

Vertical cell movement is a primary response of intertidal benthic biofilms to increasing light dose

Rupert G. Perkins, Johann Lavaud, Joao Serôdio, Jean-Luc Mouget, Paolo Cartaxana, Philippe Rosa, Laurent Barillé, Vanda Brotas, Bruno Jesus

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5	Perkins, R.G. ^{*1} , Lavaud, J. ² , Serôdio, J. ³ , Mouget, J-L. ⁴ ; Cartaxana, P. ⁵ , Rosa, P. ⁶ , Barille,
6	L. ⁶ , Brotas, V. ⁵ , Jesus, B.M. ^{5,7}
7 8	* corresponding author
9 10 11 12	*1. School of Earth, Ocean and Planetary Sciences, Cardiff University, Main Building, Park Place, CF10 3YE Cardiff, UK E-mail: PerkinsR@cf.ac.uk, Tel.: +44-29-20874943, Fax: +44-29-20874326
12 13 14	2. UMR CNRS 6250 'LIENSs', Institute for Coastal and Environmental Research (ILE), University of La Rochelle, 2 rue Olympe de Gouges, 17042 La Rochelle Cedex, France.
15 16 17	3. Departamento de Biologia, Universidade de Aveiro, Aveiro, 3810-193, Portugal
17 18 19 20 21	4. Laboratoire de Physiologie et Biochimie Végétales, Faculté des Sciences et Techniques, Université du Maine, EA2663, Av. O. Messiaen, 72085 Le Mans Cedex 9, France
21 22 23 24	5. Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, 1749-016 Lisboa, Portugal.
24 25 26	6. Université de Nantes, EA 21 60 "Mer Molécules Santé", Faculté des Sciences et Techniques, 2, rue de la Houssinière, BP 92 208, 44322 Nantes cedex 3, France
27 28 29 30 31 32 33 34	7. Centro de Biodiversidade, Genómica Integrativa e Funcional (BioFIG), Faculdade de Ciências, Universidade de Lisboa, Lisboa, Portugal
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- 38 Abstract
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40 Intertidal soft sediment microphytobenthic biofilms are often dominated by diatoms 41 which are able to regulate their photosynthesis by physiological processes (e.g. down 42 regulation through the xanthophyll cycle, referred to as non-photochemical quenching, 43 NPQ) and behavioural processes (e.g. vertical cell movement in the sediment – biofilm 44 matrix). This study investigated these two processes over a 6 h emersion period using 45 chemical inhibitors under two light treatments (ambient light and constant light at 300 μ mol m⁻² s⁻¹). Latrunculin A (Lat A) was used to inhibit cell movement and dithiothreitol 46 47 (DTT) to inhibit NPQ. HPLC analysis for chlorophyll a and spectral analysis 48 (Normalised Difference Vegetation Index, NDVI) indicated that Lat A significantly 49 inhibited cell movement. Photosynthetic activity was measured using variable 50 chlorophyll fluorescence and radiolabelled carbon uptake and showed that the non-51 migratory Lat A treated biofilms were severely inhibited as a result of the high 52 accumulated light dose (significantly reduced maximum relative electron transport rate, rETR_{max}, and light utilization coefficient, α) compared to the migratory DTT and control 53 treated biofilms. No significant patterns were observed for ¹⁴C data, although a decrease 54 55 in uptake rate was observed over the measurement period. NPQ was investigated using 56 HPLC analysis of xanthophyll pigments (Diatoxanthin, DT and the percentage de-57 epoxidation of Diadinoxanthin, DD), chlorophyll fluorescence (change in maximum 58 fluorescence yield) and the second order spectral derivative index (Diatoxanthin Index, 59 DTI). Patterns between methods varied, but overall data indicated greater NPQ induction 60 in the non-migratory Lat A treatment and little or no NPQ induction in the DTT and 61 control treatments. Overall the data resulted in two main conclusions: firstly the primary

62	response to accumulated light dose was vertical movement, which when inhibited			
63	resulted in severe down regulation / photoinhibition; secondly diatoms down regulated			
64	their photosynthetic activity in response to accumulated light dose (e.g. over an emersion			
65	period) using a combination of vertical migration and physiological mechanisms, which			
66	may contribute to diel and/or tidal patterns in productivity.			

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Keywords: benthic, diatom, down regulation, migration, photophysiology, productivity

72 Introduction

73

74 Microphytobenthic biofilms at the surface of intertidal estuarine sediments are 75 highly productive (Brotas and Catarino, 1995; MacIntyre et al., 1996; Underwood and 76 Kromkamp, 1999). The regulatory mechanisms controlling the magnitude and periodicity 77 of this productivity are partly understood as involving sun angle and tidal patterns 78 (Pinckney and Zingmark, 1991) as well as changes in light dose exposure (Kromkamp et 79 al., 1998; Serôdio and Catarino, 1999; Perkins et al. 2002, Jesus et al. 2005). For the latter 80 it has been hypothesised (Kromkamp et al., 1998; Serôdio and Catarino, 1999; Perkins et 81 al. 2002; Jesus et al., 2006a) that cells optimise their position within the surface layers of 82 a sediment biofilm, utilising sediment light attenuation to provide an optimal light 83 environment; this is the concept of microcycling. The importance of vertical movement to 84 regulate light exposure has recently been demonstrated thoroughly using chemical 85 inhibition of movement (Cartaxana and Serôdio, 2008; Cartaxana et al. 2008). However, 86 this is the first study to directly compare the roles of vertical movement, a behavioural 87 form of photosynthetic down regulation (e.g. Perkins et al. 2002), with physiological 88 down regulation in the form of non-photochemical quenching (NPQ; e.g. Lavaud, 2007). 89 Effectively diatom cells move vertically through the sediment matrix utilising 90 extracellular polymers in response to changes in light environment: too much light, cells 91 move downwards, not enough light, cells move upwards. This is a simplification however 92 as the cumulative effect of light exposure over time modifies this response (Perkins et al. 93 2002, 2006; Jesus et al., 2006b). It should be emphasised as well, that this microcycling 94 movement over short time scales is distinct from the bulk movements of cells as vertical migration (Underwood and Kromkamp, 1999; and see the review by Consalvey et al., 95

