

Physiological steady state of phytoplankton in the field? An example based on pigment profile of *Emiliana huxleyi* (Haptophyta) during a light shift

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Abstract

A calcifying strain of *Emiliana huxleyi* was used to study the photoacclimation process during a shift from low (LL) to high (HL) photon flux density (PFD) under nutrient-replete and pH- and [CO₂]-controlled continuous cultures. Physiological steady states were obtained after culturing the alga in each PFD for more than a month, and pigment profiles and cell volume changes were monitored for 25 d after the light shift. Fucoxanthin was the major carotenoid in LL, while under HL this role was assumed by 19'hexanoyloxyfucoxanthin (19Hex). The photoprotective pigments diadinoxanthin and diatoxanthin (Dd+Dt), normalized to chlorophyll *a* (Chl *a*), increased with increasing PFD, while Chl *a* content per cell and Chl *c*'s and fucoxanthin, normalized to Chl *a*, decreased with increasing PFD. The sum of all carotenoids normalized to Chl *a* and the 19Hex+Fuco:Chl *c* ratio were remarkably constant from LL to HL conditions. The results confirm that the total amount of carotenoids was synthesized/catabolized in tandem with Chl *a* to a genetically predefined level independent of PFD. When normalized to a per cell basis, Chl *a* content reached the long-term HL steady state after 17–20 d, while Chl *c*, Fuco, and Dd+Dt, normalized to Chl *a*, reached the long-term HL steady state after 5–7 d. Growth rate adjustment was completed within 3 d after the transition to HL. When Chl *a* was normalized to cellular volume, the transition to a fully acclimated HL state was complete within 3 d. The results highlight the need for the critical evaluation of the normalization “currency” (i.e., cell number, volume, Chl *a*) to which mass is expressed during the photoacclimation process and suggest that natural phytoplankton populations are unlikely to ever be in true physiological steady state.

Photoacclimation to different light intensity occurs on a number of levels that operate on different time scales. Photoacclimation, following the definition of Falkowski and La Roche (1991), operates on time scales of hours and days, with cells typically responding with selective synthesis and degradation of pigments and electron transfer proteins (Sukenic et al. 1988) and/or Calvin cycle enzymes (Orellana and Perry 1992) rather than changing the efficiency of photochemistry and excitation energy transfer to reaction centers that operate on much shorter time scales (seconds to minutes). Morphologically photoacclimation is accompanied by changes in cell volume (Thompson et al. 1991), pigment content (Leonardos and Harris 2006), and number and density of thylakoid membranes (Post et al. 1984). Some of the photoacclimation processes are thought to occur on time scales shorter than or comparable to the cell's generation time (Cullen and Lewis 1988). A typical strategy of phytoplankton photoacclimation to high light involves a decrease in pigment content and an increase in carbon content (MacIntyre et al. 2002) when expressed in a per cell basis. Although pigment turnover times have been

reported to be on the order of days to weeks, depending on the growth of the cultures (Goericke and Welschmeyer 1992), to date little attention has been placed in ensuring that photoacclimation kinetics are investigated on fully acclimated cultures. Numerous kinetic studies have been carried out on batch cultures, which introduce uncertainty on whether the culture can be fully acclimated to any condition. The available data also reveals considerable within-species variability in response to light (MacIntyre et al. 2002). Photosynthetic and accessory pigments are expected to be influenced foremost by photon flux density (PFD) (MacIntyre et al. 2002). Indeed, in *Emiliana huxleyi*, some components of the photosynthetic apparatus exhibit considerable plasticity as a function of light, such as the ratio of the major carotenoid fucoxanthin (Fuco) to chlorophyll *a* (Chl *a*); however, other components demonstrate a very restricted stoichiometry, such as the sum of 19'hexanoyloxyfucoxanthin and Fuco normalized to Chl *c* and the ratio of the sum of all carotenoid normalized to Chl *a* (Leonardos and Harris 2006).

