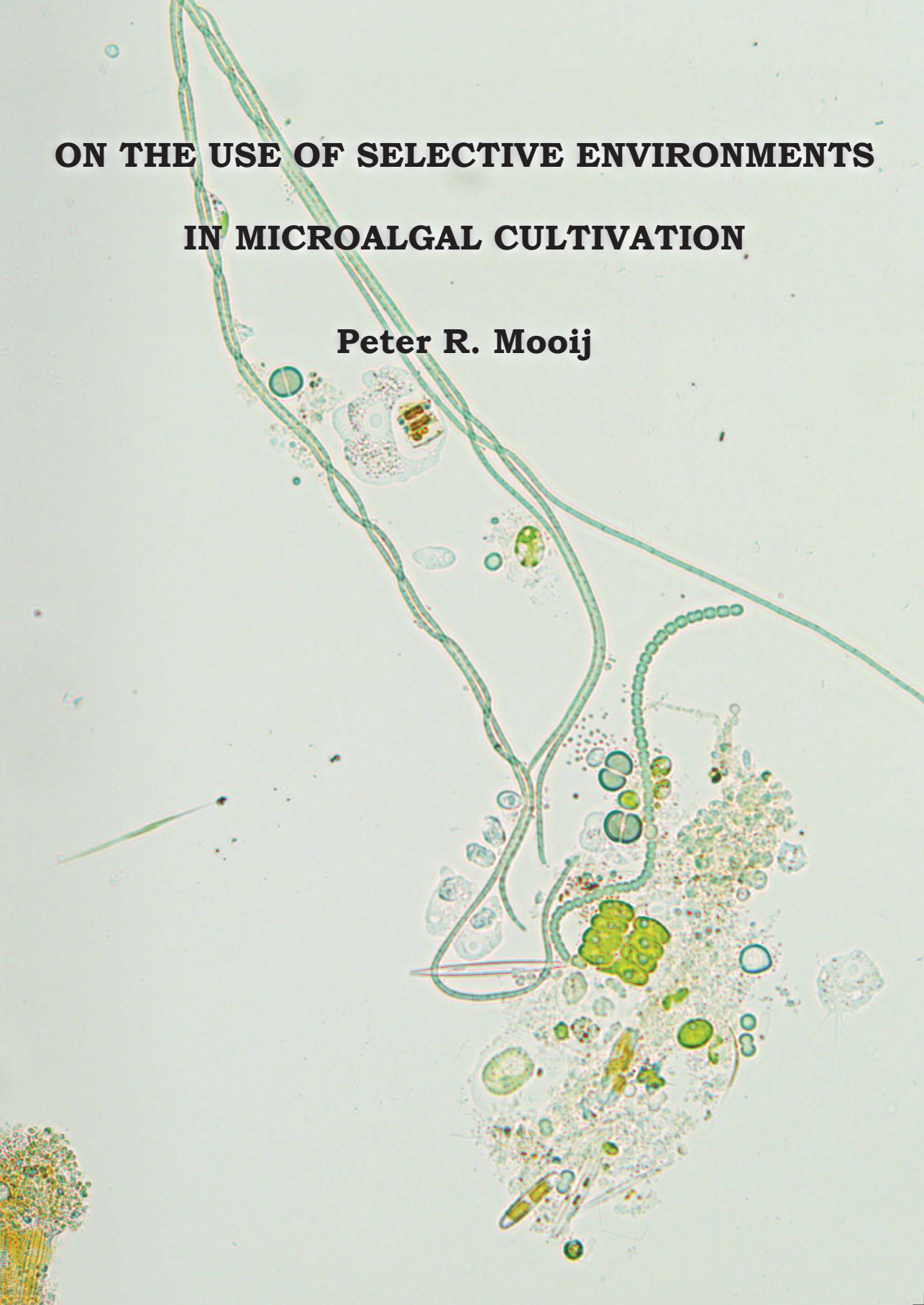
ON THE USE OF SELECTIVE ENVIRONMENTS IN MICROALGAL CULTIVATION

Peter R. Mooij



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Proefschrift

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'Nauwelijks is een winstgevende bereiding van een of ander gistingsproduct tot stand gekomen, of de chemici stellen zich doorgaans met succes tot taak dit product te synthetiseren, daarbij uitgaande van de 'goedkope koolstof' zoals deze in de fossiele organische grondstoffen als steenkolen, petroleum of aardgas aanwezig is.

Voor de microbioloog blijft dan de magere troost, waarop ik 27 jaren geleden bij mijn ambtsaanvaarding reeds wees, dat het reservoir dezer fossiele grondstoffen eens zal zijn uitgeput en dat de mensheid dan weer voor de grondstoffen van zijn organische industrieën op de voortbrengselen der recente groene plantenwereld zal zijn aangewezen. Bij de verwerking van de daarin aanwezige koolhydraten zal de microbioloog dan weer nieuwe triomfen kunnen vieren.'

Albert Jan Kluyver - 107de gedenkdag der Technische Hogeschool, 1949

CONTENTS

List of Abbreviations and Definitions

Abbreviations

ADI	applikon dependable instruments
ASW	artificial sea water
ATP	adenosinetriphosphate
DMSO	dimethylsulfoxide
C-mol	carbon-mol
DOT	dissolved oxygen tension
GAP	glyceraldehyde-3-phosphate
PCR-DGGE	polymerase chain reaction - denaturing gradient gel electrophoresis
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
P/O-ratio	phosphate / oxygen ratio
VSS	volatile suspended solids
VER	volume exchange ratio
TAG	triacylglyceride
TSS	total suspended solids
UF	urea-formamide

Definitions

microalgae	The term 'microalgae' has no formal taxonomic standing. In this thesis, and in most scientific literature, the term microalgae covers all microorganisms capable of doing oxygenic photosynthesis. This includes cyanobacteria.
biomass	The term biomass is, in this thesis, defined as the organic dry weight minus glucose polymers and minus storage lipids. Biomass therefore comprises proteins, DNA, organelles, membranes and other cell constituents. The term 'residual biomass' is used as synonym.

SUMMARY

This thesis deals with selective environments in microalgal cultivation. As explained in Chapter 1 microalgae have changed the course of life on Earth dramatically by performing oxygenic photosynthesis. In oxygenic photosynthesis electrons from water are used to reduce carbon dioxide to carbohydrates or lipids using solar energy. As a waste product oxygen is produced. The production of carbohydrates and particularly lipids by microalgae attracts currently considerable scientific interest as microalgal lipids can be converted to yield biodiesel. As the CO_2 emitted upon combustion of this biodiesel has recently been withdrawn from the atmosphere by the microalgae, no net increase in atmospheric CO_2 level takes place. Microalgae offer advantages over other lipid production platforms as microalgae are able to reach high intracellular lipid contents and need little freshwater and arable land.

Chapter 2 describes the advantages and limitations of the application of a selective environment to obtain a certain functionality in a system. A selective environment aims to give a competitive advantage to a microorganism displaying the desired functionality. By rewarding microalgae for displaying a certain functionality it becomes in the interest of microalgae themselves to display this characteristic. The best reward in nature is an increased chance of survival. A selective environment therefore tries to couple the desired characteristic to an increased chance of survival.

Microalgal cultivation based on selective environment fundamentally differs from cultivation of pure cultures. Maintaining the desired culture is the goal of the latter, whereas a microalgal cultivation process based on selective environments aims to maintain a functionality in a system. The species, or multiple species, displaying the desired functionality are expected to differ at different geographical places, with changing climate conditions and over time. Under any condition the species that thrives under these specific conditions by displaying the desired characteristic is enriched.

Interesting microalgal functionalities from an industrial point of view include a high carbohydrate and lipid productivity. Both of these storage compounds are produced by microalgae to endure dynamic growth conditions. By limiting the presence of the essential microalgal nutrient nitrogen (in the form of NH_4^+ or NO_3^-) to the dark phase solely an environment is created in which production of storage compounds in the light period is an advantageous strategy. Production of these compounds in the light period will allow microalgae to metabolise the available nitrogen in the dark period by supplying carbon skeletons and energy in the dark. Chapter 3 shows that such an environment enriches carbohydrate producing microalgae from a natural inoculum.

Chapter 4 shows that both the moment of nitrogen addition as the amount of nitrogen dosed per microalgae had significant influence on the metabolic behaviour of marine microalgal cultures enriched using the procedure described in Chapter 3. Carbohydrate and lipid productivity proved maximal if ammonium was supplied at the start of the dark period rather than the light period, irrespective of the amount of nitrogen dosed

per microalgae. Increasing the amount of nitrogen dosed per microalgae, by increasing the volume exchange ratio from 33 to 50 percent per cycle, induced a decrease in storage compound production if ammonium was supplied in the light period whereas the storage compound productivity was comparable when ammonium was supplied in the dark period.

Chapter 5 shows that the enriched microalgal community was highly dependent on an environmental parameters as the presence of silicate. If silicate was present at non-limiting concentrations the enriched culture was dominated by diatoms, whereas green algae were dominant if silicate was absent. Both cultures showed however the same functionality of producing large amounts of carbohydrates in the light period to be able to consume the supplied nitrogen source in the dark period. These results, together with the data obtained under marine conditions, showed that carbohydrate production can be achieved under various conditions, as long as a carbon fixation in the light period is uncoupled from nitrogen uptake in the dark.