2004) driven by tidal and sun angle driving forces as originally outlined by Pinkney and
Zingmark (1991).

98 Why do diatom cells require an optimum light environment to maximise their 99 photosynthetic potential? It is well known that excess light can lead to photodamage by 100 production of free radicals and superoxides which may lead to protein breakdown in 101 photosystem II reaction centres, e.g. the D1 dimer (Olaizola et al., 1994; Materna et al, 102 2009). Cells can hence prevent such damage through two processes, both of which 103 effectively down regulate photosynthetic activity. Firstly, cells can migrate downwards 104 away from high light that could result in a photodamaging light dose. This is effectively a 105 behavioural form of down regulation (Kromkamp et al., 1998; Serôdio and Catarino, 106 1999; Perkins et al. 2001; Mouget et al., 2008). Secondly cells can down regulate by 107 diverting excess light energy away from PSII reaction centres via alternative energy 108 pathways (Ting and Owens, 1993; Lavaud et al., 2002a; Goss et al., 2006; Lavaud, 2007; 109 Serodio et al., 2008). This physiological process of down regulation is often referred to 110 as non-photochemical quenching (NPQ) as it quenches the energy using energy 111 conversions with no photochemical output. The process utilises energisation of the 112 thylakoid membrane by generation of a proton gradient, which induces de-epoxidation of 113 diadinoxanthin to diatoxanthin (DT) known as the xanthophylls cycle (Lavaud et al., 114 2002a, 2004, 2007; Goss et al., 2006). DT competes for light energy with chlorophyll 115 pigments in the light harvesting complexes, hence diverting the energy away from the 116 pathway that would lead to generation of harmful reducing agents created by over 117 excitation of PSII reaction centres (Lavaud, 2007; Ruban et al., 2004). Diatoms are 118 known to have highly effective xanthophyll cycle and are able to rapidly induce NPQ in

response to increasing light levels (Goss et al., 2006; Lavaud, 2004, 2007; Ruban et al.,
2004; Serodio et al., 2005, 2008) even so far as to induce short term photoacclimation
through NPQ induction in the time required for 30 second rapid light curves, e.g. over a 4
minute period (Perkins et al., 2002; Perkins et al., 2006; Cruz and Serodio, 2008).

123 Diatom cells in surface biofilms can therefore respond to changing light 124 environments, and hence accumulated historical light doses, through two mechanisms, 125 vertical cell movement within the sediment matrix, or NPQ induction. These processes 126 are now well understood, e.g. Consalvey et al. (2004), Kromkamp et al. (1998), Perkins et 127 al. (2002), Spilmont et al. (2007) and Mouget et al. (2008) regarding light induced cell 128 movement and Lavaud (2007) and Perkins et al. 2006 regarding NPQ. Also how does the 129 down regulation effect net productivity and how does this vary in response to the light 130 dose over a low tide emersion period? This study aimed to address these questions 131 through manipulative experiments using engineered biofilms treated with chemical 132 inhibitors, under two different light dose regimes. Chemical treatments comprised 133 inhibition of cell motility using Latrunculin A which inhibits actin filaments involved in 134 diatom movement without affecting photosynthetic activity (Cartaxana et al. 2008) and 135 also the use of DL-dithiothreitol which inhibits the de-epoxidation of DD to DT, and 136 hence inhibits NPQ induction (Lavaud et al., 2002b). These treatments were compared to 137 controls over a 6 hour emersion period under two light treatments, ambient light and a 138 constant low light environment. Thus the roles of cell movement and NPQ induction 139 were compared as functions of the increasing photodose accumulated over the emersion 140 period.

141

142 Methods

145 Experimental design and sampling

Surface mud to a depth of approximately 1 cm was collected on the 1st July 2008 from 146 147 Alcochete mudflat, located on the eastern shore of the Tagus Estuary (38 44' N, 9 08' W), 148 composed of slightly gravelly mud (Jesus et al 2006c). All experimental measurements were carried out on the following day, 2nd July 2008. The mud and surface biofilm was 149 150 returned to the laboratory where a sub-sample was examined by light microscopy to 151 determine the dominance of epipelic diatoms in the biofilm. The remainder of the surface 152 mud was thoroughly mixed by hand and then evenly spread in trays to a depth of 5 cm. A 153 shallow depth of site water (< 2 cm) was carefully added so as not to re-suspend the mud 154 and the trays were left overnight in the laboratory. The following morning, at the start of 155 the low tide emersion predicted for the original sample site, the shallow depth of site 156 water was removed and a spectroradiometer (see below) was used to monitor the 157 establishment of surface biomass in one of the sample trays. Plastic cores ($2 \text{ cm} \times 2.5 \text{ cm}$ 158 diameter) were then carefully inserted into the mud to isolate minicore sediment samples 159 in each sediment tray for the following chemical treatments: controls (addition of filtered 160 site water only), Latrunculin A (Lat A, dissolved in site water) to inhibit cell motility and 161 DL-dithiothreitol (DTT, in site water) to inhibit conversion of DD to DT and hence 162 inhibit non-photochemical quenching (NPQ). Full details of these treatments are given 163 below. Three replicates for each chemical treatment were used to provide independent 164 samples for the following measurement: rapid light response curves using PAM-165 fluorescence, spectroradiometry, sampling for pigment analysis using HPLC (minicore set 1) and ¹⁴C radiolabelled measurement of primary productivity (minicore set 2). Hence 166

