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

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Abstract

The following is an extended summary of the project “A model system approach to biological climate forcing: the example of *Emiliania huxleyi*,” that was carried out within the framework of the Dutch National Programme on Air Pollution and Climate Change (NRP) – phase II.

In this report the progress is described that has been made in the formulation of the Dynamic Energy Budget (DEB) Theory that now includes photosynthesis, nutrient limitation and calcification. On top of this the next level of organisation was explored in the adaptation of the model towards a small ecosystem comprising an autotrophic and a heterotrophic organism together with a decomposer, -the dynamics of biomass in vertical profiles has been analysed. At the same time experiments were conducted with *E. huxleyi* in order to be able to validate the DEB-models at the organismic level. The experiments involved exploration of the genes that control calcification, photosynthesis and nutrition, a detailed description of growth based on chemostat experiments, and an inventory of environmental factors that determine the production of dimethylsulfoniopropionate, the precursor of the biogenic gas dimethylsufide (DMS) that regulates climate once it has reached the atmosphere acting as a source of cloud condensation nuclei.

Preface

The project was supported financially by NWO through the Dutch National Research Programme on Global Air Pollution and Climate Change (NRP) – phase II. It was initiated by Prof. Dr P. Westbroek of the University of Leiden (LU) and Prof. Dr S.A.L.M. Kooijman of the Free University of Amsterdam (VUA).

The institutes that collaborated in the project were

- Department of Theoretical Biology – Free University of Amsterdam (VUA)
- Netherlands Institute for Sea Research (NIOZ), Texel
- Department of Marine Biology – University of Groningen (RUG)
- Leiden Institute of Chemistry – University of Leiden (LU)

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1. EXECUTIVE SUMMARY

1.1 English summary

The ultimate goal of Dynamic Energy Budgets **(**DEB) is to formulate a coherent and predictive theory of life. Expressed as a nested suite of compatible models for all levels of organisation, it will demolish the boundaries between biology, physics and chemistry. With the theory for organisms as a base, DEB theory presently moves down to the molecular level and up to the ecosystems and the biosphere. This study describes the progress that has been made in the formulation of the Dynamic Energy Budget (DEB) theory that now covers photosynthesis, nutrient limitation and calcification as well. In addition, the next level of organisation was explored in the adaptation of the model towards a small ecosystem comprising an autotrophic and a heterotrophic organism together with a decomposer. The dynamics of biomass in vertical profiles has been studied, including nutrient and organic matter cycling.

At the same time experiments were conducted with *Emiliania huxleyi* in order to be able to validate the DEB-models at the organismic level. The three major mechanisms that are distinguished by which the marine pelagic biota may influence the global climate system are combined in the unicellular marine alga *E. huxleyi*: (1) the drawdown of CO_2 from the upper mixed water layer and the atmosphere to intermediate and deep waters by the formation, export and remineralisation of particulate organic carbon (the organic carbon pump); (2) the production, downward transport and partial dissolution of calcium carbonate (the carbonate pump); and (3) large-scale albedo effects mainly owing to the formation of highly reflective clouds following gaseous emissions of dimethylsulphide (DMS) from algal blooms. Therefore, *Emiliania huxleyi* is exceptionally well suited for providing the kind of data we need. The experiments involved exploration of the genes involved in calcification, photosynthesis and nutrition, a detailed description of growth based on chemostat experiments and an inventory of environmental factors that determine the production of dimethylsulfoniopropionate the precursor of the climate active gas dimethylsufide (DMS).

Microalgal growth models aim at predicting growth rate as well as cellular composition, given the environmental conditions (such as temperature, nutrient availability, light etc.). This generic purpose poses strong requirements to the structure of any model attempting to comply with it. Evaluation of contemporaneous modelling methodology revealed that models are not

formulated at the level of the individual and that cell cellular characteristics such as the Chl:C ratio are often treated as model input. Furthermore, steady state growth is mostly taken as point of departure while dynamic models are potentially best suited to study the regulation of growth in response to variations in the environment. Therefore, a dynamic model for light-limited growth, without taking either chlorophyll or the Chlorophyll: C ratio as model input was formulated. Then the gap between existing models and our dynamic models was bridged by the construction of a cell-based model for the chlorophyll *a* to carbon ratio in phytoplankton. The process of photosynthesis was studied in more detail by an analysis of the effect photo-acclimation and photo inhibition on photosynthesis-irradiance curves in phytoplankton including the so-called dark reaction.

Nutrient limited growth was modelled using variable internal stores. This model had a number of advantages (it can be extended to include more nutrients) but the model behaviour caused concern for further application within a larger ecosystem framework. The concept of the synthesising unit as model for the stoichiometric fusion and branching of metabolic fluxes was postulated to solve the problems. The next step was to study how model ecosystems behave that are based on the models developed. To this end model-algae in competition were studied as well as a canonical ecosystem comprising of an autotrophic producer, a heterotrophic consumer and a hetetrophic decomposer. Implementation of the modelling strategy into a spatially heterogeneous environment was studied by the performance of a one-species community of a mixotrophic organism. The mixotroph is able to photosynthesise, as well as to consume dead biomass. The choice for a further simplification of the model ecosystem was motivated by the future implementation in ocean circulation models. At the same time, as this model is thoroughly based in DEB theory, it is still relevant for reality.

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 (NIOZ, Texel). The results confirm earlier findings that *E. huxleyi* has a high growth affinity for light (51 10^{-3} m^2 s μmol^{-1} d^{-1}) which is comparable to oceanic specialists such as *Synechococcus* and *Prochlorophyte*s. 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 a major calcifier in the world oceans is controlled by phosphorus rather than nitrogen.
Together with the new insights in the ecophysiology of *E. huxleyi* obtained with these chemostat cultures a data set was generated that could be used for the underpinning of the DEB theory, samples were stored to be investigated with the newly developed molecular techniques in Leiden, and for two steady states DMSP data were obtained that were used in the study performed in Groningen.

With the purpose of generating a data set that can be used to underpin the DEB theory at the molecular level, the goal of the project in Leiden was to produce a molecular genetic tool for the rapid characterisation of the metabolic activity in coccolithophorids in laboratory cultures. In this study, several genes were successfully cloned and characterised and the first mRNA-expression patterns that were obtained were very promising.
Isolated genes assumed to play a function in calcification included two calcification genes, gpa and ale. Another gene, hpp that is indirectly involved in calcification by encoding a phosphate-repressible phosphate permease, was cloned from a cDNA library of phosphate limited cells. Expression profiles of this gene are very interesting, because the parallel study by Riegman et al. indicated that *E. huxleyi's* success may result from its remarkably high affinity with phosphate and that phosphate limitation leads to increased calcification rates. The combined expression levels of pcna and fcp can be used to quantify cell division rate and nutrient status. Expression of the gene fcp was investigated to monitor rates of photosynthesis. It turned out that fcp expression is also a good indicator to study nutrient (nitrate) status and cell growth. However, it is not possible to distinguish between these properties on the basis of fcp expression pattern alone. To study cell division the gene encoding a proliferating cell nuclear antigen (pcna) is a good candidate. Expression levels of pcna relate directly to cell division activity.
Although gene identification and expression studies are far from complete, the results obtained during this NOP-II research period provided enough body so that the first

characterisations of samples from continuous cultures could be started. With the extant data we have started with the validation of the theoretical models developed by Kooijman et al.

It has been recognised for some time that Prymnesiophytes are the main natural producers of dimethylsulfoniopropionate (DMSP), the precursor of the biogenic gas dimethylsulfide (DMS) that regulates climate once it has reached the atmosphere by acting as a source for cloud condensation nuclei. Still information on the effects of environmental factors on its production are scarce. In Groningen the production of DMSP by *E. huxleyi* strain L was studied under different light, salinity, nutrient, temperature and UV-stress conditions. As was expected (but never investigated, let alone published) the cellular DMSP concentration was directly coupled to the salinity of the environment. This result is interesting from a physiological point of view because it confirms the osmotic role of DMSP in the cell but it has little relevance for *E. huxleyi* in its environment, where salinity is almost constant. More relevant are effects of light and temperature since both factors change on a daily basis as well as on longer time scales. In batch cultures there was no measurable effect of light (range 10-140 µmol photons m^{-2} s^{-1}) but in continuous cultures at 100 µmol photons m^{-2} s^{-1} a slightly higher concentration was observed compared to low light conditions (6 µmol photons m^{-2} s^{-1}). Temperature, however, especially at the low end of the growth range resulted in substantially higher concentrations of DMSP in the cell. This result compares to findings with macroalgae that accumulate DMSP in their tissues at low temperatures. DMSP seems to be especially effective at low temperatures as a compatible solute in algae. If the response of *E. huxleyi* is characteristic for all other DMSP producing microalgae, a decrease of DMSP concentration in the cells must be expected upon a temperature rise, for example when climate changes in the expected direction of global warming. However, changes in processes that determine the abundance of DMSP producing algae, the activity of DMSP consuming bacteria, and physical sea-to-air transfer of DMS, all of these as a result of ocean warming, are hard to predict. Therefore, it is impossible to foresee what the over-all effect of global warming on the actual sea-to-air emission of DMS will be. Nevertheless, our observation of less DMSP production at elevated temperatures, assumed to result in less DMS emission from the ocean, is in line with observations of lowest concentrations of the major oxidation product of DMS methanesulphonate (MSA) in ice cores taken on Antarctica during interglacial periods.

Preliminary results of studies of the effects of N versus P limitation on the production of DMSP showed that cells produce most DMSP when both nutrients are present in Redfield ratio quantities. Continuous cultures steady states are needed to confirm this observation that

contradicts the hypothesis that at N limitation DMSP could be produced as a substitute for the N-analog glycine-betaine. Another hypothesis in the literature that suggests that DMSP production could be enhanced at stress conditions could not be confirmed when UV stress was induced in *E. huxleyi* cells. The cellular DMSP concentration was not affected.
During the studies on the different environmental effects it became evident that in physiological studies it is important to express the DMSP content of cells as a concentration (DMSP per biovolume) rather than as an amount per cell. Due to changes in cell volume the outcome of both expressions is often different en sometimes even contradictory.

Altogether the project has provided valuable new knowledge of the ecophysiology of *E. huxleyi* that helps to explain the success of these coccolithophorids. Such knowledge is essential for understanding the role that these calcifying marine organisms play in the drawdown of CO_2 from the atmosphere to the deep ocean. Environmental factors that play a role in DMS(P) production have been identified and the results can be used in the modelling of DMS production by *E. huxleyi*.
Molecular techniques have been developed to further study the process of calcification and photosynthesis at the molecular level. These results can be used to validate the Dynamic Energy Budget theory at the molecular level. At the same time DEB at the organismic level has broadened to incorporate autotrophic calcifying organisms and has evolved towards the next level of organisation.

1.2 Dutch summary

Het uiteindelijke doel van de Dynamische Energie Budget (DEB)-theorie is om een coherente theoretische formulering te vinden waarmee door biota bewerkstelligde stof- en energiestromen te beschrijven en te voorspellen zijn. De theorie heeft een modulaire opbouw. Met een modelformulering voor het individu als uitgangspunt is het mogelijk om binnen de DEB-theorie van het moleculair niveau tot ecosysteemniveau, ja zelfs biosfeerniveau, te gaan. In dit rapport wordt de uitbreiding in de formulering van de DEB-theorie beschreven, met de processen fotosynthese, nutriëntlimitatie en calcificatie. Deze uitbreiding is een essentiële voorwaarde om te kunnen komen tot uitspraken over de biosfeer. Verder is een begin gemaakt met de stap naar het eerste organisatieniveau boven dat van het individuele organisme, namelijk het ecosysteem. Het bestudeerde ecosysteem bestond uit een primaire producent (plankton), een consument (ciliaat) en een mineraliseerder (bacterie). Ook is gekeken naar de implicaties van de DEB-theorie voor nutriëntprofielen in de oceaan. De dynamica van biomassa is beschreven, inclusief de recycling van nutriënten en organische stof. Tegelijkertijd zijn experimenten uitgevoerd met de mariene microalg *Emiliania huxleyi*. Deze hadden tot doel de ontwikkelde DEB modellen te valideren met gegevens van waarnemingen en metingen.

De drie belangrijkste mechanismen in het mariene milieu die van invloed zijn op het klimaat komen samen in *E. huxleyi*: (1) De 'biologische pomp', waarbij CO_2 uit de bovenlagen van de oceaan en uit de atmosfeer wordt gefixeerd als organisch koolstof, en naar diepere waterlagen wordt getransporteerd; (2) de 'carbonaat pomp' waarbij kalk wordt gevormd in de bovenlaag dat voor een deel naar dieper water wordt getransporteerd, en (3) grootschalige effecten op de reflectie van zonlicht door wolken die geïnitieerd worden door emissies van dimethylsulfide (DMS) afkomstig van algenbloeien. Het feit dat al deze drie mechanismen bestudeerd kunnen worden aan *E. huxleyi* maakt deze soort buitengewoon geschikt als modelorganisme.

De experimenten die uitgevoerd zijn betroffen de verkenning van sleutelgenen betrokken bij fotosynthese, calcificatie en opname van voedingsstoffen om een gedetailleerde database op het moleculaire niveau mogelijk te maken. Het betreft hier noodzakelijk voorwerk om te komen tot een integratie van moleculaire en organismale biologie. Ten behoeve van validatie van het DEB-model op het niveau van individuele cellen werd gestreefd naar een gedetailleerde beschrijving van groei m.b.v. chemostaten. Deze experimenten zijn ook belangrijk voor het verkrijgen van inzicht in de oorzaken voor het succes van deze alg. Als laatste onderdeel van het experimentele werk is geïnventariseerd welke omgevingsfactoren

van invloed kunnen zijn op de productie van dimethylsufoniopropionaat (DMSP), specifiek bedoeld als basis (op individu-niveau) voor toekomstige modellering van effecten van het biogene gas DMS in klimaatmodellen.

Algengroeimodellen zijn er doorgaans op gericht om algengroei (of biochemische biomassa-samenstelling) te beschrijven als functie van omgevingsfactoren zoals licht, temperatuur etc. Dit doel stelt strenge randvoorwaarden aan de manier waarop dergelijke modellen geformuleerd worden. Evaluatie van de huidige modellen liet zien dat deze niet uitgaan van cellen maar ook van chemische karakteristieken zoals de chlorophyll a: koolstof ratio als basis in plaats van als uitkomst. Verder gaan veel modellen uit van een stabiele ('steady-state') omgevingssituatie. Zo'n situatie is echter eerder uitzondering dan regel. Dynamische modellen zijn veel beter geschikt om de respons op fluctuerende omgevingsfactoren te beschrijven.
Voor de constructie van modellen die aan alle randvoorwaarden voldoen is allereerst een dynamisch model geformuleerd voor lichtgelimiteerde groei zonder de hoeveelheid chlorofyl of de Chl a:C ratio als uitgangspunt te nemen. Daarna is een op het individu (de cel) gebaseerd model gebruikt voor de beschrijving van de Chla:C ratio om vergelijking met bestaande modellen te vergemakkelijken. Op die manier kon de Chla:C ratio uitstekend beschreven worden, in overeenstemming met experimentele gegevens. Het fotosynthese-proces zelf is in meer detail onderzocht met name het effect van lichtsterkte op de fotosynthesesnelheid inclusief foto-acclimatie, foto-inhibitie en de donkerreactie.
Nutriëntgelimiteerde groei is gemodelleerd door gebruik te maken van een variabele interne reserveopslag. Het model heeft als voordeel dat het eenvoudig uit te breiden was naar meer dan één voedingsstof maar heeft tot nadeel dat het problemen zou geven wanneer het als basis zou worden gebruikt voor het DEB-model op ecosysteem niveau. Het probleem is gelegen in de veronderstelling dat slechts één nutriënt van invloed is op de groeisnelheid. Dit probleem is ondervangen door de formulering van een nieuw concept, de 'Synthetiserende Unit.' Een SU staat voor één of meerdere enzymen die een bepaalde functie vervullen. Zo kan men bijvoorbeeld de Calvin-cyclus beschrijven als een enkele SU. Het concept komt uitstekend van pas in de formulering van modellen die metaboliet fluxen beschrijven. Een modelformulering voor calcificatie was bijv. afhankelijk van het SU-concept.
De volgende stap was om de geformuleerde modellen in te bouwen in ecosysteemniveau-modellen. Op deze manier zijn succesvolle formuleringen voor algencompetitie en voor een mini-ecosysteem (autotroof, heterotroof en mineraliseerder) tot stand gekomen. De

modelleringstrategie is verder getest door te onderzoeken hoe een mixotrofe populatie zich gedroeg in een ééndimensionale omgeving, d.w.z. een omgeving met alleen een dieptestructuur, waarbij de mixotroof in staat wordt gesteld om te fotosynthetiseren en dode biomassa te mineraliseren. De keuze van een dergelijke simplificatie van een ecosysteem was gemotiveerd door de wens een dergelijk model in te kunnen bouwen in oceaan-circulatiemodellen. De keuze blijkt ook gerechtvaardigd in die zin dat de voorspelde diepteprofielen realistisch zijn.

Een uitgebreide studie naar de ecofysiologie van *Emiliania huxleyi* is gedaan m.b.v. licht- en substraatgelimiteerde continucultures met als doel om data te genereren voor ondersteuning van het DEB-model en ter vergroting van het inzicht in de ecofysiologie van calcificerende microalgen (NIOZ, Texel). De resultaten bevestigen eerdere observaties van een hoge affiniteit voor licht (51 10^{-3} m^2 s μmol^{-1} d^{-1}), vergelijkbaar met specialisten zoals *Synechococcus* en *Prochlorococcus*. De affiniteit voor nitraat was laag (0.37 L μmol^{-1} h^{-1}) terwijl die voor fosfaat exceptioneel hoog was (20 L μmol^{-1} h^{-1}). Nog nooit werd een hogere waarde voor microalgen gerapporteerd. Een andere unieke eigenschap kwam aan het licht met de ontdekking van de twee verschillende alkaline-fosfatasen die door deze alg geproduceerd kunnen worden wanneer fosfaat limiterend is voor groei. Deze unieke eigenschappen maken *E. huxleyi* bij uitstek geschikt om de competitie om fosfaat te winnen van andere algen. Bloeien van *E. huxleyi* kunnen daarom vooral verwacht worden wanneer er veel licht en nitraat in de omgeving is en fosfaat alleen beschikbaar is in lage hoeveelheden als organisch fosfaat. Fixatie van CO_2 door deze belangrijke calcificeerder zal daarom vooral optreden in P-gecontroleerde gebieden. Gedurende het ecofysiologische onderzoek is een waardevolle databank ontstaan die gebruikt kan worden (en al gebruikt is) voor de DEB-modellen (Vrije Universiteit van Amsterdam); er zijn monsters genomen om de nieuw ontwikkelde moleculaire technieken (Universiteit van Leiden) toe te passen en de gegevens van twee steady-states zijn gebruikt in het DMSP-onderzoek aan de Rijksuniversiteit van Groningen.

Ondersteuning van de Dynamic Energy Budget theorie op het moleculaire niveau kan alleen door karakterisatie van metabole activiteit in reactie op omgevingsfactoren. Een moleculaire genetische methode om dergelijke metingen uit te kunnen voeren is ontwikkeld aan de Rijksuniversiteit Leiden. Een aantal sleutelgenen zijn opgespoord en gecloneerd. De eerste mRNA expressiepatronen die met deze methode verkregen zijn lijken bruikbaar.

Twee genen die betrokken zijn bij calcificatie zijn gecloneerd: gpa en ale. Een ander gen, hpp, indirect betrokken bij calcificatie omdat het een fosfaatpermease codeert, is gecloneerd uit een cDNA bank van fosfaat-gelimiteerde cellen. De expressieprofielen die verkregen kunnen worden met deze methode zijn met name interessant vanwege de uitkomsten van het NIOZ deelproject van een hoge fosfaataffiniteit die gepaard gaat met een toename in calcificatie onder fosfaatlimitatie.
Expressie van fcp is een goede maat voor fotosynthese gebleken en expressie van pcna een goede maat voor celdeling terwijl de combinatie van expressieniveaus van pcna en fcp kunnen worden gebruikt om een indruk te krijgen van de mate van substraatlimitatie.
Ondanks het feit dat ontwikkeling en gebruik van deze nieuwe methode nog verre van compleet is kon worden vastgesteld dat voldoende vooruitgang is geboekt om met karakterisatie van de continuculturemonsters (afkomstig van het NIOZ deelproject) te starten. In het huidig onderzoek wordt deze aanpak gecontinueerd.

Het is bekend dat Prymnesiofyten voor het grootste deel verantwoordelijk zijn voor de productie van dimethylsulfoniopropionaat (DMSP), de precursor van het biogene gas dat zorgt voor wolkvorming na emissie in de atmosfeer. Toch ontbreekt veel elementaire kennis, zeker wat betreft de invloed van omgevingsfactoren op de productie van DMSP. De omgevingsfactoren licht, temperatuur, zoutgehalte en de verhouding waarin P en N voorkomen in het milieu zijn onderzocht op hun invloed op DMSP productie door *E. huxleyi* in het deelproject aan de Rijksuniversiteit Groningen. Zoals verwacht bleek de zoutconcentratie van het medium rechtstreeks van invloed op de concentratie van DMSP in de cellen. Deze waarneming bevestigt het vermoeden dat DMSP in de cel fungeert als osmoliet. Hoewel deze functie voor *Emiliania* nog niet eerder echt was bewezen is de waarneming van weinig ecologisch belang omdat *E. huxleyi* vooral voorkomt in milieus waar de saliniteit nauwelijks fluctueert. Interessanter zijn de factoren licht en temperatuur, die niet alleen variëren door de dag maar ook op langere termijn. Er is geen effect van lichtsterkte (range 10-140 µmol photons m^{-2} s^{-1}) in batchcultures gevonden maar er was een klein verschil tussen twee ‘steady states’ in de continuculture waarbij de hoogste lichtsterkte (100 versus 6 µmol photons m^{-2} s^{-1}) een hogere cellulaire DMSP-concentratie had. Temperatuur bleek van grotere invloed waarbij de hoogste concentraties bij lage temperatuur gevonden werden. DMSP is klaarblijkelijk een osmoliet dat probleemloos opgehoopt kan worden in algencellen, vooral bij lage temperaturen. Een vergelijkbare respons is eerder waargenomen bij macroalgen. Mocht deze fysiologische eigenschap karakteristiek zijn voor (micro)algen in het

algemeen dan is zeker geen toename in de DMSP-concentratie te verwachten als de zeeën warmer zouden worden door het broeikaseffect. Een terugkoppelingsmechanisme zoals voorspeld in de GAIA-theorie waarbij een toename van algen en DMSP wordt verwacht bij toenemende temperatuur met als uiteindelijk resultaat een reductie van zoninstraling door wolkvorming lijkt niet waarschijnlijk op grond van de hier gepresenteerde gegevens. Toch is het moeilijk om op grond van deze waarneming te stellen dat er minder DMS geproduceerd zal worden als gevolg van het broeikaseffect. Het is onmogelijk te voorzien hoe de andere processen die de uiteindelijke emissie van DMS naar de atmosfeer bepalen beïnvloed zullen worden door aardse opwarming (competitie tussen algen, activiteit van DMSP-consumerende bacteriën, overdracht van DMS uit water naar atmosfeer). Uitkomst van het onderzoek aan de Rijksuniversiteit Groningen komt wel goed overeen met de relatief lagere concentraties van MSA (methaansulfonaat, het belangrijkste oxidatieproduct van DMS) aangetroffen in ijslagen die ontstaan zijn tussen ijstijden.