The interpretation of photoacclimation responses depends critically on the currency in which mass is expressed. In many ecological studies Chl *a* is the most commonly used measure of mass, quite simply because of the specificity of this readily measured parameter, although cell number, particulate C, biovolume, and protein concentration have also been used (MacIntyre et al. 2002). Examining photoacclimation parameters on a per cell basis makes intuitive sense since the smallest population unit is a cell, and some mechanisms of photoacclimation cannot be interpreted on the basis of Chl *a* data (Anning et al. 2000). These parameters may simply correlate with one another, as they may merely be

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Acknowledgments

I would like to thank John Bartington Associates for manufacturing the pH signal amplifiers, Kevin Oxborough for the pH-stat software, and Sue Corbett for media preparation. I would also like to thank A. Sciandra and J. P. Gattuso for their help with the design of the pH-stat system, R. J. Geider and M. Ragni for useful discussions, and two anonymous reviewers whose comments significantly improved this manuscript. This study was funded by a NERC (UK) grant NERE/A/S/2003/00441 to R. J. Geider.

a manifestation of the same cellular function, but this is not always the case (Thompson et al. 1991). Here we focus on what constitutes a physiological steady state, how long it takes to be achieved, and what the ecological implications for populations in the field are. The results were obtained when studying a shift from LL to HL in the prymnesiophyte *E. huxleyi* under nutrient-replete and pH- and [CO₂]-controlled continuous culture conditions.

Materials and methods

Unialgal turbidostat-cyclostat cultures (which we will refer to from now on as turbidostats) of *E. huxleyi* (calcifying strain PML-B11) were grown at 15°C (Sanyo Gallenkamp UK), using 0.2- μm -filtered artificial seawater (ESAW; Berges et al. 2001, 2004) with metals and vitamins added to achieve f/2 medium concentrations (Guillard and Ryther 1962). Culture medium was prepared with nitrate added to 200 $\mu\text{mol L}^{-1}$ N ($\pm 10\%$) and phosphate adjusted to 40 $\mu\text{mol L}^{-1}$ P to achieve an N : P ratio of 5 (mol mol⁻¹). The continuous cultures were operated as manual turbidostats. The nutrient-replete (Leonardos and Harris 2006) cell density of 1,000 cells μL^{-1} ($\pm 10\%$) (i.e., about half the maximum yield for the inflow nitrate level of 200 $\mu\text{mol L}^{-1}$) was the target density, and the inflow medium flow rate was adjusted once daily to maintain a steady biomass. The cultures were incubated using a 14:10 light:dark (L:D) cycle at PFDs of 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (high light [HL]) and 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (low light [LL]). Culture vessels were cylindrical 3-L round-bottom borosilicate glass that were illuminated from the side. PFD was measured in the center of a similar vessel containing 3 L of distilled water using a 4 π Biospherical Instruments probe, model QSL 100. Illumination was via cool-white fluorescent tubes (Lumilux CoolWhite, OSRAM). The desired light intensity levels were obtained using neutral-density filters.

The cultures were incubated at 360 ppmv CO₂ using a pH-stating approach. The system operates on the following principle. The pH of the culture is continuously monitored, to a level of accuracy of 0.004 pH units, by a pH probe (Thermo Orion Ross pH electrodes, model 8103 BN, Thermo Corp. USA) that is mounted on the culture vessel. The pH signal is amplified (amplifier manufactured by John Bartington Associates), and the pH is logged in a computer system. If the pH of the culture increased above the predetermined target level, the computer opened a solenoid valve (SIRAI Elettromeccanica) that feeds CO₂ into the culture. Conversely, if the pH decreased below the required value, the computer controlled a solenoid switching valve (SIRAI Elettromeccanica) to direct the air through a CO₂ scrubber that resulted into feeding CO₂-free air into the culture. When the pH was within 0.01 pH units of the target range, the computer switched the solenoid valve to supply laboratory air to the culture. This mixture of gases was used to maintain the culture at a constant pH and for mixing. This culture system was tested for long-term stability over a period of 4 months, and the pH of the culture was maintained at the desired range ± 0.01 pH units.

