

Photoacclimation involves modulation of the photosynthetic oxygen-evolving reactions in *Dunaliella tertiolecta* and *Phaeodactylum tricornerutum*

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Abstract. Net energy accumulation by marine microalgae at very low photon fluxes involves modulation of several attributes related to both the growth and photosynthetic physiology of these organisms. Here we studied flash-induced oscillatory patterns in oxygen evolution by previously dark-adapted cells of the green alga *Dunaliella tertiolecta* (Butcher) and the diatom *Phaeodactylum tricornerutum* (Bohlin). The activity of the oxygen-evolving complex was found to be species-specific and influenced by photoacclimation. Results from measurements of oxygen flash yield obtained for these organisms grown under light-saturating conditions are directly comparable to those previously reported in the literature for other microalgae and higher plants. However, similar measurements on cells grown in low-light and/or light-starved conditions indicate an increased level of backward transitions (double misses) leading to the formation of super-reduced states (i.e. S_{-1} and S_{-2}). Thus, in this communication, we present the first evidence that super-reduced states can be generated *in vivo* and speculate on how they may be physiologically important.

Keywords: backward transitions, *Dunaliella tertiolecta*, eigenvalue model, Kok parameters, microalgae, oxygen evolution, *Phaeodactylum tricornerutum*, photosystem II, phytoplankton.

Introduction

Light is not only the primary source for photosynthetic energy conversion but also an essential regulatory factor for microalgal growth. Optimising photosynthetic efficiency under fluctuating irradiance involves regulation of pigment composition, light-harvesting complexes, turnover of proteins and nucleic acids. While much of the photoacclimation literature deals with changes at the level of the photosynthetic electron transport chain and the reaction centres, little attention has been focused on the role of the water-splitting reaction. This reaction sequence occurs in the oxygen-evolving complex (OEC) and is dependent on a continuous supply of photons. Yet microalgae frequently grow in light-limited conditions, particularly during intense mixing of the euphotic zone. The focus of the present study was therefore to investigate constraints imposed on oxygen

production at the level of the water-splitting reaction in order to elucidate whether charge recombination in PSII plays a role in limiting photosynthesis at low irradiances. That is to say, at very low irradiance, is the photon flux low enough to prevent advancement of the S-state cycle (see below) before a backward transition occurs? Such a form of ‘slippage’ has been postulated to define the lower limit of light for growth of photoautotrophs (Raven and Beardall 1981, 1982; Raven *et al.* 2000).

The earliest investigations into the mechanism of water oxidation were those of Pierre Joliot (Joliot *et al.* 1969) and Bessel Kok (Kok *et al.* 1970; Forbush *et al.* 1971). At about the same time, they independently reported that in previously dark-adapted cells exposed to a sequence of single-turnover flashes, the oxygen yield oscillated in a curious but reproducible fashion. (Note that in this manuscript the term ‘dark-adapted’ refers not to genotypically adapted cells, but to

Abbreviations used: Chl *a*, chlorophyll *a*; HL, saturating photon flux for growth; LL, limiting photon flux for growth; OEC, oxygen-evolving complex; PQ, plastoquinone; Q_A and Q_B , primary and secondary quinone acceptors in PSII; Y_D , reduced form of the redox-active tyrosine residue located on the reaction centre protein D2; Y_D^{OX} , oxidised form; Y_n , oxygen yield on the *n*th flash; Y_{SS} , steady-state oxygen flash yield; α , miss parameter; β , double-hit parameter; γ , single-hit parameter; δ , backward-transition parameter.

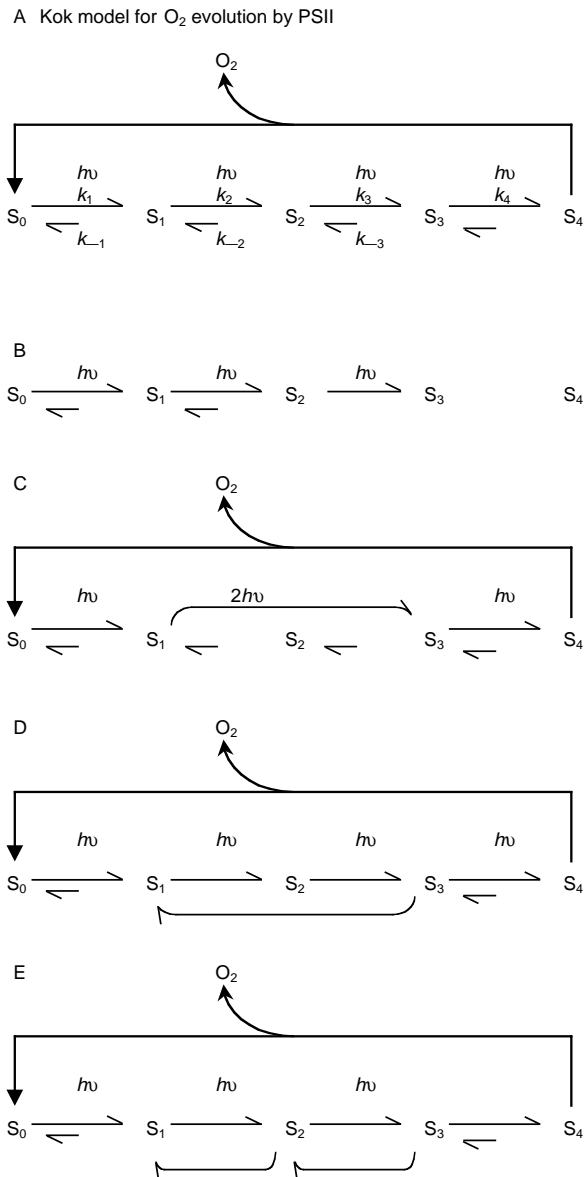


Fig. 1. (A) The OEC consists of a manganese-containing unit whose redox states are known as the S_n -states (Kok cycle). Sequential forward electron-transfer reactions are driven by the absorption of a photon by the PSII reaction centre, each leading to the release of an electron from the OEC. Once four electrons are removed, two water molecules are oxidised and an oxygen molecule is evolved. (B) Some fraction of the OECs will undergo rapid back-reactions between flashes (a miss), so the S_n states do not advance. (C) Some fraction of traps may absorb more than one photon during a flash (double hit), and advance by more than one S_n state. Backward transitions (or double misses) represent S_n states actually going backwards in the cycle owing to deactivations. These can be achieved by (D) a single hit combined with two deactivations from S_3 to S_1 , or (E) a failure of the S_n state to advance upon giving a flash, followed by one-step deactivation, either from S_3 to S_2 or S_2 to S_1 (Packham *et al.* 1988; Meunier 1993). OECs can attain redox states below the S_0 level such that super-reduced states are formed (S_{-3} , S_{-2} and S_{-1}) from D and E. These are reoxidised with similar (S_{-1}) or identical (S_{-2}) kinetics to those of S_0 (Messinger and Renger 1993).

