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Temporal variation in cyanobacteria species composition and photosynthetic activity in experimentally induced blooms

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Temporal variation in the photosynthetic activity of bloom-forming cyanobacteria was evaluated by the maximum quantum yield of photosystem II (F_v/F_m) using a 3′-(3,4-dichlorophenyl)-1′, 1′-dimethyl urea (DCMU) methodology. To evaluate the F_v/F_m of cyanobacteria in phytoplankton communities containing several algal groups, phycocyanin was directly excited at 590 nm, and *in vivo* fluorescence at 680 nm emission was measured before and after DCMU addition. Using a cultured *Microcystis* strain, we first tested the relevance of the method and found that F_v/F_m values measured under phycocyanin excitation approximately corresponded to those under chlorophyll *a* (chl *a*) excitation (440 nm). Second, we monitored temporal variations in F_v/F_m for induced cyanobacterial blooms in experimental ponds. Cyanobacterial F_v/F_m was usually lower than overall phytoplankton F_v/F_m measured under chl *a* excitation, suggesting that potential photosynthetic activity of bloom-forming cyanobacteria was lower than in other algal groups, such as green algae and diatoms. We also found that temporal variations in F_v/F_m explained the shift of dominant species from *Microcystis aeruginosa* to *Aphanizomenon issatschenkoi*. Our results consistently demonstrated that this method is simple and useful for evaluation of cyanobacterial potential photosynthetic activity in natural phytoplankton communities.

KEYWORDS: cyanobacterial bloom; DCMU [3′-(3,4-dichlorophenyl)-1′,1′-dimethyl urea]; maximum quantum yield; *Microcystis aeruginosa*

INTRODUCTION

Cyanobacterial blooms are an adverse effect of eutrophication in lakes and reservoirs. Blooms result in low transparency, odour release, the production of cyanotoxins and aquatic hypoxia during degradation (Pearl and Huisman, 2008). Most cyanobacterial species include toxic strains that produce hepatotoxins and neurotoxins, and potentially create serious problems for management of water resources (Codd *et al.*, 2005). The World Health Organisation established a guideline value of 1 μg L^{-1} in drinking water only for the major toxin microcystin-LR (Falconer *et al.*, 1999), although most toxins and their cumulative risks are still unclear. As water treatment engineering for toxin removal is technically difficult and economically costly (Vasconcelos and Pereira, 2001;

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Baptista and Vasconcelos, 2006), monitoring and study of potentially toxic cyanobacterial blooms remain important for reducing the risk of human exposure.

Quantification of chlorophyll *a* (chl *a*) concentrations and algal cell volumes has conventionally been used for accurate detection of bloom-forming algal biomass. Furthermore, for *in situ* real-time monitoring, *in vivo* fluorescence of chl *a* has been applied for simple and rapid evaluation of phytoplankton biomass. Several types of sensors and instruments have been commercially offered and used to study and monitor harmful algae. Since cyanobacteria uniquely have phycobilisomes as light-harvesting complexes for photosystem II (PS II), *in vivo* fluorescence of pigments such as phycoerythrin and phycocyanin in the phycobilisome has also been used as a specific indicator of their biomass (Lee *et al.*, 1994, 1995). This simple and sensitive method is now applied widely for ecological studies and monitoring of cyanobacterial blooms (Beutler *et al.*, 2002; Gregor *et al.*, 2005, 2007b; Izydorczyk *et al.*, 2005, 2009).

In terms of early detection and regulation of harmful algae, it is advisable to evaluate the growth potential or bloom-forming probability of *Microcystis* before any dense blooms appear. The maximum quantum yield of PS II (F_v/F_m) has commonly been used to assess direct factors of photosynthetic activity (Kolber *et al.*, 1988; Kim and Watanabe, 1994; Vaillancourt *et al.*, 2003; Yentsch *et al.*, 2004; Goto *et al.*, 2008). An instrument based on the spectrofluorometric technique has already been offered commercially and used for estimation of photosynthetic status in natural phytoplankton communities and for eco-physiological studies of algal photosynthesis (Körner and Nicklisch, 2002; Juneau *et al.*, 2003; Lippemeier *et al.*, 2003; Young and Beardall, 2003; Springer *et al.*, 2005; Gregor *et al.*, 2007a; Schmitt-Jansen and Altenburger, 2008). However, such instruments are expensive and the parameters are difficult to set for measurement. Moreover, these instruments usually underestimate cyanobacterial F_v/F_m, since dark adaptation of the samples cannot completely eliminate cyanobacterial non-photochemical quenching due to state transition (Campbell *et al.*, 1998).