To determine the pH that would reflect ambient (360 ppmv) CO₂ conditions, under a measured alkalinity, we used the CO₂ system calculator program, which takes two parameters of the CO₂ system in seawater (total alkalinity, total C, pH, and pCO₂) and calculates the other two at a set of input conditions (v. 01.05; Lewis and Wallace 1998). Alkalinity was monitored daily, and the pH of the cultures was adjusted accordingly to maintain a constant [CO₂] for each system. Alkalinity was measured as in Bradshaw et al. (1981), using Thermo Orion Ross pH electrodes, model 8103 BN (Thermo Corp. USA) calibrated with seawater buffers (Dickson and Goyet 1994) with data interpretation using the Alkalinity calculator (Rounds 2003). Alkalinity of the inflow medium was adjusted with NaHCO₃ addition to 2.60 (± 0.03) meq L⁻¹, while alkalinity of the cultures was 2.2 meq L⁻¹ (± 0.25) for all cultures throughout the experiment.

Three pH-stat systems were used. Two served as long-term controls of LL and HL conditions, and the third was used to carry out the switch from LL to HL. This culture had been growing under LL conditions for ~ 2 months before the start of the experiment. Samples from the control cultures were obtained at four discrete steady states over a period of continuous culturing of 1 month. Samples for pigment analyses were obtained only after steady state was achieved. Steady state was operationally defined in our experiments when daily cell density varied by $< 10\%$ for at least three consecutive days. The dilution flow rate at steady state in conjunction with the $< 10\%$ changes in cell density was used to calculate the growth rate (μ) at each condition. The daily growth rate was calculated as

$$\mu = \frac{\ln(d_1) - \ln(d_0)}{t_1 - t_0} + D$$

where $d_{(0, 1)}$ = cell density at time (0, 1) $t_1 - t_0$ = time difference (days), and D = dilution rate between times 0 and 1.

The biomass in the cultures was monitored daily using improved Neubauer haemocytometers. Cell volume was measured using a Coulter Counter Z2 particle counter using Accucomp 1.4 software. The dilution flow rate was adjusted manually daily to achieve a steady biomass with a target cell density of 10³ cells $\mu\text{L}^{-1} \pm 10\%$. Samples for monitoring and pigment analyses were obtained at the middle of the light phase to avoid effects of diel rhythms (Ahn et al. 2002). Pigment sampling details and the high-performance liquid chromatography protocol were carried out exactly as in Leonardos and Harris (2006). At least four replicate samples were used to calculate pigment ratios, which are expressed on a mass basis after each pigment was individually quantified using the respective standard.

Results

E. huxleyi exhibited a long-term LL-specific growth rate (μ) of 0.09 d⁻¹, whereas at HL μ was 0.59 d⁻¹. The evolution of daily growth rate during the light shift experiment is illustrated in Fig. 1. The pH-stated culture achieved the long-term HL growth rate after 3 d of culturing under HL conditions. The manual turbidostats

