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Dark-induced reduction of the plastoquinone pool in zooxanthellae of scleractinian corals and implications for measurements of chlorophyll *a* fluorescence

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Abstract

Fluorometric measurements of maximum quantum yield (F_v/F_m) and fast induction curves (FICs) require coral samples to be dark-adapted (DA). Pathways causing dark-reduction of the plastoquinone (PQ) pool are shown here to be active in corals. Early morning sunlight and far-red light successfully increased F_v/F_m and lowered the O and J steps of FICs in corals that were darkened overnight. The thick-tissued massive coral, *Cyphastrea serailia*, was shown to be more prone to reduction of the PQ pool, with significant reductions in F_v/F_m occurring after 10 min of DA, and elevated J steps occurring within 200 s following a far-red flash. In thinner-tissued branching species, *Pocillopora damicornis* and *Acropora nobilis*, elevation of the J step also occurred within 200 s of DA, but a drop in F_v/F_m was only manifested after 30 min. Pre-exposure to far-red light is an effective and simple procedure to ensure determination of the true maximum quantum yield of Photosystem II (PSII) and accurate FICs which require a fully oxidised inter-system electron transport chain and open PSII reaction centres.

Keywords: Chlorophyll *a* fluorescence, fast induction curves, OJIP, chlororespiration, far-red, coral bleaching

1. Introduction

Scleractinian corals are symbiotic organisms, containing single celled dinoflagellates (genus *Symbiodinium*) within the endodermal tissue of the cnidarian host. These photosynthetic algae are known as zooxanthellae and provide the host with organic compounds, while the host provides the symbionts with nitrogen and phosphorus as organic and inorganic compounds. Host respiration also supplies a portion of the CO_2 required for photosynthesis (Titlyanov and Titlyanova, 2002).

A common tool for investigating the photosynthetic properties of zooxanthellae (whether they are *in hospite*, freshly isolated, expelled or cultured) is chlorophyll (chl) *a* fluorescence (Iglesias-Prieto, 1995; Jones et al., 1998; Jones et al., 2000; Ralph et al., 2001; Bhagooli and Hidaka, 2003; Hill et al., 2004a,b; Hill and Ralph, 2005; Robison and Warner, 2006). This technique provides detailed information on Photosystem II (PSII) activity through the measurement of the fluorescence rise from F_o (minimum fluorescence) to F_m (maximum fluorescence) during the application of a saturating pulse of light. Changes in the variable fluorescence ($F_v = F_m - F_o$), relative to F_m (F_v/F_m), gives an indication of PSII photochemical efficiency, known as the maximum quantum yield of PSII (Schreiber, 2004).

Pulse Amplitude Modulated (PAM) fluorometers are commonly used (Warner et al., 1999; Jones et al., 2000; Fitt et al., 2001; Ralph et al., 2001; Bhagooli and Hidaka, 2003; Hill et al., 2004b), and to a lesser extent, fast induction curve (FIC) measurements, using either the Plant Efficiency Analyser (PEA) (Hill et al., 2004a; Hill and Ralph, 2005; Ulstrup et al., 2005) or a double modulation fluorometer (Photon System Instruments) (Hill and Ralph, 2006) to study the photophysiology of corals. PAM fluorometers are frequently used to measure F_v/F_m through the application of multiple-turnover saturating pulses of light, while PEA and double modulation fluorometers, which are equipped with rapid photodetectors, can measure the fluorescence rise from F_o to F_m (FICs). The majority of research using these instruments has focused on the impacts of coral bleaching

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events on specific locations of the zooxanthellae photosynthetic apparatus. Mass coral bleaching events occur on a global scale and are characterised by the expulsion of zooxanthellae from the coral host and a reduction in the concentration of photosynthetic pigments within the zooxanthellae (Kleppel et al., 1989). The animal tissue turns pale in colour, often white (hence the term 'bleaching'), and can lead to the death of the colony (Hoegh-Guldberg, 1999). The main environmental factors responsible for these events are elevated temperatures in combination with intense light (Hoegh-Guldberg, 1999; Jones et al., 2000; Fitt et al., 2001).

In theory, in order to determine the maximum quantum yield of PSII, a period of dark-adaptation (DA) (generally between 5 and 60 min) is required for oxidation of the plastoquinone (PQ) pool and for the PSII reaction centres (RCs) to open (Schreiber, 2004). It is assumed that once DA is complete, an application of a saturating pulse of light would allow for the determination of the maximum quantum yield of PSII. In reality, this may not be the case, because of the reduction of the PQ pool in the dark. Previous work on higher plants, algae and cyanobacteria has demonstrated the link between photosynthetic and respiratory electron transport chains and the potential for PQ pool reduction in the dark (Gans and Rebeille, 1990; Schreiber et al., 1995; Feild et al., 1998; Haldimann and Strasser, 1999). In addition, the formation of a proton gradient across the thylakoid membrane during darkness has been attributed to an ATP-dependant electrogenic pump, which also has the potential to reduce the acceptor side of PSII (Bennoun, 1982; Bennoun, 2004).

In corals, the consumption of oxygen by mitochondrial respiration in the host during DA has the potential to create hypoxic conditions within the coral tissue (Ulstrup et al., 2005). Hypoxia is defined here as a reduced oxygen content within the holobiont. The reduction of the PQ pool can occur during this time with the operation of chlororespiration, which has been suggested to operate in zooxanthellae under such conditions (Jones and Hoegh-Guldberg, 2001; Hill and Ralph, 2005; Ulstrup et al., 2005; Hill and Ralph, 2006). In addition, post-illumination enhanced respiration would increase the rate of respiration in the zooxanthellae, contributing to the formation of hypoxic conditions during DA (Edmunds and Davies, 1988; Beardall et al., 1994). Previous studies have alluded to the potential for both these mechanisms to affect fluorescence measurements following DA and the importance of further research in this area (Jones and Hoegh-Guldberg, 2001; Ulstrup et al., 2005; Hill and Ralph, 2006). It is therefore imperative that a clear understanding of the role that these processes play in the photophysiology of corals is obtained to ensure that commonly employed fluorescence techniques generate meaningful data. In addition, methods to prevent or reverse dark-reduction of the PQ pool need to be identified which allow for the collection of accurate results.

