays typically applied to such experiments require destructive sampling of algal communities on NDS. This approach greatly complicates the logistics of measuring responses through time because many additional replicates are required to account for attrition caused by destructive sampling. In experiments designed for a single sampling event, destructive sampling encourages use of longer-than-necessary incubation times because investigators do not want to err by sampling too early in the colonization and growth sequence when time might not have been sufficient for nutrient-induced differences in growth rate to cause measureable differences in algal biomass. Excessive incubation times unnecessarily delay experimental completion, prevent experimenters from measuring algal responses at fine temporal scales, and may result in algal sloughing from NDS, which can mask any response to nutrient addition.

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Active fluorometry can be used to measure photosynthetic performance and, under certain conditions, microalgal biomass nondestructively (Karsten et al. 1996, Serôdio et al. 1997, Honeywill et al. 2002, Consalvey et al. 2005). This method typically is used with macrophytes (Schreiber et al. 1986, Seaton and Walker 1992, Gilmore et al. 1995), but also has been used with algae (Mauzerall 1972, Samuelsson and Öquist 1977). Various portable fluorescence methods are increasingly popular alternatives for measuring algal biomass and productivity without disturbing these communities.

The 2 main methods of fluorometry currently used for in situ measurements are fast repetition rate fluorometry (FRRF) and pulse amplitude modulated (PAM) fluorometry (Kromkamp and Forster 2003). Both methods work by first measuring the quantity of photons fluoresced from Photosystem II (PSII) under ambient light conditions (the minimal fluorescence). If measurements are made on a sample held in ambient light, minimal fluorescence is denoted as F′. If measurements are made after the sample has been held for a suitable period in complete darkness (dark-acclimated), it is denoted as F_o (Table 1). In the 2nd phase of active fluorometry, 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 quantity of photons fluoresced when all available photocenters are saturated. This measurement is termed the maximum fluorescence and is denoted as either F_m' or F_m, depending on whether the sample was exposed to ambient light or was dark-acclimated. PAM uses a single pulse of light typically lasting 300 to 1200 ms (Suggett et al. 2003), whereas FRRF uses a series of alternating flashes and pauses, typically 50 to 1000 ms (Kromkamp and Forster 2003) to achieve saturation. Measurements on dark-acclimated samples can be combined to calculate photosynthetic efficiency, also known as the Genty parameter, from the formula: $(F_m - F_o)/F_m$ (Genty et al. 1989). The Genty parameter measures the maximum possible light utilization efficiency of PSII and is denoted F_v/F_m (Kromkamp and Forster 2003, Consalvey et al. 2005). Effective light utilization efficiency (measured on samples exposed to ambient light) can be calculated in a similar manner as $(F_m' - F')/F_m'$ and is denoted F_q'/F_m' (Consalvey et al. 2005).

TABLE 1. Summary of fluorescence terms. A more detailed discussion of these terms is available in the paper by Consalvey et al. (2005). PSII = photosystem II.

Term	Dark-acclimated	Light-acclimated
Minimal fluorescence	F_o	F'
Maximum fluorescence	F_m	F_m'
PSII efficiency	F_v/F_m	F_q'/F_m'

No general consensus exists regarding whether PAM or FRRF is superior. Few investigators have compared these 2 methods directly, and Kromkamp and Forster (2003) concluded that PAM and FRRF were correlated, but FRRF-based F_m measurements could be ~50% higher than corresponding PAM-based F_m measurements. Suggett et al. (2003) examined phytoplankton and concluded that the 2 methods gave comparable results, but that the more-sensitive FRRF method was better suited to open-ocean studies, whereas PAM was best suited to inland freshwaters, which typically have greater algal densities.

Light exposure of algal samples has a strong effect on measured fluorescence values and their interpretation. Investigators assume that samples held in complete darkness 10 to 15 min before measurement are dark-acclimated and that all photocenters initially will be open during measurement of F_o and F_m. The measured value of F_o is determined primarily by the amount of chlorophyll *a* in the sample, and F_o is correlated with chlorophyll *a* (Kiefer et al. 1989, Kolber and Falkowski 1993, Serôdio et al. 1997, Honeywill et al. 2002, Sylvan et al. 2007). However, factors such as species composition, sample temperature, and the thickness of the periphyton layer can alter this relationship (Serôdio et al. 1997). In contrast, investigators assume that samples measured in steady-state ambient light are light-acclimated and that some photocenters will be closed during measurement of F′ and F_m' because of photoquenching by ambient light. Therefore, the measured value of F′ is a function of the amount of chlorophyll *a* and the degree of photocenter closure. Thus, F′ may not be a good measure of biomass.

