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Temporal characteristics of chlorophyll fluorescence quenching and dissolved oxygen concentration as indicators of photosynthetic activity in *Chlorella emersonii*

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Abstract

A simple chlorophyll fluorescence (CF) measuring system has been implemented to study temporal characteristics of chlorophyll fluorescence induction (CFI) in dark-adapted freshwater algal cultures of *Chlorella emersonii*. There were two different decay time constants describing the CF quenching: τ_0 (the faster) and τ_1 (the slower) with amplitudes A_0 and A_1, respectively. The relative amplitude of the faster quenching component decreased once the sample was subject to deprivation from dissolved oxygen (DO). The DO concentration of samples was monitored to validate the effects of deprivation from air contact for up to 7 d and to the effect of adding DCMU to the culture (herbicide for blocking electron transport of photosystem 2). CFI analysis and DO measurements showed that the relative amplitude of A_0 to $(A_0 + A_1)$ and the DO concentration can be used as an indication of relative photosynthetic activity, thus allowing for the possibility to classify the physiological state of algal blooms into active and inactive states.

Additional key words: algal blooms; DCMU; non-photochemical quenching; photosystem 2; zero oxygen tablets.

Introduction

Photon energy absorbed by chlorophyll (Chl) molecules can undergo one of three paths: it can be used to drive photosynthesis, excess energy can be dissipated as heat, or it can be re-emitted in the form of Chl fluorescence (Kitajima and Butler 1975, Butler 1977). Although the total Chl fluorescence (CF) is very small, measurement and detection methods are very straightforward. In recent years, many pulse amplitude modulated (PAM) fluorimeters have been developed, and numerous aspects of photosynthesis have been investigated under different field conditions using these devices (Goh *et al*. 1999, Smith 2002, Hendrickson *et al*. 2005, Kaiblinger and Dokulil 2006).

We investigated a relationship between CF induction (CFI) decay time characteristics and corresponding dissolved oxygen (DO) concentration in algal suspensions of *Chlorella emersonii*. The commercial PAM fluorimeters use at least three synchronised light sources to accomplish this task. In this work, a simple fluorimeter with a single modulated radiation source was introduced to measure CF decay. A simple characterisation of CF decay provides a prediction of the environmental and physiological state of the algae.

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Abbreviations: A_0, A_1 – the relative fast and slow amplitudes of chlorophyll fluorescence decay; AD630 – balanced modulator analogue device lock-in amplifier; B_0, B_1 – the inverse of τ_0 and τ_1, respectively; CF – chlorophyll fluorescence; CFI – chlorophyll fluorescence induction; Chl – chlorophyll; DO – dissolved oxygen; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_m – maximal fluorescence yield; F_t – fluorescence quenching steady state; LED – light emitting diode; NPQ – non-photochemical quenching; PAM – pulse amplitude fluorometry; PQ – photochemical quenching; PS – photosystem; α – the total amplitudes of the fast and the slow fluorescence quenching; β – the amplitude ratio coefficient of the fast to the total quenching (A_0/α); τ_0, τ_1 – the fast and slow chlorophyll fluorescence decay time constants; ZOT – Zero Oxygen Tablet.

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Materials and methods

Culture of algae: A fresh water green strain, *Ch. emersonii*, was cultured in the laboratory. The culture medium was prepared from distilled water and nutrient minerals (Acreman 1994). The flask was sealed with a foam cover to allow respiration and placed under bright fluorescent lamps for 18 h a day. After 3–4 d, the culture was investigated.

