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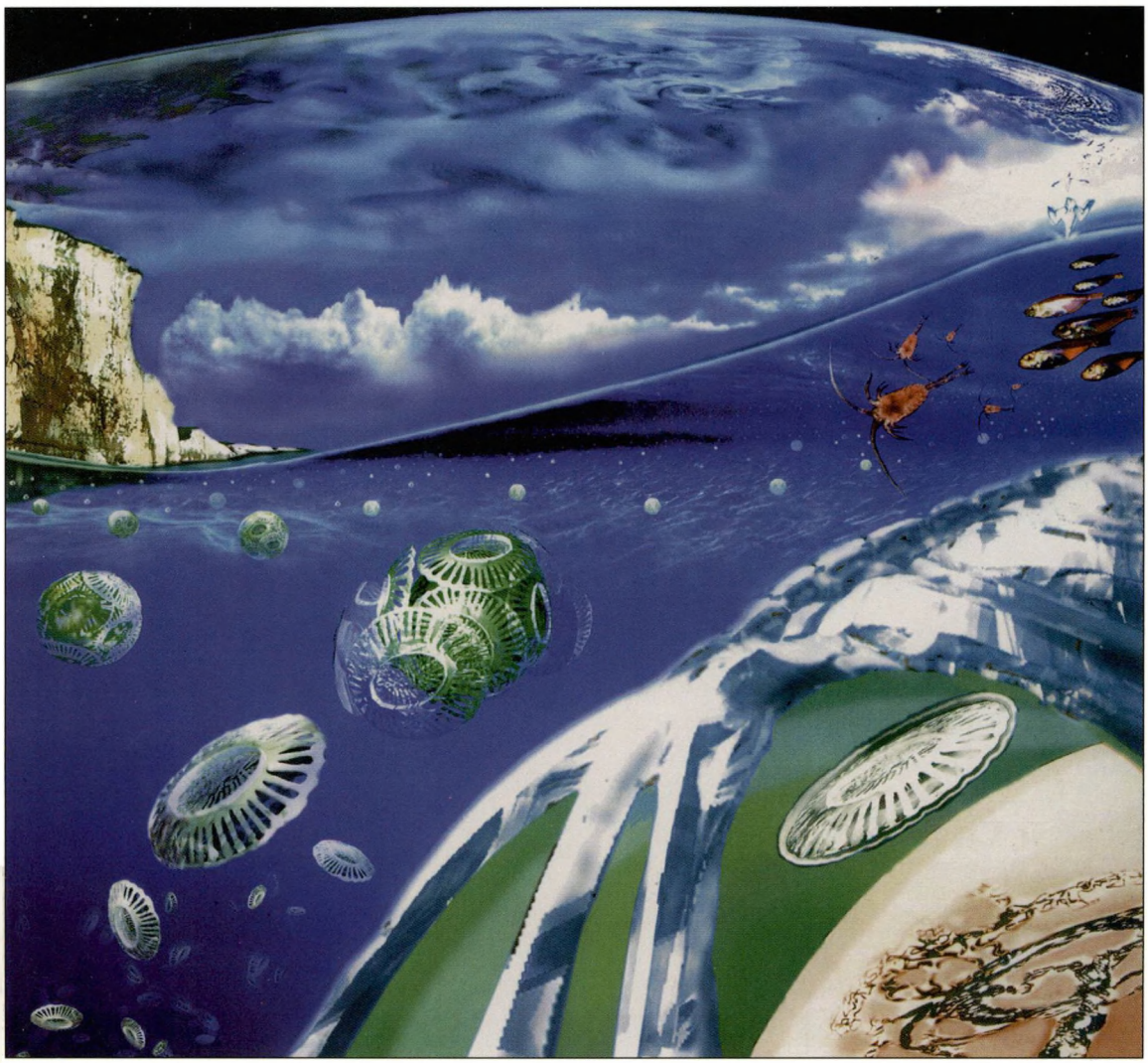
A MODEL SYSTEM APPROACH TO BIOLOGICAL CLIMATE FORCING: THE EXAMPLE OF *Emiliania huxleyi*

Final Report Subproject (b): Physiology

R. Riegman, W. Stolte and A.A.M. Noordeloos



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A model system approach to biological climate forcing: the example of *Emiliania huxleyi*.

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This project was carried out in the framework of the Dutch National Research Programme on Global Air Pollution and Climate Change; registered under nr. 013/1204.40 , entitled: "A model system approach to biological climate forcing: the example of *Emiliania huxleyi*".

1. Abstract

The coccolithophorid *Emiliania huxleyi* (Lohman) Hay and Mohler is considered to be the world's major producer of calcite. It has a worldwide distribution and forms extensive blooms in both coastal and open oceanic waters. Embedded within a larger national research program (see front page), an extensive laboratory study on the ecophysiology of *E. huxleyi* in light- and nutrient-limited continuous cultures was carried out to generate data for modelling purposes. Here, we demonstrate that *E. huxleyi* has a high growth affinity for light (27 10^{-3} m^2 s μmol^{-1} d^{-1}) which is comparable to oceanic specialists such as *Synechococcus* and Prochlorophytes. The affinity for nitrate is relatively low (0.37 L μmol^{-1} h^{-1}). However, most exceptional is its affinity for phosphate (20 L μmol^{-1} h^{-1}). This is the highest value ever recorded for a phytoplankton species. Also unique for this obligatory photo-autotrophic species is the presence of two different Alkaline Phosphatase systems under P-limitation. These properties make *E. huxleyi* an excellent competitor for P, especially when there is rapid recycling of P *via* the organic-P pool. From a mechanistic point of view, *E. huxleyi* blooms are expected to be initiated in P-controlled ecosystems, *i.e.* in the presence of high supply rates of irradiance, nitrate, and organic-P. This impression is in agreement with earlier reported field observations. The successive biomass accumulation during the development of the bloom is a consequence of cascade effects in the pelagic foodweb. The ecophysiological profile of *E. huxleyi* explains why this species mainly performs in P-controlled environments. Consequently, the removal of CO_2 from the biosphere *via* the burial of calcium-carbonate from the major calcifier in the world oceans is controlled by phosphorus rather than nitrogen.

2. Executive summary

2.1. English version

Between July 1996 and December 1998 an extensive laboratory study on the ecophysiology of *Emiliania huxleyi* was performed. In continuous cultures, light-limitation was studied at six different growth rates, and nitrogen- and phosphate-limitation was studied at four different growth rates. Actual and potential rates of photosynthesis and nitrogen- and phosphate uptake were measured at a standard temperature of 15 °C (Practical descriptions section). Cellular composition, including C,N,P, pigments, and calcite, was determined for every steady state. All analysis were carried out in duplicate or triplicate. The results (presented in Chapter 4) were compared with the ecophysiology of other marine phytoplankton species to evaluate the competitive ability of *Emiliania huxleyi* under various environmental conditions (see: 5. Discussion).
In close cooperation with Prof. S.A.L.M. Kooijman and Dr. C. Zonneveld (Department of theoretical Biology; Vrije Universiteit, Amsterdam; working within the Subproject (a): Modeling the physiological organization of *Emiliania huxleyi*), there was a continuous exchange of data and ideas to support the modelling efforts carried out in their subproject. Several parts of this final report will be published in scientific papers (for an overview: see Appendix 2).

2.2. Nederlandse versie

Gedurende de periode juli '96 tot januari '99 is er een ecofysiologisch laboratorium-onderzoek uitgevoerd aan de mariene kalkvormende alg *Emiliania huxleyi.* Met behulp van continu cultures werden licht-beperkte groei (zes steady states) en stikstof- en fosfaat-beperkte groei (ieder vier steady states) bestudeerd. De actuele en potentiële snelheden van fotosynthese en de opname van fosfaat en stikstof werden gemeten bij de kweektemperatuur van 15 °C (zie: Practical descriptions). De celsamenstelling, betreffende pigmenten, stikstof, fosfaat, koolstof en calciet gehaltes, werd in duplo of triplo gemeten voor iedere steady state. Ter vaststelling van het competitieve vermogen van *Emiliania huxleyi*, werden de resultaten (Hoofdstuk 4) vergeleken met de ecofysiologie van andere mariene algen (Hoofdstuk 5).
Gedurende het gehele project is er sprake geweest van een nauwe samenwerking met Prof. S.A.L.M. Kooijman en Dr. C. Zonneveld (werkzaam binnen het subproject (a): "Modeling the physiological organization of *Emiliania huxleyi*"). Hierbij werden gegevens en ideeën uitgewisseld ten behoeve van de tot standkoming van het mathematisch model dat de groei van *E. huxleyi* beschrijft. Verschillende delen van dit eindrapport zullen eveneens worden gepubliceerd in wetenschappelijke tijd-schriften (zie: Appendix 2).

3 Practical descriptions

3.1 Culture techniques

3.1.1 Light-limitation

The axenic *E. huxleyi* strain L. (Ietswaart et al., 1994) was cultured in continuous culture at 15 °C in continuous light (Splendor, color number 45) at different irradiances. The photon flux density was varied between 5.8 and 200 µmol $.m^{-2}.s^{-1}$ and was determined in the culture vessel without contaminating the culture using a scalar irradiance meter (QSL-101, Biospherical Instruments Inc., USA). The variation of scalar irradiance within the culture was tested before sterilizing the vessel with cell densities comparable with experimental values and was always lower than 10 % of the average scalar irradiance. The dilution rate was adjusted every day to obtain constant cell density (between 3 and $5*10^5$ ml^{-1}) at every irradiance. The volume of the culture vessel was approximately 900 ml and exactly determined just before sterilization of the vessel. Growth medium consisted of autoclaved artificial sea water to which nutrients were aseptically added according to table 1. Vitamins were filter sterilized (Sleicher & Schuell FP 030/3, pore size 0.2 µm). The pH of the medium was adjusted aseptically at 8.0 ± 0.1 before use. In contrast to other studies (Paasche, pers. commun.) no negative effects were observed of the used tygon tubing on the growth of E. huxleyi upon testing. All other connections were made with Norprene® tubing.