A reduction of the PQ pool in the dark would lead to the closure of PSII RCs, which would lower the photochemical efficiency of PSII. As a result, the quantum yield value obtained may be significantly lower than the actual maximum quantum yield and the shape and amplitude of the steps along FICs could be significantly altered. The diurnal study of FICs on three coral species during summer and winter (Hill and Ralph, 2005) showed that F_v/F_m measurements increased from pre-dawn to dawn and an increase in the speed of Q_A^- reoxidation was observed because of the oxidation of the PQ pool during sunrise. The low intensity light at dawn was found to be responsible for reversing the overnight reduction of the PQ pool, thus leading to the rise in F_v/F_m.

It is generally believed that although PSII and Photosystem I (PSI) are excited by similar wavelengths of light, PSI absorbs quanta further into the far-red region of the spectrum (Falkowski and Raven, 1997; Larkum, 2003). This allows PSI to be stimulated independently of PSII. In the case of dinoflagellates, little is known about their antenna complex, although it has been shown that they contain a unique peridinin chlorophyll *a* protein and compared to higher plants, they contain four times the amount of this carotenoid compared to chlorophyll involved in light harvesting (Iglesias-Prieto and Trench, 1996; Krueger et al., 2001; Krikunova et al., 2006). It is important to note that as the light harvesting complexes of these algae are not well understood, their photosystems may not have the same absorption spectra as other plants.

Since chl *a* fluorescence is a measure of PSII photochemical efficiency, exposing a dark-adapted sample to far-red light will not significantly, if at all, close any PSII centres or reduce the acceptor side of PSII (Egorova et al., 2005). Far-red light will predominantly activate PSI and enhance the oxidation of the inter-system electron transport chain (Havaux, 1992; Schreiber, 2004). Subsequent quantum yield or FIC measurements will be representative of a fully oxidised, inter-system chain allowing for accurate PSII maximum quantum yield determinations.

Here, the dark-reduction of the PQ pool which leads to closure of PSII RCs was investigated through the application of specific wavelengths of light prior to quantum yield and FIC measurements. Three coral species with different morphologies were examined to identify inter-species variation. Furthermore, through the simulation of coral bleaching conditions, the interaction of holobiont gas exchange and redox state of the symbiont could be explored. Tissue thickness and coral structure may influence the susceptibility of corals to dark reduction of the PQ pool due to differences in diffusion boundary layers and the rate of dissolved gas transfer. Here, the thick tissued massive coral, *Cyphastrea serailia*, and the thinner tissued branching corals, *Pocillopora damicornis* and *Acropora nobilis*, were compared.

2. Materials and Methods

Coral specimens

Coral fragments of *Pocillopora damicornis* (Linnaeus), *Acropora nobilis* (Dana) and *Cyphastrea serailia* (Forskål) were collected from the Heron Island lagoon, Great Barrier Reef, Australia (151.9°E, 23.4°S) between 15 and 28 January 2006. Samples were placed in flow-through shaded aquaria (<100 µmol photons m^{-2} s^{-1}) at the lagoon temperature of 28°C for at least 2 days prior to experimentation. Four replicate fragments of each species were used for each experiment.

Fluorescence measurements

A double modulation fluorometer (Photon Systems Instruments, FL-3300, Brno, Czech Republic) was used to apply excitation light and measure fluorescence ($\lambda \geq 700$ nm) emissions over 5 s. The measuring head contained both blue (415–520 nm; λ_{max} = 455 nm) and red (590–670 nm; λ_{max} = 640 nm) light emitting diodes (LEDs) for PSII excitation (3700 µmol photons m^{-2} s^{-1}) and a far-red LED (710–780 nm; λ_{max} = 740 nm) for PSI excitation (see Fig. 1a for specific wavelength emissions) and a PIN photodiode for fluorescence detection in volts (V).

The FluorWin software (Photon Systems Instruments, Brno, Czech Republic) was used to create protocol files controlling the activation, intensity and length of each flash for each set of LEDs. The speed of fluorescence measurements was also controlled by this program, with measurements recorded every 10 µs for the first 2 ms, every 1 ms up until 1 s, and then every 500 ms up to 5 s. The O step was defined as the fluorescence intensity at 0.05 ms, J at 2 ms, I between 0.07 and 0.1 s (depending on the timing of the inflection) and P between 1 and 2 s (depending on the time of maximum fluorescence).

For detailed descriptions of the processes involved in the fluorescence rise from the O to P step and the reduction of the PSII acceptor side, see Govindjee (1995), Hill et al. (2004a) and Schreiber (2004). In our experiments, the number of open PSII RCs may be affected by the extent of dark-reduction of the PQ pool. This has implications for the determination of F_o (the O step) and it is therefore important to note that in some cases the O step may not be representative of exclusively open PSII RCs.

Experimental protocol

Experiment 1

In the evening (0 µmol photons m^{-2} s^{-1}), corals were placed outside, in flow through (2.8 l min^{-1}) DA chambers (see Hill et al., 2004b) and left overnight in complete darkness. Corals were placed inside the DA chambers with approximately 2–5 mm of water between the coral surface