167 6 minicores were needed for each treatment for each time sampling point (n = 3, T1, 2) 168 and 3 equally spaced 2 h apart). Finally the number of minicores was duplicated in a 169 second sample tray to enable two light treatments to be investigated, ambient light and constant light (300 μ mol m⁻² s⁻¹). Note that all light levels referred to were measured with 170 171 a Licor cosine corrected light meter and refer to photosynthetically active radiation, 400 -172 700 nm. The constant light was provided by a quartz white light source (400W HPI-T Pro 173 Philips). The experimental set up is summarized in Table 1. Ambient light treatment 174 (Amb) and constant low light treatment (Con) were identical other than their respective 175 light dose exposures calculated by integration over time of light measurements taken 176 using a Licor cosine corrected light meter every 30 minutes during the experimental 177 period. Finally, all treatments were applied once the biofilm had established at the 178 sediment surface as assessed by the stabilization of the NDVI reflectance readings; hence 179 chemical and light treatments were applied to established surface biofilms rather than 180 prior to upward cell migration. Measurements using the following methodologies were 181 taken at equal time intervals of 2 h at T1, T2 and T3, hence covering a 6 h exposure 182 period typical for the original sample site. Experiments were carried out under ambient 183 light on the roof of the Instituto de Oceanografia de Lisboa, Lisbon, Portugal. Engineered 184 biofilm trays were incubated in temperature controlled water tanks to minimise potential 185 over-heating (maximum temperatures measured at the sediment surface during the 186 experimental period were 35°C, comparable to those measured in situ). Light dose was 187 calculated for each sampling point T1, T2, T3 by integrating the light measurements 188 (using a Licor cosine corrected light meter) over the preceeding time period.

191 Controls – 400 μL of filtered site water was added to all cores to mimic chemical
192 treatments but without addition of DTT or Lat A (see below).

193 DTT - DL-dithiothreitol (Sigma) was prepared as a fresh stock on the morning of the 194 experimental period. A stock solution of 20 mM (in ethanol) was diluted 100 times in 195 freshly filtered site water to reach a final concentration of 200 µM. 400 µL of this 196 solution were added to each core in order to cover the whole surface of the sediment. 197 Given the dimensions of the cores, the amount of DTT added in each core was 0.17 198 umoles. This amount of DTT was previously determined to be sufficient to virtually fully inhibit the conversion of DD in DT in a 10 μ g Chl *a* mL⁻¹ suspension *Phaeodactylum* 199 200 tricornutum (100% inhibition with 0.2 µmol DTT) (Lavaud et al., 2002b).

201 Latrunculin A - A concentrated Latrunculin A solution (1 mM) was prepared as a fresh 202 stock on the morning of the experimental period by dissolving purified Lat A (Sigma-203 Aldrich) in dimethylsulfoxide. A solution of 12.5 µM Lat A was prepared by dissolving 204 the appropriate amount of the concentrated stock solution in filtered water collected at the 205 sampling site. Small volumes of this solution (total of 300 µL) were applied to 206 undisturbed sediment samples by carefully pipetting directly onto the sediment surface, 207 until forming a continuous thin layer that completely covered the sample. The amount of 208 Lat A used was previously determined to be sufficient to virtually inhibit diatom 209 migration in benthic biofilms (Cartaxana and Serôdio, 2008). The inhibitor was applied 210 after the formation of the biofilm at the sediment surface during the period coinciding 211 with the beginning of low tide at the sampling site.

212 Spectral reflectance

213 Spectral reflectance was measured with a USB2000 (Ocean Optics, Dunedin, 214 USA) with a VIS-NIR optical configuration controlled by a laptop using OOIBase32[™] software. The spectroradiometer sensor was positioned at a 45° angle pointing at the 215 center of the minicore and measuring an approximate area of 1 cm². Reflectance spectra 216 217 of the target surface were calculated by dividing the upwelling spectral radiance from the 218 sediment surface (L_u) with the reflectance of a clean white polystyrene plate (L_d) both 219 spectra were corrected for dark noise (D_n) (electronic signal measured at total darkness):

220 (Equation 1)

221 $Reflectance = (L_u - D_n)/(L_d - D_n)$ (1)

The polystyrene plates differed less than 3% from a calibrated 99% reflectance 222 223 standard plate (Spectralon) (Forster and Jesus, 2006). The normalized vegetation index 224 (NDVI) was calculated as follows:

225 (Equation 2)

226
$$NDVI = (InfraRed - Red)/(InfraRed + Red)$$
 (3)

227 where InfraRed is the average reflectance of the rage 748-752 nm and Red the 228 average reflectance of the range 673-677 nm.