PSII efficiency measurements represent the capacity for efficient light use for photosynthesis as the effective efficiency under current illumination (F_q'/F_m') or as maximum potential efficiency (F_v/F_m) (Consalvey et al. 2005). Effective PSII efficiency measurements can be used to calculate photosynthetic electron transport. Thus, efficiency measurements are correlated with algal photosynthesis as measured by ^{14}C uptake or O_2 production, although quantitative interconversion of rates is difficult (see Barranguet and Kromkamp 2000, Glud et al. 2002, Hancke at al. 2008). ^{14}C-based measurement of photosynthetic activity under nutrient-enriched conditions has been used as a bioassay for planktonic and benthic microalgal nutrient limitation (Hameed et al. 1999, Francoeur et al. 2003). Thus, perhaps

fluorescence-based measures of photosynthetic activity could be used in a similar fashion.

The nondestructive nature of active fluorometry could be a great advantage when measuring benthic algal biomass and photosynthetic responses on NDS by facilitating both measurements early in the colonization and growth process and repeated measurements through time. Our goal was to evaluate the utility of PAM fluorometry to provide rapid and repeated in situ measurements of benthic algal biomass and photosynthetic activity in nutrient-enrichment experiments by measuring algal communities incubated on varying nutrient treatments in a freshwater wetland for 3 wk.

Methods

Study site

We conducted this study at Eastern Michigan University's Loesell Wetland in Ypsilanti, Michigan, USA (lat 42.25563°N, long 83.66071°W). This 1.62-ha 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 2007, we filtered water samples (0.7-µm glass-fiber filters GF/F) for NO_3^-, NH_4^+, and soluble reactive P (SRP) analysis. We ran analyses on a Seal AQ-2 discrete analyzer (Seal Analytical, Hampshire, UK) according to EPA-approved manufacturer's protocols for NH_4^+ and NO_3^-/NO_2^- (Seal Analytical 2005). We analyzed SRP with the molybdate blue method (Lind 1985). We used a YSI 63 meter (Yellow Springs Instruments, Yellow Springs, Ohio) to measure conductivity, water temperature, and pH in situ.

NDS construction and experiment

We constructed nutrient-diffusing substrata following the methods of Francoeur et al. (2013). We used a 2% agar solution with 4 different nutrient treatments: 0.5 M N as $NaNO_3$ (N), 0.05 M P as $NaHPO_4$ (P), both 0.5 M N and 0.05 M P (B), or a control with no additional nutrients (C). We poured agar solutions into 75-mL acid-washed polypropylene jars. After the agar had solidified, we covered it with a nitrocellulose filter (pore size = 0.8 µm) and capped the jars with lids through which a 38.1-mm-diameter hole had been bored. We replicated each nutrient treatment 5 times. We attached 1 jar of each type to a 1-m-long piece of angle-iron with ~25 cm between jars. We suspended the NDS in the wetland pool at a depth of 10 to 15 cm below the water surface, just above the sediment, from a boardwalk running through the wetland on 29 June 2007 and left them in the wetland for 24 d. The jars were continuously submerged, except when removed for measurement. During the course of the experiment, 2 of the P replicates and 1 of the C replicates were lost.

We made measurements on 6, 12, and 22 July 2007 with a Walz Diving PAM Fluorometer (Walz, Effeltrich, Germany). We made light-acclimated measurements on cloudless days in direct, ambient sunlight during the midday hours. We made dark-acclimated measurements immediately after light-acclimated measurements by covering jars with Al cylinders for 15 min. We added a spacer to the end of the fluorescence sensor to maintain a constant distance of 1 cm between the sensor and NDS surface. We used a black plastic hood to cover each jar, the PAM fluorometer, and the investigator before making the dark-acclimated measurement. Measurements in each light treatment consisted of the minimal fluorescence (F_o or F'), the maximum fluorescence (F_m or F_m'), and the PSII efficiency (F_v/F_m or F_q'/F_m').

Spectrophotometric chlorophyll a *analysis*

Immediately after the final PAM measurements on 22 July, we cut 18-mm-diameter disks from the filter of each NDS jar and froze them for later spectrophotometric chlorophyll *a* analysis with hot 90% ethanol extraction and acidification to correct for phaeopigments (Biggs and Kilroy 2000). At this time, the periphyton accumulated was <0.50 mm thick, and the texture of the filter NDS substrata could be seen through the covering of attached algae.

Statistical analysis

We used repeated measures and 1-way analyses of variance (ANOVAs) with subsequent post hoc Tukey's Honestly Significant Difference tests and Spearman's rank correlations in SYSTAT 11™ (Systat Software, Chicago, Illinois) to test for differences in chlorophyll *a* concentration and photosynthetic activity among nutrient treatments and relationships among biomass response variables.