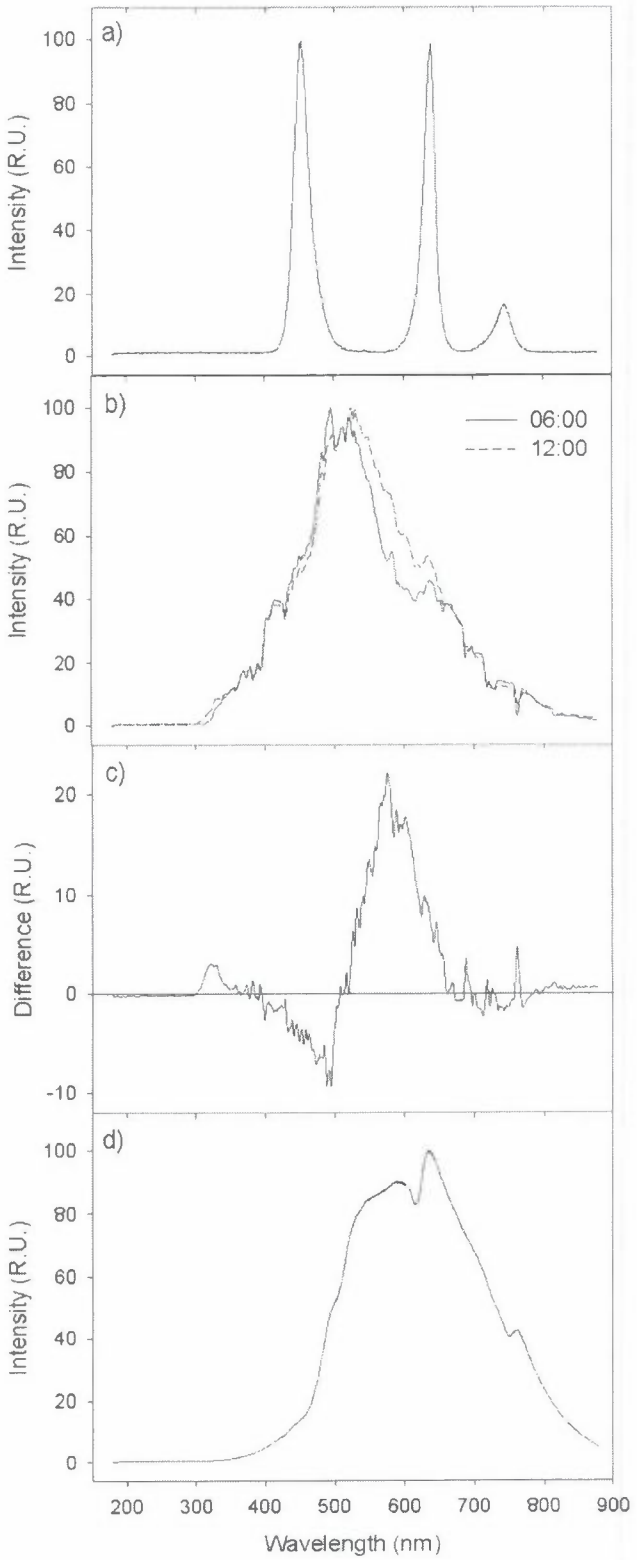

Figure 1. The relative intensity of light between the wavelengths of 180–880 nm for light sources used throughout the experiments. a) Wavelengths from the PSI double modulation fluorometer's blue, red and far-red LEDs; b) the solar spectrum at 06:00 (solid line) and 12:00 (dashed line) hrs; c) the difference between the 12:00 and 06:00 hrs solar spectra; d) the spectrum of the halogen lights used in Experiment 3.

and the aperture allowing for light entry. At pre-dawn (04:00 hrs; 0 μmol photons m^{-2} s^{-1}) and dawn (06:00 hrs; 50 μmol photons m^{-2} s^{-1}), FICs were measured on corals which had different light treatments before the measurement. At 04:00 hrs FIC measurements were taken on corals without any light exposure, on corals exposed to 10 s of far-red light, or on corals exposed to 10 s blue and red light (100 μmol photons m^{-2} s^{-1}). Between the 04:00 and 06:00 hrs measurements, the aperture of the DA chambers was left open, exposing corals to ambient light intensities during sunrise. At 06:00 hrs, all corals were given 5 min DA and then the same three light treatments applied prior to the FIC measurement. The FIC measurements were taken 0.1 s after the light treatment was completed. From these FICs, the F_v/F_m ($(F_m-F_o)/F_m$) was calculated, where F_o is the value at 0.05 ms and F_m is the maximum value along the curve. The light intensity in all experiments was measured using a photometer (Li-1400, Li-Cor, Lincoln, Nebraska, USA), with a Li-190SA quantum sensor. The solar spectrum (180-880 nm) was measured at 06:00 and 12:00 hrs using a portable spectrophotometer (USB2000-UV-VIS, Ocean Optics Inc, Florida, USA) and both curves were normalised to a minimum of 0 and a maximum of 100 units (Fig. 1b). The difference in the spectrum between these two time measurements (12:00 – 06:00 hrs) is shown in Fig. 1c.

Experiment 2

The effect of length of DA on the shape of FICs and on F_v/F_m values was investigated by taking measurements on corals exposed to complete darkness and on corals exposed to 10 s of far-red light prior to FIC curve measurement. Dark adaptation lengths of 5, 10, 20, 30 and 60 min were used. F_v/F_m values were derived from FICs as described above.

Experiment 3

Further experiments on the impact of length of DA on FICs were performed on corals under non-stressful and bleaching conditions. FICs were measured at different lengths of time following 10 min of DA and one of the following 1) a further 10 s darkness, 2) 10 s far-red light and 0.1 s darkness, 3) 10 s far-red light and 1 s darkness, 4) 10 s far-red light and 10 s darkness, 5) 10 s far-red light and 200 s darkness. These light manipulations were completed for the three species of coral under control conditions (28°C and 100 μmol photons m^{-2} s^{-1}) and treatments 1), 2) and 5) were also completed for corals exposed to 5 h of bleaching conditions (32°C and 350 μmol photons m^{-2} s^{-1}). The spectrum of the halogen exposure lights used in the bleaching experiments is shown in Fig. 1d.

Statistical analysis

In Experiment 1, the amplitude of the O, J, I and P steps, and the F_v/F_m values calculated from the FICs were subjected to one-way analysis of variance (ANOVA) tests and Tukey's post hoc comparisons ($\alpha = 0.05$). The cases where significant differences occurred in the F_v/F_m data, letters are used to indicate where the differences exist. Similarly, in Experiment 2, one-way ANOVA tests were used to establish whether differences between the various darkness treatments and far-red treatments existed. In all cases where differences were found, the F_v/F_m value from the far-red treatment was significantly higher and an asterisk (*) has been placed above this group of measurements. For Experiment 3, the derived F_v/F_m values for each of the different times following far-red light application for the control and bleaching treatments was tested, as was the amplitude of the O, J, I and P steps along the FICs. The assumptions of normality and equal variance were tested using the Kolmogorov-Smirnov test and Levene's test, respectively, and were satisfied in most cases. If these assumptions were not met, arcsine (on the F_v/F_m data) and $\log_{10}$ (on the O, J, I and P data) transformations were performed. The SPSS statistical software package (version 11.0.0, 2001, Chicago, Illinois, USA) was used for statistical analyses.

3. Results

The results from the three independent experiments are described in detail below.

Experiment 1

The FICs for *C. serailia* at 04:00 and 06:00 hrs are shown in Fig. 2a and b, respectively, with the corresponding F_v/F_m values shown below in Fig. 2c and d. Following complete darkness overnight, the FIC at 04:00 hrs (Fig. 2a) showed a large rise from the O step to the J step, followed by a sharp decline in the curve before rising again to the I step and then to the maximum fluorescence at the P step (where J = P). At this same time, corals provided with 10 s far-red light prior to FIC measurement showed a continuous rise from the O step, through the J and I step and the maximal fluorescence, P. Following blue and red light exposure, the FIC showed a similar rise from O to P as the far-red exposed coral. The amplitude of the O and J steps were significantly higher in the dark treatment, compared to the other two treatments ($P = 0.047$ and 0.013) while the I and P steps were higher in the far-red treatment compared to the blue and red light treatment, with the dark treatment falling in both groups ($P = 0.048$ and 0.037, respectively). F_v/F_m values were calculated from the FICs (Fig. 2c) and it is shown that the darkness treatment ($F_v/F_m = 0.493$) and the blue and red light treatment ($F_v/F_m = 0.490$) were significantly lower than the far-red light treatment ($F_v/F_m = 0.598$) ($P = 0.006$).