3.1.2 Phosphate-limitation

Emiliania huxleyi strain L was cultured in continuous culture as described in 3.1.1, with slight modifications. Light was always saturating (200 µmol $.m^{-2}.s^{-1}$) and the phosphate concentration in the continuous culture reservoir was reduced to 1 $\mu mol.l^{-1}$. The dilution rate was set at a constant value of approximately 0.15, 0.30, 0.45 and 0.6 d^{-1}. When the cultures were in steady state, samples were taken for cell composition and the determination of phosphate uptake kinetics.

Table 1: *Final concentration of the various compounds in the culture medium.*

		added aseptically after sterilization			
Major salts	**(g/l)**	**Nutrients**	**(µM)**	**Vitamins**	**(µg/l)**
NaCl	24.5	$NaHCO_3$	4500	Biotin	0.04
$MgCl_2.6H_2O$	9.8	$NaNO_3$	300 [1)]	Thiamin-HCl	20
$CaCl_2$. 2H2O	0.53	NaH_2PO_4	25 [2)]	Cyanocobalamine	0.8
Na_2SO_4	3.22				
K_2SO_4	0.85	**Trace elements (1)**	**(µM)**	**Trace elements (2)**	**(µM)**
		$FeCl_3.6H_2O$	5.86	KBr	92.4
		$Na_2EDTA.2H_2O$	5.85	$SrCl_2.6H_2O$	13.4
		$CuSO_4$	0.02	$AlCl_3$	0.1
		$ZnSO_4$	0.038	LiCl	0.071
		$CoCl_2.6H_2O$	0.021	KI	0.06
		$MnCl_2$	0.46	H_3BO_3	3.2
		Na_2MoO_4	0.012	RbCl	0.36

[1)] For nitrate limited cultures only 25 µM was added.
[2)] For phosphate limited cultures 1 µM was added.

3.1.3 Nitrate limitation

Emiliania huxleyi strain L was cultured in continuous culture as described in 3.1.1, with two modifications. The nitrate concentration in the continuous culture reservoir was 25 µM and the phosphate concentration was 25 µM.

3.2 Chemical and biological analyses

3.2.1 Cell characteristics

Cell number was determined daily by flow cytometry (Coulter Epics XL-MCL). Also, chlorophyll fluorescence and a measure for cell size (forward scattering) were obtained by flow cytometry. At time of sampling, cell volume distribution was determined using an electronic particle counter (Elzone). Microscopical observations were done to check for calcification. Cells without any visible coccoliths were only present in very low abundances (<10 %). The abundance of bacteria was checked by epifluorescence microscopy after staining with acridine orange (Hobbie et al., 1977). In one occasion, bacteria were detected and the culture was cleaned, sterilized, and started over again.

3.2.2 Particulate carbon and nitrogen

For total particulate carbon (TPC) and nitrogen, 20 ml culture was filtered gently (<10 mm Hg) on precombusted GF/F (Whatman) filters (12 mm ∅) and rinsed with artificial seawater without inorganic carbon or sulfate of the same osmotic value. The samples were stored at -50 °C until analysis. Filters were combusted and analyzed using a Carlo-Erba instruments NA 1500 series 2 CNS analyzer. For particulate organic carbon (POC),

filters were acidified with 200 µL H_2SO_3 prior to analysis. Particulate inorganic carbon (PIC) was calculated as the difference between TPC and POC.

3.2.3 Dissolved nutrients

Dissolved ammonia (Helder & De Vries 1979), nitrate and nitrite (Grasshoff, 1983) and phosphate (Mangelsdorf, 1972) were analyzed using a TRAACS autoanalyser. Chl-a concentration was determined fluorometrically (Holm-Hansen et al., 1965) after extraction in 90 % acetone of filters (GF/F, Whatman) with 5 or 10 ml gently filtered culture. Other pigments were extracted in methanol and analyzed using the method of Gieskes & Kraay (1983).

3.2.4 Photosynthesis-Irradiance curves

Photosynthesis rates were determined as oxygen evolution using a Clarke-type O_2-electrode at 9 different light intensities for 3 minutes per light intensity in a temperature-controlled (Lauda RMS 6;. ±0.01 °C) reaction vessel (Hansatech DW2/2, UK). The oxygen electrode was calibrated at 0 and 100 % air saturation in sea water at 15°C assuming a saturation concentration of 254 µM and a linear electrode response. A correction was made for the oxygen consumption of the electrode at appropriate oxygen concentration. Light and oxygen measurements were stored into a computer every 2 seconds using a custom made software program. Photosynthetic rates were estimated as the slope of the oxygen concentration in time using linear regression. Dark respiration (R_d), maximum photosynthetic rate (P_{max}), and half-saturation photon flux density (K_i) were estimated using non-linear regression (Solver routine, Microsoft Excel).

3.2.5 Nutrient uptake kinetics

Nutrient uptake rates were measured as the decrease of the dissolved nutrient in the seawater medium. Samples were taken at appropriate time intervals by filtering through 0.2 µm Gelman Acrodisc filters. Directly after sampling, the samples were measured using a Skalar™ Auto-analyzer.

3.2.6 Alkaline phosphatase activity determination

Alkaline phosphatase was measured using the artificial substrate methyl-fluorescinephosphate (Perry, 1978). Concentrations of 0 to approximately 100 µM of the substrate were added to a phosphate-free, washed cell suspension at pH 8. The increase of fluorescence was followed during one minute. Enzyme rates were estimated by linear regression of the fluorescence signal.

3.3 Theoretical considerations and simple model formulations.

3.3.1 Photosynthesis

Photosynthesis rates, measured as oxygen production (section 3.2.4) are dependent on the applied photon flux density. Several relationships have been proposed by different authors. For an overview of the theory we refer to Zonneveld (1996). We found that a hyperbolical relationship fitted our data best in most cases. We therefore used the equation:

$$P(O_2) = R_d + \frac{P_{max} * I}{I + K_i} \qquad \text{(Eqn. 1)}$$

to obtain the characterizing parameters.

Dark respiration (R_d), maximum photosynthetic rate (P_{max}), and half-saturation photon flux density (K_i) were estimated using non-linear regression (Solver routine, Microsoft Excel).

3.3.2 Phosphate uptake

Since algae have at least two different regulation mechanisms for phosphate uptake (Riegman 1985), the uptake experiments were designed in such a way that the kinetics of both could be studied. At 10 µM phosphate, the uptake system of *Emiliania* was saturated by the external concentration. Under this condition, uptake follows first order kinetics due to feedback inhibition of the uptake system by phosphate which has been taken up by the cells during the time course of the experiment (Riegman 1985):

$$X^{-1} * dS/dt = V_{max}\left(1 - \frac{\Delta Q}{Q_{maxt}}\right) \qquad \text{(Eqn. 2)}$$

where X is the cell concentration, X^{-1}*dS/dt the cellular uptake rate at time t during the time course of the transient state, V_{max} the maximum cellular uptake rate measured immediately after the saturating pulse is given (at t=0), ΔQ equals the cellular amount of phosphate which is taken up at t, and Q_{maxt} represents the maximum amount of phosphate which a cell can accumulate after a saturating pulse of phosphate.
This equation is only valid if the external phosphate concentration remains at saturating levels during the entire transient state. The first order inhibition constant k equals V_{max}/Q_{maxt} (Riegman & Mur 1984). k also can be calculated directly from the decrease in the external phosphate concentration (S) after the pulse:

$$S_t = (S_0 - S_\infty)e^{-kt} + S_\infty \qquad \text{(Eqn. 3)}$$

where S_t is the external phosphate concentration at time t, S_0 is the external phosphate concentration just after the pulse

is given (t= 0), S_∞ is the external phosphate concentration at t= ∞, i.e. when phosphate uptake has finished due to complete inhibition by accumulated P in the cells.

When a non-saturating pulse is given, the external phosphate concentration has an effect (additional to the feedback inhibition mechanism) on the activity of the uptake system:

$$V_t = V_{max}\left(1 - \frac{\Delta Q}{Q_{maxt}}\right)\left(\frac{S}{Km + S}\right) \quad \text{(Eqn. 4)}$$

where the cellular uptake rate equals X^{-1}*dS/dt and Km is the halfsaturation constant for uptake.

Values for V_{max} and Q_{maxt} were calculated from the 10 µM pulse experiment. From the S_t against t relationship after a 5 µM pulse, Km was estimated by iteration of Eqn. 3 using a least square fitting procedure with a program written in STEM (Simulation Tool for Easy Modeling; Resource Analysis, Delft, The Netherlands). Enschede, The Netherlands).

Since the steady state external phosphate concentration in the continuous cultures ($\bar{s}$) was too low to be measured accurately, $\bar{s}$ was calculated according to:

$$\bar{S} = \frac{\mu \bar{Q} Km}{(V_{max} - \mu \bar{Q})} \quad \text{(Eqn. 5)}$$

where µ is the specific growth rate, and $\bar{Q}$ the steady state cell P quotum.

3.3.3 Nitrate uptake

Nitrate uptake kinetics is most probably regulated by the transport system, in combination with the rate of assimilation. Interactions with ammonium and other nitrogen sources make the kinetics very complicated (Flynn, 1991). Since no simple, physiologically correct model was available to fit the decrease of nitrate concentration during uptake experiments, all data were fitted with simple Michaelis-Menten kinetics.

$$\frac{d(NO_3)}{dt} = V_{max} \frac{[NO_3]}{[NO_3] + K_m} \quad \text{(Eqn. 6)}$$

The parameters V_{max} and K_m were estimated using a numerical integration and subsequent iterating routine procedure available in the STEM software package (Resource Analysis, Delft, The Netherlands).

It must be mentioned that the maximum uptake rate (V_{max}) may not be the maximum transport rate, but could be the result of several feedback mechanisms, that we cannot distinguish with the used techniques.

4 Results

4.1 Light-limitation.

4.1.1 Cell composition

At lower PFD, there was a decrease in cell volume. Consequently, cell organic and inorganic carbon, and cell nitrogen decreased (Table 2). The steady states at 12.5 and 50 µmol $m^{-2}.s^{-1}$ showed high standard deviations for various parameters, which make these results less reliable that those from the other steady states.