Diatoms have interesting characteristics for large-scale microalgal cultivation. These include a relative easy solid-liquid separation after cultivation, increased resistance to predators and the possibility to synthesize lipids under silicate limitation. Supplying NH_4^+ in a pulse, either at the start of the light or the dark period under non-limiting silicate levels, enriched a culture fully dominated by the diatom *Nitzschia palea* from a natural inoculum, as described in Chapter 6. The metabolic behaviour of the enriched culture was highly influenced by the moment of nitrogen addition. Biomass was the main photosynthetic product in the light period if nitrogen was dosed at the start of the light period, whereas carbohydrates were the main photosynthetic product if nitrogen was dosed at the start of the dark period. Subjecting the enriched cultures to prolonged periods of nitrogen or silicate limitation induced different metabolic responses. Cell numbers increased four times and carbohydrates were the main storage compounds under nitrogen limitation, while cell division abruptly ceased and lipids were the preferred storage compound under silicate limitation.

In all experiments carried out in this thesis carbohydrates were the preferred microalgal storage compounds. Uncoupling of carbon fixation in the light from nitrogen uptake in the dark enriched under variable conditions (freshwater, marine, under high silicate concentrations) carbohydrate producing microalgae from a natural inoculum. Intracellular carbohydrate levels typically increased from 10 to 50 % of organic dry weight in the light period. Although no liquid and gas flows leaving and entering the systems were sterilised and despite regular cleaning of the systems the enriched cultures were highly stable in time. This shows that if carbohydrate productivity is aimed for a proper selective environment has been identified and tested.

A better understanding of the ecological role of lipids and carbohydrates in microalgae will help creating selective environment for lipid production. Besides drawing general conclusions, Chapter 7 elaborates more on possible strategies to enrich lipid producing microalgae. The strategy advocated in this thesis, rewarding a microalga for displaying a functionality by coupling it to an increased chance of survival by imposing a selective environment, will prove a valuable tool if the ecological role of lipids is better understood.

SAMENVATTING

Het onderwerp van dit proefschrift is het gebruik van selectieve milieus bij het kweken van microalgen. Hoofdstuk 1 beschrijft de ingrijpende verandering die microalgen op Aarde teweeg gebracht hebben door het uitvoeren van oxygene fotosynthese. In dit proces worden elektronen uit water gebruikt om CO_2 te reduceren tot koolhydraten en lipiden. Dit proces wordt gedreven door energie uit zonlicht. Zuurstof wordt geproduceerd als afvalproduct in oxygene fotosynthese. Zoals beschreven in Hoofdstuk 1 heeft het ontstaan van zuurstof op Aarde verstrekkende gevolgen gehad.

De productie van koolhydraten en met name lipiden door microalgen is een onderwerp dat tegenwoordig op veel wetenschappelijke belangstelling kan rekenen. De reden hiervoor is dat lipiden uit microalgen omgezet kunnen worden in biodiesel. De hoeveelheid CO_2 die vrijkomt bij het verbranden van deze biodiesel komt overeen met de hoeveelheid CO_2 die door de microalgen aan de atmosfeer onttrokken is door middel van fotosynthese. Het gebruik van biodiesel leidt dus niet tot een toename in de atmosferische CO_2 concentratie. Dit voordeel geldt echter ook voor het verbranden van olijfolie, raapzaadolie of elke andere olie die door recente fotosynthese is geproduceerd.

Microalgen bieden twee voordelen ten opzichte van andere biologische olieproductieplatformen. Ten eerste kunnen microalgen na de kweek voor een relatief groot gedeelte uit lipiden bestaan. Daarnaast heeft men voor het kweken van microalgen relatief weinig zoetwater en landbouwgrond nodig.

Hoofdstuk 2 beschrijft de mogelijkheden en beperkingen van het gebruik van selectieve milieus in het verkrijgen en behouden van een specifieke eigenschap in een systeem. Het doel van het gebruik van een selectief milieu is het creëren van een competitief voordeel voor een micro-organisme met de gewenste eigenschap. Door het gewenste gedrag van een microalg te belonen wordt het vertonen van dit gedrag voordelig voor de microalg zelf. De beste beloning in de natuur is een grotere overlevingskans. Een selectief milieu streeft er daarom naar het gewenste gedrag te koppelen aan een toegenomen overlevingskans.

Het kweken van microalgen gebaseerd op het gebruik van selectieve milieus is fundamenteel verschillend van het kweken van microalgen in pure culturen. Het doel van deze laatste methode is het behouden van één soort microalgen. Het kweken van microalgen gebaseerd op selectieve milieus tracht één eigenschap in een systeem te behouden. De soort microalg, of de soorten, die deze eigenschap vertonen, zullen onder verschillende kweekcondities, op verschillende plaatsen en in de tijd kunnen verschillen. Zolang echter het selectieve milieu in stand gehouden wordt zal onder alle kweekcondities een microalg verrijkt worden die onder deze specifieke condities, door het vertonen van het gewenste gedrag, kan groeien en bloeien.

Vanuit een toepassingsoogpunt is met name een hoge koolhydraat- en lipide-productiviteit een interessante eigenschap voor microalgen. Koolhydraten en lipiden dienen als energie- en koolstofopslag in microalgen, en zijn in dat opzicht te vergelijken met de rol

van glycogeen en vet in mensen. Microalgen produceren deze energetische opslagverbindingen om dynamische groeicondities, zoals donkerperiodes, te kunnen doorstaan.

Centraal in dit proefschrift staat een selectief milieu waarin het produceren van energetische opslagverbindingen voordelig voor microalgen is. Dit milieu wordt gecreëerd door de beschikbaarheid van de voor microalgen essentiële nutriënt stikstof, in de vorm van NH_4^+ of NO_3^-, tot de donkerperiode te limiteren. Het produceren van opslagverbindingen in het licht geeft microalgen de energie en koolstof die nodig is om de aanwezige stikstof in de donkerperiode te metaboliseren. Hoofdstuk 3 beschrijft dat een dergelijk milieu inderdaad koolhydraatproducerende microalgen verrijkte uit een natuurlijke ent.

Hoofdstuk 4 laat zien dat zowel het moment van stikstof dosering als de hoeveelheid stikstof die per alg gedoseerd werd significante invloed had op het metabole gedrag van verrijkte mariene microalgen. De productie van lipiden en koolhydraten was maximaal als NH_4^+ aan het begin van de donkerperiode, in plaats van aan het begin van de lichtperiode, gedoseerd werd. Dit was onafhankelijk van de hoeveelheid stikstof die per alg gedoseerd werd. Het verhogen van de gedoseerde hoeveelheid stikstof per microalg, door het verhogen van de hoeveelheid vloeistof die per cyclus ververst werd met groeimedium van 33 naar 50%, leidde tot minder energetische opslagverbindingen als NH_4^+ aan het begin van de lichtperiode gedoseerd werd. De productie van energetische opslagverbindingen bleef vergelijkbaar als NH_4^+ aan het begin van de donkerperiode gedoseerd werd.

Hoofdstuk 5 laat zien dat de verrijkte microalgen cultuur zeer afhankelijk was van milieuparameters zoals de aanwezigheid van silicaat. Diatomeeën domineerden de verrijkte cultuur als silicaat voldoende aanwezig was, groene algen waren dominant als silicaat afwezig was. Allebei de verrijkte culturen vertoonden echter hetzelfde gedrag; grote hoeveelheden koolhydraten werden in het licht geproduceerd om de aanwezige stikstof in het donker te metaboliseren. Deze resultaten, samen met de verkregen data onder mariene condities, laten zien dat de eigenschap van productie van energetische opslagverbindingen in de lichtperiode verkregen kan worden onder variabele milieucondities, zo lang het fixeren van CO_2 in de lichtperiode is losgekoppeld van de opname van stikstof in de donkerperiode.

Diatomeeën zijn een groep microalgen die silicaat gebruiken om hun celwand te bouwen. Deze celwand van silicaat leidt tot eigenschappen die diatomeeën extra interessant maken voor het grootschalig kweken van microalgen. Zo zijn diatomeeën door hun relatieve zware soortelijke gewicht relatief makkelijk af te scheiden na de kweek. Verder zijn diatomeeën meer resistent tegen predatoren en hebben diatomeeën de mogelijkheid om lipiden te synthetiseren onder silicaat limiterende condities. Het pulsgewijs doseren van NH_4^+, aan het begin van de licht- of donkerperiode onder niet-limiterende silicaat concentraties, verrijkte een volledig door de diatoom *Nitzschia palea* gedomineerde cultuur uit een natuurlijk inoculum. Dit wordt beschreven in Hoofdstuk 6. Het metabole gedrag van de verrijkte cultuur was afhankelijk van het moment van het toevoegen van NH_4^+. Biomassa was het belangrijkste product uit de fotosynthese als NH_4^+ gedoseerd werd aan het begin van de lichtperiode, terwijle polymeren van koolhydraten geproduceerd werden in de lichtperiode als NH_4^+ gedoseerd werd aan het begin van de donkerperiode. Het onderwerpen van de verrijkte culturen aan stikstof- of silicaat-limitatie leidde tot een andere metabole respons. Onder stikstof limitatie waren koolhydraten de belangrijkste energetische opslagverbinding en nam het aantal cellen een factor vier toe. Als silicaat

limiterend was bleef het aantal cellen gelijk en werden vooral lipiden geproduceerd.