cells that have been kept in darkness for a short period before measurements are made.) Considerable effort since then has been focused on elucidating the biochemical mechanism responsible for the water-splitting reaction (Wydrzynski 1982; Renger 1999). The precise details of the process, however, remain contentious. Figure 1 is a simplified scheme for water oxidation in the OEC. The S_0 and S_1 states are stable in the dark, whereas the S_2 and S_3 states are metastable, deactivating to S_1 with a lifetime dependent on the redox state of the PSII electron acceptors (Kok *et al.* 1970; Forbush *et al.* 1971). The S_4 state is transient, reacting to produce oxygen and the S_0 state (Fig. 1A). Damping of the oxygen flash yield pattern at higher flash numbers has been attributed to misses (Fig. 1B) and double hits (Fig. 1C). Backward transitions (Figs 1D, E) (or double misses) lead to the formation of the over-reduced state (S_{-1}) and the super-reduced states (S_{-2} and S_{-3}) (Messinger and Renger 1993; Messinger *et al.* 1997). The consequence of these backward transitions is an increased population of PSII units retaining a reduced primary quinone (Q_A) during the time of the S_n -state advancement. For these reasons, backward transitions can be used as an indicator of the reactivity of the OEC as well as the reducing power of the cells (Packham *et al.* 1988).

Two marine microalgae were chosen for this study on the basis of earlier evidence that their minimum requirements for light differ by an order of magnitude (Quigg 2000; Quigg and Beardall in press). *Phaeodactylum tricoratum* is able to sustain growth at even 10^{-4} of full sunlight, whereas *Dunaliella tertiolecta* has a light compensation point for growth of around $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. We hypothesised that differences in the characteristics of their OECs may define the environment of lowest light that these microalgae may exploit. Oxygen flash yield patterns were analysed by the Kok model (Kok *et al.* 1970; Forbush *et al.* 1971; Messinger *et al.* 1997) or the modified 'eigenvalue method'. The latter method allows for the direct calculation of backward transitions given the miss probability (Packham *et al.* 1988) and, with modifications, estimation of double hits (Meunier 1993; Meunier *et al.* 1995, 1996). Analyses were used to determine (1) the distribution of transition probabilities and the S states (both methods); (2) S_3 deactivation kinetics; and (3) presence (or absence) of super-reduced states and backward transitions (modified eigenvalue method only). In addition, we examined the effect of light starvation on *D. tertiolecta* and *P. tricoratum*. Prolonged darkness (>24 h) is thought to be sufficient time to scrub cells of endogenous reductants such as NAD(P)H generated in the light, and hence lower the reactivity of the OEC. Whilst OEC stability allows *P. tricoratum* to exploit extremely low photon fluxes, the flexibility of the OEC in *D. tertiolecta* seems better equipped for dealing with high photon fluxes.

Materials and methods

Culture growth conditions

Dunaliella tertiolecta (CS-175) and *P. tricornutum* (CS-29) were obtained from the CSIRO Culture Collection of Microalgae (CSIRO Division of Oceanography, Australia), and grown in semi-continuous batch cultures (250 mL) at $20 \pm 2^\circ\text{C}$ in the artificial seawater medium PHK (a modified 'D' medium; Provasoli *et al.* 1957). They were grown with continuous illumination, for at least 5–10 generations (depending on the growth rate) before sampling. *D. tertiolecta* and *P. tricornutum* were acclimated to a photon flux that saturated ('high light', HL) their growth rates: $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The limiting ('low light', LL) photon flux used to grow *P. tricornutum* ($3 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) was an order of magnitude lower than that for *D. tertiolecta* ($30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). These values are close to the light compensation points for the two species (Quigg and Beardall, in press). Cultures were maintained at $0.5\text{--}1.0 \times 10^6$ cells mL^{-1} for *D. tertiolecta* and $1.0\text{--}1.5 \times 10^6$ cells mL^{-1} for *P. tricornutum* to avoid self-shading of cells. Cell counts were performed with an Improved Neubauer haemocytometer (Superior, Berlin, Germany).

Sample preparation

Before each experiment, cultures (50 mL) were harvested (10000 g, 10 min), resuspended in 1 mL of PHK medium, and kept in the dark at 20°C . A small aliquot (10 μL) was removed for chlorophyll *a* (Chl *a*) analysis. The equations of Porra *et al.* (1989) were used to calculate the amount of Chl *a* in *D. tertiolecta* (extracted in 80% acetone with 25 mM HEPES buffer at pH 7.5) and *P. tricornutum* (extracted in 100% methanol). *D. tertiolecta* and *P. tricornutum* were then used at concentrations of 4 mg Chl *a* L^{-1} and 2 mg Chl *a* L^{-1} , respectively. For 'light-starvation' experiments, cultures were placed in the dark for at least 24 h on an orbital shaker (to prevent an anaerobic environment developing in the flasks) before harvesting. Although longer dark periods have been shown to be required for light starvation in *Chlorella* sp. (D. Mauzerall, pers. comm.), here we are simply presenting preliminary and comparative findings on the effect(s) of light starvation in the two algal species.