Zowel N- als P-limitatie hadden een verlaging van de DMSP concentratie tot gevolg; de hoogste concentraties werden gevonden bij een verhouding gelijk aan de Redfied-ratio (de verhouding die doorgaans in algen wordt aangetroffen). Dit resultaat weerlegt de hypothese dat meer DMSP geproduceerd wordt onder N-limitatie ter vervanging van de N-analoog glycine-betaine.

Een andere, meer recente geformuleerde, hypothese stelt dat DMSP-overproductie kan optreden wanneer cellen gestresst zijn en daardoor overgaan tot eiwitrecyling. Hierdoor ontstaat een overmaat aan zwavelhoudende aminozuren die in de vorm van DMSP weggewerkt zou kunnen worden. Stress geïnduceerd door UV-straling bleek geen enkel effect op de DMSP-concentratie te hebben in *E. huxleyi*.

Gedurende het onderzoek naar deze milieufactoren is gebleken dat het voor fysiologische interpretatie noodzakelijk is om DMSP uit te drukken als DMSP per hoeveelheid biovolume (maat voor concentratie). De hoeveelheid DMSP per cel levert vaak een ander en soms zelfs tegengesteld beeld op omdat het celvolume ook door milieufactoren beïnvloed wordt.

Het voorliggende onderzoek heeft waardevolle kennis opgeleverd voor het begrip van het succes van coccolithoforiden die zo belangrijk zijn voor het fixeren van CO_2 uit de atmosfeer. De belangrijkste milieufactoren zijn onderzocht op hun betekenis voor DMSP-productie waarbij een niet met de gangbare ideeën strokende rol van temperatuur werd gevonden. Verder zijn moleculaire technieken ontwikkeld die gebruikt kunnen worden voor het registreren van metabolische activiteit in de cel. De schat aan gegevens ontstaan in het project

kan goed gebruikt worden om Dynamische Energie Budget modellen te ondersteunen. De DEB-theorie zelf is nu ook geschikt gemaakt voor autotrofe calcificerende organismen en de eerste resultaten op ecosysteemniveau zijn behaald.

2. INTRODUCTION

2.1 Dynamic Energy Budgets (DEB) and the Global *Emiliania* Modelling (GEM) Initiative

There is no doubt that life is a major participant in the climate system. Yet, current global climate models seem to play down the overwhelming evidence. The highly non-linear behaviour of living systems seems to preclude the formulation of models that can represent the biological component of climate in a reliable way. We believe that an adequate representation of biology in the Global Climate Models (GLMs) is the major challenge for climatology. Can we discover a deep logic of life, consistent with the principles of physics and chemistry, to which we can adapt the models?

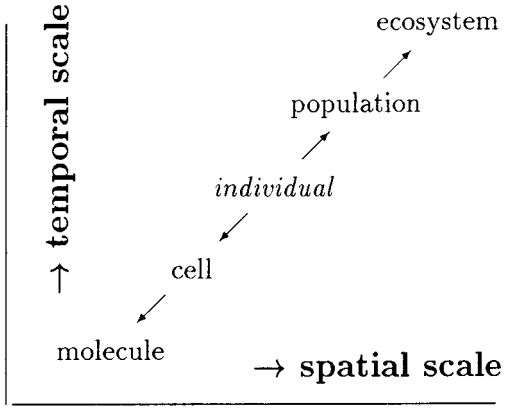

Figure 1. The DEB theory specifies a model for the individual, which is used to model populations in terms of individuals and ecosystems in terms of populations. We simplify while increasing the level of integration, and incorporate new processes that typically operate at the level of organisation. The model for the individual serves as a constraint for modelling sub-organismal processes that specify details of the (physiological) behaviour of the individual, such as regulation processes. This approach implies a link between scales and time, which excludes rapid processes at large spatial scales and slow processes at small spatial scales.

We cannot be sure, but the theory of Dynamic Energy and Mass Budgets (DEB) by Kooijman and his collaborators provides the best possible point of departure (Kooijman, 2000). This theory predicts that life cannot be understood as a single entity, but is cast into at least four

'levels of organisation' with increasing magnitude – biomolecules, organisms, ecosystems and the biosphere (Fig. 1). Together, these levels form a nested complex, whereby the dynamics at one level determines the boundary conditions under which the ones above and below can operate. For instance, the range of chemically possible biomolecular combinations forms the upper limit to cellular diversity, whereas ecological viability determines which of these possible cells are actually realised. Thus, if we wish to model the climatic effects of life at the global level, we cannot avoid taking the underlying levels into consideration.

What appears to be an impossible task may be feasible after all. In the first place, DEB is solidly founded on at least 20 years of experience. But more importantly, the work so far revealed a highly remarkable simplicity underlying the diversity of life, at least at the level of individual organisms. It turns out that simple models can reliably represent the individual's metabolism, and that widely different organisms obey to the same basic rules. It is like a phase change in physical systems: precisely at the organismic level, the circuitous maze of metabolic pathways snaps into a simple pattern. If simplicity were also to emerge at the levels above and below the organism, we are in good shape for climate modelling.

This is the ultimate goal of DEB: to formulate a coherent and predictive theory of life. Expressed as a nested suite of compatible models for all levels of organisation, it will demolish the boundaries between biology, physics and chemistry. With the theory for organisms as a base, DEB presently moves down to the molecular level and up to the ecosystems and the biosphere. Although considerable theoretical advances are being made, we need, at all levels, a suitable experimental database for validation. This is the goal of GEM, the Global Emiliania Modelling Initiative, of which this project forms a part.

The three major mechanisms that are distinguished by which the marine pelagic biota may influence the global climate system are combined in the unicellular marine alga *Emiliania huxleyi*: (1) the drawdown of CO_2 from the upper mixed water layer and the atmosphere to intermediate and deep waters by the formation, export and remineralisation of particulate organic carbon (the organic carbon pump); (2) the production, downward transport and partial dissolution of calcium carbonate (the carbonate pump); and (3) large-scale albedo effects mainly owing to the formation of highly reflective clouds following gaseous emissions of dimethylsulphide (DMS) from algal blooms (Fig. 2). Therefore, *Emiliania huxleyi* is exceptionally well suited for providing the kind of data we need. The organism is cultured in the laboratory and studied in microcosms, in the ocean at large, from satellites and in the geological record. The formation of its 'coccoliths', highly organised skeletons of calcium

carbonate, is one of its characteristic features with implications for the global carbon cycle and climate. GEM uses *Emiliania* as a model organism to help in the development of DEB theory and global climate modelling in general.

The development and validation of a model on the organismic level with *Emiliania huxleyi* was the focus of this NOP-II research project. There was a theoretical part in which the DEB model was further developed, and an experimental part that provided opportunities to validate the model.

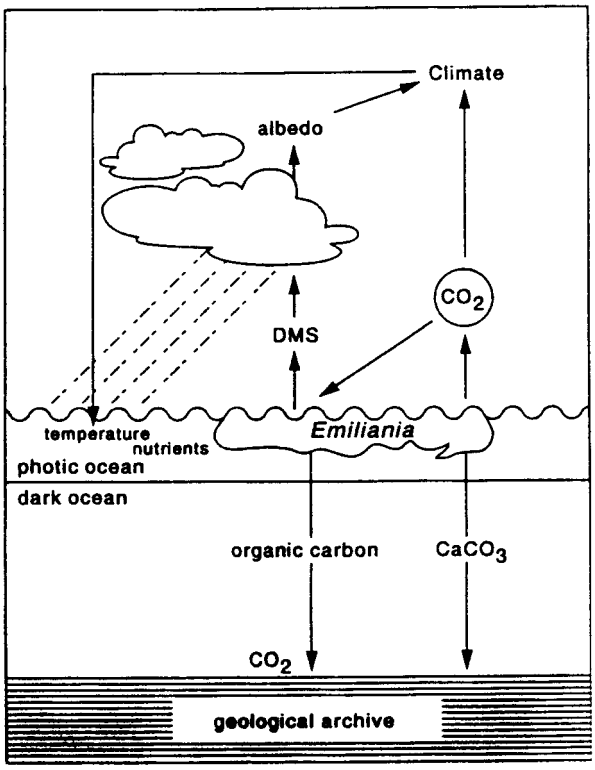

Figure 2. Coupling of three climatic effects of *Emiliania* blooms: organic carbon pump (leading to lowering of atmospheric CO_2), $CaCO_3$ counter pump (increasing atmospheric CO_2) and DMS emissions leading to the formation of white clouds, increasing the earth's albedo.

2.2 Theoretical part: the next step in DEB

In the last few decades, a steady flow of information became available on coccolithophore biology and climate forcing. Since 1989, when GEM was created, the information stream was greatly amplified by the concerted effort of more than 60 scientists in some 30 institutions world-wide (Including the NOP I project: phytoplankton and the oceanic carbon cycle;

Emiliania huxleyi as a model system, Westbroek et al. rapport 410100023). Some major results of this research were the following:

– GEM has provided experimental evidence that coccolith biosynthesis allows the cell to utilise HCO_3^- as a carbon source when CO_2 is in short supply, a condition which may commonly occur in bloom situations. The problem is particularly important as calcification reduces the alkalinity and hence influences the speciation of dissolved inorganic carbon in the surrounding ocean water.

– *E. huxleyi* blooms are major contributors to DMS fluxes into the atmosphere above the open ocean, particularly in the N. Atlantic. A spatial and temporal correlation between the blooms and albedo increase has been detected.

– In situ measurement of coccolith (export) production in various *E. huxleyi* blooms indicates that the total annual oceanic uptake of atmospheric CO_2 in these settings is reduced by 17-25% as a result of calcification.

– GEM's geological program indicates that the geographical distribution of *Emiliania* blooms is very sensitive to climate change.

The existing models on algal growth to date do not suffice for GEM (e.g. Arrigo & Sullivan 1994; Droop 1983; Kiefer & Mitchell 1983; Laws & Chalup 1990; Morel 1987; Sakshaug et al. 1989). A considerable number of these are restricted to steady state growth (e.g. Droop 1983; Kiefer & Mitchell 1983; Sakshaug et al. 1989). Although these models have provided considerable insight, they necessarily neglect the important dynamic environmental fluctuations in nutrient and light availability that may dominate algal growth dynamics in natural environments. Models based on variable internal stores (VIS-models; e.g. Shuter 1979; Laws & Chalup 1990) are potentially more suited to deal with such fluctuations. However, even these VIS-models assume nutrient saturation in the environment, a situation which is unlikely to be always fulfilled in the environment. Models dealing with light and nutrient limitation either state different equations for both situations (e.g. Kiefer & Mitchell 1983) or neglect internal stores and possible interactions between light and nutrient metabolism (e.g. Arrigo & Sullivan 1994).

On top of all this GEM needs a model at the organismic level that can easily be extended to the ecosystem and biosphere level. The research reported herein forms a limited, but central component of the GEM project extending the Dynamic Energy Budget model of Kooijman (1993) to include autotrophic and calcifying systems.

2.3. Experimental approach

2.3.1 *Emiliania huxleyi* chemostat steady state values

Modelling of *E. huxleyi* growth requires a strictly unambiguous data set, based on chemostat cultivation under continuous illumination. However, the considerable body of physiological information published on *E. huxleyi* was predominantly derived from less constrained experimental conditions, mostly batch cultures or semi-continuous cultures (e.g. Balch et al. 1992; Van Bleijswijk et al 1991; Nimer & Merrett 1992). The primary objective of this subproject was to provide a data set required for underpinning of the modelling study described above. The impact of three variables, viz. nitrate concentration, temperature and light availability which are considered to be the principal factors determining the growth, and $Ca(CO_3)_2$ production of *E. huxleyi* were investigated in chemostat experiments.

2.3.2 Calcification, photosynthesis and nutrition: down to the level of genes

Coccolith production by *Emiliania huxleyi* beautifully illustrates the need for DEB theory in climate modelling. All calcium carbonate in the ocean is formed under the influence of biological systems. This implies that the production of calcium carbonate is not determined by physico chemical factors, such as the level of supersaturation of the ocean water. The control is biological, as it depends on the outcome of the competition between the calcifiers and the non-calcifying organisms.

Thus, an in-depth investigation of biological calcification is immediately relevant for climate research. We need to know what advantages and disadvantages calcium carbonate production has for organisms; how the calcifying machinery fits in the cellular organisation; and how and when this machinery is switched on and off. Furthermore, we wish to understand how carbonate crystallisation is controlled at the molecular level. Calcification has emerged many times in evolution. Do all these calcifying machinery's revert to the same basic principle, or are they all different? Can we use *E. huxleyi* as a representative model organism, or do we need to study many calcifiers in parallel? The implementation of DEB requires that we find answers to questions such as these.

Continuous cultivation was applied in order to characterise the response of the organism to a range of external conditions, such as variations in light intensity and nutrient availability. In

the past, the response patterns could only be characterised in a gross way, ignoring specific molecular aspects. The molecular genetic approach introduced in this research permits following of the response in unprecedented detail.

The idea underlying this project is to sequence genes encoding many key proteins, and to quantitatively monitor the expression of these genes at the mRNA level as a function of the growth conditions. As metabolic regulation is not always reflected in expression at the level of mRNA, we also envision quantification of the activity of a number of known enzymes.

The intention of this project was to lay the foundation for this biomolecular approach. In order to contribute to the comparison between different coccolithophore species we concentrated not only on *E. huxleyi* but also on *Pleurochrysis carterae*, a related organism extensively studied at the physiological and biochemical levels.

2.3.3 DMSP production by *Emiliania huxleyi*

Ever since the discovery in 1972 of the missing link in the global sulphur cycle (Lovelock et al.), which was found to be the volatile component dimethylsulphide (DMS), scientists have been speculating about the role of this gas in climate regulation. Most DMS has its origin in seas and oceans; it is produced during interactions in the plankton food web, and a variable percentage is emitted to the atmosphere (Fig. 3). After emission, DMS is known to initiate the formation of cloud condensation nuclei that affect cloud reflectiveness (albedo). The possible impact of DMS sea-to-air fluxes on climate is illustrated by the CLAW feedback hypothesis formulated in the eighties by, and named after, Charlson, Lovelock, Andreae and Warren (1987). It states that if an increase in ocean temperature (e.g. due to global warming) leads to an increase in the production of the DMS precursor dimethylsulphoniopropionate (DMSP) and thus to DMS, this would result in a cooling effect of the planet by an increase in cloud albedo.

Marine microalgae are the main producers of DMSP (Keller 1991). The amount of DMS that is released directly from intact cells has been reported to be quantitatively insignificant (Turner et al. 1988, Stefels en van Boekel 1993, Wolfe and Steinke 1996). There is evidence for a physical separation in the cell of DMSP and DMSP-lyase (the enzyme that cleaves DMSP into DMS and acrylate). The substrate is most likely present in the cytoplasm while the enzyme is located externally in the case of *Phaeocystis globosa* (Stefels and Dijkhuizen 1996), and in vacuoles in *Emiliania huxleyi* (Steinke et al. 1998).

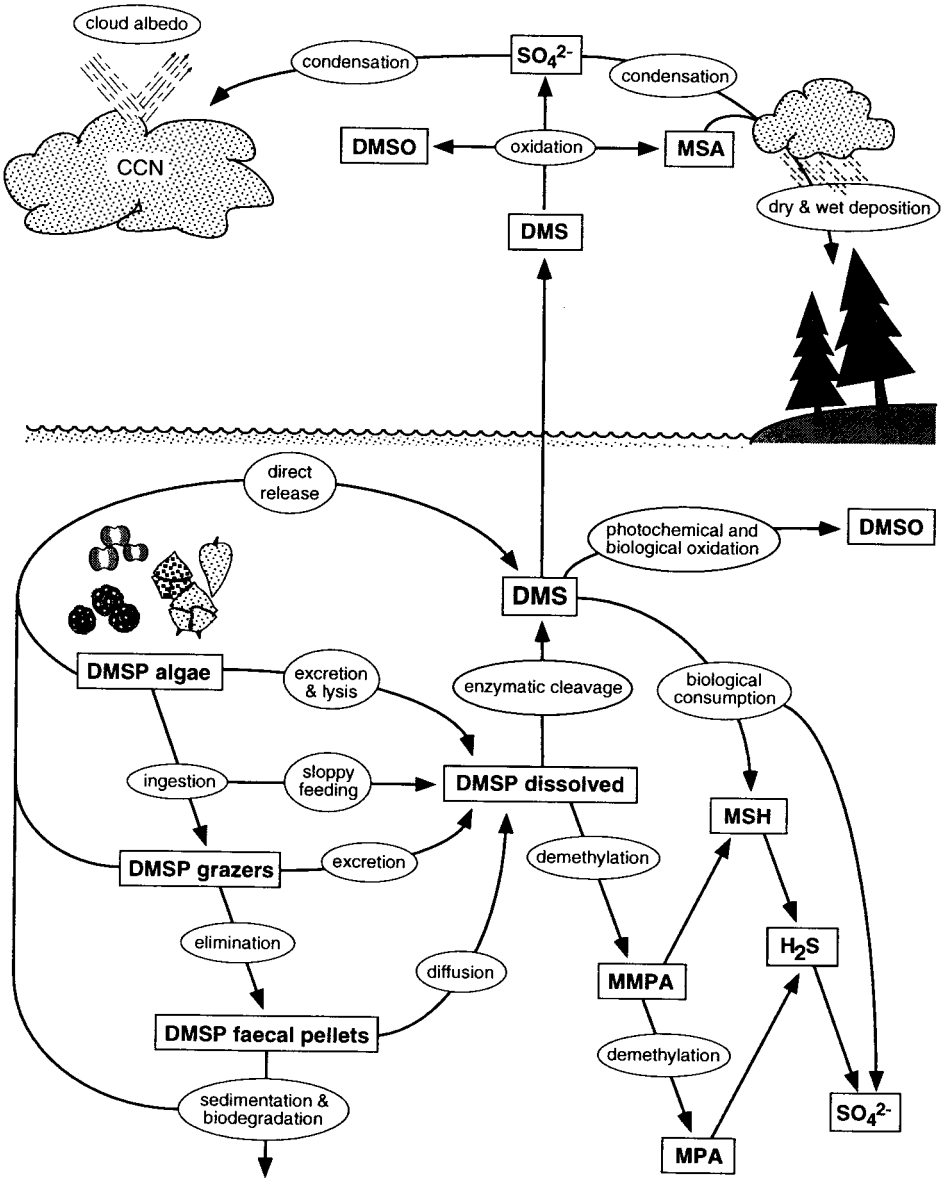

Figure 3 Schematic representation of the processes involved in the marine sulphur cycle. The right-hand side of the marine processes are covered by bacteria, the left-hand side by phyto- and zooplankton. Phytoplankton and bacteria can both be responsible for the enzymatic cleavage of dissolved DMSP (J.Stefels 1997).

Most of the DMSP that is produced by the algae can enter the environment via cell death (resulting in release of its contents), viral mediated lysis (Bratbak et al 1995) or via grazing (Levasseur et al 1996, Wolfe and Steinke 1996, Wolfe et al 1994). A small fraction is excreted directly (Laroche et al 1999). The dissolved DMSP is degraded by bacteria either via a DMSP-lyase pathway or via a demethylation pathway that does not result in DMS

production (Fig. 3). In fact a recently formulated ‘availability theory’ (Kiene et al 2000) states that DMSP is metabolised mainly via the demethylation pathway but when DMSP is produced in high amounts such as in algal bloom situations the cleavage pathway will prevail and more DMS will be formed.

The role of algae in DMS production in the ecosystem seems to be confined to the production of the precursor only. Prerequisite for a model that describes DMSP output as a function of environmental factors is the identification of factors such as nutrient concentration, light and temperature that are relevant for the model. Since the most important global change issue is the expected future temperature increase, emphasis was put on the influence of temperature on *E. huxleyi's* DMS(P) production in our experimental efforts.

3. PROGRESS IN THE DYNAMIC ENERGY BUDGET THEORY

Mircoalgal growth models aim at predicting growth rate as well as cellular composition, given the environmental conditions (such as temperature, nutrient availability, light etc.). This generic purpose poses strong requirements to the structure of any model attempting to comply with it. First, cellular characteristics should be modelled, rather than taken as model input. Second, if one interprets 'cellular composition' as nutrient cell quota, rather than as nutrient ratios, models should be formulated at the level of the individual cell. Third, modelling should proceed by making assumptions about processes, rather than about states.

At the onset of our project, we evaluated contemporaneous modelling methodology in the light of these desiderata. We concluded that current modelling could be improved upon considerably to fulfil requirements posed by generic purposes (Zonneveld 1998b). Models that chiefly aim to predict primary production are mostly formulated as steady state carbon budgets. These models lack state variables that are necessary to describe variation in cellular composition and often treat the Chl:C ratio as an input variable instead as a state variable. The basic point of departure of optimisation models is that cell partitions its recourses in such a way that the growth is maximised. Application of this principle is restricted to steady state growth. Whether this point of departure can also be applied to cells in a fluctuating environment remains unclear, yet a fluctuating environment will be the rule rather than the exception. Dynamic models are potentially best suited to study the regulation of growth in response to variations in the environment. However, these models did not take the cell as their basic point of departure.

Our primary aim in this project, then, is to bring modelling methodology more in line with the generic purpose. To comply with this aim, we propose models that explicitly obey mass conservation of all nutrients considered at the cellular level. Once such modelling strategy is available, it will be possible to make sensible prediction of organic and inorganic carbon production in relation to environmental conditions.

3.1. Nutrient limited growth

First, we paid attention to nutrient limited growth based on a model with variable internal stores (Zonneveld 1996). We described the kinetics of both limiting and non-limiting nutrients (Fig. 1). The need for this arises as soon as the model is to be applied in a variable environment. In such an environment one does not know beforehand which nutrient is

limiting, so all potentially limiting nutrients should be monitored. Concerning the limiting nutrient, the model provides an dynamic reconstruction for the well-established Droop equation for the limiting nutrient at steady state (Droop 1983). However, it also shows that there is no meaningful basis for the Droop equation for the non-limiting nutrient. Advantages of the model are that it can be extended to include more nutrients. The main difference between carbon and other nutrients is the photosynthetically driven uptake of CO_2. Once taken up, the kinetics of carbon may be similar to that of other nutrients.

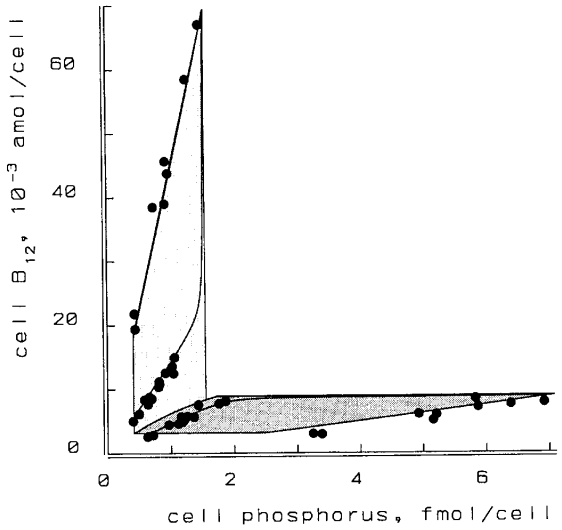

Figure 1 Relationship between cell quotas. The shaded area represents the set of all possible steady states of phosphate and vitamin B_{12} quotas on basis of parameter estimates (Zonneveld 1996). The lightly shaded area indicates a phosphorus limitation, the dark shaded area a vitamin limitation. The data of the four experiments (Droop 1974) are described by the model prediction. Points may lie outside the shaded area due to measurement errors.

