Photosynthetic impact of hypoxia on in hospite zooxanthellae in the scleractinian coral *Pocillopora damicornis*

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ABSTRACT: Shallow water coral reefs may experience hypoxia under conditions of calm weather coldtrums. Anaerobic responses of endosymbionts (i.e. zooxanthellae) within *Pocillopora damicornis* coral colonies were tested using both slow and fast chlorophyll a fluorescence induction kinetics. Zooxanthellae were examined in hospite when exposed to control conditions (26°C, 200 μmol photons m⁻² s⁻¹, 100% air-saturation, 4 cm s⁻¹ flow) and to 2 treatments of reduced air content (40 and 0%), achieved by controlling the N₂:O₂ ratio in water circulating at 2 cm s⁻¹. Furthermore, the impact of water flow on photosynthesis was examined at 0% air saturation by turning off the flow entirely (0 cm s⁻¹), thereby mimicking the environmental conditions of calm weather coldtrums. Corals exposed to depleted air content (0% with and without flow) showed a significant decrease (p < 0.001) in effective quantum yield (φₑ) in comparison with controls. Maximum quantum yield was significantly reduced when gas exchange was inhibited (0% without flow), whereas non-photochemical quenching (NPQ) was not affected. Fast polyphasic fluorescence transients of chlorophyll a fluorescence showed a significant increase in minimum dark-adapted fluorescence, Fₗ, when corals were exposed to anaerobic conditions. Furthermore, an increase in the J peak (2 ms) corresponding to the reduction of the primary electron acceptor, Q_A, was observed in 0% air-saturation with flow. We found that the most sensitive parameters for detecting physiological change associated with hypoxia were φₑ using slow (pulse-amplitude modulation) fluorescence kinetics, as well as an increase in the O peak, φₙ, non-photochemical quenching before Q_A, and an elevation of the J peak on a double-normalised transient using fast (Plant Efficiency Analysers) induction kinetics.

KEY WORDS: Fluorescence · Fast-induction kinetics · Pulse-amplitude modulation · Photosystem II · PSII

INTRODUCTION

The effect of hypoxia (defined as the absence of air or free oxygen) on Photosystem II (PSII) in photosynthesising aquatic organisms is largely unexplored (Schreiber & Vidaver 1974, Kuhl et al. 1995, Schreiber et al. 2002). This is paradoxical, especially in corals, considering the frequent and sometimes prolonged periods of calm weather often encountered in shallow water environments such as reef flats and lagoons (e.g. Lesser et al. 1994, Nakamura & van Woesik 2001, Nakamura et al. 2003). In situ flow conditions measured in the reef environment range between 1 and 39 cm s⁻¹ (Dennison & Barnes 1988, Patterson et al. 1991). An increase in the thickness of the diffusive boundary layer (DBL) is linked to a reduction in water flow across an organism’s surface (e.g. Patterson et al. 1991, Kuhl et al. 1995) leading to reduced gas-exchange, which can induce anaerobiosis (Schreiber & Vidaver 1974, Kuhl et al. 1995). Anaerobiosis is the physiological condition induced by hypoxic water. Coral-algae symbioses are complex systems where both components are inter-related and are mutually affected by abiotic factors, such as dissolved gas con-
tent which fluctuates with flow (Dennison & Barnes 1988, Kuhl et al. 1995, Nakamura & van Weesik 2001, Brown et al. 2002, Nakamura et al. 2003). Thus, water motion has been found to increase both the respiration of the host and photosynthesis of the algae known as zooxanthellae (Symbiodinium spp.) (Patterson et al. 1991). Coral colonies are more prone to bleaching (whitening of corals due to loss of either symbiotic algae or their pigment, or both) when enduring low-flow conditions (Nakamura & van Weesik 2001, Nakamura et al. 2003). Under such conditions, zooxanthellae or their pigment, or both) when enduring low-flow conditions to investigate impacts of hypoxia on PSII of zooxanthellae in hospite.