4.1.2 Growth rate.

The specific growth rate increased with increasing PFD and saturated at approximately 100 µmol.$m^{-2}.s^{-1}$.

Assuming a hyperbolic growth response, extrapolating the curve to 0 resulted in a compensation PFD for growth of 2.5 µmol .$m^{-2}.s^{-1}$. The initial slope of the µ-I curve was 0.051 $m^2.s.d^{-1}.(\mu mol^{-1})$ and the PFD at which growth was 0.5 μ_{max} was 15 µmol $m^{-2}.s^{-1}$. Generally, carbon, nitrogen and phosphorus quota increased with increasing PFD. Only at 100 and 200 µmol $m^{-2}.s^{-1}$ the quota for C and N were lower than expected extrapolating the trend obtained by the lower PAR levels (Fig.1). The cell volume at the highest PFD was lower than at 50 µmol $m^{-2}.s^{-1}$, but this accounted only for a part in the low C and N quota. Repeating the steady state at 200 µmol $m^{-2}.s^{-1}$, started from an inocculum from the old stock cultures, resulted in exactly the same values, so that we think that the observed deviation from the trend at high PFD is not an artifact of some kind.

Table 2: *Cell composition of light-limited* E. huxleyi *strain L. Organic carbon (POC), Inorganic carbon (IC), Carbon to Nitrogen ratio (N/C), Nitrogen quotum (PN), Phosphorus quotum (PP), Chlorophyll-a (Chl.-a), Maximum photosynthesis rate (Q), and Respiration rate (Rd).*

PAR	µ	cellvol	C/N	s.d.	POC	s.d.	PN	s.d.	PIC	s.d.	PP	s.d.	IC/ OC	s.d.
µmol. $m^{-2}.s^{-1}$	d^{-1}	μm^3	*mol. mol^{-1}*		*pg C. cell^{-1}*		*pg N. cell^{-1}*		*pg C. cell^{-1}*		*fmol P cell^{-1}*			
5.6	0.14	25.5	6.53	0.69	5.45	0.78	0.98	0.15	1.48	1.02	5.42	0.87	0.27	0.18
12.5	0.28	27.7	6.66	0.36	6.13	0.97	1.08	0.20	4.01	3.73	5.45	1.00	0.65	0.73
25	0.40	50.0	6.65	0.57	7.69	1.20	1.37	0.33	2.26	2.90			0.29	0.28
50	0.53	55.9	7.92	0.55	9.63	4.51	1.41	0.61	8.00	9.49	6.42	0.76	0.83	1.36
70	0.57	50.1	8.12	0.30	10.9	0.83	1.58	0.14	3.26	2.56			0.30	
100	0.65	35.9	7.94	1.03	8.00	1.41	1.00	0.09	4.08	3.19	7.70	1.33	0.51	0.22
200	0.63	48.1	8.58	1.60	9.27	1.68	1.29	0.23	4.31	5.16	10.4	0.87	0.47	0.24
400	0.61	41.0									10.5	0.88		

Table 2: *Continued.*

PAR	µ	Chl. a	s.d.	Pm	s.d.	Rd	s.d.
µmol. $m^{-2}.s^{-1}$	d^{-1}	*fg. cell^{-1}*		*pmol O_2 .cell^{-1}.h^{-1}*		*pmol O_2 .cell^{-1}.h^{-1}*	
5.6	0.14	159.89	14.56	0.0281	0.0051	0.0021	0.0008
12.5	0.28	148.93	9.42	0.0360	0.0094	0.0070	0.0028
25	0.40	152.88	5.19	0.0524	0.0093	0.0111	0.0020
50	0.53	163.90	17.99	0.0390	0.0117	0.0163	0.0044
70	0.57	130.93	18.24				
100	0.65	105.63	9.77	0.0133	0.0000	0.0071	0.0000
200	0.63	73.41	13.77	0.0128	0.0000	0.0082	0.0000
400	0.61						

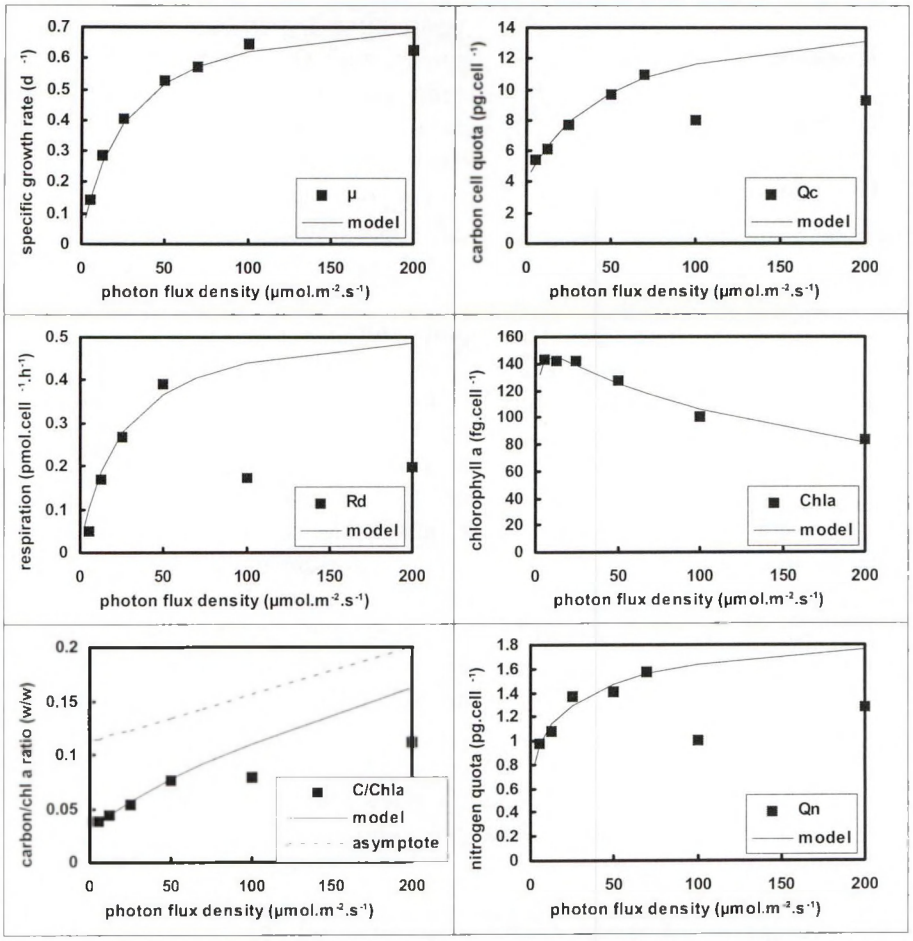

Figure 1. *Model fits for light-limited steady state data of* Emiliania huxleyi. *Data on carbon quota, nitrogen quota, dark respiration, chlorophyll a and specific growth rate are fit simultaneously using 14 parameters. Data obtained at 100 and 200 μmol.m^{-2}.s^{-1} for carbon, nitrogen and dark respiration are not used for model fits.*

4.1.3 Pigments.

Chlorophyll-a cell quota varied between 160 fg.cell^{-1} at low light and 73 pg.cell^{-1} at the highest PFD. Other pigments also varied with culture PFD (Table 3). Chlorophyll a (CHLA), chlorophyll c2 (CHLC2), chlorophyll c3 (CHLC3), fucoxanthin (FUCO) and 19'-hexanoyloxyfucoxanthin (HEXA), which have a light-harvesting function in the photosystems, were invcersely correlated with PFD. The photo-protective pigment diatoxanthin (DT) and its precursor diadinoxanthin (DD) became higher at PFD above 50 μmol *PAR* m^{-2}.s^{-1}. The chlorophyll a-normalized content of total fucoxanthin, (FUCO+HEXA) /CHLA showed a linear decrease with PFD. Since the interspecific variation of this ratio (G.W. Kraaij, pers. commun.) is much smaller than the variation due to variation in PFD (Fig. 2), it can in potential be used to estimate the light history of *E. huxleyi* cells in field situation. When this species forms almost monospecific blooms, pigments from other species will not interfere to a great extent.

Table 3. *Cellular pigment content in amol/cell at different culturing photon flux densities. (1 amol = 10^{-18} mol).*

PFD (µmol $m^{-2}.s^{-1}$)	5.8	5.8	13	13	25	25	50	50	70	70	100	100	200	200	mw	ε(mol)	λ (nm)
µ (d^{-1})	0.14	0.14	0.28	0.28	0.40	0.40	0.53	0.53	0.57	0.57	0.65	0.65	0.63	0.63		(in acetone)	
chlorophyll-c_3	39	43	37	40	35	40	34	29	27	12	21	19	14	11	653	218000	453
chlorophyll-c_2	26	28	32	32	33	34	24	21	19	10	18	14	8	6	609	227700	444
fucoxanthin	98	95	84	94	88	79	71	63	42	20	43	33	21	17	658	109000	443
19'-hex-fucoxanthin	92	97	98	98	93	105	87	77	71	30	70	61	42	37	773	109000	445
diadinoxanthin	23	28	19	27	20	28	32	29	33	13	49	40	77	65	582	130000	448
diatoxanthin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1	1	3	1	8	4	14	11	566	119000	452
chlorophyll-a	158	162	156	162	154	163	153	131	122	56	124	102	100	88	894	78000	663
α-carotene	1.7	1.6	1.8	1.9	1.9	1.8	1.5	1.2	0.5	0.3	0.8	1.2	1.0	0.6	537	145000	448
β-carotene	7.0	6.8	5.1	5.7	6.6	6.3	6.4	n.d	5.6	2.2	8.1	6.1	9.2	7.4	537	134000	454

4.1.4 Photosynthesis and dark respiration

The photosynthesis-irradiance relationship, measured as oxygen consumption/ evolution was best described using a Michaelis-Menten type of kinetics (Eqn. 1). The half-saturation constant for photosynthesis did not vary with growth rate and was at average 54 µmol *PAR* $m^{-2}.s^{-1}$. Cellular dark respiration rates (Rd), as estimated from the P-I curves increased with growth rate, but was lower at the two highest PFD's (Fig. 3). Maximum photosynthesis rates, as estimated from the P-I curves (see Section 3.3.1), was lowest at the highest culturing PFD's.