Results

Environmental measurements

All measured environmental variables remained relatively constant during the experiment (Table 2). Water temperature and pH were maximal on 6 July. Levels of NH_4^+ were below the detection limits of the methods used, but levels of NO_3^-/NO_2^- decreased from 0.238 mg/L through the course of the trial. Also,

TABLE 2. Environmental data from the Loesell wetland. * indicates data are from July 20. nd = not detected, SRP = soluble reactive P.

Variable	June 29	July 6	July 12	July 22
Water temperature (°C)	26.9	32	27	23.2
pH	6.81	7.2	6.95	6.93
Conductivity (μS/cm)	638	646	701	769
NO_3^-/NO_2^- (mg/L)	0.238	0.173	0.128	0.078*
NH_4^+	nd	nd	nd	nd*
SRP (μg/L)	14.96	44.89	59.86	49.88*

levels of SRP ranged from a low of 14.96 μg/L on 29 June to a high of 59.86 μg/L on 12 July 12.

Algal biomass and photosynthetic responses to nutrients

Repeated-measures ANOVAs of F′, F_o, F_v/F_m, and F_q'/F_m' indicated significant differences in all fluorescence variables among nutrient treatments (all $p < 0.001$) and over time (all $p < 0.006$) and significant nutrient treatment × time interactions (all $p < 0.05$, except for F_o, which was not affected by the interaction, $p = 0.178$). One-way ANOVAs considering individual variables on each sampling date revealed that PAM fluorometry could detect significant ($p = 0.003$) F_o responses to the B over the C treatment within 1 wk (6 July) (Fig. 1). This response also was significant on all subsequent sampling dates (12 July, $p = 0.031$; 22 July, $p < 0.001$). The nutrient-induced biomass patterns detected by F_o were confirmed by the traditional spectrophotometric biomass assay conducted on 22 July. Chlorophyll *a* was significantly greater on the B than the C treatment

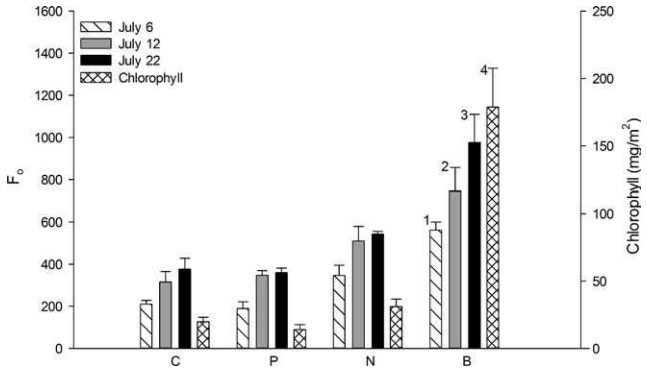

FIG. 1. Mean (+1 SE) dark-acclimated minimum fluorescence (F_o) on each date and final spectrophotometric chlorophyll *a* (collected 22 July) of periphyton on nutrient-diffusing substrates (C = control, P = P added, N = N added, B = both nutrients added). Numbers indicate where B treatment was significantly different from C treatment on that date. N or P treatments were never significantly different from the C treatment.

($p < 0.001$; Fig. 1). By the 2nd wk, F_v/F_m and F_q'/F_m' were significantly greater on the B than on the C treatment (F_v/F_m: 12 July, $p = 0.018$; 22 July, $p < 0.001$; F_q'/F_m': 12 July, $p = 0.011$; 22 July, $p < 0.001$; Fig. 2A, B). Fluorescence variables never differed between N or P treatments and C treatments.

Final (July 22) F_o measurements were highly correlated ($r_s = 0.846$, $p < 0.001$; Fig. 3A) but final F′ measurements were less strongly correlated ($r_s = 0.696$, $p = 0.002$; Fig. 3B) with spectrophotometric measurements of chlorophyll *a*. Measured values of F_o, F′, and chlorophyll *a* were clustered by nutrient treatment, and values generally were highest on the B treatment.

