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Review

The use of variable fluorescence measurements in aquatic ecosystems: differences between multiple and single turnover measuring protocols and suggested terminology

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In this review, we briefly describe the two main techniques used to measure variable fluorescence in the aquatic environment, and show how the parameters derived from this technique can be used to estimate the rate of photosynthesis. The methods estimate the photochemical efficiency of photosystem II from ratios of fluorescence levels. Flashes of light that are transiently saturating for photochemistry (i.e. they are sufficiently bright to close all PSII reaction centres) are used to obtain the maximum fluorescence level. The type of saturating flash differs between methods. In one approach, single turnover (ST) flashes are applied. This allows only one charge separation during the flash and reduces only the primary acceptor of PS II, raising fluorescence to a level $F_{m(ST)}$. In a second approach the flashes are multiple turnover (MT), which allow repeated charge separation processes until all electron acceptors of PS II are reduced. A relaxation of quenching is induced by the longer flash, and this raises the maximum fluorescence to a higher level, $F_{m(MT)}$. Application of the different approaches to an algal sample will result in differing F_m values and, as a result, different values for the photochemical efficiency of PS II, with the MT method giving higher values than ST. Several designs of equipment, based on MT or ST techniques, are available for use with phytoplankton or benthic algae. Both techniques measure variable fluorescence, but there are a number of important differences in the methods used to calculate photosynthetic rates. In our view, this necessitates the use of a different terminology in order to avoid confusion, until the underlying physiological differences are resolved. An example is given showing that combining terminology from the different approaches will result in calculation of erroneous photosynthetic electron transport rates.

Key words: absorption, fluorescence, PAM, FRRF, photosynthesis, photosynthetic electron transport, variable fluorescence, optical cross-section, functional cross-section

Introduction

Understanding the function of aquatic ecosystems requires knowledge of carbon cycles, which in turn requires a detailed knowledge of biological CO_2 fixation (Geider *et al.*, 2001). The process of gross photosynthetic carbon fixation entails the absorption of light energy by antenna pigments, followed by the process of charge separation, which produces reducing equivalents ($NADPH_2$). These are used together with photosynthetically-generated ATP to fuel the CO_2-fixing enzymatic reactions of the Calvin cycle. There are many techniques available to measure photosynthesis or primary production, and the two terms are often used interchangeably. A discussion of the different photosynthesis and production terms is outside the scope of this review; the reader is referred to Williams (1993), Platt & Sathyendranath (1993) and Sakshaug *et al.* (1997) for more information. Here, we define photosynthesis as gross carbon fixation or gross oxygen evolution.

Assessment of photoautotrophic biomass has been greatly improved since the introduction of aircraft- and satellite-mounted remote sensing equipment. Mesoscale estimates of aquatic primary production could be further improved if rates of photosynthesis could also be measured synoptically across large areas, but this would be prohibitively expensive using traditional, gas-exchange based measurements. Hence, there is a great deal of interest in alternative, optical techniques, especially chlorophyll fluorescence, which could enable measurements of photosynthesis at higher temporal and spatial resolutions.

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Correlations between the rates of photosynthesis of algal cultures, and the increase in red chlorophyll fluorescence caused by the photosystem II (PS II) inhibitor DCMU were first observed by Samuelsson & Öquist (1977). A fluorescence response index based on DCMU-enhanced fluorescence was proposed by Cullen & Renger (1979) as a proxy for the photosynthetic rates of phytoplankton. The variable component of fluorescence, measured before and after addition of DCMU, showed a close coupling with radiocarbon-based estimates of photosynthesis for freshwater phytoplankton samples incubated both *in situ* and at the surface (Vincent *et al*., 1984).

Attention has turned recently to 'active' fluorescence methods (Kolber & Falkowski, 1993), which have the advantage of being deployable *in situ* and delivering results in real time. The use of active fluorescence involves two basically similar, but significantly different ways, of measuring the variable part of chlorophyll fluorescence that were developed during the mid 1980s (Falkowski *et al*., 1986; Schreiber *et al*., 1986). The two protocols have been incorporated in various ways in the designs of commercially available fluorometers such as the PAM (Walz GmbH, Effeltrich, Germany), FMS (Hansatech Ltd, King's Lynn, England), AFM (Opti-Sciences, Tyngsboro, USA), Fast Repetition Rate Fluorometer (FRRF; Chelsea Instruments Ltd., West Molesey, England), the PSI (Photon Systems Instruments, Brno, Czech Rep) and Algae Online Analyzer (bbe Moldaenke GmbH, Kiel, Germany).

Instrumental development has increased the use of variable fluorescence as a research tool. Unfortunately, the terminology used in papers describing fluorescence results is not always clear and consistent. During a meeting in Bangor, UK (2002), celebrating the 50th anniversary of the invention of the ^{14}C-technique (Steeman-Nielsen, 1952), we noticed that the terminology originally developed for the two different fluorescence techniques was now being used interchangeably because of the similarities between the approaches. However, we feel that this may cause confusion and is not justified by current physiological evidence. Therefore, we will briefly describe the two approaches and highlight their differences, and suggest using separate terminologies until the underlying physiological differences between the methods have been resolved.