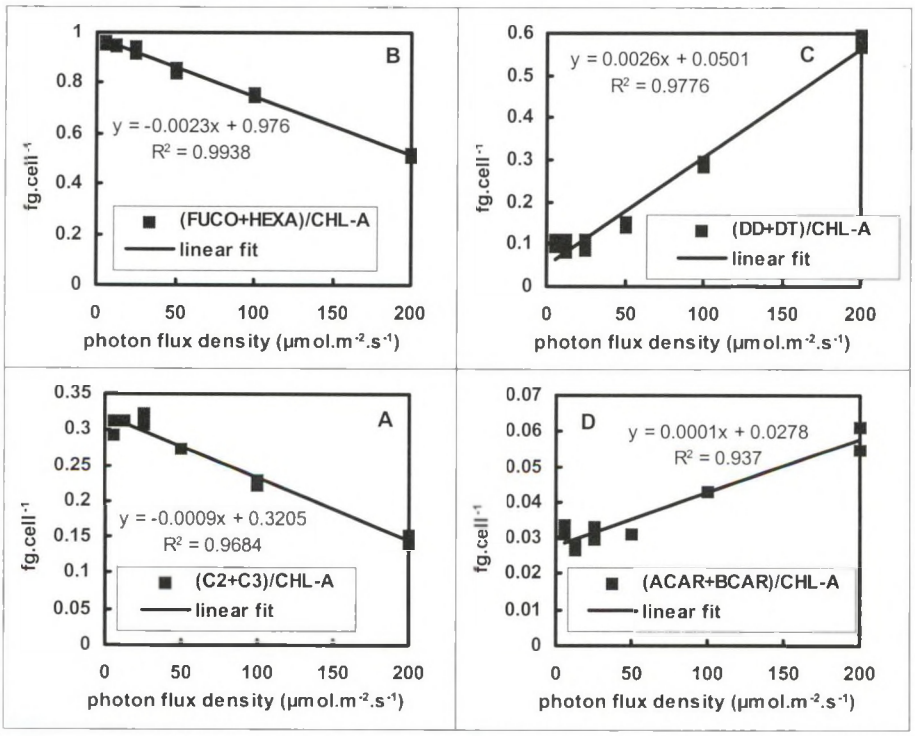

Figure 2. *Pigment ratios of* E. huxleyi *plotted against photon flux density at steady state light limitation.*

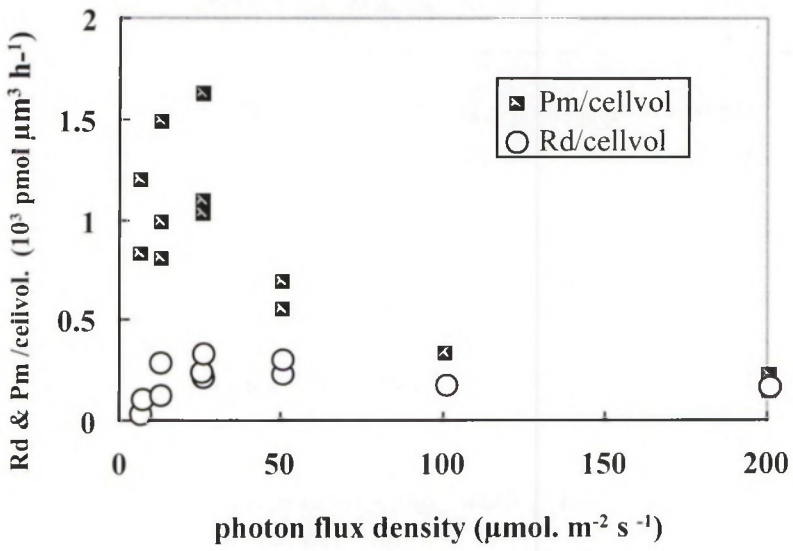

Figure 3. *Respiration (Rd) and maximum photosynthesis rate (Pm) of light-limited* E. huxleyi *grown at various PFD.*

4.2 Phosphate-limitation.

4.2.1 Cell composition

The cellular composition reflected the adaptation of *E. huxleyi* to P-limiting growth conditions. In severely limited (i.e. slowly growing) cultures, these adaptations were more pronounced than in moderately limited cultures (Table 4). At lower growth rates, the cells contained more organic and inorganic carbon, and less phosphorus. The total nitrogen content per cell volume (0.028 pg N fL^{-1}) remained unaffected by the extent of P-limitation. In the culture with the lowest growth rate (0.13 d^{-1} , i.e. 16% of μ_{max}) the average cell volume was 37% higher than in faster growing cultures. These cells also expressed the highest coccolith coverage of the cell surface as was observed microscopically.

Table 4. *Chemical composition of P-limited* Emiliania huxleyi *at various growth rates. Values are given for particulate organic carbon (POC), particulate inorganic carbon (PIC), total cellular nitrogen and phosphorus (cell N and cell P, respectively), and the N and P cell quota.*

µ	Cell volume	POC	PIC	PIC /POC	cell N	cell P	POC cellvol.$^{-1}$	N cellvol.$^{-1}$
d^{-1}	fL	pg C cell^{-1}	pg C cell^{-1}	ratio	pg N cell^{-1}	fmol P cell^{-1}	pg C fL^{-1}	pg N fL^{-1}
0.14	67.6 ±2.0	15.1 ±1.1	7.6 ±1.3	0.50	1.5 ±0.2	2.6 ±0.1	0.22	0.028
0.29	49.5 ±1.1	10.5 ±0.8	4.4 ±1.2	0.42	1.4 ±0.1	2.6 ±0.2	0.21	0.028
0.44	52.2 ±3.0	10.6 ±0.5	3.2 ±1.2	0.30	1.3 ±0.3	3.3 ±0.4	0.20	0.025
0.59	47.3 ±1.2	8.9 ±0.6	2.2 ±0.6	0.25	1.3 ±0.1	2.9 ±0.2	0.19	0.028
0.63	49.0 ±2.8	9.0 ±2.3	2.1 ±0.6	0.23	1.4 ±0.3	3.7 ±0.3	0.18	0.029

Table 5. *Pigment composition of P-limited* E. huxleyi *at various growth rates. Values in amol/cell.*

PFD (µmol $m^{-2}.s^{-1}$)	200	200	200	200	200	200	200	200	mw	e(mol)	l (nm)
µ (d^{-1})	0.14	0.14	0.3	0.3	0.44	0.44	0.59	0.59			(in acetone)
chlorophyll-c_3	5.9	4.8	7.0	4.8	9.7	7.0	9.3	10.7	653	218000	453
chlorophyll-c_2	3.2	2.7	3.5	2.9	7.2	5.1	6.2	7.9	609	227700	444
fucoxanthin	7.5	5.4	8.6	4.7	11.0	10.0	14.9	21.5	658	109000	443
19'-hex-fucoxanthin	22.3	17.1	21.1	15.2	33.6	28.7	30.6	35.7	773	109000	445
diadinoxanthin	55.4	40.0	31.7	17.5	38.0	34.8	29.1	22.8	582	130000	448
diatoxanthin	11.1	8.5	6.4	4.0	6.4	4.5	4.3	4.0	566	119000	452
chlorophyll-a	77.2	58.8	54.3	39.3	79.5	60.9	64.8	76.2	894	78000	663
a-carotene	0.0	0.0	0.0	0.0	0.0	0.8	0.8	1.2	537	145000	448
b-carotene	5.8	5.6	4.3	3.5	5.9	5.2	4.4	4.2	537	134000	454

4.2.2 Pigments.

P-limitation induced a reduction in the antenna pigments Chl.-c_2 and c_3, Fucoxanthin, 19'-hexa-fucoxanthin, and α-carotene. Higher values were observed for the photoprotective pigments and their precursors, β-carotene, diadinoxanthin and diatoxanthin (Table 5). Chl.-a was rather variable with an average of 64 amol $cell^{-1}$.

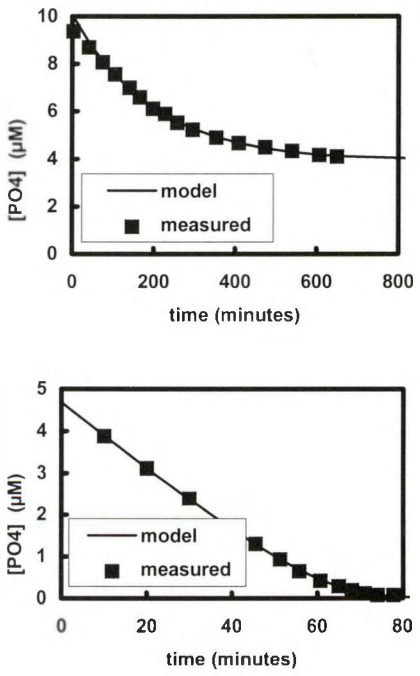

Figure 4. *Two examples of phosphate uptake by* Emiliania huxleyi strain L. *after addition of 10 µM to a 5 times diluted culture (A) and after addition of 5 µM to an undiluted culture (B). The time course of the phosphate concentrations is modeled after numerical integration using equation 4.*

4.2.3 Phosphate uptake by P-limited cultures.

Together with a reduction in cellular P, the induction of the phosphate uptake system was more pronounced at lower growth rates. Addition of 10μM phosphate to P-limited cultures resulted in a rapid uptake.