In the present study, we aimed to establish a simple, low-cost method for evaluation of cyanobacterial F_v/F_m in natural phytoplankton communities that contain several algal groups such as green algae, diatoms and dinoflagellates. We first tried to combine the *in vivo* fluorescence method for quantification of cyanobacteria with the conventional 3′-(3,4-dichlorophenyl)-1′, 1′-dimethyl urea (DCMU) method for determining F_v/F_m. The F_v/F_m of cultured *Microcystis aeruginosa* was measured using different excitation and emission wavelengths and DCMU concentration combinations, and the validity of the method was assessed. Second, we monitored cyanobacterial F_v/F_m of cyanobacterial blooms induced in outdoor ponds. The consistency of variations in cyanobacterial F_v/F_m and the species composition of bloom-forming cyanobacteria are discussed.

METHOD

Variations in F_v/F_m at different excitation and emission wavelengths and DCMU concentrations

To evaluate the sensitivity and availability of F_v/F_m obtained at each excitation wavelength, we measured F_v/F_m of the *M. aeruginosa* NIES-843 strain with different DCMU concentrations. *Microcystis aeruginosa* was cultured in CT medium (Watanabe and Ichimura, 1977) at 25°C and 30 μmol m^{-2} s^{-1} photon flux density. To reduce the effects of non-photochemical quenching, except for state transitions, samples taken from a mass culture were dark-adapted for 30 min, and *in vivo* fluorescence was measured with a spectrofluorophotometer (FP-750, Jasco Co., Japan; 10-nm widths for excitation and emission) before and 30 s after the addition of DCMU to each sample.

In this experiment, we tried to measure F_v/F_m for excited phycocyanin. The absorption spectrum and fluorescence characteristics of phycocyanin, as well as the excitation and emission spectra of cyanobacteria, have been reported previously (e.g. Lee *et al.*, 1994; Ziegmann *et al.*, 2010). On the basis of previous studies, F_v/F_m was measured using the following three combinations of excitation and emission wavelengths: (i) excite chl *a* and detect fluorescence from chl *a* (excitation at 440 nm and emission at 680 nm), (ii) excite a shorter part of the absorption wavelength of phycocyanin and detect fluorescence from phycocyanin (excitation at 590 nm and emission at 645 nm) and (iii) excite a shorter part of the absorption wavelength of phycocyanin and detect fluorescence from chl *a* (excitation at 590 nm and emission at 680 nm). DCMU was added to adjust to final concentrations of 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 1 and 15 μM. The F_v/F_m for each excitation wavelength was obtained from the following equation:

$$\frac{F_v}{F_m} = \frac{(F_m - F_0)}{F_m},$$

where F_0 and F_m denote *in vivo* fluorescence before and after addition of DCMU, respectively. The relationship between DCMU concentration and maximum quantum yield at each excitation wavelength was analysed.

Experimental ponds and monitoring

We used two field ponds (ponds A and B) located at the Center for Ecological Research, Kyoto University, Japan (34°58′2.24″N, 135°57′38.93″E). Each square pond (10 m × 10 m) had a gradual slope, a maximum depth of 1.7 m, and a volume of 70 m^3. Those ponds were filled with underground water and then left uncontrolled prior to the experiment. The trophic status of the pond water was meso-eutrophic and affected by inputs of allochthonous organic matter such as terrestrial plant debris and/or water bird faeces.

To study various aspects of potentially harmful blooms using the experimental ponds, we induced cyanobacterial blooms. On 20 August 2009, ~3 m^3 of lake water was collected from Lake Biwa, Japan, where *M. aeruginosa* blooms were found, and concentrated using a 5-μm mesh plankton net. The concentrated sample was added to one pond (A) but not to the other one (B). We also added MA medium (Ichimura, 1979) to the two ponds, adjusted to 2% of the original concentration. Since the nitrogen-to-phosphorus (N:P) ratio (N:P = 6.1:1 molar ratio) of MA medium is low, the inorganic nitrate [$Ca(NO_3)_2 \bullet 4H_2O$, KNO_3, and $NaNO_3$] concentration was increased to produce three times the original concentration (final concentration, 118.6 μM for dissolved inorganic N, DIN; 6.5 μM for dissolved inorganic P, DIP). Inorganic N [$Ca(NO_3)_2 \bullet 4H_2O$, KNO_3, and $NaNO_3$] and P (KH_2PO_4) were added to the ponds every 2 weeks to final concentrations of 29.6 and 1.6 μM, respectively.