Alle experimenten beschreven in dit proefschrift leiden tot systemen waarin koolhydraten de voornaamste energetische opslagverbinding waren. Het loskoppelen van koolstof-fixatie in de lichtperiode van het opnemen van stikstof in de donkerperiode verrijkte, onder variabele condities (in zoet of zoutwater, met of zonder silicaat) koolhydraatproducerende microalgen uit een natuurlijk inoculum. De intracellulaire koolhydraatconcentratie nam doorgaans toe van 10 tot 50 % op basis van het organische drooggewicht in de lichtperiode. De verrijkte culturen waren zeer stabiel gedurende lange tijd. Het geregeld schoonmaken van de reactoren en het niet steriliseren van de gas- en vloeistofstromen die het systeem in en uit stroomden leidden niet tot instabiele culturen. Dit alles bewijst dat een geschikt selectief milieu is gevonden waarin koolhydraatproductie door microalgen beloond wordt.

Het beter begrijpen van de ecologische rol van lipiden en koolhydraten in microalgen maakt de weg vrij voor het creëren van lipide specifieke selectieve milieus. In Hoofdstuk 7 worden, naast het formuleren van algemene conclusies, strategieën geïntroduceerd die mogelijk tot het verrijken van lipide producerende microalgen zullen leiden. Het belonen van een microalg voor het vertonen van gewenst gedrag door middel van een selectief milieu, één van de centrale ideeën in dit proefschrift, zal een waardevolle benadering blijken als de ecologische rol van lipiden beter begrepen wordt.

1

INTRODUCTION

Peter R. MOOIJ

1

1.1. INTRODUCTION

THE onset of oxygenic photosynthesis by microalgae is arguably the second most important event in the evolution of life on Earth, only surpassed by the origin of life itself. Microalgae started using water as an electron donor for photosynthesis around 2.8 billion years ago [28]. Prior to that moment, anoxygenic photosynthesis with electron donors as H_2, H_2S, S and Fe^{2+} occurred [18], but global organic productivity increased two to three orders of magnitude from the moment water was used as electron donor [28]. The build-up of oxygen, the waste product from oxygenic photosynthesis, in the atmosphere and oceans had far-fetching effects on life on earth. These include a considerable decrease in niches suitable for obligate anaerobic life, fundamental changes in the C, N, S and Fe cycles and a possible link to periods with near-global glaciation ('snowball Earth') caused by the oxidation of the potent greenhouse gas CH_4 to the less potent greenhouse gas CO_2 [92].

On the longer term, the production of oxygen created conditions in which life could flourish. Aerobic respiration evolved as a strategy to deal with and benefit from the presence of oxygen. Aerobic respiration of a substrate yields considerably more energy than anaerobic respiration [19] [31]. This energy gain allowed complex and multicellular life-forms to arise [19] [92]. The build-up of oxygen led to the formation of the ozone layer, and the UV-protection of the ozone layer is linked to the spread and further evolution of life on land around 600 million years ago [58]. A part of the organic material produced in photosynthesis in the last few hundred millions of years became buried in deeper layers of the Earth and converted in geological time to oil, coal and other fossil fuels [7].

Around 250.000 years ago the line of *Homo*, which originated around 2.5 million years ago, saw a new descendant, the *Homo sapiens*. Through technological inventions, such as the invention of agriculture around 10.000 years ago, population numbers of humans started rising and reached 1 billion around 1700 [16]. The industrial revolution started around 1760 in Great Britain and both population numbers and the use of fossil fuels per capita increased from that moment [37]. Consequently, CO_2 levels in the atmosphere increased steadily from this point from 280 ppm in 1800 to 400 ppm in 2015 (Figure 1.1). As CO_2 traps heat in the atmosphere, an increase in CO_2 leads to an increase in temperature on Earth. Both the absolute change as the speed of the change in CO_2-levels and temperature are generally regarded a serious threat to humankind [39]. An averaged increased global temperature of 2 degrees Celsius and a corresponding CO_2-concentration of 450 ppm by the year 2100 are considered to be the upper safe limit. With an increase of 50 ppm in the period 1990-2015 there is an urgent need for CO_2-neutral alternatives to CO_2-emmiting processes.

Emissions from liquid transport fuels contributed to around 23% of global anthropogenic greenhouse gas emissions in 2010 [96]. Although electrical powered means of transportation are increasing, and although these can be carbon neutral if the electricity used is generated sustainably, it is unlikely that liquid fuels will be fully replaced in the near future. The main reason for this is that oil and its derivatives are excellent energy carriers. They are easily stored without energy losses, make use of an infrastructure based on proven technology and have a high energy density [83].

All of these advantages do apply to oil from both fossil as renewable resources. Any vegetable oil, such as rapeseed-, hemp- or microalgal-oil, can be considered a carbon-

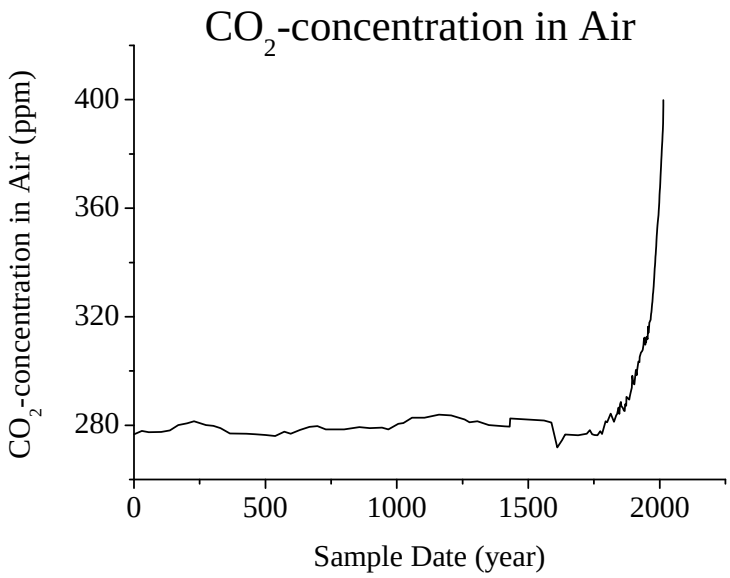

Figure 1.1: Atmospheric CO_2 concentration in time. Data based on ice core measurement before 1958 and atmospheric measurements after 1958, taken from [61]

neutral fuel, as all of the CO_2 emitted to the atmosphere upon combustion has recently been withdrawn from the atmosphere by photosynthesis. Vegetable oils are therefore carbon-neutral alternatives for fossil fuels, as long as no fossil fuels are consumed in their production processes. Technical adjustments needed to use these fuels for transportation are minor [96]. In this respect it is noteworthy that Rudolph Diesel tested several vegetable oils to run the diesel engine he invented and remarked in 1912: '*One cannot at present predict what part these oils will play in the colonies in the future. In any case, they make it certain that motor power can still be produced from the heat of the sun, which is always available for agricultural purposes, even when all our natural stores of solid and liquid fuels are exhausted.*' [29].

The choice of a suitable renewable oil production platform involves both productivity issues as dependencies on limited resources, such as freshwater and agricultural land. Concerning the productivity issue, the incident solar radiation on a surface is irrespective of the crop cultivated on that surface. As the maximum photosynthetic efficiency to convert sunlight into carbohydrate is similar in land plants and microalgae, the maximum theoretical areal carbohydrate production is comparable as well [100].

If we consider areal oil production, microalgae offer advantages and possibly higher yields, due to their high maximal oil content per weight and the likely year-round coverage of a surface area [90] [100]. As a consequence relatively little surface area is needed to supply fuel precursors compared to land-based plants. To supply Europe with transport fuel a surface area comparable to Portugal should be dedicated to microalgal cultivation [107]. As a large number of marine microalgae exists, this surface area doesn't necessarily have

to consist of arable land. Cultivation of marine strains for oil production can take place in areas not suitable for traditional agriculture and with minimal use of freshwater. The relatively high oil yield per hectare and the low agricultural- and freshwater-need make microalgae a preferable platform over land-based oleaginous crops for the production of fossil fuel alternatives.

Currently, there are no companies producing microalgal oil at a large scale. This can partly be explained by the absence of a financial reward to the carbon neutral aspect of microalgal derived fuels. There are however also practical issues hampering large-scale microalgae cultivation. These include the energy input needed in mixing and cooling during cultivation, the first solid/liquid separation after cultivation and the productivity losses due to contamination [59] [100]. Whereas contaminants can relatively easy be removed in traditional agriculture, microalgal contaminants are more difficult to eliminate by traditional means as they become an integral part of the microalgal culture and are instantly and continuously spread by mixing of the cultivation system. The traditional agricultural approach of choosing a preferred strain and fighting any contaminants therefore proves difficult in large-scale microalgal cultivation [73]. The work in this thesis explores a different approach. By focussing on a desired characteristic, such as the production of lipids, rather than on a specific microalgae the issue of contamination can be overcome and can even become of value. Obtaining and sustaining a characteristic in an open system requires a selective environment, explaining the title of this thesis [77].