229 Reflectance derived indices are susceptible to background noise and are not 230 sensitive enough to detect the didinoxanthin (DD) to diatoxanthin (DT) pigment conversion that occur during the xanthophyll cycle. Using diatom cultures Jesus et al. 231 232 (2008) showed that the conversion of DD to DT causes a reflectance decrease at 508 that 233 is proportional to DT content. However, this decrease was so small that only an index 234 based on the second derivative spectrum was appropriate to detect it. Their DT index 235 (DTI) used the second derivative peak at 508 nm normalized by the second derivative 236 peak at 630 nm and showed very promising results in the determination of diatom DT content. Thus, DTI was used in the current study as a proxy for the DT present at thesediment surface.

239 The derivative spectra (δ) were calculated using a finite approximation method 240 (Louchard et al. 2002), after smoothing the reflectance spectra with a natural cubic spline 241 function (60 nodes). The second derivative ($\delta\delta$) was chosen because in theory it 242 eliminates the background effects and strongly enhances minute changes in the 243 reflectance spectra. This would be ideal in intertidal estuarine sediments where the 244 background signal can be strongly influenced by organic matter, sediment type and 245 moisture. The second derivative spectra were only calculated for the ambient light 246 treatment due to the high noise spectra generated by the lamps used in the constant light 247 treatment.

248

249 Rapid light response curves

250 Rapid light response curves were obtained using a Walz Water-PAM fluorimeter 251 and following the methodology of Perkins et al. (2006) except that 20 second light step 252 increments were used rather than 30 seconds due to time constraints. Settings on the 253 Water-PAM were as follows: saturating pulse at setting 10 (approximately 8,600 µmol m⁻ ² s⁻¹ PAR) for 600 ms duration; light curve settings of 20 second light step duration 254 covering $0 - 1035 \,\mu\text{mol m}^{-2} \,\text{s}^{-1}$ PAR (previously determined as adequate to produce fully 255 256 saturated light curves for biofilms from this site); due to time restrictions during the 257 experimental period, increasing light level steps using the Water-PAM programming 258 were used rather than preferred decreasing light steps using Win Control (Perkins et al., 259 2006). Light curve measurements were taken in a random order between chemical 260 treatments, however at each time point, ambient light measurements were made prior to constant light measurements. Once spectral reflectance and fluorescence measurements
had been made, the same minicores were destructively sampled for pigment analysis (see
below) with care to ensure that the area sampled was not that exposed to the light dose
applied by the rapid light curve.

265 Analysis of rapid light curves also followed that described by Perkins et al. (2006) 266 with curve fitting following the iterative solution of Eilers and Peeters (1988) to 267 determine coefficients a, b and c. Following this, light curve parameters of relative 268 maximum electron transport rate (rETR_{max}), coefficient of light use efficiency (α) and 269 light saturation coefficient (E_k) were calculated from the parameters a, b and c following 270 the equations in Eilers and Peeters (1988). The software used for curve fitting and 271 regression analysis to determine curve parameters was Sigmaplot V11. Non-272 photochemical quenching (NPQ) was calculated as the change in maximum fluorescence yield (NPQ = $(F_m - F_m')/F_m'$), where Fm was taken as the initial value recorded in the 273 274 rapid light curve (e.g. after 30 seconds of darkness).

275

276 Pigment analysis

Approximately 50 mg of freeze-dried sediment were extracted in 95% cold buffered methanol (2% ammonium acetate) for 15 min at -20° C, in the dark. Samples were sonicated (Bransonic, model 1210) for 30 s at the beginning of the extraction period. Extracts were filtered (Fluoropore PTFE filter membranes, 0.2 µm pore size) and immediately injected in in a Shimadzu HPLC with photodiode array and fluorescence (Ex. 430 nm; Em. 670 nm) detectors (Cartaxana and Brotas, 2003). Chromatographic separation was carried out using a C18 column for reverse phase chromatography 284 (Supelcosil; 25 cm long; 4.6 mm in diameter; 5 μ m particles) and a 35 min elution 285 programme. The solvent gradient followed Kraay et al. (1992) with a flow rate of 0.6 mL min⁻¹ and an injection volume of 100 µL. Pigments were identified from absorbance 286 287 spectra and retention times and concentrations calculated from the signals in the 288 photodiode array detector or fluorescence detectors. Calibration of the HPLC peaks was 289 performed using commercial standards from Sigma-Aldrich and DHI (Institute for Water 290 and Environment). Samples were analysed for the xanthophyll pigments DD (the 291 epoxidised form) and DT (the de-epoxidised form). The state of de-epoxidation (DEP in 292 %) was calculated as $DT/(DD+DT) \ge 100\%$.

293

294 Radiolabelled carbon uptake

Total primary productivity (µg C [µg Chl a]⁻¹ h⁻¹) was measured from sub-295 samples of ¹⁴C-labelled biofilm. Minicores were incubated in situ with labelled ¹⁴C 296 297 sodium bicarbonate. One mL (370 Bq) of label was added to each core and allowed to 298 diffuse in the dark for 30 min. After dark diffusion (Smith and Underwood 1998) a 30 299 min incubation was carried out in both the ambient light and the constant light treatments, 300 terminated by addition of 5% gluteraldehyde. The surface 2 mm depth (approximately) of 301 each minicore was extracted and transferred to an Eppendorf. Sediment samples were 302 later freeze-dried and had inorganic label driven off by addition of concentrated HCl for 303 24 h. After addition of scintillant cocktail (Optiphase Safe, Fisons, Loughborough, UK), 304 carbon uptake rates were calculated from counts obtained from a Packard Tricarb460C 305 scintillation counter (LKB, Cambridge, UK) with internal quench correction. Counts were corrected for self-quenching by the sediment using radiation standard curves withand without sediment addition. Self quenching reduced counts by 2 to 5%.