**MATERIALS AND METHODS**

*Pocillopora damicornis* (Linnaeus) colonies were collected from Heron Island lagoon (<2 m deep) (152° 06′ E, 20° 29′ S) over the period 26 January to 4 February 2003, and transported to the University of Technology, Sydney where they were allowed to acclimate for 2 mo in a 500 l aquarium supplied with recirculating artificial seawater [carbonate 140 ppm] and synthetic sea salt [Aquasonic ‘Ocean Nature’] to 33 ppm in reverse-osmosis water, 26 ± 1°C and 200 μmol photons m⁻² s⁻¹. A nubbin was broken from 4 P. damicornis colonies and held in the 500 l aquarium to acclimatise for 14 d before experimentation. Induction-curve analysis (PAM) and fast-induced kinetic techniques (PEA) were performed for each coral replicate (n = 4).

**Experimental protocol.** One nubbin from each of the 4 colonies was transferred into a purpose-built dark-acclimation chamber (2.8 l; see Hill et al. 2004b) with continuously circulating 100% air-saturated seawater at 26 ± 1°C at a flow rate of 4 cm s⁻¹ (231 l min⁻¹ into an 11 cm diameter chamber). This was deemed to be an intermediate flow rate around a *Pocillopora damicornis* colony by Lesser et al. (1994). Each sample was acclimated for 10 min under 200 μmol photons m⁻² s⁻¹ (1.8 W m⁻²) with actinic light provided by a halogen lamp (12 V, 150 W with UV filter). A pilot study showed that 10 min of dark incubation was sufficient to fully dark-adapt the samples. Water temperature was maintained using a temperature-regulated water bath (TH3 Thermoregulator, Ratek Instruments) connected to the dark-acclimation chamber. Samples were dark-adapted for 10 min after which fast-induced-kinetic transients (PEA) were obtained. Actinic light of 200 μmol photons m⁻² s⁻¹ was re-applied for a further 10 min, after which the nubbin was dark-acclimated for 10 min. Finally, induction-curve analysis (PAM) was performed. This procedure was repeated for the con-
trol (100% air-saturation with flow) and each of the 3 treatments: 40 ± 5% air content with a flow regime of ca. 2 cm s⁻¹, 0 to 2% air content with a flow regime of approximately 2 cm s⁻¹, and 0% air content with no flow. Air saturation was regulated by adding N₂ to the flow stream (Schreiber & Vidaver 1974) and measured using oxygen-sensing 100 μm optodes (Precision Sensing). It was assumed that CO₂ flushing, due to the volume of the chamber, the recirculation of water and the buffering capacity of bicarbonate in seawater. The optodes were calibrated linearly to 100% air saturation in 200 ml of seawater, aerated with an air stone for at least 10 min, and 0% air saturation in seawater, which had been flushed with N₂ for at least 10 min.

**Slow (PAM) fluorescence measurements.** Fluorescence measurements were performed using a Diving-PAM—Walz settings: saturating intensity (SI) = 8, saturating width (SW) = 0.8 s, actinic intensity (AI) = 3 (200 μmol photons m⁻² s⁻¹), actinic width (AW) = 300 s, absorption factor (AF) = 1, gain (G) = 4, damping (D) = 2, induction delay (ID) = 40 s, induction width (IW) = 20 s. The Diving-PAM employs a 3 μs pulse from a red light-emitting diode (LED) with a peak emission at 650 nm as the measuring light (0.15 μmol photons m⁻² s⁻¹). Saturation pulses were of 4500 μmol photons m⁻² s⁻¹ and actinic light was of 100 μmol photons m⁻² s⁻¹. Chlorophyll fluorescence was detected at wavelengths above 710 nm.

Dark-light transition curves (induction curves) demonstrate the capacity of a tissue to regulate photosynthesis at a known level of irradiance. Tissue was dark-adapted initially for 10 min, then actinic light of 200 μmol photons m⁻² s⁻¹ was applied for 5 min. In order to monitor the effective quantum yield (φₑₑₑ) and quenching parameters, a saturating pulse was applied every 20 s. φₑₑₑ and non-photochemical quenching (NPQ) were determined according to the following equations (Schreiber 2004):

\[
\text{NPQ} = \left( \frac{F_{m} - F_{n}}{F_{m}} \right),
\]

\[
\phi_{e} = \left( \frac{F_{m}^\prime - F_{i}}{F_{m}^\prime} \right),
\]

where \( F_{m} \) is maximum dark-adapted fluorescence, \( F_{m}^\prime \) is maximum light-adapted fluorescence and \( F_{i} \) is minimum light-adapted fluorescence. Maximum non-photochemical quenching (NPQmax) was recorded 4 min into the induction curves of all treatments.

