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TECHNICAL COMMUNICATION

Applying Pulse Amplitude Modulation (PAM) fluorometry to microalgae suspensions: stirring potentially impacts fluorescence

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Abstract The use of microalgae suspensions in PAM-fluorometers such as the Water-PAM (Walz GmbH, Germany) presents the problem of maintaining a homogeneous sample. The Water-PAM is marketed with an optional accessory for stirring the sample within the cuvette while in the emitter–detector (ED) unit. This stirring device can help to prevent cells from settling out of suspension over the time-course of chlorophyll-*a* fluorescence measurements. The ED unit was found to provide a vertically heterogeneous light environment and, therefore, cells within a single sample can exist in different quenched states. Enhancing cell movement by stirring was found to substantially influence measured fluorescence yield while performing induction curve and rapid light curve analyses. This is likely to result from relatively unquenched cells outside the main light-path moving into a higher light region and thus emitting disproportionately more fluorescence than quenched cells. Samples containing cells with high sinking rates or motile species may encounter similar (but reduced) problems. This effect can be mitigated by: (a) reducing analysis time to minimise the distance cells can sink/swim during the measurement procedure and avoiding the necessity of stirring; (b) limiting the proportion of sample outside the light path by minimising sample volume or; (c) by activating the stirrer only for short periods between saturation pulses and allowing enough time after stirring for quenching to stabilise before activation of the saturation pulse. Alternatively, modifications to the instrument providing a vertical dimension to the LED-array could resolve the issue by providing a more homogeneous light environment for the sample.

Keywords Chl-*a* fluorescence · Electron transport rate · Methodology · Non-photochemical quenching · PAM · Quantum yield

Abbreviations

ED unit	emitter–detector unit
F	fluorescence yield
F_m	maximum fluorescence yield in the dark-adapted state
F'_m	maximum fluorescence yield in the light-adapted state
$F'_{m\ m}$	maximum F'_m value
F_v/F_m	maximum photochemical yield in the dark-adapted state
LED	light-emitting diode
NPQ	non-photochemical quenching
PSII	photosystem II
PAM	pulse amplitude modulation
rETR	relative electron transport rate
$rETR_{max}$	maximum relative electron transport rate
RLC	rapid light curve

Introduction

Chlorophyll-*a* fluorescence serves as a valuable probe of the primary biophysical events in photosynthesis (Falkowski et al. 1986; Hill and Ralph 2005; Lazár et al. 2005), including the efficiency of PSII photochemistry (Beardall et al. 2001). The development of

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highly sensitive Pulse Amplitude Modulation (PAM) fluorometers in the early 1990s (Schreiber et al. 1993), and further refinement of the electronics since then, has resulted in a recent surge in the application of Chl-*a* fluorescence in scientific research (Samson et al. 1999; Villareal 2004; Jakob et al. 2005). Instruments with sufficient sensitivity to measure variable Chl-*a* fluorescence of marine phytoplankton in coastal waters are now available (Schreiber et al. 1993; McMinn and Hegseth 2004).

The Water-PAM (Walz GmbH, Germany) was designed for the assessment of phytoplankton in natural surface waters. Light-emitting diodes (LEDs) provide non-actinic measuring light (3 LEDs, spectral peak at 650 nm), actinic light/saturation pulses (12 LEDs, spectral peak at 660 nm) and far-red light (3 LEDs, spectral peak at 730 nm). These LEDs are contained in the ED unit and are arranged in a ring which circles the 15 mm Ø quartz sample cuvette. When in place, the base of the sample cuvette rests upon the detector (Fig. 1a).

Accurate and precise fluorescence measurement with the Water-PAM requires cell distribution to remain homogenous during sampling and that all cells experience the same irradiance. This basic principle also applies to other PAM-fluorometers of similar design, such as the PHYTO-PAM (System II PHYTO-ED configuration) and Toxy-PAM (Walz GmbH, Germany). The Water-S stirring device (supplied as an optional accessory) is aimed at maintaining sample homogeneity and preventing cells from settling to the bottom of the cuvette (Fig. 1a). Observations during use of the Water-PAM fluorometer have indicated that the act of stirring the sample can, however, have a profound effect on fluorescence readings and the derived parameters. This paper presents evidence for this and suggests a design modification that the authors feel could negate this effect.