The use of selective environments to obtain a characteristic, and in many cases one microbial species best in displaying in this characteristic, was introduced and pioneered by Beijerinck around 1900 [5], further developed by Kluyver and Baas Becking [2] and proves until this day an excellent strategy to obtain a certain characteristic in microbial systems. Baas Becking summarized the enrichment approach as '*Everything is everywhere, but the environment selects.*' [2]. The first part of this tenet should be interpreted as that any natural sample holds likely a great microbial diversity, the second part explains that the imposed cultivation conditions will enrich a microorganism from the inoculum which fits best in the created environment. Recent large-scale applications of this methodology in bacterial dominated systems include the aerobic granular sludge process for cleaning waste-water treatment [27] and biopolymer production from organic waste-streams [52].

This thesis deals with the use of selective environments in microalgal cultivation. Chapter 2 describes the rationale behind using ecology-based selective environments to obtain and sustain desirable microalgal characteristics in open cultivation systems. The approach advocated in Chapter 2 can widely be applied in microalgal cultivation. Desirable microalgal properties range from the production of high-value carotenoids to an increased solid-liquid separation after cultivation and from the production of low-value carbohydrates and lipids to cultivation at high dissolved oxygen concentrations. The application of the principle introduced in Chapter 2 is throughout this thesis however limited to the production of energetic storage compounds, such as carbohydrates and lipids. Chapter 3 describes how to obtain a culture of highly productive carbohydrate producing microalgae from a mixed culture. This approach not only holds in freshwater, but also under marine condition, as presented in Chapter 4. One of the advantages of

Beijerinck isolated in 1890, likely as the first person in the world, pure cultures of *Chlorella vulgaris* and *Scenedesmus acutus* [4]

the method proposed is that the desired functionality, such as carbohydrate production, is independent of the microalgal species present. Chapter 5 demonstrates that the presence of silicate has a major influence on the microalgal community present, which is dominated by diatoms if silicate is present and by green algae if silicate is absent. The functionality of storage compound production is however present with and without silicate, as it a consequence of the imposed environment. Chapter 6 investigates the influence of environmental factors on the type and amount of storage compounds produced by an enriched culture of diatoms. General conclusions on the work conducted are drawn in Chapter 7. Besides, future research directions based on the general procedure of Chapter 2, are outlined in this chapter.

2

ECOLOGY-BASED SELECTIVE ENVIRONMENTS AS SOLUTION TO CONTAMINATION IN MICROALGAL CULTIVATION

Peter R. MOOIJ, Gerben R. STOUTEN, Mark C.M. VAN LOOSDRECHT and Robbert KLEEREBEZEM

Large-scale production of energetic storage compounds by microalgae is hampered by competition and evolution. Both phenomena result in contamination and arise due to a mismatch between the desired productive microalgal strain and the constructed environment. The prevailing approach to solve this issue involves increasing the survival potential of the desired strain, for example by working in closed systems or at extreme conditions. We advocate to adjust the environment in such a way that lipid production, or any other desired characteristic, gives a competitive advantage. Competition and evolution become a value rather than a threat to processes in which the desired characteristic is ensured by a selective environment. Research and cultivation efforts will benefit from this approach as it harnesses the microalgal diversity in nature.

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2.1. INTRODUCTION

MICROALGAE have the potential to supply a biobased society with essential photosynthetic building blocks like sugars and lipids. In nature, production of these storage compounds enables algae to endure dynamic growth conditions like the alternating presence and absence of light. From a biofuel production point of view, sugars can for example be fermented to ethanol [48] or digested to produce methane containing biogas [78] whereas lipids can be transesterified to yield biodiesel [11]. Biodiesel is the preferred microalgal fuel product since it can be implemented in existing infrastructure. As a consequence, microalgal research has been mainly focussed on lipid production. At this moment the large-scale production of low-value commodities such as lipids is economically not viable. A key parameter for the economics of lipid production by microalgae is the area-specific lipid productivity [43]. Different strategies including genetic modification [86] [40] [41], wild-type screening programmes [84] and optimization of cultivation conditions [12] are applied to increase lipid productivity. Even with increased lipid productivity, the large-scale cultivation of microalgae is challenging. The phototrophic nature of microalgal cultivation leads to light limitation at relatively low biomass concentrations. Scaling up of phototrophic processes will therefore occur on surface basis and not on volume basis as is standard practice in chemotrophic processes [90]. Large surface areas and diluted cultures result in challenges concerning gas-liquid mass transfer, down-stream processing and contamination. Contamination has a detrimental effect on production [59] [97] and is therefore ranked as a major bottleneck in microalgal cultivation in open systems [95] [73] [97]. This paper will address two ways to deal with contamination: either strain driven or founded on ecology-based selective environments.

2.2. CONTAMINATION

CONTAMINATION comes in the form of herbivores (cladocerans, copepods, rotifers, etc.), pathogens (bacteria and viruses) and competing microalgae [80] [97]. Chemical and ecological methods can be applied to reduce the effect of herbivores and pathogens [59] [95] [60] [73] [97]. Competing microalgae seem to be the most difficult form of contamination to control, since the biological and physical properties of the contaminant are largely similar to those of the desired species. We therefore define contamination in this paper as the unwanted introduction of microalgal strains and functionalities in any microalgal cultivation system, open or closed.

Contamination by competing algal strains is a logical consequence of the operation of microalgal cultivation systems. Although cultivation strategies that combine biomass and lipid production are investigated [65], the standard microalgal cultivation strategy consists of a period with excess nutrients to produce biomass followed by a nutrient depleted phase in which lipids are produced [93] [87]. The first period creates an environment in which fast growth is rewarded. In the second phase lipids are produced, but this functionality is not rewarded. Consequently, the process faces two challenges. Competing microalgae, with a higher growth rate than the desired lipid producing species, can outcompete the desired species and threaten the process from the outside. Even if the desired species is maintained by properly operating under axenic conditions, the desired characteristic can be lost through evolution [67]. In this case evolution may lead to an

increased growth rate at the cost of a decreased lipid production capacity [14]. This allows a mutated strain to outcompete the original high productive strain. Evolution can therefore be classified as a danger from the inside and is as such coined 'strain degeneration'.

With a mismatch between the desired lipid producing species and the constructed cultivation environment, two approaches emerge. One could either increase the survival potential of the desired species or create an environment that favours lipid production. Figure 2.1 gives a schematic overview of these two lines of thought and points out where current research is focussed.

2.3. INCREASING THE SURVIVAL POTENTIAL OF THE DESIRED STRAIN

THE majority of measures taken to avoid contamination aims at increasing the survival potential of the desired strain (left part of Figure 2.1). Axenic cultures are established in closed cultivation systems by imposing a physical barrier between the desired and competing microalgae. In these systems the risk of evolutionary loss of required functionalities is imminent if continuous cultivation is applied. It is furthermore questionable if the energetic and economic requirements for axenic cultivation can be justified for the phototrophic production of low value commodity chemicals [55] [24] [11]. Various studies underline that open systems are required for large-scale production of bulk products [93] [104] [73].

The survival potential of the desired microalgae in open systems (e.g. open ponds) can be increased by various means. Process development can for example aim for application of extremophiles. The high salt concentration and high irradiance, in the beta-carotene production process by *Dunaliella salina*, create an environment in which only a few microalgae can survive [11]. *D. salina* is the most salt-tolerant algae known to exist, competition is therefore minimal above a certain salinity [10]. Strain degeneration however still forms a threat to beta-carotene production. Nature offers many strategies to deal with high irradiance [57]. As long as *D. salina* produces beta-carotene as photoprotection, cultivation under high irradiance and high salinity ensures beta-carotene production [10]. Other strategies to deal with a high irradiance, such as enhanced DNA repair mechanisms or synthesis of other photoprotective substances, could arise in the culture and diminish the beta-carotene production capacity. Strain degeneration therefore imposes a threat to beta-carotene productivity. The same holds true for application of herbicide-resistant microalgal strains in combination with herbicides to prevent competing microalgae [11] [40]. Competition might initially be limited, but herbicide-resistant strains with increased growth rate and decreased lipid potential cannot be excluded to develop through evolution.

Changing the operation of the system can diminish the loss of productivity caused by strain degeneration. Cultivation systems can be operated as batch processes, with large inocula of the desired strain [11] possibly combined with crop-rotation strategies as used in traditional agriculture [95]. Large inocula will, as shown in chemotrophic biotechnological processes, ensure both the desired microalgae and the desired functionality for a certain time. Nevertheless, axenic batch operation, inocula preparation and process

control are cost factors that will remain challenging for the production of low value bulk products such as lipids.

