Chlorophyll *a* Fluorescence in Aquatic Sciences

Methods and Applications

Developments in Applied Phycology 4

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Methods and Applications

Springer

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Preface

It is unquestionable that Chlorophyll *a* fluorescence is quite literally a global phenomenon. Fluorescence merely describes an optical phenomenon where light absorbed at one wavelength is re-emitted at another (longer) wavelength; it exists passively in nature and occurs wherever light exists to be absorbed by Chlorophyll *a* molecules. These molecules are a common property of all photoautotrophic organisms on land and in water; thus Chlorophyll *a* fluorescence is essentially ubiquitous in nature (Fig. 1). It is incredible that such a natural phenomenon has been exploited by such a wide variety of researchers and across the biological and environmental sciences, and perhaps is testament to the importance we place on understanding photoautotrophic activity. We have long known that Chlorophyll *a* fluorescence of photosynthetic organisms varies as a result of changes in the amount (biomass), as well as function (quantum yield), of Chlorophyll *a* present. At operational temperatures that exist in most natural environments, Chlorophyll *a* fluorescence is largely derived from the Chlorophyll *a* associated with photosystem II (PSII), i.e. the oxygen evolving complex; as such, changes in the quantum yield of fluorescence directly relate to changes in photosynthetic (O_2 evolving) capabilities. Thus, by actively inducing changes in Chlorophyll *a* fluorescence using an actinic light source, we can perturb the physiological *status quo* of (PSII) photoautotrophy itself. Packaging of technology to enable induction and measurement of such Chlorophyll *a* fluorescence perturbations has entirely made possible examination of processes associated with plant and algal ecology, physiology and productivity, and at scales from the single cell to the entire planet (van Kooten and Snel 1990). Therefore, it is hard to imagine a future that does not continue to exploit the properties of Chlorophyll *a* fluorescence, not only for research but also in how we continue to sustainably exploit our ever-changing environment.

The history of using fluorescence to investigate biomass, photosynthetic physiology and primary productivity has been covered in several comprehensive publications, most recently by Papageorgiou and Govindjee (2005) (and chapters therein); however, it is of course important to note the place of aquatic studies in this history, at least for the context of the following chapters. Whilst many major developments in using *variable* Chlorophyll *a* fluorescence have arguably come from studies on terrestrial (vascular) plants, free-living microalgae (chlorophytes in particular in particular) and cyanobacteria have proved to be important laboratory organisms in examining principal photobiological mechanisms. Examining such aquatic organisms under controlled laboratory conditions is a perhaps an obvious step; aside from the relative ease of probing photosynthetic machinery of single celled compared to multi-cellular organisms, microalgae and cyanobacteria dominate photosynthetic activity of much of the Earth's aquatic realm. However, in contrast to working on terrestrial plants, extending such laboratory-based observations to the 'real world' has proven to be the greatest challenge for aquatic scientists and one that has been largely led by technology and engineering. In overcoming the technical challenges, exciting and important discoveries, such as the confirmation of iron limitation of ocean productivity (Behrenfeld et al. 1996) and the discovery of aerobic anoxygenic bacteria (Kolber et al. 2001), have followed.

The earliest application of Chlorophyll *a* fluorescence to aquatic system research (*in situ*) is well recognized as from Carl Lorenzen (1966) who first pumped seawater through a shipboard fluorometer. Such a convenient, rapid approach was quickly adopted by both

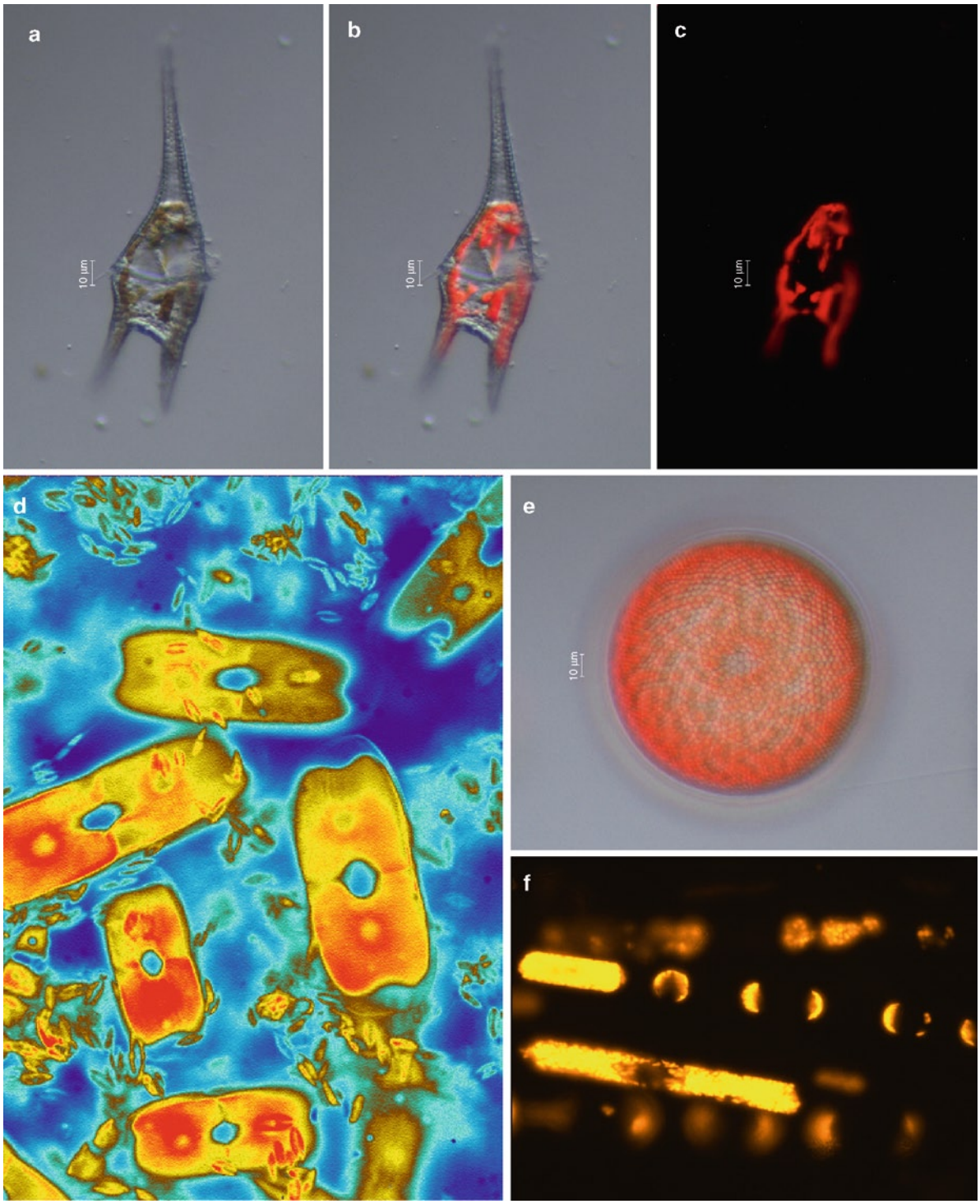

Fig. 1 Fluorescence in action: (**a–c**) Chloroplast fluorescence in the dinoflagellate *Ceratium* sp. (Photo: L. Novoveska); (**d**) False colour high resolution fluorescence image of cells of the diatom *Nitzschia dubia*. Fluorescence emanating from the chloroplasts becomes restricted to the area of the pyrenoid as light intensity increases (Photo: R. Perkins); (**e**) Chloroplast fluorescence in the centric diatom *Coscinodiscus* sp. (Photo: L. Novoveska); (**f**) Delayed fluorescence in the colonial diatom *Rhizosolenia* (Photo: M. Berden-Zrimek)

oceanographic and limnological communities; this, not surprisingly quickly led to a wealth of highly novel studies linking physical and biological processes, in particular, the distribution of phytoplankton with ocean turbulence (Platt 1972) and the discovery of the deep chlorophyll maximum of stratified waters (Cullen and Eppley 1981). The major challenge for aquatic scientists to evolve to *in situ* studies was 'simply' to package complex and innovative technology into a system that could withstand the constraints of working in water, especially in marine environments where salts and pressure rapidly build. It wasn't until the 1970s that technology caught up with concept and the first profilable *in situ* fluorometers were truly developed (see Falkowski and Kolber 1995). Ever since, such fluorometers have become smaller and better integrated to sensor arrays, and essentially a routine yet fundamental tool for aquatic scientists. However, despite their rapid adoption by the aquatic community, these fluorometers were still generally restricted to assaying a single chlorophyll fluorescence yield, which was set according to the excitation intensity of the instrument in question, and thus could only ever provide some approximate measure of Chlorophyll *a* biomass *in situ*. An important step to aquatic research was thus in producing fluorometers that induced a variable Chlorophyll *a* excitation (and hence fluorescence emission) protocol (Fig. 2).