A drawback of the model is the discontinuity of uptake of nutrients when a previously non-limiting nutrient becomes limiting. If a population of algae is simulated in a chemostat environment, the behaviour of the model can become erratic: a steady state may either be absent, or multiple steady states are possible (for certain values of the dilution rate and supplied nutrients). Although not relevant for most laboratory applications, the model behaviour caused concern for further application within a larger ecosystem framework. The problem hinges on the distinction between nutrients that are either fully limiting, or not limiting at all. Mathematically this means that a minimum-operator is part of the model. To solve the problems associated with such models, the concept of the synthesising unit as model for the stoichiometric fusion and branching of metabolic fluxes was postulated.

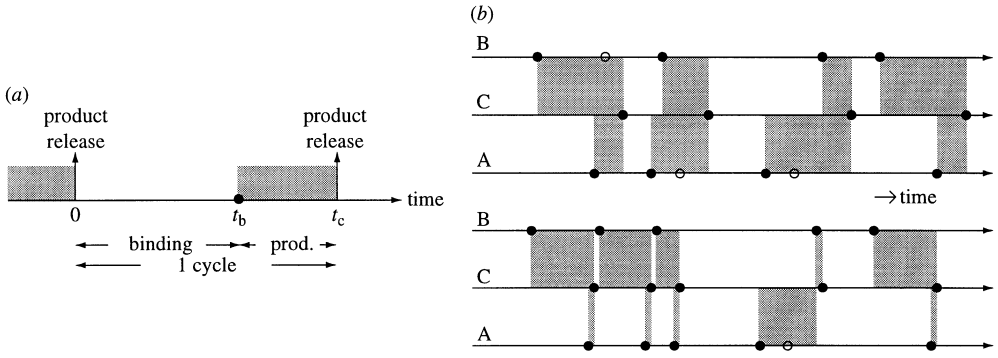

Figure 2 (a) This illustrates that the Synthesizing unit (SU) goes through a cycle consisting of a binding phase and a production phase. The production rate is inverse to the cycle time. (b) The transformation A + B → C, where A and B are bound at different sites on a SU. The SU switches to the binding phase upon the release of product C. The upper diagram represents a slow SU, the lower diagram a faster one: both experience the same substrate arrival process. The time axis labeled C shows the production events; the other time axes show arrival events of substrates. Filled dots stand for acceptance, open dots for rejection. The grey areas indicate periods during which the SU cannot bind substrate. Note that the fast SU still has substantial blocked periods due to the stochasticity of the (Poisson) arrival processes (Kooijman 2001).

The Synthesising Unit (SU) binds given numbers of substrate molecules of several types of substrate to produce a product molecule or set of product molecules. Irreversible binding results in relatively simple and explicit expressions for the rate of product formation (Fig. 2). Reversible binding can be implemented with relative ease in the carrier-SU complex, where the products of a set of carriers (a special type of SU) serve as substrate for an SU or set of SUs. A simple and parameter sparse approximation is presented for the production rate of a generalised compound, i.e. a rich mixture of compounds that does not change in composition (Kooijman 1998). An analysis of Droop's data on the growth of a haptophyte on phosphate and vitamin B12 reserves illustrated the application of SUs (Kooijman 1998, Fig. 5).

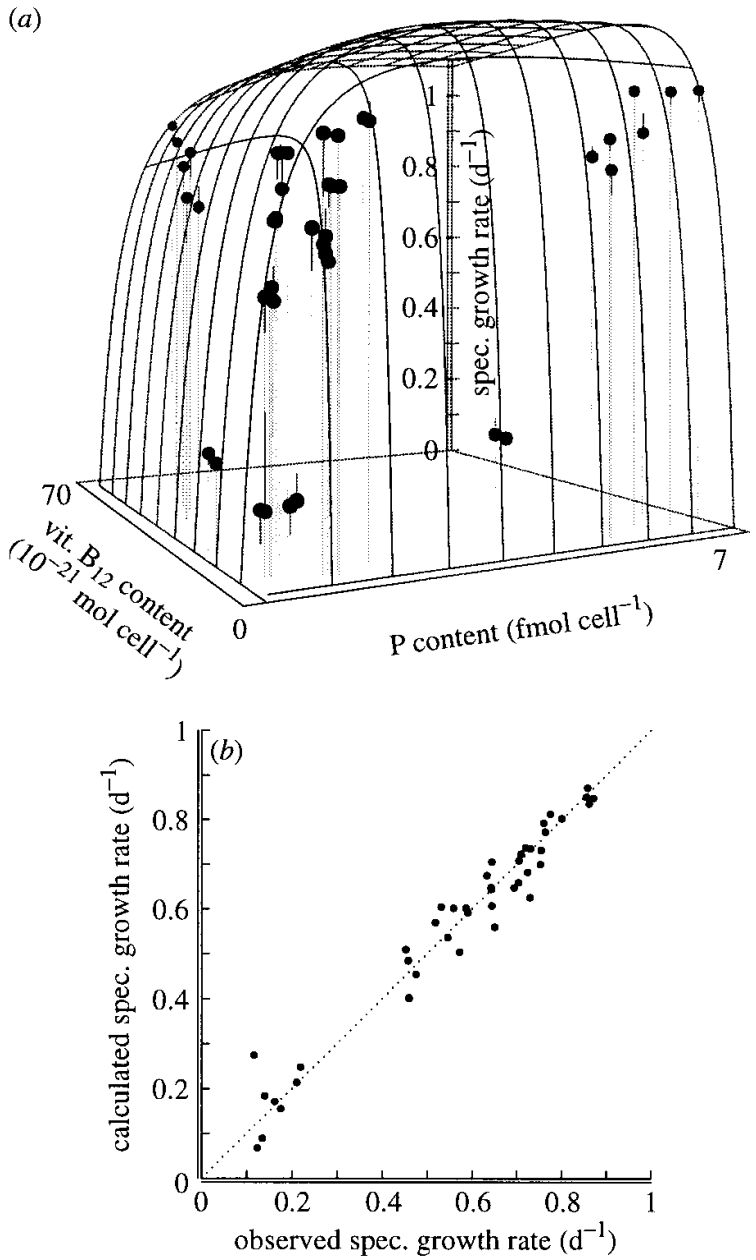

Figure 3 Specific growth rate of the haptophyte *Pavlova lutheri* as a function of intracellular reserves of phosphorus and vitamin B_{12} at 20 °C. Data from Droop (1974). The surface is based on kinetics of a fast SU for two complementary substrates, where the arrival rates are proportional to the reserve densities (as follows from the reserve kinetics in the absence of maintenance costs). Because the reserve turnover rates are equal, and the phosphorus and B_{12} concentrations in the structure are known, the surface has three parameters. (a) The difference between the data points and the surface is marked with line-segments. (b) Calculated values are plotted against observed ones to judge the goodness of fit. The absence of data points for high values of both reserves illustrates the damming up of one reserve due to growth limitation by the other (from Kooijman 2001).

3.2 Light limited growth

We constructed a model for light-limited growth, without taking either chlorophyll or the Chlorophyll: C ratio as model input (Zonneveld et al. 1997). This model is capable of accounting for variations in carbon cell quota with photon flux density. Two components were distinguished in the cell: permanent biovolume and a transient pool. In addition assimilation, maintenance and growth were distinguished as processes. The model is basically formulated in a dynamic way and accurately described the complete carbon budget of three different phytoplankton species (Fig. 5). In essence it can be regarded as a generalisation of the well-known Droop model for nutrient-limited growth. This indicates that light-limited and nutrient-limited growth are less different than previously thought. (This model was used to describe the light-limited data obtained with *E. huxleyi* in turbidostat, Chapter 4, Stolte et al. in prep).

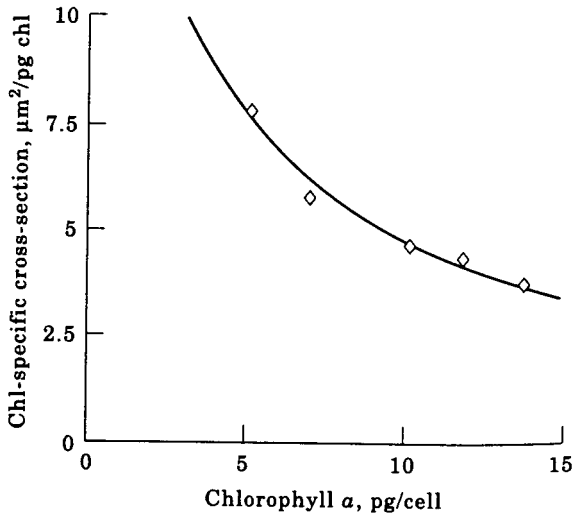

Figure 4 Dependence of chlorophyll specific absorption cross section on cellular chlorophyll concentration, in the diatom *Thalassiosira weisfloggi*. The maximum cellular cross section equals 63.4 μm^2 $cell^{-1}$, and the true chlorophyll-specific absorption cross section equals 1.93 dm^2/mg chl *a*. Zonneveld (1997), data from Falkowski et al. (1985).

The process of photosynthesis was studied in more detail by an analysis of the effect photo-acclimation on photosynthesis-irradiance curves in phytoplankton (Zonneveld 1997). Four parameters are presumably affected by photo-acclimation: (1) the size of a photosynthetic unit; (2) the number of photosynthetic units per cell; (3) the average turnover time of a photosynthetic unit; and (4) the chlorophyll-specific absorption cross-section. Prezelin's well-

known conceptual model of photo-acclimation deals with variation in size and number of photosynthetic units, but not with the other two parameters (Prezelin, 1981). Prezelin's model predicts that the photosynthesis-irradiance curve is differentially affected by variations in size or number of photosynthetic units. By contrast, our analysis showed that if photo-acclimational variation in the turnover time is also accounted for, only the cellular chlorophyll concentration is relevant; variation in the size vs. number of photosynthetic units is immaterial.

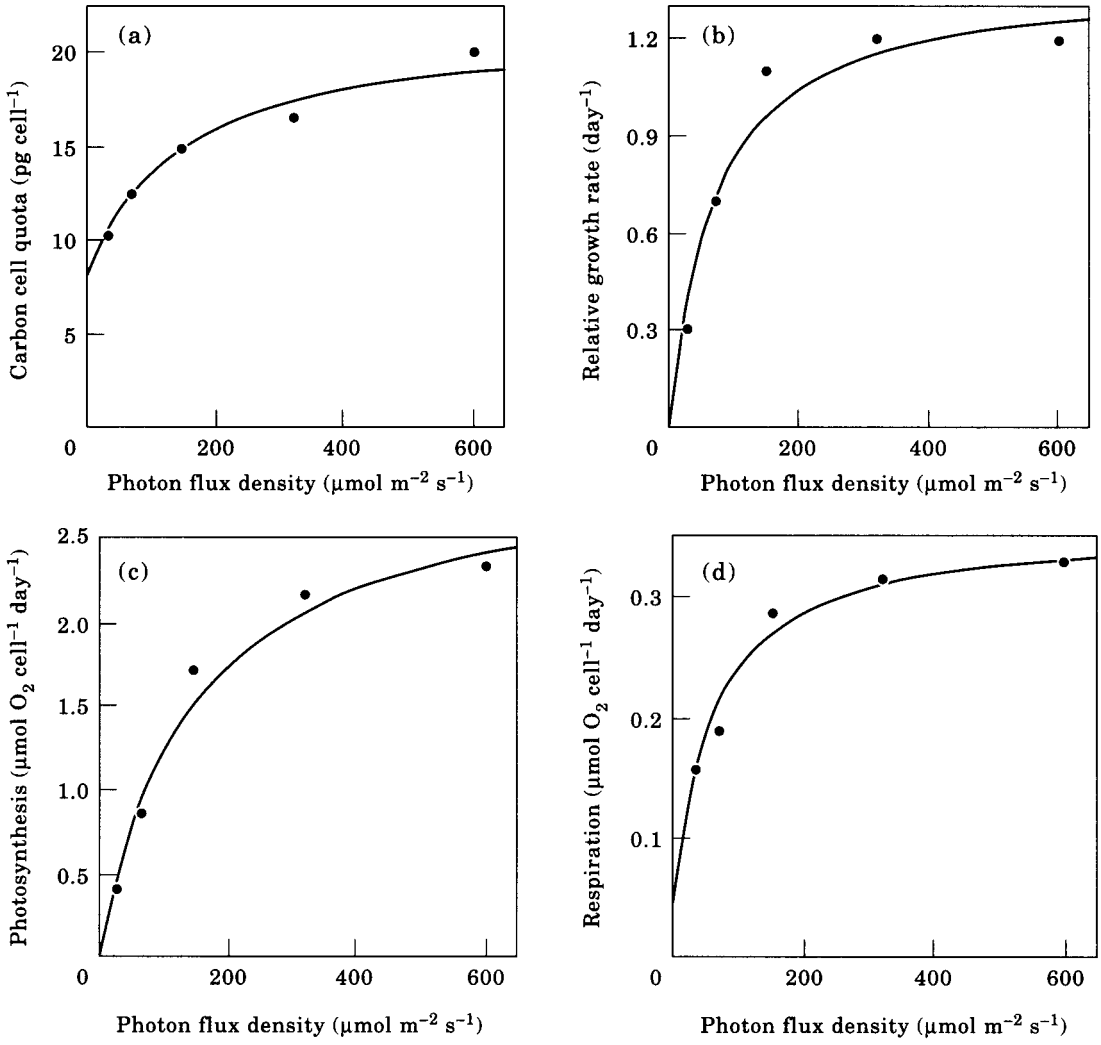

Figure 5 (a) Light dependence of carbon cell quota, (b) specific growth rate, (c) gross photosynthesis, (d) and respiration for *Isochrysis galbana* (Zonneveld et al. 1997, data from Falkowski et al., 1985)

The photo-acclimation response of the chlorophyll-specific absorption cross-section allows a simple description consistent with experimental data (Fig. 4). This description was used to derive a single expression for the rate of photosynthesis as dependent on irradiance and the cellular chlorophyll concentration (it does take chlorophyll as model input; in a different paper we describe how chlorophyll cell quota can be modelled). This model for the photosynthesis-irradiance-curve accounts for the photo-acclimational alterations of the above mentioned parameters. Changes in these parameters occur at the time scale of about one day. On a much shorter time scale, high irradiances can lead to diminished rates of photosynthesis, a phenomenon referred to as "photo-inhibition". Photo-acclimation and photo-inhibition both affect the PI-curve, but existing models for the PI-curve did not account for both phenomena at once. Therefore, we described changes in the photosynthetic machinery relevant at the short time scale characteristic of photo-inhibition (Zonneveld 1998a). Photo-acclimation is accounted for by variations in parameter values in response to changes in the chlorophyll content of the cells, the hallmark of photo-acclimation. The rate of photosynthesis depends on the photon absorption rate rather than on irradiance. The photon absorption rate is the product of the irradiance and the cellular absorption cross section. Photo-acclimation affects the cellular absorption cross section through its effects on the chlorophyll content of the cells. As a result, photo-acclimation affects the photon absorption rate, and so indirectly the sensitivity to photo-inhibition at a particular irradiance.

Many mathematical formulations aim to describe the photosynthesis-irradiance curve. However, none of them refers explicitly to the enzymatic part of the entire process, the so-called dark reaction. This involves the enzymatic binding of CO_2, driven by ATP en NADHP generated by the light reactions. As a final element in our study on photosynthesis we formulated a model that explicitly accounts for the enzymatic nature of the dark reactions (Zonneveld submitted). This results in a photosynthesis-irradiance curve that saturates much more steeply than the often-used hyperbola. This allows a much more accurate description of experimental data regarding the photosynthesis-irradiance curve. The approach combines well with the earlier study on photo-acclimation.

Finally, we bridged the gap between existing models and our dynamic models by the construction of a cell-based model for the chlorophyll *a* to carbon ratio in phytoplankton. The model describes carbon and chlorophyll a cell quota, and thus the chlorophyll a to carbon ratio, in nutrient-limited as well as light-limited growth (Zonneveld 1998c, Fig. 6,7). The model predictions are in line with experimental data. Variations in Chl:C result, among others, from photo-acclimation. The analysis in this paper shows that the impact of photo-acclimation on phytoplankton growth is rather small, despite large variations in Chl:C. This is mainly due to the package effect, a phenomenon that can be easily accounted for especially in cell-based models.

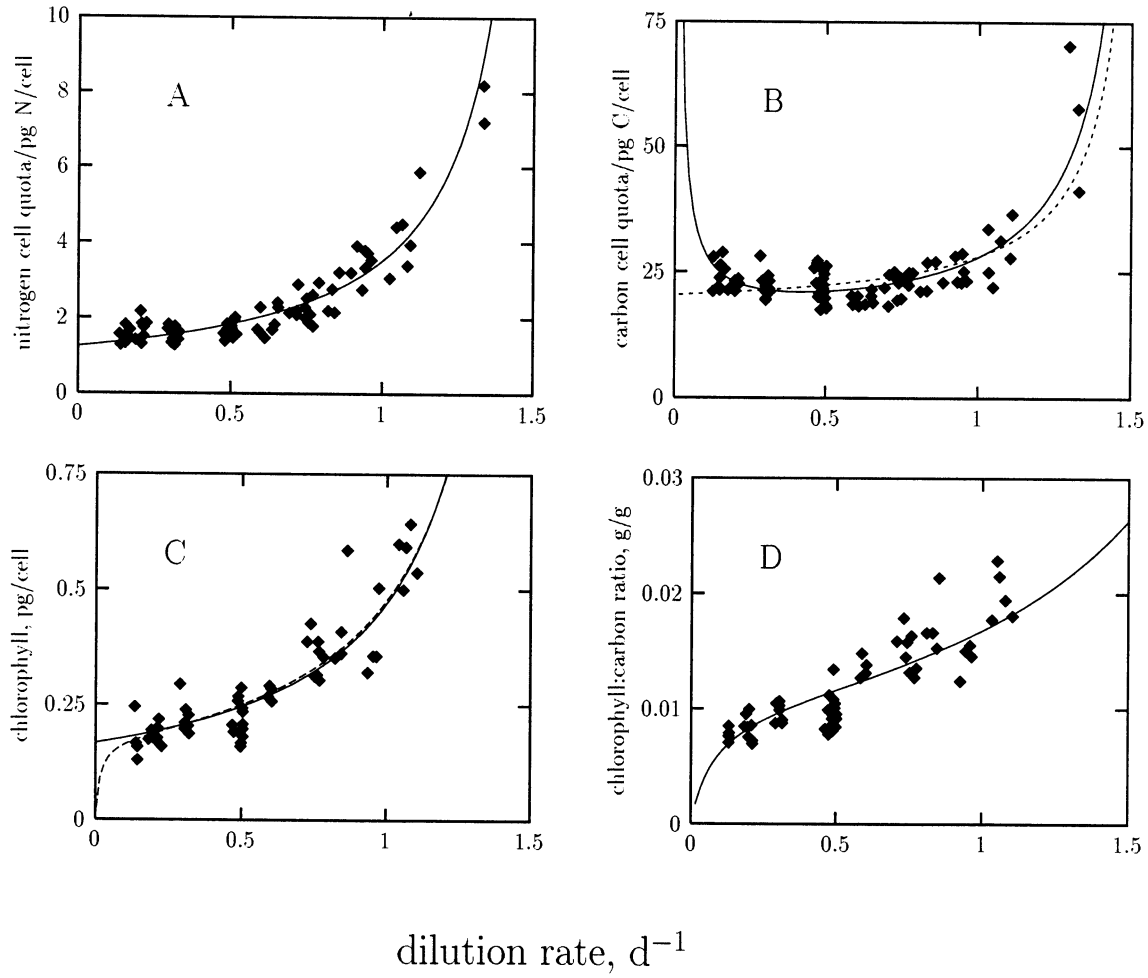

Figure. 6 (a) Nitrogen cell quota, (b) carbon cell quota, (c) chlorophyll cell quota and (d) Chl:C in nitrogen-limited *Dunaliella tertiolecta*. The parameter values were simultaneously estimated from the data on nitrogen, carbon and chloprophyll cell quota. Zonneveld (1998), data from Goldman and Peavey (1979) and Goldman (1980).

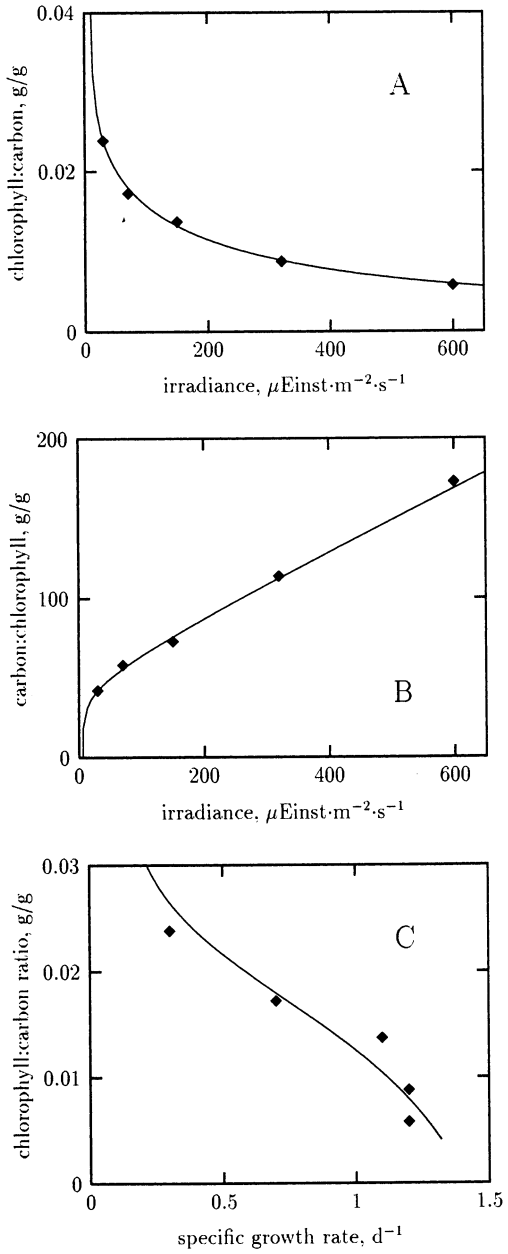

Fig. 7 (a) Chlorophyll:Carbon ratio; (b) Carbon : chlorophyll ratio, both as dependent on irradiance; and (c) chlorophyll: carbon ratio as dependent on the specific growth rate of *Isochrysis galbana*. Zonneveld (1998), data from Falkowski et al. (1985).

3.3. Calcification

An important characteristic of Coccolithophorids in general is their ability to utilise bicarbonate in the production of calcium-carbonate. The function of this ability is still a matter of debate. One important hypothesis holds that the primary aim of calcification is to produce CO_2 for photosynthesis. In the marine environment most of inorganic carbon is

present as bicarbonate, with only about 1% present as CO_2. This molecule is thus in rather short supply, and the additional calcification-derived CO_2 might well give Coccolithophorids a competitive advantage over other groups. This attractive hypothesis is mute with respect to the intricate shape of the coccoliths, but can explain several observations about physiology.

