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► **To cite this version:**

Rupert G. Perkins, Jean-Luc Mouget, Sébastien Lefebvre, Johann Lavaud. Light response curve methodology and possible implications in the 2 application of chlorophyll fluorescence to benthic diatoms. *Marine Biology*, 2006, 149, pp.703-712. 10.1007/s00227-005-0222-z . hal-01094638

HAL Id: hal-01094638

<https://hal.science/hal-01094638v1>

Submitted on 12 Dec 2014

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2 Light response curve methodology and possible implications in the
3 application of chlorophyll fluorescence to benthic diatoms

4

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23

24 **Abstract**

25

26 Chlorophyll *a* fluorescence has been increasingly applied to benthic microalgae,
27 especially diatoms, for measurements of electron transport rate (ETR) and
28 construction of rapid light response curves (RLCs) for the determination of
29 photophysiological parameters (mainly the maximum relative ETR ($rETR_{max}$), the
30 light saturation coefficient (E_k) and the maximum light use coefficient). Various
31 problems with the estimation of ETR from the microphytobenthos have been
32 identified, especially *in situ*. This study further examined the effects of light history of
33 the cells and light dose accumulation during RLCs on the fluorescence measurements
34 of ETR using the benthic diatom *Navicula phyllepta*. RLCs failed to saturate when
35 using incremental increases in irradiance, however curves with decreasing irradiance
36 did saturate. Patterns indicating photoacclimation in response to light histories were
37 observed, with higher $rETR_{max}$ and E_k , and lower α , at high light compared to low
38 light. However these differences could be negated by increasing the RLC irradiance
39 duration from 30 to 60 s. It is suggested that problems arose as a result of rapid
40 fluorescence variations due to ubiquinone, Q_A , oxidation and non-photochemical
41 chlorophyll fluorescence quenching, NPQ, which depended upon the light history of
42 the cells and the RLCs accumulated light dose. Also, RLCs with irradiance duration
43 of 10 s were shown to have an error possibly specific to the fluorimeter programming.
44 It is suggested that RLCs, using a Diving-PAM fluorimeter on benthic diatoms,
45 should be run using decreasing irradiance steps of 30 s duration.

46

47 **Introduction**

48 Benthic microalgae communities, mainly composed of diatoms, inhabit
49 shallow estuarine intertidal sediments where they are responsible for the major part of
50 the photosynthetic primary production (MacIntyre et al., 1996). The light environment
51 to which the microphytobenthos are exposed is highly variable due to the tidal regime,
52 which expose the cells to a wide range of, and rapid changes between, levels of
53 irradiance, together with a high spatial and temporal frequency of light fluctuations.
54 Therefore, one the main challenges for microphytobenthic algae is to cope with
55 fluctuations in irradiance, largely through avoidance of energy imbalance within the
56 photosynthetic apparatus, and maintainance of an optimal irradiance to maximise
57 photosynthetic productivity (Underwood and Kromkamp, 1999, Perkins et al 2002,
58 Consalvey et al. 2005a). For this purpose, algae have evolved a number of
59 mechanisms referred to as photoacclimation (MacIntyre et al. 2000, Raven and
60 Geider, 2003).

61 To investigate the photosynthesis of the microphytobenthos, chlorophyll *a*
62 (Chl *a*) fluorescence measurements of electron transport rate (ETR) and related
63 photophysiological parameters are being increasingly applied (Consalvey et al.
64 2005a). Studies have been used to compare measurements of primary productivity
65 using different methodologies, principally carbon uptake (radio-labeled ^{14}C), electron
66 transport rate (Chl *a* fluorescence) and oxygen evolution (oxygen electrodes)
67 (Flameling & Kromkamp 1998, Hartig et al. 1998, Barranguet and Kromkamp 2000,
68 Perkins et al. 2001, 2002). Others have been confined solely to the use of Chl *a*
69 fluorescence as a proxy for primary productivity by measuring ETR (Kromkamp et al.
70 1998; Serôdio and Catarino 2000; Serôdio 2003; Serôdio et al. 2005a; Underwood et
71 al. 2005) or as a proxy for algal biomass (Serôdio et al. 1997; Honeywill et al. 2002).

72 The recent technique of rapid light response curves (RLCs), defined as very
73 short (tens of seconds) light steps of different intensity, has been widely applied for
74 the determination of ETR versus irradiance on different photosynthetic aquatic
75 organisms like macro-and micro-algae, seagrasses and corals (Schreiber et al. 1997,
76 Ralph et al. 1999, Kühl et al. 2001, Glud et al. 2002, Ralph and Gademann 2005). The
77 short duration of each light step is an attempt to minimize the confounding effects of
78 light acclimation encountered with 'steady-state' traditional light curves (Serôdio
79 2004, Serôdio et al. 2005a). RLCs have been used with success on microphytobenthos
80 assemblages isolated from the field or directly *in situ* (Perkins et al. 2002, Serôdio et
81 al. 2005a). However, the assessment of RLCs on intact biofilms can be disturbed,
82 which may alter the calculation of ETR as a function of light intensity (Perkins et al.
83 2002). The two major sources of disturbance that have been identified are the
84 attenuation of light in the sediment and the depth-integration of fluorescence emitted
85 from sub-surface layers (Forster and Kromkamp 2004, Serôdio 2004), and the
86 migration of the cells within the biofilm (Kromkamp et al. 1998, Perkins et al. 2002,
87 Serôdio 2004). In addition, rapid as well as endogenous changes in photosynthesis
88 activity, which modulate the Chl *a* fluorescence emission, can potentially affect ETR
89 measurements (Serôdio et al. 2005a). In particular, the red-ox state of Q_A , the primary
90 PSII electron acceptor, and non-photochemical Chl *a* fluorescence quenching (NPQ)
91 have been shown to influence the fluorescence measurements in microphytobenthic
92 diatoms (Consalvey et al. 2004, Serôdio et al. 2005a, 2005b). In this context, technical
93 features of the RLCs, such as the length of each irradiance step, have been shown to
94 be important for the assessment of ETR (Serôdio et al. 2005a).

95 This study aimed to further improve the use of Chl *a* fluorescence
96 measurements for the construction of RLCs for application to benthic algae. We

97 especially focused on the ability of the RLCs to relate the photoacclimation status
98 through the assessment of ETR. By using algal cultures of the benthic diatom,
99 *Navicula phyllepta* Kützing, we investigated the effect on the ETR/light relationship
100 of: 1) the light history of the cells, 2) the RLCs light step duration and order (i.e.
101 increasing or decreasing irradiance). The data obtained were compared with RLCs
102 performed on separate replicates of the culture for each light step ('non sequential'
103 light curves, N-SLCs) to assess the potential cumulative effect of rapid
104 photoacclimation of the cells during the light curve itself. The results raise important
105 questions with regard to potential errors in the measurement and interpretation of
106 RLCs for cultured benthic diatoms, errors which may equally apply for *in situ*
107 measurements.

108

109 **Methods**

110

111

112 *Navicula phyllepta* cultures

113

114

115 *Navicula phyllepta* was obtained from the microalgal culture collection of the
116 Laboratoire de Biologie Marine (ISOMer, Nantes, France). Stock cultures were grown
117 in an artificial seawater medium (Harrison et al., 1980) at low irradiance (20 μmol
118 $\text{photons m}^{-2} \text{s}^{-1}$, 6h/18h, light/dark photoperiod). The original medium was
119 complemented following De Brouwer et al. (2002), with the addition of Fe-NH₄-
120 citrate (1.37 μM final concentration), CuSO₄ 5H₂O (0.04 μM f.c.), folic acid (0.18 nM
121 f.c.), nicotinic acid (0.0325 μM f.c.), thymine (0.95 μM f.c.), Ca-d-pantothenate (8.39
122 nM f.c.) and inositol (1.11 μM f.c.). Experiments were run with cultures grown in
123 semi-continuous culture mode (volume: 250 mL, temperature: 15 \pm 1°C) in
124 Erlenmeyer flasks (500 mL) illuminated from below (100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$,

125 14h/10h, light/dark photoperiod) by a high intensity discharge lamp (Osram HQI BT,
126 400 W).