Discussion

Nutrient effects on algal biomass and photosynthesis

Our results suggest that traditional spectrophotometric measurement of biomass accrual and active fluorometry-based optical measurement of biomass accrual and photosynthetic activity are equivalent in their ability to detect responses to nutrient addition. Benthic algae in the Loesell wetland displayed colimitation by N and P because biomass accrual (measured by spectrophotometric chlorophyll *a* assay) was stimulated by simultaneous addition of N and P but not by individual N or P treatments. The colimitation observed through spectrophotometric chlorophyll *a* analysis was reflected in F_o and F′. These results support the theoretical and empirical arguments that F_o is an accurate indicator of algal biomass (Serôdio et al. 1997, Honeywill et al. 2002, Kromkamp and Forster 2003) and indicate that in situ optical measurements of biomass can be used in conjunction with NDS to evaluate algal responses to nutrient addition in aquatic ecosystems. If samples are measured in steady-state ambient light, some photocenters already will be closed during the measurement of F′. F′ may not be a good measure of biomass because F′ is a function of the amount of chlorophyll *a* and the degree of photocenter closure because of photoquenching by ambient light.

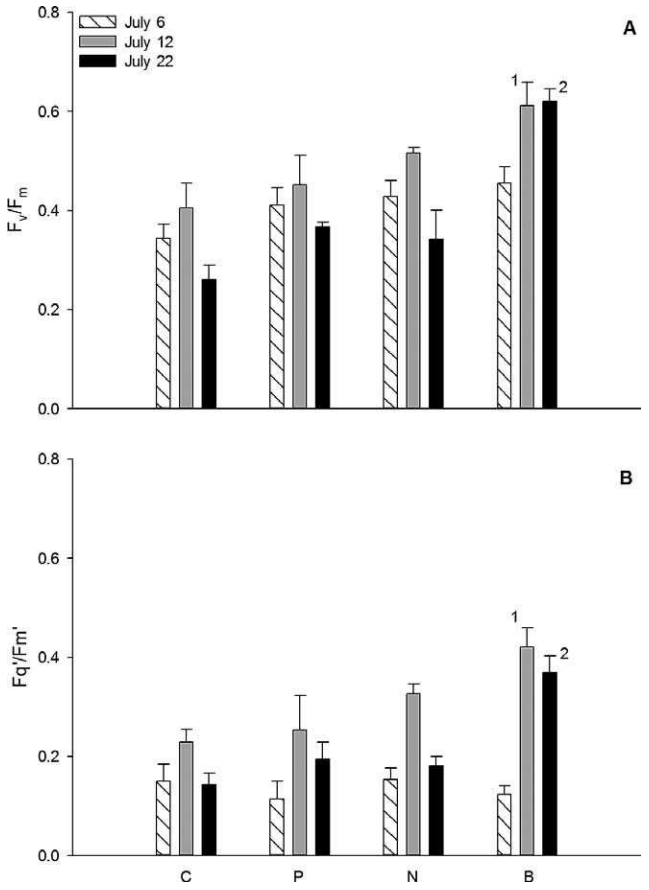

FIG. 2. Mean (+1 SE) maximum (F_v/F_m) (A) and effective (F_q'/F_m') (B) light utilization efficiency of photosystem II (PSII) by nutrient treatment (C = control, P = P added, N = N added, B = both nutrients added). Numbers indicate where B treatment was significantly different from C treatment at that date ($p < 0.05$). N and P treatments were never significantly different from the C treatment. See Table 1 for explanation of fluorescence variables.