Methods

Nannochloropsis oculata CS179 (Droop) Hibberd (Eustigmatophyceae) was chosen as the study organism as it is considered hardy (not physically impacted by stirring) and has a slow sinking rate resulting in little to no cell movement over the duration of a rapid light curve (RLC) or induction curve. A vertical flat plate photobioreactor (5.5 l) was used to grow *N. oculata* in semi-continuous culture at 25 ± 2°C at an irradiance of 540 μmol quanta m^{-2} s^{-1} with a 12 h:12 h light/dark cycle provided by cool white fluorescent lamps set along both sides of the reactor. Mixing was provided by bubbling air at a rate of 1.0 l min^{-1}. The culture was maintained in logarithmic-phase by daily dilutions based on cell density. Culture media consisted of double-filtered seawater (charcoal-treated then filtered through Whatman filter paper followed by 0.45 μm MFS mixed cellulose ester) enriched with f/2 − Si nutrients (modified from Guillard and Ryther 1962).

Samples were collected from the photobioreactor and placed into darkened vials for 10 min dark adaptation. Dark adaptation of 10 min had previously been found to yield the peak F_v/F_m (data not shown). Samples were then transferred to the sample cuvette (in a darkened room), which was immediately inserted into the ED unit and the stirrer unit fitted. Sample volume was maintained at 2.5 ml unless otherwise specified. Far-red (FR) light was subsequently applied for 5 s to ensure full oxidation of Q_A. Once fluorescence had stabilised after applying FR light, either the

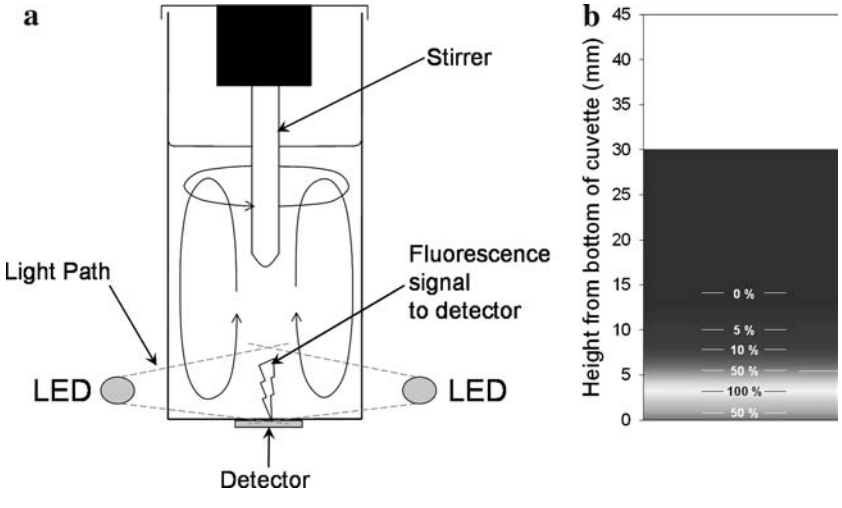

Fig. 1 (a) Basic line drawing (not to scale) of Water-PAM cuvette with stirring device in place. Stirring is both in the horizontal plane and the vertical plane; (b) Light distribution measured in the centre of the cuvette when mounted within the ED-unit of the Water-PAM

induction curve or rapid light curve (RLC) programme (within WINCONTROL software, Walz GmbH) was initiated. Two treatments were used: (1) not stirred and; (2) stirred. For both treatments the stirring device was in place, however, it was not activated during treatment 1.

Chlorophyll *a* content was calculated spectrophotometrically using the equations of Jeffrey and Humphrey (1975) after extraction of filtered samples (Whatman GF/F) in 90% acetone. All measurements were performed both at 'high' (140.9 ± 11.9 μg Chl-*a* l^{-1}; mean ± se, n = 3) and 'low' (6.3 ± 0.27 μg Chl-*a* l^{-1}; mean ± se, n = 3) chlorophyll concentrations.

Light distribution in Water-PAM

The relative distribution of light in the Water-PAM was measured using the spherical micro-quantum sensor attachment (US-SQS/WB, Walz GmbH). The ED-unit actinic light was activated and irradiance measured at 1 mm intervals from the bottom of the cuvette. Actinic light was turned off between measurements to avoid changes in LED temperature.

Rapid light curves

Rapid light curves were performed with 10 s exposure duration to each of eight incremental irradiances (50, 76, 118, 176, 271, 406, 579 and 806 μmol quanta m^{-2} s^{-1} measured at the base of the cuvette with US-SQS/WB sensor) after an initial quasi-dark measurement (~1.5 μmol quanta m^{-2} s^{-1}) to provide estimates of F_o and F_m.

Rapid light curves were constructed by calculating relative electron transport rate (rETR, Eq. 1) through PSII for each level of actinic light:

$$rETR = \left(\frac{F'_m - F}{F'_m}\right) \times PAR \times 0.5 \quad (1)$$

where $(F'_m\text{-}F)/F'_m$ estimates the effective quantum yield of PSII, PAR is the actinic irradiance in μmol quanta m^{-2} s^{-1}, 0.5 is a multiplication factor based on the assumption that 50% of the absorbed quanta are distributed to PSII (Beer et al. 1998b).