Numerous laboratory studies by the 1970s and early 1980s had already demonstrated important concepts linking variable Chlorophyll *a* fluorescence to photosynthetic physiology in aquatic algae (e.g. Mauzerall 1972; Ley and Mauzerall 1982; but note an ISI Web of Science search yields >125 publications in the 1970s alone!), however, modification of these techniques to *in situ* aquatic studies to thus add a physiological component (the variable fluorescence 'transient') to measures of fluorescence yield (biomass) was not straightforward. Here, the development of actinic light sources that could deliver the intensity and/or frequency of excitation required to induce variable fluorescence remained an even greater technological challenge to the pre-existing in water operational constraints. Solving this problem essentially had to occur twice since variable fluorescence techniques have already evolved into two parallel but distinct paths (Chapter 3 by Huot and Babin, this volume): Pulse Amplitude Modulation (PAM; Schreiber et al. 1986), where fluorescence is induced by a weak modulated light source evaluated independently from a relatively long yet moderate intensity light pulse; and Pump and Probe (PP; Mauzerall 1972; Falkowski et al. 1986; but see also Kolber and Falkowski 1993), where variable fluorescence is measured by a weak 'probe' actinic flash before and after a saturating 'pump' flash. PP later evolved into Fast Repetition Rate (FRR; Kolber et al. 1998), where a complex fluorescence transient could be induced by initially delivering a series of subsaturating high intensity flashlets followed by a series of more widely spaced 'probing' flashlets that examined the subsequent fluorescence decay. All subsequent variable fluorometers have essentially followed one (or a combination) of these paths. Importantly, this new generation of fluorometers not only opened new possibilities for examining photoautotrophic physiology but also a potential revolution in how aquatic scientist would determine primary productivity (Kolber and Falkowski 1993, Kromkamp and Forster 2003; Suggett et al., Chapter 6, this volume).

Evolution of both PAM and FRR (PP) was originally driven from the pioneering laboratory work using microalgae; as such, the first *in situ* variable fluorometers in the 1980s and 1990s were essentially restricted to working on natural phytoplankton suspensions in lakes and oceans. Technical improvements in overall signal resolution since then has enabled researchers to investigate ever more oligotrophic waters of oceans and nutrient impoverished lakes. However, subtle technological changes in sensitivity and the optical configurations within a few years of PAM and FRR fluorometer introduction enabled the photophysiology of benthic autotrophs (corals, microphytobenthic mats, seagrasses and macroalgae) to be examined (see Chapter 9 by Enríquez and Borowitzka, Chapter 10 by Warner et al., and Chapter 11 by Shelly et al., this volume). More recent additional but relatively small optical alterations to the PAM and FRR 'model' to examine far red fluorescence (>800 nm) has introduced more new research opportunities, e.g. bacteriochlorophyll *a* (Kolber et al. 2001) and Photosystem I (PSI) variable fluorescence (Dual PAM, e.g. see Sukenik et al. 2009). Modification of the spectral quality of fluorescence excitation and emission detection has also added the potential for variable fluorometers to taxonomically discriminate bulk fluorescence properties (Schreiber 1998; Beutler et al. 2002; Chapter 7 by MacIntyre et al., this volume). All of these advances have unquestionably facilitated the explosion of interest in the use

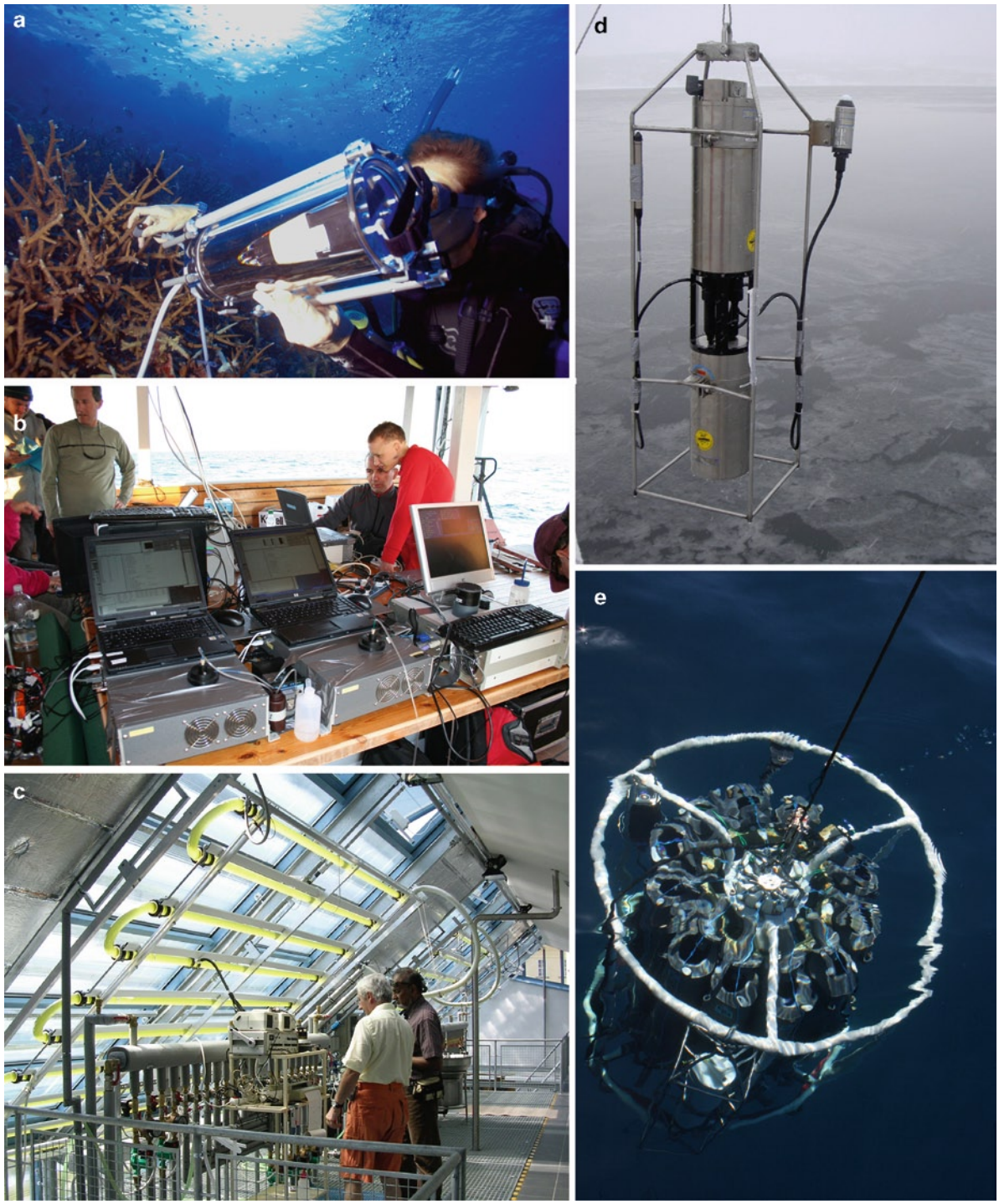

Fig. 2 Fluorometers in action: (**a**) Diving PAM used to measure fluorescence signal in corals, Wakatobi Marine National Park, Indonesia (Photo: D. Smith); (**b**) Fluorometer comparisons at GAP Workshop, Eilat, Israel, 2008 (Photo: D. Suggett); (**c**) *In-situ* measurement of fluorescence quenching using the fluorometer PAM 101-103 (H.Walz, Germany) in the *Haematococcus* culture grown in solar photobioreactor at the Centre of Biological Technologies, University of South Bohemia in Nové Hrady, Czech Republic (Photo: J. Masojidek); (**d**) FRRF being deployed in winter, Bedford Basin, Canada (Photo: D. Suggett); (**e**) Fasttrack II attached to a CTD frame in water column sampling in Eilat, Israel (Photo: D. Suggett)

of fluorometers for aquatic research in recent years; arguably, compared to 20 years ago, aquatic research investigations are incomplete without some form of fluorescence examination.

Variable fluorometers have clearly provided a platform for aquatic scientists wishing to conveniently assay photosynthetic physiology non-invasively and more accurately scale changes of photosynthesis to the environment. Current acceleration of environmental variability via climate change perhaps provides very real justification for further investing in tools such as fluorometers that have the capacity to link ecosystem processes with environmental regulation. Fluorescence-based technological development (including delayed fluorescence; Chapter 14 by Berden-Zrimec et al., this volume) combined with research has publically produced a tool that can potentially inform stakeholders of the photosynthetic 'viability' (or 'health') of their associated aquatic environment; certainly, a tool that is less labour intensive and costly in the long term than conventional (and destructive) assays that require water or organisms to be removed and analysed in the laboratory. Such applications to those wishing to monitor and subsequently manage ecosystem function was an obvious step in exchanging the knowledge beyond pure research but also necessary for commercial manufacturers to invest further in instrument production. Key examples to date come from the monitoring of lakes and coastal waters for (harmful) algal blooms (Cullen et al. 1997) and coral reefs for pollution and coral bleaching (Jones 1999). PAM Fluorometry has also been demonstrated in action for two BBC documentaries – by Prof Ove Hoegh-Guldberg examining coral bleaching for the BBC documentary *State of the Planet* and by Dr Rupert Perkins investigating stromatolites for *Oceans*) Furthermore, recent developments of algae as biofuels will inevitably require application of fluorometers to optimize and also continually monitor yields (Kromkamp et al. 2009; Sukenik et al. 2009) and thus further move fluorometers from a purely ecological to an industrial monitoring tool.

Despite the potential growth industry that obviously exists for chlorophyll fluorescence, it is clear that the previous growth of fluorometer technological development and the subsequent array of commercially available fluorometers have somewhat superseded our fundamental understanding of the fluorescence signals generated. It is perhaps quite ironic that technological developments have already enabled us to collect vast fluorescence data sets, however, we are only recently arming ourselves with the key knowledge required to interpret and consequently apply these data into informed opinion. Examining the growth of citations for (variable) fluorescence-based papers over the past decade is perhaps more testament to our confidence in interpreting the data as opposed to reduced constraints in collecting the data itself. Armed with a decades worth of what is arguably 'fluorescence exploration', It is really only now that we are beginning to gain maximum benefit of using fluorescence as a tool to address fundamental research questions in the aquatic sciences.