**Fast (PEA) fluorescence measurements.** The OJIP transient nomenclature by Strasser et al. (1995) can be divided into 2 phases, the fast rise from the minimum fluorescence (O → F₁) to J and onwards to the intermediate step (II, and a slower rise to F₃ [see P]). The fluorescence rise from O to J corresponds to the reduction of the primary electron acceptor of PSII, QA, to Qₐ⁻ (Strasser et al. 1995, Hill et al. 2004a). Fast-induction kinetics provide detailed information on the photochemical state of PSII, as well as the filling of the PQ pool (Govindjee 1995).

Fast-induction kinetics were measured using the Plant Efficiency Analyser (PEA) with coral samples after 10 min dark adaptation. The array of 6 red LEDs (peak wavelength 650 nm) provided the saturating illumination (3200 μmol photons m⁻² s⁻¹) and focused on an area 4 mm in diameter. A PIN-photodiode (shielded by a long-pass filter >720 nm) detected the fluorescence signal from the coral. The fluorescence signal was obtained over 2 s and recorded every 10 μs for the first 2 ms, every 1 ms for the first 1 s of sampling, and every 100 ms thereafter. The base fluorescence (\( F_{b} \)) was measured at 0.05 ms (O), and \( P (F_{P}) \) was recorded as the maximum fluorescence reached after a 1000 ms sampling period. Curves for each treatment were normalised to \( F_{b}/(F_{b}/P) \) to illustrate the change in variable fluorescence (\( F_{v} \)). Furthermore, the relative variable fluorescence was calculated by double normalising to O and \( P \) (see Fig. 1) and \( Q_{A} (Q_{A}) \) (Haldimann & Strasser 1999). The efficiency of electron transport before \( Q_{A} \) was calculated using the following formula (Lazar 1999):

\[
\phi_{p} = 1 - \left( \frac{F_{i}}{F_{m}} \right),
\]

where \( F_{i} \) is minimum dark-adapted fluorescence and \( F_{m} \) is maximum dark-adapted fluorescence.

**Statistical analyses.** One-way analysis of variance (ANOVA, Glantz 2002) tests were used to determine if significant differences were present among the different air-content treatments in \( F_{i}/F_{m} \), \( \phi_{e} \), and \( \text{NPQ}_{max} \) (see Fig. 1), as well as 6 individual PEA parameters (O, J, I, P, \( F_{i} \), and \( \phi_{e} \), see Table 1) and OJIP points (see Fig. 3A) along the curve. Where the assumptions of normality and equal variance failed (\( p < 0.05 \)), data was transformed using natural log. Transformed data successfully met the assumptions of normality and equal variance. Post-hoc comparisons were performed using the Holm test, which is less conservative than Tukey's HSD or Bonferroni and also controls the overall risk of a false-positive conclusion at the nominal level (Glantz 2002).

**RESULTS**

In a pilot study, the air content at the coral surface under 4 cm s⁻¹ flow rates was monitored using optodes. Results showed that this was reduced from 100 to 35 ± 5% air-saturation after 10 min in darkness. This was the lowest air content achievable (under flow) without N₂ manipulation, thereby defining the 40% intermedi-
ate air treatment. In a subset (n = 2) where the flow was re-established (100% air), the coral was able to completely reverse the impact of turning off the flow.

**Slow (PAM) fluorescence**

Maximum ($F_{m}/F_{n}$) and effective ($\Phi_{oq}$) quantum yield, as well as NPQ, of the 4 different treatments, are shown in Fig. 1. $F_{m}/F_{n}$ was significantly reduced ($p = 0.017$) in the 0% air-saturation without flow treatment compared to the 100, 40 and 0% air-saturation treatments with flow. $\Phi_{oq}$ values for the severely air-depleted treatments (0% air-saturation with and without flow) were significantly lower ($p < 0.001$) than those for the 100 and 40% air-saturation treatments. NPQ was not significantly different ($p = 0.536$) among all 4 treatments.