Pulse amplitude modulated (PAM) instrument (Fig. 1) consists of photodiode, lock-in amplifier, and a data acquisition system (using an 8-bit A/D USB converter). Modulating irradiance was obtained from blue LED's (λ_{max} = 450 nm) of about 1 600–2 000 μmol m^{-2} s^{-1}. Its intensity was sufficient to reach maximum CF by exciting all photosystem reaction centres. The LED's array circuit was driven by a square wave generator. The oscillation frequency of the generated pulses was adjusted to 460 Hz. Measurement chamber was a simple glass cylindrical cuvette of 22 cm length and 3 cm diameter with the LEDs mounted at the final 2 cm of the cuvette. Photo-detection system consisted of an optical band-pass interference filter of type *Melles Griot* (*03FIV325*, New York, USA) of 92 % transmission that was inserted at the input of the photo-detection system to avoid contamination by the excitation radiation while allowing capture of the peak of fluorescence emission around 680±10 nm. The silicon photodiode detector was based on a large active area (1 cm^2) followed by a high gain op-amp. The signal was then processed and filtered through an *AD630* modulator/demodulator configured as a lock-in amplifier (*Analog Devices*, Ireland). Interfacing was carried out *via* an A/D converter using the *USB-1208LS* (*Measurement Computing Co.*, Norton, USA).

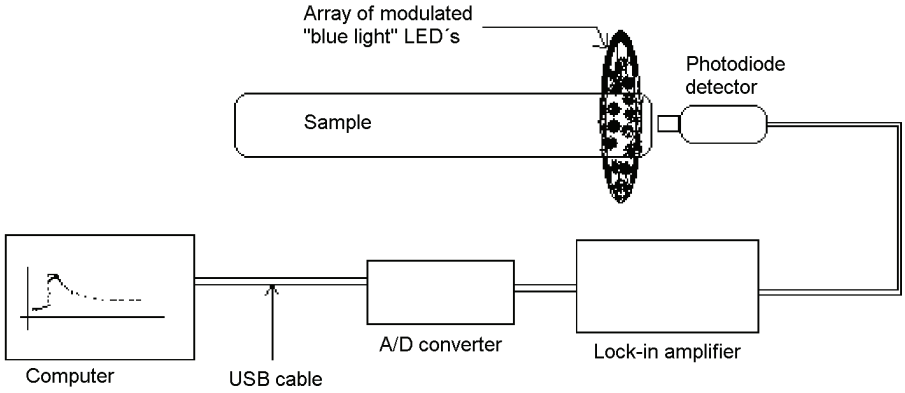

Fig. 1. Schematic representation of the developed fluorimeter set-up used for CF measurements.

CF and DO measurements: One algae culture was divided into four sub-samples, A, B, C, and D, each subjected to different physiological conditions: Sample A was sealed and kept in the dark to prevent photosynthesis from taking place and additional oxygen from entering the sample (A2, sealed and kept in the dark for 2 d; A7, sealed and kept in the dark for 7 d). Sample B was sealed also to prevent air contact and kept under bright ambient continuous irradiation [B7, for 7 d; B4, for 4 d; B4+1, sub-sample B4 sealed and dark-adapted for 1 d to re-activate photosystem 2 (PS2) reaction centres]. Sample C was maintained under the same conditions as sample B, but its DO concentration was brought down sharply to a minimum by adding a zero oxygen tablet (ZOT) to the sample (*Mettler Toledo Co.*, Dublin, Ireland): C1, sample C+ZOT (after 1 h); C2, sub-sample C1 sealed and dark-adapted for 3 d. Sample D was used to study the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (an inhibitor of electron transport at the PS2 site) (Calvayrac *et al*. 1979) on CF quenching.

All samples were kept at room temperature and monitored accordingly. Before taking any CF measurement, the sample under study was first dark adapted for 20 min to allow any photosynthetic activity to settle down. Then the sample was irradiated with the modulated radiation and fluorescence measured for about 2 min until a near steady state of the quenching process is observed. In these conditions, the fluorescence signals are well described by the Kautsky kinetics as shown in Fig. 2 (Bulgarea and Boukadoum 2001).