127

128 *Rapid light response curves*

129

130

131 Fluorescence measurements were made using a Diving-PAM fluorimeter (Walz,
132 Effeltrich, Germany). Sub-samples of *N. phyllepta* cultures were incubated at either
133 low or high light (LL or HL; 25 or 400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD respectively), in a
134 stirred, temperature controlled (15 °C) chamber, for 60 min. This light acclimation
135 period was staggered, so that each sub-sample had been exposed for exactly 60 min
136 prior to measurement of each RLC. After light acclimation, the culture sub-sample
137 was transferred into a temperature controlled (15 °C) Hansatech DW2 chamber, with
138 continuous stirring to prevent settling. The Diving-PAM fibre optic probe was applied
139 to the top aperture of the chamber so that measurements were taken from cells
140 exposed to the actinic light level applied from the halogen internal light source, thus
141 minimising any light gradient effect (other apertures were darkened). Cultures were
142 dark-adapted for 5 min prior to RLCs, with measurements at 10, 30 and 60 s at each
143 light intensity. RLCs were performed with either incremental increases ('up') or
144 decreases ('down') in the actinic light intensity between 0 and 1850 $\mu\text{mol photons m}^{-2}$
145 s^{-1} PPFD. Light levels were measured using the Diving-PAM quantum meter,
146 corrected against a calibrated Li-Cor LI-189 quantum meter with a Q21284 quantum
147 sensor.

148 At each light level, effective photosystem II (PSII) quantum efficiency
149 (F_q'/F_m' , Oxborough et al. 2000; Lawson et al. 2002; Perkins et al. 2002) was
150 measured by the saturation pulse technique, whereby a saturating light pulse of
151 7,600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD was applied for 400 ms to measure the maximum

152 fluorescence yield, F_m' . F_q'/F_m' is equivalent to $\Delta F/F_m'$ (Genty et al., 1989), however
153 F_q' is preferred as it represents, not a change in fluorescence yield, but a difference
154 resulting from photosynthetic quenching of yield, hence the suffix q (Oxborough et
155 al., 2000). F_q'/F_m' was calculated as $(F_m' - F') / F_m'$, where F' is the fluorescence
156 yield in the light adapted state, just prior to the application of the saturating pulse.
157 Hence F_q'/F_m' is equivalent to the Genty parameter in the light adapted state (Genty et
158 al. 1989). Relative electron transport rate (rETR) was then calculated as the product of
159 F_q'/F_m' and PPFD/2 (Sakshaug et al. 1997; Perkins et al. 2001 2002).

160 The Diving-PAM has internal programmes allowing 8-step RLCs with
161 increasing light levels. Therefore, RLCs were performed using the remote control
162 functions in the WinControl software (Walz, Effeltrich, Germany) from a laptop
163 computer, so as to apply decreasing as well as increasing light levels covering 12 light
164 increments. This also enabled examination of the transient fluorescence kinetics, by
165 monitoring the F' signal in the chart mode, allowing determination of F' 'steady-
166 state' as well as examining changes in F' in response to application of each actinic
167 light level.

168 Prior to all sets of light curves, the Diving-PAM auto-zero function was set
169 using the Hansatech chamber filled with an equivalent volume of clear media. Light
170 calibration was also carried out before and after all light curves due to an observed
171 10 % variation in halogen output over time, despite running the Diving-PAM from a
172 mains supply. As a result light levels differed between curves by up to 10 %, with this
173 variation accounted for in calculations of rETR.

174 Over-estimation of photochemical efficiency can occur when using the
175 Diving-PAM with low biomass culture which results in an F' signal below
176 130 relative units (Walz Diving-PAM handbook). This low signal strength may also

177 occur at exposure to high light due to high levels of non-photochemical fluorescence
178 quenching (NPQ). Therefore the Diving-PAM gain setting was set to a maximum of
179 12 to avoid low values of F' . However at such a high gain, the auto-zero function can
180 also result in over-estimation of quantum efficiency. A large auto-zero value will be a
181 greater proportion of F' compared to F_m' , when F' decreases as actinic light level
182 increases. Mathematically, the same percentage change in F' and F_m' will therefore
183 result in different values of F_q'/F_m' due to the weighted influence of the auto-zero.
184 Therefore, only measurements with a low auto-zero (< 40 relative units) were used in
185 the construction of RLCs.

186

187 *Non-sequential light response curves*

188

189 To remove the cumulative effect of light history experienced during a RLC, the above
190 methods were modified by using a different replicate sub-sample of culture for each
191 actinic light level. The range of light intensities was also extended up to 3200 μmol
192 $\text{photons m}^{-2} \text{s}^{-1}$. In addition a further RLC data point was added, with measurements
193 made when the fluorescence signal F' reached a constant level (as observed on the
194 Win Control software chart function). This value of F' , defined here as 'steady-state',
195 was probably not a true steady-state due to time limitations and so was not defined as
196 F_s .

197 Non-sequential light response curves (N-SLCs) and calculations were
198 otherwise the same as for rapid RLCs, except that a first set of curves used cells
199 maintained at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD, with no photo-acclimation to high or
200 low light. A second set of N-SLCs were then obtained using a different original semi-
201 continuous culture of *N. phyllepta*, but incorporating the HL and LL photoacclimation

202 period. These curves were not directly comparable to the preceding datasets, due to
203 the change in source culture.

204 NPQ was calculated during N-SLCs, as $(F_m - F_m') / F_m'$ (Krause and Weiss
205 1991; Lavaud et al. 2002a). F_m' was measured as described above, using application
206 of the saturating pulse after 10, 30 and 60 s at each irradiance, and when F' reached
207 an approximate 'steady-state'. The maximum fluorescence yield in the dark adapted
208 state, F_m , is more problematic to measure for diatoms, due to NPQ being maintained
209 in the dark through processes such as chlororespiration (Jakob et al. 2001; Dijkman
210 and Kroon 2002; Lavaud et al. 2002b), thus suppressing F_m below its true value
211 (Mouget and Tremblin 2002). The calculated values of NPQ therefore show relative
212 changes, using an approximation of F_m obtained after 5 min dark adaptation prior to
213 each RLC.

214

215 *Statistical analysis*

216

217 RLCs of rETR against light intensity (PPFD) were constructed using the model of
218 Eilers and Peeters (1988), estimating the maximum electron transport rate (rETR_{max}),
219 the maximum light use efficiency (α) and the light saturation coefficient (E_k)
220 calculated as (rETR_{max} / α).

221 Curve fitting was achieved using the downhill simplex method of the Nelder-
222 Mead model, and standard deviation of parameters was estimated by a bootstrap
223 method under Fortan 77 code (Press et al. 2003). All fittings were tested by analyses
224 of variance (P<0.001), residues being tested for normality and homogeneity of
225 variance, and parameters significance by Student t-test (P<0.05). RLCs and

226 photosynthetic parameter comparisons were achieved using the method of Ratkowski
227 (1983) for non-linear models.

228

229 **Results**

230

231 *Rapid light response curves*

232

233 Rapid RLCs for low light (LL) and high light (HL) acclimated cultures of *N.*
234 *phyllepta* showed saturation and down regulation when irradiance was reduced from
235 1850 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 1A,B: 10, 30 and 60 down). In contrast, when
236 irradiance was incrementally increased, light saturation and photoinhibition (Fig.
237 1A,B: 10, 30 and 60 up) did not occur. When irradiance was increased or reduced,
238 rETR above 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ increased in proportional to the increase in
239 length of time at each light level.