Although several other hypotheses concerning the function of calcification are possible, we only modelled the one just described. Kooijman (2000; p 167-168) formalises this hypothesis to arrive at, among others, quantitative formulations about relationships between light intensity and calcification. This submodel is currently under investigation. Preliminary results suggest that to accurately describe the data on the organic:inorganic carbon ratio, it might be necessary to include an additional state variable in the model, namely the internal CO_2 pool of the cell.

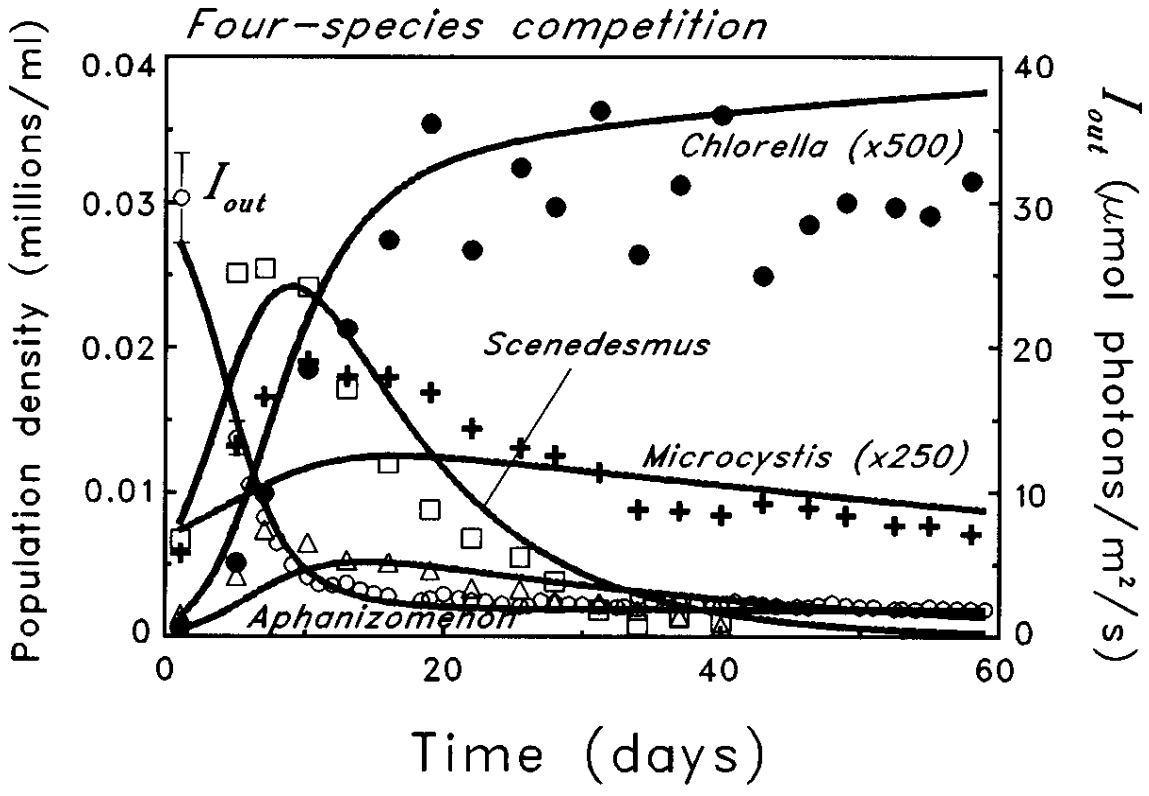

Fig. 10 Competition for light between four species: *Chlorella* (closed circles), *Scenedesmus* (open squares), *Aphanizomenon* (triangles), and *Microcystis* (plusses). Open circles indicate the light intensity penetrating through the cultures. Solid lines show the time course of competition predicted by the model. For details see Huisman et al. (1999).

3.4. Towards ecosystem modelling

The direct aim of our project was to develop a modelling strategy that allows accounting for the impact of environmental variation on cellular characteristics. However, the project is part of our research program, which ultimate aim is to make confident predictions concerning the interactions between biota and climate. The modelling strategy is a necessary prerequisite to fulfil this ultimate aim. A next step in the direction of our aim is to study how model ecosystems based on the models developed behave. We did two such studies as the final part of our project.

First, we investigated the properties of our model-algae in competition experiments (Huisman et al. 1999, Fig. 8). In line with the distinction between nutrient-limited and light-limited growth competition was also studied for these two limitations separately. Especially the study on competition for different nutrients exposed the weakness of the use of the minimum operator, as steady states were often not attained. Despite this drawback, we were able to specify whether competition is possible or not. Although current theory on competition can be refined somewhat, its major results remain untouched.

Second, we studied a so-called canonical ecosystem in a closed and homogeneous environment: life in a closed bottle (Kooijman and Nisbet 2000, the system is closed for nutrients, but open for energy.) This ecosystem contains three populations: an autotrophic producer, a heterotrophic consumer and a hetetrophic decomposer; for example, a phytoplankton – ciliate – bacteria system. Of direct interest are the partitioning of various nutrients over the three populations, and the rate of cycling between them. Furthermore, we studied the impact of light on these characteristics.

Third, and final, we made a first step to implement the modelling strategy into a spatially heterogeneous environment by studying the performance of a one-species community of a mixotrophic organism (Kooijman et al. submitted). The hypothetical mixotroph is able to photosynthesise, as well as to consume dead biomass. The choice for a further simplification of the ecosystem involved is motivated by future plans for implementation into ocean circulation models. At the same time, as this model is thoroughly based in DEB theory, it is still relevant for reality. Only if we understand the most basic situations, we might hope for an understanding of more complex systems. The study shows vertical profiles for the distribution of living and dead biomass, as well as the nutrients involved, and how light triggers nutrient cycling.

4. PHYSIOLOGICAL STUDIES ON *EMILIANIA HUXLEYI* AT STEADY STATE CONDITIONS UNDER LIGHT, PHOSPHATE OR NITROGEN LIMITATION

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. Cellular composition, including C, N, P, pigments, and calcite, was determined for every steady state (Tables 1, 2, 3). All analysis were carried out in duplicate or triplicate. The results were compared with the ecophysiology of other marine phytoplankton species to evaluate the competitive ability of *E. huxleyi* under various environmental conditions. More details can be found in Riegman et al. (1998).

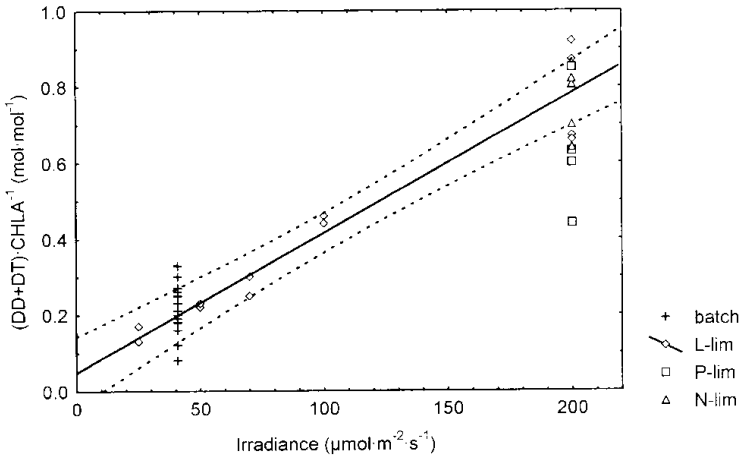

Figure 1 (DD+DT)/Chl *a* ratios as function of photon flux density used to culture *Emiliania huxleyi*. Line and 95 % confidence interval fitted through light-limitation data. N- and P-limitation data are included as well as batch culture data (Stolte et al. 2000).

4.1. 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 (Stolte et al. 2000). 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, Fig. 1). 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.

The DT/(DD+DT) ratio has been used (Brunet et al. 1993) as an estimate of 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 that 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 may 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 (Fig. 2). 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 that the continuous light regime negatively affects μ in our strain (Chapter 6).

A compilation of data from the existing literature gives the impression that *E. huxleyi* has a low compensation irradiance level for growth (I_c) and a relatively (but not exceptionally) high affinity (dμ/dI) for light (Riegman et al. 1998). These characteristics make *E. huxleyi*

comparable to typical oceanic species such as *Prochiorococcus* and *Synechococcus* WH8103. In fact, it could be stated that *E. huxleyi* shows a comparable performance as the latter two species, but 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 that originally was isolated from the Oslo fjord. There may very well be a difference in temperature dependence amongst different isolates of one and the same algal species (Paasche et al. 1996).

Table 1 Cell composition of Light limited *Emiliania huxleyi* strain L. Organic carbon (POC), Inorganic carbon (IC), Carbon to nitrogen ratio (N/C), Nitrogen quotum (PN), Phosphorus quotum (PP), Chloropyll a (Chl. a), Maximum photosynthesis rate (Pm), and respiration rate (Rd).Riegman et al. 1998.

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		

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						

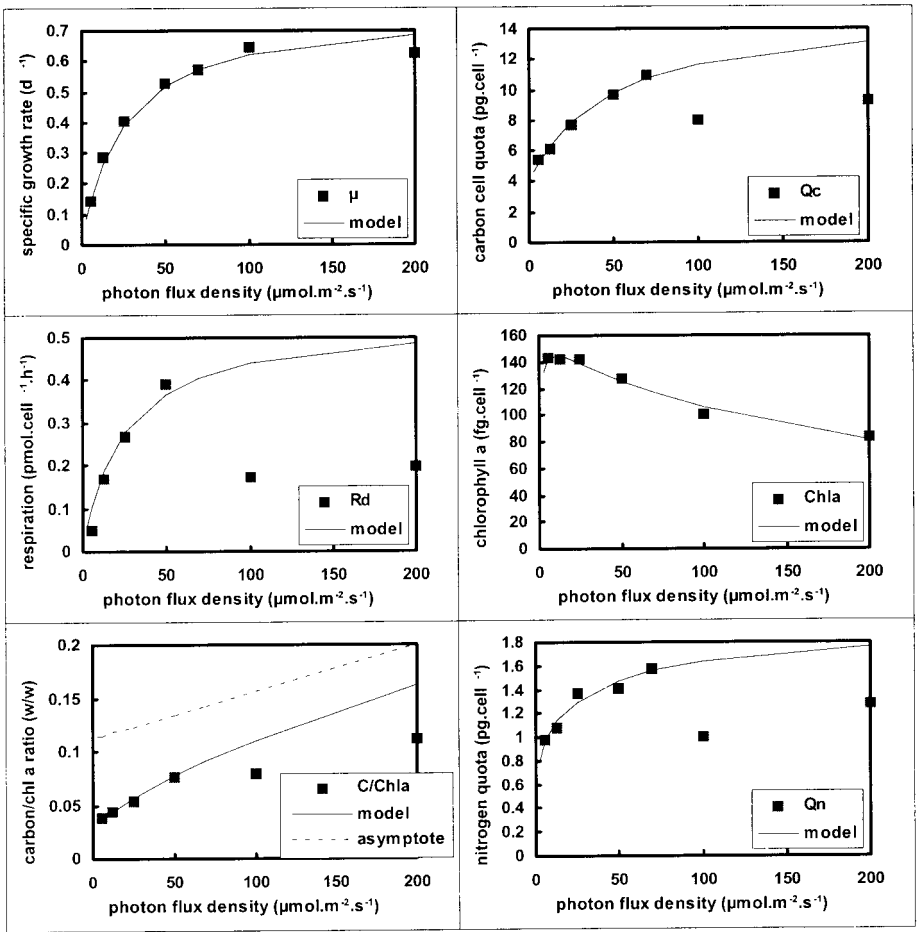

Figure 2. 12 Model fits for light limited steady state data of *Emiliania huxleyi*. Data on carbon quota, nitrogen quota, dark respiration, chlorophyll *a* and specific growth rate were fit simultaneously using 14 parameters. Data obtained at 100 and 200 µmol· m^{-2}· s^{-1} for carbon, nitrogen and dark respiration were not used for model fits (Stolte et al. in prep).

4.2 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 (Riegman et al. 1998, 2000).

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.

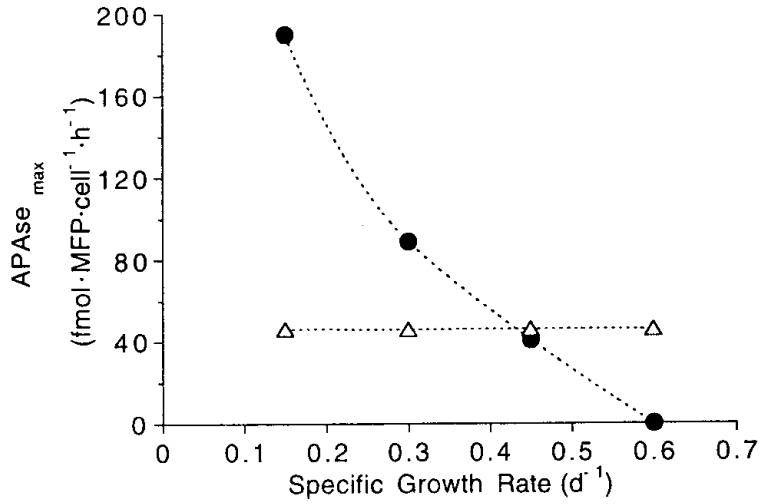

Figure 3. Calculated V_{max} for the two Apase systems of *Emiliania huxleyi*; the constitutively synthesised system (open triangles), and the inducible system (closed circles). Riegman et al. (2000).

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 (Fig. 3). One of them is synthesised constitutively, with a cell-P based affinity for MFP of 8.7 L μmol^{-1} h^{-1}. The inducible 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 auto lysis 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_{maxt}

and Q_{min} will be important selection criteria for algae which are competing for phosphorus. The cell-P normalised maximum phosphate uptake rate of *E. huxleyi* is within the same range as the V_{max} of other algae. The coefficient for luxury uptake, i.e. the ratio Q_{maxt}/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 $\mu = 0.14\ d^{1}$) 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 2 Cell composition of P limited *Emiliania huxleyi* strain L. abbreviations as in Table 1. Riegman et al. (1998).

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

4.3 N-limited growth

With respect to N-limited growth, *E. huxleyi* showed less exceptional properties (Riegman et al. 1998, 2000). In comparison with most other algal species, 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* (Riegman 1998). For *Thalassiosira allenii, Monochiysis 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.

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 *Pleurochivsis, Prvmnesium* and *Amphidinium* species that they posses cell-surface L-amino acid oxidases enabling them to utilise 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 demineralisation 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.

Table 3 Cell composition of N limited *Emiliania huxleyi* strain L. abbreviations as in Table 1. Riegman et al.(1998).

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

4.4 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 (Fernandez et al. 1994), are nitrate levels above 4 μM,

phosphate below 0.2 µM, and a stratified water column 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 water column is generally characterised 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. After a physical disturbance of the water column, 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 remains P-controlled during this transient state, the high affinity of *E. huxleyi* for Pi and its high APase activity might favour rapid growth.

It must be remembered 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 potential to control the biomass of microalgac (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 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 between glacial and interglacial periods is still 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 optimally in P controlled environments.

The removal of CO_2 from the biosphere *via* the burial of calcium-carbonate from a major calcifier in the world's oceans may be controlled by phosphorus rather than nitrogen.

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.

5. COCCOLITHOPHORID GENES INVOLVED IN CALCIFICATION, NUTRITION, AND PHOTOSYNTHESIS

In order to characterise the response of *Emiliania huxleyi* to a range of external conditions, such as variations in light intensity and nutrient availability continuous cultivation was applied. In the past, the response patterns could only be characterised in a gross way, ignoring specific molecular aspects. The molecular genetic approach introduced in this research permits following the response in unprecedented detail.

The idea underlying this project is to sequence genes encoding many key proteins, and to quantitatively monitor the expression of these genes at the mRNA level as a function of the growth conditions. As metabolic regulation is not always reflected in expression at the level of mRNA, we also envision quantification of the activity of a number of known enzymes.

The intention of this project was to lay the foundation for this biomolecular approach. In order to contribute to the comparison between different coccolithophore species we concentrated not only on *E. huxleyi* but also on *Pleurochrysis carterae*, a related organism extensively studied at the physiological and biochemical levels.

From physiological experiments it is known that *E. huxleyi* cells calcify in the light and that calcification is arrested in the dark. We produced two c-DNA libraries, one from illuminated and the other from non-illuminated cells. In addition, a c-DNA library of calcifying *P. carterae* cells was constructed. These libraries were used for the isolation of the genes that were studied in this project. Some of the genes could be obtained directly from these libraries, others were isolated using differential hybridisation of the libraries from illuminated and non-illuminated *E. huxleyi* cells. Using these and additional methods, we identified and characterised several genes involved in calcification, photosynthesis and cell division.

This work is a continuation of long-standing research in the laboratory in Leiden University and elsewhere. In our previous research we used biochemistry and cytology to characterise the mechanism of calcification in these species. This older work resulted in a hypothesis on the mechanism of coccolith production.

5.1 Genes involved in calcification

5.1.1 GPA, a calcium binding protein

In the past, minute quantities of intermediates in the biosynthesis of a polysaccharide involved in coccolith biosynthesis could be isolated in our laboratory (Borman et al 1987). In addition to polysaccharide moieties, they contained an acidic protein. An antiserum against one of the intermediates was raised (Nanninga et al 1996). This antibody was used to screen the c-DNA library from illuminated *E. huxleyi*. This led to the isolation of a gene, gpa, which was assumed to be involved in calcification (Corstjens 1998). We then produced recombinant GPA, the corresponding protein, in a prokaryotic system. Sequencing of gpa revealed that GPA is rich in glutamic acid, proline and alanine (hence the name, Table 1), and that it contains a binding site for calcium ions (Fig. 1). Milligram amounts of recombinant GPA were produced and the calcium-binding function was corroborated in *in vitro* studies. An antiserum was raised against this material. Immunocytological localization on whole cells indicated that GPA is localised in the cell surface of illuminated *E. huxleyi* cells.

Table 1 Amino acid composition of the gpa-encoded *Emiliania. huxleyi* protein. Corstjens et al. (1998).

Amino acid		Mole %	Amino acid		Mole %
A	Ala	23.0	M	Met	0.3
C	Cys	0.6	N	Asn	0.6
D	Asp	6.1	P	Pro	12.2
E	Glu	18.0	Q	Gln	1.4
F	Phe	0.8	R	Arg	1.1
G	Gly	4.4	S	Ser	6.7
H	His	0.0	T	Thr	3.0
I	Ile	1.9	V	Val	8.9
K	Lys	5.8	W	Trp	0.0
L	Leu	3.6	Y	Tyr	1.7

These data allowed us to suggest that GPA is a component of the calcifying vesicle, and plays a role in the transport of calcium to the site of calcification. It would assume its extracellular localisation after the termination of coccolith formation when the calcifying vesicle fuses with the plasmalemma.

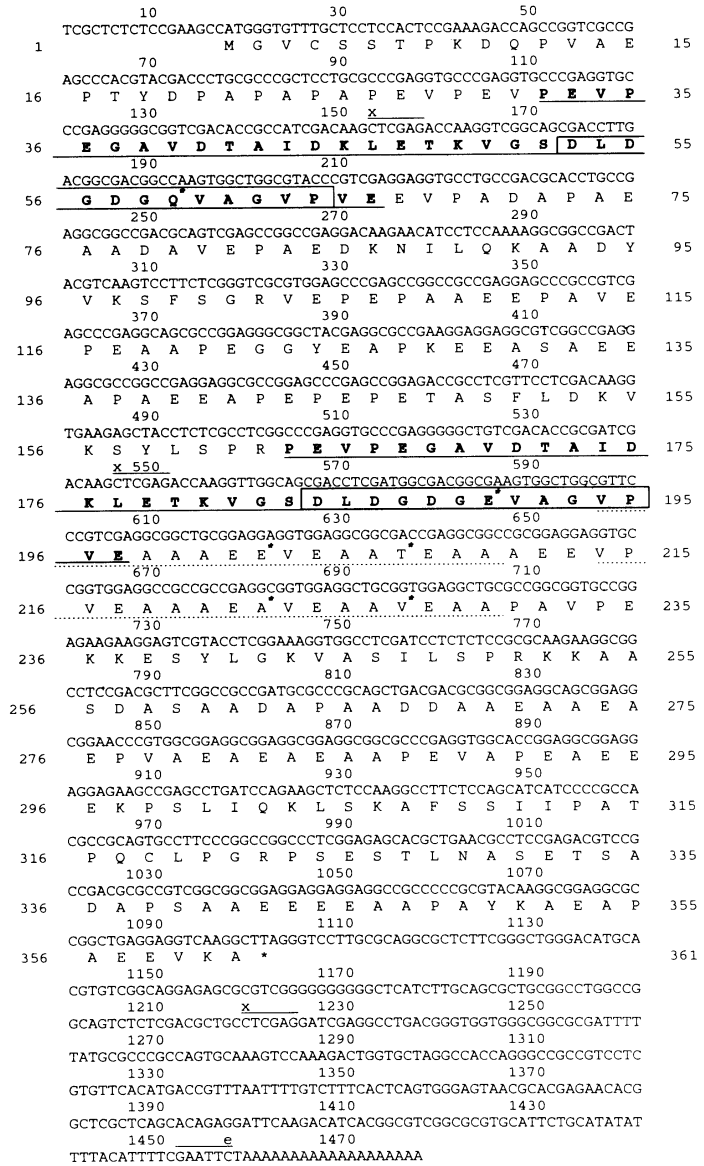

Figure 1 Nucleotide sequence of the *gpa* gene and amino acid sequence of the encoded GPA. The 35-amino acid repeat is underlined and shown bold; the 17-amino acid repeat is indicated by dotted underlining; deviations in both repeats are marked by asterisks. The two boxed stretches represent the sequences homologous to the Ca^{2+}-binding loop of the EF-hand motif. The *Xho*I (x) and *Eco*RI (e) restriction sites are indicated. The 5' and 3' multiple cloning sites (MCS) adjacent to the 3'- and 5'-UTR of *gpa* are not shown; the 5' MCS contains a *Pst*I and an *Eco*RI site; the 3' MCS contains a *Xho*I site. Corstjens et al. (1998).

5.1.2 ale, another gene involved in calcification

The second gene thought to be involved in calcification is ale. This gene corresponds to one of the gene-clones originally isolated by means of differential hybridisation from cDNA libraries of *E. huxleyi* (Corstjens et al. 1995). Nucleotide sequence analysis revealed that ale encoded a protein with some features similar to GPA. The protein is called ALE. Like GPA, it is an acidic protein composed for a large part of only few amino acids. In case of ALE, the most abundant amino acids are alanine, leucine and glutamic acid.

Because the original cDNA-clone only contained a small part of the ALE-encoding open reading frame, we isolated clones with larger inserts from the cDNA libraries for further nucleotide sequence information. The nucleotide sequence analysis of these new clones showed the presence of a putative membrane-spanning region. Based on the amino acid sequence of ALE and because ale is (differentially-) expressed during periods in which we find active calcification, we speculate that this protein has a function in anchoring the coccolith polysaccharide to the membrane of the coccolith vesicle.

Similar to the strategy followed for GPA, antibodies will be produced against recombinant ALE to determine the cellular location of the native protein. Expression studies at protein and mRNA level will then be used to study the hypothesised link with active calcification. This work is being continued by J.P.M. de Vrind (Geophysiology, Leiden University).