Why the need for AQUAFLUO? — Rapid growth of using active fluorescence across the aquatic science disciplines has inevitably led to divergence in approach and terminology (see Chapter 1 by Cosgrove and Borowitzka, this volume, for recommended terminology). Even though many of us have been attempting to answer similar questions, this divergence has resulted in a lack of consistency required to facilitate information exchange; consequently, the field was not evolving as quickly as originally envisaged. Arguably, the aquatic sciences still communicate fluorescence-based studies in numerous dialects that are often not easily inter-comparable or reconcilable.

Using fluorescence as a non-invasive means for assaying processes, such as (harmful) bloom detection and primary productivity, is still heralded as a key breakthrough for aquatic research and not surprisingly has attracted much funding and research time investment. However, efforts to capitalise on these larger process-scale problems have somewhat overshadowed our need to understand the fundamental nuances of fluorescence measurements using different instrumentation, protocols and for the array of aquatic primary producers that exist. On many occasions, the interpretation of data sets has been confounded by what is real in nature *versus* an artifact of instrument use. Conversations amongst the aquatic sciences community over recent years have increasingly identified the need for conformity in the application and operation of (active) fluorometers, not only to standardise and reconcile existing data sets but also to ensure that fluorometry remained 'accessible' to the ever-growing new user community. Such a step is indeed critical if fluorometry is ever to evolve from a purely academic tool to an everyday, practical and informative management tool. However, despite attempts to call for a common set of approaches (the best example to date for the

aquatic sciences is Kromkamp and Forster 2003), a few researchers have adapted their own approach and/or terms to fit. Perhaps the main limitation in adapting (amongst this rapidly growing and evolving field) has been where on earth should we start?

The concept of AQUAFLUO (AQUAtic FLUOrescence) was introduced in 2005 following breakout discussions between several of us at a meeting on modeling algal growth in Villefranche, France. It was then apparent that the immense popularity of fluorometry for aquatic studies was not a transient phenomenon. Both researchers and industry were increasingly investing in development of new fluorometers; however, the fundamental operational and conceptual issues that were limiting how confidently trends in variable fluorescence could be scaled to biology had become a bottleneck in supporting (and ultimately rationalising the need for furthering) this industry growth. Therefore, an organizing committee was established from a cross section of the aquatic community and these concerns translated into the ethos for an international meeting to be held at Nové Hrady in the Czech Republic in 2007 (Prášil et al. 2008). Initial priority questions were identified, including:

1. Is it time to step back from trying to focus on using variable fluorescence as a substitute for conventional productivity (^{14}C, ^{13}C, O_2) primary productivity techniques? Should we redistribute current research efforts and instead focus on the assessment of algal physiology and the general heterogeneity of algal physiology in nature?
2. How important (for the end users) are nuances in current fluorescence induction methodologies (e.g. single or multiple turnover flashes; single band *vs*. multi-spectral)? Can we constructively use these differences in experimental techniques to get better insight into algal physiology?
3. Can we expect successful scaling from direct to remotely sensed (LIDARs and satellites) variable fluorescence?

It was immediately clear that addressing such priority questions could only be achieved by bringing together researchers from across the aquatic disciplines (microalgae, macroalgae, submerged vascular plants, corals and aerobic anoxygenic photoheterotrophs (AAPs); lakes, rivers, coasts and oceans) as well as fluorometer manufacturers and engineers. By bringing this group together, the needs of all interested stakeholders could for the first time identify the common and complimentary needs required to move the field further forward and identify new opportunities; as well, to understand the individual needs of the various aquatic disciplines and develop specific approaches that may help to bridge gaps in consistency in the fluorescence yields that were being measured. A series of talks and workshops at the AQUAFLUO 2007 meeting led by leaders in key aquatic disciplines successfully laid the foundations in exploring these questions. Targeted research activities conducted since the meeting are already beginning to demonstrate that the original ethos of AQUAFLUO and the outcomes of the 2007 meeting are being adopted. However, given the continually evolving nature of using chlorophyll fluorescence in both physiological concept and technological approach, AQUAFLUO is seen as the beginning of a long-term relationship across the aquatic community and an idea with underlying goals and priority questions that will inevitably need to be continually revisited.

The chapters of this book communicate key components of the talks and workshops conducted during the AQUAFLUO 2007. Primarily, they address what measurements can be made and how; the common and aquatic discipline specific pitfalls that may be encountered in both performing measurements and interpreting the fluorescence yields themselves. In essence the book provides a guide to making Chlorophyll *a* fluorescence measurements in the various aquatic sciences and is certainly aimed at experienced and new users alike. *This book is certainly not intended to be a comprehensive review on the subject of Chlorophyll a fluorescence*; several key aspects are not considered in depth, notably remote sensing of variable fluorescence and examination of AAPs.

Each chapter summarises the progress specific to that discipline, the journey that Chlorophyll *a* fluorescence has taken in both approach and application; consequently, the scientific information that can be obtained. Importantly, these chapters also mark the output of that meeting: The first targeted effort to amalgamate the concerted efforts of leaders of the various fields/aquatic disciplines to identify what the next major conceptual (physiological, ecological, biogeochemical) questions that fluorescence measurements can contribute? What are the technological challenges

we need to overcome to realize these contributions? The following chapters highlight fundamental areas for research focusing (a) on a range of organisms from corals and macroalgae to microphytobenthos, and (b) scales, from photosynthetic physiology from the cellular level to mass culture. Importantly, these chapters aim to not only target experienced users but also present best practice, which represents optimisation through past (and often frustrating) research, to those new to the field. Of course, since AQUAFLUO 2007 and preparation of this book, our understanding of active fluorescence will have inevitably evolved even further.

Finally, we would like to thank the contributers and the many reviewers of the chapters for their valuable input.

David J. Suggett
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Contents

1 Chlorophyll Fluorescence Terminology: An Introduction 1
Jeff Cosgrove and Michael A. Borowitzka

2 *In Situ* Measurement of Variable Fluorescence Transients 19
Samuel R. Laney

3 Overview of Fluorescence Protocols: Theory, Basic Concepts, and Practice 31
Yannick Huot and Marcel Babin

4 Fluorescence as a Tool to Understand Changes in Photosynthetic Electron Flow Regulation 75
Peter J. Ralph, Christian Wilhelm, Johann Lavaud, Torsten Jakob, Katherina Petrou, and Sven A. Kranz

5 Microscopic Measurements of the Chlorophyll *a* Fluorescence Kinetics 91
Ondrej Komárek, Kristina Felcmanová, Eva Šetlíková, Eva Kotabová, Martin Trtílek, and Ondrej Prášil

6 Estimating Aquatic Productivity from Active Fluorescence Measurements 103
David J. Suggett, C. Mark Moore, and Richard J. Geider

7 Taxonomic Discrimination of Phytoplankton by Spectral Fluorescence 129
Hugh L. MacIntyre, Evelyn Lawrenz, and Tammi L. Richardson

8 Flow Cytometry in Phytoplankton Research 171
Heidi M. Sosik, Robert J. Olson, and E. Virginia Armbrust

9 The Use of the Fluorescence Signal in Studies of Seagrasses and Macroalgae 187
Susana Enríquez and Michael A. Borowitzka

10 Chlorophyll Fluorescence in Reef Building Corals 209
Mark E. Warner, Michael P. Lesser, and Peter J. Ralph

11 Assessing Nutrient Status of Microalgae Using Chlorophyll *a* Fluorescence 223
Kirsten Shelly, Daryl Holland, and John Beardall

12 The Application of Variable Chlorophyll Fluorescence to Microphytobenthic Biofilms 237
R.G. Perkins, J.C. Kromkamp, J. Serôdio, J. Lavaud, B. Jesus, J.L. Mouget, S. Lefebvre, and R.M. Forster

13 Chlorophyll Fluorescence Applications in Microalgal Mass Cultures 277
Jiří Masojídek, Avigad Vonshak, and Giuseppe Torzillo

14 Delayed Fluorescence 293
Maja Berden-Zrimec, Luka Drinovec, and Alexis Zrimec

15 The Study of Phytoplankton Photosynthesis by Photoacoustics 311
Yulia Pinchasov Grinblat and Zvy Dubinsky

Index 317

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Chapter 1
Chlorophyll Fluorescence Terminology: An Introduction

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1 Introduction

The terminology used to describe the various components of variable chlorophyll fluorescence has evolved as our understanding of variable chlorophyll fluorescence has increased. For the newcomer to *in vivo* chlorophyll *a* (chl-*a)* fluorescence studies one of the most confusing aspects can be the large number of terms and notations used, many of which often refer to the same parameter. Several proposals to standardise fluorescence notation, most notably by van Kooten and Snel (1990) and Maxwell and Johnson (2000), have reduced the extent of this variation in more recent publications, but some variation still occurs (Baker and Oxborough 2004). Furthermore, in recent years the notation used necessarily has become more complex as new instruments have allowed researchers to apply several techniques within a single study. On such occasions it is essential that notation also distinguishes between techniques (e.g. single turnover vs. multiple turnover) or method (e.g. steady-state light curve vs. non-steady-state light curve).

This chapter introduces the basic nomenclature associated with the study of variable chl-*a* fluorescence and provides some background on the parameters measured and/or derived. Here we present guidelines for the application and interpretation of chl-*a* fluorescence terminology, with the aim of enhancing communication and translation between methods.

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Since chlorophyll fluorescence is reliant on the prior absorption of photon energy, a section outlining the basics of light absorption and its estimation has been included. The accurate estimation of the amount of photosynthetically active radiation absorbed by LHCII and funnelled to RCII is essential for the calculation of fluorescence parameters such as electron transport rate through PSII.