RLC data were fitted to the model of Platt et al. (1980) in order to obtain values for the initial slope (α), inhibition term (β), light saturation parameter (I_k) and maximum relative electron transport rate ($rETR_{max}$). I_k was derived from $rETR_{max}$ and α (Eq. 2):

$$I_k = rETR_{max}/\alpha \quad (2)$$

Non-photochemical quenching (NPQ) was calculated following Serôdio et al. (2005) since F'_m values were found to be higher than F_m in stirred samples (Eq. 3):

$$NPQ = \left(F'_{m\,m} - F'_m\right)/F'_m \quad (3)$$

where $F'_{m\,m}$ is the maximum F'_m value and was replaced by F_m when the dark-adapted value was the highest.

An additional observation was made by plotting the fluorescence trace of a light curve (LC) with a longer duration at each actinic irradiance (3 min). Sample homogeneity was maintained during the extended time period of this curve by stirring. Stirring was performed for 10 s, beginning approximately 10 s after each saturation pulse, leaving over 2.5 min for fluorescence to stabilise before the next saturation pulse.

Induction Curves

Induction curves were measured with a delay of 30 s between determination of F_v/F_m and onset of actinic illumination (406 μmol quanta m^{-2} s^{-1} measured at the base of the cuvette). A saturation pulse, measuring quantum yield and NPQ, was performed immediately after the onset of actinic illumination and every 20 s thereafter until deactivation of actinic light 4 min later. A trace of the fluorescence signal was recorded for each treatment and changes in NPQ and quantum yield were calculated as previously described.

Diving-PAM

RLC and induction curve data were also obtained using a Diving-PAM (Walz, Germany) to use as a reference against Water-PAM data to assist in discerning between 'typical' and 'atypical' fluorescence patterns. *Nannochloropsis oculata* was filtered onto a Whatman GF/C filter paper under low pressure and left damp. In a darkened room the Diving-PAM's 8-mm fibre-optic probe was positioned ~3 mm above the filter paper and RLC and induction curve programmes activated. The fibre-optic probe was repositioned to a fresh, darkened portion of the filter between each measurement.

Results

The impact of stirring was similar at both chlorophyll concentrations, however more background noise was

evident when analysing the 'low' chlorophyll samples (higher photomultiplier gain settings used). For this reason only results for 'high' chlorophyll concentration samples are presented here.

Light distribution in Water-PAM

Irradiance measurements indicate that the single ring of LEDs produces a narrow band of light with maximum irradiance measured just over 3 mm above the base of the cuvette (Fig. 1b). Just ~2.1 mm further above or below this point irradiance was 50% of maximum. The use of the Water-S stirring accessory promotes mixing of the sample both horizontally and vertically (Fig. 1a) and, as a consequence, cells move through different irradiance fields.

Rapid Light Curves

Representative plots of the fluorescence trace for each treatment are presented in Fig. 2. These fluorescence data indicate a substantial influence of stirring on the dynamics of fluorescence measurements. In particular, F'_m remains high and F increases with each increase in PAR in stirred samples compared to unstirred samples (Fig. 2b). This resulted in higher effective quantum yield values, hence α was significantly higher in the stirred treatment compared to the unstirred treatment (Table 1). While $rETR_{max}$ was significantly higher in stirred samples (Table 1, Fig. 3a) as a result of enhanced F'_m values, the effect was limited by increasing F at higher PAR (Fig. 2b). NPQ in stirred samples was severely diminished by artificially high F'_m values (Table 1, Fig. 3b).

The fluorescence chart from the extended LC (3 min at each irradiance) clearly illustrates an impact of stirring on the fluorescence signal (Fig. 4). There is also an indication that the impact of stirring becomes greater at higher actinic irradiances, with negligible impact observed during the first actinic light level (50 μmol quanta m^{-2} s^{-1}).

Induction Curves

Induction curve data were adversely impacted by stirring samples during chlorophyll-*a* fluorescence measurements in the Water-PAM. Representative plots of the fluorescence trace for each treatment are presented in Fig. 5. Induction curve data obtained from the unstirred sample follow the typical 'slow phase' kinetics of fluorescence induction (Lazár 1999). While NPQ appeared active in samples that were not stirred, data from samples that were stirred gave an impression of severely limited NPQ development (Fig. 6a). NPQ was significantly lower in stirred sampled compared to unstirred samples ($F_{0.05(1),138} = 422.29$, $P < 0.001$). Effective quantum yield data was also impacted by stirring (Fig. 6b) with stirred samples displaying significantly higher measures than unstirred samples ($F_{0.05(1),138} = 12.582$, $P = 0.001$). This increase in effective quantum yield resulted in a similar increase in rETR values (data not shown).