308

309 Statistical analysis

310 Significant difference was determined using two factor ANOVA with chemical treatment 311 (Lat A, DTT or controls) nested within light treatment (ambient or constant light) nested 312 within time (T1, 2, and 3). This resulted in 3 replicates for each of the 3 chemical 313 treatments nested within 2 light treatments within 3 time points. Normality and 314 homogeneity of variance of data were tested using the Kolmogorov-Smirnov test 315 followed by Bartletts or Levenes test (for normal or non normal data respectively). If data 316 did not have equal variance then a log transformation was applied (Zar, 1999). In all 317 cases data were normal and non-parametric testing was not required. All tests were 318 applied using Minitab V15 software.

319

320 **Results**

321 Accumulated light dose

The light dose calculated for ambient light and the constant light treatment (Table 2), showed a slightly higher accumulated dose for the constant light treatment at T1, which was reversed by T2. However it was not until T3 that the difference in light dose between the two treatments was significantly large, with a light dose under ambient light being 2.6 times that under constant light.

327 Migration

328 Visual observation of the biofilms showed clear downward migration of cells over 329 the experimental time period except for the Lat A treatment which showed no difference 330 in appearance (Authors pers. obs.). This was largely corroborated by the pigment data 331 (Chl a) which showed clear declines in surface biomass by time T3 (Figure 1) for 332 controls and DTT treatments under constant light ($F_{2,26} = 25.90$, p < 0.01) and under ambient light ($F_{2,26} = 14.05$, p < 0.01), but with no pattern of decline for the Lat A 333 334 treatment. Migration monitored using the spectral reflectance NDVI index (Figure 2) 335 showed a similar result, with a decrease in surface biomass under ambient light for all three chemical treatments ($F_{2,26} = 23.4$, p < 0.01) between T2 and T3, although the 336 337 percentage decline for Lat A was only half that of the DTT treatment and the controls. 338 Under constant light, no data were obtained for T1, however between T2 and T3 there was a significant ($F_{2,17} = 18.6$, p < 0.05) decline for the controls and DTT treatment, but 339 340 no decline for the Lat A treatment. Overall, the Lat A clearly inhibited cell vertical 341 migration compared to the other two treatments.

342

343 Fluorescence data

There was a significant decrease ($F_{2,26} = 8.403$, p < 0.01) in rETR_{max} over the experimental period for all treatments, although the magnitude of the decline was lower in treatments under constant light compared to those under higher ambient light (**Figure 3**). There was no significant difference in rETR_{max} between treatments at time T1 or T2, however by T3 the Lat A treatment showed a significantly lower ($F_{2,26} = 7.444$, p < 0.05) value than controls and the DTT treatment for both light treatments. The magnitude of this difference was clearly larger under ambient light compared to constant light. There was no significant difference between controls and the DTT treatment under either lightenvironment.

353 Under constant light, α showed no significant pattern over time (Figure 4), 354 although in general slight decreases (noticeable most for the DTT treatment) were observed. However under ambient light, α significantly decreased (F_{2,26} = 6.281, p < 355 356 0.05) in all three treatments, with the decrease for the Lat A treatment being significantly greater ($F_{2,26} = 6.810$, p < 0.05) than either controls or DTT treatments. The value for the 357 358 Lat A treatment at T3 was essentially zero (0.0005 rel. units compared to an initial value 359 of 0.25 real. units). The light saturation coefficient (E_k) followed exactly the same 360 patterns as described above for rETR_{max}, due to the magnitude of change in rETR_{max} dominating the shape of the light response curves, rather than that of α (note E_k = 361 362 rETR_{max} $/\alpha$).

363 *Productivity* (¹⁴*C* uptake rate)

364 Due to a high level of variation in values between replicates of the same 365 treatment, no significant differences were observed between chemical treatments in either ambient light or constant light (Figure 5). There was also no significant difference 366 between light treatments, however over time, all data showed a significant decrease (F_{2.26} 367 368 = 15.08, p < 0.01). Productivity did not correlate with rETR_{max} uptake within chemical 369 treatments, although the temporal decline for all data showed a significant correlation (r = 0.63, n = 27, p < 0.05) with rETR_{max} (Figure 6). It should be noted that the ${}^{14}C$ has a 370 371 lower resolution than the fluorescence methodology, with measurements effectively 372 integrated over the surface 5 mm of the sediment rather than restricted to surface and near 373 surface analysis for the latter method.

NPQ calculated from the change in maximum fluorescence yield $(F_m - F_m')/F_m'$, 375 376 surprisingly showed negligible induction. In all cases the decline in quantum efficiency $(\Delta F/F_m)$ was the result of an increase in F' relative to F_m ' (Figure 7), F_m ' initially 377 378 declined before showing an asymptotic increase. Such a pattern resulted in small values 379 of NPQ (< 0.20) at low light, followed by a decrease to near zero, or often less than zero, at light levels at and above 320 μ mol m⁻² s⁻¹ PAR (data not shown). DT measured by 380 381 spectral analysis showed little change in the three treatments by T1 and T2 (Figure 8), 382 however by T3 the DTI values were greater for the non-migratory Lat A treatment 383 compared to the migratory biofilms in both controls and the DTT treatments. This 384 method is under development, but clearly shows a treatment effect for the Lat A treatment regarding NPQ induction compared to the other two treatments. This overall 385 386 pattern was corroborated by concomitant samples analysed by pigment analysis (Figure 387 **9**). Data for pigment analysis expressed as DD de-epoxidation (%), DT/Chl a and 388 DT+DD/Chl a are shown in comparison with corresponding spectral derivative analysis. 389 These data showed little (non-significant) change under constant low light, however 390 under high light, both DD de-epoxidation and DT/Chl a showed significantly higher values by T3 ($F_{2,26} = 157.67 \text{ p} < 0.001$) for the Lat A treatments compared to controls and 391 392 the DTT treatment.