During the time course of the experiment the uptake of phosphate gradually declined to almost zero (Fig. 4) although the external phosphate concentration of 4 μM was potentially still high enough to support a maximum uptake rate. This gradual decline in uptake activity illustrated the feedback of accumulated P on the activity of the uptake system. Transient state PO_4^{3-} uptake followed first order kinetics (Eqn. 3) when the external concentration remained at saturating levels. The inhibition constant k was found to increase with growth rate (Table 6). This implied that severely limited cells showed a smaller feedback inhibition by accumulated P on the uptake system then moderately limited cells. When a lower pulse was provided to a dense cell suspension, the external PO_4^{3-} concentration declined to nM levels. Below 1 μM, the uptake rate decreased (Fig. 4) since the external PO_4^{3-} concentration had reached non-saturating levels. The half saturation constants for uptake (Ks) were 0.44, 0.47, 0.36, and 0.34 μM, respectively, for cells growing at μ= 0.14, 0.29, 0.44, and 0.59 d^{-1}. Considering the standart error of the estimation, these differences in Ks were not significant (Table 7).

V_{max} declined with growth rate (Fig.5), and was in all cultures much higher than the actual cellular phosphate uptake rate in the continuous culture ($\bar{v}$).

At the lowest growth rate, the maximum phosphate uptake rate exceeded the actual uptake rate more than 1000-fold (Table 6). The cell specific affinity of the uptake system, represented by the initial slope of the V/S curve (dV/dS at P approaching zero; Healy 1980), varied between 52 $.10^{-9}$ L $cell^{-1}$ h^{-1} at μ= 0.14 h^{-1}, and 36.10^{-9} L $cell^{-1}$ h^{-1} at μ= 0.59 h^{-1} (Table 6). On the basis of cellular P, these values for the affinity of P-uptake were 19.8 and 12.4 L μmol^{-1} h^{-1}, respectively.

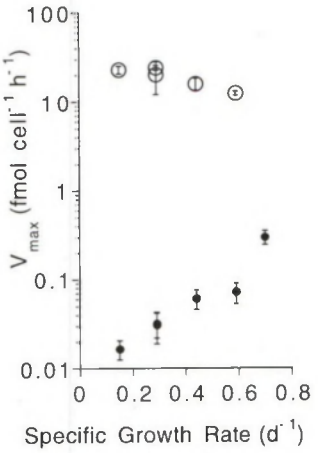

Figure 5. *Effect of specific growth rate on the actual cell specific phosphate uptake rate (closed symbols) of* E. huxleyi, *and on the induction of the phosphate uptake system, as indicated by the maximum phosphate uptake rate (V_{max}; open symbols).*

So with respect to phosphate uptake in the light, both V_{max} and affinity declined with growth rate. Transient state phosphate uptake in the dark at saturating concentrations was lower than in the light for all cultures. This V_{max} in the dark was not influenced by growth rate, except for the culture with the lowest growth rate (Table 7). Below .5 μmax, when cells were severely limited, the affinity of phosphate uptake in the dark and in the light were similar. At higher growth rates, the affinity of P uptake in the dark increased with a factor 2 or even higher.

Table 6. *Physiological adaptation of phosphate uptake by* E. huxleyi, *grown at different growth rates (μ): the ratio between maximum and steady state phosphate uptake rate ($V_{max}/\bar{V}$) and between the maximum amount of phosphate which is taken up after a saturating pulse and the steady state P quotum of the cells ($Q_{maxt}/\bar{Q}$); the inhibition constant k which describes transient state P uptake according to first order kinetics; the affinity of the phosphate uptake system (dV/dS when PO_4^{3-} approaches zero); and the ratios between alkaline phosphatase activity and phosphate uptake with respect to the V_{max} and affinity values of both systems.*

						Ratio APAse act. /PO_4^{3-} uptake	
μ	V_{max}	$V_{max}/\bar{V}$	$Q_{maxt}/\bar{Q}$	k	dV/dS at $P \rightarrow 0$	Max. rates	dV/dS at $S \rightarrow 0$
d^{-1}	fmol P $cell^{-1}$ h^{-1}	-	-	h^{-1}	10^{-9} L $cell^{-1}$ h^{-1}	-	-
0.14	23.0±2.3	1384	30.5 ±6.1	0.29±0.15	52±27	10.0	0.73
0.29	22.2±4.7	705 ±214	22.0 ±4.3	0.38±0.19	47±12	6.3	0.66
0.44	15.9±2.5	260 ±32	13.4 ±5.1	0.36±0.09	44±26	5.0	0.58
0.59	12.4±0.7	172 ±42	10.4 ±2.3	0.41±0.11	36±14	3.6	0.62

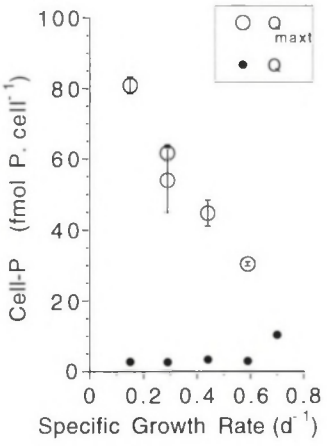

Figure 6. *Steady state cell P quota (closed symbols) and surplus P uptake capacity (open symbols) of P-limited* E. huxleyi, *grown at different dilution rates.*

Table 7. *Effect of light (200 μE m^{-2} s^{-1}) and darkness (0 μE m^{-2} s^{-1}) on the kinetics of phosphate uptake by* E. huxleyi, *after cultivation at various growth rates.*

μ	V_{max}		Ks		Affinity (dV/dS)	
d^{-1}	h^{-1}		μM PO_4^{3-}		L μmol^{-1} PO_4^{3-} h^{-1}	
	Light	Dark	Light	Dark	Light	Dark
0.14	8.9 ±1.3	4.6 ±0.3	0.44 ±0.2	0.27 ±0.11	20	17
0.29	8.5 ±2.7	3.8 ±1.2	0.47 ±0.1	0.22 ±0.09	18	17
0.44	4.8 ±0.8	3.9 ±0.8	0.36 ±0.1	0.19 ±0.03	13	20
0.59	4.3 ±0.6	3.9 ±1.1	0.34 ±0.2	0.10 ±0.02	12	39

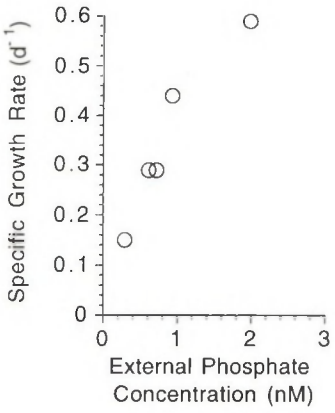

Figure 7. *Correlation between the calculated actual phosphate concentration (s) in the continuous cultures and the specific growth rate of* E. huxleyi.

The steady state cell quota for P followed Droop kinetics (Fig. 6). The minimum (at μ=0) and maximum cell quota Q_{max} (at $\mu=\mu_{max}$=0.76 d^{-1}) were 2.6 and 11.2 fmol P $cell^{-1}$, respectively. Surplus P uptake, exhibited after a PO_4^{3-} pulse to P-limited cells, yielded transient state cell quota up to 82 fmol P . $cell^{-1}$ (note that this is more than seven times Q_{max}). At growth rates higher than 25% of μ_{max}, the surplus storage of P (Q_{maxt} in Fig. 6) was less pronounced.

The steady state external phosphate concentration ($\bar{s}$) in the chemostats was too low to be measured accurately.

Calculation of $\bar{s}$ (according to Eqn. 5) yielded values in the nM range (Fig. 7). Only in the culture growing close to μ_{max} (at μ=0.7 d^{-1}), $\bar{s}$ was much higher, i.e. 1.3 μM (not shown in Fig. 7).

Obviously, the $\mu/\bar{s}$ relationship could not be described by Monod kinetics. The calculated external phosphate concentration at $\mu=0.5*\mu_{max}$ was 1.1 nM. Especially at growth rates below 50% of μ_{max}, a more linear relationship between μ and the calculated $\bar{s}$ was observed.

4.2.4 Alkaline phosphatase activity

APA activity was dependent on the concentration of the substrate and was related to the particulate fraction. No or very low activities could be detected in culture filtrate. Heat-treated cells showed no activity. Furthermore, no APA could be detected in light- or nitrate-limited cultures of *E. huxleyi* strain L (data not shown).

Severely limited cells expressed a higher APase activity than moderately limited cells .

For each culture, the kinetics of APase activity was not according to Michaelis-Menten. Instead, a more linear relationship was observed at MFP concentrations below 5 μM. Since one explanation might be the presence of two APase systems, the data were fit according to an equation which describes the sum of the activity of two enzyme systems, each of them obeying MM kinetics. This yielded a good fit for the data from all cultures, and indicated the presence of a system being constitutively synthesized (Fig. 9). At all growth rates, the V_{max} and halfsaturation constant were 43 fmol MFP $cell^{-1}$ h^{-1} and 1.9 μM, respectively. Additionally, an inducable APase system, with a halfsaturation constant of 12.2 μM (independent of the growth rate) was also present.