Diving-PAM

Diving-PAM RLC fluorescence trace data (Fig. 7a) indicated a decline in F'_m compared to F_m similar to that observed in the unstirred Water-PAM samples. Similarly, induction curve chlorophyll-*a* fluorescence data obtained with the Diving-PAM (Fig. 7b) resemble the Water-PAM data from the unstirred treatment (Fig. 5a).

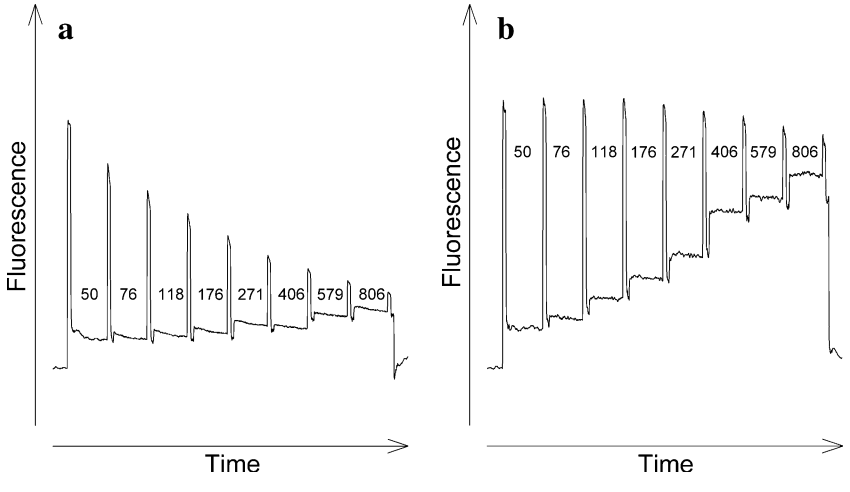

Fig. 2 Fluorescence traces of 10 min dark-adapted samples of *N. oculata* for the duration of a RLC. Numbers indicate actinic irradiance in μmol quanta m^{-2} s^{-1} applied for each 10 s period between saturation pulses. (a) Stirrer not activated, (b) stirrer activated

Table 1 RLC parameters as measured in the Water-PAM. * = significant difference between treatments ($P < 0.05$). Values are mean ± se (n = 5)

	*$rETR_{max}$	*α	I_k	*Max. NPQ
No stirring	42.9 ± 2.0	0.270 ± 0.005	159.4 ± 8.3	0.876 ± 0.017
Stirring	52.8 ± 1.4	0.331 ± 0.011	160.0 ± 3.3	0.073 ± 0.003
t, *P*(0.05)	−4.082, 0.004	−5.210, 0.001	−0.064, 0.950	46.165, <0.001

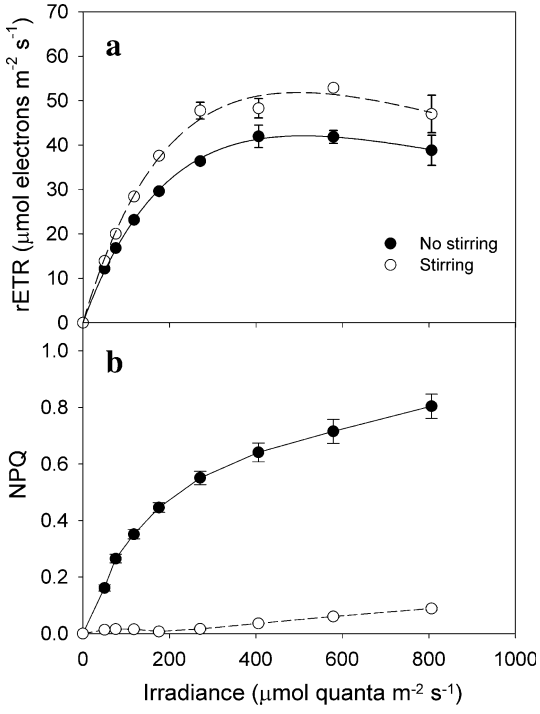

Fig. 3 RLC data illustrating the impact of stirring on (a) rETR and, (b) NPQ (n = 5, error bars indicate standard error)

Discussion

PAM-fluorescence measurements have become popular for assessing the photochemical status of crops (Schäfer and Björkman 1989), corals (Schreiber et al. 1997; Beer et al. 1998a; Hill et al. 2004), seagrass (Ralph et al. 2002; Campbell et al. 2003; Durako et al. 2003), macroalgae (Häder et al. 1998; Beach et al. 2003; Beer and Axelsson 2004) and microalgae (Kromkamp and Peene 1999; Gilbert et al. 2000; Suggett et al. 2003). With the advent of sensitive and portable equipment such as the Water-PAM (Walz GmbH, Germany) the application of chlorophyll-*a* fluorescence measurements to assess phytoplankton in natural surface waters can be expected to further increase in popularity. Despite the efforts of some to detail methodological guidelines for PAM-fluorescence studies (Ralph and Gademann 2005), some points of debate still remain regarding specific procedures.