5.1.3 hpp, a high affinity phosphate-repressible phosphate permease gene

Low phosphate concentrations are known to affect growth and calcification rates *in E. huxleyi*. Using a differential hybridisation method (Corstjens et al. 1995), a gene, hpp, encoding a high affinity phosphate-repressible phosphate permease (as determined by amino acid sequence homology) was isolated and analysed. In fungi and yeast, regulation of this phosphate permease is at the transcriptional level. Hence, low phosphate concentrations in the environment trigger the induction of this permease. It has often been demonstrated that in *E. huxleyi* low environmental phosphate conditions lead to higher calcification rates. Hence, a link between hpp-expression and calcification seems probable.

The permease has strong sequence similarity with several retrovirus permease receptors. This may provide a clue for the rapid degeneration of many natural *E. huxleyi* blooms. Depletion of phosphate in these blooms would lead to the accumulation of phosphate permease in the cell membrane, and make the cells more susceptible for viral attack.

In collaboration with Riegman (NIOZ) we shall test the mRNA expression level of hpp (using RT-PCR) on culture material obtained from the phosphate limited continuous cultures provided by Dr. Stolte (Chapter 4).

5.1.4 VAP, a V-type H(+) ATPase

A side effect of the production of calcium carbonate is acidification of the mother fluid whom which the crystals are formed. In coccolith vesicles, the protons may accumulate and bring calcification to a halt. For the calcification process to be completed, these protons must be removed from the site of crystallisation. A V-type H(+) ATPase (VAP) would be capable of fulfilling this function. Using a strain of the coccolithophorid *P. carterae*, we cloned a vap-gene (Fig. 2), and immuno-localised the corresponding VAP protein in the cells. The presence of the V-type H(+) APTase in the membrane of the calcifying vesicle could be unambiguously demonstrated. A paper on this subject was published in the Journal of Phycology (Corstjens et al. 2001).

Expression studies of vap mRNA were performed in *P.carterae*. Expression of vap mRNA was studied in Northerns (slot-blot analysis) and by RT-PCR. For RT-PCR experiments expression levels were normalised to level of Actin encoding act-mRNA (the cloning of act to determine the necessary sequence information is described in Corstjens and Gonzalez (1999). A paper describing the effects of N and P limitation on vap expression is in preparation (Corstjens and Gonzalez in prep). So far, after normalisation to actin, expression results indicate that vap is expressed at a constitutive level independent of growth rate, nitrate- and phosphate concentration. However, since in some cases with Northern slot-blot analysis variations in vap expression were observed, it may be necessary to reconsider the validity of actin-normalisation. Nevertheless, the hypothesis that the level of vap-mRNA expression is a measure for the synthesis-rate of calcifying vesicle-membrane was not yet verified with these experiments. If vap expression indeed turns out to be constitutive, it is still possible that VAP protein expression is a measure for calcifying vesicle synthesis. In order to be able to study protein expression levels we have raised antibodies against a synthetic peptide covering the most variable part of the vap-encoded 16 kD proteolipid of the V-type H+-ATPase. This antibody was also used to verify the location of the vap-encoded protein in purified *Pleurochrysis* cell-fractions. (Corstjens et al. 2001).

PCR amplification of the most variable vap-region from genomic DNA of diverse coccolithophorids demonstrated that all *Pleurochrysis* strains had an intron sequence in that specific vap-region. A similar intron was missing from all *Emiliania* strains. The intron

sequence can be used as an identification marker for *Pleurochrysis*. Nucleotide sequence variation in the intron can be used to study strain phylogeny (Corstjens et al., 1997).

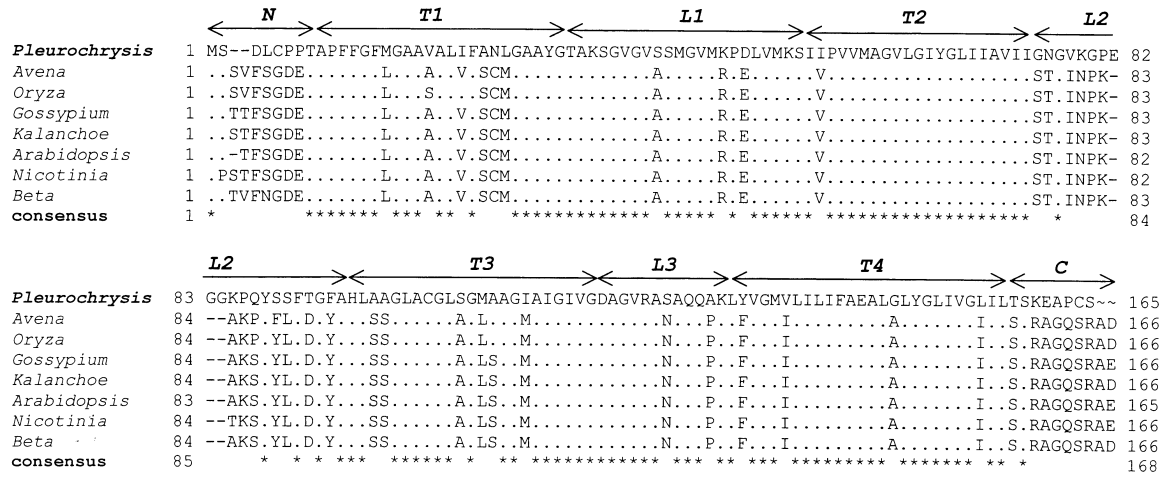

Fig. 2 Alignment of amino acid sequence for the 16-kDa proteolipid subunit of higher plant V-type H^+-ATPases. The structural domains are indicated above the dequence:N, the N-terminal domain; T(1-4), the *trans*-membrane domains; L(1-3), the extramembrane domains; and C, the C-terminal domain. Amino acis sequences were compiled from the Swissprot database, and accession numbers of the sequences are as follows: *A. sativa* (Avena) no. P13957; *O. sativa* (Oryza) no. U27098; *G. hirsutum (Gossypium*) no. U13670; *K. daigremobtiana (Kalanchoe)* no. U16244; *A. thaliana (Arabidopsis*) no. Q39039; *N. tabacum (Nicotiana)* no. 95751; *B. vugaris (*Beta) no. 98851; *Pleurochrysis* sp.*(Pleurochrysis)* no. U81519. Corstjens et al. (2001).

5.2 fcp a gene involved in photosynthesis

The process of photosynthesis was studied in both *Emiliania* and *Pleurochrysis*. Several (partial-) genes of the fucoxanthin-encoding multi-copy gene family of *E. huxleyi* were isolated and sequenced. Isolation of these genes was accomplished by differential hybridisation of two cDNA libraries, one specific for mRNA molecules present in the light period (L1Light) and one specific for mRNA molecules present in the dark period (L2Dark).
The fucoxanthin-encoding genes are generally called fcp. They encode the chlorophyll a/c binding proteins of the light harvesting protein. Using sequence data obtained from *E. huxleyi* fcp genes, a fragment of a fcp gene from *Pleurochrysis* was obtained. In the latter organism a

positive correlation between the expression of fcp mRNA, active cell growth and the external concentration of nutrient (N-source) was demonstrated using Northern slot-blot analysis. The level of fcp expression was shown to increase with the growth rate (cell division) and external nitrate concentrations.

Our experiments demonstrate that the expression pattern of fcp follows a pattern similar to the production of chlorophyll *a*. In actively growing and dividing batch cultures, fcp expression is high and the total amount of chlorophyll increases until nutrient levels become limiting. In non-proliferating cells exposed to high concentrations of an N-source, a reservoir of chloroplast material is formed. An increase can be measured of both the level of chlorophyll per cell and the expression rate of fcp. As soon as the nutrients become depleted, fcp expression is inhibited and the amount of chlorophyll per cell decreases.

We conclude that the expression of fcp is a useful marker to study growth and nutrient status. However, it is not possible to conclude from high fcp levels that cells are actively dividing; to study cell-division pcna expression (see below) is preferred.

Part of this expression work is presented in a paper describing expression of the coccolith vesicle-proton pump in *Pleurochrysis carterae* (Corstjens and Gonzalez, in preparation).

5.3. pcna, a gene involved in cell division

In collaboration with Prof. Senjie Lin (Stoney Brook, NY), a gene encoding the Proliferating Cell Nuclear Antigen (PCNA) was isolated from a cDNA library of *P.carterae*. Beside molecular characterisation, we were able (using RT-PCR) to demonstrate that expression of this gene is closely linked to growth rate. Immunofluorescence with a heterologous antibody (from rat) was used to demonstrate the presence of PCNA in the nucleus. Cross-reactivity of the rat-antibody with protein material produced from the cloned *P.carterae* gene was used to validate that the cloned gene indeed encoded a PCNA. Since PCNA is a conserved protein, the corresponding gene is likely to be a useful probe for cell division in *E. huxleyi*. It could then be used in (natural) samples of the latter species to study growth rates (Northern and Southern analysis in *P. carterae* as well as *E. huxleyi* are underway). A paper describing the isolation, the molecular characterisation, expression profile and localisation of this gene/-product in coccolithophores is in preparation (Lin et al.).

5.4. Miscellaneous genes

A number of additional genes were identified, isolated and sequenced. The ones that are now available now for expression studies include the following.

- Some *P. carterae* genes encoding proteins involved in carbon metabolism.
- Genes from the gene-family encoding the elongation factor 1-alpha from *E.huxleyi* (protein biosynthesis).
- Several *E. huxleyi* genes specifically expressed in the light-period; for these genes so far no homology in data bases has been found.
- An *E. huxleyi* gene specifically expressed in the dark (no homology in data bases found).

Sequences from these genes have not yet been posted in the GenEMBL databank but are available upon request. Furthermore, several uncharacterised partial sequences have been determined which eventually will end up in a sequence-tag based library of *E. huxleyi*.

6. AN INVENTORY OF ENVIRONMENTAL FACTORS THAT AFFECT DMSP PRODUCTION BY *EMILIANIA HUXLEYI*

6.1 Salinity

If we are to move forward into the direction of a model that will include the output of the cell in terms of DMSP production it is necessary to understand how environmental factors will affect DMSP production. Unfortunately, the function of DMSP is not fully understood yet (Stefels 2000). There is no doubt that DMSP, that is a compatible solute, contributes to the osmotic balance of algal cells in salt water (Kirst 1996). The cellular DMSP content is often found to be coupled to salinity (Kirst 1996, Vairavamurthy et al. 1985, Dickson et al. 1982, Dickson et al. 1987 a,b; Stefels 2000, Karsten et al. 1991a, 1992, Reed, 1983).

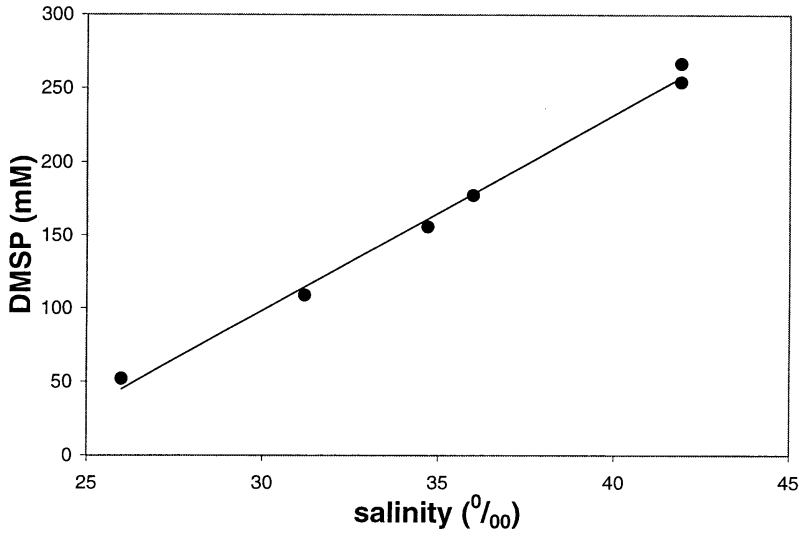

Figure 1 The amount of DMSP, expressed as DMSP per biovolume (mM), in *Emiliania huxleyi* batch cultures at different salinity's (van Rijssel and Buma, submitted).

The relationship between salinity and the cellular DMSP concentration measured in this project (unpublished results) is given in Fig. 1. As expected there was an increase in the concentration of DMSP upon an increase in the environmental salinity: concentration (mM) = 13.4 salinity (‰) – 303 , r^2 = 0.994 at 15°C and a continuous irradiance of 200 µmol photons $m^{-2} s^{-1}$. All samples were taken at the end of the exponential phase of growth, in addition a sample at mid-exponential phase was taken at the highest salinity. There was not

much difference in the concentration of DMSP in the cells between the two phases in growth. The cell size, however, was different (35 and 43 μm^3), resulting in a different DMSP content, resp. 8.9 and 11.5 fmol DMSP per cell. The amount of DMSP that was found in the medium increased from 4 up to 12.5 % of the total DMSP in the culture with increasing salinity.
Release of DMSP into the medium could be the result of autolysis or of exudation. Abiotic conversion of DMSP into DMS and acrylate is slow in natural and bacterial degradation was excluded since no bacteria were present in the cultures. In all cultures DMS was detected in the supernatant indicating the action of a DMSP lyase. Wolfe et al (1996) found that DMSP and the DMSP-lyase in *Emiliania huxleyi* are segregated within the cells, therefore the presence of DMS in the cultures suggests autolysis as a way of DMSP release. However, Laroche et al (1999) estimated the DMSP exudation rate of *Phaeocystis globosa* between 3 and 11 % d^{-1} of its DMSP quota using a modelling approach. For *Phaeocystis* DMSP release via exudation seemed as important as autolysis. Combined exudation and autolysis DMSP release for *Emiliania huxleyi* strain L was no more than 6% at 33 ‰ (natural salinity).

If the function of DMSP is confined to the osmolyte function only, modelling would be simple for *Emiliania huxleyi* since large fluctuations in salinity are not to be expected in its environment. However, apart from the osmolyte function there are reports indicating other functions for DMSP. Variables such as light regime, nutrient limitation, and temperature have been reported to affect DMSP content as well.

6.2 Temperature

DMSP is most effective as compatible solute (Gröne and Kirst 1991) at low temperatures (Nishiguchi and Somero, 1992, Karsten et al. 1996), one reason why at low temperatures macroalgae accumulate DMSP in their tissues (Karsten et al. 1990a, 1992, 1996). Microalgae seem to have a similar response to temperature: the amounts of DMSP per cell in *E. huxleyi* (Meyerdierks 1997) *and Tertraselmis subcordiformis* (Sheets and Rhodes, 1996) are higher at low temperatures. It is unfortunate that the DMSP content in microalgae is often expressed on a per cell or per unit chlorophyll *a* basis because both parameters are known to vary during changes in the environment. To understand the role of DMSP as a compatible solute, an expression per cell volume gives more insight in the concentration in the cell and its physiological functions (Stefels, 2000, Keller, 1991). Our results (van Rijssel and Gieskes accepted, Fig 2) not only confirm the increase of the amount of DMSP per cell at low

temperatures reported before, but also show an increase in cellular volume. Nevertheless, the DMSP concentration in the cell expressed as DMSP per volume (in mM) doubled at low temperatures. For the first time proof was obtained that in a miroalga the concentration of DMSP changed upon a decrease in temperature.

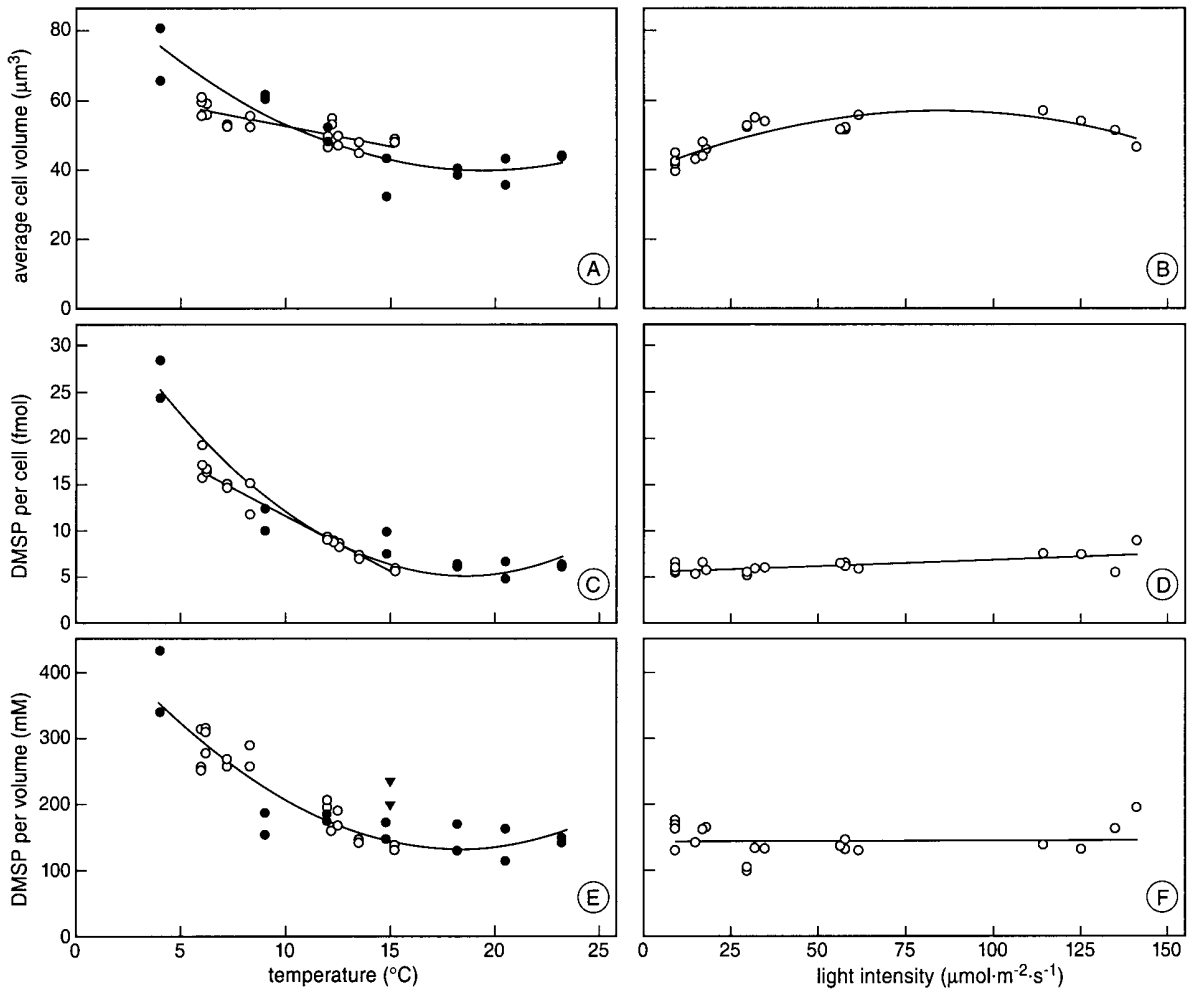

Figure 2 The effect of temperature and light intensity on average cell volume, DMSP per cell and DMSP per volume at the end of the batch culture experiments. The dependent variables were standardised for 20 µmol photons m^{-2} s^{-1}(temperature graphs) and 15°C (light intensity graphs) using the regression models (van Rijssel and Gieskes, accepted). Each point represents one culture value, (●) day-night regime and (○) continuous light regime.

The two-fold drop in DMSP per volume found in the temperature range 5-15°C in cultures is highly relevant since this is the temperature range of the surface waters where *Emiliania* blooms occur (Brown and Yoder, 1994). Although adjustment of the DMSP concentration is

not a quick process, cells are likely to be able to adapt to small temperature changes within days. Weather conditions during a bloom, therefore, will determine the total DMSP present at the end of the bloom (the total amount of biomass formed in the cultures remained constant and was most likely coupled to the amount of nutrients that were supplied at the beginning of the batch cultures). When the bloom collapses and the DMSP is released into the water column a mismatch in DMSP availability and the bacterial reduced sulfur demand occurs and superfluous DMSP is cleaved into DMS and acrylate facilitating escape of DMS to the atmosphere (Kiene et al. 2000). The colder the bloom conditions the more DMS will be able to escape to the atmosphere.

If the response of *E. huxleyi* is characteristic for all other DMSP producing microalgae, a decrease of DMSP concentration in the cells must be expected upon a temperature rise. This result does not necessarily contradict the feedback mechanism proposed by Charlson et al. (1987). Only a small fraction of the total DMSP that is produced in microalgal blooms will escape to the atmosphere. Changes in processes that determine abundance of DMSP producing algae, the activity of DMSP consuming bacteria and physical sea-to-air transfer of DMS, as a result of ocean warming are hard to predict. Therefore, it is impossible to foresee what the over-all effect of 'global warming' on the actual sea-to-air emission of DMS will be. However, our observation of less DMSP production at elevated temperatures, assumed to result in less DMS emission from the ocean, is in line with observations of lowest concentrations of the major oxidation product of DMS methanesulphonate (MSA), in ice cores taken on Antarctica during interglacial periods (Legrand, 1997).

6.3 Light regime

Light is a factor that is continuously changing in the environment of microalgae and was also considered (and studied) as a candidate for the model. A higher light intensity and longer day lengths induce elevated DMSP contents in macroalgae (Karsten et al 1990b, 1991b, 1992). For microalgae the relationship with light is less apparent because of the use of different expressions for DMSP content (or production rates). Illustrative is the study of Wilhelm et al (1997) where increasing amounts of DMSP per chlorophyll *a* in *Prymnesium parvum* were found with increasing irradiance. The amount of DMSP per cell, however, was independent of irradiance. The production rates of DMSP per chlorophyll *a* by *Phaeocystis sp*. have been reported to be a function of irradiance (Matrai et al 1995). Different light intensities used in

the culturing of four phytoplankton species, including *E. huxleyi*, did not yield significant differences in the amount of DMSP per cell (Keller and Korjeff-Bellows, 1996).

In our study (van Rijssel and Gieskes accepted) different combinations of temperature and light were analysed for their effect on the concentration of DMSP in *E. huxleyi* (Fig. 2). Apparently, DMSP per cell was influenced by light conditions but this was mostly due to cell size differences. For DMSP expressed as amount per biovolume neither light regime nor light intensity (range 10-140 µmol photons m^{-2} s^{-1}) were found to be of influence in batch cultures. There was an increase (less than 20 %) between two steady states in a turbidostat at two light intensities (6 and100 µmol photons m^{-2} s^{-1}). Nanninga and Tyrell 1996, reviewed the literature and observed that blooms occur usually in stratified waters with a mixing depth of less than 20 m and a surface light intensity of 530 µmol photons m^{-2} s^{-1}. The average light intensity in the mixed layer is 350 µmol photons m^{-2} s^{-1} assuming a attenuation coefficient of 0.1 m^{-1}. Therefore, growth of *E. huxleyi* is not expected to be light limited during bloom conditions (Chapter 4) In contrast, growth of *E. huxleyi* can be inhibited by continuous light (Fig. 3) The 20% difference in DMSP concentration observed in the turbidostat must be interpreted as the maximal effect possible.