2 Light and Absorption

Radiant energy, or irradiance, is expressed as energy incident per unit time and area; in earlier studies of photosynthetic irradiance was usually denoted with the symbol *'I'*, however, as *I* is also used for radiant intensity (W sr^{-1}), the symbol *E* is recommended by the International Union of Pure and Applied Chemistry (IUPAC) to denote irradiance (W m^{-2}) (Braslavsky 2007). Incident radiance in the 400–700 nm waveband is generally considered the photosynthetically active component of total spectral irradiance ($E_{(\lambda)}$) and is termed Photosynthetically Active Radiation (PAR or E_{PAR}) or photosynthetic photon flux density (PPFD or E_{PPFD}). Algae can use irradiance at wavelengths as low as 350 nm for photosynthesis, however, difficulties of measuring the 350–400 nm waveband and its very small contribution (~5–7%) to total irradiance (either solar or from commonly used emission sources) means this is usually ignored (Geider and Osborne 1991; Sakshaug et al. 1997). Since a photon of any wavelength between 400 and 700 nm is equally competent at generating charge separation, E_{PAR} is generally given in units of mol quanta m^{-2} s^{-1} or mol photons m^{-2} s^{-1} rather than

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Table 1 Summary of various terms and abbreviations used (see also Tables 2 and 3 for terms relating to the Kautsky curve and variable fluorescence)

Parameter	Other notations	Definition	Units
E	I	Irradiance	$W.m^{-2}$
$E_{(\lambda)}$	$I_{(\lambda)}$	Spectral Irradiance	$W.m^{-2}$
$\mathring{E}$		Scalar Irradiance	$W.m^{-2}$
PAR	PPFD	Photosynthetically active radiation – or	mol quanta m^{-2} s^{-1}
E_{PAR}	E_{PPFD}	-Photosynthetically active photon flux density[a]	mol photons m^{-2} s^{-1}
PSII	PS2	Photosystem II	
PSI	PS1	Photosystem I	
LHC I	LHC2	Light Harvesting Complex II	
RCII	RC2	Reaction Center II	
Φ_{PSII}		Quantum yield (efficiency) of electron transport through PSII	
Φ_{O2}		Quantum yield (efficiency) of oxygen evolution	mol O_2 evolved. absorbed $quanta^{-1}$
Φ_{CO2}		Quantum yield (efficiency) of carbon fixation	mol CO_2 evolved. absorbed $quanta^{-1}$
Φ_f		Quantum efficiency of fluorescence	
a^{chl}		Chlorophyll-*a* specific absorption coefficient	m^{-2} (mg chl-*a*)$^{-1}$
$\tilde{a}^{chl}$		Average chlorophyll-*a* specific light absorption coefficient	m^{-2} (mg chl-*a*)$^{-1}$
PUR^{chl}		Absorbed, chlorophyll-specific, photosynthetically usable radiation	µmol quanta (mg chl-*a*)$^{-1}$ s^{-1}
Q_{phar}		Absorbed photosynthetically usable radiation	µmol quanta m^{-2} s^{-1}
σ_{PSII}		Functional absorption cross-section for PSII	$Å^2$ $quantum^{-1}$; or m^2 mol $RCII^{-1}$
τ		PSII turnover time	s
η_{PSII}		Density of functional PS II centres	mol PSII (mol chl *a*)$^{-1}$
a_{PSII}^{chl}		chl *a*–specific light absorption for PSII photochemistry	m^2 (mg chl *a*)$^{-1}$

[a] The Joint Global Ocean Flux Study (JGOFS) Task Team for Photosynthetic Measurements recommends avoidance of the use 'photon flux density' (Sakshaug et al. 1997)

Einsteins m^{-2} s^{-1} (For easily quotable unit sizes standard practice is to convert from mol to µmol; e.g. 70 µmol photons m^{-2} s^{-1}). For phytoplankton cells which collect radiant energy equally for all sides the photosynthetically active scalar irradiance, $\mathring{E}_{PAR}$, measured with a spherical (4π) sensor should be used.

In order to obtain accurate estimates of the quantum yields (efficiency) of electron transport through PSII (Φ_{PSII}), oxygen evolution (Φ_{O2}) or carbon fixation (Φ_{CO2}), one must know the amount of E_{PAR} that is absorbed by the study organism. Difficulties associated with accounting for the scattering component of attenuation in optical measurements have limited the determination of absorbed irradiance as distinct from incident irradiance (Geider and Osborne 1991).

Spectrophotometric techniques are used for the determination of optical absorption coefficients and a number of techniques have been developed to estimate absorption of light by phytoplankton cells or macrophyte tissue.

Conventional spectrophotometers measure transmittance (T) and/or absorbance (A, also often referred to as O.D. (optical density)):

$$A(\lambda) = \log_{10}\left(\frac{E_\lambda^0}{E_\lambda}\right) = -\log_{10} T(\lambda) \qquad (1)$$

where E_λ^0 is the incident (prior to absorption) spectral irradiance and E_λ is the transmitted spectral irradiance.

Working with phytoplankton techniques that have negligible losses due to scattering, such as the opal glass method (Shibata et al. 1954), use of a diffusing plate and minimal sample-detector distance (Bricaud et al. 1983), or an integrating sphere (Bricaud et al. 1983; Maske and Haardt 1987), allows absorptance (A) to be calculated as follows:

$$A = -\log_{10}(1-\alpha) \qquad (2)$$

This parameter, used extensively for characterising the absorption of a cell suspension, is the absorption coefficient (a) with units of m^{-1}. The absorption coefficient is expressed as an exponential function of the absorptance (α) and the path length (l) in metres through the suspension:

$$a = -(1/l)\log_e(1-\alpha) \quad (3)$$

Rearranging the above equations allows a to be calculated directly from A:

$$a = 2.303 \cdot A/l \quad (4)$$

When expressed per unit mass of chlorophyll-a, the chlorophyll-a specific absorption coefficient a^{chl} is:

$$a^{chl} = a/[\text{chl-}a] \quad (5)$$

where [chl-a] is the chlorophyll-a concentration ($mg.m^{-3}$). a^{chl} has been found to vary from 0.004–0.043 m^2 (mg chl-a)$^{-1}$ (Geider and Osborne 1991).

The E_{PAR} that is absorbed and photosynthetically useable can be estimated by a number of different methods: two of the more commonly applied methods will be described here. Calculation of the absorbed, chlorophyll-specific, photosynthetically-usable radiation (PUR^{chl}) with units of µmol quanta (mg chl-a)$^{-1}$ s^{-1} can be calculated from Eq. 6. However, the parameter Q_{phar} (Eq. 7), which provides an estimate of absorbed photosynthetically usable radiation (PUR) in units of µmol quanta m^{-2} s^{-1} is used preferentially for the calculation of absolute Electron Transport Rate (ETR) (Gilbert et al. 2000a, b; Toepel et al. 2004; Wilhelm et al. 2004; Jakob et al. 2005). Estimation of either PUR^{chl} or Q_{phar} requires knowledge of both emission and absorption spectra.

$$PUR^{chl} = \sum_{\lambda=400}^{\lambda=700} E(\lambda) a^{chl}(\lambda) \quad (6)$$

$$Q_{phar} = \int_{400nm}^{700nm} E(\lambda) - E(\lambda) \cdot e^{\left(-a^{chl}(\lambda)\cdot[\text{Chl-}a]\cdot d\right)} d\lambda \quad (7)$$

where $E(\lambda)$ = photosynthetically available (incident) spectral radiation (µmol quanta m^{-2} nm^{-1} s^{-1}); $a^{chl}(\lambda)$ = Chl-a specific *in vivo* absorption coefficient of the cell suspension at wavelength λ in [m^2 mg^{-1}(chl-a)]; [chl-a] = Chl-a concentration in [mg (chl-a) m^{-3}]; d = optical path length (m).

Not all of the absorbed photosynthetically usable radiation is necessarily actively used in photosynthesis. Absorption by photosynthetic pigments is described by the photosynthetic cross-section (Suggett et al. 2003, 2004; Macintyre and Cullen 2005). The functional absorption cross-section for PSII (σ_{PSII}; $Å^2$ quanta^{-1}) is the product of the light-harvesting capability (optical absorption coefficient) of the photosynthetic pigments and the efficiency of excitation transfer to the reaction centre, and provides a measure of the effectiveness of incident light capture and conversion to electron transfer in PSII (Mauzerall and Greenbaum 1989; Kolber and Falkowski 1993; Wood and Oliver 1995). Decreasing growth irradiance and the onset of nutrient limitation lead to increases in σ_{PSII} (Kolber et al. 1988; Falkowski and Kolber 1995; Wood and Oliver 1995). On the other hand, non-photochemical dissipation of excitation energy, such as heat dissipation by the xanthophyll cycle, will decrease σ_{PSII} (Olaizola et al. 1994; Babin et al. 1996; Barranguet and Kromkamp 2000).

Algae may experience threefold changes in magnitude of σ_{PSII} without any changes in quantum efficiency of PSII as they adapt to different growth irradiances, although the quantum yield of O_2-evolution may be altered (Olaizola et al. 1994). All in all, measurements of σ_{PSII} in natural phytoplankton communities are highly variable, with data from Kolber and Falkowski (1993) suggesting a range from 250–1,000 $Å^2$ quanta^{-1} and an average of ~500 $Å^2$ quanta^{-1}. Relatively few measurements of σ_{PSII} have been obtained from individual taxa under controlled conditions (Suggett et al. 2004). However, a recent analysis of multiple ship cruise and controlled culture data-sets (Suggett et al. 2009) has highlighted the influence of phytoplankton community structure, including taxonomic composition (pigments) and cell size, on recorded σ_{PSII}.