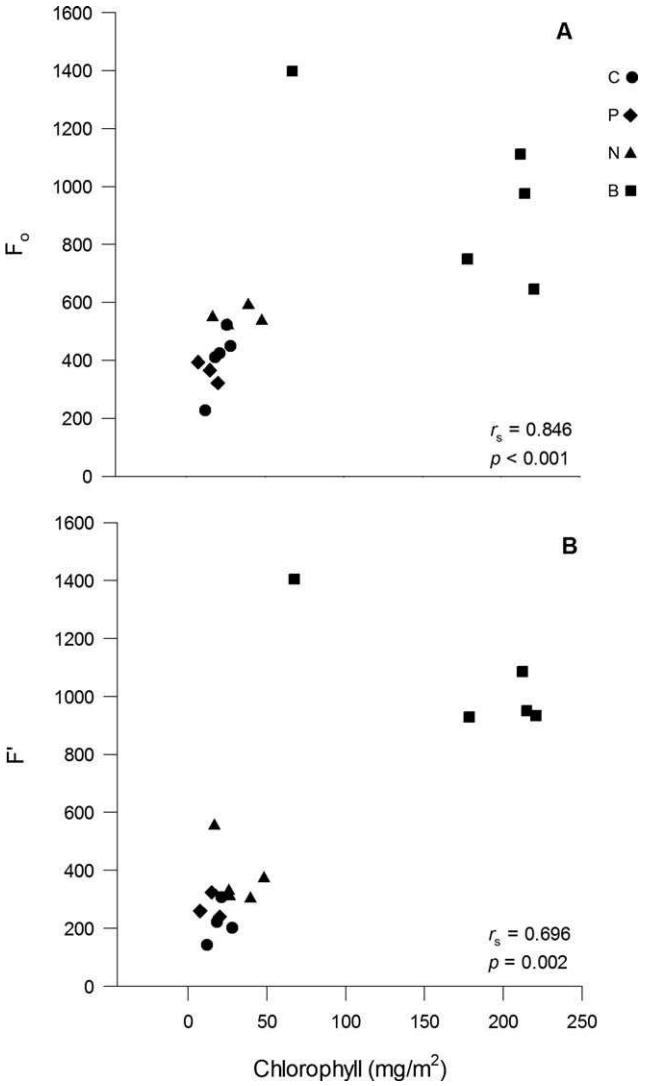

FIG. 3. Scatterplots and Spearman's rank correlation of traditional spectrophotometric chlorophyll *a* assay results with dark-acclimated minimal fluorescence (F_o) (A) and light-acclimated minimal fluorescence (F′) (B) of periphyton on nutrient-diffusing substrates (C = control, P = P added, N = N added, B = both nutrients added) on July 22.

F_o was able to detect a significant biomass response to nutrient addition after only 1 wk of growth. We do not know whether spectrophotometric analysis also would have detected a significant biomass response after only 1 wk, but the nondestructive nature of the fluorometric assay allowed us to investigate the possibility of an early biomass signal without the need to deploy multiple sets of replicate substrata. Thus, use of active fluorometry could facilitate shorter NDS experimental durations and improve the temporal resolution of their conclusions.

Very high biomass could uncouple F_o and algal community biomass because fluorescence signal from deep within a periphyton mat might be quenched by overlying algae before it reaches the fluorometer (Serôdio et al. 1997, Consalvey et al. 2005). However, this limitation is not a fatal flaw because the main utility of the active fluorescence measurements in the context of an NDS experiment is to detect a biomass signal as soon as it develops, not after the community has reached maximum biomass. Investigators have attempted to quantify the relationship between the thickness of a periphyton mat and its true minimal fluorescence value when the photoquenching associated with the deeper cells has been taken into account (Forster and Kromkamp 2004, Serôdio 2004). Forster and Kromkamp (2004) found that photoquenching can occur when a microalgal cell is as shallow as 0.10 to 0.15 mm below sediment surface. The direct applicability of this estimate to our data is uncertain

because our periphyton layer did not include sediments. However, the periphyton accumulated on our NDS jars was never >0.50 mm.

Effective ($p < 0.001$) and maximum ($p < 0.001$) PSII efficiencies were significantly stimulated by nutrients. These results indicate that the responses of these variables to nutrient addition can be used as a bioassay for algal nutrient limitation, as has been done with traditional ^{14}C-based photosynthesis measurements (Hameed et al. 1999, Francoeur et al. 2003). One would expect that elevated photosynthesis would be an important mechanism driving nutrient-induced increases in microalgal biomass. Active fluorometry assays can be superior to other assay methods because their nondestructive nature allows a manageable number of experimental substrata to be measured repeatedly throughout the entire study and because they provide the opportunity to make in situ measurements of microalgal photosynthesis on fine time scales (hourly, daily; Juneau et al. 2001). This capability encourages early measurements and leads to rapid detection of microalgal biomass and photosynthetic responses to nutrient enrichment. In contrast, destructive analyses encourage use of longer incubation to ensure that responses to nutrient enrichment will have had sufficient time to occur and reach a measurable level.

Several investigators have shown that nutrient addition can rapidly (min) alter fluorescence variables (e.g., F_v/F_m, F_m) of nutrient-stressed phytoplankton, a phenomenon known as nutrient-induced fluorescence transients (Wood and Oliver 1995, Beardall et al. 2001). Our measurements were made after prolonged exposure (d to wk) of algae to nutrient additions and, thus, do not reflect these short-term transient responses. Our experiment was similar to longer-term nutrient-addition experiments in which laboratory algal cultures (Beardall et al. 2001) and natural phytoplankton communities (Sylvan et al. 2007) displayed increased F_v/F_m after relatively long (d) exposure to nutrients. Results of a single-species chemostat experiment suggested that shifts in algal F_v/F_m reliably indicated nutrient stress under unbalanced growth conditions but not under balanced growth conditions (Parkhill et al. 2001), and Kruskopf and Flynn (2006) strongly criticized the use of F_v/F_m to infer algal nutrient status. Sylvan et al. (2007) suggested that the findings of Parkhill et al. (2001) could have been caused by individualistic responses of the diatom species used in their experiment. However, balanced nutrient limitation appears to be rare in natural algal communities (see Berman-Frank and Dubinsky 1999), probably because of the multispecies nature of natural algal communities and the temporal fluctuations of environmental conditions in the field.