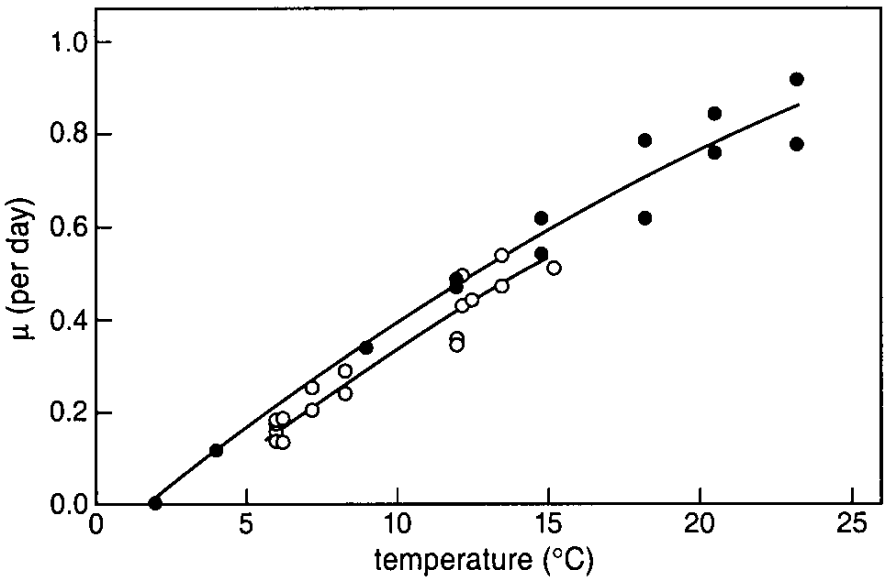

Figure 3. Growth rates of *Emiliania huxleyi* in batch cultures at two regimes, (●) day-night regime and (o) continuous light regime as a function of the incubation temperature (van Rijssel and Gieskes, accepted).

6.4 DMSP production as an overflow mechanism for excess reduced sulphur or carbon

An additional, more complex role for DMSP in the cell was proposed by Stefels (2000). DMSP production as an overflow mechanism for excess reduced sulphur and as a means of dissipating energy excess. Within this concept DMSP levels in the cell are regulated to match sulphur and nitrogen metabolism. If the cell is short in nitrogen or stressed in some other way that prevents balanced growth, DMSP can be overproduced and/or excreted. It is interesting to investigate nitrogen verses phosphorus limitation in this respect. In some algae nitrogen limitation did result in elevated DMSP contents (Dacey et al 1987, Turner et al 1988, Gröne and Kirst 1992, Hanson et al 1994). Another natural stress factor that can be considered is UV-B radiation.

6.4.1 Nitrogen versus phosphorus limitation

Turner et al. 1988 observed that *E. huxleyi* produced less internal DMSP (on a per cell basis) in a nitrogen supplemented culture. The mean cytoplasmic concentration of sea samples in "high nitrogen areas" (> 0.2µM NO_3^-) of 37 mM was significantly lower than the 95 mM observed for "low nitrogen area's" (<0.2µM NO_3^-). It is tempting to speculate that DMSP, that is the sulphur analog of the nitrogen containing glycine betaine (also known as an osmolyte), is produced when nitrogen is the growth limiting nutrient. However, glycine betaine was not present in *E. huxleyi* strain ccmp370 (Macdonnald et al 1996). During the course of this project Keller et al. (1999 a &b) found that DMSP was far more dominant in *E. huxleyi* ccmp378 compared to glycine betaine making a reciprocal relationship between the two osmolytes highly unlikely.

Table 1 Nutrient concentrations and N/P ratio's used in batch cultures of *E. huxleyi* to induce N or P limitation at the end of exponential growth phase.

N/P ratio	NO_3^- (initial concentration, µM)	PO_4^{3-} (initial concentration, µM)
75	300	4
30	300	10
18.8	300	16

12	200	16.6
4	100	25
1	25	25

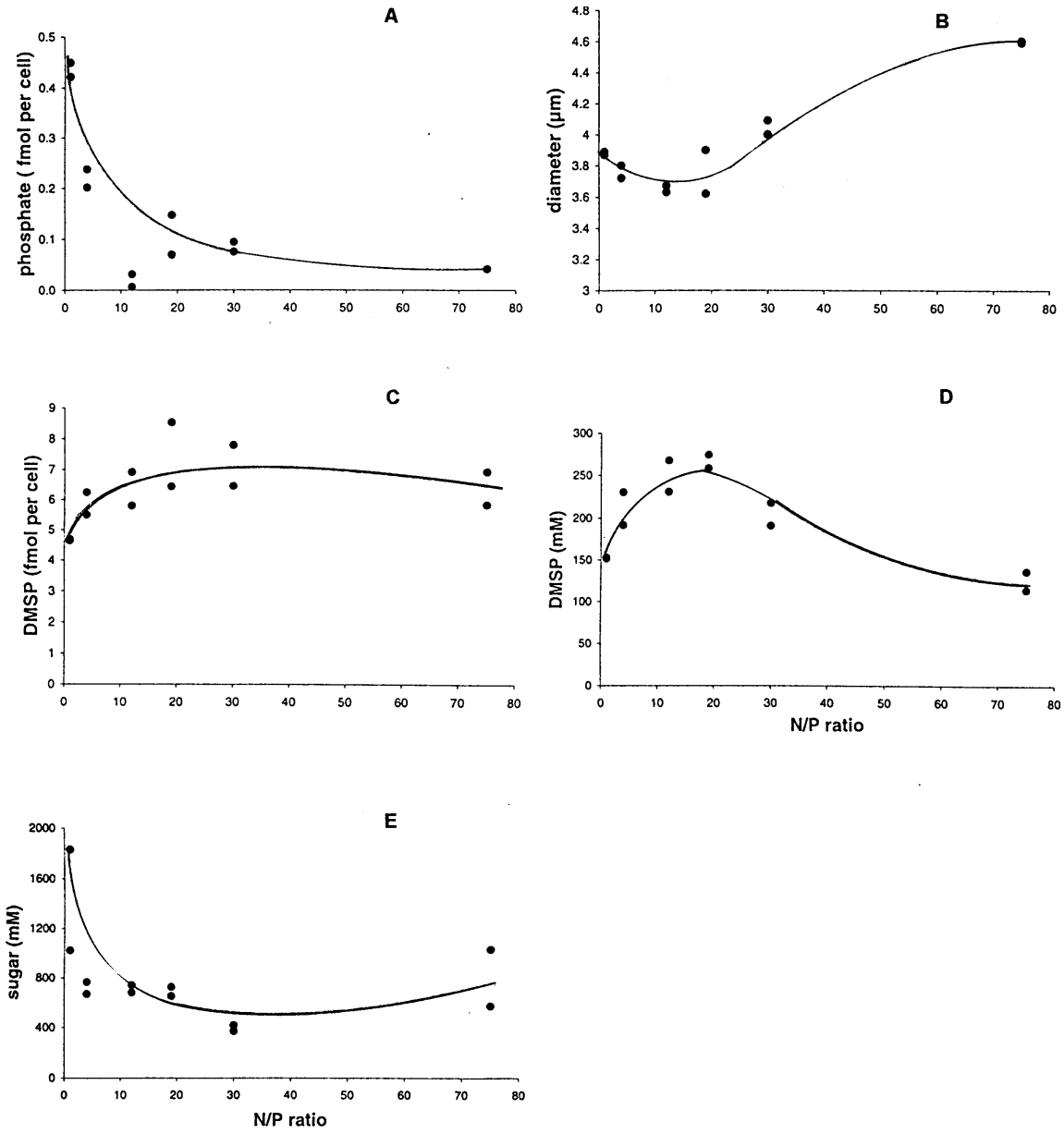

Figure 4 The effect of different initial concentrations of N and P, expressed as N/P ratio, on end values of batch cultures of *Emiliania huxleyi*, (a) phosphorus quota, (b) average cell size, (c) DMSP per cell, (d) DMSP per biovolume, (e) sugar per biovolume (van Rijssel, unpublished results). Curves are drawn by hand to illustrate the trends.

Still the effects of nutrient supply had to be considered as environmental condition relevant to the DMSP content in *E. huxleyi*. To this end batch cultures with different amounts of nitrogen

and phosphorus were inoculated (Table 1). As a result the cells at the stationary phase of growth would have a difference in nutrient limitation which was confirmed by the phosphorus quota of the cells (Fig. 4). The quota were high in batches with an initial low N/P ratio-medium and decreasing towards higher values and were in the same range as those measured in the chemostats (chapter 4). Cell size was affected, a minimum (25 μm^3) was found between N/P 12 and 19, suggesting a minimal size around Redfield Ratio. The amount of DMSP per cell seemed to increase with increasing phosphorus limitation but this was due to cell size differences because the concentration of DMSP was at an optimum around 270 mM DMSP. The conclusion must be that at nutrient stress (both phosphorus and nitrogen) the concentration of DMSP decreases. The result again shows that cell size differences should be taken into account when considering effects on the DMSP concentration of the cells.

6.4.2 UV-B induced stress

When a cell is at stress conditions proteases come into action that facilitate the production of better adapted enzymes by the reuse of amino acids present in enzymes that are not needed at adverse conditions. DMSP production then serves as a means reallocate nitrogen from methionine to the synthesis of new amino acids. In addition, Stefels (2000) postulated that DMSP could be used as an overflow of carbon when carbohydrate production exceeds the cells needs.

It is indisputable that solar ultraviolet radiation (UVR: 280-400) negatively affects marine microalgae, judging from the many field experiments that have demonstrated UVBR-related decreases in primary production (Smith et al. 1992, Helbling et al. 1994, Neale et al. 1994, Prezelin et al. 1994, Boucher and Prezelin 1996, Prezelin et al. 1998, McMinn et al. 1999. On top of naturally occurring UVBR stress, enhanced UVBR as a result of springtime ozone reduction is estimated to cause an additional inhibition of water column productivity (Helbling et al.1994). Ozone depletion extends from polar into subpolar and temperate regions including those where *E. huxleyi* forms massive blooms. *E. huxleyi* is rather sensitive to UVBR, as concluded from laboratory studies (Buma et al 2000). DNA damage and complete growth inhibition already occurred at a daily weighted UVBR dosis of 400 $J.m^{-2}.d^{-1}$ ($BED_{DNA300nm}$). Therefore, it is possible that increased levels of UVBR as a result of ozone depletion induce damage and unbalanced growth in *E. huxleyi* and DMSP production by could be affected as well.

Cultures of *E. huxleyi* were exposed to a range of UVBR doses for two consecutive days. During and after these treatments growth, cell size, DNA damage, sugar accumulation and DMSP concentrations were followed (Fig. 5) The vulnerability of *E. huxleyi* for relatively low UVBR doses was demonstrated by the inhibition of growth and the simultaneous occurrence

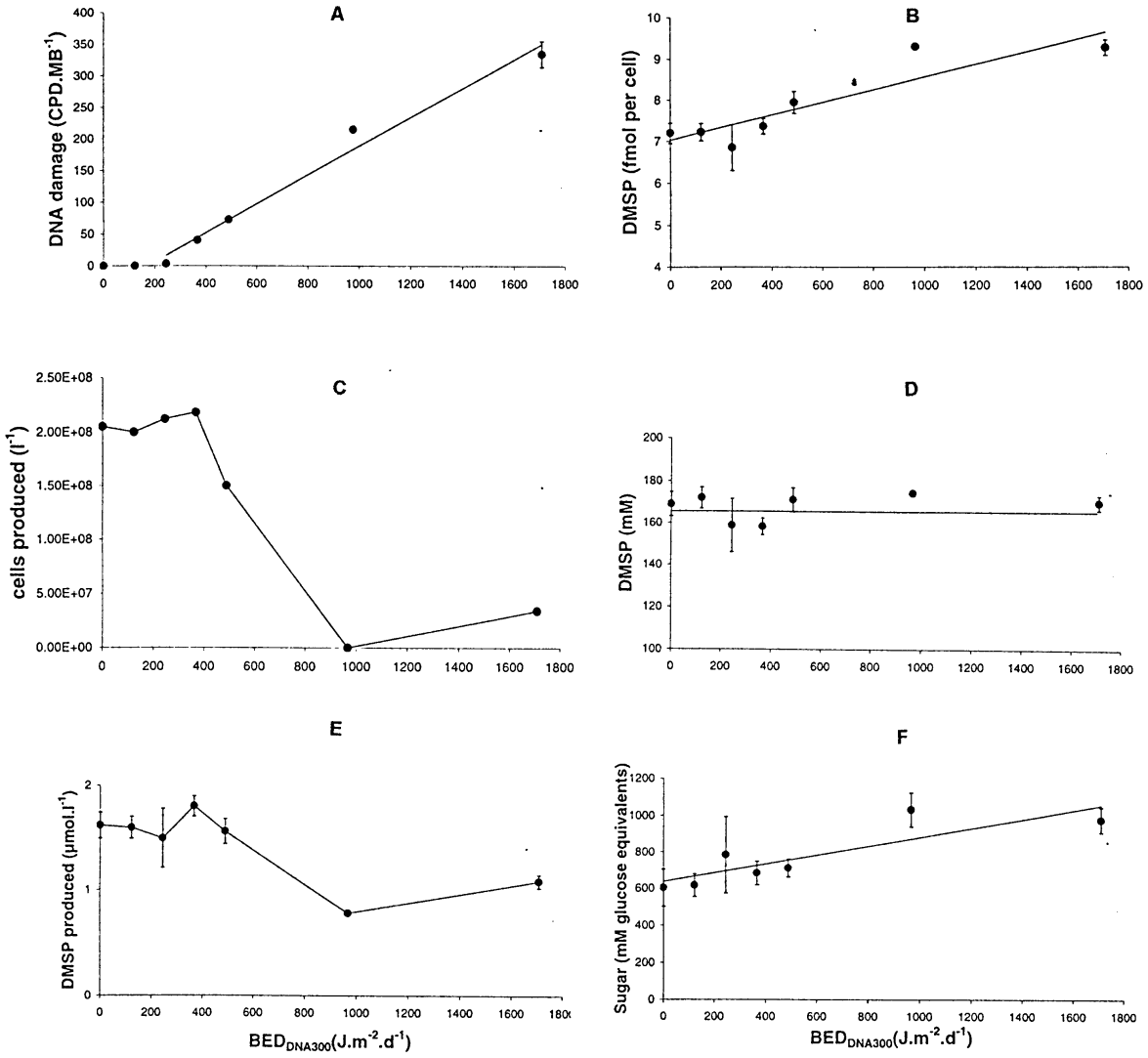

Figure 5 Effect of UV-B radiation on a batch culture of *Emiliania huxleyi* after two daiys of exposure. (a) DNA damage, (b) amount of DMSP per cell, (c) growth , (d) amount of DMSP per biovolume, (e) total amount of DMSP produced in the cultures, (f) amount of sugars per biovolume. Van Rijssel and Buma, submitted.

of DNA damage. Also, mean cell size increased and sugars accumulated as a result of the UVBR treatments. In contrast, no effect was observed on the optimal quantum yield of PSII, a measure for the efficiency of photosynthesis. With increasing UVBR dose, cellular DMSP

content increased, but due to the accompanying cell size increase intracellular DMSP concentrations remained constant. Although UVBR stress causes unbalanced growth in *E. huxleyi*, DMSP, in this case of stress, was not used as an overflow metabolite.

The increase in cellular DMSP content did not compensate for the decreased growth rates. Therefore, UVBR related decreases in growth rates caused an overall decrease in the total amount of DMSP produced in the cultures. The UVBR effects as induced in this study are assumed to be severe as compared with natural solar conditions, especially because high in situ UVAR (315-400 nm) may ameliorate UVBR damage by activation of photorepair. Yet, the here presented results imply that when (increased) UVBR causes growth rate reduction of *E. huxleyi* in situ, DMS fluxes are likely to be reduced too.

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APPENDIX 1. PROJECT DESCRIPTION.

Applicant

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2. **Contracting organization**

Vrije Universiteit Amsterdam (VUA)
Mr. J.G. van der Zee, De Boelelaan 1087, 1081 HV Amsterdam
Telephone 020-4447008; Fax 020-4447123; email jgvdzee@bio.vu.nl

3. **Title** A model system approach to biological climate forcing
– the example of *Emiliania huxleyi*

subprojects: (a) Modelling the physiological organisation of *E. huxleyi*
(b) Physiological characterisation of *E. huxleyi*

4. **Collaborating Scientists from other institutes**

(1) Dr. P. Westbroek, Geobiochemistry, Gorlaes Laboratory, Leiden
(2) Dr. R. Riegman & Dr. M. Veldhuis, NIOZ, Texel
(3) Dr. W.W.C. Gieskes, Department of Marine Biology, RU Groningen

5. **NRP theme** Theme 1, Subtheme A; Sources and Sinks in the ocean.

6. **Duration** 4 years, **starting date** 01-01-96

7. **Project costs in kf** –NOP :1485
–Contribution of participants : :1610
–Overall project costs :3095
–Total man years involved : 28

8. **Short 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 Modelling Initiative (GEM: an international collaborative effort between many research institutes) provides the strategy and infrastructure which 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 modelling study of a single representative organism, the calcifying alga *Emiliania huxleyi*, relating the cellular organisation of this phytoplankter to its ecology and to the fate of its products (CaCO3, organic carbon, and DMS). To make the resulting models more generally

applicable, little additional experimentation and minor alterations in model structure are required.

Here, we address the central problem of GEM -- to establish a model of the *E. huxleyi* cell, defining how environmental factors affect the production of the climatically relevant products. An interactive experimental and modelling approach is proposed. To provide the required data base, an extensive programme of laboratory cultures in chemostats is envisaged. Further validation requires a molecular genetical analysis of crucial metabolic processes.

9. **Rationale**

A. General introduction

Three major mechanisms are distinguished by which the marine pelagic biota may influence the global climate system: (1) the drawdown of CO_2 from the upper mixed water layer and the atmosphere to intermediate and deep waters by the formation, export and remineralisation of particulate organic carbon (the organic carbon pump); (2) the production, downward transport and partial dissolution of calcium carbonate (the carbonate pump); and (3) large-scale albedo effects mainly owing to the formation of highly reflective clouds following gaseous emissions of dimethyl sulphide (DMS) from algal blooms.

The climatic effects of these mechanisms vary geographically and through time, as they depend on the abundance of the participating organisms. Many species of algae, protozoa, bacteria, viruses and animals interact with the physico-chemical environment and with each other, and it is from all these interactions that the climatic effects emerge. In view of these complexities, the crucial question is: Can we formulate general rules that adequately describe these interactions and that at the same time are simple enough to be useful for climate modelling? The Global Emiliania Modelling Initiative (GEM) addresses this problem. We are convinced that at all levels of organization, biological systems obey a limited set of fundamental rules. GEM is an attempt to track down some of these rules and to implement them in models of biosphere-climate interactions.

GEM - a system's approach

The concept underlying the Global Emiliania Modelling Initiative (GEM) is clear and attractive: a single dominant organism, the coccolithophore alga *Emiliania huxleyi,* is selected as an exemplary model system, much as the bacterium *Escherichia coli* became a key to the biochemistry of all living organisms. Climate forcing by *E. huxleyi* is studied in depth in an interactive modelling and experimental investigation and the acquired understanding of this

system then serves as a key to investigate the wider implications of biological climate forcing. Fig. 1 is an overview of the major oceanic and atmospheric processes associated with the *E. huxleyi* phenomenon. *E. huxleyi* is a major component of virtually all open marine plankton world-wide, and forms very large blooms, particularly at mid latitudes. The figure shows that in *E. huxleyi* all three climate forcing functions are coupled, and that its history can be reconstructed from the archive at the floor of the deep sea. Major assets of the GEM strategy are the following: (1) it offers a key to the problem of biological diversity, as in-depth information on *E. huxleyi* may be extended to include a wide range of other organisms; (2) it highlights the highly non-linear character of the biological intervention in the climate system; (3) it allows the intimate coupling of the three forcing functions to be modelled; (4) it optimally exploits the excellent accessibility of *E. huxleyi* for laboratory and field research; and (5) the integration of biological and stratigraphic studies permits quantification of carbonate fluxes to the ocean floor as well as extensive model validation by comparing biological responses to diverse climate regimes.

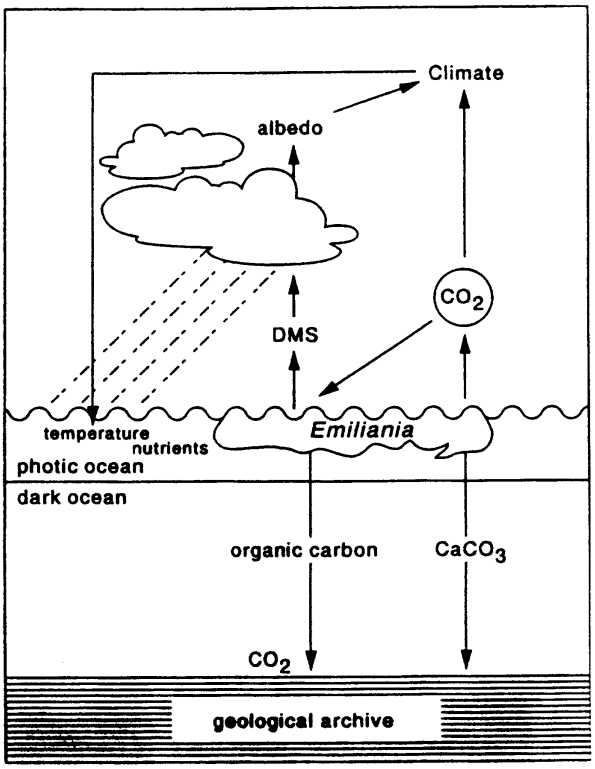

Figure 1. Coupling of three climatic effects of *Emiliania* blooms: organic carbon pump (leading to lowering of atmospheric CO_2), $CaCO_3$ counter pump (increasing atmospheric CO_2) and DMS emissions leading to the formation of white clouds, increasing the earth's albedo.

Structure of GEM and position of proposed programme

A simplified overview of the GEM research strategy is worked out in Fig. 2, which shows how a combination of dedicated physiological and molecular genetical research on *Emiliania huxleyi* leads to a model simulating the CaCO3, CH_2O and DMSP output of a single cell, as it depends on physico-chemical environmental conditions (model 1) (NB. DMSP is the precursor of DMS as it is produced by the alga). *The correct formulation of model 1 is essential for the entire GEM strategy and forms the prime goal of this grant proposal.* Laboratory studies additional to the work on *E. huxleyi* on the major represented organisms lead to a generalised model of $CaCO_3$, CH_2O and DMSP productivity (model 2) by the calcifying biota.

By combining model 2 with a global ocean environment model, the potential production of $CaCO_3$, CH_2O and DMSP by the calcifying plankton in general is predicted. To account for the effects of competing organisms (e.g. diatoms, flagellates and cyanobacteria) which modify the physico-chemical conditions, an extra model must be included. Furthermore, special models must be developed to implement the effects of consumers on the proliferation of the calcifiers and on the fate of their products. These interacting factors are combined in model 3**,** which predicts the actual global production of $CaCO_3$, CH_2O and DMS by the calcifying biota. The model is validated by laboratory cultures in chemostats, mesocosms in the Norwegian fjords, *in situ* studies of blooms in the ocean and by remote sensing.

Models 1 - 3, which apply to production in the photic ocean, provide an input for export production and transformation models (mainly simulating CaCO3 and biomarker sedimentation and dissolution fluxes in the dark ocean and the top sedimentary layer) and subsequent carbonate palaeoflux (not shown in the figure). The latter models allow, vice versa, an interpretation of the geological archive in terms of global productivity of carbonate, organic carbon and DMS by the calcifying biota in the geological past. This geological part of GEM research is funded by NWO (GOA) and concentrates on laboratory work of coccolith dissolution and biomarker degradation, as well as sediment trap and core profiling at chose localities in the N. Atlantic and the Pacific.