Fluorescence parameters

F_o, F, F_m

Chlorophyll fluorescence (mainly originating from PSII; Krause & Weis, 1991) is often used as a proxy for chlorophyll concentrations, and many ships are equipped with a fluorometer for this purpose. However, the fluorescence emission per unit chlorophyll is not constant, and varies in relation primarily to the rate of photosynthesis, but also in response to other factors such as prior exposure to excess irradiance. When the light source used by the fluorometer is sufficiently low not to drive photosynthesis, and in the absence of solar irradiance, a low level of fluorescence emanating from the pigment bed is measured. In the dark condition, the primary electron acceptor Q_A is oxidized and is a strong quencher of fluorescence. The fluorescence emission under these conditions is termed F_o. Upon illumination of algal cells, Q_A becomes increasingly reduced and as a result the fluorescence will rise to an intermediate level F, depending on the irradiance used (see Table 1 for explanation of the abbreviations used).

When a dark-adapted sample is exposed to a high-energy single turnover (ST) flash, i.e. an amount of light short enough and just sufficient to cause a single reduction of all the primary acceptor, Q_A, the fluorescence rises from F_o to a maximum fluorescence level. This point has been variously called I_1 or J by previous authors (Strasser *et al*., 1995; Schreiber *et al*., 1995b), and will be termed $F_{m(ST)}$ for the purposes of this review. A longer, multiple turnover (MT) flash, typically 50–1000 ms will cause a further increase in fluorescence, often via an inflexion (termed I or I_2 by Strasser *et al*., 1995 and Schreiber *et al*., 1995b) to a higher maximum level, which we term $F_{m(MT)}$ (both Strasser *et al*., 1995 and Schreiber *et al*., 1995b called this level P). The rise in fluorescence after the primary photochemical phase has finished can be large and, as a result, the $F_{m(MT)}$ level reached by the MT-flash can be 50% higher than the $F_{m(ST)}$ level reached by a ST-flash (Fig. 1). The ratio of the two F_m levels is not constant, and will vary according to species composition (see next section), the immediate irradiance history of the algal material (Koblížek *et al*., 1999) or the cell-cycle stage of an algal culture (Strasser *et al*., 1999). Reduction of the secondary electron acceptors, Q_B, and the plastoquinone pool occurs upon prolonged irradiation, and it is thought that these changes cause a relaxation of fluorescence quenching at PSII. The exact mechanisms for the increase in fluorescence from $F_{m(ST)}$ to $F_{m(MT)}$, or the *thermal phase* of the fluorescence rise, are however unknown (see Samson *et al*., 1999 for a detailed review). The rise from I_2 to $F_{m(MT)}$ (see Fig. 1) is of much smaller magnitude than that from $F_{m(ST)}$ to $F_{m(MT)}$ and, as the two levels become indistinguishable in the presence of background light (Schreiber *et al*. 1995b), the difference will not be considered further here.

Table 1. Fluorescence parameters and their meaning. As far as possible, the recommendations of van Kooten & Snel (1990) and Koblížek *et al.* (2001) have been followed for the MT-measuring protocol, and those of Kolber & Falkowski (1993) and Gorbunov *et al.* (2001) for the ST-protocol.