The induction curves for $\Phi_{oq}$ and NPQ showed similar shapes for the 100 and 40% air-saturation treatments, but differed for the air-depleted samples (0% air saturation with and without flow) (Fig. 2). The most conspicuous difference between these 2 groups was the fast down-regulation of $\Phi_{oq}$ at the dark-light transition for the air-depleted samples (Fig. 2C,D), most likely due to a large rise in $F_{o}$ and a smaller decline in $F_{m}$. NPQ declined after 100 s actinic light in the 100 and 40% air-saturation treatments, whereas it increased or remained steady in the 0% air-saturation treatments (with and without flow).

![Fig. 1. Pocillopora damicornis. Effects of air-saturation and flow on maximum quantum yield, $F_{m}/F_{n}$ (black bars), effective quantum yield, $\Phi_{oq}$ (light grey bars) and maximum non-photochemical quenching, NPQ (dark grey bars) during induction curve analysis (n = 4). The 4 treatments, 100% air-saturation, 40% air-saturation, 0% air-saturation with flow and 0% air-saturation without flow are shown including standard error bars. Values with different letters were found to be significantly different ($p < 0.05$) by the Holm test.](image)

![Fig. 2. Pocillopora damicornis. Induction-curve analysis of the 4 treatments, (A) 100% air-saturation, (B) 40% air-saturation, (C) 0% air-saturation with flow and (D) 0% air-saturation without flow. The curves represent mean values, including standard error bars (n = 4) of $\Phi_{oq}$ (EQY) and non-photochemical quenching (NPQ). A representative curve showing $F_{o}$ is superimposed on the data. SP: saturating pulse](image)
Fast (PEA) fluorescence

The fast-induction kinetics of the 4 treatments are shown in Fig. 3. When exposed to 100 and 40% air saturation, corals showed a classical OJIP curve (Fig. 3A) with no significant differences in the amplitudes of the peaks (Table 1). An analysis of the amplitudes of the O, J, I and P peaks allowed the detection of significant differences with the other 2 treatments (Table 1). The O peak was found to increase under anaerobic conditions (p < 0.001) and the Holm post-hoc test revealed that there were also significant differences between the 0% air-saturation treatments, where the treatment with flow had a higher O (Table 1). A significantly higher J peak amplitude was also observed in the 0% air-saturation with flow treatment, compared to the other 3 treatments (p < 0.005). Following the J peak, the corals exposed to 0% air saturation with and without flow showed a pronounced dip, which was not apparent in the 100 and 40% treatments (Fig. 3A). The I and P peaks were greatly reduced (p < 0.05) in the 0% air-saturation without flow treatment compared to the other treatments. Fig. 3B shows the fast-induction curves of the 4 treatments normalised to F_0. This presentation of the data (also see Table 2) allows a clearer understanding of the change in variable fluorescence \( I F = F_{m} - F_{0} = P - O \) among the treatments, where \( F_{m} \) is the value for the 0% air-saturation with and without flow treatments were significantly lower than those for the 100 and 40% treatments. When double-normalised (Fig. 3C), the J peak was considerably higher in the 0% air-saturation with and without flow treatments than in the 100 and 40% treatments.

Variation in the time at which each peak occurred was also investigated. Table 1 shows that the timing of the O, J and P peaks remained constant among treatments (0.05 ms, 2 ms and 1000 ms, respectively), but the timing of the I peak varied. In the 100 and 40% air-saturation treatments, the I peak occurred at 50 ms, whilst in the 0% with and without flow treatments, the I peak took longer to reach (150 ms). An analysis of the electron transport efficiency before O_2 (\( \Phi_{o} \)) also separated the treatments into the 2 groups of with air (100 and 40%) and without air (0%) with and without flow (Table 2: p < 0.05). This shows that under conditions of 0% air, the electron transport efficiency before O_2 had declined.