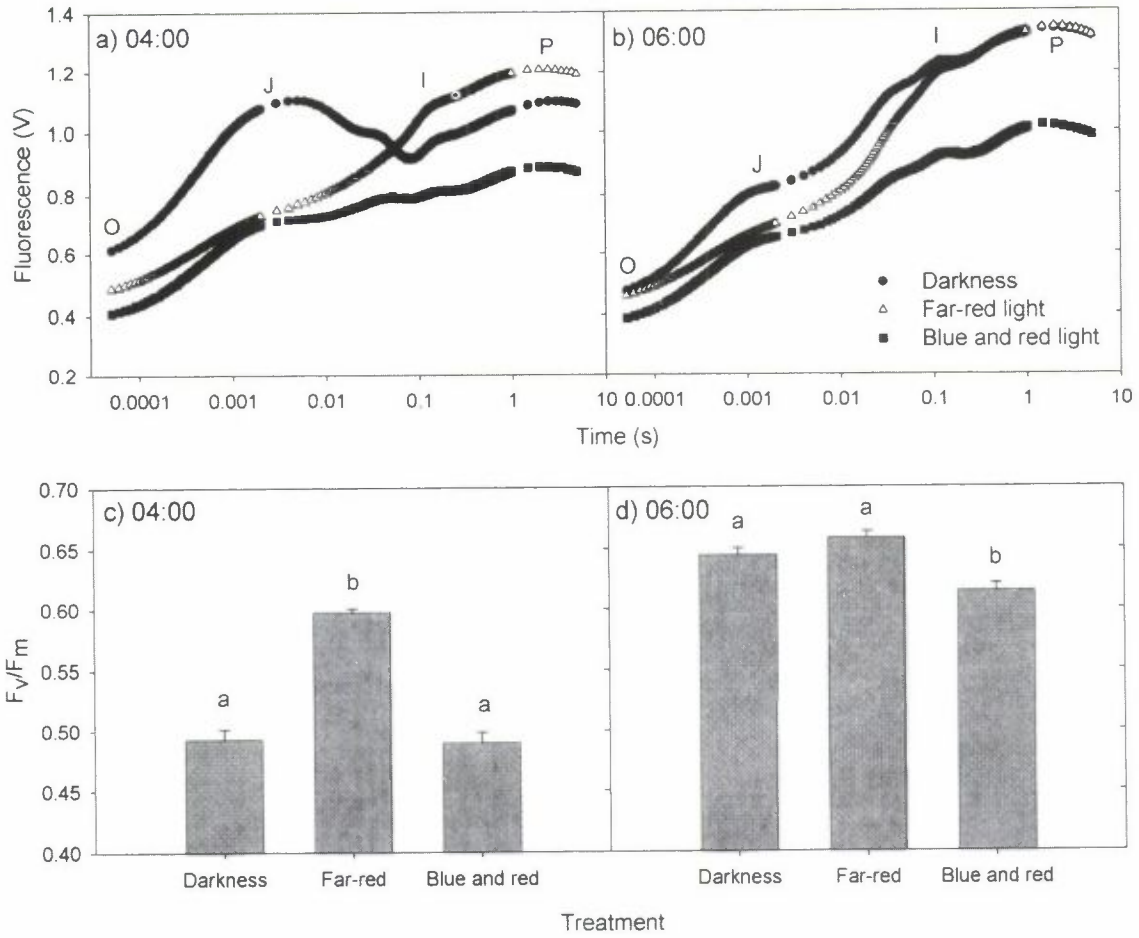

Figure 2. Fast induction curves at (a) 04:00 hrs, and (b) 06:00 following darkness (•), 10 s exposure to far-red light then 0.1 s of darkness (Δ), and 10 s exposure to blue and red light then 0.1 s darkness (■) for *Cyphastrea serailia*. Average curves are shown (n = 4). The corresponding F_v/F_m value for each light treatment is shown for (c) 04:00 hrs, and (d) 06:00 hrs. Averages ± standard error of mean shown (n = 4). The letters above the columns in c) and d) are the result from Tukey's post hoc comparisons test.

Following dawn, at 06:00 hrs (Fig. 2b), the same measurements were performed on these corals. The FICs for all three treatments showed the usual rise from the O step, through J and I and to the maximum fluorescence amplitude, P. At the O step, no significant differences between the three treatments were found. At the J step, the treatment with 5 mins of DA was significantly higher than the other two treatments (P = 0.009). The I and P steps for the blue and red light treatment were significantly lower than the other two treatments (P = 0.004 and 0.008, respectively). The F_v/F_m data derived from these FICs showed that the blue and red light treatment was significantly lower than the dark and far-red light treatments (P = 0.002) (Fig. 2d).

The solar spectrum was measured at 06:00 hrs and at midday (Fig. 1b) and the difference between the two curves (12:00 – 6:00 hrs) were compared (Fig. 1c) in order to identify changes in the spectral composition. When the difference curve in Fig. 1c enters positive values, this indicates that there was a greater amount of this particular wavelength at midday than at 06:00 hrs, and when it enters negative values, these wavelengths were more abundant at 06:00 hrs. At 12:00 hrs, there were higher levels of light in the ultra violet (UV) region of 300–350 nm, and in the visible region of 520–665 nm. At 06:00 hrs, there was greater visible light in the region 400–510 nm. In the far-red/infrared wavelengths, 700–780 nm, there was considerable fluctuation around 0, although, 77% of these wavelengths were greater in abundance at 06:00 hrs. The remaining wavelengths showed similar abundance at both times (i.e., the curve hovered around 0).