Parameter	Synonym	Protocol	Meaning	Units
a*		MT	Optical absorption cross section	m^2 mg chla^{-1}
a^*_{PSII}		MT/ST	Optical absorption cross section of PSII	nm^2
σ_{PSII}		ST	Functional cross section of PSII	nm^2
σ'_{PSII}		ST	Functional cross section of PSII in ambient light	nm^2
n_{PSII}		ST	Number of functional PSII centres	mol PSII (mol chl a)$^{-1}$
F_o		ST/MT	Minimal fluorescence after dark acclimation	dimensionless
F_o'		ST/MT	Minimal fluorescence in light acclimated state	dimensionless
F_{DCMU}		ST/MT	Maximum fluorescence after dark acclimation, addition of DCMU, then exposure to saturating irradiance	dimensionless
$F_{m(ST)}$	J, I_1	ST	Maximum fluorescence after dark acclimation then measurement during exposure to single turnover flash	dimensionless
$F_{m(MT)}$	P	MT	Maximum fluorescence after dark acclimation then measurement during exposure to a multiple turnover flash of saturating irradiance	dimensionless
F	F', F_s *or* F_t	ST/MT	Steady-state fluorescence in the light	dimensionless
$F_{m'(ST)}$		ST	Maximum fluorescence in light acclimated state using ST flash	dimensionless
$F_{m'(MT)}$		MT	Maximum fluorescence in light acclimated state using MT flash	dimensionless
F_v		MT	Variable fluorescence after dark acclimation using MT flash $F_v = (F_{m(MT)}\text{-}F_o)$	dimensionless
F_v'		MT	Variable fluorescence in light acclimated state using MT flash. $F_v' = F_{m'(MT)}\text{-}F_o'$	dimensionless
F_v/F_m	Φ_P°	MT	Maximum PSII efficiency measured using a MT flash. $F_v/F_m = (F_{m(MT)}\text{-}F_o)/(F_{m(MT)})$	dimensionless[1]
$\Delta\Phi_m$		ST	Maximum PSII efficiency measured using a ST flash. $\Delta\Phi_m = (F_{m(ST)}\text{-}F_o)/(F_{m(ST)})$	dimensionless[1]
$\Delta F/F_m'$	Y, F_q'/F_m'	MT	Effective PSII quantum efficiency measured using a MT-flash. $\Delta\Phi/F_m' = (F_{m'(MT)}\text{-}F)/F_{m'(MT)}$	dimensionless[1]
$\Delta\Phi$		ST	Effective PSII quantum efficiency measured using a ST flash. $\Delta\Phi = (F_{m'(ST)}\text{-}F)/F_{m'(ST)}$	dimensionless[1]
Φ_e		ST/MT	Electron yield of O_2 production	$O_2.e^-$
q_P	F_q'/F_v'	ST/MT	$(F_m'\text{-}F)/(F_m'\text{-}F_o')$	dimensionless
q_N		ST/MT	$1-((F_m'-F_o')/(F_m\text{-}F_o))$	dimensionless
NPQ		ST/MT	$(F_m - F_m')/F_m'$	dimensionless
ETR_{ST}		ST	Electron transport rate	mol e^- mg chla^{-1} s^{-1}
ETR_{MT}		MT	Electron transport rate	mol e^- mg chla^{-1} s^{-1}
P^B		ST/MT	Photosynthetic rate	mol O_2 mg chla^{-1} s^{-1}

[1]Although mathematically dimensionless, the yield parameters are functionally equivalent to 1 electron produced by the process of charge separation per photon absorbed.

Thus, the major factor in determining the maximum fluorescence level is the choice of flash used to transiently close PS II reaction centres. At present, the most commonly used types of commercially-available equipment utilize either short flashlets of single turnover nature, such as in the fast repetition rate fluorometer (FRRF), whereas MT or 'fat flashes' are inherent in the design of PAM-type fluorometers.

Dark adapted PS II yield: F_v/F_m, and $\Delta\Phi_m$

The maximum photochemical efficiency of PSII can be derived mathematically from $F_{m(MT)}$ and F_o, using the expression $(F_{m(MT)}\text{-}F_o)/F_{m(MT)}$ (Butler & Kitajima, 1978) and signifies the number of electrons produced as the result of the absorption of a photon by a single charge separation event in PSII. This parameter is usually termed F_v/F_m, where $F_v = F_{m(MT)}\text{-}F_o$ (Schreiber *et al.*, 1986), or Φ_P° (Dau, 1994) when obtained using the MT-method. Alternatively, the abbreviation $\Delta\Phi_m$ was proposed when using the ST-method (Kolber & Falkowski, 1993). Because the F_m levels obtained with a ST flash are lower, the maximum PSII quantum efficiency $\Delta\Phi_m$ is also lower than F_v/F_m when measured using a MT-flash (Schreiber *et al.*, 1995b). Comparative experiments with phytoplankton have shown that $\Delta\Phi_m$ could be up to 16% lower than F_v/F_m, with differences in the F_m levels largest for green algae and least for cyanobacteria (Koblížek *et al.*, 2001).

It should be emphasized that the terms F_v/F_m and $\Delta\Phi_m$ refer to the maximum photochemical efficiency of PS II in the absence of non-photochemical quenching processes. This condition requires strictly that the sample be darkened for a period of typically 15–30 min before measure-