Experimental design

An unmodulated Joliot-type electrode was used (Messinger *et al.* 1997). Samples were excited with a flash control unit and a xenon flash lamp (EG and G) with pulse duration of 9 μs (half-height). An orange

filter placed in front of the sample was used to reduce some flash artefacts from the flash lamp. The flash-induced O_2 amperometric signals were uncoupled from the steady background signal caused by oxygen dissolved in the flow buffer (PHK medium at pH 8.2 and 20°C) with an isolation transformer (Messinger *et al.* 1997). The first flash was given 200 ms after the start of data recording. The isolated and amplified signals were then digitised with an analogue–digital converter with a sampling rate of 1 kHz and stored on computer.

The oxygen flash yield pattern was measured on samples (10 μL) that were dark-incubated (5 min) in the oxygen electrode, and then given a train of 20 flashes. After a further 5 min in the dark, cells were given a single flash and allowed to dark-adapt for another 5 min. These steps constitute the sample 'pretreatment', which enhanced the initial S_1 population. After the third dark incubation, a 16-flash series was given at a rate of 2 Hz, and the corresponding flash sequence recorded. The final dark-incubation time required to obtain a stable peak height on the third flash was species-specific and dependent on the growth photon flux (Table 1). In order to explain the occurrence of the maximal oxygen yield after the third flash and not the fourth flash, Kok *et al.* (1970) postulated that the $\text{S}_1:\text{S}_0$ ratio equilibrated to 3:1 in the dark. The initial $\text{S}_1:\text{S}_0$ ratio was on average set to 1.9:1 and 3.4:1 for *D. tertiolecta* and *P. tricornutum*, respectively (Table 1), suggesting that dark synchronisation of catalytic centres in *D. tertiolecta* may have required a longer dark incubation. Distribution of transition probabilities, however, is not influenced by the pre-incubation time (Schmid *et al.* 1994).

S_3 deactivation kinetics

S_3 deactivation kinetics were determined by first pretreating samples as described above. After the third dark-incubation period in the electrode, two flashes were given. A final dark period was then varied from 10 s to several minutes before applying the remaining flash sequence at 2 Hz. In this way, S_3 decay curves could be generated for *D. tertiolecta* and *P. tricornutum* by plotting the normalized oxygen yield on the third flash (i.e. Y_3/Y_{SS} , where Y_{SS} is the steady-state oxygen flash yield) as a function of the variable dark time between the second and third flashes. Such decay curves are biphasic (Fig. 2) where the relative contributions of the fast and slow phases represent the relative amount of Y_D (the reduced form of the redox-active tyrosine residue located on the reaction centre D2 protein) to $\text{Y}_\text{D}^{\text{OX}}$ (the oxidised form) in the sample. The proportion of each form was estimated by extrapolating the slow phase back to the y -axis at zero dark time. The

Table 1. S_3 deactivation kinetics and Kok parameters for *D. tertiolecta* and *P. tricornutum* grown at HL and LL under 'non-starved' and 'light-starved' conditions

The S_3 lifetime was calculated using only the initial linear decay from the biphasic plots of Y_3/Y_{SS} versus dark time. The standard error associated with these measurements was 5% or less. The S_n -state transition probabilities α and β were determined by a least-squares fit of the relative oxygen yields after the first 12 flashes with a theoretical sequence based on the conventional Kok model (Messinger *et al.* 1997). Values for each of the parameters are mean \pm s.e., $n \geq 6$. Dark-adaptation times were optimised for each species and corresponding growth conditions. $\gamma = 1 - \alpha - \beta \times 100$

Cells	n	S_3 decay (k_{-3}) half-life (s)	Dark incubation time (min)	Parameters					
				S_1 (%)	S_0 (%)	$\text{S}_1:\text{S}_0$	α (%)	β (%)	γ (%)
Non-starved cells									
HL <i>D. tertiolecta</i>	6	38	5 or 7	62.7 ± 1.7	37.3	1.7:1	18.3 ± 0.4	5.2 ± 0.18	76
LL <i>D. tertiolecta</i>	11	48	5	66.8 ± 2.1	33.2	2.0:1	15.9 ± 0.7	5.4 ± 1.24	79
HL <i>P. tricornutum</i>	10	26	2 or 5	78.5 ± 3.4	21.5	3.6:1	15.1 ± 0.6	6.7 ± 0.39	78
LL <i>P. tricornutum</i>	8	46	5, 7 or 10	76.2 ± 4.4	23.8	2.7:1	12.2 ± 0.4	5.6 ± 0.16	82
Light-starved cells									
HL <i>D. tertiolecta</i>	7	104	5, 7 or 10	66.1 ± 1.2	33.9	1.9:1	16.3 ± 0.5	6.90 ± 0.86	77
LL <i>D. tertiolecta</i>	13	53	5, 7 or 10	66.2 ± 1.5	33.8	1.9:1	18.6 ± 0.7	4.64 ± 0.05	77
HL <i>P. tricornutum</i>	10	17	10	73 ± 2.6	27	2.7:1	13.9 ± 0.7	6.60 ± 0.74	80
LL <i>P. tricornutum</i>	9	67	10	82.8 ± 5.7	17.2	4.8:1	25.8 ± 8.9	6.60 ± 0.57	67