394 Discussion

395 These data clearly indicate that, for these biofilms at least, benthic diatoms 396 principally employ vertical migration as their first main mechanism in response to 397 increasing light dose exposure. This is concluded from the significant photoinhibition of 398 the Lat A treated biofilms, with probable enhanced level of physiological down 399 regulation through NPQ, when compared to the two migratory treatments, DTT treated 400 biofilms and controls. In simple terms, cells migrated vertically in response to increasing 401 light dose over time, but when vertical movement was inhibited by Lat A, NPQ induction 402 increased, but not sufficiently to prevent photoinhibition. This is in agreement with the 403 light induced vertical movement (microcycling) proposed by Kromkamp et al. (1998), 404 Serôdio and Catarino (1999) and Perkins et al. (2002), and also further emphasises the 405 role of vertical movement demonstrated in other experiments using the same chemical 406 inhibitors (Cartaxana and Serôdio 2008; Cartaxana et al. 2008).

407 Migration was significantly inhibited by the addition of Lat A (Figures 1 and 2), 408 in agreement with work by Cartaxana et al. (2008) and Cartaxana and Serôdio (2008). 409 This was apparent through analysis of Chl a pigment in the surface 2 mm (a 410 comparatively low resolution method) and the surface chlorophyll proxy, NDVI. Both 411 methods showed no major change over the experimental period, whereas for controls and 412 the DTT treated biofilms, significant decreases in biomass were observed. It should be 413 noted that there was no significant difference in biomass between DTT treatment and 414 controls, indicating that DTT did not induce an increase or decrease in cell movement 415 relative to controls. It should also be noted that patterns were largely the same between 416 ambient light and constant light, thus the magnitude of the photodose did not enhance

417 migration. This latter point could have two explanations. Firstly the magnitude of the 418 vertical migration may have been predominantly determined by an endogenous tidal 419 rhythm (e.g. Serôdio et al. 1997) rather than the light dose. Secondly micro-cycling of 420 cells (Kromkamp et al. 1998; Perkins et al. 2002) may have resulted in similar light dose 421 exposure, irrespective of the two light treatments. Thus the integrated light dose of cells 422 cycling through the surface of the sediment was not significantly greater in the ambient 423 light treatment (this being the product of light intensity and length of exposure) compared 424 to cells at lower light level in the constant light treatment. It is likely that both processes 425 played a role in the migratory pattern of the controls and DTT treated cells, however 426 differentiation between these two driving functions was not an explicit aim of this study. 427 Also it should be noted that comparison of ambient light data at T2 and constant light 428 data at T3, which related to biofilms that had been exposed to similar overall light dose, 429 showed subtle differences in fluorescence values (rETRmax and α), demonstrating that 430 light dose was not the sole driving function of the differences observed. Furthermore 431 NPQ induction were investigated over the 6 h emersion period and hence the resolution 432 of the measurements did not analyse short term patterns in NPQ induction. It is well 433 known that diatoms may rapidly induce NPQ in response to short term (10s of seconds) 434 changes in light environment (e.g. Perkins et al. 2006). The role of the comparatively 435 long term light dose effect can be noted by the fact that it was not until T3, when the 436 difference in light dose between the two treatments was greatest (Table 2), that 437 differences between the chemical treatments were of highest magnitude.

438 Over the exposure period, relative maximum electron transport rate (rETR_{max}) 439 decreased in all treatments (Figure 3). This may have been the result of an endogenous

440 diel rhythm (Underwood et al. 2005) and / or the effect of the increasing photodose. As 441 the magnitude of the decrease was greatest under ambient light, compared to the lower 442 photodose experienced under constant light, both an endogenous decrease and a 443 photodose effect seem likely. The magnitude of this decrease in $rETR_{max}$ was greatest for 444 Lat A treated biofilms, but only significantly so under ambient light, indicating the 445 inhibition of cell vertical movement resulted in photoinhibition. This pattern was also 446 indicated by the decline to effectively zero by the light use efficiency coefficient (α) for 447 the Lat A treated biofilms under ambient light (Figure 4). Clearly this higher photodose 448 induced photoinhibition (possibly photodamage) when cells were unable to migrate away 449 from the sediment surface. It should be noted that no difference was observed between 450 the DTT treated biofilms and the controls. Therefore it can be concluded that inhibition of 451 NPQ (DTT treatment) had no significant impact whereas inhibition of migration (Lat A 452 treatment) resulted in a reduction in both rETR_{max} and α , but only when the light dose 453 was sufficiently high compared to the constant light treatment.

454 It is unlikely that the decrease in photosynthetic activity over the experimental 455 period was the result of increasing environmental stress in response to experimental 456 conditions. In fact the use of the water bath may have reduced temperature stress relative 457 to in situ temperature increases, and the biofilms showed no obvious drying out for any of 458 the treatments. In situ warming and desiccation are likely to be equal to or greater than 459 those experienced during this study, thus any temporal pattern is likely to occur under in 460 situ conditions as well. In addition, as all chemical treatments were exposed to the same 461 stress, albeit a lower warming under constant light, experimental induced stresses cannot 462 explain the differences between the Lat A treatment and the controls and DTT treatment.