At first glance each fluorescence time sequence appears to be well represented by an exponential function in the form of:

$$F(t) = F_m - A\,[1 - \exp(B\,t)] \quad (1)$$

However, fitting algorithms failed to fit such a function accurately to the fluorescence sequences in Fig. 2. A function which provided a good fit in all cases using two time decay components provides a realistic fit as shown in Fig. 2 for the control samples. This function is given by

$$F(t) = F_m - A_0\,[1 - \exp(B_0\,t)] - A_1\,[1 - \exp(B_1\,t)] \quad (2)$$

where F_m is the maximum CF, A_0 and A_1 are the amplitudes of the fast and slow exponential quenching terms, B_0 (= $1/\tau_0$) and B_1 (= $1/\tau_1$) are the corresponding time constants to be estimated by the fitting of Eq. 2 for each sample, and t is the time. The DO concentration was measured using a portable DO meter, before taking the fluorescence measurements (*HQ10* Portable LDO™ Dissolved Oxygen Meter, *HACH*, *Hach LANGE*, Dublin, Ireland).

Results

Fig. 2 shows the mean of a set of typical measurements of CFI time sequences of six newly cultured fresh samples of *Ch. emersonii*, which were dark-adapted for 20 min prior to the measurement. Upon the application of modulated radiation, CF rose to a maximum value F_m followed by a quenching process, which reduced fluorescence to an eventual steady state value F_t. At this latter point the PS2 centre is assumed to be fully closed.

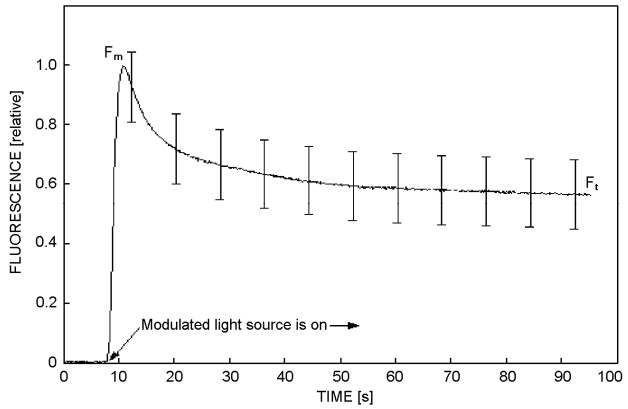

Fig. 2. Typical mean record of chlorophyll fluorescence induction decay of six new-made fresh samples of *Chlorella emersonii* after being dark-adapted for 20 min, obtained using the new designed fluorimeter. F_m and F_t are the normalised maximum and steady state fluorescence, respectively (Table 1). The measurements were conducted under the same room temperature. The standard deviation indicated as bars on F_m and F_t.

The concepts of NPQ and PQ drive the time dependence of CFI and are derived mainly from PS2 (Fig. 2). It reflects a lowering of CF below maximal levels through photochemical competition with fluorescence emission. When all PS2 centres are closed and no photochemistry can occur, photochemical quenching is zero and the fluorescence remains near maximal. When all PS2 reaction centres are open and the potential for photochemistry is maximal, photochemical quenching of fluorescence is also maximal: in this case fluorescence yield decreases from F_m to its asymptotic level F_t. NPQ is induced when the photosynthetic system cannot use the total absorbed photon energy for photochemistry. This can occur at low irradiances even under optimum conditions for photosynthesis.

For the control set of measurements shown in Fig. 2 the values of the various fitted parameters and their standard errors, after normalising CF data for $F_m = 1$, are: $F_t = 0.527\pm0.079$, $A_0 = 0.285\pm0.081$, $A_1 = 0.188\pm0.052$, $B_0 = -0.282\pm0.109\ s^{-1}$, $B_1 = -0.038\pm0.014\ s^{-1}$, $\tau_0 = 4\pm1$ s, and $\tau_1 = 26\pm3$ s. The corresponding mean DO concentration for these samples was $8.620\pm0.202\ g\ m^{-3}$. For these healthy control samples the broad conclusion is that an initial faster quenching process dominates with $\tau_0 \approx \tau_1/6$ s, $A_0 \approx 2\ A_1$.