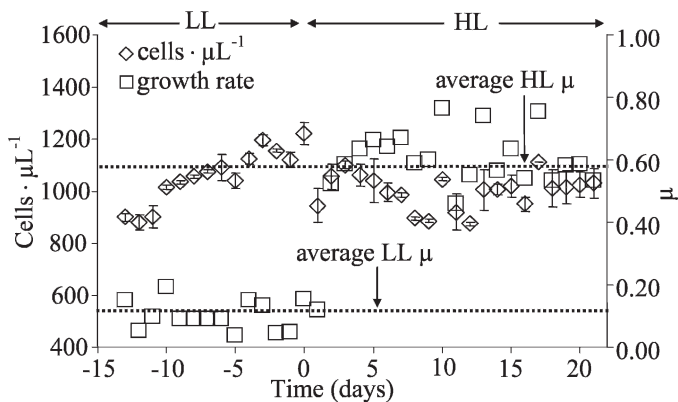


Fig. 1. Evolution of cell density and growth rate (μ) of *Emiliana huxleyi* over time during the shift from low light (LL) to high light (HL). Filled symbols are the long-term LL (plotted at day 0) and HL (plotted at day 30) values. The average HL and LL growth rate values are indicated for visual reference. Vertical lines are \pm one standard deviation. In some cases standard deviations are contained within the symbols and cannot be seen.

were operated within the target cell density of 10^3 cells $\mu\text{L}^{-1} \pm 10\%$ (Fig. 1).

Cellular content of Chl *a* decreased during the shift from LL to HL from ~ 125 to 90 fg cell $^{-1}$, while 15–17 d were required to complete this process (Fig. 2A). A concurrent with Chl *a* decrease of Chl c_3+c_2 was observed during the light shift period, from 113 to 53 fg cell $^{-1}$. However, long-term cellular Chl c_3+c_2 content was reached after 7 d into the experiment (Fig. 2A). The diadinoxanthin (Dd) content of *E. huxleyi* cells increased during the shift from LL to HL, from 16 to 40 fg cell $^{-1}$ (Fig. 2A), and similarly to Chl c_3+c_2 , long-term HL content values were reached after 7 d of the new light regime. 19Hex content of *E. huxleyi* did not significantly differ between the two light intensities used (Fig. 2B), and during the light shift experiment 19Hex content appeared to fluctuate around the mean values of 36–40 fg cell $^{-1}$. A significant reduction of Fuco content of *E. huxleyi* cells was observed (Fig. 2B) from ~ 75 fg cell $^{-1}$ under LL to ~ 20 fg cell $^{-1}$ under HL. The observed reduction of diatinoxanthin (Dt) from 12 to ~ 5.5 fg cell $^{-1}$ from LL to HL, respectively, took ~ 15 d to complete.

On examining the stoichiometry of various pigment ratios of *E. huxleyi* on a mass basis, it was found that most of the long-term HL-acclimated values were achieved after 5–7 d into the light shift experiment. This was observed for Chl c_3+c_2 :Chl *a*, Fuco:Chl *a*, and the sum of the photo-protective pigment Dd+Dt:Chl *a* (Fig. 3A) and the sum of 19Hex+Fuco:Chl *a* (Fig. 3B). However, 19Hex:Chl *a* (Fig. 3A), 19Hex+Fuco:Chl c_3+c_2 , and total carotenoids:Chl *a* (Fig. 3B) did not significantly differ between the light conditions and consequently did not significantly change during the light shift experiment and were growth rate independent (data not graphically illustrated).

The changes of the average biovolume of *E. huxleyi* cells during the light shift experiment, together with the values exhibited by the long-term HL- and LL-acclimated cultures, are presented in Fig. 4A. At LL the average cell volume was $62 \mu\text{m}^3$, while at HL *E. huxleyi* biovolume was