Experiment 2

The FICs following different lengths of exposure to DA (5, 10, 20, 30 and 60 min), plus an extra 10 s of darkness or 10 s of far-red light, are shown in Figs. 3a–c, for the three coral species. The only curve shown following far-red light

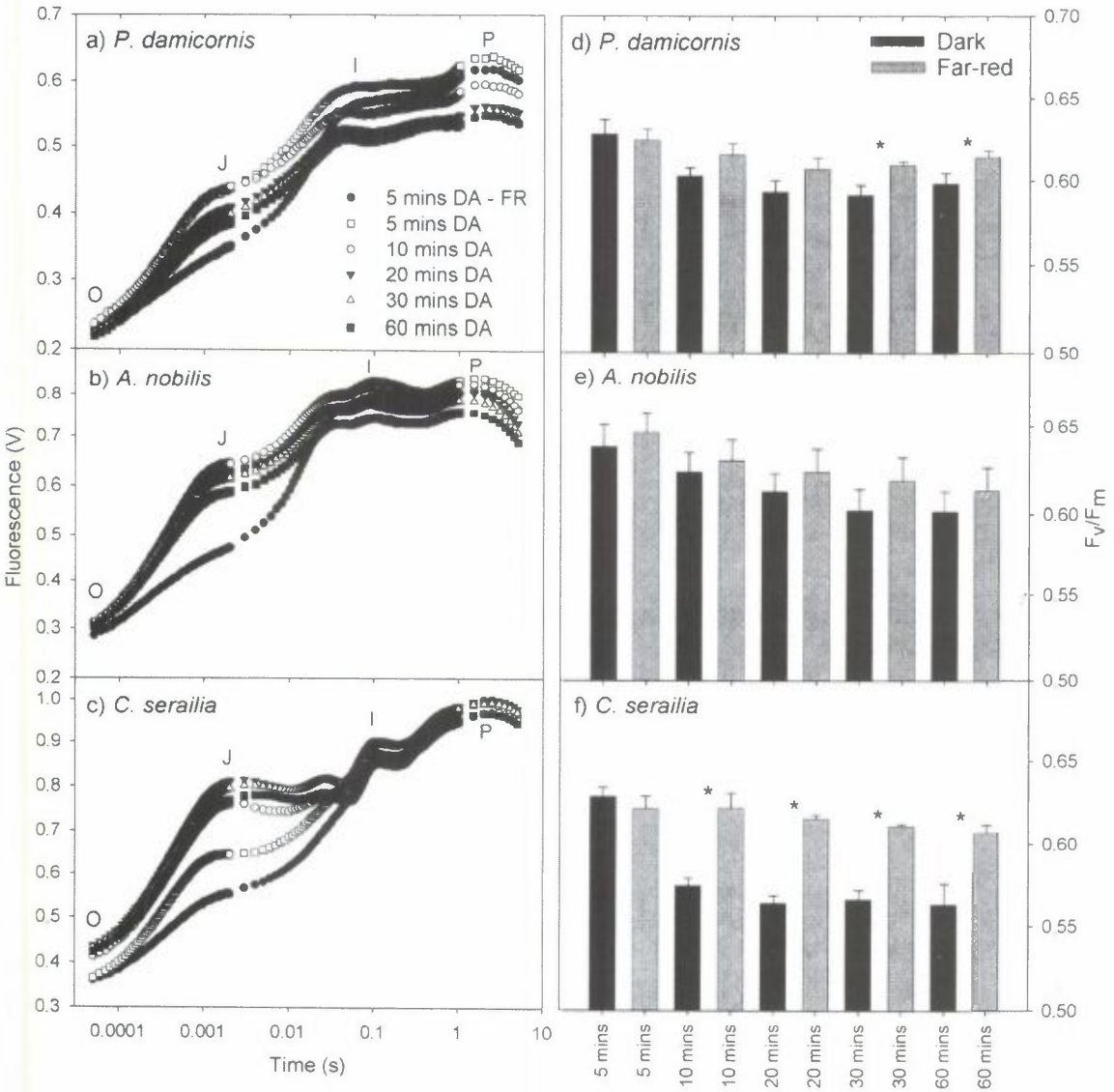

Figure 3. Effect of length of dark-adaptation (DA) on fast induction curves and F_v/F_m values. Fast induction curves for (a) *Pocillopora damicornis*, (b) *Acropora nobilis*, and (c) *Cyphastrea serailia* following 5 min of DA, then 10 s far-red light and 0.1 s darkness (●), 5 min DA, (□), 10 min DA (○), 20 min DA (▼), 30 min DA (Δ), and 60 min DA (■). Average curves are shown ($n = 4$). The corresponding F_v/F_m value for each light treatment is shown for (d) *P. damicornis*, (e) *A. nobilis*, and (f) *C. serailia*. Averages ± standard error of mean shown ($n = 4$). Asterisks (*) indicate where the far-red light treatment had a significantly higher F_v/F_m than the darkness treatment.

exposure was the 5 min dark-adapted corals. The other far-red FICs are not shown as they were very similar to this curve. For each species, the FICs taken after far-red light exposure had lower J steps, without any dip following J, compared to the dark-adapted curves. Following 20 mins of DA, the J step reached its maximum amplitude for all three species and remained at this level through to the 60 min DA treatment. The F_v/F_m values derived from the FICs showed no significant change between different lengths of DA in the far-red treatments for all three species. However, when comparing between complete darkness and far-red exposed treatments, *P. damicornis* showed a significantly lower F_v/F_m in dark-adapted samples after 30 and 60 min, compared to those given a 10 s flash of far-red (Fig. 3d). This can be attributed to the lower P step (F_m) in the FICs for the 30 and 60 min DA treatment. In contrast, *A. nobilis* showed no significant change in F_v/F_m over time or between dark and far-red measurements (Fig. 3e). Although the FICs for *A. nobilis* showed clear variation at the J step (Fig. 3b), this was not evident in the derived F_v/F_m values. *C. serailia* showed significant declines in F_v/F_m following 10, 20, 30 and 60 min DA ($P < 0.05$ in all cases) (Fig. 3f) and this drop can be attributed to the rise in the O step (F_o) in the FICs (Fig. 3c).