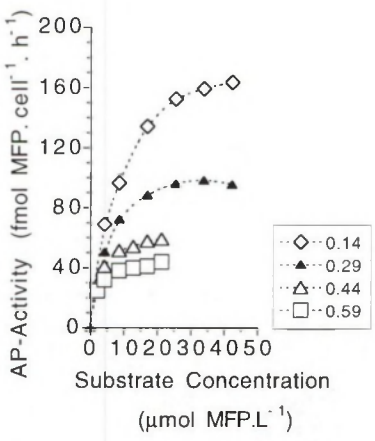

Figure 8. *Alkaline phosphatase activity of* E. huxleyi, *at various dilution rates (D).*

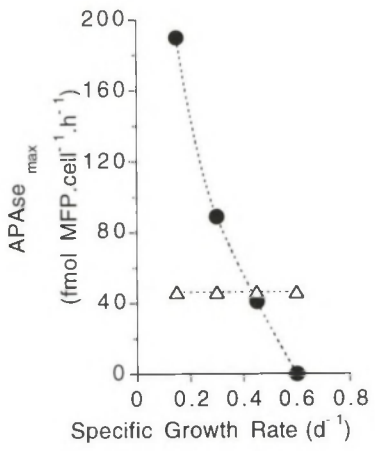

Figure 9. *Calculated V_{max} for the two APase systems of* E. huxleyi*; the constitutively synthesized system (open triangles) and the inducable system (closed circles).*

The combined activity of both systems resulted in an affinity for MFP of 38.2, 30.8, 25.7, and 22.2 10^{-9} L $cell^{-1}$ h^{-1} for cells grown at 0.14, 0.29, 0.44, and 0.59 d^{-1}, respectively. Comparison with the PO_4^{3-} uptake system indicated that the affinity of *E. huxleyi* for the organic substrate MFP was about 60% of the affinity for inorganic phosphate (Table 6). Consequently, the halfsaturation constant for potential growth on MFP in the absence of inorganic phosphate was estimated to be 1.5 nM. At saturating MFP concentrations, e.g. higher than 50µM, APase activity exceeded up to 10 times the V_{max} for inorganic phosphate. To exclude the possibility that the observed deviation from MM kinetics was a consequence of diffusion limitation of the APase system, causing linearity at low MFP concentrations, the temperature dependence of APase was established. At both low, and saturating MFP concentrations, it was found that APase activity increased with temperature with a breakpoint at 37-38 °C (Fig. 10). This indicates that these APases are lipid membrane bound enzymes (Rivkin & Swift 1980). Sonified cells maintained their APase activity, and microscopical observations after application of the ELF-100 substrate (Molecular Probes, Leiden, The Netherlands) indicated that APase of *E. huxleyi* is in the plasmalemma (data not shown). For the Q_{10}, two different sets of values were found (Fig. 11). At non-saturating MFP, the Q_{10} of the APase system was close to 1.26. At saturating substrate concentrations, the Q_{10} was slightly higher, i.e. 1.42. At all MFP concentrations tested, the Q_{10} was significantly higher than 1 (the value to be expected if a physical rather than an enzymatic process is determining the rate limiting step).

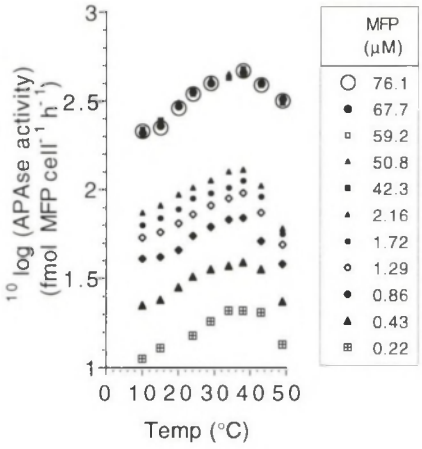

Figure 10. *Effect of incubation temperature on Apase activity of P-limited* E. huxleyi.

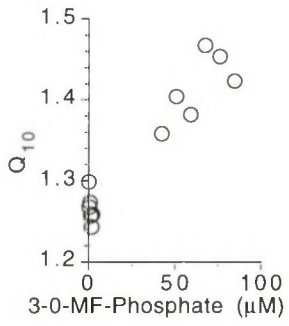

Figure 11. *Q_{10} values for Apase activity at low and high substrate concentrations.*

4.3 Nitrate limitation

4.3.1 Cell composition

The cell volume of *E. huxleyi* was much lower under severe N-limitation than under P-limitation (Table 8). There was no significant variation in cellular organic and inorganic carbon with growth rate under N-limitation. On the average, the cellular organic and inorganic carbon was 7.83 ±1.26 pg C and 3.30 ± 0.14 pg C, respectively.

Cellular N decreased with growth rate to a minimum cell quotum of 0.6 pg N $cell^{-1}$.

Table 8. *Chemical composition of N-limited* Emiliania huxleyi *at various growth rates. Values are given for particulate organic carbon (POC), particulate inorganic carbon (PIC), total cellular nitrogen and phosphorus (cell N and cell P, respectively), and the N and P cell quota.*

μ	Cell volume	POC	PIC	PIC /POC	cell N	cell P	POC cellvol.$^{-1}$	N cellvol.$^{-1}$
d^{-1}	fL	pg C $cell^{-1}$	pg C $cell^{-1}$	ratio	pg N $cell^{-1}$	fmol P $cell^{-1}$	pg C fL^{-1}	pg N fL^{-1}
0.15	32.8 ±1.1	7.2 ±1.2	3.1 ±0.5	0.5	0.6 ±0.1	1.8 ±3	0.22	0.018
0.30	28.5 ±1.2	6.5 ±0.8	3.3 ±0.5	0.5	0.7 ±0.1	3.9 ±4	0.23	0.025
0.45	42.0 ±2.1	8.2 ±0.9	3.4 ±1.2	0.4	1.0 ±0.1	6.0 ±3	0.20	0.024
0.60	50.6 ±3.2	9.4 ±0.1	3.4 ±0.1	0.4	1.3 ±0.2	-	0.19	0.026

4.3.2. Pigments in N-limited cells.

N-limitation induced a reduction in all investigated pigments, except for α-carotene which was highest at the lower growth rates (Table 9). Obviously, the increase of diadinoxanthin and diatoxanthin under P-limitation was not observed under N-limitation.

Table 9. *Pigment composition of N-limited* E. huxleyi *at various growth rates. Values in amol/cell.*

PFD (μmol $m^{-2}.s^{-1}$)	200	200	200	200	mw	e(mol)	l (nm)
μ (d^{-1})	0.14	0.3	0.44	0.60		(in acetone)	
chlorophyll-c_3	1.00	2.73	8.12	8.63	653	218000	453
chlorophyll-c_2	2.36	5.18	7.71	8.19	609	227700	444
fucoxanthin	1.50	5.06	8.06	8.57	658	109000	443
19'-hex-fucoxanthin	8.72	17.5	26.9	27.4	773	109000	445
diadinoxanthin	12.8	23.6	32.0	35.2	582	130000	448
diatoxanthin	1.54	4.09	4.94	5.27	566	119000	452
chlorophyll-a	19.3	33.5	57.5	58.5	894	78000	663
a-carotene	0.20	0.21	0.05	0.08	537	145000	448
b-carotene	1.77	2.02	3.20	3.56	537	134000	454

4.3.3. Nitrate uptake by N-limited cells.

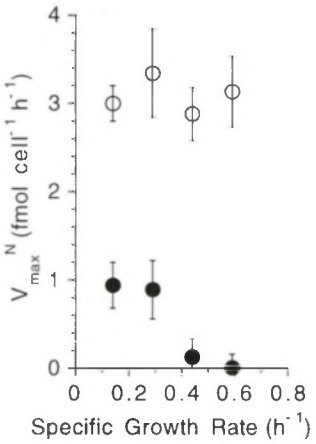

Fig. 12. *Effect of specific growth rate on the maximum cell specific nitrate uptake rate of* E. huxleyi *in the light (open symbols), and in the dark (closed symbols).*

In the N-limited cultures, the external nitrate concentration was close to the detection limit of 0.02 µM. The half saturation constant for nitrate uptake (Ks^N) was 0.22, 0.18, 0.24, and 0.23 µM, respectively, for cells growing at μ= 0.15, 0.30, 0.45, and 0.60 d^{-1}. The maximum cellular nitrate uptake rate (V_{max}^N) was independent on the growth rate (Fig.12), and in all cultures higher than the actual nitrate uptake rate ($\bar{v}$).

At the lowest growth rate, V_{max}^N was 11.5 times higher than the actual uptake rate. The nitrate uptake rate in the dark was 30% of the uptake rate in the light. The affinity of the uptake system, on the basis of cellular N, varied between 0.15 and 0.37 L µmol^{-1} h^{-1}, respectively.

5. Discussion

5.1. Light-limited growth.

Although the cellular pigment content of *E. huxleyi* was highly variable between strains (G.W. Kraaij, pers. commun.), certain pigment ratios are less subject to genetic variation within the tested strains. Especially the total fucoxanthin/chl-a ratio proved to be very constant between strains. In the turbidostat experiments, the ratio between the total carotenoids and chlorophyll-a was almost invariable. The reason for this is not clear. There is no evidence in the literature that fucoxanthin could be replaced by diadinoxanthin directly. However, in this species we find an almost stochiometric decrease of (FUCO + HEXA) associated with an increase of (DD + DT).

The ratio ((HEXA+FUCO)/CHL-A) proved to be best correlated with culturing PFD (R^2 = 0.99; p=0.0005). In a monospecific bloom of *E. huxleyi*, this could provide a useful tool to obtain information about the light history of the cells. When *E. huxleyi* co-occurs with cyanobacteria, the ratio could be disturbed by their chlorophyll-a. In that case, the ratio between (HEXA+FUCO)/(DD+DT) could be a better measure for light history, since cyanobacteria do not contain any of these pigments.

DT/(DD+DT) ratio has been used (Brunet 1993) as an estimate for light-adaptation of phytoplankton in the field. It was found to correlate well with the turbidity of the water. In the light, DD is de-epoxycated to DT, which can act as a non-photochemical quencher. DT is enzymatically converted to DD in the dark, a process which can be very fast (in the order of minutes). Since we did not take special precautions to freeze the filters immediately after filtering, part of the DT might have been converted to DD before freezing. For this reason, DT+DD might be more useful as an indication to longer term photoacclimation than DT alone.

The turbidostat experiments in this study were all performed in continuous light. Since this is not the natural condition the exact physiological response of cells in nature is additionally influenced by photoperiodicity. In nature, a steady state will therefore never be reached. However, it can be argued that phytoplankton cells in nature are continuously adapting towards the steady state situation that appears from this study. The quantitative importance of the role of photoperiodicity in the physiology of *E. huxleyi* remains to be studied.

The final response in physiological acclimation of pigment content, and photosynthetic activity is reflected in the specific growth rate achieved at non-saturating irradiance levels. Intercomparison with other marine phytoplankton species is somewhat hampered by the lack of uniformity in the experimental set-up. It should be kept in mind, that in general a higher temperature will enhance μ (within the 0 to 25 °C range) and a Light: Dark cycle less than 12:12 h: h will reduce the specific growth rate.