When collecting chlorophyll-*a* fluorescence measurements using the Water-PAM it is important that: (i) cells do not settle to the bottom of the cuvette during measurements; (ii) all cells experience the same light environment and; (iii) the cells are evenly distributed throughout the sample (homogeneous). To aid in fulfilling these criteria a stirrer is sold as an optional accessory with the Water-PAM. We have shown, however, that stirring samples during RLC or induction curve measurements may significantly impact results. The PHYTO-PAM (System II PHYTO-ED configuration) and Toxy-PAM (Walz GmbH, Germany) ED units have similar geometry and light fields to that of the Water-PAM ED unit and stirring is likely to have a similar impact in each of these fluorometers. Different results may be apparent with units varying in geometry and light environment.

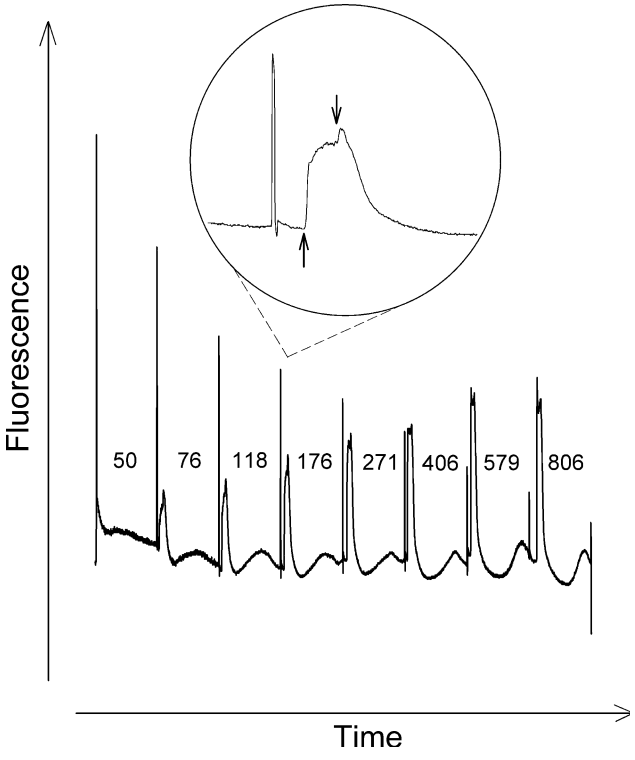

Fig. 4 Fluorescence trace of a light curve with 3 min exposure to each successive irradiance (intensities indicated). Measurements were performed with dark-adapted *N. oculata*. Magnified region details 10 s delay after saturation pulse before stirrer was turned on (up arrow), fluorescence rise during 10 s stirring, and transient fluorescence peak before a rapid decline once the stirrer was turned off (down arrow)

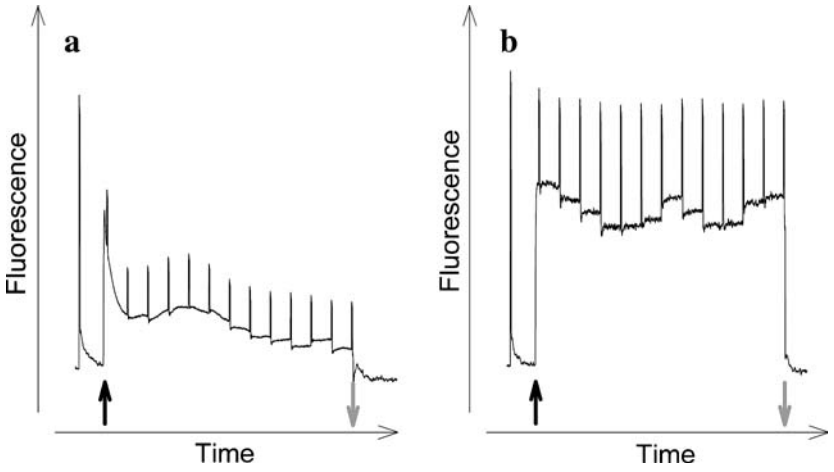

Fig. 5 Fluorescence traces of 10 min dark-adapted samples for the duration of an induction curve. (a) Stirrer not activated, (b) stirrer activated. Up arrows and down arrows indicate activation and deactivation of actinic light (406 μmol quanta m^{-2} s^{-1}) respectively