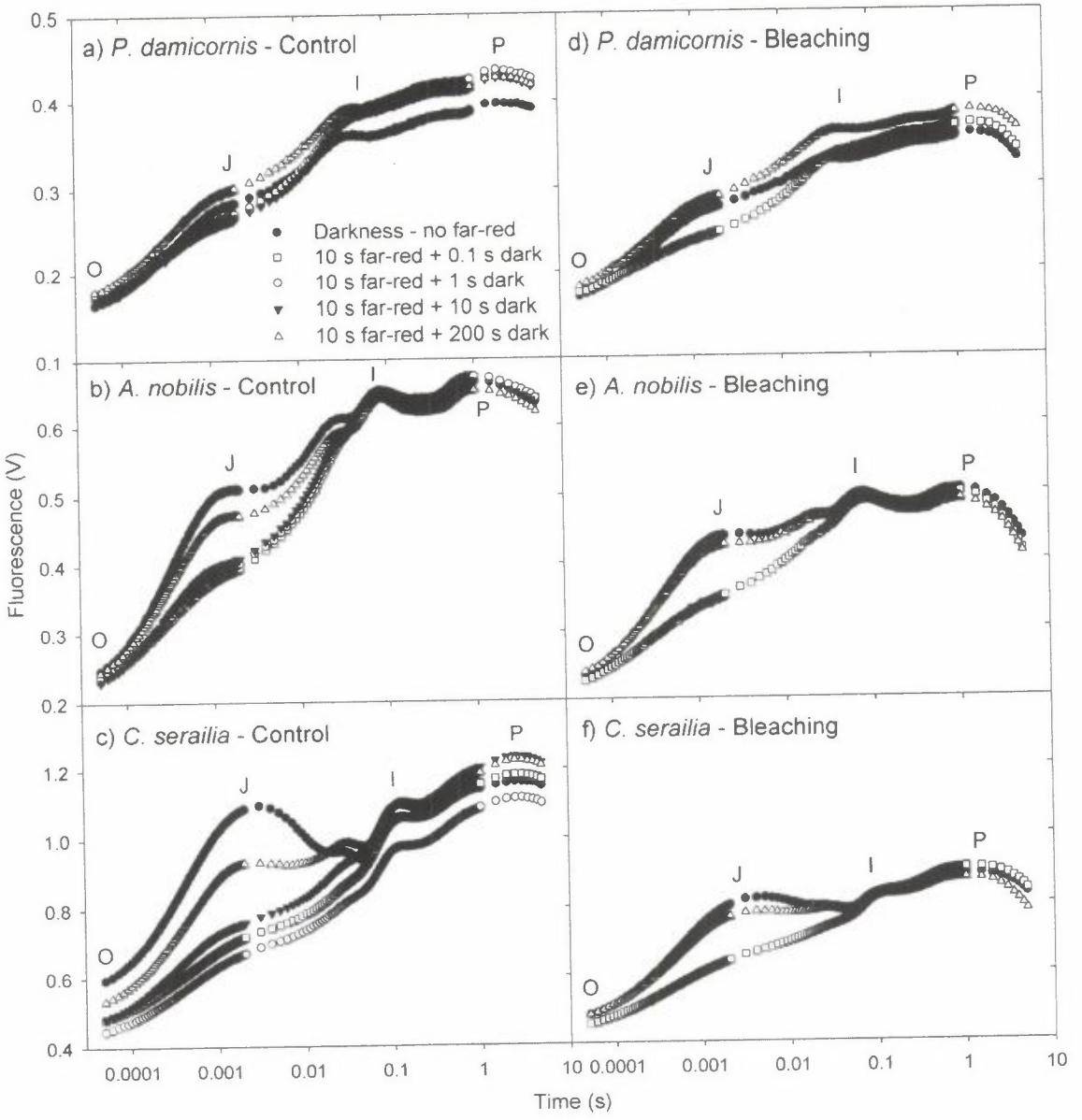

Figure 4. Fast induction curves for (a) *Pocillopora damicornis*, (b) *Acropora nobilis*, and (c) *Cyphastrea serailia* during control conditions, and for (d) *P. damicornis*, (e) *A. nobilis*, and (f) *C. serailia* following 5 h under bleaching conditions. Corals were given 10 min dark-adaptation (DA) (●), 10 min DA, 10 s far-red light and 0.1 s darkness (□), 10 min DA, 10 s far-red light and 1 s darkness (○), 10 min DA, 10 s far-red light and 10 s darkness (▼), and 10 min DA, 10 s far-red light and 200 s darkness (Δ). Average curves are shown ($n = 4$).

Experiment 3

Changes in FICs following far-red light exposure were investigated here with different lengths of darkness between the application of far-red light and FIC measurement (Fig. 4). Similar responses in the curves were observed under control and bleaching conditions. In *P. damicornis*, no significant differences were found between any of the O, J, I or P steps along the FICs in the controls (Fig. 4a) or the bleaching treatment (Fig. 4d). In addition, no significant changes in F_v/F_m (data not shown) were found between the different times following far-red light application for the control or bleaching treatments, although significantly lower F_v/F_m values were found in the bleaching treatment (0.528) compared to the control (0.590) ($P < 0.001$).

The only differences in the FICs in the control for *A. nobilis* (Fig. 4b) were found at the J step, where the dark and 200 s treatments were significantly higher than the other treatments ($P = 0.006$). At the other steps, no differences were found. In the dark and 200 s curves, an

extra step can be seen between the J and I steps at 0.03 s, although this step was removed by the far-red light exposure in the other curves. The bleaching treatment (Fig. 4e) showed a significantly higher J step in the dark and 200 s treatment compared to the 0.1 s treatment ($P = 0.047$), and no change in any other steps along the curves. The F_v/F_m data derived from these FICs (data not shown) did not significantly vary between any of the treatments in either the control or bleaching data sets. However, F_v/F_m was significantly lower in the bleaching treatment (0.534) compared to the control treatment (0.643) ($P < 0.001$).

In *C. serailia*, both the control (Fig. 4c) and bleaching (Fig. 4f) treatments showed the same response with regard to changes in the FICs for the different lengths of darkness between far-red light application and FIC measurement. The only significant differences in the FICs were at the J step, where the dark and 200 s treatments had a higher amplitude than the other treatments (P values <0.05). An extra peak between the J and I steps, similar to the one observed in *A. nobilis*, was detected in the dark and 200 s curves. A significant drop in F_v/F_m was found (data not shown) under bleaching conditions (0.463) compared to the controls (0.570) and this can be attributed to a lower fluorescence amplitude at the P step (F_m). Within the controls, the dark treatment had a significantly lower F_v/F_m than the other treatments ($P = 0.002$), whereas under bleaching conditions, there was no significant difference between any of the treatments.