As σ_{PSII} reflects light harvesting capacity of the PSII antenna and exciton transfer efficiency, the time interval between exciton arrivals at the PSII reaction centre can be calculated by:

$$\tau = \left(\sigma_{PSII} \times E_{PAR}\right)^{-1} \quad (8)$$

where τ is the PSII turnover time (s). τ can increase markedly as cells adapt to lower growth irradiance and the ability of the dark carbon fixation pathways to match the pace of the light-driven Electron Transport Chain (ETC) (when in saturating irradiances) is reduced (Kolber and Falkowski 1993) .

Importantly, for those studying chlorophyll fluorescence, σ_{PSII} multiplied by incident irradiance and the density of functional PS II centres, η_{PSII} (mol PSII (mol chl *a*)$^{-1}$), provides a measure of total photon flow available for charge separation at PSII (E_{PSII}) (Kromkamp and Forster 2003).

$$E_{PSII} = E_{PAR} \times \sigma_{PSII} \times \eta_{PSII} \quad (9)$$

Therefore the parameters σ_{PSII} and η_{PSII} combine to provide the chl *a*–specific light absorption for PSII photochemistry (a^{chl}_{PSII}; m^2 (mg chl *a*)$^{-1}$) (Suggett et al. 2004):

$$a^{chl}_{PSII} = \sigma_{PSII} \times \eta_{PSII} \times 0.00674 \quad (10)$$

where the constant 0.00674 is a conversion factor based on conversion of σ_{PSII} units to m^2 (mol PSII)$^{-1}$ and η_{PSII} to mol PSII (mg chl-*a*)$^{-1}$ (i.e. $\sigma_{PSII} \times 6023$ and $\eta_{PSII}/893490$).

Unfortunately η_{PSII} is difficult or impractical to measure and assumed values are commonly used (Suggett et al. 2004). Values of 1.6×10^{-3}–2×10^{-3} mol PSII (mol chl *a*)$^{-1}$ based on the measurements of total photosynthetic unit (PSU) size by Emerson and Arnold (1932) and Gaffron and Wohl (1936) are common. These authors measured between 2,000 and 2,500 chlorophyll molecules associated with each PSU and η_{PSII}^{-1} was considered to be one quarter of the PSU size due to the requirement for four electrons to pass through PSII for each O_2 molecule evolved (Ley and Mauzerall 1982). However, η_{PSII}^{-1} values ranging from 260 to 800 have been used (Kromkamp and Forster 2003 and references therein).

Adopting an assumed value for η_{PSII} may produce inaccurate results since η_{PSII} is known to change as a result of photoacclimation, photoinhibition and nutrient limitation (Suggett et al. 2004).

3 Fluorescence

Fluorescence is the re-emission of energy in the form of a photon (light) as an electron returns to ground state from a singlet excited state. In the case of chl-*a* fluorescence a chlorophyll molecule can become excited and achieve singlet state 1 (S_1) after absorbing a photon of less than 670 nm wavelength (Bolhàr-Nordenkampf and Öquist 1993). If the energy is not utilised in charge separation, heat dissipation, or resonance energy transfer, fluorescence will occur as the electron drops out of the excited state. As some energy is also given off as heat, the photon is red-shifted with an emission peak of ~685 nm. If the absorbed photon is of a shorter wavelength (e.g. blue light at about 420 nm) the extra energy excites the chlorophyll molecule to the singlet state 2 (S_2) and heat is emitted as it rapidly decays to the S_1 state. It is commonly considered that, at ambient temperatures, nearly all fluorescence (~90–95%) originates from PSII at 685 nm (Krause and Weis 1991; Papageorgiou et al. 2007) and represents 0.6% to ~10% of the absorbed light (Nicklisch and Köhler 2001). This is because the 680 nm absorption peak of chl-*a* molecule at the core of PSII is red-shifted only 5 nm from the absorption peak of the lowest singlet excited state of chl-*a* in most antenna systems. With such a small difference, energy can escape from RCII back into the pigment bed, hence PSII is known as a "shallow trap" and most fluorescence is emitted from the PSII antennae molecules (Krause and Weis 1988). The absorption maximum for PSI is sufficiently red-shifted (25 nm) relative to its antenna so that there is much less chance for energy to escape (Falkowski and Raven 1997).

Carl Lorenzen introduced the technique of *in vivo* chlorophyll fluorescence analysis to biological oceanography in 1966 (Lorenzen 1966). Since then a large number of different fluorometers have been designed to measure variable chl-*a* fluorescence under a wide range of conditions and for various applications. Each of these fluorometers is based on one of a few basic operational principals and can be classified as one of the following:

1. Pulse Amplitude Modulation fluorometer (PAM)
2. Fast Repetition Rate Fluorometer (FRR)
3. Fluorescence Induction and Relaxation System (FIRe)
4. Pump and Probe Fluorometer (PandP)
5. Induction Fluorometer/Continuous Excitation Fluorometer

The application and underlying theory of most of these methods for the *in situ* measurement of phytoplankton fluorescence has recently been reviewed (Babin 2008; Huot and Babin Chapter 3).

Light energy absorbed by a photosystem and its LHC can be used/dissipated through one of three competing pathways: (1) photochemistry (primary charge

separation and photosynthetic electron transfer), (2) thermal dissipation (non-radiative decay) or, (3) fluorescence emission (Falkowski et al. 1986; Seaton and Walker 1990; Kolber and Falkowski 1993; Nicklisch and Köhler 2001). It is assumed that the sum of the quantum yields of each of these processes is unity. Thus, changes in fluorescence yield reflect changes in the complementary pathways. It is important to note that the above-mentioned fluorometers measure fluorescence yield (which may vary up to a factor of 5 or 6), not fluorescence intensity (which may by a factor of several thousand), as it is the former that carries information on photosynthesis (Dau 1994; Schreiber 2004).

The quantum efficiency of fluorescence (Φ_f) is simply the ratio of quanta fluoresced to total quanta absorbed:

$$\Phi_f = Q_f / Q_a \tag{11}$$

where Q_a is the quanta absorbed and Q_f the quanta fluoresced. If Φ_f is known, then this can be used to determine fluorescence emission on a per unit chlorophyll basis:

$$F = E \times \bar{a}^{chl} \times \Phi_f \tag{12}$$

where F is the chlorophyll-specific fluorescence, E = incident irradiance, $\bar{a}\tilde{a}^{hl}$ = average chl-specific light absorption coefficient (m^{-2} (mg chl)$^{-1}$) (Estrada et al. 1996).

Higher Φ_f, and therefore higher F, is correlated with a closure of RCIIs since energy must then be dissipated to a greater extent by pathways other than primary charge separation (Ralph and Gademann 2005). As the redox state of the quinone Q_A determines whether RCII is open (Q_A) or closed (Q_A^-), it is also the main controlling factor determining chl-*a* fluorescence yield (Schreiber et al. 1998).

The Kautsky curve, also referred to as the fluorescence induction curve or the fluorescence transient, describes the characteristic changes in chl-*a* fluorescence yield upon illumination of a dark-adapted alga or leaf (Fig. 1). This pattern was first described by Kautsky and Hirsch (1931), but has been elaborated on since then (see Govindjee and Papageorgiou 1971; Govindjee 1995 for details) The fluorescence induction curve is a complex phenomenon that can be broken down into two primary phases: a *fast phase* (up to ~1 s) followed by a *slow phase* (up to several minutes) (Krause and Weis 1984; Govindjee 1995) (Fig. 1).

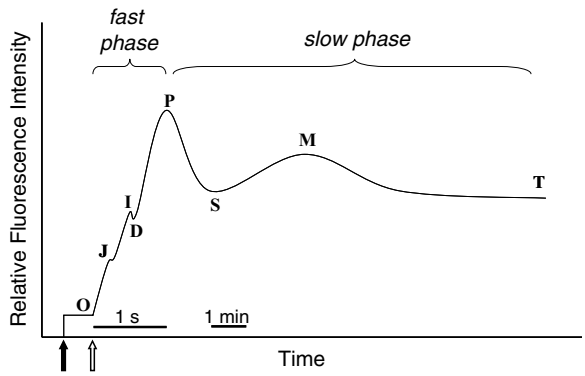

Fig. 1 Stylised representation of the chl-*a* fluorescence induction curve. Closed arrow represents activation of non-actinic measuring light. On application of strong actinic light (open arrow) fluorescence rises from the origin (O) to a peak (P) via two inflections (J and I). A dip (D) may occur after I. This O-P rise is known as the *fast phase* and reflects primary photochemistry and redox state of Q_A. After P fluorescence declines due to formation of a transthylakoid pH gradient and associated thermal quenching. The remainder of the transient (S-M-T) is called the *slow phase* and is the result of induction of Calvin cycle enzyme activity and its subsequent interaction with the electron transport chain (via NADPH) and photochemical and non-photochemical quenching

3.1 Fast Phase (O-J-I-P)

The fast phase of the fluorescence rise begins upon illumination of dark acclimated samples as fluorescence rises rapidly from the origin (O) to a peak (P) via an inflection (I) and dip (D) of variable magnitude (Fig. 1, Table 2). If the actinic light is strong another inflection (J) can be observed between O-I (Govindjee 1995; Strasser et al. 2004). The fast phase is often named after these cardinal points and referred to as the O-J-I-P curve, although other features may appear under certain experimental conditions (see *additional features* below), and reflects changes in the redox state of the RCII coinciding with the primary processes of photosynthesis (Govindjee and Papageorgiou 1971; Büchel and Wilhelm 1993). The fast phase has also been labelled the 'polyphasic fluorescence rise'.