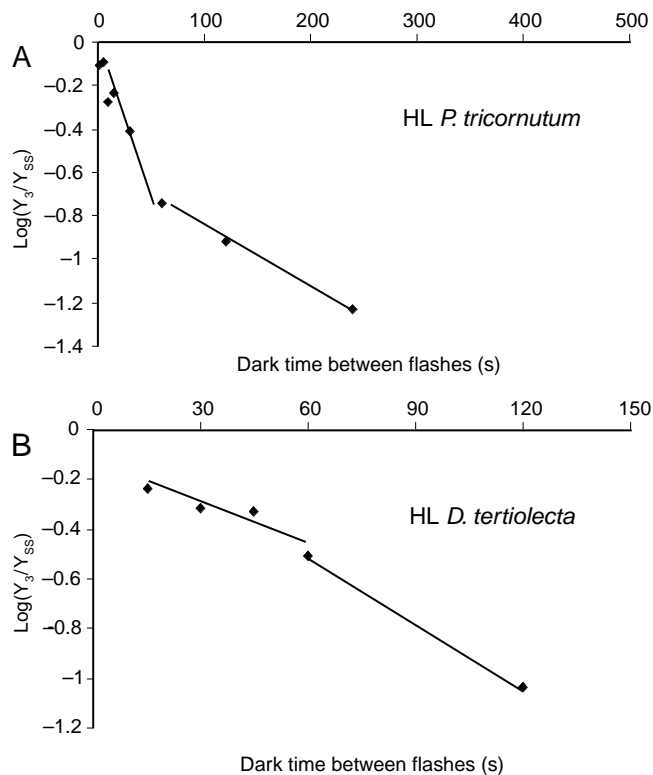


Fig. 2. Semi-log plots of the S_3 -state deactivation kinetics for HL-grown (A) *P. tricornutum* and (B) *D. tertiolecta* were used to calculate the S_3 lifetime (determined using only the first phase of the decay curve). The variation of the S_3 -state population is given by the oxygen yield of the third flash (Y_3) as a function of the dark time separating the second and third flashes. The equations describing the biphasic decays are, (A) for *P. tricornutum*, $y = -0.01x - 0.09$ ($r^2=0.97$) and $y = -0.003x - 0.59$ ($r^2=0.99$); and, (B) for *D. tertiolecta*, $y = -0.005x - 0.14$ ($r^2=0.87$) and $y = -0.009x + 0.02$ ($r^2=1$).

intersection on the ordinate gave the proportion of Y_D^{OX} , while the difference between this value and 1.0 gave the proportion of Y_D . S_3 decay kinetics were calculated from only the first decay component.

Generation of backward transitions

Samples prepared as described above were acclimated for increasing dark periods (15, 20, 30, 40 and 60 min) in the electrode (voltage off during incubation) and then pretreated before the 16-flash series was given. Only oxygen flash sequences from cultures that had not been light-starved were analysed by the modified eigenvalue method (Table 2), as light-starved cells did not produce sequences that could be analysed by either the 5-step or 6-step model (Meunier 1993; Meunier *et al.* 1995, 1996). The eigenvalue method recognised the participation of the additional redox states S_{-1} and S_{-2} by an extension of the S_n -state population vectors and a transition matrix (Meunier 1993), and readily analysed the flash sequences. The prediction of negative values for S_2 and S_3 (Table 2) is a common problem of the method; however, it has been shown not to affect the conclusions reached regarding S_{-2} and S_{-1} (Meunier *et al.* 1995).

Results and discussion

Reduction of the S_n -states in PSII has been identified as one of the reactions having the potential to decrease the efficiency of photosynthesis at extremely low photon fluxes (Radmer and Kok 1977; Rutherford and Inoue 1984; Raven *et al.* 2000). The $S'_3 \rightarrow S_4 \rightarrow S_0$ transition is associated with the release of oxygen and represents the overall rate-limiting forward reaction (Kok *et al.* 1970), where S'_3 is the light-activated S_n -state before the forward reaction occurs. If not driven forward by a light quantum, S_3 deactivation occurs.

S_3 decay kinetics

S_3 decay exhibits biphasic kinetics (Fig. 2). The fast kinetic phase involves oxidation of the Tyr-160 electron donor (Y_D) of the D2 peptide of PSII (Messinger *et al.* 1995). *D. tertiolecta* ($70 \pm 4\%$) was enriched with Y_D^{OX} (based on

Table 2. S_n -state transition probabilities (α , β , γ), including backward transitions (δ , when present) were determined for 'non-starved' cells by the modified eigenvalue method

The modified eigenvalue method comes from Meunier (1993) and Meunier *et al.* (1995, 1996). Values for each of the parameters are mean \pm s.e., $n \geq 6$. The initial distribution of S_n -states in the dark was based on oxygen flash yield sequences fitted with a 6-step model for HL *D. tertiolecta* and a 5-step model for LL *D. tertiolecta* and HL and LL *P. tricornutum*. The total distribution between S_n -states should always equal 100%. Standard deviations (RSD) were estimates of the true deviation between the model and the experimental data. z is the sum of probabilities (Meunier and Popovic 1990)

	Percentage of each S_n -state						Total (%)
	S_{-2}	S_{-1}	S_0	S_1	S_2	S_3	
HL <i>D. tertiolecta</i>	5.59 ± 0.91	27.6 ± 6.98	33.97 ± 3.24	31.30 ± 4.58	1.53 ± 1.63	-0.010 ± 0.193	99.998
LL <i>D. tertiolecta</i>		7.21 ± 2.11	73.18 ± 10.64	17.85 ± 9.93	1.78 ± 1.45	0.0015 ± 0.0007	100.69
HL <i>P. tricornutum</i>		21.90 ± 2.50	41.49 ± 0.58	34.58 ± 3.23	-0.44 ± 0.76	2.50 ± 0.16	100.05
LL <i>P. tricornutum</i>			17.11 ± 0.51	86.79 ± 1.10	2.51 ± 0.48	0.79 ± 0.29	99.999
	Transition probabilities (%)						
	α	β	γ	δ	RSD	z	
HL <i>D. tertiolecta</i>	8.55 ± 1.85	7.12 ± 2.16	80.12 ± 4.88	0	2.63	0.92	
LL <i>D. tertiolecta</i>	16.15 ± 4.10	7.25 ± 2.73	67.75 ± 4.54	5.02 ± 3.79	0.0015	0.94	
HL <i>P. tricornutum</i>	16.10 ± 1.48	3.31 ± 1.15	79.98 ± 1.70	1.92 ± 1.68	0.74	0.98	
LL <i>P. tricornutum</i>	17.34 ± 1.57	2.86 ± 2.21	79.96 ± 2.13	2.19 ± 1.77	2.18	0.95	

the fast-phase components in Fig. 2) compared with Y_D^{OX} in *P. tricornutum* ($36 \pm 6\%$). The oxidation level of plastoquinone (PQ) in *D. tertiolecta* is higher than in other species, a result of a greater number of charge separations at PSI than at PSII (Meunier and Popovic 1989).