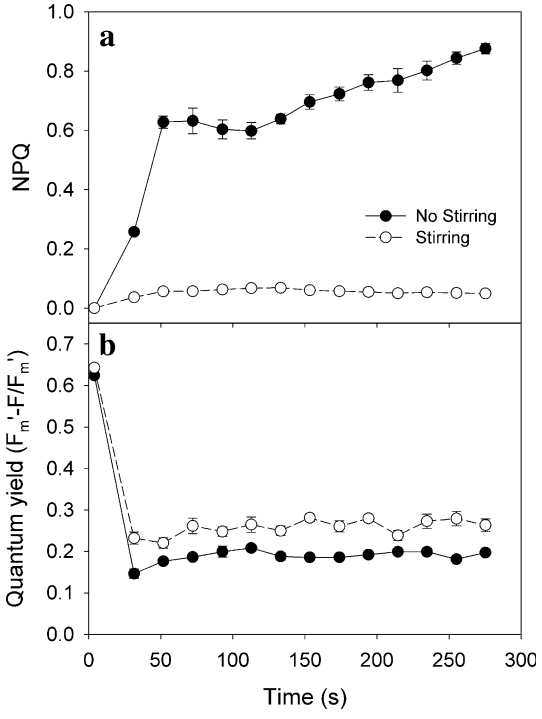

Fig. 6 (a) NPQ and (b) effective quantum yield measurements, taken from successive saturation pulse data during the course of an induction curve (actinic light = 406 μmol quanta m^{-2} s^{-1}). Error bars indicate standard error (n = 5)

Stirring samples within the ED-unit during chlorophyll-*a* fluorescence measurements resulted in significantly higher RLC parameters $rETR_{max}$ and α, while NPQ readings were significantly negatively impacted (both RLC and induction curve). Artificially high F'_m values recorded while samples were stirred appear to be the cause of these changes. Concomitant increases in F tend to moderate increases in effective quantum yield and the derived rETR values.

Comparison of fluorescence patterns observed in the Water-PAM against those obtained using a Diving-PAM revealed a close similarity between the pattern of the Diving-PAM fluorescence data and the unstirred Water-PAM data. This suggests that the unstirred data were 'typical' rather than 'atypical'. While changes in F during the course of a RLC recorded with the Diving-PAM did appear to deviate from that observed with the Water-PAM, the pattern observed (Fig. 7a) was very similar to dark-adapted pea leaf RLC fluorescence patterns observed by White and Chritchley (1999) using a MINI-PAM (Walz GmbH, Germany).

Irradiance measurements show that there is a heterogeneous light environment within the ED unit (Fig. 1b). The LED array is located in a ring near the bottom of the cuvette (Fig. 1a); providing the maximum PAR irradiance just over 3 mm above the base of the cuvette and 50% PAR ~2.1 mm above or below this point. The fluorescence signal received by the detector is likely to be predominantly from those cells within this narrow beam. It is suggested that uneven illumination of the sample within the cuvette may be the underlying cause of artificially high F'_m and F values when the sample is stirred. Cells outside the main light path would experience less quenching of fluorescence and by activating the stirrer these cells become vertically mixed and pass into the 'high light' zone. The relatively unquenched state of these cells would then yield an increase in measured fluorescence. This could potentially also happen (to a lesser extent) in unstirred samples if cells are motile or sink significantly during the course of a RLC or induction curve. Both of these situations would result in cells moving across a gradient of light intensities resulting in an impact on their quenched state. Sinking rate data from Bienfang (1980) suggest that cells within a sample of natural phytoplankton may sink 0.34–1.66 mm within the 87 s it takes to run a RLC with a 10-s exposure at each irradiance. Our measurements indicate that a cell sinking 1.66 mm to the point of maximum light