Pond water was sampled every 3 or 4 days. Two litres of surface water were collected and stored in the dark at nearly *in situ* water temperature conditions. Chemical and biological analyses of water samples were conducted within 2 h of sampling. Aliquots of water samples were filtered through pre-combusted Whatman GF/F filters (3 h at 420°C), and filters were stored frozen at −20°C until the analysis of chl *a*. The chl *a* concentration was determined following the method of Welschmeyer (Welschmeyer, 1994) using a spectrofluorophotometer (RF-5300 Shimadzu) after extraction with 10 mL of *N*,*N*-dimethylformamide.

For determining phytoplankton density, 100 mL water samples were preserved with 1% glutaraldehyde or Lugol solution to enumerate all species of cyanobacteria and Cryptomonaceae, which potentially have phycobili proteins. For *Microcystis*, the numbers of single cells and colonies in samples fixed with glutaraldehyde solution were enumerated with a Fuch-Rosenthal haemocytometer (Hirschmann, Germany). At least six fields were counted. Average cell numbers of *Microcystis* colonies were separately counted on a slide at ×400 magnification under a light microscope, and the total cell density was calculated from the cell and colony density and the average cell number of the colony. Trichomes of filamentous cyanobacteria and *Cryptomonas* cells of the samples fixed with Lugol's solution were enumerated using a Sedgwick-Rafter cell with an inverted microscope, and trichome and cell numbers of a sample of >1.2 mL were counted. Algal biovolumes were calculated from the density and average cell or trichome volume. Cell volumes were estimated by approximation to the nearest simple geometric solid after measurement of at least 20 cells or trichomes (Hillebrand *et al.*, 1999).

Spectrofluorometric detection of phytoplankton biomass and photosynthetic activity

To reduce the effects of non-photochemical quenching, samples collected from the ponds were dark-adapted for >30 min in a thermal container. For separate evaluation of total phytoplankton and cyanobacterial biomass, *in vivo* fluorescence (F_0) was measured at 440 and 590 nm excitation wavelengths following Gregor *et al.* (2007b). The emission wavelength was fixed at 680 nm for chl *a*. After measurements of *in vivo* fluorescence, maximum fluorescence yield (F_m) of the sample was measured 30 s after the addition of DCMU (final concentration 15 μM) for each excitation wavelength, and F_v/F_m for each excitation wavelength was calculated.

RESULTS

Variations in F_v/F_m with different excitation and emission pigment combinations

The F_v/F_m values determined at the 680 nm emission wavelength increased consistently with DCMU concentration when chl *a* (440 nm) or phycocyanin (590 nm) was excited. Both excitation wavelengths showed similar F_v/F_m values at the same DCMU concentration (Fig. 1). On the other hand, the *in vivo* fluorescence of phycocyanin determined at 645 nm hardly increased at varying DCMU concentrations when phycocyanin was directly excited at 590 nm. Only 0.7% of the *in vivo* fluorescence of phycocyanin increased after addition of 15 μM DCMU.

Phytoplankton biomass and *Microcystis* abundance

In both ponds, chl *a* concentrations increased during the first week after addition of MA medium and then

decreased until Day 11 (Fig. 2A). Concentrations of chl *a* oscillated with the addition of inorganic nutrients.

A cyanobacterial bloom was observed in pond A, to which condensed Lake Biwa water had been added (Fig. 2B). In this pond, *Cryptomonas* spp. initially dominated. *Microcystis* cells were first detected on Day 21, with a density of $2.0 \times 10^5\ \mu m^3\ mL^{-1}$. *Microcystis* cell density reached a maximum ($4.3 \times 10^6\ \mu m^3\ mL^{-1}$) on Day 63, rapidly decreased by Day 70 and remained low ($<8.0 \times 10^5\ \mu m^3\ mL^{-1}$) thereafter. *Aphanizomenon issatschenkoi* also increased and dominated after the *Microcystis* bloom. In pond B, some species of Oscillatoriaceae (*Phormidium* spp. and *Oscillatoria* spp.) and *Cryptomonas* spp. dominated until Day 70 (Fig. 2C).