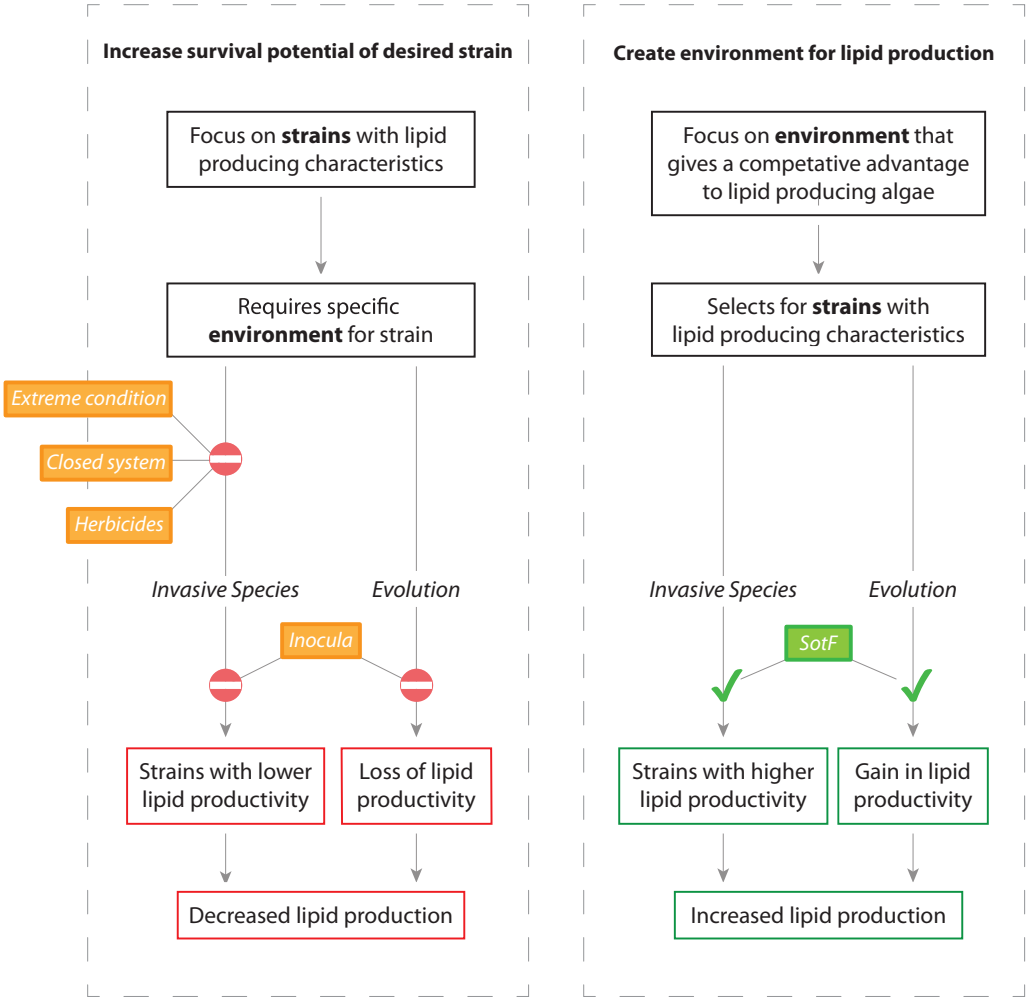

Figure 2.1: Two ways to achieve large scale lipid production using microalgae. Left panel: Strain based approach. The point of action of working with closed systems (closed systems), using a herbicide-resistant strain (herbicides), under extreme conditions (extreme conditions) or with regular inoculation (inocula) with the desired strain is highlighted. Right panel: Ecological approach. The point of action of the 'Survival of the Fattest' approach (SotF) is highlighted. Do not enter sign indicates: aims to block, checkmark indicates: benefits from.

2.4. CREATING AN ECOLOGY-BASED SELECTIVE ENVIRONMENT FOR LIPID PRODUCTION

ALTERNATIVELY, we can aim for developing algae cultivation conditions that provide a competitive advantage for the desired characteristic (in our case lipid production) (right part of Figure 2.1). To create such an environment it is of paramount importance to identify and use the ecological role of lipids and other storage compounds. Storage compounds are consumed at times when the intracellular energy and carbon demand exceeds extracellular supplies. In the natural day-night cycle a certain amount of storage material is produced in the light and consumed in the dark [66]. When this innate behaviour is amplified in designed processes, algae with high storage compound productivity will be selected. A way to enhance storage compound consumption in the dark is to limit the availability of an essential growth nutrient other than carbon to the dark period [76] (or Chapter 3). Such cultivation conditions will favour growth of algae strains with the capacity to produce storage compounds in the light, which are required for nutrient uptake and heterotrophic growth on internal storage compounds in the dark.

This ecological concept, which we recently introduced as 'Survival of the Fattest', allowed the enrichment of microalgae with a high starch productivity, reaching 57 % of glucose polymers on organic dry weight after 8 hours of light [76] (or Chapter 3). These values are comparable to the highest values reported in literature for pure cultures [72]. In our experiments we used the nitrogen source (ammonium, nitrate) as limiting nutrient during the light, because ammonium and nitrate levels can be relatively easily monitored. This selective environment provides a competitive advantage for storing carbon and energy during the light period, but provides no control on the type of storage compound produced. Apparently, starch is the preferred storage compound under the applied conditions. The challenge that remains is to define the cultivation conditions that provide a competitive advantage for lipid production, instead of carbohydrate production. Clues could come from intrinsic differences between carbohydrates and lipids, such as their density. For example, an environment in which selection on buoyancy is combined with the 'Survival of the Fattest' principle could favour lipid over starch-producing microalgae. Another intrinsic difference between carbohydrates and lipids is the energy content per carbon. The higher energy content per carbon in lipids may provide a competitive advantage over carbohydrates during prolonged dark and cold periods. In general, a better understanding of carbon allocation in microalgae will help in designing selective environments that favour lipid over carbohydrate production. Microalgal engineering and synthetic biology approaches [41] [23] [26] will prove crucial in our comprehension of carbon allocation.

The proposed cultivation strategy can be applied to both open and closed systems. In closed systems it may induce directed evolution towards increased storage compound productivity. We believe however that the 'Survival of the Fattest' approach is especially suited for open systems. Theoretically all microalgae can enter an open cultivation system. With an estimated 300.000 different microalgal species [90] [40] with great interspecies differences [40] [46] an enormous potential can be unlocked. Three main advantages of using nature's diversity can be distinguished.

Firstly, using natural selection makes use of all biodiversity present. A similar ap-

proach providing a selective environment for polyhydroxyalkanoate (PHA) producing bacteria enabled the enrichment of a previously unknown PHA-producing bacterium [62] [52]. This strain has the same storage capacity but a much higher storage rate than the strains that were available through conventional strain selection and genetic modification techniques. Just as for bacteria, the microalgal biodiversity is largely unexplored.

Secondly, if the selective environment is ensured, the approach will work under variable conditions. The 'Survival of the Fattest' principle has been shown to work under fresh water and marine conditions [75] (or Chapter 4) and will likely also work for different temperatures, pH and other variations that might exist in different geographic regions. Each time, potentially a different organism will be selected, but it will be the one with the highest storage compound productivity in this environment. This allows 'contaminants' native to the ponds to take over the system, as was suggested by the Aquatic Species Programme [93].

The third advantage is that optimisation is intrinsically included in the process design. If competition or directed evolution results in better storage compound productivity, the new organism or strain will outcompete the present organism; this results in a more productive process. If a new organism or strain has a lower productivity it will be outcompeted automatically, resulting in a stable system.

As a concluding remark, it is good to note that the above mentioned advantages hold true for any characteristic obtained by creating a selective environment and are not limited to the production of storage compounds.

2.5. SELECTIVE ENVIRONMENTS FOR OTHER CHARACTERISTICS

BESIDES productivity, other characteristics are desired for large-scale microalgal cultivation. Natural selection can be a good principle to exploit microbial diversity for these characteristics as well. The crux lies in the design of an environment that couples the desired characteristic to a competitive advantage of a species. Table 2.1 lists a selection of desirable microalgal functionalities and the environmental conditions that may allow for their enrichment.

A desirable microalgal characteristic other than storage compound productivity is being easily harvestable. Biomass concentrations during phototrophic cultivation are typically 0.1 – 4.0 g / L [10]. In chemotrophic processes biomass concentrations of 100 g / L can be achieved. Therefore, the solid-liquid separation after microalgal cultivation is a costly step. From this point of view, the characteristic of fast-settling, and therefore easily separable biomass, would be beneficial. Fast-settling granules can be enriched for in bacterial systems by selectively removing non-settling biomass [8]. A comparable approach has been used in mixed cultures of microalgae and bacteria, yielding microalgal-bacterial flocs [44].

Some desired characteristics cannot be obtained using a selective environment, since they do not give a competitive advantage to the microalgae. Synthetic biology approaches can be used to create species displaying these characteristics. Examples of functionalities that are obtained using microalgal engineering are the inhibition of lipid catabolism [101] and having a specific lipid composition which is more suitable for biodiesel production [41]. Cultivation of these strains will face the challenges of competition and strain degeneration. As such, the advantages of the desired characteristic should outweigh the

Table 2.1: Desired microalgal characteristics, derived from [11], and possible selective environments for these traits

Desired microalgal characteristic	**Possible selective process conditions for this characteristic**	**reference**
High growth rate	Batch mode or chemostat with high dilution rate	-
High starch productivity	'Survival of the Fattest' approach	[76]
High lipid productivity	'Survival of the Fattest' combined with for example selection on low density cells	[14]
Staying in suspension	Periodic removal of biofilms	-
Being easily separable from the liquid phase	Provide competitive advantage to algae that form aggregates through inclusion of a settling period	[44]
Tolerating marine conditions	Cultivation under marine conditions	-
High shear tolerance	Cultivation under high shear conditions	-
Low sensitivity of Rubisco to high O2 concentrations	Cultivation under high oxygen concentration	-
Tolerating broad temperature range	Cultivation under fluctuating temperature	-
No lipid catabolism	-	-
Specific lipid composition for biodiesel production	-	-

cultivation efforts.