In the experiments reported in Fig. 3, the parameters extracted from fitting the model of Eq. 2 to normalised CF data for samples A to D as described above are shown in Table 1. It demonstrates the effect of reducing DO concentration in freshwater *Ch. emersonii* by the separate physiological conditions on the CFI model parameters. In these experiments, all the samples were kept for 20 min at 22 °C in darkness prior to measurements. Two additional parameters derived from the function parameters are also useful in describing the results. These are total quenching amplitude which is described by α, where $\alpha = (A_0 + A_1)$ and the relative amplitude of the faster quenching is described by β, where β is the ratio: A_0/α. For the control samples $\beta = 0.597\pm0.117$ or the fast quenching represented somewhat more than half the total quenching amplitude. For sample A, for which the photosynthesis process was effectively suspended, the important conclusion here is that as long as the DO concentration

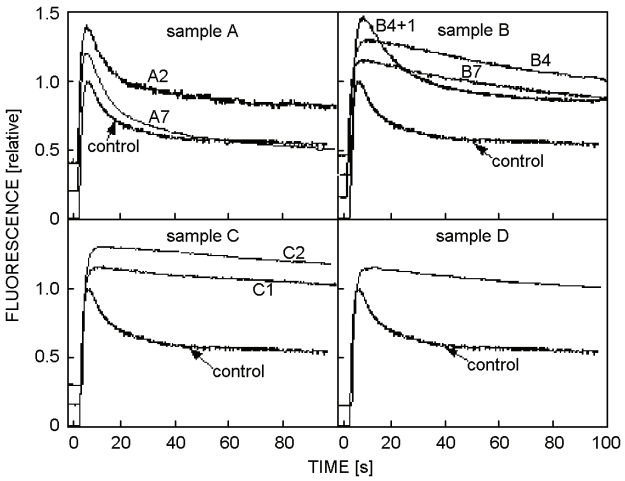

Fig. 3. Profiles of normalised chlorophyll fluorescence kinetics of the four samples: A, B, C, and D (multiple sample kinetics curves are stepped upwards for clarity). The different curves in each figure represent the result when a dark-adapted sample was excited with a strong "blue modulated light". The temperature was ≈ 22 °C. For sample description see the text.

Table 1. The concentration of dissolved oxygen (DO) [g m^{-3}] and the measured normalised chlorophyll fluorescence (CF) parameters F_t, A_0, A_1, τ_0, τ_1, α, and β from Eq. 1, represent: steady state CF, amplitude of the fast CF, amplitude of the slow CF, the fast CF decay time constant, the slow CF decay time constant, the fast and slow CF amplitudes, and the amplitude ratio coefficient, respectively. $F_m = 1$ in all variants. ND – not determined (because of the risk of damaging the DO sensor).

Sample	Sub-sample	F_t	A_0	B_0 [s^{-1}] (τ_0) [s]	A_1	B_1 [s^{-1}] (τ_1) [s]	$\alpha = A_0 + A_1$	$\beta = A_0/\alpha$	DO
Control		0.527	0.285	–0.282 (4)	0.188	–0.038 (26)	0.473	0.597	8.60
A	A2	0.303	0.499	–0.133 (8)	0.315	–0.018 (56)	0.814	0.613	8.86
	A7	0.414	0.410	–0.153 (7)	0.244	–0.021 (48)	0.654	0.627	8.97
B	B7	0.726	0.061	–0.597 (2)	0.450	–0.012 (84)	0.511	0.119	7.63
	B4	0.713	0.089	–0.074 (14)	0.470	–0.017 (58)	0.559	0.159	7.62
	B4+1	0.404	0.521	–0.081 (12)	0.154	–0.016 (62)	0.675	0.772	9.38
C	C1	0.874	0.025	–0.069 (15)	0.317	–0.004 (230)	0.342	0.073	3.30
	C2	0.874	0.012	–0.444 (2)	0.644	–0.003 (362)	0.656	0.018	4.30
D	D	0.772	–6.28	–0.012 (86)	6.517	–0.012 (86)	0.229	–27.458	ND

remains constant and the sample is not exposed to any additional stress, the algae behave after 2 and 7 d in a manner which is not significantly different to the control sample.