The origin (O) of the fluorescence induction curve represents a minimum value and is fluorescence emitted from excited chl-*a* molecules in the antennae complex

Table 2 Nomenclature used in characterising features of the fluorescence induction curve

Parameter	Synonym	Definition	Time (ms)
O		Origin, Constant fluorescence, minimum fluorescence, initial fluorescence	0 or 0.05
J	I_1		~2–5
I	I_2	Inflection or intermediate	~30–50
D		Dip	
P	M	Peak	~500
L			~0.1–0.2
K			~0.3–0.4
H		Hump	
G			
S		Semi-steady state , stationary level	
M		Maximum	
T		Terminal steady-state	
F_i		Fluorescence yield at *i*, where *i* equals any of the parameters above (e.g. F_P equals yield at P)	

before excitons have migrated to the reaction centre (Krause and Weis 1984). This point is also given the terms *dark fluorescence*, *constant fluorescence*, *initial fluorescence* or *fluorescence minimum*. Fluorescence yield at O is commonly given the notation F_o (subscript o representing "origin"), F_0 (subscript 0 representing minimum) or simply F_{min}.

The rapid O-J rise in fluorescence represents the photochemical phase (Govindjee 1995), with fluorescence yield increasing proportionally with reduction of Q_A. Consequently the slope and height of this phase is dependent on incident light intensity (Lazár 2006). The inflection J occurs after ~2–5 ms of illumination (Lazár 2006; Papageorgiou et al. 2007), reflects a momentary maximum of Q_A reduction and is equivalent to the less common notation of I_1, of Neubauer and Schreiber (1987) and Schreiber and Neubauer (1987).

The remaining rise in fluorescence yield from J to its peak (P) represents the thermal phase, as it is impacted by temperature within the physiological range (slower at lower temperatures) (Lazár 2006). This phase of the fast fluorescence rise is shaped by the two-step reduction of Q_B ($Q_B \rightarrow Q_B^- \rightarrow Q_B^{2-}$) and a heterogeneity in the reduction of the plastoquinone (PQ) pool (Lazár et al. 1999). The second inflection I, also known as I_2 (Neubauer and Schreiber 1987; Schreiber and Neubauer 1987), occurs some 30–50 ms after illumination (Lazár 2006; Papageorgiou et al. 2007) and is thought to reflect a temporary maximum of $Q_A^-Q_B^{2-}$ (Govindjee 2004) , however other interpretations are presented in recent literature (Heredia and Rivas 2003; Ilík et al. 2006; Lazár 2006). The fluorescence yield may dip (D) after the J and/or I inflection(s) as electrons begin to move from one quencher to the next (e.g. Q_A^- to Q_B), resulting in transient reoxidation of the primary quencher (Krause and Weis 1984; Schreiber et al. 1998). If multiple dips occur it is standard to label each dip with a numeric subscript (e.g. O-J(D_1)-I(D_2)-P) (Govindjee 1995). Fluorescence yield then continues to rise as a lack of reductants on the PS II acceptor side becomes limiting, reaching the peak (P) when the PQ pool becomes fully reduced and $Q_A^-Q_B^{2-}$ concentration reaches a second maximum. In this situation, under saturating excitation irradiance, P is equivalent to F_m (maximum fluorescence yield).

3.1.1 Additional Features

The O, J, I, D and P features of the fast fluorescence transient are those that are most commonly observed under normal conditions. However, additional features can be observed in certain experimental conditions. Labelling of each of these features has been conducted such that they run in reverse alphabetical order between O and P.

The earliest feature is the L-band. This covers an area of the initial rise from O about 0.1–0.2 ms after the onset of illumination. Curvature of this portion of the fluorescence transient is related to PS II connectivity, with a shift to a more hyperbolic transient indicating greater connectivity (Oukarroum et al. 2007).

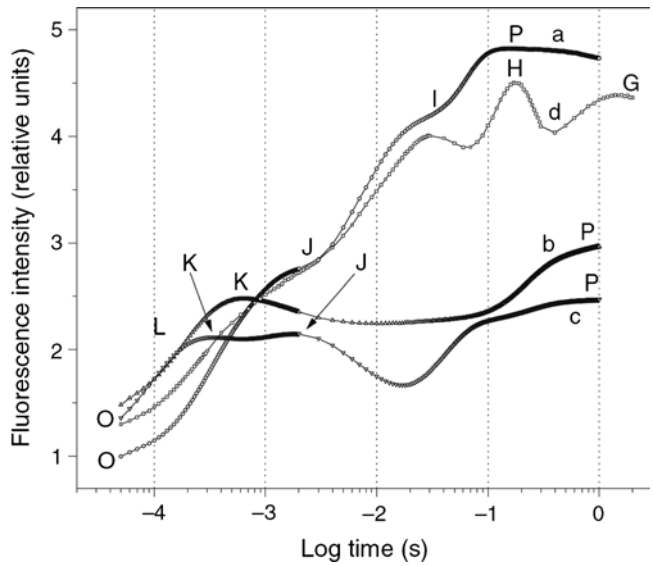

Fig. 2 Chl-*a* fluorescence rise measured with dark adapted pea leaves (curve a, not treatment; curve b, leaf incubated at 47°C in water for 5 min), potato leaf (curve c, leaf incubated at 44°C in water for 13 min), and the lichen *Umbilicaria hirsuta* (curve d, no treatment) by PEA fluorometer under high intensity of excitation light [3,400 μmol photons m^{-2} s^{-1} of red light]. Steps O, L, K, J, I, H, G and P are labelled (Figure modified from Lazár 2006)

Samples under stress, such as heat or drought stress or nitrogen limitation (Strasser et al. 2004; Oukarroum et al. 2007), can experience donor-side inhibition of PS II. When this occurs the K-band becomes apparent, typically ~0.2–0.4 ms after the onset of illumination. The K-band may even be associated with dissociation of the oxygen evolving complex (Oukarroum et al. 2007).

In some taxa P is effectively split into two separate peaks, H and G (curve d, Fig. 2). Ilík et al. (2006) proposed that this might be due to the combined actions of rapidly activated PS I reoxidation (ferredoxin-$NADP^+$ oxidoreductase activation) or an active Mehler-peroxidase reaction causing transient reoxidation of the PQ pool, Q_B^{2-} and Q_A^- (dip from H) before limitation of electron flow at cyt b_6/f causes a rise in fluorescence to the secondary peak, G.

3.2 Slow Phase (S-M-T)

Following the fast phase there is a polyphasic decline in chl-*a* fluorescence that ends at a terminal steady state level (T). This phase of the curve is known as the *slow phase* and may include a secondary peak (M) (refer to Walker (1981) for a discussion on the occurrence and dynamics of the M-peak) after a trough (S) (Fig. 1). This slow phase is primarily related to the balancing of a number of processes and several oscillations may be observed as overcompensation occurs in the regulation of reductive power and thylakoid energisation (Renger and Schreiber 1986). This results in successive S troughs and M peaks, each labelled with successive numeric subscripts (e.g. $S_1M_1S_2M_2$…T). In dark-adapted cyanobacteria, the S-M-T transient may dominate the fluorescence induction pattern, with fluorescence yield increasing above P as a result of State 2 → State 1 transition (Papageorgiou et al. 2007).

The decay in fluorescence from P → S is a complex phenomenon that has been described as the least understood part of the fluorescence transient (Govindjee and Papageorgiou 1971). Current understanding suggests that fluorescence yield decreases with the combined effects of enhanced PSI activity and ΔpH formation (Govindjee and Satoh 1986; Krause and Weis 1991). The faster passage of electrons through PSI, with the activation of ferredoxin-$NADP^+$ oxidoreductase results in a build up of qP as Q_A^- becomes partially reoxidised (Renger and Schreiber 1986). Formation of a transthylakoid pH gradient (ΔpH) results in protonation of acidic amino acids leading to conformational changes in core PSII antenna complexes that promote non-radiative dissipation of excess energy (Strasser et al. 2004; Holub et al. 2007; Papageorgiou et al. 2007). State transition quenching ($q(T_{1\rightarrow 2})$) is also

thought to contribute to the P-S decline . Such processes, and therefore the P → S feature of the fluorescence induction curve, depend upon an intact chloroplast envelope (Papageorgiou et al. 2007).

The S-M rise, when present, has been explained by LHC II dephosphorylation and a return to State 1 (Holub et al. 2007) and a lack of reductant ($NADP^+$) on the acceptor side of PSI (Govindjee and Satoh 1986; Renger and Schreiber 1986; Krause and Weis 1988). In phycobilisome-containing cyanobacteria the State 2 → 1 change, as the photosynthetic electron transport chain become reoxidised after reduction in the dark by components of the respiratory electron transport chain, can result in the S-M rise being the dominant feature of the fluorescence induction curve (see Figure 2 in Papageorgiou et al. 2007).

The decline from M to T is generally recognised as a reflection of activation of the Calvin-Benson Cycle enzymes. The resulting increase in CO_2 fixation rate and NADPH reoxidation has a flow-through effect back up the photosynthetic electron transport chain, yielding a higher qP (Renger and Schreiber 1986).

Due to the complex nature of the numerous interactions influencing the chl-*a* fluorescence induction curve there has been some question over the level of interpretation of the data that is possible (Holzwarth 1993; Trissl et al. 1993; Falkowski 1994; Trissl 1994), however, it is generally acknowledged that analysis of the induction curve remains a useful tool that will only improve as our knowledge of photosynthetic (and interacting) processes advances (Govindjee 1995).