The slow phase reflects reduction of the OEC through charge recombination with reduced Q_B (the exchangeable PQ in the PSII), which is dependent on the redox state of the PQ pool (Rutherford and Inoue 1984; Meunier and Popovic 1989; Messinger and Renger 1993). The redox level of the acceptor side of PSII may vary between species so that S_3 lifetimes will be species-specific. S_3 lifetimes in LL-grown *D. tertiolecta* and *P. tricornutum* were significantly longer than those for HL-grown cultures (Table 1). This prolongation of the S_3 lifetime can be interpreted in terms of an overall decrease in reduction reactivity of the S_n -states (Schmid *et al.* 1994), findings supported by the even longer S_3 lifetimes in light-starved LL *D. tertiolecta* and *P. tricornutum* (Table 1). HL-grown, light-starved *D. tertiolecta*, however, had the longest of all the S_3 lifetimes (Table 1), suggesting a strongly reduced reactivity of the S_n -states. *D. tertiolecta* growing at saturating photon fluxes has been previously shown to have a low tolerance for prolonged darkness (Berges and Falkowski 1998). Future experiments should examine species-specific responses in the reactivity of the OEC in *D. tertiolecta* and *P. tricornutum*, tracking changes in S_3 lifetimes and OEC activity with increasingly longer dark (light-starvation) incubations.

Analysis of transition probabilities

The probability of a single hit (γ), miss (α) and double-hit (β) for oxygen flash sequences analysed by the Kok model are typically 80, 15 and 5% (with a 10- μ s xenon flash), respectively, for all algal cells examined (Kok *et al.* 1970; Forbush *et al.* 1971; Thibault 1978; Wydrzynski 1982). Both models predicted that single hits would occur about 80% of the time in *D. tertiolecta* and *P. tricornutum* (Tables 1, 2). Similar probabilities have been previously observed for *Chlorella* (72–86%; Thibault 1978), *D. tertiolecta* (82%; Meunier and Popovic 1989) and the cyanobacteria *Synechocystis* sp. PCC 6803 (77%; Meunier *et al.* 1995, 1996) and *Oscillatoria chalybea* (74%; Schmid *et al.* 1994).

The probability of a double hit did not differ significantly between *D. tertiolecta* ($5.6 \pm 0.6\%$) and *P. tricornutum* ($6.4 \pm 0.3\%$) (Table 1) for sequences analysed by the Kok model (Wydrzynski 1982). Half as many double hits were predicted for *P. tricornutum* ($3.1 \pm 1.7\%$) as for *D. tertiolecta* ($7.2 \pm 2.4\%$) by the modified eigenvalue method (Table 2). Meunier's (1993) evaluation of several models suggested that the computations used in the Kok model might include backward transitions within the estimated double hits. This would explain the higher-than-predicted proportion of double hits calculated by the Kok model than by the

modified eigenvalue method, as well as why backward transitions (Table 2) were deconvoluted from the oxygen flash yield sequences for *D. tertiolecta* but not for *P. tricornutum* (below).

Although the predicted probability of a miss for algal cells is usually around 15% by the Kok model (Wydrzynski 1982), closer examination of the literature revealed that there has been a wide range of probabilities reported. Misses calculated here for *D. tertiolecta* and *P. tricornutum* by either method (Tables 1, 2) fell within the range (10.6–20%) reported by Thibault (1978) for *Chlorella* sp. Others have reported miss probabilities of 10% in *Chlorella sorokiniana* (Lavergne and Rappaport 1998) and 12.5% in *D. tertiolecta* (Meunier and Popovic 1989, 1990). Variation in the miss probability occurs because this parameter is most sensitive to perturbations on the acceptor side of PSII and to the physiological status of the OEC (Kok *et al.* 1970; Meunier and Popovic 1990; Lavergne and Rappaport 1998).

Shifts in the flash sequences

Shifts in the oxygen flash yield oscillations were the first indication that the OEC activity of *D. tertiolecta* and *P. tricornutum* was responding to the physiological status of these microalgae. Experimental oxygen flash yields were directly comparable to the theoretical fit data (Fig. 3A), with the first maximum occurring on the third flash, and thereafter on the seventh and eleventh flashes. This maximum, however, could be reproducibly shifted to the fourth or later flashes in a species-specific manner (Figs 3B–D). The maximum oxygen yield for HL *P. tricornutum* could be shifted to the fourth flash by increasing the dark period to 20 min (Fig. 3B). Longer dark periods, however, could not shift this to higher flash numbers. For LL *D. tertiolecta* (Fig. 3C), the highest oxygen flash yield could be seen on the third, fourth or fifth flash using dark periods of 20, 30 and 40 min, respectively. In addition to the shifts seen in LL cultures, the highest oxygen flash yield was also observed on the fourth and fifth flashes together for HL *D. tertiolecta* (Fig. 3D) after 40 min of dark incubation. LL *P. tricornutum* and light-starved cultures never showed a shift beyond the third flash. The only exception, light-starved HL *D. tertiolecta*, had a maximum oxygen yield on the fourth flash (data not shown).

Shifts in the initial flash sequence have been previously reported for eukaryotes (references in Wydrzynski 1982) and cyanophytes (Myers *et al.* 1983). These were taken as evidence for a substantial reduction of the S_n -states and the PQ pool in darkness. The chlororespiratory chain is thought to be responsible for the reduction of PQ in the dark (Diner and Mauzerall 1973a; Meunier and Popovic 1990; Field *et al.* 1998; Beardall *et al.* in press), with the redox state of PQ thought to determine the number of misses on the first flash (Meunier and Popovic 1990). PQ has been implicated in provision of the pool of reducing equivalents needed to

regulate the S_n -states for the following reasons: (1) it is a component of the electron transport chain; (2) it is able to undergo reversible oxidation and reduction in the light; and (3) there is a close correlation between the kinetics of its oxidation and reduction and the turnover times of photosynthesis (Diner and Mauzerall 1973a, 1973b). Hence, the redox state of PQ represents the competition between oxidation caused by flashes and the reduction caused by chlororespiration (Meunier and Popovic 1990; Beardall *et al.* in press).

Backward transitions

Until now, the addition of exogenous reducing agents such as hydrazine or hydroxylamine has been required for super-reduced states to accumulate in higher plant thylakoids, *Oscillatoria chalybea* and microalgae (Kretschmann and Witt 1993; Messinger and Renger 1993; Schmid *et al.* 1994; Messinger *et al.* 1997). These reductants are thought to change not only the redox poise of the system but also the enzymatic conditions (e.g. redox conformation),

so there is dissipation of oxidising redox equivalents in the OEC with S_n -state dependence (Messinger and Renger 1993; Schmid *et al.* 1994; Vener *et al.* 1997).