For most applications a combination of the desired characteristics is preferred. By combining selective process conditions, multiple desired characteristics can be obtained. Running an enrichment culture under high oxygen concentration while removing biofilm will select for microalgae with a high oxygen tolerance that stay in suspension. Other desired characteristics might be incompatible, such as the characteristic of being easily harvestable with the trait of staying in suspension. Finally it should be realised that numerous, unintentional selective environments are applied to everyday microalgal cultivation. In many processes part of the biomass is harvested and the non-harvested part is retained in the reactor. This creates a clear selective advantage for the characteristic of being non-harvestable [14].

2.6. CONCLUSION

LARGE-SCALE microalgal cultivation for low value products will take place in large, open ponds. Both competition and strain degeneration are a threat to stable storage compound production in these systems. Working at extreme conditions, in closed systems or with herbicides could solve the competition aspect, but the problem of strain degeneration is not addressed in these approaches. Operating a cultivation system with large inocula of the desired strain diminishes the effect of both threats but only for a limited time and at considerable cost. We agree that ecological principles should be the basis for

improving microalgal cultivation [59] [95] [60] [80] [97] and therefore advocate an ecology based solution in which both competition and evolution are improving the process. This is achieved by creating a selective environment in which storage compound production is rewarded by directly linking it to growth. A similar approach can be applied to enrich other desirable characteristics. With a clear list of desired traits and a vast, unexplored microalgal diversity we encourage and endorse the further exploration of ecology-based selective environments in future algal research.

3

SURVIVAL OF THE FATTEST

Peter R. MOOIJ, Gerben R. STOUTEN, Jelmer TAMIS, Mark C.M. VAN LOOSDRECHT and Robbert KLEEREBEZEM

Liquid fuels have excellent properties in terms of storage, logistics and energy density compared to gaseous fuels or electricity. A major disadvantage of liquid fuels is that a vast majority of them is derived from fossil resources. Currently, the consumption rate of fossil fuels by far outcompetes the natural production rate, resulting in elevated atmospheric CO_2 concentrations. Photosynthetic organisms (plants and algae) fixate atmospheric CO_2 using solar energy. CO_2 consumption and emission would be balanced if liquid fuels would be derived from plants or algae. However, growing terrestrial plants for biofuel production means less agricultural land and fresh water remains available for food production. Microalgae can grow under marine conditions and outcompete terrestrial plants in terms of areal productivity. On the other hand, cultivation of microalgae introduces new challenges. Species control is, compared to terrestrial plants, much more difficult. Any cultivation system is prone to contamination by undesired algal species threatening stable production. In this study we show that we can overcome this hurdle by creating a selective environment. Our approach allows for large scale, stable production of biofuel precursors and is therefore a substantial step forward in the production of renewable fuels.

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3.1. SURVIVAL OF THE FATTEST

FOR quite some time, storage compounds produced by photosynthetic microalgae are regarded as a potential resource for future fuel requirements [93] [99]. The main fuel precursors produced by algae are starch and triacylglycerol. Starch is used as substrate for bioethanol, CH_4- or H_2-production whereas triacylglycerol is used as precursor for biodiesel production [87] [13]. Since all algal species have different characteristics some species produce more storage compounds than others. This explains the 'species-based' approach used in the majority of algal research, the choice of a species is regarded as a starting point for an experimental- or process design. Unfortunately, storage compound production is not linearly related to growth rate [87]. Many productive strains, either naturally occuring or genetically modified, are outcompeted by faster growing algae when these are present in the system. A solution is to prevent other species from entering the system, as can be achieved in closed photobioreactors by sterilising incoming and outgoing flows. However, sterilisation comes at a cost [40] and maintaining a large scale monoculture is practically troublesome [59]. Contamination possesses therefore a serious risk to stable storage compound production in any microalgae cultivation system [40] [59]. This hurdle is inherent to the 'species-based' approach. To come up with an alternative we question the use of a defined monoculture in the first place. As mentioned before the main argument for using a certain species are the interesting characteristics it offers. If in the end we are interested in a characteristic, why don't we focus on this specific property from the start?

Now we enter the field of Environmental Biotechnology which aims at enriching and maintaining a characteristic or a functionality instead of a specific species in a system. Here, we argue that maintaining of a functionality rather than a species can be an attractive alternative for the pure culture approach for large scale algae cultivation. The remaining question is how to establish and maintain the desired characteristic of producing storage compounds in any microalgae cultivation system. We can solve this by using ecological principles and the endless microbial diversity nature has to offer. Utilizing these, we show how to obtain a stable, open system with a population of storage compound producing microalgae. Besides, we explain why the inevitable contamination by invading species becomes a value instead of a threat to the production process.

Approximately 50 microalgal strains are used for the majority of microalgae research, while a few billion years of evolution has resulted in 40.000 identified and a multitude of unidentified microalgal strains [49]. Statistically speaking it is likely that many more suitable candidates are present in nature. Therefore, we propose a new method to enrich for naturally occurring microalgae with a high storage compound production capacity by means of natural selection. The proposed method acts as a high throughput screening system enriching the most efficient strains from the inoculum. This approach is inspired by the principle defined by Lourens Baas Becking: '*Everything is everywhere, but, the environment selects.*' [2]. Applied to microalgae this tenet can be interpreted as follows. The first part tells us that in any natural water sample many algal strains are present. The second part explains that we can enrich specific microalgae from this group by creating a selective environment.

A selective environment that emphasizes the ecological role of storage compounds should enrich a culture with the optimal storage compound potential. Successful exam-

ples are using a feast-famine regime to enrich for polyhydroxyalkanoate producing bacteria or alternating between anaerobic and aerobic conditions to select for polyphosphate-accumulating organisms [52] [91] [108]. It is widely accepted that microalgae produce storage compounds in absence of an essential growth nutrient [6] [49]. Exploiting the cyclic absence of an essential nutrient is therefore the basis of the proposed strategy.

To this end, a photobioreactor was operated with cycles of 24 hour. Each cycle was divided into a light and dark period. During the light period CO_2 was supplied while the nitrogen source was absent. With light and CO_2 present microalgae are able to produce storage compounds. During the dark period the nitrogen source was supplied. Since nitrogen assimilation consumes energy and carbon skeletons [102], only microalgae with internally stored energy and carbon can assimilate nitrogen in the dark period. By this means, microalgae that have produced storage compounds during the light period have a competitive advantage over non-storing microalgae. Every cycle, part of the biomass was harvested and fresh medium was added. Non-storing microalgae were washed out while an enrichment of storage compound producing microalgae was obtained by repeating this cycle over and over.

A photobioreactor with an inoculum consisting of samples from several surface waters was operated for three months under the above described conditions. All operational parameters can be found in section 3.2. After six weeks a steady state was reached in which the limiting nutrient (NH_4^+) added during the dark period was depleted at the start of the light period. In steady state offgas CO_2, O_2 and pH profiles were comparable for every daily cycle.

Typical profiles can be found in section 3.3. During steady state, samples were taken at the transition from dark to light and from light to dark. Samples were analysed for biomass, lipids and polyglucose (Figure 3.1). Figure 3.1 shows that carbon fixation and nitrogen uptake are indeed separated in time. During the light period nitrogen was absent and the fixed CO_2 is converted to storage compounds. The amount of polyglucose increased from 10 ± 2 % to 57 ± 2 % on VSS basis. This is comparable to the highest reported values in literature [13] for pure culture studies. It is likely that an even higher productivity can be achieved with the proposed method, since light intensity and duration, CO_2 concentration and other parameters have not been optimised in this study. At the end of the light period 72% of the energy-rich biomass was harvested. The remaining 28 % of the biomass could divide in the dark phase on the stored storage polymers and supplied oxygen. The nitrogen supply was adjusted such that it was fully depleted during the dark period. At the end of the dark period the algae had the initial cell composition again, allowing for a new round of carbon fixation.

Light microscopic observation of the inoculum indicated at least 30 morphologically different algal strains. After enrichment, DNA analysis and microscopic observation revealed that the culture was highly dominated by *Chlorella luteoviridis*. A small side population of *Nitzschia frustulum* coexisted. Staining techniques indicated that *Chlorella luteoviridis* primarily produced starch as storage compounds, while *Nitzschia frustulum* produced neutral lipids (Figure 3.2).