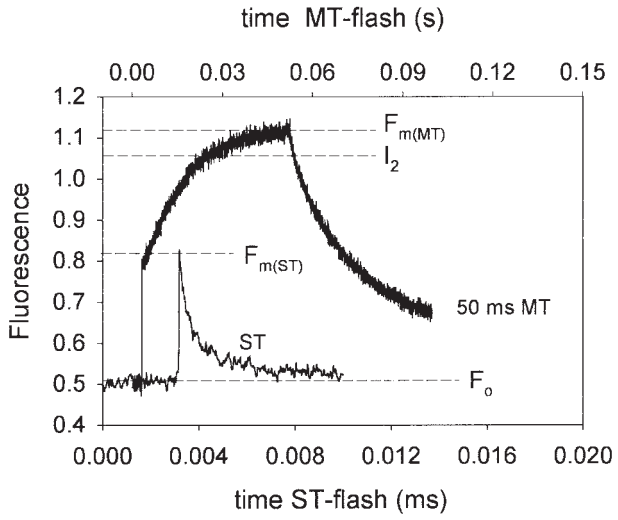

Fig. 1. Fluorescence induction curves obtained for the green alga *Ankistrodesmus* using either a Xenon-single turnover flash ('ST'; lower X-axis 0–0.02 ms), or a Xenon MT-turnover flash of 50 ms ('50 ms MT'; top X-axis 0–0.15 s). Note the different time scales for X-axis. Measurements were made on a suspension of algae in a temperature-controlled cuvette after 10 min dark acclimation, using a PAM 101 fluorometer, with custom-written software for instrument control and data acquisition. In order to confirm that both flashes were sufficient to reach $F_{m(ST)}$ or $F_{m(MT)}$, the flash intensities were varied by changing the distance from the light source to the cuvette.

ment. For cyanobacteria, a special protocol has been recommended (Campbell *et al.*, 1998).

Dark-adapted F_v/F_m or $\Delta\Phi_m$ estimates the optimal photochemical efficiency of PS II, and has been used widely as an 'algal health' parameter, which is responsive to the short-term (hours) light and nutrient history of the cells (Berges *et al.*, 1996; Olaizola *et al.*, 1996). However, F_v/F_m does not necessarily indicate the potential of the plant for photosynthesis, as limitations may occur elsewhere in the photosynthetic chain, particularly in the dark reactions, without affecting PS II efficiency. Diurnal measurements have shown that substantially lowered F_v/F_m may occur simultaneously with highest rates of photosynthetic electron transport (Ensminger *et al.*, 2001). It is also evident that phytoplankton can acclimate to nutrient stress over several cell generations without a noticeable decrease in F_v/F_m (Parkhill *et al.*, 2000). F_v/F_m of healthy cells is however not always maximal in the dark. Many algae show chlororespiration in the dark (Dijkman & Kroon, 2002; Schreiber *et al.*, 1995a). In this process, electrons are donated to O_2 in the thylakoid membrane by a plastoquinone-oxidoreductase via PQ (Nixon, 2000). The resulting reduction of PQ causes some quenching of $F_{m(MT)}$ and, as a result, the maximum F_v/F_m is reached in low light conditions.

F_o', F_m', q_P, q_N, NPQ

The use of modulated fluorometers allows the measurement of maximum and minimum fluorescence levels in the presence of actinic (i.e. photosynthetically active) irradiance; the parameters thus measured are distinguished with a superscript ''. In the light adapted state, the maximum fluorescence, F_m', is often lower than F_m, and in prolonged high irradiance the minimum fluorescence F_o' can also be reduced to a level substantially below F_o, a phenomenon especially prevalent in algae. F_o' is the most difficult fluorescence parameter to measure. F_o' should be measured immediately after darkening the sample, preferably following far-red irradiation to increase the oxidation rate of Q_A^-. When using a halogen source for actinic irradiance, however, it is important to be aware that such lamps 'glow' for several seconds after turning off. This makes accurate determination of F_o' difficult, especially in the field. Alternatively, F_o' can be estimated indirectly from other fluorescence parameters using the method of Oxborough & Baker (1997).

Steady state fluorescence in the light-acclimated state, denoted as F, varies between F_o' and F_m', depending on the redox state of the Q_A and/or PQ-pool. The ratio of the effective to the maximum trapping efficiency is called q_P (from photochemical quenching) and can be calculated as:

$$q_p = (F_m' - F)/(F_m' - F_o') \qquad (1)$$

In the case of unconnected reaction centres, where transfer of excitation energy between units is not possible, q_P gives a direct measure of the fraction of reaction centres that are open. Otherwise, when some degree of 'connectivity' between reaction centres is present, the relationship between q_P and reaction centre closure will be curvilinear. From the degree of curvilinearity, the connectivity can be calculated (Lavergne & Trissl, 1995).

The reason for the reduction in F_m' and F_o' is that non-photochemical quenching of fluorescence is induced at moderate to high irradiances, typically commencing shortly before photosynthesis becomes light-saturated. There are a variety of causes, such as downregulation of photosynthesis by the xanthophyll-cycle (in which absorbed light energy is dissipated as heat by the carotenoids zeaxanthin in green algae, or diatoxanthin in chromophyte algae), state transitions (redistribution of absorbed light energy among the photosystems), or chronic photoinhibition (structural damage to the PSII reaction centre), which are discussed in detail elsewhere (Horton & Ruban, 1994; Horton *et al.*, 2000). The extent

of non-photochemical quenching can be calculated as:

$$q_N = 1 - ((F_m' - F_o')/(F_m - F_o)) \quad (2a)$$

or $$NPQ = (F_m - F_m')/F_m' \quad (2b)$$

Quantum yield of steady-state photosynthesis in the light: $\Delta F/F_m'$ *and* $\Delta\Phi$