Productivity, when measured by ¹⁴C uptake rate, showed no chemical or light 463 464 treatment effects, indeed the only significant pattern observed was an overall temporal 465 decline over the experimental period for the whole dataset. This decline correlated with 466 that of $rETR_{max}$ (Figure 6) supporting the statement above that a combination of diel 467 rhythm and light dose exposure resulted in a decrease in photosynthetic activity. The lack 468 of any chemical treatment effect could be due to two reasons. Firstly the method 469 effectively integrates the productivity measurement over the surface 5 mm depth of 470 sediment, hence resulting in a weighted average value dependent upon the biomass 471 distribution over this depth. Secondly the chemical treatments may not have been fully 472 active at depth despite the pre-measurement 30 minute incubation period, hence resulting 473 in cell migration towards the surface of cells able to replenish the surface biofilm with 474 photosynthetically active cells. The former seems more probable as an explanation as the 475 latter would have resulted in a surface biomass enrichment in the Lat A treatment (i.e. 476 cells would have migrated to the surface and then been unable to migrate back down due 477 to the chemical treatment), which was not observed.

478 Analysis of the data indicating induction of non-photochemical quenching (NPQ) 479 is not so clear cut. In all biofilms, the quenching of the photochemical efficiency $(\Delta F/F_m)$ was the result of an increase in F' and not a quenching of the F_m ' yield. This 480 indicates a low level or even lack of induction of NPQ as indicated by the calculated 481 values (NPQ = $(F_m - F_m')/F_m'$). For the migratory biofilms the data must be interpreted 482 with care as downward migration between measurements of F_m and F_m ') results in an 483 484 increase in the calculated value of NPQ solely due to the increased distance between the 485 cells and the fluorimeter probe (e.g. Consalvey et al. 2005; Perkins et al. in press). 486 However this would have increased the magnitude in difference between the non-487 migratory (Lat A) and migratory (controls and DTT) treatments. In comparison, both the 488 spectral derivative (Figure 8) and the pigment analysis (Figure 9) for biofilms under 489 ambient light indicated a greater level of NPQ induction in the Lat A treated biofilms. 490 Under constant light there was no difference between controls and DTT treated biofilms 491 and no difference between chemical treatments. Thus a photodose effect was observed 492 whereby the higher ambient light photodose induced a greater level of NPQ when cell 493 vertical movement was inhibited. Diadinoxanthin de-epoxidation as well as the relative 494 Diatoxanthin (DT) concentration (DT/Chl a) both showed the same patterns. Interestingly 495 there was no increase in (DD+DT) concentration, indicating no de novo synthesis but a 496 conversion of DD to DT as the primary NPQ mechanism. This is an expected result in 497 response to high light exposure (Lavaud et al., 2004; Schumann et al. 2007). The lack of 498 any significant effect of DTT treatment compared to controls may imply that the DTT 499 dose was insufficient to inhibit NPQ induction. Certainly under ambient light, pigment 500 data show an induction of NPQ in both these treatments relative to the constant light 501 treatment. However the spectral derivate did not show this pattern, nor did fluorescence 502 data indicate NPQ induction for any treatment. In addition the magnitude of NPQ 503 induction in controls and DTT treatments was significantly less than in the Lat A 504 treatment. Therefore the overall pattern in the combined datasets indicate that cell vertical 505 movement was more important in optimizing photosynthetic activity, rather than NPQ 506 induction.

507 In conclusion, this study has two main findings. Firstly optimization of 508 photosynthetic activity in response to an increasing exposure to light (i.e. an accumulated

509 light dose response) is largely due to vertical cell migration. Cells position themselves in 510 the sediment surface layer such that the attenuation of light provides an optimal light 511 environment for their photochemistry. This is in agreement of the microcycling and light 512 induced vertical migration responses reported by Kromkamp et al. (1998), Serodio and 513 Catarino (2000) and Perkins et al. (2002). In addition, it goes towards explaining the fact 514 that integrated biofilm light response curves examined in literature seem to saturate at 400 - 800 µmol m⁻² s⁻¹ PAR (see Perkins et al., 2002, 2006; Serôdio et al., 2003; 515 516 Consalvey et al., 2005; Jesus et al., 2005, 2006 and others), significantly lower than 517 ambient light levels at the sediment surface on a sunny day. It seems logical then that cells would position themselves in a light environment nearer to 800 μ mol m⁻² s⁻¹ PAR or 518 519 lower, rather than expose themselves to the potentially photodamaging light intensities at 520 the sediment surface. This cell migration may well be more energetically favorable than 521 physiological down regulation processes such as NPQ induction. It is hypothesized from 522 this data that NPQ is a secondary response to light dose and / or a response to more rapid 523 changes in light environment rather than a longer term increase in light dose. Secondly 524 these data suggest that a probable combination of vertical migration and physiological 525 mechanisms result in a diel and/or tidal pattern of down regulation. Underwood et al. 526 (2005) reported diel down regulation at the single cell level, and other studies suggest 527 probable tidal patterns for integrated biofilm measurements (Perkins et al., 2001; Jesus et 528 al., 2005, 2006). Again it is logical that after adequate light exposure for photosynthate 529 production, cells would down regulate their photosynthetic activity. Hence this diel 530 pattern may be a response to integration of the light dose over time, rather than an 531 endogenous rhythm. Hence this study has shown overall, the importance of cell vertical

- 532 movement as a driving function optimizing photosynthetic activity in response to light
- 533 dose for benthic biofilms dominated by diatoms.