Algae from the genus *Chlorella* are often suggested as candidates for the production of storage compounds [49] [13]. The specific species *Chlorella luteoviridis* is however less documented in this respect. This indicates that our enrichment method is an

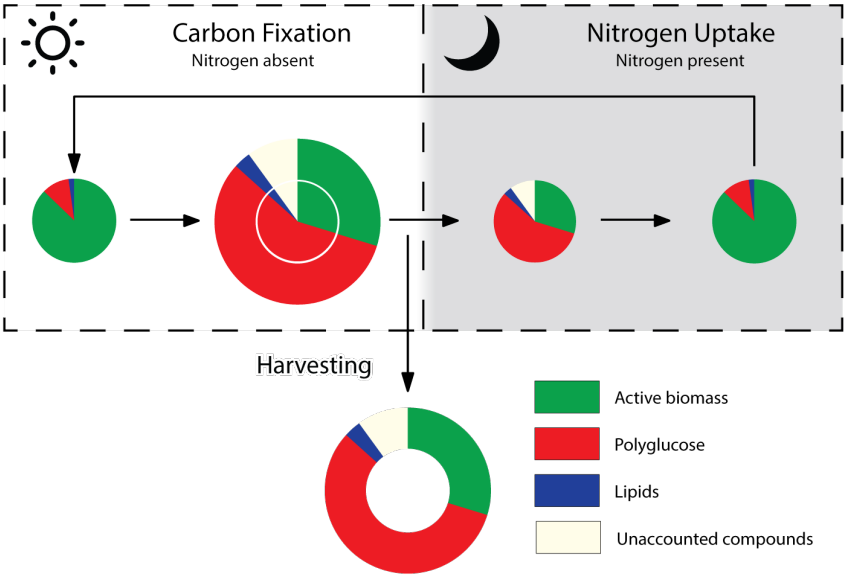

Figure 3.1: Fraction of biomass (green), lipids (blue), polyglucose (red), and unaccounted compounds (white) during light–dark cycles in the steady state after six weeks of cultivation. Pie-area is linearly related to total amounts,which are an average of three cycles. Data used for constructing the figure can be found in section 3.3.

advantageous tool for selecting lesser-known species specialised in producing storage compounds.

A new, ecology based method to effectively enrich microalgae with a high storage compound productivity has been proposed above. By cyclically uncoupling carbon fixation and nitrogen uptake microalgae that produce storage compounds have an advantage over non-storing species. The majority of the storage compound rich biomass is harvested every cycle. Future research could focus on selecting specifically lipid or starch producing microalgae. Elucidating on the ecophysiological roles of these compounds in more detail will likely make it possible to develop compound selective algal selection strategies.

Although now tested under certain experimental conditions (fresh water, 28 °C, etc) we believe that our system will under all possible conditions select for microalgae that thrive in these conditions by producing storage compounds.

We believe that our findings are essential to large scale microalgae cultivation. The above described method can be used as a high throughput screening method to enrich for the most suitable microalgae. Compared to traditional screening methods we directly select microalgae based on the functionality we are aiming for, i.e. the capacity to produce storage compounds.

Invading species are one of the main vulnerabilities threatening a stable production of energy-rich biomass, regardless of the cultivation method [40]. In our process however, invading species or mutations that result in a lower storage compound productivity will

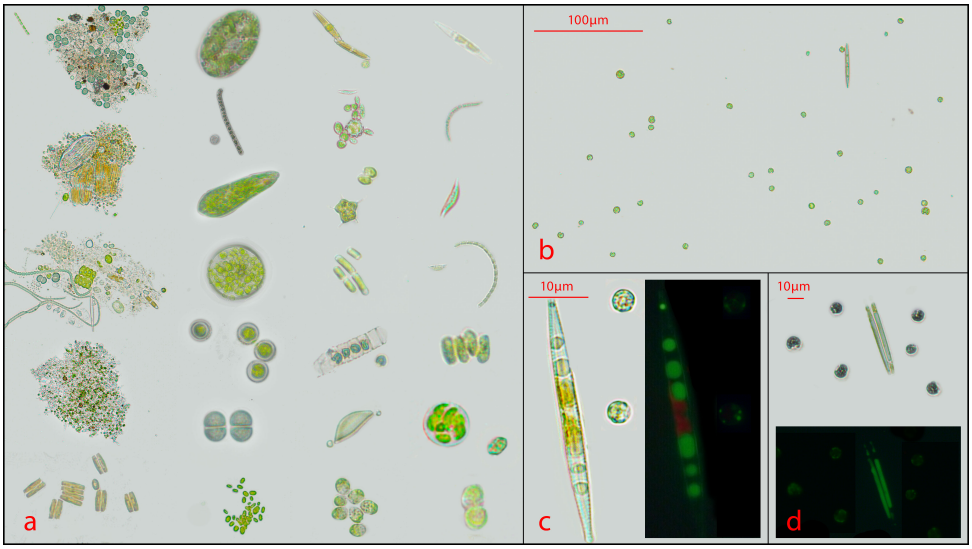

Figure 3.2: (a)Overview of the different microalgae present in an inoculum (not to scale), (b) microscopic view of the enriched culture in the steady state. Detail pictures of *Nitzschia frustulum* and *Chlorella luteoviridis* under normal and fluorescence light stained using stains (c) for lipids and chloroplasts (Bodipy 505/515) and (d) for lipids, chloroplasts (Bodipy 505/515) and starch (Lugol's solution).

be outcompeted. On the contrary, invading strains with an improved storage compound productivity will become dominant.

The functional characteristics of the process will hardly change or slowly improve over time [53]. Even though the robustness of the methodology proposed here requires experimental confirmation, the functional stability of environmental biotechnology processes in general has firmly been established. Maintaining the characteristic of storage compound production in the above described method comes down to ensuring that all of the limiting nutrient NH_4 has been consumed during the dark period. We firmly belief that in large scale microalgae cultivation this is much more easily achieved than sterilising all flows leaving and entering the system.

As a final remark we want to emphasize the fundaments underlying the above described method. Nature offers an overwhelming microbial diversity of which we are only able to see the contours. This diversity offers great opportunity in solving many problems mankind faces. The first step should be to understand what microbial characteristic will overcome a certain problem. Are we for example looking for a 5-carbon sugar utilizing microbe? Secondly we should ask ourselves in which conditions this functionality would be beneficial for the microorganism so it can outcompete other microorganisms in the system. A medium with solely 5-carbon sugars will be beneficial only to those who are able to utilize them.

In the end, all that is left for us is creating a selective environment, add an inoculum and let nature do the job. About 150 years ago Darwin revolutionized our way of thinking about life by realizing the potential of natural selection through 'survival of the fittest'. We believe that the same mechanism can have a huge potential for the realization of the

biobased economy, by the '*Survival of the Fattest*'.

3.2. MATERIAL AND METHODS

A non-sterile 1,5 L bioreactor (Applikon, Schiedam, The Netherlands) with a diameter of 11 cm and height of 17 cm was run in a sequenced batch mode for three months with cycles of 24 hour. Figure 3.3 describes the operation cycle.

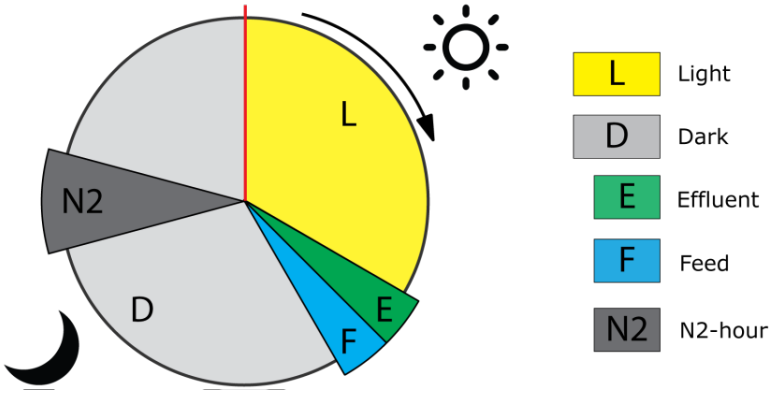

Figure 3.3: overview of the operational cycle of the photobioreactor

During the N_2 period the reactor was sparged with N_2 gas to decrease protozoa growth. The reactor was run using the following operational parameters: volume 1320 mL, cycle length 24 h, light period 8 h, dark period 16 h, solid retention time 33.3 h, temperature 28 °C, stirrer speed 200 rpm, gas flow reactor in and out 40 mL/min, gas recycle over reactor 1500 mL/ min, gas composition 5% CO_2 in air and 5% CO_2 in N_2 during N_2 period, average light intensity at inner reactor surface 650 µmol m^{-2} s^{-1} provided by HPS lamps, pH setpoint 7.5, base 1 M $NaHCO_3$, acid 0.5 M HCl. All liquid and gas flows entering and leaving the system were not sterilised. The reactor was cleaned once a week in a non-sterile environment to remove any biofilm. Several samples for surface water were used as inoculum.

A Bio Controller ADI 1030 (Applikon, Schiedam, The Netherlands) controlled Masterflex pumps (Cole-Parmer, Vernon Hills, IL, USA) and mass flow controllers (Brooks Instruments, Ede, The Netherlands). The Bio Controller itself was controlled by a PC with MFCS_win software (Sartorius Stedim Systems, Goettingen, Germany)

A modified COMBO-medium was used. The final nitrogen concentration was 10 mg N / L. To ensure N limitation all concentrations except for the N-source were multiplied by a factor twelve. The following modifications were made to the recipe: NH_4Cl instead of $NaNO_3$, no $NaHCO_3$, No KCl, no animal trace elements, no vitamins, addition of 10 mg / L allylthiourea to prevent nitrification. The N-source was dosed separately from the rest of the medium in peaks throughout the night.

pH and DOT were continuously measured by the Bio Controller ADI 1030. Offgas CO_2 and O_2 were analysed using a Rosemount NGA offgas analyser (Emerson, USA). NH_4^+ was determined spectrophotometrically using Dr. Lange LCK 403 NH_4^+ cuvette tests (Hach

Lange, Dusseldorf, Germany).