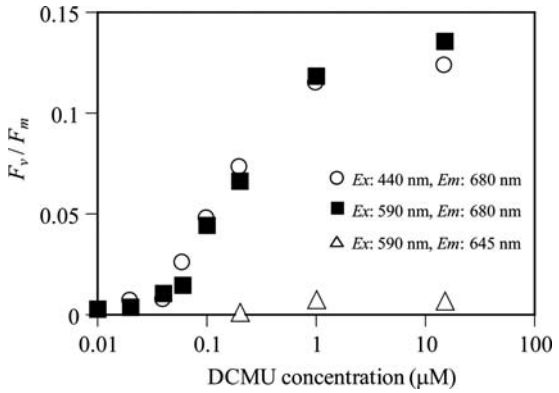

Fig. 1. Variations in maximum quantum yield of photosystem II (F_v/F_m) under different excitation and emission wavelengths and DCMU concentrations.

Fluorometric detection of cyanobacterial biomass and F_v/F_m

In vivo fluorescence at excitation wavelengths of 440 and 590 nm indicated total phytoplankton and cyanobacterial biomass, respectively (Fig. 3). Variations in *in vivo* fluorescence at 440 nm excitation were almost coincident and significantly correlated with chl *a* concentrations in both ponds [pond A, chl *a* ($\mu g\ L^{-1}$) $= 12.6x + 32.0$, $r^2 = 0.714$, $P < 0.001$; pond B, chl *a* ($\mu g\ L^{-1}$) $= 9.6x + 26.9$, $r^2 = 0.800$, $P < 0.001$]. *In vivo* fluorescence at 590 nm excitation was also significantly correlated with chl *a* concentrations [chl *a* ($\mu g\ L^{-1}$) $= 23.2x + 5.18$, $r^2 = 0.405$, $P < 0.01$; pond B, chl *a* ($\mu g\ L^{-1}$) $= 23.5x + 33.1$, $r^2 = 0.472$, $P < 0.01$], although determinant coefficients at 590 nm excitation were lower than those at 440 nm excitation in both ponds.

In pond A, variations in F_v/F_m were different among the excitation wavelengths, and F_v/F_m values at 440 nm were higher than those at 590 nm. At 590 nm, F_v/F_m values were relatively stable, at ~0.27 ($\pm$0.04 SD) until Day 60 (Fig. 4A); they then decreased to 0.06 between Days 63 and 76 and again increased after Day 81. The minimum F_v/F_m value was detected when the dominant species shifted from *M. aeruginosa* to *A. issatschenkoi* (Fig. 2B). However, F_v/F_m at 440 nm remained relatively high until Day 18 (0.43 ± 0.05) and then fluctuated between 0.20 and 0.40 after Day 21. In addition, changes in F_v/F_m at 440 nm seemed to be independent of nutrient addition.

In pond B, F_v/F_m at 440 nm was also higher than at 590 nm (Fig. 4B). However, unlike F_v/F_m at 440 or