Comparison of the geological and biological data with model predictions allows extensive validation of the GEM models. Optimisation of the models finally leads to increasingly realistic predictions of (paleo)fluxes and climate forcing by the marine calcifying plankton.

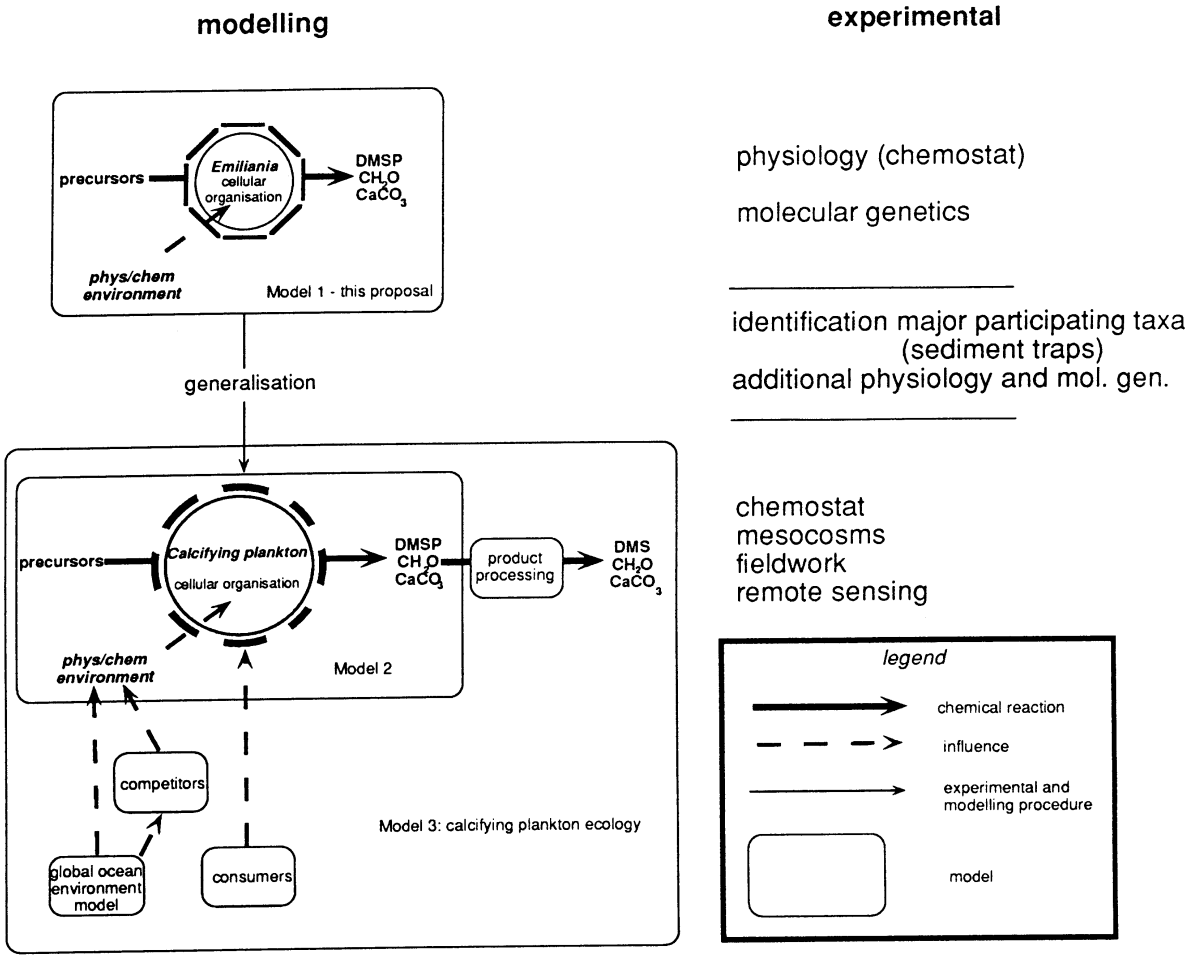

Figure 2. Schematic overview of the GEM project. See text for explanation

GEM - State of the art

In the last few decades, a steady flow of information became available on coccolithophore biology and climate forcing. Since 1989, when GEM was created, the information stream was greatly amplified by the concerted effort of more than 60 scientists in some 30 institutions world-wide. Some major results of this research are the following:

– GEM has provided experimental evidence that coccolith biosynthesis allows the cell to utilise HCO_3^- as a carbon source when CO_2 is in short supply, a condition which may commonly occur in bloom situations. The problem is particularly important as calcification reduces the alkalinity and hence influences the speciation of dissolved inorganic carbon in the surrounding ocean water.

– *E. huxleyi* blooms are major contributors to DMS fluxes into the atmosphere above the open ocean, particularly in the N. Atlantic. A spatial and temporal correlation between the blooms and albedo increase has been detected.
– In situ measurement of coccolith (export) production in various *E. huxleyi* blooms indicates that the total annual oceanic uptake of atmospheric CO_2 in these settings is reduced by 17-25% as a result of calcification.
– GEM's geological program indicates that the geographical distribution of *Emiliania* blooms is very sensitive to climate change.

The information gathered so far allows us to propose an overall structure of the models. The precise formulation of the models is the next step in this project and this is what this proposal is about. Chemostat studies of *E. huxleyi* under precisely defined conditions are urgently needed, as well as new techniques by which the state of the cellular regulation can be monitored.
Comparison of the geological and biological data with model predictions allows extensive validation of the GEM models. Optimisation of the models finally leads to increasingly realistic predictions of (paleo)fluxes and climate forcing by the marine calcifying plankton.

B. Specific information on this project

Central research question
The development and validation of the grey-box model of *E. huxleyi* is the focus of the research put forward in this grant proposal. We propose a theoretical part in which the model is developed, and an experimental part that provides opportunities to validate the model.

Subproject. (a) Modelling the physiological organization of *E. huxleyi*

State of the art
To date, a plethora of models on algal growth exists (e.g. Arrigo & Sullivan 1994; Droop 1983; Kiefer & Mitchell 1983; Laws & Chalup 1990; Morel 1987; Sakshaug et al. 1989). A considerable number of these are restricted to steady state growth (e.g. Droop 1983; Kiefer & Mitchell 1983; Sakshaug et al. 1989). Although these models have provided considerable insight, they necessarily neglect the important dynamic environmental fluctuations in nutrient and light availability that may dominate algal growth dynamics in natural environments.

Models based on variable internal stores (VIS-models; e.g. Shuter 1979; Laws & Chalup 1990) are potentially more suited to deal with such fluctuations. However, even these VIS-models assume nutrient saturation in the environment, a situation which is unlikely to be always fulfilled in the environment. Models dealing with light and nutrient limitation either state different equations for both situations (e.g. Kiefer & Mitchell 1983) or neglect internal stores and possible interactions between light and nutrient metabolism (e.g. Arrigo & Sullivan 1994).

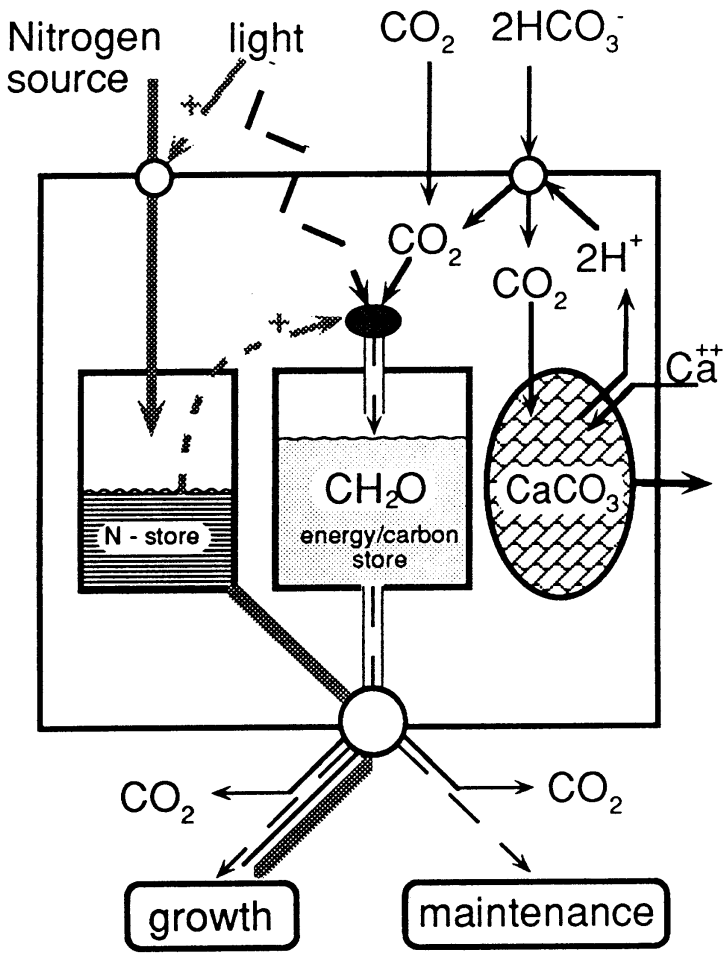

Figure 3. Structure of the model that aims to describe mass and energy fluxes in *Emiliania huxleyi*. Dashed arrows indicate energy fluxes; solid black arrows indicate carbon fluxes; solid grey arrows indicate nutrient fluxes; white dashed grey arrows indicate feedbacks.

Methodology

The philosophy behind our approach is rather different from contemporary ones. Starting point will be the scheme in Fig. 3. The state variable approach guarantees that the model will be able to cope with a changing environment. The cell will be described by a small number of state variables, e.g. the nutrient and carbon stores, and cell size. These change in time, in response to changes in the input variables, e.g. medium nutrient concentration, light. The output of the cell (growth, $CaCO_3$, and DMSP) depends on the state and input variables. For a complete model, it is necessary to formulate how the state variables change in response to the input variables.

Additional assumptions are necessary to link the state variables to observed variables. An advantage of this approach is that new experimental methods do not require a new model formulation in terms of the measured variables. An additional assumption on how newly measured variables relate to the state variables is sufficient.

This approach is a very powerful one. Since we start with concepts that are not species-specific, the model for *E. huxleyi* can be used for other autotrophic organisms with only minor adjustments. An example of such a general model is that of Kooijman (1993), who made a single model for growth of both animals and micro-organisms. He used only two state variables, viz. energy storage and volume. Despite the simplicity of the basic structure, he was able to describe microbial product formation in a chemostat under aerobic and anaerobic conditions. Also, the dynamics of a chemostat food chain of myxamoebae feeding on *E. coli* feeding on glucose were nicely captured by this model (Kooi & Kooijman 1994). In the latter case, the model for myxamoebae and the bacteria differed only in the parameter values.

Subproject (b) Physiological characterisation

Modelling of the physiology of *E. huxleyi* requires a strictly unambiguous data set, based on chemostat cultivation under continuous illumination. However, the considerable body of physiological information published on *E. huxleyi* at present, is predominantly derived from less constrained experimental conditions, mostly batch cultures or semi-continuous cultures (e.g. Balch et al. 1992; Van Bleijswijk et al 1991; Nimer & Merrett 1992). The primary objective of this subproject is to provide the data set required for the modelling study described under (a). The impact of four variables, viz. nitrate concentration, Dissolved Inorganic Carbon, temperature and light availability which are considered to be the principal

factors determining the growth, $CaCO_3$ and DMSP production of *E. huxleyi* will be investigated in chemostat experiments.

Environmental variables like the concentration of nutrients, light intensity, and temperature affect four physiological key processes in microalgal growth: calcification, photosynthesis, cell-division and nutrient metabolism. Application of the envisaged model to other photosynthetic calcifying organisms will only become feasible when the interactions between the different key-processes are known. To study interactions between cellular processes it is necessary to know which enzymes, and on what time scale, are switched on in response to specific external changes. Molecular genetics will be used for this purpose because it supplies us the tools to look at the primary response, viz. the appearance of specific mRNA molecules rather than the corresponding enzymes, of the cell (Hildebrand et al. 1993). Collaboration with experts in the field of photosynthesis, nutrient metabolism and calcification is warranted, respectively with Prof. dr. C. Astier (CNRS, Gif-sur-Yvette), Prof. dr. Y. le Gal (College de France, Concarneau) and Dr. C. Brownlee (MBA, Plymouth).

Little is known about the environmental conditions that induce *E. huxleyi* to produce the DMS precursor DMSP, and that determine the rate of the processes that degrade DMSP to DMS. It is necessary to generate a large data set that allows the development of a model by which it will possible to predict how DMS output depends on environmental factors such as nutrient concentration, light and temperature. The output depends on the values of the state variables (cellular nutrient pools) and input variables (light, temperature, medium nutrient concentrations). Since the most important global change issue is the expected future temperature increase, the influence of temperature on *E. huxleyi's* DMSP production will feature prominently in our experimental efforts.

Methodology

The experimental underpinning of the model of **subproject a** will initially be based on data obtained from chemostat steday states. For the input variables studied, viz. nitrate, light, DIC, temperature, a limited number of values will be chosen, each at different throughput rates. Measurements will be made on a number of variables, e.g. cell quota of nitrogen, P-I curves, nutrient uptake kibnetics etc. (for details, see research plan).

Similar continuous cultures of *E. huxleyi* will be run with respect to the DMS work. Special attention will be given to possible shifts in the production of DMSP and glycine-betaine in response to nitrogen limitation.

cDNA libraries are the starting material for the genetical work. They represent the mRNA-

pool of the cells at the moment of cell harvesting, and the mRNA-pool represents the enzymatic activity of the cell. The different libraries represent a calcifying and a non-calcifying strain, harvested at different periods in the light/dark cycle. Photosynthesis is confined to the light period, calcification is strongly light-dependent, and cell-division is confined to the dark period (Linschooten et al. 1991). Consequently the libraries from cells harvested during the light period should contain genes functional in calcification and photosynthesis, those resulting from cells harvested during the dark period should contain genes functional in cell-division. Two type of experiments will be performed: (1) the isolation of genes encoding enzymes which are already known, using DNA probing, PCR and antibody screening; (2) the isolation of novel genes, using differential hybridisation and subtracted probes.

Connections between subprojects

Initially, the model development will be based on general concepts and knowledge from the literature. The assumptions and model predictions will be tested against experimental evidence from **subproject b**. The simultaneous execution and continuous interaction of both subprojects is essential for the success of the whole GEM project. While the modelling will steer the experimental programme, this itself provides a feedback and will correct erroneous assumptions.

Innovative aspects

Biological forcing is an essentially unknown factor in climate modelling. GEM offers a strategy by which this enormous problem can be overcome. Primarily, GEM addresses the calcifying plankton in the ocean. Ultimately, this strategy has much wider implications as it is easily extended to include all oceanic plankton. It relates many scales of biological organisation (from molecular to global) and time (milliseconds to tens of millennia).

The research proposed herein forms a limited, but central component of the GEM project. A major innovating aspect is that it extends the Dynamic Energy Budget model of Kooijman (1993) to include autotrophic and calcifying systems. Novel molecular approaches in biology, such as molecular genetics, are insufficiently used in marine ecology. In our programme, they play a fundamental role.

Relevance for NOP II

The application of GEM is essential for the improvement of climate models as it helps in the implementation of biological climate forcing.

Links with NOP-I research

NOP-I funding has allowed us to develop the major insights as well as the international infrastructure for realising the GEM approach. The present proposal builds on from a general picture of the *E. huxleyi* cell which was created in this first period. The difference with the NOP-I phase is that we are now in a position to develop a general model of *E. huxleyi* cell dynamics. This new system's approach to the *E. huxleyi* cell is the cornerstone of GEM's goal to understand the role of calcifying ocean plankton in global climate, and in addition permits generalisation of the result to include a much wider variety of plankton. NOP II aims at a higher level of organisation than NOP I.

10. **Objectives and expected results**

The objective of the proposed research is to construct the central model for the Global Emiliania Modelling Initiative. Primarily it will describe the ecological behaviour of the *Emiliania* cell. In association with other models it will be capable of simulating biosphere-climate interactions. More specifically, the objectives are (1) to develop a dynamic model of the physiology of *E. huxleyi* as far as climatically relevant products are concerned; (2) to generate a solid data base on the steady state performance under various nutrient, light and temperature levels; (3) to implement this model in an ocean environment; and (4) to obtain insight in the applicability of our results to other calcifying organisms through molecular genetical studies.

Expected results are: (1) a model succinctly describing the relationships between environmental circumstances, cellular characteristics, and the production of new biomass, DMSP and $CaCO_3$; (2) precise experimental evidence on the dependence of cellular characteristics on environmental circumstances.

11. **Relevance and use for policy making**

The importance of biological calcium carbonate production for climate modelling, especially over time scales of more than a few hundred years, was recently underlined by Maier-Reimer

et al. (Nature, 1994). In *Emiliania* this process is coupled to the organic pump and to DMS-derived cloud production, which operate on much shorter time scales. With the blooms shifting polewards and their surface area decreasing upon further global warming, the cooling effect of DMS may decrease and a positive feedback re-enforcing the greenhouse effect may be set in operation. Quantification of such important relationships require a thorough understanding of the physiology of the participating organisms.

12-13 **International collaboration**

GEM was created in the Netherlands in 1989, with financial support from NWO (CO2), NOP and NIOZ. It was realised that, in order to appropriately address the huge complexity of the problem, an international network of dedicated investigators was required. In five international symposia organised by the Dutch team at Château de Blagnac, near Bordeaux, France the GEM research strategy was worked out in detail, results were discussed, funding was organized and the international team erected. Presently, about 50 investigators from 7 countries, including the US, Canada and Japan, participate in GEM. Elaborate common experimental and field programmes were carried out (e.g., cruises in the N. Atlantic area and 2 mesocosm experiments, 6 weeks each, in the Norwegian fjords). The communication between the participating projects is facilitated by an extensive modelling programme. Funding obtained from the European project MAST II supports extensive collaboration in Europe. Important contributions were also obtained from national agencies, particularly in the Netherlands and in Norway. GEM was part of the JGOFS experiment in the N. Atlantic and a special 1990 BOFS cruise was dedicated to *E. huxleyi* blooms S. of Iceland.

14. **Relevant expertise/experience of the research groups**

The Geobiochemistry Unit at Leiden University has a long-standing experience with biochemical and molecular genetical research on ecologically important biochemical processes. The laboratory is internationally highly respected and very well equipped for this research. Dr. P. Westbroek is the head of this department.
Kooijman has been research director at the department of environmental toxicology of TNO-MW from 1977 till 1985. Since 1985 he is professor of the department of theoretical biology, VUA. This group represents a substantial expertise in the kind of models necessary for this project. The tenure staff consist of two mathematical biologists, a statistician, a computer

scientist, and a systems theorist, all specialised in applying mathematics to biology. The group participates in the MAST-II project and in the NWO-program 'Verstoring Aardsystemen' collaborating with the group of Dr. Mur, Dr. van Stam (UVA), and van Gemerden (RUG). This program links up well with the present proposal.

Dr. Riegman is actively involved in research for 13 years. During a period of 2 years het was head of the Microbiology Laboratory of the Food Inspection Service.

Dr. Gieskes received his Ph.D. at the university of McGill, Montreal. From 1970 till 1987 he worked at the NIOZ. From 1987 onwards het is associate professor at the university of Groningen. He is coordinator of the european DMS program. The group of Dr. Gieskes has built up a internationally recognized expertise in the field of DMS research, partly funded by NOP-I and partly with EG funds. Both at NIOZ and at RUG there is a thorough expertise in running and manipulating chemostat cultures of *E. huxleyi* cultures. Immunochemistry applied on algal cells, necessary for detecting DMSP lyase, has been a hot item at the Department of Marine Botany since a few years.

15. **Long term research strategy of the institutes**

The present proposal is well embedded in other work of the department of Theoretical Biology VUA. In the past 10 years a dynamic energy budget theory has been developed that has, among other things, been successfully used in the modelling of product formation in micro-organisms, and in the description of the dynamics of chemostat based food chain experiments (for many of the results, see Kooijman 1993). Several Ph.D. students are working on related topics, such as microbial physiology, simultaneous limitations in autotrophic systems, and population and food chain dynamics (financed by the NWO priority program `Non-linear and dynamic systems' and the NWO priority program `Verstoring Van Aardsystemen'. The central issue is in mass and energy fluxes through biota, and more in particular, the relationships between different levels of biological organisation and their characteristic time scales.

`Geobiochemistry' has been a recognised line of research since 1970, when the geobiochemistry unit was founded at the Biochemistry Laboratory. The University has an informal commitment to continue this research until 2002 (retirement Westbroek).

The long term strategy of the NIOZ is to unravel the cycles in the marine environment with a high degree of relevance to society and to the connected political management. The phytoplankton research focusses on topics related to algal bloom associated carbon and nutrient fluxes, and its impacts on global change.

16 **Description of research plan**

(a) Modelling the physiological organisation of *E. huxleyi*

starting date 01-01-1995 **final date** 31-12-1998

input Literature on microalgal growth models; literature on experimental evidence of the processes to be modelled. More specifically, the latter point concerns literature on nutrient uptake, photosynthesis, the interaction of these two processes, the dependence of calcification on photosynthesis and nutrient status, and the effects of temperature on all processes mentioned. For both subjects a considerable literature data base is already available (over 150 highly relevant references).

activities The first will be the identification of input, state and output variables. Next, the connections between the variables have to be defined. Finally, precise assumptions We will model mass fluxes of a limited number of chemical species, for instance 2 nutrients and carbon (see Figure 3), especially paying attention to the law of mass conservation. Any model not satisfying this constraint is useless in an ecological context.

We will distinguish between structural biomass and a small number of nutrient storages. Any component has a constant composition, but the various components (may) differ in composition. Growth is determined by the states of the storages. The storages are replenished by uptake from the environment. This uptake depends on the nutrient concentrations in the environment, and may be driven by light.

Several routes in modelling will be followed. The various models will be tested against experimental evidence, partly taken from the literature. The data to be generated by subproject (b) will provide the final test for the model. This also allows the estimation of the values of the model parameters specifically for *Emiliania huxleyi*.

products

– Review of existing approaches in microalgal growth modelling.
– Model for growth on two substrates, under light-saturating conditions.
– Model for growth on DIC under various light regimes, other nutrients at saturating concentrations.
– Model for growth on one nutrient and light, DIC saturating.

– Integrated model that describes growth and $CaCO_3$ as dependent on nutrient concentrations and light.
– Incorporation into the model of the effects of temperature.
– Implementation of integrated model in mesocosm environment.

responsible scientist Prof. Kooijman

organization Vrije Universiteit Amsterdam

(b) Physiological characterisation of *Emiliania huxleyi*

starting date 01-01-1995 **final date** 31-12-1997

input At present *E. huxleyi* is studied in continuous cultures at the Geobiochemistry group in Leiden (investigation of the formation of polysaccharides) at the NIOZ (uptake of carbon dioxide/bicarbonate), and at Groningen (DMS). These experience will be used in the present project.