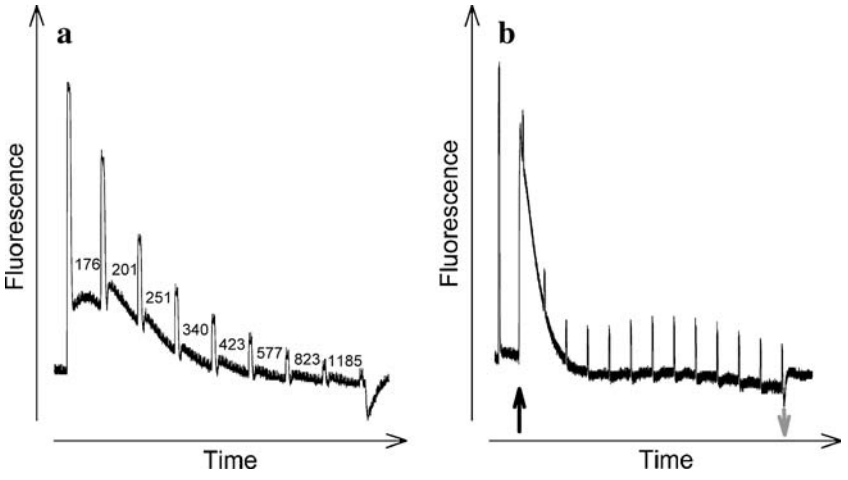

Fig. 7 Diving-PAM (8 mm fibre-optic probe) fluorescence traces taken from *N. oculata* filtered onto Whatman GF/C filter paper. (a) RLC trace with figures indicating actinic irradiance in μmol quanta m^{-2} s^{-1}, and (b) induction curve where up and down arrows indicate activation and deactivation of actinic light (423 μmol quanta m^{-2} s^{-1}) respectively

intensity would experience a 64% increase in irradiance. Actively swimming cells have the potential to experience even greater variation in irradiance. Even the moderately paced cryptophyte, *Plagioselmis nannoplanctica* (Clegg et al. 2003), could swim a distance of ~7 mm over the duration of a similar RLC. Phototactic responses of motile species could also influence quenching data simply due to accumulation (or dispersion) of cells into (or away from) the narrow beam of light.

One would expect the rise in F upon activation of the stirrer to become more substantial as the actinic irradiance setting is increased. The fluorescence trace recorded during the course of a LC with 3 min exposure at each irradiance displays both a rise in the stirrer-induced fluorescence peak and a greater amplitude to the recovery-wave with each successive increase in actinic irradiance. These data indicate that stirring will have a negligible influence on fluorescence data when only the measuring light or low irradiance actinic light (approx. <150 μmol quanta m^{-2} s^{-1}) is activated.

Induction curve data obtained from unstirred samples (Fig. 5a) and filtered samples (Diving-PAM, Fig. 7b) follow typical fluorescence induction patterns (Lazár 1999), however, stirred samples are atypical and experience elevated F and F'_m values (Fig. 5b). This impact on F and F'_m values is not transient and persists for the period of the induction curve. Again, the fluorescence parameter most influenced by this change is NPQ (Fig. 6).

Significant methodological questions still remain when stirring samples to maintain sample homogeneity when using the Water-PAM ED-unit and other similar units. Users should be mindful of the need to stir their sample (particularly if the cell sinking rate is moderate to high), the susceptibility of the study organism(s) to stirrer-generated shear and enhanced 'noise' caused by stirring. Some users stir the sample by placing the entire ED unit on a modified orbital shaker, effectively eliminating stirrer-generated shear forces and reducing vertical mixing (P. Ralph, personal communication).

One might suggest that by using minimal sample volume, thereby reducing the proportion of sample outside the light path, the impact of stirring on the fluorescence signal could be reduced. While this approach does reduce the magnitude of the impact a substantial change in fluorescence remains (data not shown). We propose that providing a vertical dimension to the LED-array, such as the addition of a second ring of LEDs at a higher level relative to the cuvette, plus restriction of sample volume to 2 ml, would result in more even illumination of the whole sample and significant mitigation of the described fluorescence artefacts.

References

Beach K, Walters L, Vroom P, Smith C, Coyer J, Hunter C (2003) Variability in the ecophysiology of *Halimeda* spp. (Chlorophyta, Bryopsidales) on Conch Reef, Florida Keys, USA. J Phycol 39:633–643

Beardall J, Young EB, Roberts S (2001) Approaches for determining phytoplankton nutrient limitation. Aquat Sci 63:44–69

Beer S, Axelsson L (2004) Limitations in the use of PAM fluorometry for measuring photosynthetic rates of macroalgae at high irradiances. Eur J Phycol 39:1–7

Beer S, Ilan M, Eshel A, Weil A, Brickner I (1998a) Use of pulse amplitude modulated (PAM) fluorometry for in situ measurements of photosynthesis in two Red Sea faviid corals. Mar Biol 131:607–612

Beer S, Vilkenkin B, Weil A, Veste M, Susel L, Eshel A (1998b) Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. Mar Ecol Prog Ser 174:293–300

Bienfang PK (1980) Phytoplankton sinking rates in oligotrophic waters off Hawaii, USA. Mar Biol 61:69–77

Campbell S, Miller C, Steven A, Stephens A (2003) Photosynthetic responses of two temperate seagrasses across a water quality gradient using chlorophyll fluorescence. J Exp Mar Biol Ecol 291:57–78

Clegg MR, Maberly SC, Jones RI (2003) Behavioural responses of freshwater phytoplanktonic flagellates to a temperature gradient. Eur J Phycol 38:195–203