4. Discussion

This study explored the potential for dark-induced reduction of the PQ pool in zooxanthellae of scleractinian corals, which has been identified as an important topic requiring further research (Jones and Hoegh-Guldberg, 2001; Ulstrup et al., 2005; Hill and Ralph, 2006). The extensive use of chl *a* fluorescence as a means of detecting changes in the photosynthetic efficiency of PSII in the zooxanthellae of corals highlights the need for a detailed investigation into the assumptions made regarding the redox state of the electron acceptors of PSII and the PQ pool during DA. Maximum quantum yield or FIC measurements taken after DA are assumed to be representative of open PSII RCs. However, reductant-forming pathways can become active during dark conditions, thus leading to PQ pool reduction and PSII RC closure. Through the application of far-red light, which preferentially excites PSI and ensures oxidation of the inter-system electron carriers, increases in quantum yield values and specific changes in FICs, will provide evidence of processes responsible for dark-induced reduction of the PQ pool and the PSII electron acceptors.

Early morning increases in F_v/F_m (Jones and Hoegh-Guldberg, 2001; Hill and Ralph, 2005) and rises in the P step along FICs (Hill and Ralph, 2005) have been documented in corals following overnight darkness, which indicates an increase in PSII photosynthetic capacity upon exposure to the low intensity morning light. Here, specific wavelengths of light were used as pre-exposure treatments at 04:00 hrs (pre-dawn) and 06:00 hrs (dawn) in *C. serailia* (Fig. 2). FICs from corals kept in overnight darkness until 04:00 hrs showed a typical response of a coral exposed to hypoxic conditions (Ulstrup et al., 2005) (Fig. 2a). The elevated O and J steps and large dip following J, indicate an accumulation of Q_A^-. In higher plants, these responses have been shown to be characteristic of hypoxic conditions and subsequent dark reduction of the PQ pool (Feild et al., 1998; Haldimann and Strasser, 1999).

Application of far-red light prior to FIC measurement at 04:00 hrs lowered the O and J steps and removed the dip beyond the J step (Fig. 2a) by effectively exciting PSI and oxidising the PQ pool and the accumulated Q_A^- to Q_A (Schreiber, 2004). The FIC continued to rise from J, to I and then P (F_m). The reduction in the O step and increase in the P step resulted in an increase in the maximum quantum yield (Fig. 2c) as a greater proportion of PSII RCs were open. In comparison, excitation of PSII by blue and red light contributed to the partial closure of PSII centres and reduction of electron acceptors. However, this light allowed for the removal of the hypoxic conditions (as shown by the low O and J step and lack of a dip following J) which have the potential to reduce PSII electron acceptors and the PQ pool in corals (Ulstrup et al., 2005). The 10 s of 100 µmol photons m^{-2} s^{-1} blue and red light would have been sufficient to induce non-photochemical quenching (Ralph et al., 2005) and would be responsible for the lower amplitude of the I and P steps. As a result, F_v/F_m was also significantly lower than the far-red light treated corals (Fig. 2c).

The symbiotic relationship of scleractinian corals is primarily responsible for the formation of hypoxic conditions during the night as the animal host consumes oxygen through mitochondrial respiration, as do the zooxanthellae (Kühl et al., 1995; Gardella and Edmunds, 1999). Without any oxygen production through photosynthesis (as occurs during daylight hours), the only source of O_2 is from the water column. The diffusive boundary layer can limit the efficiency of O_2 transfer to the coral tissue, the efficiency of which generally depends on water flow and coral morphology (Patterson et al., 1991; Kühl et al., 1995; Bruno and Edmunds, 1998; Ulstrup et al., 2005). The availability of O_2 has consequences for the operation of the oxygen dependent process, known as chlororespiration, which involves the transportation of electrons from intraplastidic NAD(P)H to O_2 in the lumen. This has been shown to be highly active in algae (Büchel and Wilhelm, 1990) and once oxygen becomes limiting, the electron sink for this process is no longer available. As a result, electrons accumulate in the inter-system electron transport chain, resulting in the reduction of the PQ pool

(Garab et al., 1989; Bennoun, 1982, 1994; Feild et al., 1998; Peltier and Cournac, 2002; Beardall and Quigg, 2003).

The overnight reduction of the inter-system electron transport chain was reversed once the early morning light reached the corals (06:00 hrs). This low intensity light was sufficient to excite the photosystems, process the trapped electrons, produce molecular oxygen and alleviate the hypoxic conditions. Although the J step was significantly higher in the 5 min DA treatment without any pre-exposure to light, the O step was similar to the other treatments, and there was no dip in the curve following the J step (Fig. 2b). The curve following far-red light exposure only differed from the dark curve by having a lower J step due to the complete oxidation of the inter-system chain (Haldimann and Strasser, 1999; Schansker and Strasser, 2005). As F_o and F_m were the same for these treatments, there was no difference in their F_v/F_m values (Fig. 2d). The blue and red light exposed corals showed a reduced I and P step due to the onset of non-photochemical quenching, which resulted in a lower F_v/F_m (Hill et al., 2004a; Ralph et al., 2005).

The spectral properties of the sunlight at 06:00 hrs was measured, and compared to the solar spectra at midday on a fine, cloudless day, in order to identify any specific wavelengths which may have been responsible for the removal of hypoxic conditions at 06:00 hrs (Fig. 1b and c). Blue light (400–510 nm) and most of the far-red light wavelengths (700–780 nm) were in greater abundance at 06:00 hrs (see Robertson, 1966; Chambers and Spence, 1984). We suggest these wavelengths at low intensity (50 µmol photons m^{-2} s^{-1} at 06:00 hrs) would be sufficient to turn over both photosystems, produce oxygen and alleviate the tissue hypoxia (Larkum, 2003; Schreiber, 2004). The 5 min of DA at 06:00 hrs were insufficient for the formation of hypoxic conditions, thus dark-induced reduction of the PQ pool was minimal.

These series of FICs and F_v/F_m values from dark, far-red, and blue and red light exposed corals (Fig. 2), indicate that for accurate maximum quantum yield determinations after prolonged periods of darkness (e.g., overnight) a far-red light flash is required prior to chl *a* fluorescence measurements during a saturating pulse. A simple F_v/F_m measurement on a coral at pre-dawn with some degree of inter-system electron transport chain reduction will underestimate the maximum quantum yield of PSII by up to 18%.