240 Calculated values of rETR_{max} , α and E_k obtained from RLCs with decreasing
241 light levels were compared between HL and LL cultures (Fig. 2). 10, 30 and 60 s
242 RLCs for HL cultures showed rETR_{max} and E_k higher and α lower than LL cultures
243 ($P < 0.001$; Fig. 2A,B,C). Thus photoacclimation occurred within the 1 h light
244 treatment period. rETR_{max} increased significantly with the length of irradiance step for
245 low light and high light ($P < 0.01$) acclimated cultures, whereas α showed no
246 significant correlation with length of irradiance step. E_k , due the nature of its
247 derivation from ($\text{rETR}_{\text{max}} / \alpha$) showed the same increase as rETR_{max} as a function of
248 lengthening irradiance step ($P < 0.001$).

249 RLCs with increasing light levels did not saturate for LL (Fig. 1A: 60 s) and
250 HL cultures (Fig. 1B: 10, 30 and 60 s), preventing calculation of rETR_{max} and E_k .
251 Estimation of α indicated photoacclimation patterns similar to the decreasing

252 irradiance RLCs. LL cultures had significant higher values of α compared to HL
253 cultures ($P < 0.001$). 10 and 30 s LCRs for LL cultures just reached saturation, and
254 showed the same pattern in $rETR_{max}$ observed for decreasing RLCs, with a significant
255 increase in $rETR_{max}$ (165 to 235 rel. units from 10 to 30 s respectively), an increase in
256 E_k (319 to 439 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD), but no change in α (0.52 rel. units).

257

258 *Non-sequential light response curves*

259

260 Separate replicate *N. phyllepta* cultures used for each light level (Fig. 3)
261 resulted in N-SLCs with similar patterns as rapid RLCs (Fig. 1). $rETR$ increased
262 significantly with length of irradiance step ($P < 0.001$) at irradiances above 300 μmol
263 $\text{photons m}^{-2} \text{s}^{-1}$, with correspondingly higher $rETR_{max}$. 10, 30 and 60 s RLCs all
264 showed light saturation and down regulation, whereas light curves when F' was
265 allowed to reach an apparent 'steady-state' at each light intensity, did not saturate. No
266 steady state data point was possible at 1850 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ as photosynthetic
267 down regulation (presumably NPQ) resulted in a Chl *a* fluorescence yield (F') below
268 the minimum value of 130 relative units required for accurate measurement of
269 F_q'/F_m' .

270 *N. phyllepta* (a different culture from that used above and so not directly
271 comparable) was then acclimated to either low (25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or high
272 (400) light as above, prior to RLCs with different replicate cultures used for each light
273 intensity (Fig. 4). There were significant differences between the curves ($P < 0.001$),
274 HL and LL cultures showed a significant increase in $rETR$ proportional to an increase
275 in time at each irradiance step, except between 10 and 30 s HL, 30 and 60 s HL and
276 between steady state HL and LL RLCs.

277 Despite changing replicate cultures for each light level, the same patterns were
 278 observed as for the rapid RLCs (Fig. 1). HL acclimated cultures had significant
 279 greater $rETR_{max}$ ($P < 0.001$, Fig. 5A), and higher E_k ($P < 0.001$, Fig. 5C) than LL
 280 acclimated cultures. There were no significant differences for α ($P > 0.05$; Fig. 5B).
 281 However the difference in $rETR_{max}$ between HL and LL cells declined as the time at
 282 each RLC increment increased. There was a significant increase in $rETR_{max}$ and E_k
 283 with the length of irradiance step for low light and high light acclimated cultures
 284 ($P < 0.001$); α showed no significant differences for both LL and HL cultures ($P > 0.05$).

285

286 *Chl a fluorescence and NPQ kinetics*

287

288 An example of the fluorescence kinetics obtained at irradiance steps above $300 \mu\text{mol}$
 289 $\text{photons m}^{-2} \text{s}^{-1}$ is reproduced (Fig. 6) showing the position at which saturation pulses
 290 were applied. The example is for a low-light culture ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$), transferred to
 291 $370 \mu\text{mol m}^{-2} \text{s}^{-1}$. At 10 s the comparatively slow data acquisition time of the Diving-
 292 PAM fluorimeter (compared to the rapid induction of fluorescence quenching)
 293 resulted in an under-estimation of F_q'/F_m' . This resulted from the high rate of
 294 decrease in F' following the increase in actinic irradiance. F' was recorded by the
 295 Diving-PAM prior to a further decrease before the measurement of F_m' . F' and F_m'
 296 were therefore recorded at different times, resulting in under-estimation of F_m'
 297 relative to F' so that values of F_q'/F_m' were underestimated and often values of zero
 298 were reported. 10 s light curves would then result in under-estimation of $rETR$ above
 299 $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD. This was most obvious for HL cultures, which
 300 showed a more rapid decline in F' compared to LL cultures (data not shown). The
 301 magnitude of the increase in F' on the application of the actinic light and the rate of

302 decline after the peak in F' both increased as irradiance increased above 300 μmol
303 photons $\text{m}^{-2} \text{s}^{-1}$.

304 Above 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, NPQ was induced rapidly during RLCs, with
305 relatively high levels after 60 s at each irradiance, for both LL and HL acclimated
306 cultures (Fig. 7). While the amplitude of NPQ increased with illumination time for LL
307 cultures, it remained relatively similar for HL cultures. LL cultures (Fig. 7A) had
308 similar or lower levels of NPQ compared to HL cultures (Fig. 7B) after 10, 30 and 60
309 s, but higher NPQ at 'steady-state' (insert, Fig. 7B).

310

311 **Discussion**

312

313 The aims of the study were to determine the effects of light history prior to, and
314 accumulated light dose during, a light response curve (RLC) obtained using Chl *a*
315 fluorescence. It was expected that the light dose experienced by the algal cells over
316 different time scales during RLCs, would affect the Chl *a* fluorescence measurements
317 obtained, and hence modify the resulting RLCs and photophysiological parameters
318 derived. The data obtained raise important questions with regard to the measurement
319 and interpretation of Chl *a* fluorescence rapid RLCs. The primary question is, what is
320 being measured for light curves of different length of actinic irradiance steps?

321 The data reported here suggest two stages of photoacclimation. Firstly, the
322 acclimation resulting from exposure to low and high irradiance prior to RLCs,
323 effectively the type of photoacclimation that a RLC attempts to detect. Secondly, the
324 'acclimation' during the RLCs themselves; which RLC methodology should avoid. In
325 addition, the limitations of available methodology must also be considered.

326

327

328 *The effect of accumulated light dose on RLCs interpretation*

329

330 RLCs with incremental decreases in irradiance showed saturation and a decrease in
331 $rETR_{max}$ at higher irradiance due to photoinhibition (Fig. 1). In contrast, RLCs with
332 increasing irradiance often did not saturate. This raises the first issue of RLCs
333 methodology: the effect of accumulated light dose during the light curve, dependent
334 upon the order of irradiances ('up' or 'down') and the duration of each step (from 10 s
335 to 2-3 min).