![Diagram of Pocillopora damicornis Fast-induction kinetic transient curves under 100% air-saturation (●), 40% air-saturation (□), 0% air-saturation (▲) and 0% air-saturation without flow (▼).](image_url)
ANAEROBICITY RESULTED IN AN INCREASE IN $F_o$ AFTER A SATURATING PULSE IN THE DARK (SEEN IN FIG. 2C,D), AND THIS FLUORESCENCE SIGNAL WAS SLOWLY QUENCHED WHEN EXPOSED TO MODERATE ECTONIC LIGHT (200 nMOL PHOTONS m$^{-2}$ s$^{-1}$). THIS IS EXPLAINED BY THE REDUCTION OF PSI REACTION CENTRES, WHICH IS LINKED TO DARK REDUCTION OF THE PQ POOL, A PHENOMENON KNOWN AS CHLORORESPIRATION (HARRIS & HEBER 1983). IN LIGHT, FLUORESCENCE IS SLOWLY QUENCHED DUE TO PHOTOSYSTEM I (PSII) GRADUALLY RE-OXIDISING PSI (OPENING REACTION CENTRES) VIA THE ELECTRON TRANSPORT CHAIN (KALTANEN ET AL. 1999).

The fast-induction curves revealed several physiological changes, which occur in corals when exposed to anaerobic conditions (Fig. 3). For the 100 and 40% air-saturation treatments (Table 1), no significant differences were observed in the amplitudes of the O, J, I or P peaks, nor in the timing of each peak. This indicates that the various components of the photosynthetic apparatus are functioning similarly under these conditions. However, under conditions of 0% air-saturation with and without flow, changes in the fast-induction curves became apparent (Fig. 3). When the air-saturation was reduced to 0%, a significant rise in the O peak was seen (Fig. 3A). This rise was maximal under 0% air-saturation with flow (Table 1). An increase in $F_o$, which has been suggested to occur as a result of the dissociation of the light-harvesting complex of PSII (LHC II) from the PSII reaction centres (ARMOOD ET AL. 1978, YAMANE ET AL. 2000), was also observed by Schreiber & Vidaver (1975), who proposed that the recovery of photosynthesis, as manifested in $F_o$, is dependent upon the dark-limiting steps of the re-oxidation of the PSII primary acceptor pool. The increase in $F_o$ is thought to be linked to the water-splitting enzyme, hydrogenase, catalysing the endogenous electron reactions on the donor side of PSII, which is slowly induced during anaerobic stress (SCHREIBER & VIDAYER 1974).
electron acceptors of PSI in the dark. Another possible contributor to a rise in \( F_0 \) is the decline in efficient energy trapping by PSI (Havaux 1993, Yamane et al. 2000).

A greater J peak amplitude occurred under the 0% air-saturation with flow treatment compared to the other 3 treatments, which all had similar J peak amplitudes (Fig. 3A). The J peak is indicative of the photochemical phase, where \( Q_A \) is reduced to \( Q_A^- \) under illumination (Govindjee 1995, Hill et al. 2004a). The increase in J indicates that there was a large increase in the rate of \( Q_A^- \) reduction under 0% air-saturation with flow. This response is probably due to the accumulation of redox components in the electron transport chain, which become reduced in the dark (Haldimann & Strasser 1999). The probable mechanism responsible for this dark pre-reduction of the electron transport chain is chlororespiration or PSI cyclic electron transport (Yamane et al. 2000). An elevated J peak was only observed in the 0% air-saturation with flow treatment. The lack of a similar response in the 0% treatment without flow suggests that elevation of the J peak may not be a stable biomarker of anaerobiosis in zoanthellae in hospite (Fig. 3A).