The impact of length of DA on F_v/F_m values and on the shape of FICs was investigated for *P. damicornis*, *A. nobilis* and *C. serailia* (Fig. 3) in order to determine the presence and severity of dark-induced reduction of the inter-system electron transport chain and to identify any species-specific variation in sensitivity to processes involved in such reduction. FICs and F_v/F_m values from corals held in complete darkness and corals given a 10 s flash of far-red light were compared. *Cyphastrea serailia* showed a significant deviation between the far-red and dark measurements. After 10 min of DA, a significant decline of 8% in F_v/F_m was found and this trend continued up to the 60 min DA treatment (Fig. 3f). Following 5 min DA, the J step significantly increased in amplitude as did the O step by 10 min. Closure of PSII RCs would be responsible for the rising O step which resulted in the decline of F_v/F_m (Feild et al., 1998; Haldimann and Strasser, 1999; Ulstrup et al., 2005). *Pocillopora damicornis* showed a significant 3% decline in F_v/F_m after 30 and 60 min of DA when far-red light was not applied. Accumulation of electrons in the inter-system chain due to the onset of hypoxia resulted in the lowering of the P step (F_m) (Haldimann and Strasser, 1999; Ulstrup et al., 2005). Although *A. nobilis* did not show any significant change in F_v/F_m over time or between dark and far-red light treatments (Fig. 3e), the characteristic lower J step in the far-red pre-exposed FIC was observed (Fig. 3b) indicating that some degree of inter-system electron transport chain reduction was occurring in the dark treatments (Haldimann and Strasser, 1999; Ulstrup et al., 2005).

It has been shown here that measurements of F_v/F_m on corals following DA can produce a value significantly lower than the true maximum quantum yield. The length of DA which will lead to a reduction of the inter-system electron transport chain is dependent on the species being studied. The three coral species investigated had varying morphological characteristics and we suggest that thicker tissued corals (such as the massive *C. serailia*; Loya et al., 2001) are more prone to dark reduction of the PQ pool, as hypoxic conditions develop faster. In addition to tissue thickness, massive corals have a larger diffusion boundary layer and lower rates of dissolved gas transfer because of their mound-like structure (Bruno and Edmunds, 1998). The thinner tissued branching corals, *P. damicornis* and *A. nobilis* (Loya et al., 2001), may be less susceptible to hypoxia during darkness because of a greater potential for oxygen diffusion from the water column through to the coral tissue (Bruno and Edmunds, 1998). A greater diffusion of O_2 would eliminate the build-up of electrons in the inter-system electron transport chain, as the chlororespiratory pathway would have a continuous electron sink (i.e., molecular oxygen) (Garab et al., 1989; Bennoun, 1982, 1994; Feild et al., 1998; Peltier and Cournac, 2002; Beardall and Quigg, 2003).

These results demonstrate the difficulty of obtaining a suitable length of DA in corals. It must be sufficient to ensure the oxidation of the PSII electron acceptors and the PQ pool from electrons produced during photosynthesis, but not too long, leading to hypoxia within the coral tissue and the reduction of the inter-system electron transport chain. The thicker tissued, massive corals may be more susceptible to the dark-reduction of the PQ pool and for these species in particular, special care should be taken in deciding on the length of DA. If a far-red light source is

available in the fluorometer used to take the measurements, brief exposure of the sample to far-red light prior to maximum quantum yield or FIC measurements is recommended.

The results from Experiment 2 indicate that although FICs can significantly change with increasing length of DA, in comparison, F_v/F_m values are more robust and can withstand greater lengths of DA without producing significantly lower values (Fig. 3). This is because the O and P steps (which are used to determine F_v/F_m) remain stable, while amplitudes of J and I vary. The sensitivity of these FICs to the length of DA highlights the need for an in-depth analysis of the speed at which changes can occur to FICs. Fig. 4 shows the FICs measured during darkness or after different length of time following far-red light exposure. Corals maintained under optimal temperature and light conditions were compared to those exposed to bleaching conditions (elevated temperature and high light). The reduction in F_v/F_m between the two treatments is a characteristic stress response observed in corals during bleaching events (Iglesias-Prieto, 1995; Warner et al., 1996; Jones et al., 2000; Bhagooli and Hidaka, 2003; Hill et al., 2004a,b; Hill and Ralph, 2006). The use of chl *a* fluorescence as a tool for studying bleaching impacts warrants the need for a detailed comparison in FIC and F_v/F_m values between optimal and stressful environmental conditions. In *A. nobilis* and *C. serailia*, both the control and bleaching treatments resulted in the elevation of the J step when exposed to complete darkness and 200 s following a far-red flash of light. The amplitude of the J step provides an accurate measure of the reduction of Q_A to Q_A^- (Govindjee, 1995; Strasser et al., 1995; Haldimann and Strasser, 1999; Zhu et al., 2005) and these results indicate that 200 s of darkness prior to FIC measurement are sufficient for the accumulation of Q_A^-. FIC measurements taken after shorter periods of time following a far-red flash reveal a lower J step, suggesting that far-red light application is effective in oxidising the dark-induced reduction of the PSII electron acceptors and the PQ pool.

After 200 s of darkness, an elevation in the J step can be detected and this has considerable implications for measurements of FICs where DA exceeds 3 min. In such cases, the observed amplitude of J may be overestimated due to the sensitivity of the J step to the redox state of the electron carriers in the inter-system chain. We recommend that for determination of a J step which reflects open PSII RCs and a fully oxidised inter-system electron transport chain, a far-red flash must be applied prior to FIC measurement.

The extra peak observed in the dark and 200 s curves between the J and I steps at 0.03 s in *A. nobilis* (Fig. 4b and e) and *C. serailia* (Fig. 4c and f) can be attributed to PQ pool redox state and PSI activity (Hill et al., 2004a; Ilik et al., 2006). PQ pool reduction in the dark may be reversed upon PSI excitation during the saturating pulse resulting in PQ reoxidation and formation of the extra step. With the far-red light stimulation of PSI and subsequent PQ pool oxidation, this extra peak was not visible upon FIC measurement. This further demonstrates the importance of a far-red flash prior to FIC measurement to ensure that features that are uncharacteristic of an oxidised inter-system electron transport chain are not inadvertently detected.

In summary, the potential for misinterpretation of chl *a* fluorescence data collected from dark-adapted corals, because of dark-induced reduction of the PQ pool, is significant and needs to be considered in studies using this technique. The assumptions of an oxidised inter-system electron transport chain and open PSII RC following DA cannot be made, especially for thick-tissued massive corals because they become anoxic very quickly in darkness. These issues are equally significant for corals in optimal and stressful (i.e., bleaching) conditions. A far-red light pulse given prior to F_v/F_m or FIC measurement is an effective and simple method to ensure oxidation of the inter-system electron transport chain which then allows for the accurate determination of the maximum quantum yield and FICs.

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