336 With increasing irradiance, there is an accumulative effect of light dosage
337 resulting in progressive induction, occurring on a time scale of 10's of seconds, of the
338 different components of the photosynthetic apparatus. Ubiquinone (Q_A) oxidation, the
339 rate limiting step in electron transport (Dau 1994) would have been faster for
340 increasing irradiance light curves due to induction of more rapid photochemical
341 energy transfer. Also, induction of non-photochemical Chl *a* fluorescence quenching,
342 NPQ will have increased proportionally to the light dose experienced during the RLC.
343 In diatoms, the photosynthetic translocation of protons across the thylakoid membrane
344 has been linked to energy dependent NPQ (Ting & Owens 1993; Lavaud et al. 2002c)
345 associated with xanthophyll pigment synthesis (Arsalane et al. 1994; Olaizola et al.
346 1994; Lavaud et al. 2002a 2003 2004; Serôdio et al. 2005b). This process generates a
347 photoprotective dissipation of excess energy in photosystem II (PSII) reducing the Chl
348 *a* fluorescence yield, on a time scale of 10's of seconds (MacIntyre et al. 2000;
349 Lavaud et al. 2002a 2004; Raven and Geider 2003). Hence, Q_A oxidation and NPQ
350 will affect in complex ways, the measurement of effective PSII quantum efficiency
351 and resultant calculation of (r)ETR.

352 Increasing the duration of each incremental irradiance step was also seen to
353 induce photoacclimation. As the length of each step increased from 10 to 60 s rETR
354 increased proportionally, often resulting in a lack of saturation for RLCs with
355 increasing irradiance. Above 500 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, NPQ increased in importance,
356 and the duration for each irradiance step increased the extent of NPQ (Fig. 7). In
357 *N. phyllepta*, Q_A oxidation was the primary cause of the change in fluorescence yield
358 for RLCs with 10 s steps, however for 30 s and above, the level of NPQ became most
359 significant. As discussed below, the relative importance of Q_A oxidation and NPQ is
360 species and light history dependent, and in diatoms the relationship between PSII
361 redox-state and NPQ is species specific (Ruban et al. 2004; Lavaud unpublished
362 results).

363 For decreasing irradiance steps, the accumulative light dose effect described
364 above was reduced relative to increasing irradiance RLCs. However, decreasing the
365 irradiance and hence immediate exposure to high irradiance did not appear to induce
366 photodamage. Two observations support this, the low amplitude of NPQ for 10 and
367 30 s illumination duration at high irradiances, and the fact that rETR below 500 μmol
368 $\text{photons m}^{-2} \text{ s}^{-1}$ was similar to that obtained with increasing irradiance. The use of
369 decreasing irradiance therefore reduces the over-estimation of rETR and is likely to be
370 more representative of the photophysiological state of the diatom cells prior to
371 application of the RLC, which is the state that the RLC aims to ascertain.

372 Despite changing the culture used for each irradiance step (N-SLCs), the same
373 patterns in data were observed as for rapid RLCs (Fig. 3, 4). rETR increased as a
374 function of the length of irradiance during the light curve, such that even when F' was
375 allowed to reach an approximation of 'steady-state', RLCs failed to saturate. The N-
376 SLCs indicate that the effect of light dose on the fluorescence measurements occurred

377 rapidly, within the duration of each irradiance step. This observation confirms the
378 impact of combined Q_A redox state and NPQ on the Chl *a* fluorescence kinetics and
379 acquisition of F' and F_m' for the calculation of rETR (see Fig. 6).

380 Ralph and Gademann (2005) conducted a recent similar investigation on the
381 higher aquatic plant *Zostera marina*. They did not report a lack of saturation of the
382 rETR curves, but in some cases did report a lack of down regulation post saturation.
383 This lack of down regulation occurred for plants after low light treatment, and Ralph
384 and Gademann (2005) suggested that their capacity for down regulation was
385 exceeded. This differs from the data reported in the present study, where a lack of
386 saturation in rETR was greater for high light compared to low light treatments, despite
387 a higher capacity for NPQ in the former. We suggest that the lack of saturation of
388 RLCs observed with *N. phyllepta* is an additional indication (see also Ruban et al.
389 2004) that rapid photoacclimatory processes occur in diatoms which can greatly affect
390 fluorescence measurements, and especially the velocity of fluorescence transients
391 (Ruban et al. 2004). Rapid processes known to occur to a higher extent and with more
392 rapid induction kinetics in diatoms are the xanthophyll cycle (Jakob et al 2001;
393 Lavaud et al 2004), NPQ (Lavaud et al. 2002a; Ruban et al 2004, Serôdio et al.
394 2005b) and the PS II electron cycle (Lavaud et al 2002b).

395

396 *The effect of light history on RLC interpretation*

397

398 The effects of the order and the duration of each RLC irradiance step were greatest for
399 high light (HL) acclimated cultures (Fig. 1, 4). The light history, to which the cells
400 were exposed, modified the speed of response of photochemistry to short changes in
401 irradiance. HL cells had a greater capability to respond quickly to an increase in

402 irradiance, most likely due to a greater availability in electron acceptors from
403 photochemical reactions, increasing the speed of Q_A oxidation. The more rapid
404 kinetics for NPQ in HL cells can be explained by the basal level of NPQ developed
405 after 1 h exposure at $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, which did not relax during the 5 min
406 dark adaptation (Lavaud et al. 2002c; Ruban et al. 2004) In diatoms, NPQ amplitude
407 and kinetics are closely related to the amount of xanthophylls (Casper-Lindley and
408 Bjorkman 1998; Lavaud et al. 2002a 2002c), and are dependent upon light history
409 (Willemoes and Monas 1991; Mouget et al. 1999 2004; Lavaud et al. 2003), the state
410 of growth (Arsalane et al. 1994) and the species (Lavaud et al. 2004; Serôdio et al.
411 2005b). Thus, all these aspects have to be taken into account in the potential effects of
412 NPQ during the RLC acquisition and interpretation.

413

414 *Consequences for photophysiological parameters calculated from the RLCs*

415

416 An expected pattern was observed when comparing $rETR_{\text{max}}$, α and E_k
417 between HL and LL cultures (Fig. 2). The 1 h acclimation resulted in higher $rETR_{\text{max}}$
418 and E_k and lower α for HL compared to LL cells. Higher $rETR_{\text{max}}$ and E_k are typical
419 for high light acclimated cells which have modified their light harvesting to utilise the
420 high levels of light to which they are exposed. Conversely, low light acclimated cells
421 modify their photophysiology to maximise light harvesting efficiency and hence have
422 higher values of α . However the light dose effect experienced during the RLCs
423 reduced or even negated these differences in $rETR_{\text{max}}$. Similarly, for N-SLCs, the
424 differences in photophysiological parameters reduced as a function of increasing
425 length of each irradiance period. As a result, no difference was observed in $rETR_{\text{max}}$
426 by 'steady-state' (Fig. 5). This implies that long irradiance steps caused a photo-dose

427 effect that reduced or even negated the real level of 1 h photoacclimation. It remains
428 surprising though that cultures of *N. phyllepta* acclimated to 25 or 400 $\mu\text{mol photons}$
429 $\text{m}^{-2} \text{s}^{-1}$ did not saturate at 1850 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. *N. phyllepta* may have a high
430 capacity to respond quickly to high light, probably through regeneration of ADP and
431 NADP^+ and as a result of alternative electron pathways known to be active in diatoms
432 (Caron et al. 1987; Lavaud et al. 2002b; Wilhelm et al. 2004). This is possibly a
433 common feature of benthic diatoms, which may often experience rapid variations in
434 incident light intensity.

435 Although the dataset was obtained from measurements on diatom cells in
436 culture, the data suggest possible ecological implications with regard to diatom
437 acclimation to light environment fluctuation *in situ* (Serôdio et al. 2005b). It would
438 appear that *N. phyllepta* has a high ability to acclimate quickly to increasing
439 irradiance. This would obviously be an advantage to cells inhabiting an open mudflat
440 environment in which rapid changes could occur, e.g. as a result of cloud induced
441 light flecking. Any energy dependent photoprotective acclimation, being more rapid
442 than downward migration, would not only precede migration (Underwood et al.,
443 2005) should high irradiance persist, but would also prevent wasteful short-term
444 migrations requiring production and excretion of extracellular polymeric substances
445 (carbohydrates generically described as EPS) used in migration (Consalvey et al.
446 2005b and references there-in).