3.3 The Saturation Pulse Method

The fast phase of the induction curve is commonly used to estimate photochemical quantum yield. The method applied is the *saturation pulse method* (Fig. 3) and involves analysis of the quenching components. Among the first to apply this method were Bradbury and Baker (1984) with refinement by Schreiber et al. (1986), and the technique has been further described (Schreiber et al. 1995b, c; Schreiber 2004). Minimum fluorescence yield (F_o) will occur when all RCIIs are open (Q_A in all RCIIs is oxidised) and quantum energy reaching the reaction centre has the maximal chance of being utilised photochemically and a negligible chance of being dissipated as heat or fluorescence. This state is generally considered to be achieved after adaptation to the dark and dissipation of any transthylakoid pH gradient

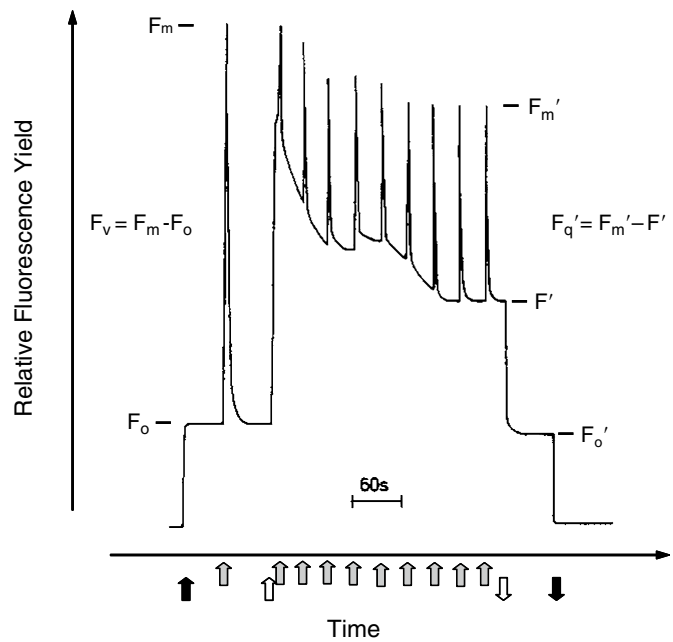

Fig. 3 Fluorescence induction kinetics including application of the saturation pulse method and associated nomenclature (refer to text). Dark arrows indicate measuring light on (*up*) and off (*down*); Grey arrows indicate application of a short pulse of saturating light; open arrows indication activation (*up*) and deactivation (*down*) of actinic light (Modified from Büchel and Wilhelm 1993)

(see *Quenching* below). In order to measure such a state the fluorometer's measuring light must be weak enough (<0.5 µmol quanta m^{-2} s^{-1}) so as not to induce reduction of Q_A and closure of any reaction centres. When a pulse of high intensity light sufficient to close all RCIIs (reduce all Q_A) is applied to a sample, a condition is induced where photochemistry is reduced to zero and fluorescence yield is maximal. If the sample was dark-adapted prior to application of the saturation pulse, non-photochemical quenching will be negligible and fluorescence yield will reach its true maximum (F_m). However, if the sample was not dark-adapted, non-photochemical quenching will act to quench the fluorescence yield and the achieved maximum value will be lower (F_m'; where the ′ denotes that the sample was not dark adapted). Hence a drop in F_m to F_m' can be used as a measure of non-photochemical quenching. These measures assume that no non-photochemical quenching is induced by the short saturation pulse (Schreiber et al. 1995a; Schreiber 2004). We can therefore use these principles to estimate photochemical quenching (see Fig. 3 and Table 3). It should be

Table 3 Fluorescence parameters, their definition and their synonyms (following Baker et al. (2001)

Parameter	Synonyms	Definition	Derivation
ETR	J_e	Electron Transport Rate (through PSII)	
ETR_m	ETR_{max}	Maximum ETR	
rETR		Relative Electron Transport Rate (through PSII)	$F_q'/F_m' \times E_{PAR}$ $(F_q'/F_m' \times E_{PAR} \times 0.5)$
$rETR_m$	$rETR_{max}$	Maximum rETR	
F_o	F_0, F_{min}, Φ_{Fo}, Φ_f^{min}	Fluorescence yield at O; Minimum fluorescence yield; dark fluorescence yield (dark adapted, all RCIIs open)	
F_m	F_{max}, Φ_{Fm}, Φ_f^{max}, (F_P)	Maximum fluorescence yield (dark adapted, all RCIIs closed with no NPQ), Fluorescence yield at point P (F_P) of the fluorescence induction curve is equivalent to F_m if irradiance is saturating.	
F_v	Φ_{Fv}	Maximum variable fluorescence yield (qN = 0)	$F_m - F_o$
F_v/F_m	Φ_{Po}, Φ_P^{max}, Φ_{PSII}^{max}, $\Delta\Phi_m$	Maximum photochemical efficiency (quantum yield) of open RCIIs. [The term $\Delta\Phi_m$ is the equivalent term for single turnover saturation pulse measurements.]	$(F_m - F_o) / F_m$
F′	F, F_t, Φ_F, F_v	Fluorescence yield in actinic light; fluorescence yield at time *t* (F_v is from Schreiber et al. (1986))	
F_s	Φ_{Fs}, F_T	Steady-state fluorescence yield in actinic light. F_T represents fluorescence yield at point T of the fluorescence induction curve.	
F_o'		Minimum fluorescence yield in light-acclimated state (Usually measured with the application of far-red light)	
F_m'		Maximum fluorescence yield in actinic light	
F_v'		Variable fluorescence yield in actinic light	$F_m' - F_o'$
$F_m'_m$		The maximum value of F_m'	
F_q'	ΔF	Difference between fluorescence yields F_m' and F′	$F_m' - F'$
F_q'/F_m'	$\Delta F / F_m'$, Φ_{PSII}	Effective photochemical efficiency of RCIIs in actinic light	$(F_m' - F') / F_m'$
F_v'/F_m'		Maximum PSII photochemical efficiency or quantum yield in actinic light.	$(F_m' - F_o') / F_m'$
NPQ		(Stern-Volmer) Non-photochemical quenching	$(F_m - F_m') / F_m'$
q_E	qE, q(E)	Energy-dependent non-photochemical quenching	$(F_v - F_v') / F_v$
q_I	qI, q(I)	Photoinhibitory non-photochemical quenching	
q_N	qN, q(N)	Non-photochemical quenching	$(F_m - F_m') / (F_m - F_o)$
q_P	q_Q, F_q' / F_v', qP, q(P)	Photochemical quenching	$(F_m' - F') / (F_m' - F_o')$
q_T	qT, q($T_{1\to2}$)	State-transition related non-photochemical quenching	

All parameters are dimensionless

noted here that there is the potential for induction of non-photochemical quenching pathways in the dark (possibly linked to chlororespiration) (Jakob et al. 2001) which would impact particularly the F_m value. The potential error caused by this can often be overcome by applying a short pulse of far-red light to ensure full reoxidation of the PQ pool (P. Ralph, personal communication) or by correcting F_o and F_m based on measured non-photochemical quenching (Ting and Owens 1993). However, in some experimental situations it may be necessary to use the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to be confident of measuring true F_m.[1]

The measured F_m (or F_m') is dependent on the measuring technique. Some fluorometers, such as the FRR type, apply a rapid flash (also called a "flashlet") that is short enough (10–100 μs) to result in only a single reduction of Q_A to close the RCII and cause a rise in fluorescence through the photochemical phase of the induction curve, but not the thermal phase. Thus, F_m measured using this 'single turnover' technique is roughly equivalent to F_J of the fluorescence induction curve, although with the application of DCMU it can be significantly higher than F_J due to a reduction in quenching by Q_B and the PQ pool. Other fluorometers, such as the PAM type, apply an extended flash (usually between 0.4 and 1.0 s) of high intensity light that results in multiple turnovers of the reaction centre to saturate Q_A, Q_B and PQ to close all RCIIs (i.e. both the photochemical and thermal phases are induced) and F_m is equivalent to F_P of the fluorescence induction curve. The Fluorescence Induction and Relaxation (FIRe) system by Satlantic employs both single- and multiple-turnover protocols. A comprehensive comparison of these two saturation techniques and their respective advantages and disadvantages has been provided by Kromkamp and Forster (2003) and Babin (2008), however, it is fundamentally important to note that direct comparison of results between single turnover and multiple turnover techniques cannot be made as they yield different results. Even direct comparison of results within the same fluorometer type, PAM fluorometers for example, should be done with some caution since system geometry and the position of the detector relative to the cuvette/sample (Mouget and Tremblin 2002) or stirring in the Water-PAM (Cosgrove and Borowitzka 2006) can affect results. It is for these reasons, amongst others, that researchers should carefully detail their equipment and methodology used and nomenclature should, where confusion could occur, specify the method or technique used.

Examples of the used of well applied nomenclature aimed at distinguishing methodologies are included here. Rascher et al. (2000) compared effective quantum yield ($\Delta F/F_m'$) calculated from saturation pulse measurements taken under ambient conditions with those calculated from a light curve protocol. An extra subscript term (i.e. $\Delta F/F_{m'amb}$ or $\Delta F/F_{m'LC}$) was used to indicate which method was used. This approach was also employed by Kromkamp and Forster (2003) and Kromkamp et al. (2008) to distinguish parameters derived from single turnover (ST) flashes from those derived from multiple turnover (MT) flashes, although on this occasion the added descriptor was enclosed in brackets when other subscript notation was present (e.g. $F_{m(ST)}$ vs. $F_{m(MT)}$ or ETR_{ST} vs. ETR_{MT}). This nomenclature has, in principle, been followed by others such as (Röttgers 2007), who removed the brackets from the method descriptor (e.g. $F_{m,ST}$ *vs.* $F_{m,ST}$; $rETR_{m,ST}$ vs. $rETR_{m,MT}$). For clarity we recommend the use of brackets for the method descriptor.

Similarly, a number of authors (Kühl et al. 2001; Serôdio et al. 2006; Ulstrup et al. 2007; Cruz and Serôdio 2008) have calculated fluorescence parameters and photosynthetic electron transport from both Rapid Light Curves (RLCs) and steady-state light curves (LCs; also referred to as SSLCs by Ulstrup et al. (2007). Serôdio et al. (2006) and Cruz and Serôdio (2008) used the notation to clearly differentiate between the methods (e.g. $ETR_{m,RLC}$), while other workers have chosen to do so in the text.