In a key paper, Genty *et al.* (1989) demonstrated using the MT-protocol that the effective quantum efficiency of PSII is the product of q_P and the maximum efficiency of PSII in the light adapted state (given by $F_v'/F_{m'(MT)}$):

$$q_P \, x \, F_v'/F'_{m(MT)} = (F_{m'(MT)} - F)/F_{m'(MT)} = \Delta F/F_{m'(MT)} \quad (3a)$$

In words, the overall efficiency of PS II in the light is equal to the number of open reaction centres multiplied by the efficiency of those reaction centres that are open. The 'Genty' formula thus accounts for the effects of both photochemical and non-photochemical quenching processes on the light-acclimated quantum yield of photosynthesis. The difficult-to-measure F_o' parameter is thus eliminated from the equation, enabling the use of a simple 'point and flash' MT fluorescence protocol for both field and laboratory measurements, a feature which has been exploited by a number of manufacturers (Maxwell & Johnson, 2000). The popularity of the MT method is shown by the number of citations of the original paper (Genty *et al.*, 1989) – over 1240 citations to date.

In a similar way, Kolber & Falkowski (1993) demonstrated that the effective quantum efficiency could be measured using a ST flash with algae in actinic light:

$$\Delta\Phi = (F_{m'(ST)} - F)/F_{m'(ST)} \quad (3b)$$

However, it should again be emphasized that, because F_m' measured with a MT-flash will be higher than that measured with a ST-flash, $\Delta F/F_m'$ will be greater than $\Delta\Phi$ measured at the same irradiance. Note that the steady state fluorescence F is independent of the ST or MT-protocol (see also Fig. 1).

A further issue involves the misuse of the fluorescence parameters listed above. Whilst publications on higher plants have tended to follow the nomenclature of van Kooten & Snel (1990) or the more recent recommendations of Baker *et al.* (2001), these have not been followed in some recent publications on phytoplankton and macroalgae. For example, the Genty factor has been used to describe dark-adapted F_v/F_m measurements (Defew *et al.*, 2002). The two yield measurements, F_v/F_m and $\Delta F/F_m'$, reflect fundamentally different processes, namely the potential maximum efficiency of PS II, and the efficiency of whole-chain photosynthetic electron transport, respectively. The dark-acclimated yield measurement reflects only processes acting on the PSII reaction centre or its antenna pigments, whereas the light-acclimated yield also integrates all the processes downstream of PSII which influence the redox state of the PQ-pool and thus the degree of PSII closure, as well as the amount of non-photochemical quenching of fluorescence associated with dynamic and chronic photoinhibition. Consequently, F_v/F_m and $\Delta F/F_m'$ cannot be used interchangeably. As true *in situ* measurements can be difficult to perform (perhaps because the sample is underwater), 'effective quantum yield' measurements have been made after removal of algae from the experimental irradiance to which they were acclimated. This will involve transfer to a (usually) lower irradiance in the laboratory (e.g. Cabello-Pasini *et al.*, 2000), during which time both q_P and non-photochemical quenching processes will change rapidly. F_v'/F_m' may be preferred to describe this type of yield measurement (Sagert *et al.*, 1997; Ralph *et al.* 1999). It will indicate the efficiency of open PSII reaction centres, and its value will be intermediate between $\Delta F/F_m'$ and dark-adapted F_v/F_m.

Calculation of photosynthetic electron transport rates

MT method

The introduction of a simple-to-use fluorescence parameter for measuring photosynthetic efficiency in the light by Genty *et al.* (1989), and subsequent findings that this parameter agreed well with quantum yields of other photosynthetic processes such as oxygen evolution and CO_2 fixation (for algal examples, see Masojídek *et al.*, 2001), have led to the widespread use of protocols for converting the quantum yield of PSII into actual rates of electron flow. 'Electron transport rates', or *ETR*, as originally described by Schreiber *et al.* (1994) can be measured in the field from knowledge of $\Delta F/F_m'$ and photosynthetically-active irradiance.

The rate of light absorption per mg chlorophyll *a* by algae can be calculated as the product of the optical cross section a^* and the irradiance E. Thus, the rate of linear photosynthetic electron transport (*ETR*) by PSII is (using the MT-method):

$$ETR = E \, x \, a^* \, x \, 0.5 \, x \, \Delta F/F_m' \quad (4a)$$

This assumes that the quantum yield of electron transfer of trapped photons within a reaction

centre is 1 (Kolber & Falkowski, 1993), that 50% of the absorbed light goes to PSII and 50% to PSI in order to achieve a balanced excitation pressure on both photosystems (Gilbert *et al.*, 2000), and that no cyclic electron transport by PSI is occurring. Whilst in higher plants, and presumably in green algae, the ratio of the PSI:PSII cross section may be close to unity (Boichenko, 1998), the ratios in other algal groups may deviate significantly. The functional cross-section of PSII compared to PSI can vary substantially among species and with growth irradiance (Dubinsky *et al.*, 1986; Fietz & Nicklisch, 2002). If the absorption cross section of PSII (a^*_{PSII}) and the number of PSII (n_{PSII}) per mg of chlorophyll *a* are known, then *ETR* can be more accurately calculated as:

$$ETR = E \; x \; a^*_{PSII} \, x \, n_{PSII} \, x \, \Delta F/F_m' \qquad (4b)$$