447

448 *Specificity of the RLCs acquisition with the Diving-PAM methodology*

449

450 Analysis of the fluorescence kinetics from *N. phyllepta* indicated an aspect of
451 methodology, which may be particular to the Diving-PAM, and which can generate an

452 error in the measurement of F_m' and hence rETR for 10 s irradiance steps (see the
453 description in the Results section, Fig. 6). To summarise, the time delay between
454 measurement of F' and the corresponding F_m' used in calculation of the quantum
455 efficiency resulted (during this study) in an under-estimation of F_m' relative to F' and
456 hence a falsely low efficiency. The example illustrated was for a low light culture at
457 $25 \mu\text{mol m}^{-2} \text{s}^{-1}$, transferred to $370 \mu\text{mol m}^{-2} \text{s}^{-1}$. This is an abrupt change, however the
458 effect was greatest for HL acclimated cultures and at high irradiances, when the rate
459 decay in F' (presumably resulting from interaction between Q_A oxidation and NPQ
460 induction) was greatest. As such the level of this error is a function of light dose and
461 hence the light history to which the cells were exposed. This problem was not
462 encountered with the higher aquatic plant *Zostera* (Ralph and Gademann 2005)
463 presumably because the transients in fluorescence yield in plants are slower than in
464 diatoms, although NPQ relaxation is slower in diatoms (Ruban et al. 2004).

465

466 *Conclusions*

467 The present work indicates that light dose and light history strongly affect
468 fluorescence measurements used in RLC acquisition. These features have to be taken
469 into account in the acquisition and interpretation of Chl *a* fluorescence RLCs. For
470 cultured benthic diatoms, and presumably for *in situ* mixed biofilms for the same
471 reasons, extreme care should be taken in choice of light curve methodology.
472 Parameters measured will be functions of light history and light exposure during the
473 light curve itself and the extent of this will in turn be dependent upon the light history
474 prior to the RLC. It is suggested that it is not always possible to answer the question
475 posed above: what is being measured for light curves of different length of actinic
476 irradiance? It is not possible to extrapolate these results to make general comments for

477 all diatom species, nor to say that the changes suggested will occur for *in situ*
478 measurements. Indeed, no methodology is perfect and in many cases measurement
479 itself induces a change, thus making the commonly stated “non-intrusive” nature of
480 fluorescence measurements incorrect. For example, an increasing RLC induces
481 photoacclimation, however a decreasing RLC will allow dissipation of
482 photophysiological state (e.g. high light induced NPQ) as light level is reduced:
483 neither method is devoid of experimental error. However it is suggested that the
484 changes induced during a RLC should be carefully considered during interpretation of
485 results. In general, RLCs of 60 s or those with increasing incremental irradiance steps
486 may not detect differences in photophysiological state caused by light history,
487 differences that RLCs aim to determine. Conversely RLCs with short irradiance steps
488 may result in errors due to rapid NPQ induction and Q_A oxidation, especially for
489 diatom cells exposed to high light or a high-accumulated light dose history. Overall,
490 re-programming of the Diving-PAM fluorimeter to enable RLCs with decreasing
491 irradiance levels is advised, and RLCs of 30 s at each irradiance step may be optimal
492 for use with benthic diatoms.
493

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725 **Figure Legends**

726

727 Figure 1. Rapid light response curves for *N. phyllepta* cultures grown at an irradiance
728 of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and exposed to 1 h light acclimation period of low (A, 25
729 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or high (B, 400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) light. Light response
730 curves were run with irradiance durations at each light curve increment of 10, 30 and
731 60 s and with either increasing (up) or decreasing (down) irradiance steps. Curves
732 were constructed using the model of Eilers and Peeters (1988) followed by curve
733 fitting following the Nelder-Mead model (Press et al., 2003).

734

735 Figure 2. Rapid light response curve parameters for light curves obtained using
736 decreasing irradiance steps, shown in Fig. 1. (A) maximum electron transport rate,
737 $r\text{ETR}_{\text{max}}$; (B) maximum light use coefficient (α); (C) light saturation coefficient (E_k).

738

739 Figure 3. Non-sequential light response curves for *N. phyllepta* cultures grown at 100
740 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Light curves were run using different sub-samples of culture for
741 each light curve step and with irradiance durations of 10, 30 and 60 s, followed by a
742 final measurement when F' reached approximate 'steady-state' after 2 to 3 minutes.
743 Curves were constructed using the model of Eilers and Peeters (1988) followed by
744 curve fitting following the Nelder-Mead model (Press et al., 2003).

745

746 Figure 4. Non-sequential light response curves (mean \pm s.e., $n = 3$) for *N. phyllepta*
747 cultures grown at an irradiance of 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and exposed to 1 h light
748 acclimation period of low (A, 25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or high (B, 400 $\mu\text{mol photons}$
749 $\text{m}^{-2} \text{ s}^{-1}$) light. Light curves were run using different sub-samples of culture for each
750 light curve step, and with irradiance durations of 10, 30 and 60 s, followed by a final

751 measurement when F' reached approximate 'steady-state' after 2 to 3 minutes. Curves
752 were constructed using the model of Eilers and Peeters (1988) followed by curve
753 fitting following the Nelder-Mead model (Press et al., 2003).

754

755 Figure 5. Non-sequential light response curve parameters (mean \pm s.e., $n = 3$) for
756 light curves obtained using different sub-samples of *N. phyllepta* culture for each
757 irradiance step, shown in Figure 4. (A) maximum electron transport rate, $rETR_{max}$; (B)
758 maximum light use coefficient (α); (C) light saturation coefficient (E_k).

759

760 Figure 6. Example of fluorescence kinetics obtained for a sub-sample of *N. phyllepta*
761 low-light culture used in a non-sequential light response curve step. Application of
762 saturating pulses are indicated by downward arrows after 10, 30 and 60 s and when F'
763 reached approximate 'steady-state' after 2 to 3 minutes. The actinic light increase was
764 from 25 to 370 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

765

766 Figure 7. Non-photochemical Chl *a* fluorescence quenching (NPQ) calculated as $(F_m -$
767 $F_m') / F_m'$ for non-sequential light curves (Fig. 4) of *N. phyllepta* exposed to 1 h light
768 acclimation period of low (A, 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or high (B, 400 $\mu\text{mol photons}$
769 $\text{m}^{-2} \text{s}^{-1}$) light. NPQ was calculated using F_m' obtained from saturating pulses after
770 irradiance durations of 10, 30 and 60 s, followed by a final measurement when F'
771 reached approximate 'steady-state' after 2 to 3 minutes. The insert shows the change
772 in NPQ at 560 and 3200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ over time.