A compilation of data from the existing literature gives the impression that *E. huxleyi* has a low compensation irradiance levels for growth (I_c) and a relatively (but not exceptionally) high affinity ($d\mu/dI$) for light (Table 10). These characteristics make *E. huxleyi* comparable to typical oceanic species such as *Prochlorococcus* and *Synechococcus* WH8103. In fact, it could be stated that *E. huxleyi* shows a comparable performance as the latter two species, but than at a lower temperature. This agrees with the general impression,

that coccolithophorid blooms are usually dominated by *E. huxleyi* in moderate and polar climatological zones, whereas near the equator other coccolithophorids may dominate. On the other hand, these results may be the consequence of the fact that we worked with a strain which originally was isolated from the Oslo fjord. There very well may be a difference in temperature dependence amongst different isolates of one and the same algal species.

Table 10. *Intercomparison of the growth characteristics of light-limited marine phytoplankton species. Data are presented on the specific growth rate* (μ_{max}), *the affinity of growth for light* ($d\mu/dI$), *the irradiance compensation intensity, at which respiration equals photosynthesis and where consequently* $\mu = 0$ (I_c), *the growth temperature* (T), *and the light: dark cycle* (L:D) *which was applied.*

	μ_{max}	$d\mu/dI$	I_c	T	L:D	
Species	d^{-1}	10^{-3} m^2 s $\mu mol^{-1} d^{-1}$	μmol $m^{-2}s^{-1}$	°C	h: h	Reference
Gyrodinium aureolum	0.38	7	10	20	12:12	Garcia & Purdie 1992
Alexandrium excavatum	0.56	4.9	35	15	14:10	Langdon 1987
Prorocentrum micans	0.18	1	15	18	24: 0	Falkowski et al. 1985
Skeletonema costatum	1.59	2.2	1.8	15	14:10	Langdon 1987
Olisthodiscus luteus	0.87	7.3	9	15	14:10	Langdon 1987
Thalassiosira weisflogii	1.8	12	22	18	24: 0	Falkowski et al. 1985
T. oceanica	1.2	17	17	15	24: 0	Sakshaug et al. 1987
T. pseudonana	1.47	12	17	15	24: 0	Sakshaug et al. 1987
T. nordenskioeldii	0.33	5		0.5	24: 0	Sakshaug et al. 1991
Phaeodactylum tricornutum	1.38	42	0.5	23	24:0	Geider et al. 1985
Chaetoceros furcellatus	0.33	5		0.5	24: 0	Sakshaug et al. 1991
Isochrysis galbana	1.2	10	15	18	24: 0	Falkowski et al. 1985
Pavlova lutheri	1.0	7	28	20	24: 0	Goldman 1986
Synechococcus WH8103	1.0	20	5	25	14:10	Moore et al. 1995
Prochlorococcus marinus	0.6	30	1.3	25	14:10	Moore et al. 1995
Emiliania huxleyi	0.8	10		15	24: 0	Nielsen 1997
E. huxleyi	0.8	51	2.5	15	24:0	This study

5.2. P-limited growth.

Emiliania huxleyi showed some special features with respect to its ability to compete for phosphorus. Induction of its phosphate uptake system under P-limiting growth conditions is a common feature amongst phytoplankton species. However, the affinity of the phosphate uptake system is remarkably high. In fact, *E. huxleyi* has an affinity for inorganic PO_4^{3-} which is the highest ever recorded for a phytoplankton species (Table 11).
This enables *E. huxleyi* to outcompete other algae at levels of Pi down to the nM range. In most field studies, Pi is determined as Soluble Reactive Phosphorus (SRP) with detection levels down to 0.1 µM. Since many organic phosphates also react with the molybdenum-blue reagent, Pi is generally over-estimated. Isotope studies reveal indeed nM concentrations of Pi in P-controlled ecosystems (Thingstad et al. 1993). $^{32}P\text{-}PO_4^{3-}$ uptake in laboratory cultures of the freshwater cyanobacterium *Anacystis nidulans* indicated a threshold concentration for P-uptake at 2 nM (Wagner et al. 1995; Falkner et al. 1996). The presence of such a threshold concentration for P-uptake by *E. huxleyi* can not be excluded from our data.

Maintenance of *E. huxleyi* in P-controlled systems is additionally supported by its APase activity. Kuenzler & Perras (1965) reported the highest APase production rates for *E. huxleyi* in comparison with 10 other algal species. Here, we provide evidence, that *E. huxleyi* is in the unique possession of two different enzymatic APase systems. One of them is synthesized constitutively, with a cell-P based affinity for MFP of 8.7 L μmol^{-1} h^{-1}. The inducable APase is somewhat more complex in structure (according to its higher Q_{10}), can reach a five-fold higher V_{max} than the constitutive system, and has a slightly lower affinity (6.0 L μmol^{-1} h^{-1}). Together, they allow a cleavage rate of an organic substrate at non-saturating levels which equals up to 73% of the inorganic P uptake rate capacity at nM levels. This implies, that if Pi and organic P would be available at the same concentration in the nM range, roughly 40% of the growth of *E. huxleyi* would be supported by organic P. At a tenfold increase of organic P (e.g. due to autolysis of competing algae (Brussaard et al. 1995)) this value may increase up to 90%. Exact rates of cleavage will of course depend on the nature of the organic substrate.

In environments with fluctuating phosphate concentrations, V_{max} and the ratio between Q_{max} and Q_{min} will be important selection criteria for algae which are competing for phosphorus. The cell-P normalized maximum phosphate uptake rate of *E. huxleyi* is within the same range as the V_{max} of other algae (Table 11). The coefficient for luxury uptake, i.e. the ratio Q_{max}/ Q_{min}, stands for the maximum amount of cells which potentially can be produced from one P-limited cell after a single saturating phosphate pulse. The highest value for *E. huxleyi* (30.5 at μ= 0.14 d^{-1}) is low, relative to other species. *Selenastrum capricornutum* has a coefficient for luxury uptake of 56 (Braddock & Brown 1994), *Synechococcus nägeli* expresses a coefficient of 63, and for *Navicula pelliculosa* a value of 55 was reported (Brown et al. 1981). So, with respect to its P-metabolism, *E. huxleyi* is especially superior to other algal species with respect to its affinity for inorganic phosphate uptake, and its possession of two APase enzyme systems, one of them being induced under severe P-limitation.

Table 11. *Interspecific comparison of phosphate uptake kinetics of bacteria and algae.*

Species	V_{max} (h^{-1})	Affinity (L μmol^{-1} h^{-1})	Reference
Freshwater Bacteria	0.4 -13	31-146	Vadstein & Olsen 1989
Freshwater Algae	0.5-16	2-17	Vadstein & Olsen 1989
Thalassiosira pseudonana	7.8	11	Perry 1976
Pseudonitzchia multiseries	12		Pan et al. 1996
Skeletonema costatum	12	17	Tarutani & Yamamoto 1994
Phaeocystis globosa	0.22	2	Veldhuis et al. 1991
Rhodomonas sp.	3	10	Riegman, unpubl results
Nannochloropsis sp.	8	12	Riegman, unpubl results
Emiliania huxleyi	**9**	**20**	This study

Table 12. *Interspecific comparison of nitrate uptake kinetics of marine algae.*

Species	V_{max} (h^{-1})	dV/dS (L μmol^{-1} h^{-1})	Reference
Dunaliella tertiolecta	0.31	1.48	Caperon & Meyer 1972
Dunaliella tertiolecta	0.13		Laws & Wong 1978
Monochrysis lutheri	0.32	0.76	Caperon & Meyer 1972
Monochrysis lutheri	0.24		Laws & Wong 1978
Coccochloris stagnina	0.07	0.25	Caperon & Meyer 1972
Cyclotella nana	0.27	0.9	Caperon & Meyer 1972
Thalassiosira pseudonana	0.28	0.19	Dortch et al. 1991
Thalassiosira allenii	0.28		Laws & Wong 1978
Asterionalla japonica	0.30	0.23	Epply & Thomas 1969
Chaetoceros gracilis	0.49	1.64	Epply & Thomas 1969
Emiliania huxleyi	**0.07**	**0.37**	This study

5.3. N-limited growth.

With respect to N-limited growth, *E. huxleyi* showed less exceptional properties. In comparison with most other algal species (Table 12), its maximum nitrate uptake rate is rather low. Its affinity for nitrate uptake is also not extremely high, considering the high values reported for *Dunaliella tertiolecta, Monochrysis lutheri,* and *Chaetoceros gracilis* (Table 12). For *Thalassiosira allenii, Monochrysis lutheri,* and *Dunaliella tertiolecta*, it was found that V_{max}^{N} in the dark was 100, 30, and 60%, respectively, of the uptake rate in the light (Laws & Wong 1978). After correction for excretion of nitrite during the period of uptake, these values were 97, 0.5 and 53%, respectively. In our experiments, nitrite production by *E. huxleyi* during nitrate uptake in the dark was below the detection level (data not shown).

The strong reduction in nitrate uptake in the absence of light as observed for *M. lutheri* and *E. huxleyi* is not exceptional for phytoplankton species. The diatom *Skeletonema costatum* reduced nitrate uptake down to zero during the dark period (Epply et al. 1971). Obviously, a reduction of nitrate uptake in the dark will reduce the competitive ability of the latter species.

We did not investigate the ability of *E. huxleyi* to take up organic nitrogen. It is known from *Pleurochrysis, Prymnesium* and *Amphidinium* species that they posses cell-surface L-amino acid oxidases enabling them to utilize amino acids (Palenik & Morel 1990, 1991). Growth of *E. huxleyi* on amino acids has been reported, but high cell densities were obtained exclusively on alanine, leucine, and serine (Ietswaard et al. 1994). This indicates the presence of an uptake system for neutral amino acids only. Additionally, Ni dependent growth of *E. huxleyi* on hypoxanthine and other purines has been reported, as well as indications for uptake of urea, thiourea, hydroxyurea and acetamide (Palenik & Henson 1997). Thus, there seems a limited potential for the use of remineralisation products by N-limited *E. huxleyi*. To which extent this ability is relevant under natural conditions, where much lower concentrations prevail compared to the experimental conditions which were used, is unknown.