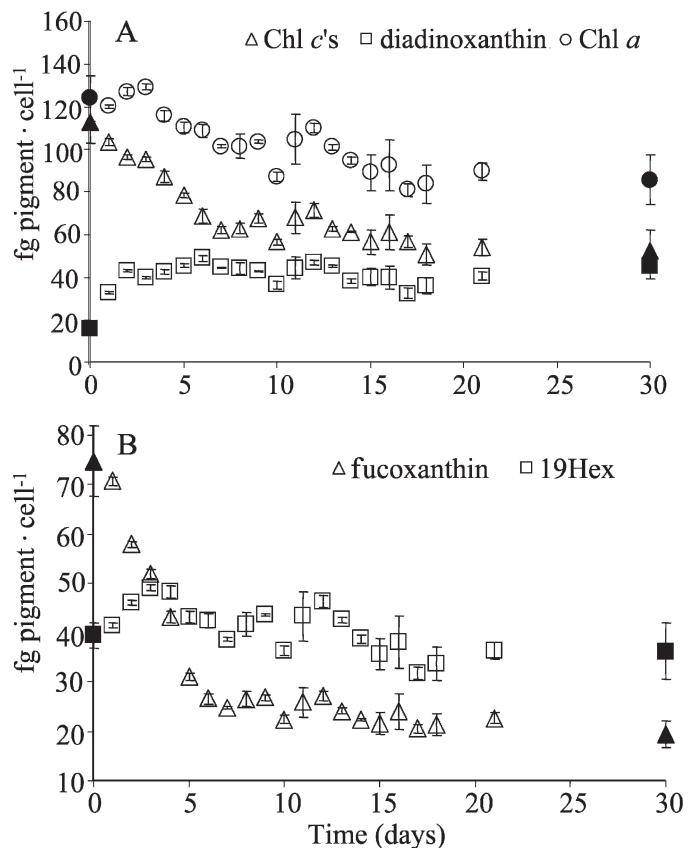


Fig. 2. Evolution of cellular content of chlorophyll *a* (Chl *a*), diadinoxanthin and Chl c_3+c_2 (A) and 19'hexanoyloxyfucoxanthin and fucoxanthin (B) of *Emiliana huxleyi* over time during the shift from low light (LL) to high light (HL). Filled symbols are the long-term LL (plotted at day 0) and HL (plotted at day 30). Vertical lines are \pm one standard deviation. In some cases standard deviations are contained within the symbols and cannot be seen.

$86 \mu\text{m}^3$. During the light shift experiment *E. huxleyi* cells overshoot the “target” volume by $\sim 25\%$ (day 4) before stabilizing to the long-term HL-acclimated volume after 17 d into the light shift experiment. When Chl *a* content of *E. huxleyi* cells was normalized to biovolume (Fig. 4B), the long-term HL-acclimated values were observed within 3–5 d after the light shift.

Discussion

Photoacclimation strategy and dynamics—In terms of the pigment content and stoichiometry in *E. huxleyi*, the results here are consistent with previous reports (Stolte et al. 2000; Leonardos and Harris 2006), and the reader is referred to them for in-depth discussion. Falkowski (1984), analyzing the kinetics of the shift to higher light in *Dunaliella tertiolecta*, suggested that about half the decrease in pigment can be accounted for by dilution through growth, whereas half appeared to have been degraded. Goericke and Welschmeyer (1992) found that neither Chl *a* nor Fuco was degraded during the adaptation to higher light in the diatom *Thalassiosira weissflogii*. The results from *E. huxleyi* suggest a much more complex picture. Under HL, *E.*