Applying the NDS bioassay to wetland habitats

The NDS technique was originally developed for use in lake littoral zones (Fairchild et al. 1985). Its extensive use in lakes and streams has led to synthesis of broad patterns regarding benthic algal nutrient limitation in these habitats (Borchardt 1996, Francoeur 2001). NDS have been used much less frequently in nonlittoral wetlands, preventing similar robust synthesis of wetland benthic algal nutrient limitation. We documented N and P colimitation of benthic algal biomass accrual and photosynthesis in a small Michigan wetland. This result agrees with results of previous wetland NDS experiments, which have documented nutrient limitation and co-limitation of wetland algal biomass accrual (Goldsborough et al. 2005, Scott et al. 2005). NDS results are most directly applicable to wetland benthic algal communities growing on relatively inert substrata (e.g., rocks, large woody debris, plant litter) because macrophytes and sediments can be sources of nutrients and inorganic C for epipelic algae (Vadeboncoeur and Lodge 1998, 2000, Goldsborough et al. 2005).

Factors affecting fluorometric measurements

The amount of ambient light exposure can affect measured values of light-acclimated fluorometry variables. We do not think that light-induced variation greatly affected our ability to discern nutrient-induced patterns because we made measurements only on cloudless days within a short period of time, thereby minimizing differences in ambient light exposure between replicates. In addition, light-induced variability resulting from random fluctuations in ambient light intensity during measurements should have increased stochastic variation in the measured values of light-acclimated fluorometry variables, making it less likely for us to detect nutrient-induced patterns. We are confident that any ambient light variability did not greatly affect our results because we observed clear patterns and significant differences among treatments.

The relative proportion of cyanobacteria to eukaryotic periphyton also can affect fluorometric measurements. The PAM fluorometer in our study used red (650 nm) excitation light and captured far-red (>700 nm) emission light, thereby efficiently inducing and detecting chlorophyll *a* fluorescence from both eukaryotic algae and cyanophytes (Walz 1998, Simis et al. 2012). Measuring cyanobacterial fluorescence with a PAM fluorometer may lead to artifactually low F_v/F_m and high minimal fluorescence readings

because the ratio of cyanobacterial PSII:PSI differs from that of eukaryotic algae and because cyanobacteria have other photosynthetic pigments that fluoresce in the far-red range (Campbell et al. 1998, Simis et al. 2012). Cursory examination of the algal communities in our study suggests that the relative abundance of chlorophytes was somewhat greater and of cyanobacteria was slightly lower in the B than in the other treatments. Thus, some of the increased F_v/F_m response in the B nutrient treatment could have been caused by a community shift away from cyanobacteria in this treatment. However, this effect probably was small in our experiment because measured F_o values increased significantly in the B treatment, a pattern opposite to that expected if among-treatment differences were caused by reduced cyanobacterial abundance in the B treatment. The close agreement of PAM and spectrophotometric biomass analyses also suggest that any potential influence of algal community composition on the PAM results was small. Nevertheless, division-level shifts in algal community composition can be induced by nutrients from NDS (Fairchild et al. 1985, Peterson and Grimm 1992), so further studies are needed to determine the degree of uncertainty caused by analysis of mixed cyanobacterial/eukaryotic algal communities.

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Literature Cited

BARRANGUET, C., AND J. KROMKAMP. 2000. Estimating primary production rates from photosynthetic electron transport in estuarine microphytobenthos. Marine Ecology Progress Series 204:39–52.

BEARDALL, J., T. BERMAN, P. HERAUD, M. O. KADIRI, B. R. LIGHT, G. PATTERSON, S. ROBERTS, B. SULZBERGER, E. SAHAN, U. UEHLINGER, AND B. WOOD. 2001. A comparison of methods for detection of phosphate limitation in microalgae. Aquatic Sciences 63:107–121.

BERMAN-FRANK, I., AND Z. DUBINSKY. 1999. Balanced growth in aquatic plants: myth or reality? BioScience 49:29–37.

BIGGS, B. J. F., AND C. KILROY. 2000. Stream periphyton monitoring manual. National Institute of Water and Atmospheric Research, Christchurch, New Zealand.

BORCHARDT, M. A. 1996. Nutrients. Pages 184–227 *in* R. J. Stevenson, M. L. Bothwell, and R. L. Lowe (editors). Algal ecology: freshwater benthic ecosystems. Academic Press, San Diego, California.

CAMPBELL, D., V. HURRY, A. K. CLARKE, P. GUSTAFSSON, AND G. ÖQUIST. 1998. Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. Microbiology and Molecular Biology Reviews 62:667–683.