5.4. On the global distribution of *Emiliania huxleyi* blooms and associated calcification.

A comparison between the specific physiological features of *E. huxleyi*, and the predominant environmental conditions which persist in areas where *E. huxleyi* blooms occur, may give an explanation for the performance of this species and associated calcification in marine ecosystems. Commonalties between conditions prior to coccolith producing *E. huxleyi* blooms in the Bjørnafjorden in Norway (Veldhuis et al. 1994), the northern North Sea (van der Wal et al. 1995), and in the NE Atlantic (Fernández et al. 1994), are nitrate levels above 4 μM, phosphate below 0.2 μM , and a stratified watercolumn which facilitates higher average PAR levels in the upper mixed layer. Prior to the bloom, maximum densities of *E. huxleyi* cells are usually found just above the thermocline. From a nutritional point of view, this location in the watercolumn is generally characterized by inorganic nutrient entrainment from below the thermocline by internal wave forcing, organic nutrient import due to the sinking of particulate matter which is produced in the upper layer, and sub-saturating irradiance levels. At sub-saturating irradiance levels, the coccolith production per cell is low (Balch et al. 1996; Paasche 1998). In fact, naked cells grow faster than calcified ones at low light (Lecourt et al. 1996). The microzoooplankton generated

turn-over in phytoplankton cells can potentially lead to a population of naked *E. huxleyi* cells near the thermocline. The maintenance of *E. huxleyi* near the thermocline at non-saturating irradiance levels is obviously facilitated by its high affinity for light (Table 10). After a physical disturbance of the watercolumn, either by a storm, or by the passage of a large Eddy, subsurface populations of phytoplankton are transported to the surface. Especially, if the upper layer of the photic zone will remain P-controlled during this transient state, the high affinity of *E. huxleyi* for Pi and its high APase activity might favor rapid growth.

It must be mentioned that biomass accumulation in natural systems is the result of a balance between gain and loss rates. Predation on *E. huxleyi* is amongst others by microzooplankton (Hansen et al. 1996). Due to their high intrinsic maximum growth rates, microzooplankton species have the potention to control the biomass of microalgae (e.g. Riegman et al. 1993). One possibility might be that surface blooms of *E. huxleyi* develop due to a cascade effect (e.g. Verity & Smetacek 1996) in the predatory foodchain. Characteristic for the periods in which *E. huxleyi* blooms have been observed, are an active microbial foodweb (including microzooplankton grazing on small algae) and associated recycling of nutrients via various organic speciations. Only a slightly enhanced biomass of copepods reducing the microzooplankton density ,thereby releasing the biomass control of *E. huxleyi*, has the potential to stimulate the blooming of the coccolithophore. Such a cascade effect has been shown to facilitate blooms of the Haptophyte *Phaeocystis* (Hansen et al. 1993). Indeed, also for *E. huxleyi* this mechanism has shown to be operational in natural communities. In 11 m^3 mesocosm experiments in Norway, Nejstgaard et al. (1997) clearly demonstrated a cascade effect by grazing of *Calanus finmarchicus* on microzooplankton, causing *E. huxleyi* to bloom.

Elevated irradiance levels at the surface will stimulate the calcification of individual cells, especially under P-limitation (Paasche & Brubak, 1994, Paasche, 1998), and subsequent satellite detection (e.g. Balch et al. 1996) is a fact. The highest sinking velocities for heavy calcified single cells is less than 2 m d^{-1} in stagnant water (Lecourt et al. 1996). This makes it most likely that the major source of calcite in sedimentary deposits is facilitated by copepods fecal pellet production (Honjo 1976) rather than the settlement of individual cells.

It has been suggested that the removal of CO_2 from the biosphere *via* primary production in the photic zone of marine ecosystems may be nitrogen controlled (e.g. Falkowski et al. 1998). These authors published the hypothesis that the reduced atmospheric CO_2 during the glacial periods was the result of enhanced N_2 fixation in the oceans. The low competitive ability of *E. huxleyi* under N-limitation explains the reduced *E. huxleyi* associated calcite deposition during glacial periods. From the ecophysiology of *E. huxleyi*, we conclude that P-limitation apparently occurred more frequently during the interglacial periods. This hypothetical alternation of N- and P-limitation in between glacial and interglacial periods is still highly hypothetical and deserves further attention with respect to its potential impact on the biogeochemical fluxes in the oceans.

In agreement with the ecophysiological profile of *E. huxleyi,* this species mainly performs in P-controlled environments. **The removal of CO_2 from the biosphere *via* the burial of calcium-carbonate from the major calcifier in the worlds oceans may be controlled by phosphorus rather than nitrogen**.

6 Conclusions and recommendations.

In summary, it can be concluded that *Emiliania huxleyi*:

- Is very capable in growing at low irradiance levels which allows this species to maintain near the thermocline,
- Has a low sensitivity to photoinhibition which prevents this species from photoinhibition after transport to surface layers,
- Has a very high affinity for phosphate which makes this species a good competitor for P.
- Induces two alkaline phosphatase systems under P-limitation which enables this species to profit from the organic P production by the microbial foodweb.

From a mechanistic point of view, *E. huxleyi* blooms are expected to occur:

- When there is an enhancement of irradiance levels, facilitating coccolith production,
- When there is P-control of the plankton community,
- When there is rapid recycling of P *via* the organic P-pool in the pelagic zone,
- When microzooplankton grazing control is prevented, for example by cascade effects in the pelagic foodweb.

These conclusions clearly indicate that, to model the bloom dynamics of *E. huxleyi*, other components of the pelagic foodweb such as predators and competitors should be included.

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Appendix 1 Project description

Biological climate forcing is important, but the huge diversity of organisms severely complicates a system's approach to global climate dynamics. The Global Emiliania Modeling initiative (GEM: an international collaborative effort between many research institutes) provides the strategy and infrastructure that is needed to overcome this problem, at least in so far as the pelagic ocean biota is concerned. GEM conducts an in-depth experimental and modeling study of a single representative organism, the calcifying alga *Emiliania huxleyi* , relating the cellular organization of this species to its ecology and to the fate of its products ($CaCO_3$, organic carbon, and DMS). To make the resulting models more generally applicable, little additional experimentation and minor alterations in model structure will be required.
Here, we address the central problem of GEM -- to establish a model for the *E. huxleyi* cell, defining how environmental factors affect the production of the climatically relevant products. An interactive experimental and modeling approach is realized. To provide the required database, and extensive program of laboratory cultures in chemostats was envisaged. The was the main task of subproject b.

Appendix 2 List of project publications, including papers in preparation

- Stolte, W., Noordeloos, A.A.M., Zonneveld, C., Riegman, R. Towards modeling the growth of *Emiliania huxleyi* I: Cell composition and photosynthesis under steady state light limitation in turbidostat at different photon flux densities. (In prep.)
- Stolte, W. Noordeloos, A.A.M., Kraaij, G.W. Riegman, R. Pigment composition of the bloom-forming phytoplankton species *Emiliania huxleyi* (Lohmann) at different growth rates during steady-state light-, phosphorus- and nitrate-limitation and the potential use as a physiological marker. (in prep.)
- Riegman, R., Stolte, W. & Noordeloos, A.A.M. (1999) Nutrient uptake and Alkaline phosphatase (EC 3:1:3:1) activity of *Emiliania huxleyi* (strain L) during N- and P-limited growth in continuous cultures. Submitted to *J. Phycol.*

Appendix 3 Coordination with other projects and programmes

"The physiology of *Emiliania huxleyi*" was subproject b from the NOP project: "A model system approach to biological climate forcing: the example of *Emiliania huxleyi*". This project is embedded within GEM which operates at the international level.

Appendix 4 Attendance at national and international meetings

1996

In 1996, no congress was visited.

1997

- 25th -26th March. Organization of an international *Emiliania huxleyi* workshop by W. Stolte at the Netherlands Institute for Sea Research, Texel
- 29-30 May. W. Stolte and A. Noordeloos visited "European Network on Integrated Marine System Analysis" (ENIMSA) in Brussel, Belgium.
- 11-15th August. W. Stolte presented an oral presentation on "Cell-size dependent nitrate and ammonium uptake kinetics in marine phytoplankton". At the 6th International Phycological Congress, Leiden, The Netherlands.
- 13-17th September. W. Stolte attended the 7th *Emiliania hux*leyi meeting, Blagnac, France.
- 21st October. Prof. Dr. Elma Gonzalez, who was visiting the University of Leiden, was invited by W. Stolte to present a lecture at NIOZ titled: "A Cellular Perspective of Coccolithophorid Calcification"
- 3rd October. W. Stolte presented a lecture at a meeting of the Dutch-Flamish Society for Diatomists at the RIZA institute in Lelystad, The Netherlands. Title: Size dependent restrictions on competition for nutrients by marine phytoplankton.

1998

- February: W. Stolte presented an oral presentation entitled "Light-limited growth and cell composition of *Emiliania huxleyi* in turbidostat" at the "Ocean Sciences meeting" of the "American Society for Limnology and Oceanography" (ASLO) in San Diego, USA.
- 8 May. W. Stolte visiting Prof. Dr. Edna Granéli of the Department of Marine Sciences of the University of Kalmar, Sweden to discuss possible cooperation in the future.
- 8-10 June. Organization of the second international *Emiliania huxleyi* workshop by W. Stolte at the Netherlands Institute for Sea Research, Texel. W. Stolte presented two papers: Titles: "Light-limited growth of *E. huxleyi*" and " Modelling phosphate uptake by phytoplankton". Doris Slezak presented a paper on " phosphate uptake kinetics and alkaline phosphatase activity in phosphate limited *E. huxleyi* ".
- 9 July W. Stolte presented an oral presentation titled "Phosphorus-limited growth of *Emiliania huxleyi*" at the Department of Marine Microbiology of the University of Bergen. Also, the results were discussed more thoroughly with Prof. Dr. T.F. Thingstad of this department.

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