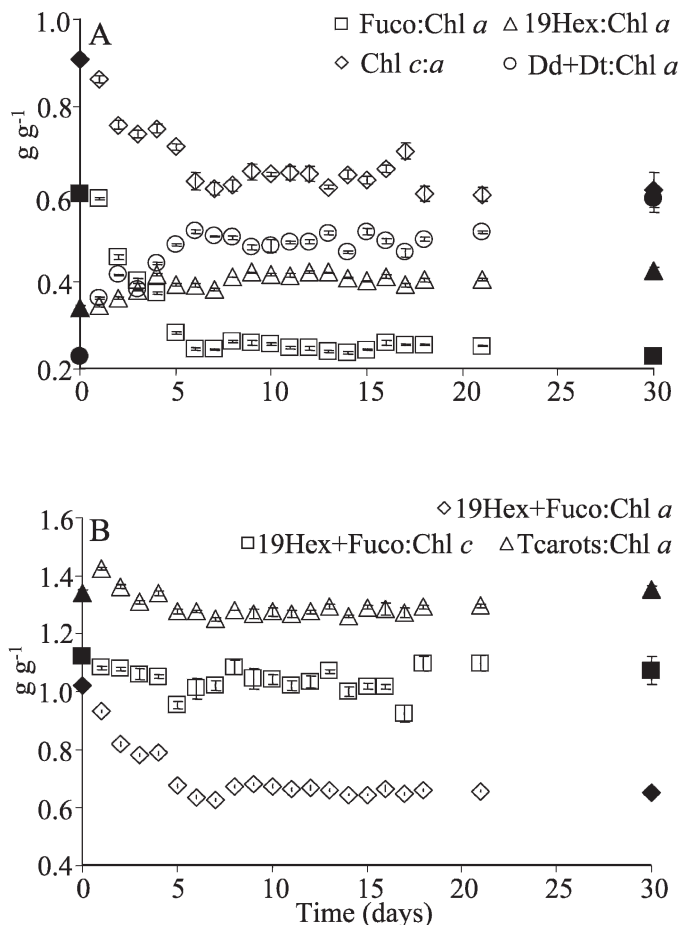


Fig. 3. Evolution of mass ratios of accessory pigments to chlorophyll (Chl) ($g\ g^{-1}$) of *Emiliana huxleyi* during the shift from low light (LL) to high light (HL). Chl c_3+c_2 :Chl a , Fuco:Chl a , 19Hex:Chl a and Dd+Dt:Chl a (A) and 19Hex+Fuco:Chl a , 19Hex+Fuco:Chl c , and total carotenoids (Tcarots):Chl a (B). Filled symbols are the long-term LL (plotted at day 0) and HL (plotted at day 30). Vertical lines are \pm one standard deviation. In some cases standard deviations are contained within the symbols and cannot be seen.

huxleyi exhibited a specific growth rate of ~ 0.6 , which translates into roughly one division every day. Therefore, if the case of *D. tertiolecta* were a typical example (Falkowski 1984), the shift to the HL steady state, with the measured $\sim 30\%$ reduction of Chl a per *E. huxleyi* cell, would have been completed in about 2.6 generations (i.e., ~ 3 d), whereas in fact, in *E. huxleyi*, the photoacclimatory process took much longer to complete (~ 17 – 21 d), in terms of Chl a content per cell. Clearly, a completely different dynamic of photoacclimation emerges when the process is considered on the basis of Chl a normalized to cell volume. In this case we observed that the full transition from LL to HL was completed within 3–4 d, which translates to three to four cellular divisions.

The redox state of the plastoquinone pool (Escoubas et al. 1995) as the first light-sensitive chemoreceptor and the other short-term (seconds to minutes) mechanisms to fluctuating light (the efficiency of photochemistry and excitation energy transfer to reaction centers), such as the