Recent funding within the MAST II-project enabled us (GeoBiochemistry group, Leiden University) to set up most of the required molecular techniques for this project. At the moment cDNA libraries of a calcifying strain and a non-calcifying 'mutant' are available (Corstjens and Westbroek 1994). These cDNA libraries will form the starting material for this research.

activities

– Installation of equipment for cultivation and preparation of analytical equipment.
– Data generation. Under all conditions, the first step is to arrive at steady state conditions of chemostat cultures under continuous illumination. *Measured characteristics under steady state conditions:* biomass (cell number, dry weight); nutrient quota (nitrogen, carbon); chlorophyll a; dissolved inorganic nutrients in effluent; DMSP and glycine-betaine production. In addition to measuring chemostat steady states characteristics, uptake kinetics of nutrients in steady state will be investigated. PI curves in all steady states will be constructed. The experiments will be performed under the following conditions:
• nitrogen as nutrient, under light-saturating conditions. Steady states at 4 dilution rates.
• light limited chemostats under nutrient-saturating conditions.

• *simultaneous* nutrient and light sub-saturating conditions.

With respect to molecular genetics, two experiments will be performed: (1) the isolation of genes encoding enzymes with are already known using DNA probing, PCR and antibody screening; and (2) the isolation of novel genes, using differential hybridisation and subtracted probes. The cDNA libraries are constructed in such a way that they can be screened by using DNA probing as well as antibody screening. If heterologous DNA-probes and antibodies cannot be used, we will use the PCR technique with DNA primers constructed against short but highly conserved DNA regions. (if these sequences are available). Differential hybridisation will be used to identify novel genes functional in calcification, photosynthesis or cell-division. First we focus on the identification of genes involved in the process of calcification. The ideal result from the experiments will be three classes of hybridising clones: (i) clones containing probably common 'household genes' functional in the light as well as the dark period; (2) clones containing genes functional only during the light period, including genes functional in calcification and photosynthesis; and (3) clones containing the specific genes functional in calcification. After identification and isolation of specific clones DNA sequence analysis will be used to characterise the genes that have been isolated. Northern blotting will then be used to determine when and how transcription of these specific genes is induced.

products The expected results of this subproject is a unique data base on a number of important characteristics of *E. huxleyi* under a diversity of relevant environmental circumstances. At present, such a complete data set *does not exist for any* species of phytoplankton. Therefore, the generation of this data set is highly important, not only to back up the model subproject, but also for phytoplankton ecology in general.

responsible scientists Dr. R. Riegman (chemostat cultures at NIOZ), Dr, M. Veldhuis (CO_2 limited growth, NIOZ), Dr. W.W.C. Gieskes (DMS at RUG), and P. Westbroek (molecular genetics, RU Leiden).

organisation NIOZ, Texel; RU Groningen; RU Leiden.

Table 1. Specification of reserach plan (milestones marked with '√').

Sub-project	Product	Time Schedule		Report date
(a) Modelling				
Post doc	Review of growth models	3 months	√	01-06-95
	Analysis model for growth on 2 nutrients	3 months		31-12-95
	Model for growth with sub-saturating light and nutrient	6 months	√	31-12-96
	Implementation of model in mesocosm environment	6 months	√	31-12-97
	Final model validation and parametrization	6 months	√	31-12-98
OIO	Orientation on subject	6 months		
	Model for growth on DIC with varying light	1 year	√	01-06-96
	Incorporation of temperature effects	1 year	√	01-06-97
	Integrated model	1 year	√	01-06-98
	Writing of PhD thesis	6 months	√	31-12-99

Table 1 (continued)**. Specification of reserach plan** (milestones marked with '√').

(b) Physiology				
NIOZ	Installation of equipment	6 months		
	Experiments with varying nutrient, light constant	6 months	√	01-01-97
	Experiments with varying light and nutrient	1 year	√	01-01-98
	Temperature effects	1 year	√	31-12-98
RU Groningen	Installation of equipment	6 months		
	Experiments with varying nutrient, light constant	6 months	√	01-01-97
	Experiments with varying light and nutrient	1 year	√	01-01-98
	Temperature effects	1 year	√	31-12-98
RU Leiden	Isolation and sequencing of genes coding for enzymes with known functions	1 year	√	31-12-96
	Isolation of novel genes	1 year	√	31-12-97

APPENDIX 2. PROJECT PUBLICATIONS

Peer-reviewed publications in scientific magazines

(including papers in preparation)

Corstjens P.L.A.M., Araki Y. & Gonzalez E.L. 2001. A coccolithophorid calcifying vesicle with a V-type ATPase proton pump: cloning and immunolocalization of the Vo subunit c(1). *J. Phycol.* 37: 71-78

Corstjens P.L.A.M. & Gonzalez E.L. (in prep). Effects of N and P limitation on expression of the coccolith vesicle-proton pump (subunit c) of *Pleurochrysis* (Haptophyta).

Corstjens P.L.A.M., van der Kooij M., Linschooten C., Brouwers, G.-J., Westbroek P. & de Vrind- de Jong. 1998. GPA, a calcium-binding protein in the Coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae). *J. Phycol*. 34: 622-630.

Huisman, J. and Jonker, R.R. and Zonneveld, C. and Weissing, F. J. 1999. Competition for light between phytoplankton species: experimental tests of mechanistic theory. *Ecology* 80: 211-222.

Kooijman, S.A.L.M. 1998. The Synthesizing Unit as model for the stoichiometric fusion and branching of metabolic fluxes. *Biophys. Chem*. 73:179-188.

Kooijman, S.A.L.M. 2001. Quantitative aspects of metabolic organization; a discussion of concepts. *Phil. Trans. R. Soc. B* 356:331-349.

Kooijman, S.A.L.M., Auger, P., Poggiale, J. C. & Kooi, B.W. (in prep). Eight quantitative steps in the evolution of endosymbiosis and homeostasis.

Kooijman, S.A.L.M., Dijkstra, H. A. &. Kooi, B. W. (submitted). Light-induced mass turnover in a mono-species community of mixotrophs. *J. Theor. Biol*.

Lin, S., Corstjens P.L.A.M. & Carpenter E.J. (in prep). Conservation and evolution of proliferating cell nuclear antigen in the coccolithophorid *Pleurochrysis carterea*.

Nisbet, R.M., Muller, E.B., Lika, K. & Kooijman, S.A.L.M. 2000. From molecules to ecosystems with Dynamic Energy Budget models, *J. Anim. Ecol*, 69:913-926.

Riegman, R., Stolte, W. & Noordeloos, A.A.M. 1999. A model system approach to biological climate forcing: the example of *Emiliania huxleyi*. *NIOZ-rapport* 1998-8

Riegman, R., Stolte, W., Noordeloos, A.A.M. & Slezak, D. 2000. Nutrient uptake and Alkaline phosphatase (EC 3:1:3:1) activity in *Emiliania huxleyi* (strain L) during N- and P-limited growth in continuous cultures. *J. Phycol*. 36:87-96.

Stolte, W., Noordeloos, A.A.M., Zonneveld, C. & Riegman, R. (in prep). Towards modeling the growth of *Emiliania huxleyi*: Cell composition and photosynthesis under steady state light limitation in turbidostat at different photon flux densities.

Stolte, W., Noordeloos, A.A.M., Kraaij, G.W. & Riegman, R. 2000. Genetic and physiological variation in pigment composition of *Emiliania huxleyi* (Prymnesiophyceae) and the potential use of its pigment ratios as a quantitative physiological marker. *J. Phycol.* 36:529-539.

Van Rijssel, M., W.W.C. Gieskes. 2001. Temperature, light, and the dimethyl-sulfoniopropionate (DMSP) content of *Emiliania huxleyi* (prymnesiophyceae). *J. Phycol*. (accepted)

Van Rijssel, M., Buma, A. (submitted). UV-B induced stress does not affect DMSP synthesis in *Emiliania huxleyi*. *Mar. Ecol. Prog. Ser.*

Zonneveld, C. 1996. Modelling the kinetics of non-limiting nutrients in microalgae. *J. Mar. Syst* 9:121-136.

Zonneveld, C. 1997. Modeling effects of photoadaptation on the photosynthesis-irradiance curve. *J. Theor. Biol*.186:381-388

Zonneveld, C. 1998. Photoinhibition as affected by photoacclimation in phytoplankton: a model approach. *J. Theor. Biol*.193:115-123

Zonneveld, C. 1998. Light-limited microalgal growth: a comparison of modeling approaches. *Ecol. Modell*. 113:41-54

Zonneveld, C. 1998. A cell-based model for the chlorophyll *a* to carbon ratio in phytoplankton. *Ecol. Modell* 113:55-70.

Zonneveld, C., Berg, H.A. van den & Kooijman, S.A.L.M. 1997. Modeling carbon cell quota in light-limited phytoplankton. *J. Theor. Biol* 188:215-226.

Zonneveld, C. (submitted). The Photosynthesis--Irradiance relationship and the dark reactions: an inconvenient marriage? submitted to Ecological Modelling

Books (book chapters)

Kooijman, S.A.L.M. 2000. Dynamic Energy and Mass Budgets in Biological Systems. Cambridge University Press, Cambridge.

Kooijman, S.A.L.M. and Nisbet, R.M. 2000. How light and nutrients affect life in a closed bottle. *In*: J¢rgensen, S.E. (ed), *Thermodynamics and Ecological Modelling*, CRC Publ., Boca Raton, FL, USA, pp 19-60

Kooijman, S.A.L.M. (submitted). Global aspects of metabolism; on the coevolution of life and its environment. In: Miller, J., Boston, P.J., Scheider, S.H. and Crist, E. Scientists in Gaia: 2000 MIT Press.

Kooijman, S.A.L.M. 2000b. Van herbivorie, via symbiose naar mixotrofie. In: Heesterbeek, H., Diekmann, O. & Metz, J.A.J. (eds) Theoretische biologie, Epsilon, Utrecht.

Stefels, J., Van Rijssel, M., Hansen, T.A., Dijkhuizen. L. & Gieskes, W.W.C. (eds). 2000 Biological and environmental chemistry of DMS(P) and the role of *Phaeocystis* in marine biogeochemical cycles. *Special issue J. Sea Res*. 43.

Riegman, R. and Stolte, W. and Noordeloos, A. A. M. 1999. A model system approach to biological climate forcing: the example of *Emiliania huxleyi*. *NIOZ-rapport* 1998-8

Published abstracts

Corstjens P.L.A.M., Westbroek P. & Gonzalez E.L. 1997. Coccolithophorid phylogeny: sequence analysis of the highly conserved proteolipid subunit of the V-type ATPase gene. *Phycologia* 36 supplement: 21-22.

Corstjens PLAM & Gonzalez EL. 1996. Cloning, molecular characterization and expression of a vacuolar H+-ATPase 16 kD proteolipid gene of *Pleurochrysis carterae*. *J. Phycol*. 32S: 13.

Published gene sequences

Corstjens P.L.A.M. & Gonzalez E.L. 1999b. Nucleotide sequence of genes encoding Actin of the coccolithophorid *Pleurochrysis carterae* str. 136.*Plant Physiol.* 120:933.

Corstjens PLAM, Araki Y, Westbroek P & Gonzalez EL. 1996. A gene encoding the 16 kD proteolipid subunit of a Vacuolar-type H(+)-ATPase from *Pleurochrysis carterae* strain 136 (accession no. U48365 and U53182). (PGR96-038) *Plant Physiol.* 111: 652.

Publications for broader public

Gieskes, W.W.C. 1997. Mariene microalgen dragen bij aan thermoregulatie van de aarde. Bionieuws 16, 11 oktober, jaargang 7.

M. van Rijssel. 2000. DMS, a biogenic climate-regulating gas. Change 51:11-13.

M. aan de Brugh. 1999. Interview met P. Westbroek: Het dier aarde. NRC handelsblad 20 nov 1999. pg 51.

P. Westbroek. 1999. Gaia en de zachte kanten van het leven.. Rede uitgesproken bij aanvaarding van het ambt van hoogleraar in de Geofysiologie aan de Universiteit van Leiden.

Publications via internet

http://www.bio.vu.nl/thb/deb/

Dynamic Energy Budget Theory.

http://www.bio.vu.nl/thb/deb/deblab

An electronic laboratory has been opened at, which presents downloadable software to encourage (international interdisciplinary) collaboration in developing the DEB theory, and its many applications. The main program is named “DEBtool”', and automizes computation on the eco-physiology of micro-organisms, algae, plants, animals and symbioses. Future developments will include the population and ecosystem levels, as have already been elaborated in the DEB theory.

APPENDIX 3 COORDINATION WITH OTHER PROJECTS AND PROGRAMMES

The program was preceded by:

The NOP I project “Phytoplankton and the oceanic carbon cycle; *Emiliania huxleyi* as a model system.”

The European *Emiliania huxleyi* program MAST II- funded program: “Coccolithophorid dynamics (EHUX)” for identification of genes involved in calcification of *Emiliania huxleyi.*

The EU program “Role and significance of biological processes in DMS release from ocean to atmosphere: a close examination of the black box.” (EV5V-CT-93-0326).

The project was embedded in**:**

The Global Emiliania Modeling Initiative (GEM) that was created in the Netherlands in 1989, with financial support from NWO (CO_2), NOP and NIOZ. Presently, about 50 investigators from 7 countries, including the US, Canada and Japan, participate in GEM. The communication between the participating projects is facilitated by an extensive modelling program and by the recent foundation of the Gaia Research Center for Biogeology (RUL).

The Dynamic Energy Budget (DEB) research program of the department of Theoretical Biology (VUA).

The project linked with:

NWO-priority program “Perturbation of Earth Systems.” Dr. Mur, Dr. van Stam (UVA), and van Gemerden (RUG).

NWO-priority program “Non-linear and dynamic systems.” Dr de Roos (UVA), Dr de Boer (VUA).

EU-MAST III program “Entangled Sulfur and Carbon cycles in *Phaeocystis* dominated Ecosystems (ESCAPE), Prof. Dr Dijkhuizen, Dr Ir Hansen, Dr Stefels, Dr. Faber (RUG).

NWO Global Change “ the partial pressure of CO_2 in seawater as a control on marine biological productivity and calcification.” Prof. Dr de Baar, Drs Buitenhuis (NIOZ).

EU-5th framework programme -“ Iron Resources and Oceanic Nutrients- Advancements of Global Environment Simulations ” (IRONAGES), Dr Stefels (RUG), Prof. Dr de Baar (NIOZ).

CAO-GOA “Effects of UV-radiation on phytoplankton.” Dr Buma, Dr Gieskes (RUG).

Note

Part of the work performed by Corstjens at the Department of Organismic Biology, Ecology & Evolution in the group of Prof. E. Gonzalez (University of California Los Angeles) was financed by a personal grant to Prof. E. Gonzalez from the Office of Naval Research, Department of Defence, USA.

APPENDIX 4 SPECIAL ACTIVITIES AND ATTENDANCE AT NATIONAL AND INTERNATIONAL MEETINGS

A.1 Organisation of meetings:

Leiden

1999/09/11-17 Global carbon and silica cycles. Les Treilles, France.

NIOZ

1997/05/25-26. 1st international *Emiliania huxleyi* workshop on modelling the growth of *Emiliania huxleyi*

1998/06/10. 2nd international *Emiliania huxleyi* workshop

RUG

1999/08/25-28. Biological and Environmental. Chemistry of DMS(P) and related compounds.

1999/08/28-30.The role of *Phaeocystis* in Marine Biochemical Cycles

A.2 Attendance at national and international meetings

Corstjens:

1997/06/5-11. Coccolithophoric calcification:Intracellular coccolith production by the unicellular algae *Emiliania huxleyi* and *Pleurochrysis carterae*. Workshop "Biomineralisation: its role in the cambian diversity of life", Fondation des Treilles, France.

1997/08/6-16. 6th International phycological congress. Leiden, Netherlands. Oral presentation: Coccolithoporid phylogeny: sequence analysis of the highly conserved proteolipid stubunit of the V-type ATP-ase gene.

Van Rijssel:

1997/03/25- 26. 1st International *Emiliania huxleyi* workshop NIOZ, Texel

1998/02/9-13. Ocean Science Meeting San Diego, USA

1998/06/10. 2nd international *Emiliania huxleyi* workshop, NIOZ, Texel

1999/08/25-28. Biological and Environmental. Chemistry of DMS(P) and related compounds. Rijksuniversiteit Groningen.

1999/08/28-30. The role of *Phaeocystis* in Marine Biochemical Cycles. Rijksuniversiteit Groningen

Gieskes:

1998/10/29. Symposium NRP-II “Global Change”, hotel 't Speulderbos, Garderen.

1999/08/25-28. Biological and Environmental. Chemistry of DMS(P) and related compounds. Rijksuniversiteit Groningen.

1999/08/28-30. The role of *Phaeocystis* in Marine Biochemical Cycles. Rijksuniversiteit Groningen

Westbroek:

1997/08/6-16. 6th International phycological congress. Leiden, Netherlands. Oral presentation: Coccolithoporid phylogeny: sequence analysis of the highly conserved proteolipid stubunit of the V-type ATP-ase gene.

1997/06/5-11. Coccolithophoric calcification:Intracellular coccolith production by the unicellular algae *Emiliania huxleyi* and *Pleurochrysis carterae*. Workshop “Biomineralisation: its role in the cambian diversity of life”, Fondation des Treilles, France.

1999/09/11-17. Global carbon and silica cycles. Les Treilles, France.

Kooijman:

1996/03/05. Theorie voor dynamische energie budgetten. Caput P. Westbroek, Gorleus Lab., Leiden.

1996/04/23. Samenhang van biologische organisatie-niveau's. Caput P. Westbroek, Gorleus Lab., Leiden.

1996/04/25. De rol van *Emiliania huxleyi* in de globale C-cyclus. ACES Symposium biogeochemie, VUA, Amsterdam.

1996/10/21. The dynamics of microbial food chains. Workshop on Mathematical Ecology, ICTP, Trieste, Italy.

1997/02/11. Scaling problems in mass and energy balances in biological systems: a dynamic energy budget modeling approach. University of California, Santa Barbara, USA.

1997/09/15. Modelling simultaneous nutrient limitation in algal growth. 7 th GEM conference, Blagnac, France.

1998/04/23. Dynamische Energie Budget theorie en toepassingen. NIOZ, Texel.

1998/04/28. General philosophy of the Dynamic Energy Budget theory for the uptake and use of substrates by organisms. Institut Universitaire Européen de la Mer, Brest, France.

1998/05/11. Mass and energy balances in relation to body size scaling relationships. Workshop “Energetic and Thermodynamic Foundations of Ecological Systems”, Santa Fee, New Mexico, USA.

1998/10/05. The Dynamic Energy Budget Model for structuring populations on the basis of physiological processes. ``First International Conference on Mathematical Ecology'', Colegio de San Ildefonso, Alcalá de Henares (Madrid), Spain.

1998/10/29. The role of phytoplankton in the global carbon cycle in relation to climate change. Symposium NRP-II ``Global Change'', hotel 't Speulderbos, Garderen.

1998/12/15. How toxicants can effect life in a closed bottle. Workshop ``predicting Population Level Effects of Toxicants'', National Center for Ecological Analysis and Synthesis, Santa Barbara, California, USA.

1999/03/22. Modelling diatom metabolism, in relation to primary production. Silica Workshop, Southampton, UK.

1999/09/13. The application of the DEB theory to analyse trophic interactions: calcifying coral/zooxantellae symbiosis. Biological participation in the global carbonate cycle. Les Treilles, France.

2000/02/17. Six lectures on Dynamic Energy Budget theory. Humboldt University, Berlin, Germany.

2000/02/28,29,30. Dynamic Energy Budget theory Physiologically structured population and ecosystem dynamics Applications of DEB theory. 5th Workshop on Mathematical Ecology, ICTP, Trieste, Italy.

2000/03/13. Heat dissipation from biological systems; the energy-mass coupling, International Onsager-Workshop, Oud Poelgeest, Leiden.

2000/05/01. The Synthesizing Unit as model for in interaction of substrates in the uptake by organisms. Lecture at Arizona State University, Tempe, USA.

2000/05/03. Stoichiometric constraints on food uptake. NCEAS workshop on stoichiometry and on predicting population level effects of toxicants, University of California, Santa Barbara, USA.

2000/05/25. The role of the individual organism in the chain from molecule to ecosystem. NWO Symposium on Nonlinear Systems, University of Twente, Enschede.

2000/05/30. Stoichiometric contraints in Dynamics Energy Budgets. Workshop on Stoichiometric constrains on carbon sequestration in ecosystems, Det Norske Videnskaps-Akademi, Oslo, Norway.

2000/06/19. Quantitative aspects of metabolism and its global impact. Chapman Conference on the Gaia Hypothesis, University of Valencia, Spain

2000/07/20. Dynamic Energy Budget theory for mixotrophs. Third World Congress of Nonlinear Analysts, International Federation of Nonlinear Analysts (IFNA), Catania, Sicily, Italy.

2001/02/19 Quantitative aspects of metabolic organization. Van Gogh exchange programme, Université Claude Bernard, Lyon, France.

2001/03/12. Multiple nutrient limitation and dynamic energy budgets. Workshop Modelling Nutrient Limitation: Liebig and Beyond. Mathematics Institute, University of Warwick, UK.

2001/06/14 The evolution of endosymbiosis and homeostasis. 21-th siminar of the French Society for Theoretical Biology, Paris, Van Gogh exchange programme

Stolte:

1997/03/25- 26. Organisation of an international *Emiliania huxleyi* workshop by W. Stolte at the Netherlands Institute for Sea Research, Texel

1997/05/29-30. W. Stolte and A. Noordeloos visited 'European Network on Integrated Marine System Analysis' (ENIMSA) in Brussel, Belgium.

1997/08/11-15. 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.

1997/09/l3-l7. 7th *Emiliania huxleyi* meeting, Blagnac, France.

1997/10/21. Prof. Dr. Elma Gonzalez, who was visiting the University of Leiden, was invited by W. Stolte to present a lecture at MOZ titled: "A Cellular Perspective of Coccolithophorid Calcification."

1997/10/3. 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.

1998/02/9-13. 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" (AS LO) in San Diego, USA.

1998/05. W. Stolte visiting Prof. Dr. Edna Graneli of the Department of Marine Sciences of the University of Kalmar, Sweden to discuss possible co-operation in the future.

1998/06/10. Organisation 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*".

1998/07/9. 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.

Zonneveld:

1996/08/11-18. First European Phycological Congress. Poster: Microalgal growth: a new modeling approach. Cologne, Germany

1996/10/31. Colloquium NIOZ.

1997/04/11, 3de Marine life sciences platform (MLP) lezingendag, Amsterdam

1997/05/20-26. International JGOFS Modelling Symposium.. Poster: A process-based model for the chlorophyll to carbon ratio in phytoplankton. Oban, Scotland

1997/08/6-16. 6th International phycological congress. Oral presentation: A process-based model for the chlorophyll to carbon ratio in phytoplankton. Leiden, Netherlands

1997/09/16-19. 1st European Ecological Modelling Conference. Oral presentation 1: Light-limited growth in phytoplankton: a critical appraisal of current modelling methodology. Oral presentation. 2: A cell-based model for the chlorophyll to carbon ratio in phytoplankton. Pula, Croatia

1998/06/10. 2nd international *Emiliania huxleyi* workshop

1998/06/21-26 5th Int Conf Math Pop Dynam. Zakopane, Poland. A cell-based model for the chlorophyll to carbon ratio in phytoplankton.

1998/09/4-8, Alcala 1st Conference on Mathematical Ecology. Phytoplankton, nutrients and resource competition: the concept of invasibility.

1998/10/29. Symposium NRP-II ``Global Change", hotel 't Speulderbos, Garderen.

1998/04/28, Universite de Bretagne Occidentale, Brest

1999/06/29 -3, 4th EMSTB meeting. Poster: Luxury consumption and resource competition among phytoplankton.