Analysis of HL *D. tertiolecta* sequences (Fig. 3D) suggested the super-reduced S_{-1} and S_{-2} states may be present (Table 2). In HL *P. tricornutum* and LL *D. tertiolecta* (Table 2; Figs 3B, C) only the S_{-1} state could be present. No super-reduced states were detected for LL *P. tricornutum* or predicted by the eigenvalue method (Table 2; Fig. 3A). The probability of a backward transition (δ) was difficult to relate to the distribution of the S_n -states (Table 2). For example, although both the S_{-2} and S_{-1} states were seen for HL *D. tertiolecta*, the probability of backward transition was predicted to be zero by the modified eigenvalue method (Table 2). For HL and LL *P. tricornutum*, the average probability of backward transition was 2%, yet the S_{-1} state was seen only in HL *P. tricornutum* (Table 2).

The generation of the S_{-1} and S_{-2} states was independent of the $S_0:S_1$ ratio, in agreement with previous findings by Kretschmann and Witt (1993), but was dependent on the

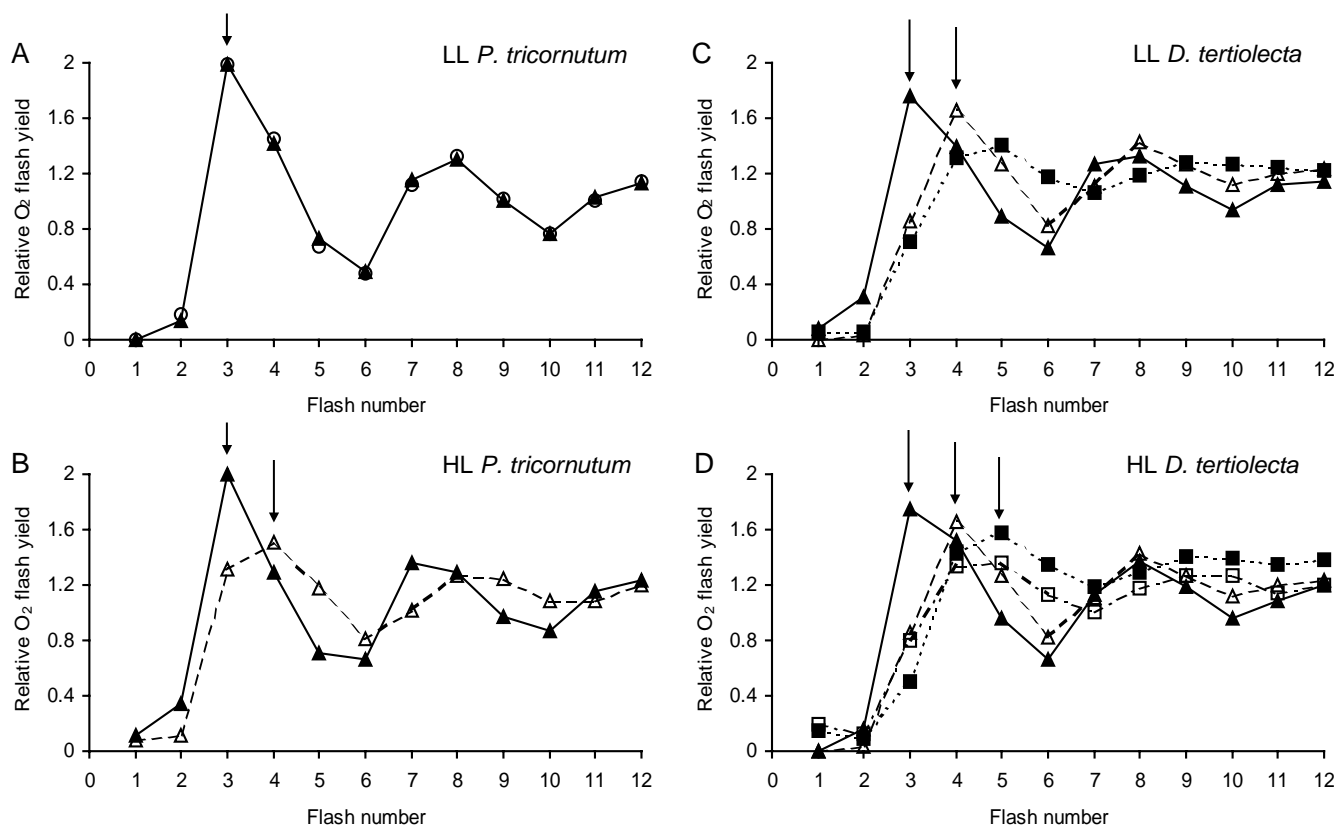


Fig. 3. The effect of successive single-turnover flashes on oxygen production following increased periods of dark acclimation is a shift to increasing flash numbers for the first maximum oxygen yield. The oxygen yield on any flash, Y_n , was normalised to the steady state yield, Y_{SS} , reached after many flashes. The experimental data are presented for (A) LL *P. tricornutum*, (B) HL *P. tricornutum*, (C) LL *D. tertiolecta* and (D) HL *D. tertiolecta*. Data analysed by the Kok model (Kok *et al.* 1970; Forbush *et al.* 1971; Messinger *et al.* 1997) are shown for LL *P. tricornutum* (A): experimental (\blacktriangle) and theoretical fit (\circ) data. The first maximum oxygen yield occurred on the third flash (\blacktriangle). However, in some cases (see B–D) first maximum oxygen yield occurred instead on the fourth (\triangle), fifth (\blacksquare), and/or the fourth and fifth (\square) flashes together. These latter flash sequences were analysed by the improved eigenvalue method (Meunier *et al.* 1996). Arrows indicate where the first maximum oxygen yield occurred. The sample treatment used to shift the maximum peak from the third flash to higher flash numbers is described in the text.

photon flux for growth in *D. tertiolecta* and *P. tricornutum* (Table 2). Approximately 25% of all S_n -states were S_{-1} after dark incubation of HL-grown *D. tertiolecta* and *P. tricornutum*. S_{-1} accounted for up to 17% of the S_n -states in dark-adapted *Chlorella vulgaris* and 60% of the S_n -states in *Euglena gracilis* (Shiraiwa and Schmid 1985).