CONSALVEY, M., R. G. PERKINS, D. M. PATERSON, AND G. J. C. UNDERWOOD. 2005. PAM fluorescence: a beginners guide for benthic diatoms. Diatom Research 20:1–22.

FAIRCHILD, G. W., R. L. LOWE, AND W. B. RICHARDSON. 1985. Algal periphyton growth on nutrient diffusing substrates: an in situ bioassay. Ecology 66:465–472.

FORSTER, R. M., AND J. C. KROMKAMP. 2004. Modelling the effect of chlorophyll fluorescence from subsurface layers on photosynthetic efficiency measurements in microphytobenthic algae. Marine Ecology Progress Series 284: 9–22.

FRANCOEUR, S. N. 2001. Meta-analysis of lotic nutrient amendment experiments: detecting and quantifying subtle responses. Journal of the North American Benthological Society 20:358–368.

FRANCOEUR, S. N., B. J. F. BIGGS, R. A. SMITH, AND R. L. LOWE. 1999. Nutrient limitation of algal biomass accrual in streams: seasonal patterns and a comparison of methods. Journal of the North American Benthological Society 18:242–260.

FRANCOEUR, S. N., E. M. ESPELAND, AND R. G. WETZEL. 2003. Short-term effects of nitrogen and extracellular protease amendment on algal productivity in nitrogen-deprived periphyton. Journal of Freshwater Ecology 18:105–113.

FRANCOEUR, S. N., S. T. RIER, AND S. B. WHORLEY. 2013. Methods for sampling and analyzing wetland algae. Chapter 9 *in* J. T. Anderson, W. C. Conway, and C. A. Davis (editors). Wetland techniques. Springer, New York (in press).

GENTY, B., J. M. BRIANTAIS, AND N. R. BAKER. 1989. The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta 990:87–92.

GILMORE, A. M., T. L. HAZLETT, AND GOVINDJEE. 1995. Xanthophyll cycle-dependent quenching of photosystem II chlorophyll *a* fluorescence: formation of a quenching complex with a short fluorescence lifetime. Proceedings of the National Academy of Sciences of the United States of America 92:2273–2277.

GLUD, R. N., S. RYSGAARD, AND M. KÜHL. 2002. A laboratory study on O_2 dynamics and photosynthesis in ice algal communities: quantification by microsensors, O_2 exchange rates, ^{14}C incubations and a PAM fluorimeter. Aquatic Microbial Ecology 27:301–311.

GOLDSBOROUGH, L. G., R. L. MCDOUGAL, AND A. K. NORTH. 2005. Periphyton in freshwater lakes and wetlands. Pages 71–89 *in* M. E. Azim, M. C. J. Verdegem, A. A. van Dam, and M. C. M. Beveridge (editors). Periphyton:

ecology, exploitation, and management. CABI Publishing, Wallingford, UK.

HAMEED, H. A., S. KILINC, S. MCGOWAN, AND B. MOSS. 1999. Physiological tests and bioassays: aids or superfluities to the diagnosis of phytoplankton nutrient limitation? A comparative study in the broads and the meres of England. European Journal of Phycology 34:253–269.

HANCKE, T. B., K. HANCKE, G. JOHNSEN, AND E. SAKSHUAG. 2008. Rate of O_2 production derived from pulse-amplitude-modulated fluorescence: testing three biooptical approaches against measured O_2-production rate. Journal of Phycology 44:803–813.

HONEYWILL, C., D. M. PATTERSON, AND S. E. HAGERTHEY. 2002. Determination of microphytobenthic biomass using pulse-amplitude modulated minimum fluorescence. European Journal of Phycology 37:485–492.

JUNEAU, P., A. EL BERDEY, AND R. POPOVIC. 2001. PAM fluorometry in the determination of the sensitivity of *Chlorella vulgaris*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardtii* to copper. Environmental Contamination and Toxicology 42:155–164.

KARSTEN, U., I. KLIMANT, AND G. HOLST. 1996. A new in vivo fluorimetric technique to measure growth of adhering phototrophic microorganisms. Applied and Environmental Microbiology 62:237–243.

KIEFER, D. A., W. S. CHAMBERLIN, AND C. R. BOOTH. 1989. Natural fluorescence of chlorophyll *a*: relationship to photosynthesis and chlorophyll concentration in the western South Pacific gyre. Limnology and Oceanography 34:868–881.

KOLBER, Z., AND P. G. FALKOWSKI. 1993. Use of active fluorescence to estimate phytoplankton photosynthesis in situ. Limnology and Oceanography 38:1646–1665.