The J peaks in the 0% air-saturation with and without flow treatments were 97 and 96%, respectively, of the maximum fluorescence (P peak). In contrast, the same rise in the 100 and 40% air-saturation with flow treatments only contributed 72 and 79%, respectively, of the maximum fluorescence. The transients of the air-depleted (0% air-saturation with and without flow) treatments were thus dominated by the O–J rise, where \( J = I + P \) (Haldimann & Strasser 1999). This indicates that the maximal fluorescence yield occurred at the J peak, due to the accumulation of electrons beyond \( Q_A \). The reduction in the efficiency of electron transport before \( Q_A^- (\Phi_{II}) \) (Table 2) indicates that the dark reduction of the electron acceptors limits the capacity for \( Q_A^- \) reduction. Also, the P peak was reduced in amplitude to the level of the J peak, due to the limited capacity for \( Q_A^- \) re-oxidation. This provides further evidence that anaerobic conditions cause the reduction of the electron transport chain in the dark.

The anaerobic (0% air-saturation with and without flow) treatments caused a significant dip to occur after the J peak (Fig. 3A). Schreiber & Vidaver (1974) proposed that this dip occurs in response to enhanced PSI activity, which functions optimally under conditions of anaerobiosis. The dip after the J peak in the fast-induction curves signifies that PSI cyclic electron transport is operating, possibly along with chlororespiration, causing the dark reduction of the electron acceptors and PQ pool (Schreiber & Vidaver 1974). We speculate that this possible increase in PSI activity could also explain the delayed appearance of the I peak \( (I_b) \) in the air-depleted treatments. In comparison, PSI becomes inhibited, which is demonstrated by the decline in \( F_0/F_m \) (Fig. 1) and \( F_0 \) (Table 2, Fig. 3B). Furthermore, I and P peak signals were lower in 0% air-saturation without flow compared to the other treatments, as a result of reduction of the PQ pool (Fig. 3A). The relative variable fluorescence (Fig. 3C) demonstrates that there is an elevation of the J peak with a decline in air saturation, due to the accumulation of \( Q_A^- \) under hypoxic conditions in the dark. From these fast-induction curves, the most sensitive biomarkers of the onset of anaerobiosis are a rise in \( F_0 \), a reduction in \( \Phi_{II} \) and an elevated J peak in double-normalised transients.

Future research is needed to demonstrate whether far-red illumination is able to re-oxidise PQ and open the PSI reaction centres. Under far-red illumination, PSI activity would be stimulated, thus removing effects of dark-reduced electron acceptors in PSI under anaerobic conditions. Introducing \( O_2 \) would also remove the anaerobiosis-dependent increase in fluorescence signal by oxidising the PQ pool (see Schreiber & Vidaver 1974).

In conclusion, we have demonstrated that hypoxia indeed induces deviations from normal functioning PSI systems, through the measurements of both fast (PEA) and slow (PAM) chlorophyll a fluorescence. The 2 instruments were shown to complement each other in giving detailed information on the response of *Pocillopora damicornis* when exposed to decreased air and flow levels. Anaerobiosis can develop rapidly under low-flow conditions (Patterson et al. 1991, Kühl et al. 1995, Nakamura & van Woesik 2001, Nakamura et al. 2003). This has important implications for coral bleaching events, which are intensified under such conditions. We observed similar photochemical impacts as those which occur during bleaching events (e.g. Jones et al. 1998, Hill et al. 2004b). Hypoxia and bleaching conditions in conjunction (i.e. doldrums, elevated temperature and high light) may intensify this response, which would suggest increased susceptibility of coral to bleaching. Furthermore, an important technical outcome of this research is that the use of dark-adapted coral samples held in low-volume facilities could lead to overestimation of the \( F_0 \) level (O peak, Table 1) and, thereby, distortion of other fluorescence parameters such as \( \Phi_{II} \), F0 and \( F_0/F_m \).

**Acknowledgements.** We thank N. Ralph for fabrication of the dark-adaptation chambers and Dr. F. Torpy and Dr. B. Kelaber for assistance in statistical analyses. Prof. A. W. D. Larkum contributed editorial comments. We are also thankful for the valuable comments of 3 anonymous reviewers. This work was performed with the permission of GBRMPA (permit number G03/97661). The Australian Research Council and the University of Technology, Sydney provided financial and logistical support.
LITERATURE CITED


Submitted: April 14, 2004; Accepted: September 30, 2004

Proofs received from author(s): January 5, 2005