Super-reduced states are thought to play an important role in photoactivation (references in Renger 1999; Ananyev *et al.* 2001). In the assembly of the manganese cluster, the super-reduced states are formed before the stable, more-oxidised S_0 and S_1 states. This may allow the manganese cluster to fall apart, so that the OEC can respond to changes in photon supply. The presence of super-reduced S_n -states (Fig. 3; Table 2) in appreciable quantities in these microalgae is linked to their capacity for the inactivation and/or reactivation reactions of PSII centres (Meunier *et al.* 1996). These responses are closely correlated with the internal redox state of the cell, so that more-reduced cells make more super-reduced states. The inability of *P. tricornutum* to generate super-reduced states may be because this species maintains a more-oxidised PQ pool, particularly at low irradiances, so that there is less reductant available for the deactivation of its S_n -states. The cells balance their requirements for stable S_2 and S_3 states. At the same time, destabilising the S_0 and S_1 states in *D. tertiolecta* permits the appearance of the super-reduced S_{-1} (and S_{-2}) states in the process of photoactivation (Meunier *et al.* 1996; Ananyev *et al.* 2001). It seems reasonable that species-specific differences in the generation of super-reduced states will occur if their manganese cluster is more or less sensitive to photo-degradation under various light regimes.

Physiological significance

The significance of the present findings is twofold. First, no reductants were added to the system. The reducing power generated in intact cells influenced the redox state of the PQ pool, the probability of back reactions, and the stability of the S_3 state, thereby permitting the appearance of super-reduced states. Hence, this provides the first evidence that the S_{-1} and S_{-2} redox states can exist *in vivo*. They can no longer be considered 'exotic' states of the OEC created only by artificial treatments. Instead, they are now known to play an important role in photoactivation (Renger 1999; Ananyev *et al.* 2001). A part of the natural system, super-reduced states provide microalgae with an additional mechanism for responding to fluctuating irradiance. The strong reducing power within cells may have been generated endogenously by PQ but also by PSII (Q_B^{2-}) (Raven *et al.* 2000), Y_D (Rutherford and Inoue 1984; Messinger *et al.* 1997), cytochrome b_{559} (Wydrzynski 1982), PSI (available in the forms of reduced ferredoxin, flavin and NADP) and NADPH (Diner and Mauzerall 1973b). Future investigations should endeavour to determine (1) the redox state of the PQ pool and stroma of cells grown at HL and LL, and (2) the kind of

messenger molecule(s) involved in the deactivation reactions and how the photon flux experienced by photosynthetic organisms regulates this molecule(s). Although it is widely believed that the NADP-malate dehydrogenase poises the stromal redox state over a wide range of environmental conditions, keeping the stroma oxidised, and that the PQ pool responds to environmental cues by becoming largely reduced, there is no experimental evidence that this is indeed the situation, particularly in microalgal cells growing at different photon fluxes. However, in *D. tertiolecta* at least, the PQ pool has been shown to be more oxidised (by a factor of ~2) when this alga is grown in LL than when grown in HL (Yibu Chen, pers. comm.). Second, the ability of *P. tricornutum* to grow at photon fluxes an order of magnitude lower than can *D. tertiolecta* may indeed be related to charge recombination of PSII. *P. tricornutum* appeared to maintain a steadier OEC at very low photon fluxes for growth, whereas 'flexibility' in the OEC of *D. tertiolecta* may allow it to deal with high photon fluxes. At the same time, this disadvantages *D. tertiolecta* when they are growing close to their light compensation points.

Conclusion

The present work is part of a larger study examining the three major *raison d'être* for light limitation on photoautotrophic growth: protein turnover (Quigg and Beardall *in press*), charge recombination in PSII as measured by S_3 decay kinetics (present study), and proton leakage and slippage. Microalgae lower their metabolic activity (protein turnover) at low photon fluxes, thereby decreasing the costs of maintenance processes. Slippage reactions have important implications for the successful exploitation of low-light environments as they imply a less-than-theoretical stoichiometry of some energy-transduction processes that may lead to proportionally larger reductions in photon efficiency at low rates of photon absorption (Raven and Beardall 1981, 1982; Raven *et al.* 2000). Collectively, the impact of these three processes is synergistic; by limiting rates of photosynthesis at low irradiances, they place a limit on the minimum photon flux necessary to sustain photoautotrophic growth.