KROMKAMP, J. C., AND R. M. FORSTER. 2003. The use of variable fluorescence measurements in aquatic ecosystems: Differences between multiple and single turnover measuring protocols and suggested terminology. European Journal of Phycology 38:103–112.

KRUSKOPF, M., AND K. J. FLYNN. 2006. Chlorophyll content and fluorescence responses cannot be used to gauge reliably phytoplankton biomass, nutrient status or growth rate. New Phytologist 169:525–536.

LIND, O. T. 1985. Handbook of common methods in limnology. Kendall–Hunt, St. Louis, Missouri.

MAUZERALL, D. 1972. Light induced fluorescence changes in *Chlorella*, and the primary photoreactions for the production of oxygen. Proceedings of the National Academy of Sciences of the United States of America 69:1358–1362.

PARKHILL, J. P., G. MAILLET, AND J. J. CULLEN. 2001. Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. Journal of Phycology 37:517–529.

PETERSON, C. G., AND N. B. GRIMM. 1992. Temporal variation in enrichment effects during periphyton succession in a nitrogen-limited desert stream ecosystem. Journal of the North American Benthological Society 11:20–36.

SAMUELSSON, G., AND G. ÖQUIST. 1977. A method for studying photosynthetic capacities of unicellular algae based on in vivo chlorophyll fluorescence. Plant Physiology 40: 315–319.

SCHRIEBER, U., U. SCHLIWA, AND W. BILGER. 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with new type of modulation fluorimeter. Photosynthesis Research 10:51–62.

SEAL ANALYTICAL. 2005. EPA-approved methods for Aq-2 Discrete Analyzer. Seal Analytical, Fareham, UK. (Available from: http://www.seal-us.com/Products/AQ2DiscreteAnalyzer/tabid/54/Default.aspx)

SCOTT, J. T., R. D. DOYLE, AND C. T. FILSTRUP. 2005. Periphyton nutrient limitation and nitrogen fixation potential along a wetland nutrient-depletion gradient. Wetlands 25:439–448.

SEATON, G. G. R., AND D. A. WALKER. 1992. Validating chlorophyll fluorescence measures of efficiency: observations on fluorimetric estimation of photosynthesis rate. Proceedings of the Royal Society of London Series B: Biological Sciences 249:41–47.

SERÔDIO, J. 2004. Analysis of variable chlorophyll fluorescence in microphytobenthos assemblages: implications of the use of depth-integrated measurements. Aquatic Microbial Ecology 36:137–152.

SERÔDIO, J., J. MARQUES DA SILVA, AND F. CATARINO. 1997. Nondestructive tracing of migratory rhythms of intertidal microalgae using in vivo chlorophyll *a* fluorescence. Journal of Phycology 33:542–553.

SIMIS, S. G. H., Y. HUOT, M. BABIN, J. SEPPÄLÄ, AND L. METSAMAA. 2012. Optimization of variable fluorescence measurements of phytoplankton communities with cyanobacteria. Photosynthesis Research 112:13–30.

SUGGETT, D. J., K. OXBOROUGH, N. R. BAKER, H. L. MACINTYRE, T. M. KANA, AND R. J. GEIDER. 2003. Fast repetition rate and pulse amplitude modulated chlorophyll *a* fluorescence measurements for assessment of photosynthetic electron transport in marine phytoplankton. European Journal of Phycology 38:371–384.

SYLVAN, J. B., A. QUIGG, S. TOZZI, AND J. W. AMMERMAN. 2007. Eutrophication-induced phosphorous limitation in the Mississippi River plume: evidence from fast repetition rate fluorometry. Limnology and Oceanography 52:2679–2685.

VADEBONCOEUR, Y., AND D. M. LODGE. 1998. Dissolved inorganic carbon sources for epipelic algal production: sensitivity of primary production estimates to spatial and temporal distribution of ^{14}C. Limnology and Oceanography 43:1222–1226.

VADEBONCOEUR, Y., AND D. M. LODGE. 2000. Periphyton production on wood and sediment: substratum-specific response to laboratory and whole-lake nutrient manipulations. Journal of the North American Benthological Society 19: 68–81.

WALZ, H. 1998. Underwater fluorometer diving-PAM handbook of operations. D-91090. Walz, Effeltrich, Germany. (Available from: www.walz.com)

WOOD, M. D., AND R. L. OLIVER. 1995. Fluorescence transients in response to nutrient enrichment of nitrogen- and phosphorus-limited *Microcystis aeruginosa* cultures and natural phytoplankton populations: a measure of nutrient limitation. Australian Journal of Plant Physiology 22:331–340.

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