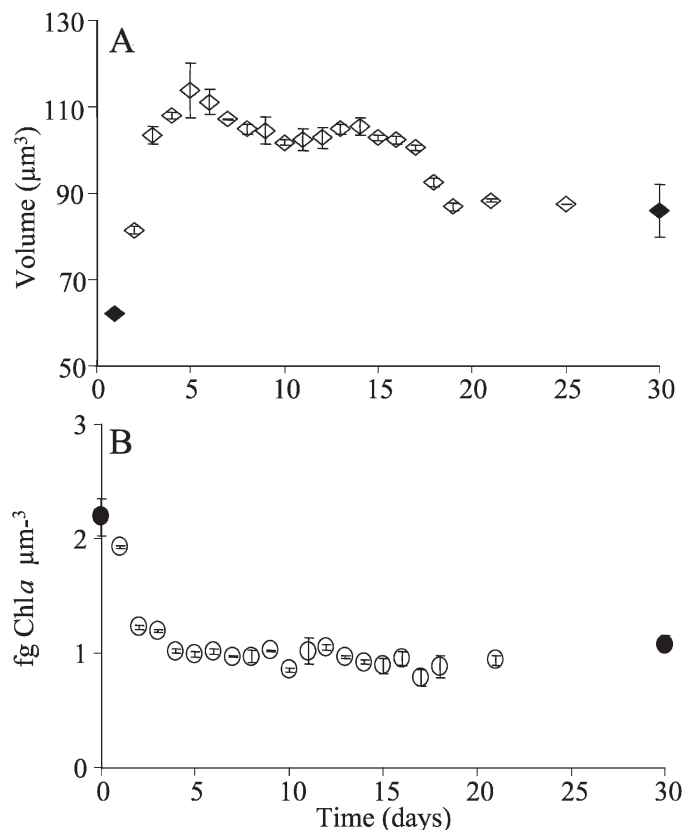


Fig. 4. Evolution of cell biovolume (μm^3) and chlorophyll a (Chl a) content normalized to biovolume (B) of *Emiliana huxleyi* over time during the shift from low light (LL) to high light (HL). Filled symbols are the long-term LL (plotted at day 0) and HL (plotted at day 30). Vertical lines are \pm one standard deviation. In some cases standard deviations are contained within the symbols and cannot be seen.

cytochrome f/b_6 complex (Pearson et al. 1993) and the proton gradient (Mühlbauer and Eichacker 1998), cannot by themselves explain the long period, up to 21 d, depending on the normalization currency, that it takes to achieve a fully light-acclimated steady state, especially since the turbidostats were also undergoing an L:D cycle. Photosynthetic activity is often believed to be the photon-sensing receptor that transfers information to the mechanism that controls gene expression. From an evolutionary perspective, Escoubas et al. (1995) studied a green alga, whereas redox signaling in haptophyte cells derived from a second endosymbiosis is thought to be clearly different (Wilhelm et al. 2006). The redox state of the plastoquinone pool operates in an order of time of micro- to milliseconds, so clearly the cell must possess other regulatory mechanisms to control long-term pigment synthesis. The photoacclimation strategy in response to a light shift is expected to ultimately require regulation of gene expression and several changes at the cellular level after the information about the change in PFD has been transferred through signal transduction pathways (Ritz et al. 2000). However, the functional regulation of these mechanisms is still not very well understood (Sukenic et al. 1987; Orellana and Perry 1992).

Table 1. Time (days) required for a full transition from a long-term low-light state to a long-term high-light state of growth rate, biovolume, and various pigment contents and stoichiometry in *Emiliania huxleyi*.

Parameter	Time (days) required for fully acclimated state
Growth rate (μ)	1–2
Chlorophyll <i>a</i> (Chl <i>a</i>) cell ⁻¹	17–21
Diadinoxanthin cell ⁻¹	5–7
Chl <i>c</i> ₃ + <i>c</i> ₂ cell ⁻¹	7–10
19'hexanoyloxyfucoxanthin cell ⁻¹	0
Fucoxanthin cell ⁻¹	7–10
Chl <i>c</i> ₃ + <i>c</i> ₂ :Chl <i>a</i>	6
Fuco:Chl <i>a</i>	6
19Hex:Chl <i>a</i>	3
Dd+Dt:Chl <i>a</i>	7
19Hex+Fuco:Chl <i>a</i>	5
19Hex+Fuco:Chl <i>c</i>	0
Total carotenoids:Chl <i>a</i>	0
Biovolume (μm^3)	18
Chl <i>a</i> : biovolume	3–5