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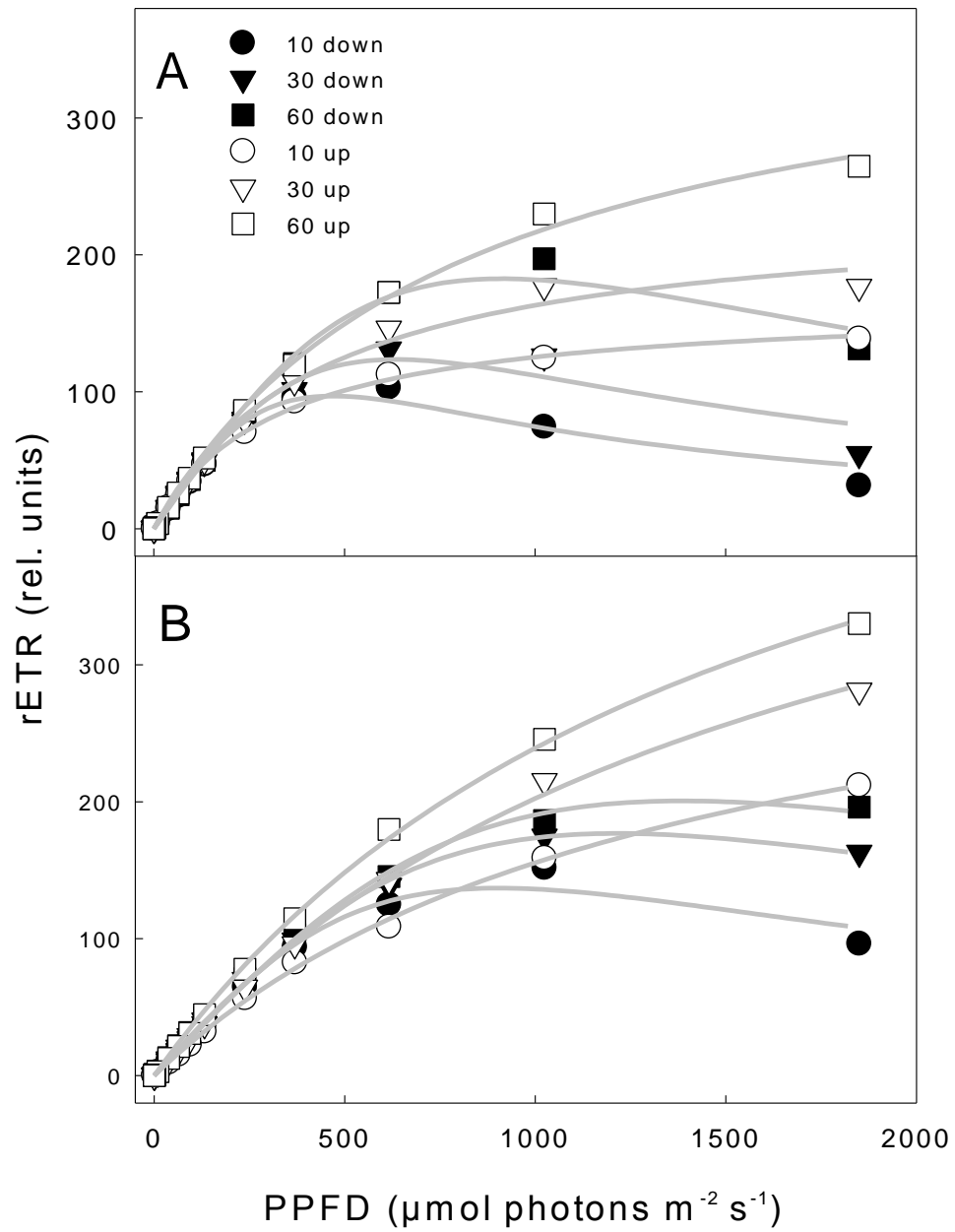
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Figure 1

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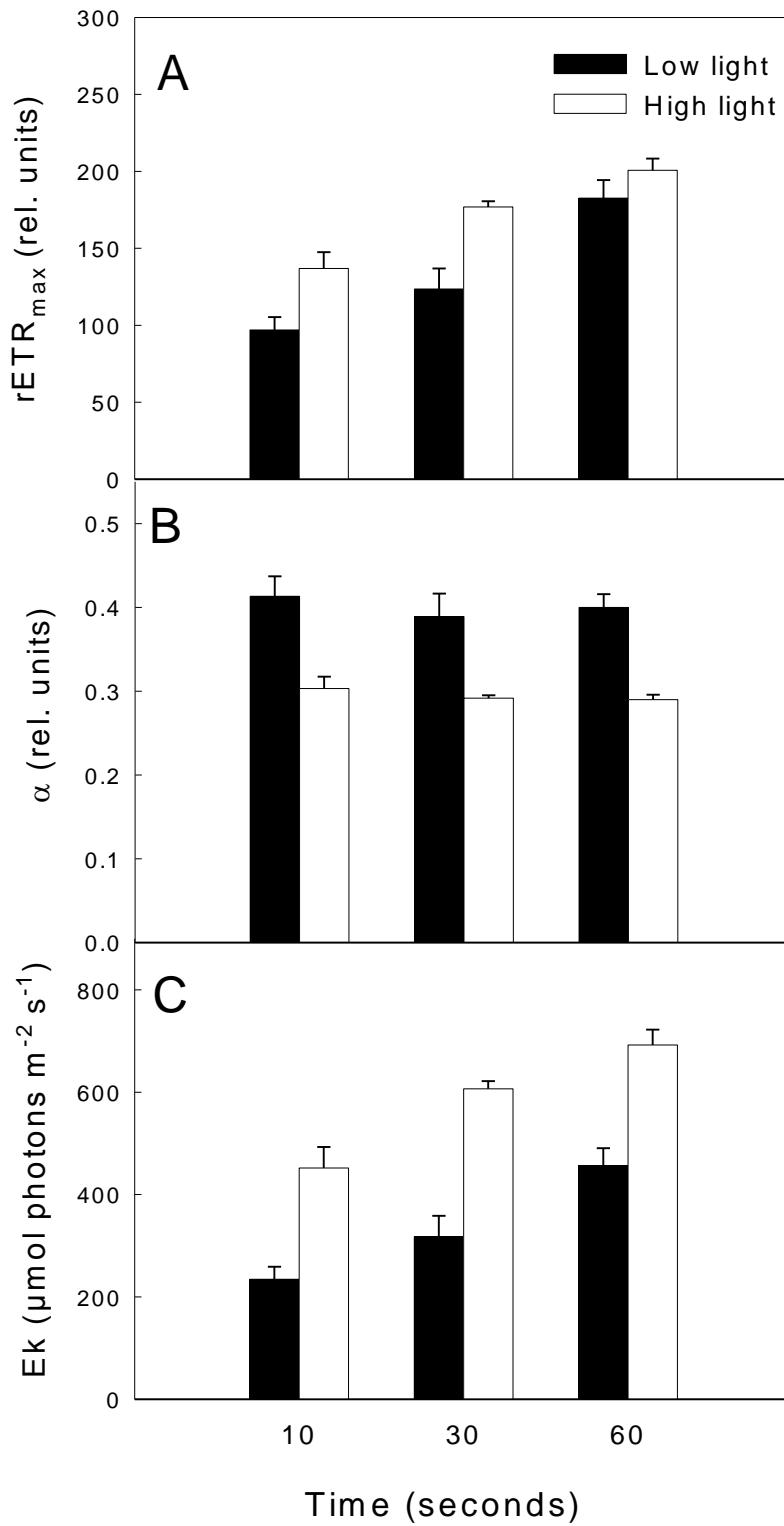