In contrast to sample A, sample B was exposed to continuous irradiation. Since continuous photosynthesis activity took place in sample B, the DO concentration in the sample was decreased from above 8.60 g m^{-3} to 7.62 g m^{-3}. Under these conditions and as shown in Fig. 3 (B7 and B4), the faster quenching process apparent in the control and sample A was no longer significant. This is indicated by the decrease from $\beta \approx 0.6$ to $\beta \approx 0.1$. This decrease in the relative amplitude of the faster CF quenching indicates the stress on the photosynthetic activity of the sample due to the reduction in the DO concentration while irradiated.

In order to investigate the influence of irradiation in inducing the stress effect seen in sample B4, this sample was resealed and placed in the dark for a further day to see if the stress effects were reversible. A complete recovery of photosynthetic activity to near control values was evident in sample B4+1. A recovery of DO level from 7.62 to 9.38 g m^{-3} was also found. Although this result is not new, it demonstrates the link between β and DO concentration and stress on the photosystem. A standard CO_2 test showing no trace of CO_2 in sample B was carried out to eliminate the option of a CO_2 effect on these results.

The effect of removing oxygen from samples C was examined by using ZOT. The tablets were introduced in order to eliminate DO from the sample without directly destroying the anatomy of the algae so as to demonstrate that lack of sufficient DO in a sample can radically affect the PS2 activity. Fig. 3, curves C1 and C2 and the corresponding entries in Table 1, particularly the values for β, show how the faster component of quenching was removed. This indicates that the electron transport process at the PS2 site was deactivated or blocked.

To test this latter hypothesis experimentally, sample D was treated with the herbicide DCMU that blocks electron transport on the acceptor side of PS2. In the presence of DCMU, the kinetics of fluorescence quenching, particularly the loss of the faster component was evident. The flow of electrons to oxygen responds rapidly to environmental and metabolic conditions and can be an important element in preventing the PS2 process. These results suggest that reducing the DO level in the sample can damage and/or inhibit PS2 and that this is again effectively indicated by the absence of the fast CF quenching. In this case exponential quenching terms are absent and the model fit is essentially linear.

Discussion

Altering the state of PS2 centres under continuous irradiation without air contact, by reducing oxygen or by applying DCMU, lead to a loss of PS2 centre activity. This can be sensed by the coefficient β, the relative amplitude of the faster CF quenching.

Our measurements of modulated CF in *Ch. emersonii* were a useful and informative indicator for characterising its physiological activity. DO concentrations in the medium together with the relative amplitude ratio of the faster component of CFI quenching $\beta = A_0/(A_0 + A_1)$, give effective indication of the photosynthesis activity of an active algal culture. The variations of both parameters β and the DO concentration of a freshwater sample of *Ch. emersonii* reinforced the case for using β as a prime indicator of photosynthetic activity.

A proposed classification scheme for describing the health of the alga based on DO and β is as follows: Healthy *Ch. emersonii* population would be characterised by $\beta \geq 0.6$ and DO≥8.6 g m^{-3}. Inactive or moderately stressed alga population can have a slightly lower DO value and similar or somewhat smaller than the healthy sample: DO ≈ 7–8 g m^{-3} and $0.1 \leq \beta \leq 0.5$. A large decrease

in DO and/or β values to those of the order of DO ≈ 4 g m^{-3} and β<0.1 can be used to identify dead or very stressed *Ch. emersonii* populations.

This work arose from a requirement to develop a measuring system to determine bloom photosynthetic status for *in-vivo* national and international monitoring programmes. Continued bloom growth depends on available nutrients, irradiance, and photosynthetic capability. An inactive bloom, perhaps nutrient deficient, may not grow further, but could settle with its trapped nutrients and affect the benthic environment. The proposed parameter β can be easily obtained *in-vivo* from analysis of the CF quenching during transition from a dark-adapted to a light-adapted state determined for any algal bloom with Chl content >60 mg m^{-3} using relatively simple apparatus. Being derived by fitting a time dependent function to a normalised time series, β can be determined without any need for absolute amplitude determination or intensity calibration.

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