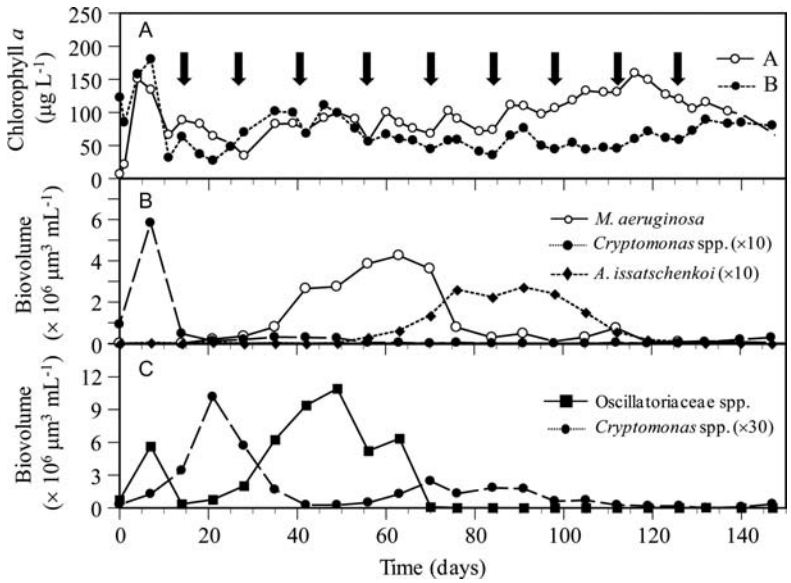

Fig. 2. Temporal variations in chlorophyll *a* (chl *a*) concentration (**A**) and species composition in pond A (**B**) and pond B (**C**). Arrows indicate the date of inorganic nutrient additions.

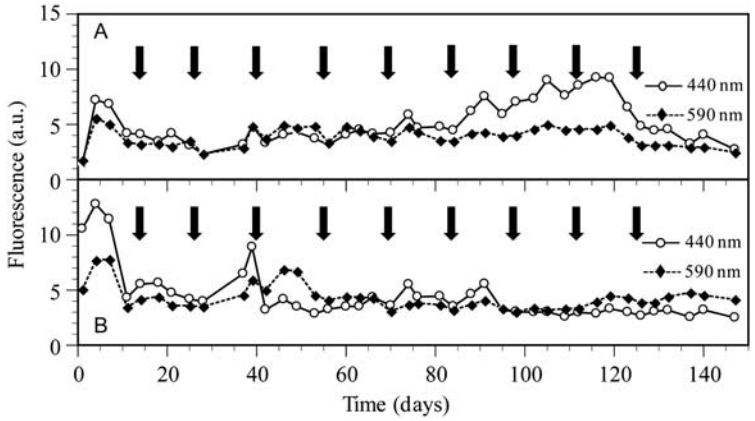

Fig. 3. Temporal variations in *in vivo* fluorescence at 440 and 590 nm excitation wavelengths in pond A (**A**) and pond B (**B**). Arrows indicate the date of inorganic nutrient additions.

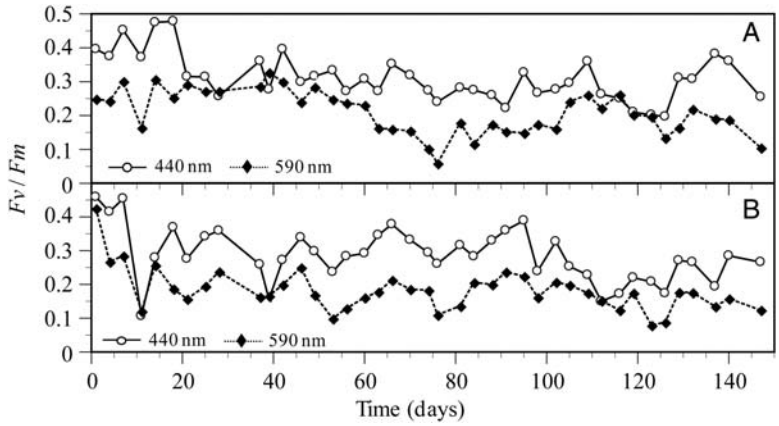

Fig. 4. Temporal variations in maximum quantum yield of photosystem II (F_v/F_m) at 440 and 590 nm excitation wavelengths in pond A (**A**) and pond B (**B**).

590 nm in pond A, the variations in pond B showed similar oscillations. In addition, F_v/F_m at 440 or 590 nm seemed to be affected by nutrient addition. However, we could not find any clear relationship between variations in F_v/F_m and succession in the cyanobacterial community.

DISCUSSION

Application of F_v/F_m under phycocyanin excitation

In the present study, phycocyanin fluorescence did not increase with DCMU addition. This result supports that of a previous study (Lee *et al.*, 1994), indicating that excess energy emitted from phycocyanin is negligible even when electron transfer from PS II to PS I is inhibited by DCMU. However, F_v/F_m values of *M. aeruginosa* determined at 680 nm increased with DCMU concentrations and did not change, irrespective of which of the two excitation wavelengths was used. This indicates that we can determine F_v/F_m specifically for cyanobacteria through measurement of fluorescence spectra under phycocyanin excitation, as determined in studies of the photosynthetic electron transport system (Mullineaux and Allen, 1990; McConnell *et al.*, 2002).