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Figure 2



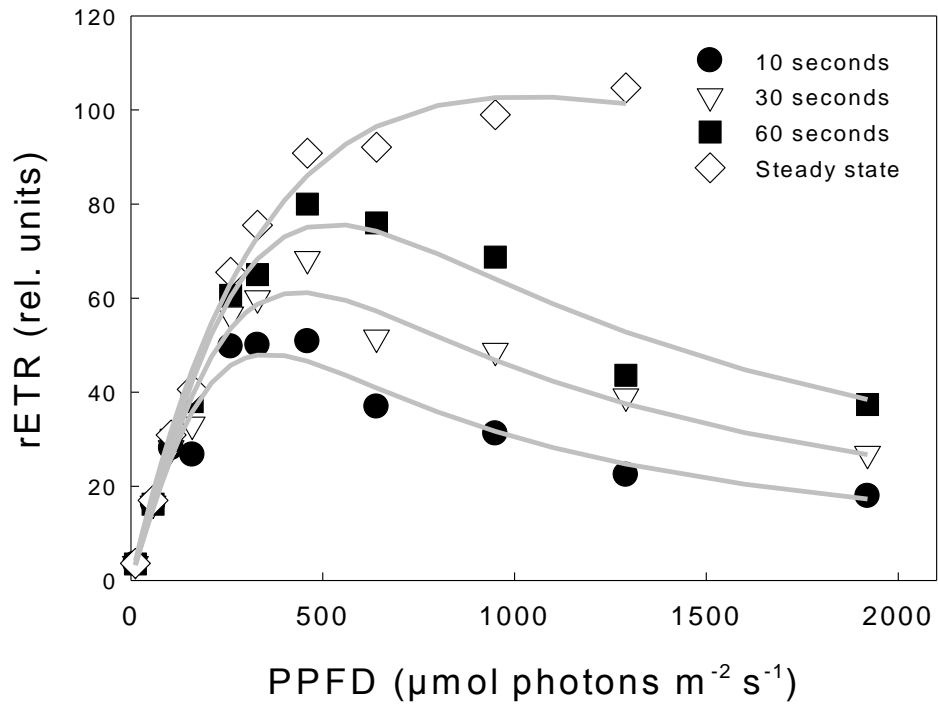
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Figure 3



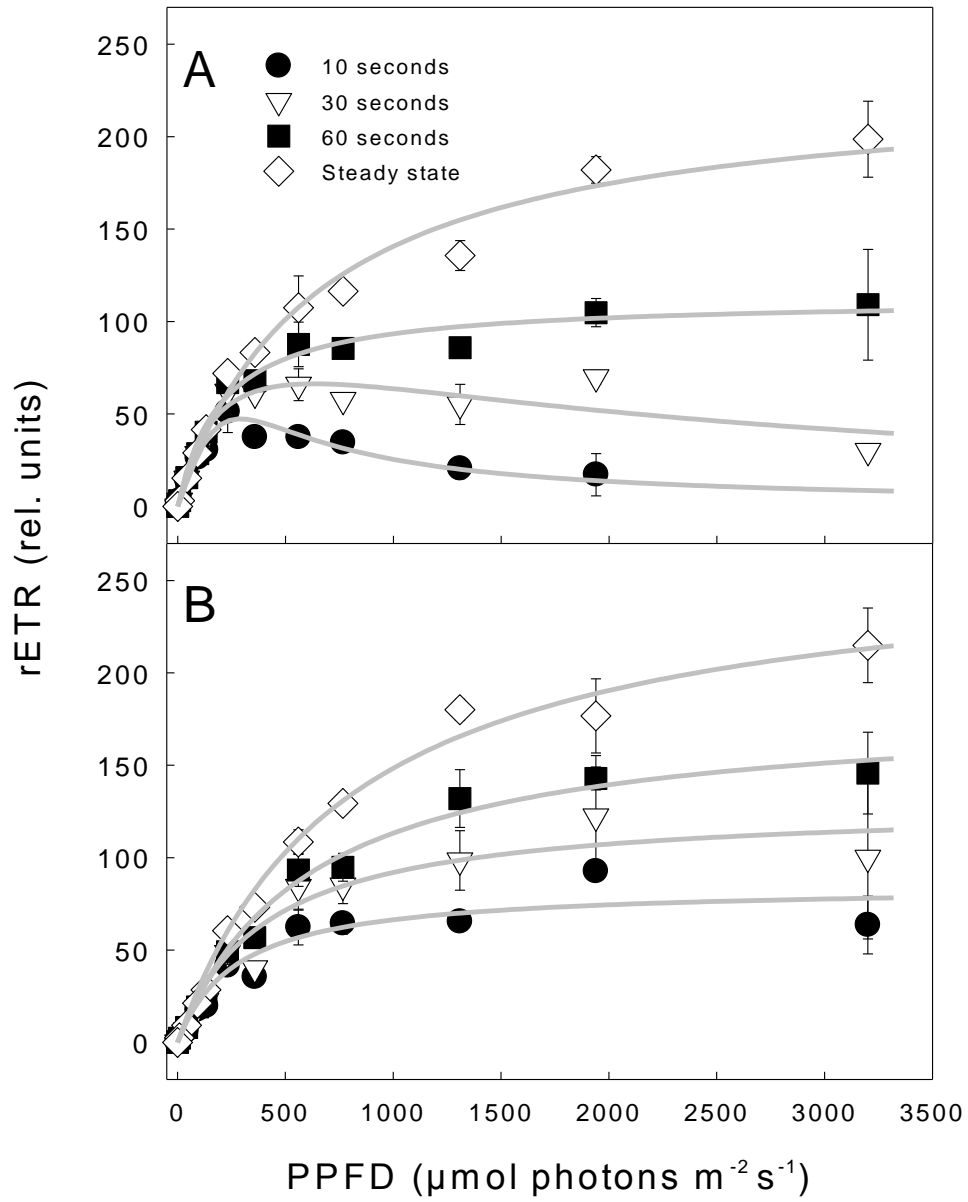
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Figure 4



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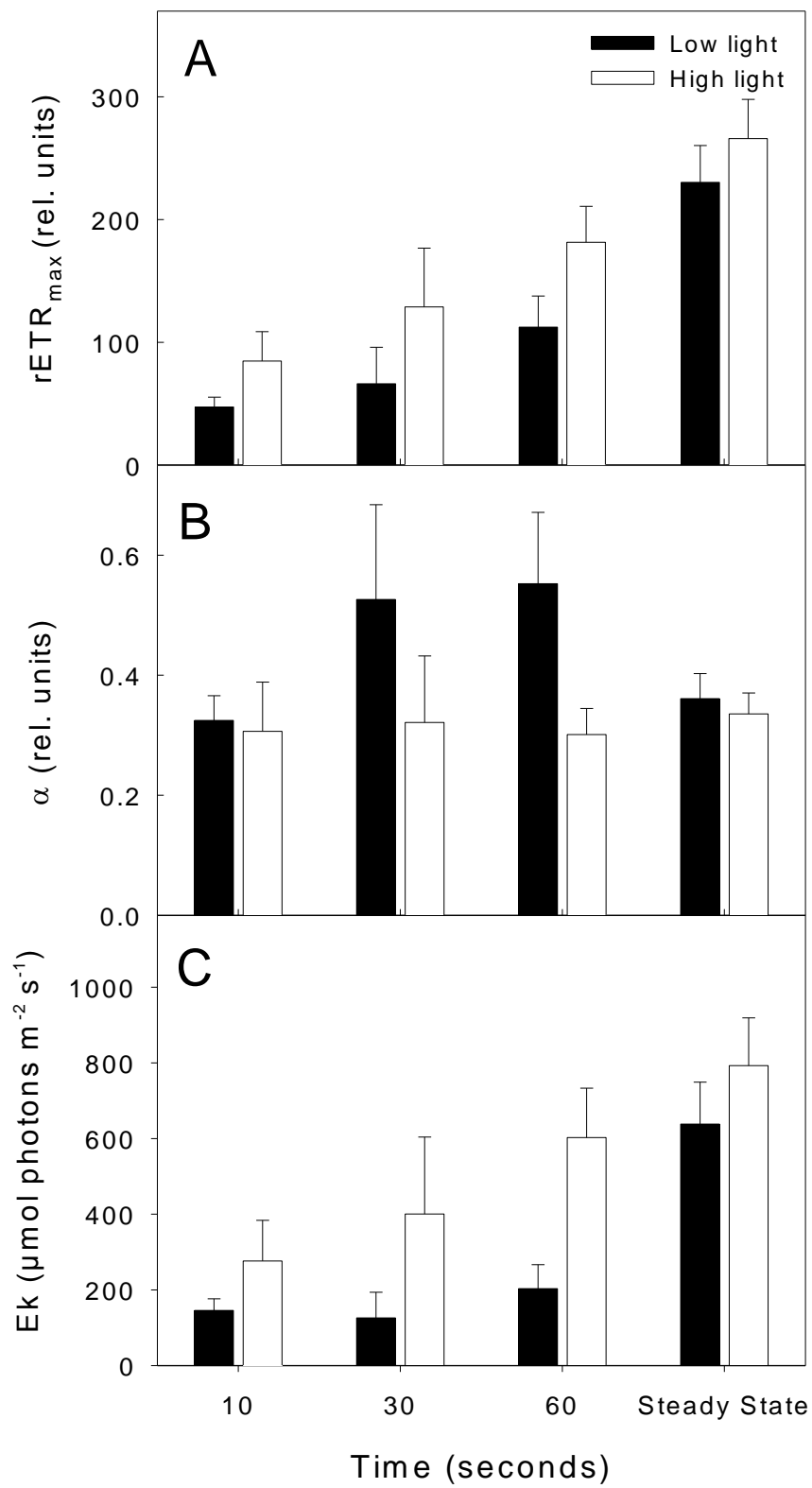
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Figure 5