Mass normalization currency (cell, particle, volume, C) and steady-state definitions—In interpreting photoacclimation responses, the currency in which mass is expressed (dry weight, C, Chl *a*, cell volume) is critical (MacIntyre et al. 2002). Very different conclusions can be reached regarding the duration and completion of the photoacclimation process in relation to the choice of currency, as is evidenced in Table 1. Depending on the choice of parameter and normalization currency, *E. huxleyi* would be deemed to be fully acclimated to the new PFD anywhere between 0 and 21 d. This poses significant questions on the interpretation of field- and laboratory-based data. A significant linear relationship between the log of PFD and carbon quota has been shown for various species (Thompson et al. 1991); however, a considerable portion of the modeling work used to characterize photoacclimation is based on the physiological variable of Chl *a*: C (Geider et al. 1997). Descriptions of photoacclimation processes are often based on data interpretation from photosynthesis-irradiance response curves, which usually involve short-term responses of some expression of photosynthetic rates and a notion of an achievable steady-state photosynthetic rate that is within the time constraints of the experiment. For example, Mouget et al. (1999) found that the diatom *Haslea ostrearia* acclimated to high irradiance and had lower maximum photosynthetic rate and maximum light utilization coefficient when expressed on a per cell basis; however, these assertions were reversed when the same data were normalized on a Chl *a* basis. Different normalization currencies and physiological steady states have been extensively used in the literature with little precision in their definition and the practical work involved. There is little understanding between the difference of a short-term, essentially transient response and a long-term manifestation of photosynthetic acclimation. More important, the concept of a steady state differs in the practical definition and accuracy in almost every published work. For example Giddings (1977) outlined the experimental steady state as “when day-to-day changes in Chl

a concentrations became fairly small,” while Ahn et al. (2002) judged that the system was in steady state when “the cell concentrations exhibited no increasing or decreasing trends and varied by no more than $\pm 5\%$ for 3 consecutive days.” Anning et al. (2000) used manual daily dilution on batch cultures acclimated for seven generations prior to the experiment. Many others give no details on how and for how long the cells were acclimated to the experimental conditions (e.g., Laws and Bannister 1980). Clearly the list is too long, and in particular it is questionable to what extent a batch culture can ever be used to describe a physiological steady state because the environment that the cells are experiencing is not stable.

As discussed previously, the kinetic response of the light shift in *E. huxleyi* substantially differs from data obtained from other species (Falkowski 1984; Anning et al. 2000). There certainly might be some species-specific differences, notwithstanding the different evolutionary histories of the species tested (Wilhelm et al. 2006); however, one cannot discount that the observed differences can also be attributed, at least partially, to not-well-defined steady states in experiments carried out in batch/semicontinuous cultures, which inadvertently introduces erroneous parameterization of the kinetic analysis. One has to remember that the definition of a steady state in culture depends on the process that is under investigation, and, as already discussed, the practical considerations of algal culturing often outweigh precise definition of a physiological steady state of the population.

Ecological implications and conclusions—It probably is central to the success of *E. huxleyi* populations in the field that the overall growth rate adjustment takes 1–2 d to complete. This is in contradistinction to the continuous adjustment of the photosynthetic apparatus, which may take place over longer periods. The results clearly show that although a long-term physiological equilibrium was not reached, the cells were able to divide at maximum division rates very rapidly after the shift to HL. Only when normalizing to the cell volume parameter did we observe a close relationship between most measured parameters (Chl *a*, growth rate). This relationship may represent the yet-unknown spatial restrictions on the intracellular enzyme-mediated biochemical reactions regulated during the photoacclimatory process. Behrenfeld et al. (1998) suggested that under certain circumstances, adaptation to high light becomes the optimal strategy for phytoplankton. However, the most striking implication of this study is that it is almost inconceivable that natural phytoplankton populations are ever in steady state, given that the environment that they experience changes rapidly and periodically, in far shorter time scales than the up to 17–21 d suggested here. So it is questionable whether relevant field studies can claim to do anything other than capture a snapshot of a population state since the true steady state for any condition is rarely well defined. Laboratory- and field-based studies are complementary in providing insights into physiological mechanisms, but considerable care must be exercised when interpreting the generated data. It would be scientifically beneficial if future research focused on how

the cells regulate their photoacclimatory responses in the field over longer time scales and what kind of cellular/metabolic signal that can reach a dynamic steady state over several generations can be used for this purpose.

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Received: 12 February 2007

Accepted: 7 July 2007

Amended: 7 September 2007