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References

- Ananyev GM, Zaltsman L, Vasko C, Dismukes GC (2001) The inorganic biochemistry of photosynthetic oxygen evolution/water oxidation. *Biochimica et Biophysica Acta* **1503**, 52–68.
- Beardall J, Quigg A, Raven JA (in press) Oxygen consumption: photorespiration and chlororespiration. In 'Photosynthesis in the algae'. (Eds T Larkum, JA Raven and S Douglas) (Kluwer Academic Publishers: Dordrecht)
- Berges JA, Falkowski PG (1998) Physiological stress and cell death in marine phytoplankton: induction of proteases in response to nitrogen or light limitation. *Limnology and Oceanography* **43**, 129–135.
- Diner B, Mauzerall D (1973a) The turnover times of photosynthesis and redox properties of the pool of electron carriers between the photosystems. *Biochimica et Biophysica Acta* **305**, 353–363.
- Diner B, Mauzerall D (1973b) Feedback controlling oxygen production in a cross-reaction between two photosystems in photosynthesis. *Biochimica et Biophysica Acta* **305**, 329–352.
- Field TS, Nedbal L, Ort DR (1998) Nonphotochemical reduction of the plastoquinone pool in sunflower leaves originates from chlororespiration. *Plant Physiology* **116**, 1209–1218.
- Forbush B, Kok B, McGloin MP (1971) Cooperation of charges in photosynthetic O₂ evolution. II. Damping of flash yield oscillation, deactivation. *Photochemistry and Photobiology* **14**, 307–321.
- Joliot P, Barbieri G, Chabaud R (1969) Un nouveau modele des centres photochimiques du systeme II. *Photochemistry and Photobiology* **10**, 309–329.
- Kok B, Forbush B, McGloin M (1970) Cooperation of charges in photosynthetic O₂ evolution — 1. A linear four step mechanism. *Photochemistry and Photobiology* **11**, 457–475.
- Kretschmann H, Witt HT (1993) Chemical reduction of the water splitting enzyme system of photosynthesis and its light-induced reoxidation characterized by optical and mass spectrometric measurements: a basis for the estimation of the states of the redox active manganese and of water in the quaternary oxygen-evolving S-state cycle. *Biochimica et Biophysica Acta* **1144**, 331–345.
- Lavergne J, Rappaport F (1998) Stabilization of charge separation and photochemical misses in photosystem II. *Biochemistry* **37**, 7899–7906.
- Messinger J, Renger G (1993) Generation, oxidation by the oxidized form of the tyrosine of polypeptide D2, and possible electronic configuration of the redox states S₀, S₋₁, and S₋₂ of the water oxidase in isolated spinach thylakoids. *Biochemistry* **32**, 9379–9386.
- Messinger J, Badger M, Wydrzynski T (1995) Detection of one slowly exchanging substrate water molecule in the S₃ state of photosystem II. *Proceedings of the National Academy of Sciences USA* **92**, 3209–3213.
- Messinger J, Seaton G, Wydrzynski T, Wacker U, Renger G (1997) S₋₃ state of the water oxidase in photosystem II. *Biochemistry* **36**, 6862–6873.
- Meunier PC (1993) Oxygen evolution by photosystem II: the contribution of backward transitions to the anomalous behaviour of double-hits revealed by a new analysis method. *Photosynthesis Research* **23**, 213–221.
- Meunier PC, Popovic R (1989) Evidence for a linear variation of the miss and single hit S-state probabilities with the flash number, measured by oxygen evolution in *Dunaliella tertiolecta*. *Photosynthesis Research* **22**, 131–136.
- Meunier PC, Popovic R (1990) Control of misses in oxygen evolution by the oxido-reduction state of plastoquinone in *Dunaliella tertiolecta*. *Photosynthesis Research* **23**, 213–221.
- Meunier PC, Burnap RL, Sherman LA (1995) Interaction of the photosynthetic and respiratory electron transport chains producing slow O₂ signals under flashing light in *Synechocystis* sp. PCC 6803. *Photosynthesis Research* **45**, 31–40.
- Meunier PC, Burnap RL, Sherman LA (1996) Improved 5-step modelling of the photosystem II S-state mechanism in cyanobacteria. *Photosynthesis Research* **47**, 61–76.
- Myers J, Graham JR, Wang RT (1983) On the O₂ flash yields of two cyanophytes. *Biochimica et Biophysica Acta* **722**, 281–290.
- Packham NK, Hodges M, Etienne AL, Briantais JM (1988) Changes in flash-induced oxygen yield pattern by thylakoid membrane phosphorylation. *Photosynthesis Research* **15**, 221–232.
- Porra RJ, Thompson WA, Kriedeman PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* **975**, 384–394.
- Provasoli L, McLachlan JJA, Droop MR (1957) The development of artificial media for marine algae. *Archives of Microbiology* **25**, 392–428.
- Quigg A (2000) A study of physiological features determining the capacity of marine microalgae to grow at low irradiance. PhD thesis, Monash University, Melbourne, Australia.
- Quigg A, Beardall J (in press) Protein turnover in relation to maintenance metabolism at low photon flux in two species of marine microalgae. *Plant, Cell and Environment*.
- Radmer R, Kok B (1977) Light energy conversion in photosynthesis. In 'Encyclopedia of plant physiology'. (Eds A Trebst and M Avron) pp. 125–135. (Springer-Verlag: Berlin)
- Raven JA, Beardall J (1981) The intrinsic permeability of biological membranes to H⁺: significance for the efficiency of low rates of energy transformation. *FEMS Microbiology Letters* **10**, 1–5.
- Raven JA, Beardall J (1982) The lower limit of photon fluence rate for phototrophic growth: the significance of 'slippage' reactions. *Plant, Cell and Environment* **5**, 117–124.
- Raven JA, Kübler JE, Beardall J (2000) Put out the light, then put out the light. *Journal of the Marine Biological Association of the United Kingdom* **80**, 1–27.
- Renger G (1999) Mechanism of photosynthetic water cleavage. In 'Concepts in photobiology: photosynthesis and photomorphogenesis'. (Eds GS Singhal, G Renger, SK Sopory, K-D Irrgang and Govindjee) pp. 292–329. (Narosa Publishing House: New Delhi)
- Rutherford AW, Inoue Y (1984) Oscillation of delayed luminescence from PS II: recombination of S₂Q_B⁻ and S₃Q_B⁻. *FEBS Letters* **165**, 163–170.
- Schmid GH, Bader KP, Schulder R (1994) A study of the lifetime of the S₃-state in the filamentous cyanobacterium *Oscillatoria chalybea*. *Verlag der Zeitschrift für Naturforschung C* **49**, 108–114.
- Shiraiwa Y, Schmid GH (1985) Effect of the CO₂-concentration during growth on the oxygen evolution pattern under flash light in *Chlorella*. *Verlag der Zeitschrift für Naturforschung C* **41**, 269–274.
- Thibault P (1978) A new attempt to study the oxygen evolving system of photosynthesis: determination of transition probabilities of a state *i*. *Journal of Theoretical Biology* **73**, 271–284.
- Vener AV, Van Kan PJM, Rich PR, Ohad I, Andersson B (1997) Plastoquinol at the quinol oxidation site of reduced cytochrome *bf* mediates signal transduction between light and protein phosphorylation: thylakoid protein kinase deactivation by a single-turnover flash. *Proceedings of the National Academy of Sciences USA* **94**, 1585–1590.
- Wydrzynski TJ (1982) Oxygen evolution in photosynthesis. In 'Photosynthesis: energy conversion by plants and bacteria'. (Ed. Govindjee) pp. 469–505. (Academic Press: New York)

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