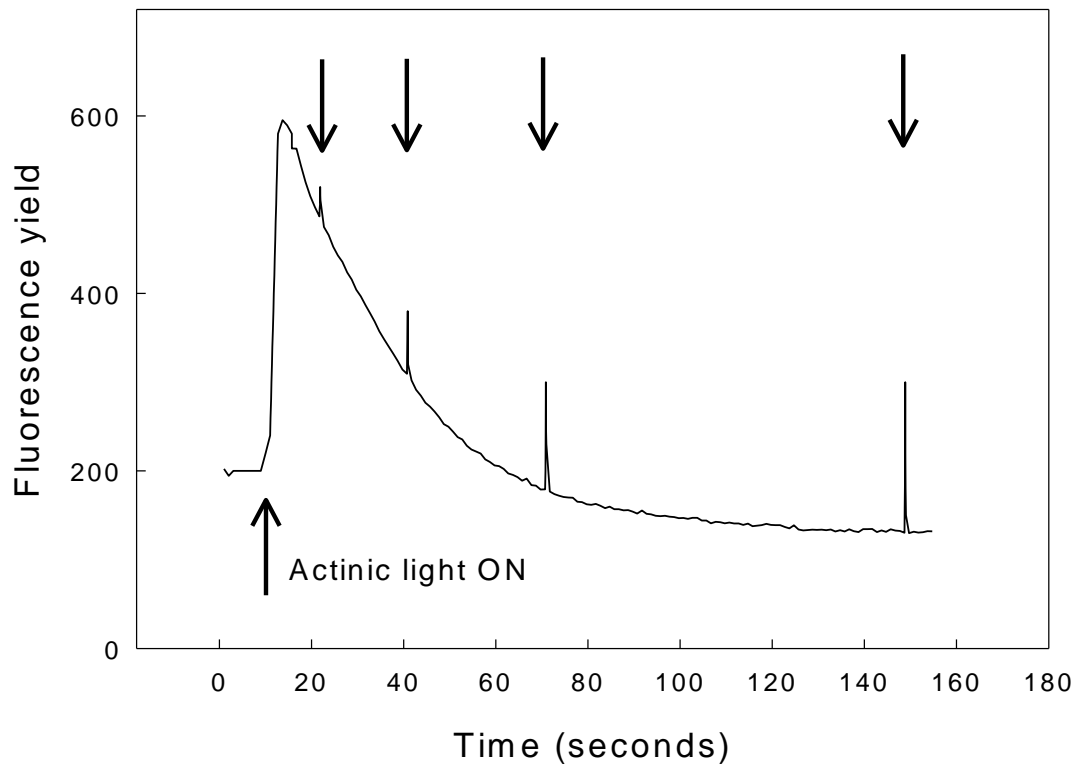


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Figure 6

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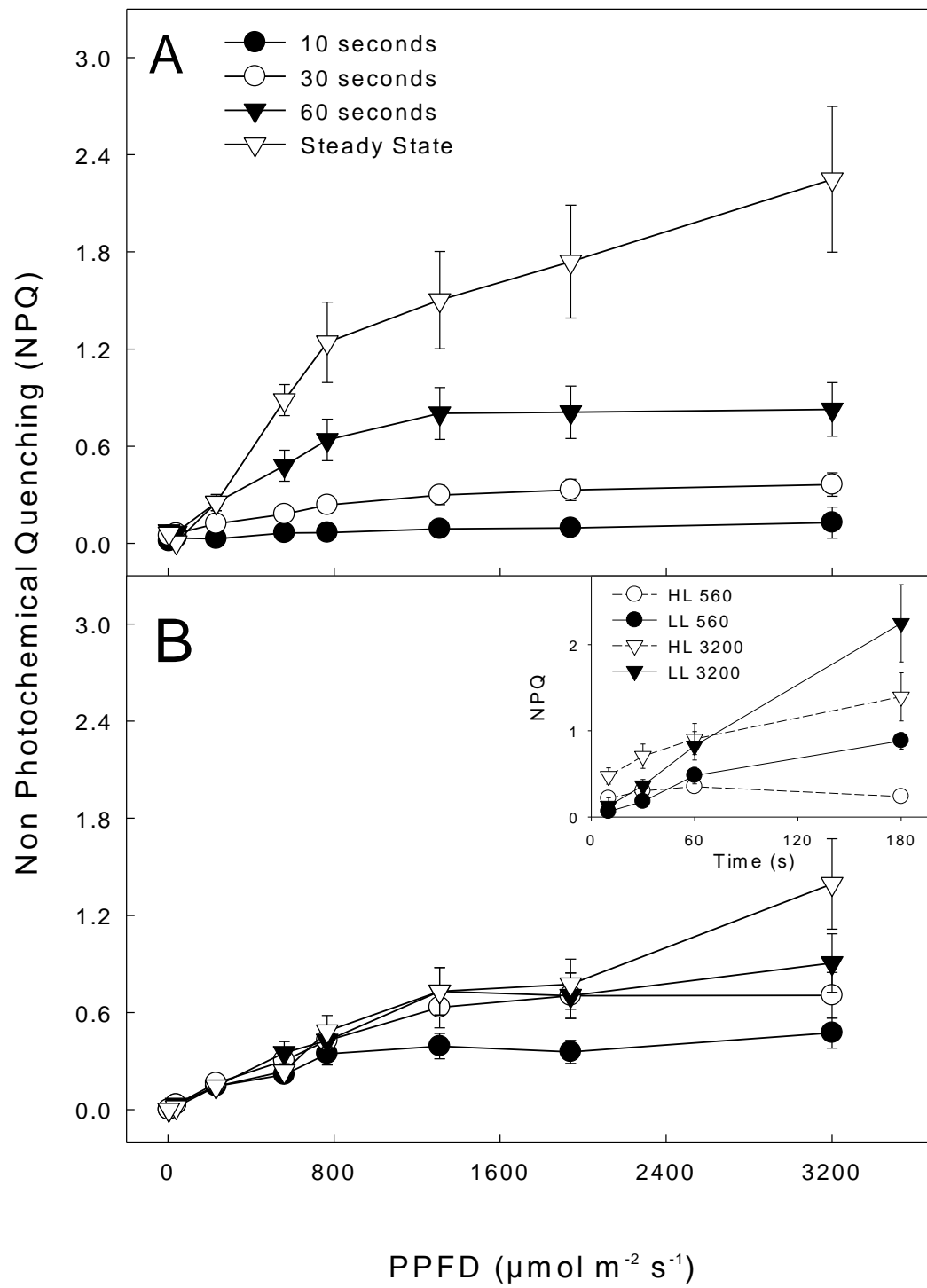
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Figure 7

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