If the electron yield of oxygen evolution (i.e. the moles of O_2 produced per mole of electrons extracted from water by PSII photochemistry; Φ_e) is known, the chlorophyll specific rate of photosynthetic oxygen evolution (P^B) can be predicted from *ETR*:

$$P^B = E \; x \; a^*_{PSII} \, x \, n_{PSII} \, x \, \Delta F/F_m' \, x \, \Phi_e = ETR \, x \, \Phi_e \qquad (5)$$

A theoretical minimum value of 4 charge separations is needed per molecule of oxygen evolved, meaning that $\Phi_e \leqslant 0.25$, (Gilbert *et al.*, 2000), although larger numbers have been found experimentally (Kromkamp *et al.*, 2001; Longstaff *et al.*, 2002). The same approach can be used to calculate gross carbon fixation, but the variability between ETR-based estimates and actual measurements will increase further due to uncertainty about the photosynthetic quotient (the ratio of oxygen evolved to carbon fixed; Kroon *et al.*, 1993). This is partly due to the fact that some photosynthetically-generated electrons are used to reduce nitrate by nitrate reductase in a light dependent step, rather than being used to reduce CO_2. An alternative approach is to make paired measurements of fluorescence and oxygen evolution on the same sample, under identical irradiance conditions, and to calculate empirical conversion factors. A summary of conversion factors for different algal species is given by Masojídek *et al.* (2001), and for microphytobenthos at different temperatures by Morris & Kromkamp (2003).

ST method

The original pump and probe technique developed by Falkowski and coworkers (Falkowski *et al.*, 1986) used a xenon-light source to produce a short (< 20 μs) ST-flash. Fluorescence levels were measured from weak probe flashes given immediately before and after the ST flash. The modern FRR instrumentation uses LED technology to deliver a sequence of short flashlets (with a duration of ~ 1 μs, which alone are not saturating), in order to reach $F_{m(ST)}$. The time required to achieve this may vary between 100 and 280 μs, depending on the specification of the instrument (Kolber *et al.* 1998; Suggett *et al.*, 2001). Both the FRRF and certain types of other fluorescence equipment (e.g. PSI fluorometer; Brno, Czech Republic) can be programmed to supply both ST and MT flashes, or flashlet sequences (Koblížek *et al.*, 2001). Recalculation of the data presented in Fig. 8A of Kolber *et al.* (1998) shows that $\Delta\Phi_m = 0.62$ for the ST flash and that $F_v/F_m = 0.71$ for the MT-flash.

Kolber & Falkowski (1993, Eq. 4) suggested the use of the following relationship between P^B and variable fluorescence measurements obtained originally with their 'pump and probe' method, but also valid for the commercially available FRRF**:**

$$P^B = E \; x \; \sigma_{PSII} \; x \; n_{PSII} \; x \; q_P \; x \; \Phi_e \; x \; f \qquad (6)$$

where σ_{PSII} is the functional cross section of PSII, and *f* the fraction of active centres (see below). The functional cross section is a measure of the fraction of light absorbed by PSII which is used for photochemistry, and is the product of the trapping efficiency and the optical absorption cross section (Mauzerall & Greenbaum, 1989). Incident irradiance multiplied by σ_{PSII} and the number of functional PS II centres, n_{PSII} (mol PSII (mol chl $a)^{-1}$) gives the total photon flow available for PSII charge separation. The fraction of inactive PSII centres, *f*, is often calculated as $\Delta\Phi_m/0.65$. Dark adaptation periods for measurement of $\Delta\Phi_m$ vary normally from 5 to 30 min, and this can be sufficient for dynamic downregulation of PSII to relax completely, but long-lasting quenching of fluorescence, mainly caused by photodamage, may take longer to relax. When this occurs, the factor *f* will be smaller than unity, and the value of 0.65 e^- photon^{-1} was chosen as the benchmark for highest photochemical efficiency following observations of phytoplankton cultures and natural samples (Falkowski *et al.*, 1986; Kolber & Falkowski, 1993). This assumption of a constant maximum photochemical value is somewhat arbitrary, as both lower and higher values can be found with the ST method (Berges *et al.*, 1996). Phytoplankton populations dominated by cyanobacteria can be expected routinely to have a lower $\Delta\Phi_m$ or F_v/F_m (Campbell *et al.*, 1998; Kromkamp *et al.*, 2001). Recently, Equation 6 has been modified and the factor *f* has been omitted (Gorbunov *et al.*, 2000, 2001). Although no arguments were given for the change,

it would appear to be a correct decision because non-photochemical quenching processes, which affect the efficiency of charge separation, are already incorporated in the equation via an effect on the functional cross section σ_{PSII}. The reason why the MT method for calculating *ETR* does not contain the factor f is that, according to the currently used radical pair model (see Dau, 1994), the effect of damaged PSII centres on the fluorescence yield is incorporated in F_v'/F_m'.