Durako M, Kunzelman J, Kenworthy W, Hammerstrom K (2003) Depth-related variability in the photobiology of two populations of *Halophila johnsonii* and *Halophila decipiens*. Mar Biol 142:1219–1228

Falkowski P, Wyman K, Ley A, Mauzerall D (1986) Relationship of steady state photosynthesis to fluorescence in eukaryotic algae. Biochim et Biophys Acta 849:183–192

Gilbert M, Domin A, Becker A, Wilhelm C (2000) Estimation of primary productivity by chlorophyll a in vivo fluorescence in freshwater phytoplankton. Photosynthetica 38:111–126

Guillard RRL, Ryther JH (1962) Studies on marine diatoms. 1 *Cyclotella nana* Husted and *Detonula confervacea* Gran. Can J Microbiol 8:229–239

Häder D, Lebert M, Figueroa F, Jiménez C, Viñegla B, Perez-Rodriguez E (1998) Photoinhibition in Mediterranean macroalgae by solar radiation measured on site by PAM fluorescence. Aquat Bot 61:225–236

Hill R, Ralph PJ (2005) Diel and seasonal changes in fluorescence rise kinetics of three scleractinian corals. Funct Plant Biol 32:549–559

Hill R, Schreiber U, Gademann R, Larkum AWD, Kühl M, Ralph PJ (2004) Spatial heterogeneity of photosynthesis and the effect of temperature-induced bleaching conditions in three species of corals. Mar Biol 144:633–640

Jakob T, Schreiber U, Kirchesch V, Langner U, Wilhelm C (2005) Estimation of chlorophyll content and daily primary production of the major algal groups by means of multiwavelength-excitation PAM chlorophyll fluorometry: performance and methodological limits. Photosynth Res 83:343–361

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton. Biochem Physiol Pflanz 167:191–194

Kromkamp J, Peene J (1999) Estimation of phytoplankton photosynthesis and nutrient limitation in the Eastern Scheldt estuary using variable fluorescence. Aquat Ecol 33:101–104

Lazár D (1999) Chlorophyll *a* fluorescence induction. Biochim et Biophys Acta 1412:1–28

Lazár Da, Ilík P, Kruk J, Strzalka K, Naus J (2005) A theoretical study on effect of the initial redox state of cytochrome b_{559} on maximal chlorophyll fluorescence level (F_M): implications for photoinhibition of photosystem II. J Theor Biol 233:287–300

McMinn A, Hegseth E (2004) Quantum yield and photosynthetic parameters of marine microalgae from the southern Arctic Ocean, Svalbard. J Mar Biol Assoc UK 84:865–871

Platt T, Gallegos C, Harrison W (1980) Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. J Mar Res 38:687–701

Ralph PJ, Gademann R (2005) Rapid light curves: a powerful tool for the assessment of photosynthetic activity. Aquat Bot 82:222–237

Ralph PJ, Polk SM, Moore KA, Orth RJ, Smith Jr WO (2002) Operation of the xanthophyll cycle in the seagrass *Zostera marina* in response to variable irradiance. J Exp Mar Biol Ecol 271:189–207

Samson G, Prášil O, Yaakoubd B (1999) Photochemical and thermal phases of chlorophyll a fluorescence. Photosynthetica 37:163–182

Schäfer C, Björkman O (1989) Relationship between efficiency of photosynthetic energy conversion and chlorophyll fluorescence quenching in upland cotton (*Gossypium hirsutum* L.). Planta 178:367–376

Schreiber U, Gademann R, Ralph PJ, Larkum AWD (1997) Assessment of photosynthetic performance of *Prochloron* in *Lissoclinum patella* in hospite by chlorophyll fluorescence measurements. Plant Cell Physiol 38:945–951

Schreiber U, Neubauer C, Schliwa U (1993) PAM fluorometer based on medium-frequency pulsed Xe-flash measuring light: a highly sensitive new tool in basic and applied photosynthesis research. Photosynth Res 36:65–72

Serôdio J, Cruz S, Vieira S, Brotas V (2005) Non-photochemical quenching of chlorophyll fluorescence and operation of the xanthophyll cycle in estuarine microphytobenthos. J Exp Mar Biol Ecol 326:157–169

Suggett D, Oxborough K, Baker N (2003) Fast repetition rate and pulse amplitude modulation chlorophyll a fluorescence measurements for assessment of photosynthetic electron transport in marine phytoplankton. Eur J Phycol 38:371–384

Villareal TA (2004) Single-cell pulse amplitude modulation fluorescence measurements of the giant diatom *Ethmodiscus* (Bacillariophyceae). J Phycol 40:1052–1061

White A, Critchley C (1999) Rapid light curves: a new fluorescence method to assess the state of the photosynthetic apparatus. Photosynth Res 59:63–72