Several types of instruments for measuring photosynthetic activity are already commercially available. State transition causes underestimation of cyanobacterial F_v/F_m without DCMU addition (Campbell *et al.*, 1998), although instruments based on this approach provide a number of useful photosynthetic parameters. The state transition is a function that distributes energy captured by phycobilisomes between PS II and PS I, and the transition from state I (excitation energy largely allocated to PS II) to state II (most excitation energy reallocated to PS I) is determined by the degree of reduction in the

plastoquinone pool (Campbell *et al.*, 1998). As cyanobacteria are prokaryotes, the electron transfer systems for photosynthesis and respiration are located on the same thylakoid membrane, sharing numerous electron transport intermediates (Mullineaux and Allen, 1986, 1990). Thus reduction in a substantial portion of the plastoquinone pool usually occurs due to respiration, which induces a state transition, even in dark conditions (Mullineaux and Allen, 1986, 1990). However, addition of DCMU completely suppresses the state transition (Mullineaux and Allen, 1986), and the DCMU methodology is clearly more applicable for measurement of cyanobacterial F_v/F_m.

Temporal variations in species composition of cyanobacteria and their F_v/F_m

We successfully induced cyanobacterial blooms with addition of nutrients and lake plankton, and observed succession from *M. aeruginosa* and *A. issatschenkoi*. Because we started the experiment in late August, water temperature was highest at the onset of the experiment and successively decreased during the experiment (data not shown). As *A. issatschenkoi* is known to dominate eutrophic lakes from summer to winter (Watanabe, 1985, 1991), the change in dominant cyanobacteria detected in the present study may be common in seasonal successions of phytoplankton in lakes. Moreover, the rise and fall of *M. aeruginosa* populations and the change in dominant species to *A. issatschenkoi* in pond A corresponded with variations in F_v/F_m under phycocyanin excitation, and F_v/F_m reached a minimum value when the dominant species changed from *M. aeruginosa* to *A. issatschenkoi* (Fig. 4A). As F_v/F_m for algae rapidly decreases during the transition from the stationary growth phase to the senescent phase (Parkhill *et al.*, 2001), this decline in F_v/F_m under phycocyanin excitation would be the result of senescent *M. aeruginosa*. In contrast, variation in F_v/F_m under chl *a* excitation seemed to be independent of variation in cyanobacterial species composition and F_v/F_m under phycocyanin excitation (Fig. 4A). This result also demonstrated that we could determine F_v/F_m that is specific to cyanobacteria in samples containing several algal groups.

Different responses of F_v/F_m to repeated nutrient addition were also observed between the two ponds. Changes in F_v/F_m at 440 nm seemed to be affected by repeated nutrient addition in pond B but not in pond A (Fig. 4). Since we evaluated the species composition of the phytoplankton community only with respect to cyanobacteria and Cryptomonaceae, no information is available for other taxonomic groups, such as diatoms and green algae. However, as all species have different nutrient uptake properties (e.g. Olsen *et al.*, 1989; Watanabe and Miyazaki, 1996; Lampert and Sommer, 1997), the difference in the F_v/F_m response at 440 nm would be caused by differences in species composition between the ponds. Particularly in pond A, variation in F_v/F_m for bloom forming cyanobacteria was stable and independent of nutrient addition (Fig. 4A), which would also contribute to stable variation in F_v/F_m for total phytoplankton.

In the present study, we successfully developed a simple and low-cost method for measuring cyanobacterial F_v/F_m in a natural phytoplankton community. On the other hand, particularly in aquatic ecology, F_v/F_m has also been used as an indirect indicator of the nutrient status and relative growth rate of phytoplankton communities. However, some studies have disputed the use of F_v/F_m as an indicator of nutrient status and growth rate. Parkhill *et al.* (2001) suggested that F_v/F_m is not a good indicator of nutrient limitation under balanced growth conditions and found that F_v/F_m decreased under extremely nutrient-starved conditions. Kruskopf and Flynn (Kruskopf and Flynn, 2006) conducted nitrogen-limited culture experiments and pointed out that variations in F_v/F_m showed no consistent pattern with growth rate and the cellular carbon-to-nitrogen ratio. Further work is needed to discern its limitations as an indicator of nutrient status and relative growth rate in phytoplankton communities.

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