Thus, the FRRF uses the **functional** absorption cross section, whereas the PAM uses the **optical** absorption cross section. What is the reason for these differences between the ST and MT methods? Both methods can calculate q_P. The functional cross section is equal to the product of the optical cross section and the trapping efficiency:

$$\sigma_{PSII} = a^*_{PSII} \; x \; \Phi_T.$$

Therefore, it is possible (assuming $f = 1$) to rearrange Eqn. 6 as:

$$P^B = E \; x \; n_{PSII} \; x \; a^*_{PSII} \; x \; \Phi_T \; x \; q_P \; x \; \Phi_e \qquad (7a)$$

Because $q_P x \Phi_T = \Delta F/F_m'$ (Genty *et al.*, 1989, see above), this can be substituted in Eqn. 7a:

$$P^B = E \; x \; n_{PSII} \; x \; a^*_{PSII} \; x \; \Delta F/F_m' \; x \; \Phi_e \qquad (7b)$$

Equation 7b is identical to Eq. 5, suggesting that the MT and ST protocols are the same, which is **not** the case. Calculated values of *ETR* will be higher using the MT method (Fig. 2) because $F_{m'(MT)}$ will be higher than $F_{m'(ST)}$. The difference will be irradiance-dependent (Samson *et al.*, 1999), with higher irradiances causing an increased reduction of the PQ pool and lessening the difference between the two F_m levels. However, small differences in yield values can still result in considerable differences in calculated electron transport rates at high irradiance, due to the multiplication by irradiance in both Eqns. 5 and 6. For the example shown (Fig. 2), the largest difference occurs at 900 μmol photon $m^{-2}\,s^{-1}$, at which point ETR_{ST} is 65% of the value for ETR_{MT}.

Consequently, the two methods differ significantly because of how F_m is measured. Recently, the MT terminology has been used to describe yield measurements made with ST flashes (Lesser & Gorbunov, 2001 for corals; Boyd & Abraham, 2001 for phytoplankton), allowing a tempting, but false, comparison with PAM-based measurements (e.g. Ralph *et al.*, 1999 for coral reef studies). Because of the underlying differences between the protocols, we suggest that the original terminology should not be hybridized, and we recommend the use of the parameters listed in Table 1 to avoid

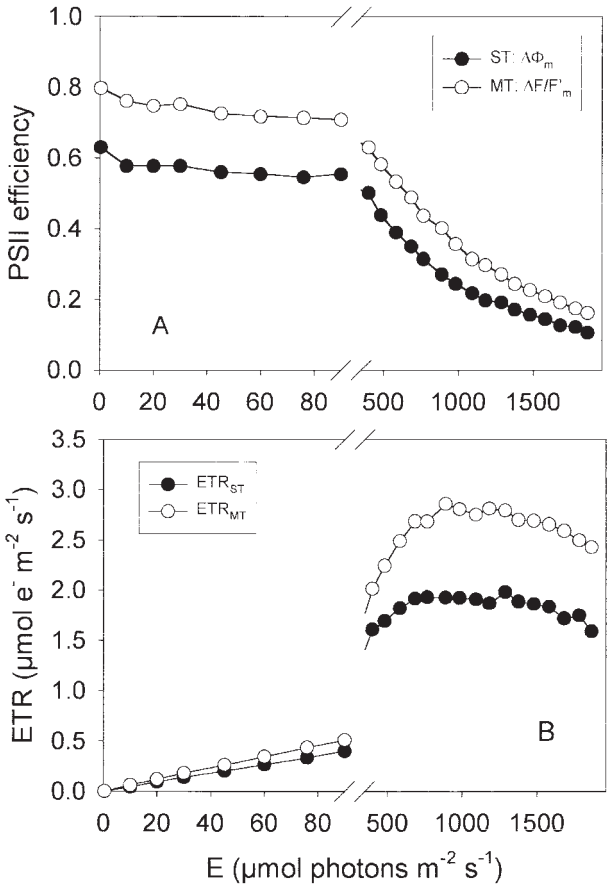

Fig. 2. Data showing PSII efficiency at different irradiance (A) and calculated relative electron transport rates (B) using the single turnover (ST) or multiple turnover (MT) protocol, and Equation 4a with $a^* = 0.016\ m^2\ (mg\ chl\ a)^{-1}$. Data for the green alga *Ankistrodesmus braunii* (modified from Schreiber *et al.*, 1995a).

confusion. The suffixes 'ST' and 'MT' should be applied to F_m and also to derived parameters such as *ETR* and P^B.