3.4 Quantum Yield for PSII (Φ_{PSII})

The efficiency of a light-dependent process is referred to as the *quantum yield* or *quantum efficiency*. In basic terms the quantum yield (Φ) can be described as the ratio of product output to gathered quanta:

$$\Phi = \frac{mol\ product\ out}{mol\ quanta\ in} \tag{13}$$

Saturation pulse analysis, as described above, can be used to estimate the quantum yield, or efficiency, of

[1]DCMU must be added in total darkness and the sample should not be exposed to any light before measurements are made: Since DCMU functions by displacing Q_B, even low light can cause quick net formation of Q_A^- artificially raising the measured F_0 as Chl fluorescence is high when Q_A^- is present (Govindjee 2004; Huot and Babin Chapter 3).

PSII photochemistry, Φ_{PSII} (Genty et al. 1989; Schreiber et al. 1995c). In this circumstance the end product can be described as primary charge separation and the passage of an electron through PSII.

When in the dark, with Q_A in a fully oxidised state and no non-photochemical quenching, the *maximum* quantum yield of PSII (Φ_{PSII}^{max}) can be estimated by normalising the variable fluorescence (F_v) to the maximum fluorescence yield (F_m) (Table 3). In the light the photochemical efficiency of PSII and closure of reaction centres (reduced Q_A) decreases due to the induction of non-photochemical quenching (and possibly photoinhibition in high light) and the parameter F_q' ($F_q' = F_m' - F'$; see Oxborough et al. (2000)), where subscript "q" signifies quenched fluorescence, is normalised to the light adapted maximum fluorescence yield (F_m') (Table 3). Thus, by applying the saturation pulse methods the quantum yield of PSII can be rapidly measured on a virtually real-time basis with high sensitivity (Kolber et al. 1994).

Maximum theoretical values for F_v/F_m are ~0.65 (Kolber and Falkowski 1993) for single turnover saturation pulses and ~0.83 for multiple turnover pulses (Magnusson 1997)}. In practice, maximum achievable F_v/F_m is known to vary between taxa as a result of differences in pigment composition and cell structure (Koblížek et al. 2001; Suggett et al. 2009). For example, smaller taxa appear to have lower F_v/F_m values (as low as 0.3–0.4 for the smallest pico-eukaryotes) along with higher σ_{PSII} values (Suggett et al. 2009). Environmental factors that impact upon PSII, directly or indirectly, will also impact measures of F_v/F_m (Greene et al. 1992). Dominant factors in this regard include light, nutrient status and temperature (Wozniak et al. 2002), however Brand (1982) also found that many marine phytoplankton species exhibit endogenous diel patterns in fluorescence parameters and suggested this may be the result of changes in cellular metabolism to "predict" environmental condition. All these factors combine to confound interpretation of F_v/F_m and other fluorescence parameters (Kroon et al. 1993).

3.5 Quenching

Fluorescence, or radiative decay, is one of three competitive pathways for the de-activation of chl-excited states in the photosynthetic reaction centres and their antennae. Thus, the other two pathways, photochemistry and non-radiative decay (heat dissipation), act to *quench* the fluorescence signal. These processes are called photochemical- and non-photochemical quenching respectively. To quantify these quenching pathways various quenching coefficients have been defined. As mentioned previously, the saturation pulse method can be used to measure each of the quenching components.

Photochemical quenching, q_P, estimates the percentage of RCIIs that are open (Magnusson 1997) or the capacity for photochemistry to compete for trapped quantum energy (Ting and Owens 1993). When all reaction centres are open $q_P = 1$ and when all centres are closed $q_P = 0$ (Schreiber et al. 1986). The RCII is considered 'open' when Q_A is oxidised and capable of accepting an electron from RCII via pheophytin. It is important to note that energy transfer between RCIIs, or "*connectivity*", modifies the linear relationship between q_P and the fraction of RCIIs that are open (Suggett et al. 2003; Schreiber 2004). Thus, q_P more accurately represents the redox state of Q_A. The quinones Q_A and Q_B where labelled "Q" because they act to *quench* fluorescence, Q_B is a secondary quencher as it acts to re-oxidise Q_A, thereby returning it to its quenching state. Similarly, the redox status of PQ may influence q_P. For example, q_P can be increased by increasing ambient dissolved inorganic carbon (DIC) concentrations as this acts to favour the Rubisco carboxylase reaction and increase the rate of linear electron flow, resulting in partial reoxidation of the PQ pool (Carr and Björk 2003). Given that these primary components influencing q_P are highly conserved across taxa, the mechanism of q_P are likely to be similar (Ting and Owens 1993).

Based on the work of Bilger and Schreiber (1986) who found that F_o could be quenched to F_o', q_P was defined by Van Kooten and Snel (1990) with standardised nomenclature (see Table 3). F_o' may be hard to measure and on occasion F_o has been used for the calculation of q_p instead (e.g. Weis and Berry (1987) or Ralph and Gademann (2005)). This risks overestimation of q_P and, as a consequence, an alternative method for deriving F_o' has been described (Eq. 14) (Oxborough and Baker 1997).

$$F_o' = \frac{F_o}{F_v / F_m + F_o / F_m'} \tag{14}$$

The total non-photochemical quenching coefficient (q_N) is a measure of the fraction of maximum dark-adapted variable fluorescence ($F_m - F_o$) that is quenched in the light (Eq. 15). This coefficient has two disadvantages in that: (1) it involves the estimation of F_o', and (2) there is evidence that it may be influenced directly by the rate constant of photochemistry (Krause and Jahns 2004). Another measure of total non-photochemical quenching (NPQ) can be calculated using the Stern-Volmer equation (see Table 3). In this case NPQ represents the relative increase in the sum of the rate constants of the non-photochemical deactivation processes (fluorescence emission, heat dissipation and spillover of excitation energy from PSII to PSI) relative to the dark-adapted state (assuming no non-photochemical reduction of the PQ pool in the dark) (Krause and Jahns 2004). The parameter NPQ (Eq. 16) is considered more robust and is often used in preference to q_N (Ralph and Gademann 2005).

$$q_N = 1 - \left(F_m' - F_o'\right) / \left(F_m - F_o\right) \tag{15}$$

$$NPQ = \left(F_m - F_m'\right) / F_m' \tag{16}$$

The primary site for the development of non-photochemical quenching is thought to be the light harvesting antennae and is largely independent of Q_A redox state (Ting and Owens 1993; Oxborough and Baker 1997). Given the diversity in composition of light harvesting complexes one may expect the mechanisms and response of non-photochemical quenching to vary between taxa.

Non-photochemical quenching of chl fluorescence has three major components, each of these components can be distinguished by careful analysis of dark relaxation kinetics, as described by Horton and Hague (1988). The dominant component of non-photochemical quenching is *energy dependent* quenching (q_E). However, the relative contribution of each NPQ component is dependent upon the light history of the sample and conditions under which the measurements were taken (Ting and Owens 1992). Energy dependent quenching is also the quickest NPQ component to relax upon return to darkness and reports on q_E relaxation time vary from 30–60 s (Ralph and Gademann 2005) to 2–3 min (White and Critchley 1999) or a $t_{½}$ of <1 min (Masojidek et al. 1999).

Energy dependent quenching can account for up to 90% of the decay in F_v on exposure to light (Krause and Weis 1991). This down-regulation of PSII photochemistry may act as a photoprotective mechanism by reducing the potential for the formation of triplet state chlorophyll ($^3chl^*$) in the RCII and the formation of reactive oxygen species (Krause and Jahns 2004) and there is overwhelming evidence indicating that photoinhibition is diminished by the development of a large q_E (Krause and Weis 1991). An early adaptive response by plants and algae in unfavourable conditions is to increase q_E (Schreiber et al. 1995b) and plants adapted to high light have been shown to have more active q_E and less PSII closure than low light adapted plants (Ralph and Gademann 2005).

The next NPQ component has a relaxation half-time of ~5–10 min and is known as state transitional quenching, or q_T (Masojidek et al. 1999). As state transitions (state I → state II) involve the movement of LHCII*b* from PSII to PSI and as comparatively little fluorescence escapes from PSI, fluorescence is consequently quenched (Schreiber et al. 1995b). In this scenario F_v and F_o are quenched by the same proportion (Krause and Weis 1991). While q_T may be of particular importance in low light environments, as light increases acidification of the lumen appears to inhibit LHCII*b* phosphorylation and the role of q_T may become negligible (Krause and Jahns 2004). However, there remain many question as to the mechanisms and function of state transitions, especially in the Chromista, hence the role and activity of q_T in many taxa remains unclear.

The slowest NPQ component to relax, taking >10 min to hours, is photoinhibitory quenching (q_I) which is related to photoinhibitory damage of PSII. There is general agreement that the primary source of q_I is damage of the PSII core protein D1 as a result of donor- or acceptor-side photoinhibition (Hill et al. 2005). However, since q_I formation may precede inactivation of D1, other mechanisms such as the presence of persistent levels of de-epoxidised xanthophyll cycle pigments in the dark (Krause and Jahns 2004) must be considered. Photoinhibitory quenching relaxes only as PSII repair mechanisms take place, such as the synthesis and replacement of damaged D1 proteins (Masojidek et al. 1999). Due to the longer recovery time q_I can be indicated by a reduction in F_v/F_m after exposure to high light. As a rule, this reduction in F_v/F_m is the result of diminished F_v, however the contribution of the F_o component to this decrease may vary (Krause and Weis 1984, 1991).