535 **References**

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649	Table 1. Overview of the experimental design showing the nesting of chemical treatments
650	(Lat A = Latrunculin A to inhibit cell motility; DTT = DL-dithiothreitol to inhibit NPQ
651	and controls) within light treatment (Amb = ambient, Con = constant) within time period
652	(T1, 2, and 3) and the measurements made (Spec = spectroradiometry to measure NPQ
653	induction and surface biomass as NDVI, RLC = rapid light curve by fluorescence, Pig =
654	pigments including Chl a, DD and DT, ${}^{14}C$ = productivity measured as labelled carbon
655	uptake rate). All measurements were made as triple replicates, i.e. 3 separate minicores.

Time period	Light	Chemical	Measurement,	Measurement,
	Treatment treatment		minicore set 1	minicore set 2
	Amb	Lat A	Spec, RLC, Pig	^{14}C
		DTT	Spec, RLC, Pig	^{14}C
T1		Controls	Spec, RLC, Pig	^{14}C
	Con	Lat A	Spec, RLC, Pig	^{14}C
		DTT	Spec, RLC, Pig	^{14}C
		Controls	Spec, RLC, Pig	^{14}C
	Amb	Lat A	Spec, RLC, Pig	14 C
		DTT	Spec, RLC, Pig	^{14}C
T2		Controls	Spec, RLC, Pig	^{14}C
	Con	Lat A	Spec, RLC, Pig	^{14}C
		DTT	Spec, RLC, Pig	^{14}C
		Controls	Spec, RLC, Pig	^{14}C
	Amb	Lat A	Spec, RLC, Pig	^{14}C
		DTT	Spec, RLC, Pig	^{14}C
T3		Controls	Spec, RLC, Pig	^{14}C
	Con	Lat A	Spec, RLC, Pig	¹⁴ C
		DTT	Spec, RLC, Pig	¹⁴ C
		Controls	Spec, RLC, Pig	¹⁴ C

Table 2. Accumulated light dose calculated from the product of light measurement and
length of exposure at each sampling time (T1, T2 and T3) for the ambient and constant
light treatments. Units of light dose are mole of photons m⁻².

Sampling	Ambient treatment light dose	Constant treatment light dose		
Time				
T1	1.82	2.16		
T2	7.83	5.40		
T3	20.00	7.56		

671 Figure legends

Figure 1. Biomass represented as the proxy of chlorophyll a (Chl a) for each chemical (D

- 673 = DTT, L = Lat A, C = control) and light treatment (Amb = ambient, Con = constant)
- over the three sampling points (T1, T2 and T3). Values are mean \pm s.e. (n = 3).
- 675

Figure 2. Surface biomass represented as the proxy of Normalised Difference Vegetation Index (NDVI) measured using the spectroradiometer. Values are represented as percentage change compared to the initial value at T1 for each chemical (D = DTT, L = Lat A, C = control) and light treatment (Amb = ambient, Con = constant) over the three sampling points (T1, T2 and T3). Values are mean \pm s.e (n = 3).

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Figure 3. Maximum relative electron transport (rETR_{max}) rate as a proxy for productivity measured using variable chlorophyll fluorescence. Values are represented as percentage change compared to the initial value at T1 for each chemical (D = DTT, L = Lat A, C = control) and light treatment (Amb = ambient, Con = constant) over the three sampling points (T1, T2 and T3). Values are mean \pm s.e (n = 3).

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Figure 4. Light utilisation coefficient (α) measured using variable chlorophyll fluorescence. Values are represented as percentage change compared to the initial value at T1 for each chemical (D = DTT, L = Lat A, C = control) and light treatment (Amb = ambient, Con = constant) over the three sampling points (T1, T2 and T3). Values are mean \pm s.e (n = 3).

Figure 5. Productivity measured as the uptake rate of labelled carbon (14 C-NaHCO₃) for each chemical (D = DTT, L = Lat A, C = control) and light treatment (Amb = ambient, Con = constant) over the three sampling points (T1, T2 and T3). Values are mean ± s.e (n = 3).

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Figure 6. Maximum relative electron transport rate ($rETR_{max}$) presented as a function of productivity (¹⁴C uptake rate) for the whole data set.

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Figure 7. Operational fluorescence yield (*F*) and maximum fluorescence yield (F_m ') during a 20 second rapid light response curve. Data shown are for a control sample, however the pattern was identical (increase in F and slight decline in Fm' followed by a curvilinear increase) for all light curves measured (all three light treatments and at all three sampling points).

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Figure 8. Diatoxanthin Index measured from the spectral second derivatives (508/630 nm) for each chemical (D = DTT, L = Lat A, C = control) under the ambient light treatment (Amb = ambient) over the three sampling points (T1, T2 and T3). Values are mean \pm s.e (n = 3).

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Figure 9. Pigment data of A: Diatoxanthin (DT) and the B: Percentage de-epoxidation (%) of Diadinoxanthin for each chemical (D = DTT, L = Lat A, C = control) and light treatment (Amb = ambient, Con = constant) over the three sampling points (T1, T2 and T3). Values are mean \pm s.e (n = 3).



Perkins et al. Figure 2









Perkins et al. Figure 5



Perkins et al. Figure 6

Perkins et al. Figure 7 \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc



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