It is clear from the above that both protocols should be thoroughly tested. To date, few comparative studies between the ST and MT protocols have been conducted in aquatic systems (but see Suggett *et al.*, 2003). Samson *et al.* (1999) carried out a comparison using tomato leaves, and observed that the relation between $\Delta\Phi$ and $\Delta F/F_m'$, and between q_P values measured with ST or MT flashes, was completely linear ($r^2 > 0.99$), and that the values obtained with the MT protocol were always higher. On the other hand, q_N showed a biphasic relationship at low to moderate irradiances, related probably to the disappearance of chlororespiration and the onset of the xanthophyll cycle. The effect of non-photochemical quenching on $F_{m'(ST)}$ and $F_{m'(MT)}$ was also species-dependent, with the diatom *Phaeodactylum* showing a similar extent of quenching on both variables, whereas $F_{m'(MT)}$ increased relative to $F_{m'(ST)}$ as quenching relaxed, following a period

of light stress, in the green alga *Scenedesmus* (Koblížek *et al*., 2001).

The fact that measurements of both q_P and the effective PSII yield with the ST or MT method were highly correlated, implies that both protocols would be equally valuable in the field. Indeed, there are good examples of both ST and MT measurements showing agreement with carbon- or oxygen-based measurements of photosynthesis (see Barranguet & Kromkamp 2000, for MT method; Suggett *et al*., 2001 for ST method).

Advantages and disadvantages of both methods

For the original ST protocol, calculation of $\Delta\Phi_m$ required determination of F_o, which required dark adaptation of the samples. The dark chamber of the currently-available model of FRRF only supplies a short dark period, which may not be enough for relaxation of downregulation by energy quenching. As a result, the fraction of inactive centres may be overestimated. In the more recent formulation of Gorbunov *et al*. (2001), however, the *f* parameter has been removed, and replaced by the light-acclimated functional cross-section, σ'_{PSII}, thus removing the need for dark acclimation.

A disadvantage of the MT technique is the use of long (> 0.2 to < 1.5 s) flash times in equipment such as the Diving-PAM. Frequent application of long MT flashes may cause build-up of non-photochemical quenching. Schreiber *et al*. (1995a) suggested that future equipment designs should use shorter (e.g. 50 ms) flashes in order to reach the I_2 fluorescence level, but avoid unwanted quenching effects.

Measurement of the photosynthe tically-absorbed irradiance (i.e. the fraction of irradiance which is absorbed by light harvesting pigments connected to a photosynthetic unit) is required to calculate absolute electron transport rates in both methods. The optical cross section can be measured using a variety of methods: the filterpad method (e.g. Mitchell & Kiefer, 1988), which requires a correction for multiple scatter; the filter-transfer-freeze method (Tassan & Allali, 2002); or by using a reflective tube (Zaneveld *et al*., 1990), which requires a determination of the scatter to absorption ratio of the water investigated. An advantage of the FRRF design is that it can measure the functional cross section, σ_{PSII}. However, the value of σ_{PSII} will be influenced by the value assigned to the connectivity parameter, which determines the degree of energy transfer between PSII reaction centres. In addition, n_{PSII}, the number of PSII units is not easy to measure, and more research is needed to determine the variability of this parameter. Kolber & Falkowski (1993) used a value of 545 molecules chl *a* per electron, but other values, ranging from 260 to 800 (see Dubinsky *et al*., 1986; Boyd *et al*., 1997) have been cited. Kromkamp & Limbeek (1993) demonstrated that, in the marine diatom *Skeletonema costatum,* n_{PSII} changes when the algae are grown in constant or fluctuating light.

Perhaps the biggest current advantage of the FRRF is that it is more sensitive, and can be used *in situ* for phytoplankton. This means that the natural irradiance and temperature conditions for photosynthesis are utilised, and that possible errors due to chromatic shifts during on-deck measurements are avoided. A drawback of all fluorometric methods is that the irradiance used to excite fluorescence may be highly selective for certain algal groups. For example, blue LED equipped fluorometers will preferentially excite the antenna pigments of chromophyte algae, and may greatly undersample those species with a low PSII cross section in the 400 – 500 nm region, such as cyanobacteria and prochlorophytes. Comparison of results obtained from different designs of fluorometer would be particularly problematic when the species composition is subject to dynamic change, as for example during large-scale ocean fertilization experiments (Gervais *et al*., 2002). In coastal waters, high levels of phaeophytin may cause an underestimation of PSII efficiency (Fuchs *et al*., 2002).

In conclusion, the methods differ because the maximum level of fluorescence is higher during a prolonged, multiple turnover flash than during a single turnover flash. In order to avoid confusion between users of the ST or MT protocols, the terminology of the similar terms should remain distinct. Otherwise, errors will be made when comparing photochemical efficiencies or photosynthetic electron transport rates measured with the different techniques.

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