Samples were taken at the transition from dark to light and from light to dark in steady-state after six weeks of cultivation. Reactors were assumed to be in steady state when offgas CO_2, O_2 and pH showed similar profiles during multiple subsequent cycles. Besides, NH_4^+ should be depleted before the end of the dark period. Three consecutive cycles were measured. Total dry weight was determined by centrifuging and freezedrying a known amount of sample. Ash content was determined by burning freezedried biomass for 1 hour at 550 degrees. Volatile Suspended Solids (VSS) was obtained by subtracting ash amount from the total dry weight.

Lipid extraction was done as described by [52]. Extracted lipids were analysed for the presence of PHB, myristic, palmitic, oleic, stearic, linoleic and linolenic acid by gas chromatography (model 6890N, Agilent, Lexington, MA, USA) equipped with a flame ionization detector on a HP Innowax column. Unidentified lipid peaks were not taken into account.

Polyglucose was measured by heating approx 5 mg freezedried biomass with 0.6 M HCl for three hours at 100 ºC. After centrifugation and filtration with a 0.45 µm pore size filter (PVDF Membrane, Millipore, Tullagreen, Ireland) the polyglucose concentration was determined by high performance liquid chromatography, using an Aminex HPX-87H column from Bio-Rad (Hercules, CA, USA) (t=60 ºC) coupled to an ultraviolet and a refractive index detector. The obtained lipid and polyglucose were multiplied with the total dry weight to yield total polyglucose and lipids. Biomass for the transition from dark to light was calculated as VSS minus polyglucose and lipids. Since it is highly likely that other storage compounds are also produced during the light period the biomass at the transition from light to dark was calculated in a different way. The biomass was assumed to be linearly related to the cell number. To calculate the biomass at the transition from light to dark the biomass at the transition from dark to light was multiplied by the increase in cell number during the light period.

Pictures were taken using a Leica DM500B light microscope (Leica Microsystems, Wetzlar, Germany) equipped with fluorescence filtercube A (excitation: UV, excitation filter: BP 340-380, dichromatic mirror: 400, suppression filter: LP 425). 1 µl BODIPY 505/515 (Invitrogen D3921, Life Technologies, Grand Island, USA) in DMSO (1 mg/ml) was used to colour lipids in 1 mL of algal cells. 10 µl Lugol's solution was used to colour starch in 1 mL of algal cells. Cell number and concentration were determined by counting at least 300 algal cells using a counter chamber (W. Schreck, Hofheim, Germany).

The microbial composition of the enriched culture was analysed using the PCR-DGGE technique. Genomic DNA was extracted using the Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The subsequent extracted DNA was used for amplifying the bacterial 16S rRNA gene using the primer pair BAC341F with a GC clamp and BAC907RM [3], eukaryotic 18S rRNA gene using the primer pair EUK1F and EUK563R with a GC clamp [30]and chloroplast 16S rRNA gene from the eukaryotes using a newly developed primer CHL21F ('5-TGGCTCAGGATGAACGCTGG-3') and CYA781RW without GC clamp. For DGGE the CHL21F-CYA781rW fragment was reamplified using the primer pair CYA359F with GC clamp and CYA781rW [81]. The temperature program for amplifying bacterial 16S-rRNA gene fragments was the same as described by [3]. For specifically amplifying the chloro-

plast 16s-rRNA gene the following program was used for the first step: starting with an initial incubation at 95 °C for 5 min. and 32 cycles of 95 °C for 30 sec., 55 °C for 40 sec., 72 °C for 40 sec. followed by an final extension of 72 °C for 30 min. The second step used a lower annealing temperature of 52 °C instead of 55 °C. The 18S and 16S rRNA gene amplicons were applied onto a 6% polyacrylamide gel with a denaturing gradient from 20 to 50% and 10 to 70% UF, respectively [89]. Individual bands were excised with a sterile razor and incubated overnight in 40 µl 10 mM tris solution water at 4 °C. Re-amplification was performed using the same primer pairs without GC clamp for 25 cycles under the same conditions as above. The purity of the PCR products was checked on an agarose gel. Subsequently, the PCR products were sequenced by Macrogen Inc. (Amsterdam, the Netherlands).

3.3. SUPPLEMENTARY MATERIAL - CYCLE DATA

Figure 3.4 gives an overview of the CO_2 and O_2 in the off gas and the pH during a typical light-dark cycle.

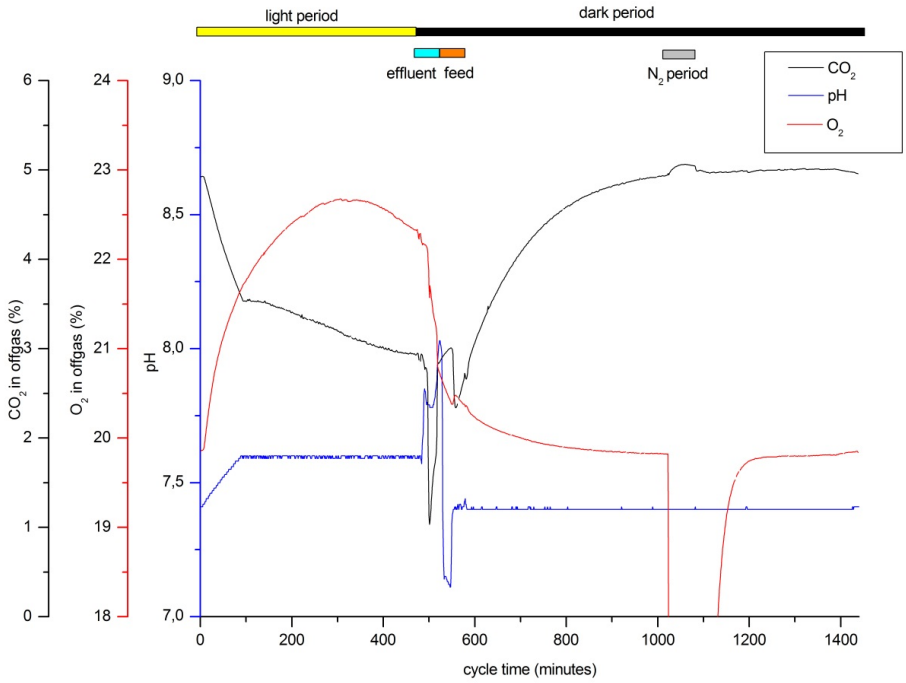

Figure 3.4: typical pH (blue), CO_2 (black) and O_2 (red) offgas profiles during a cycle

Figure 3.5 shows the amount of biomass, lipids, polyglucose and unaccounted compounds in a light-dark cycle. The data presented in Figure 3.5 has been used to construct Figure 3.1. The decrease at t=480 min is due to effluent removal and medium addition.

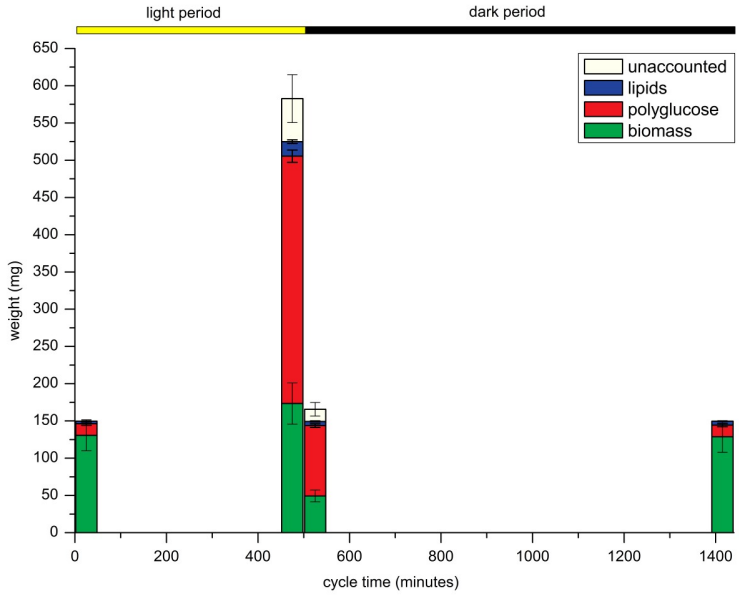

Figure 3.5: amout of biomass (green), lipids (blue), polyglucose (red) and unaccounted compounds (white) weight during a cycle

4

STARCH PRODUCTIVITY IN CYCLICALLY OPERATED PHOTOBIOREACTORS WITH MARINE MICROALGAE

Peter R. MOOIJ, Danny R. DE GRAAFF, Mark C.M. VAN LOOSDRECHT and Robbert KLEEREBEZEM

Large-scale production of microalgal storage compounds will likely involve marine microalgae. Previously we described a method to enrich microalgae with a high storage compound productivity from a natural inoculum. Here, this strategy was implemented under marine conditions in a sequencing batch reactor. The influence of the volume exchange ratio and the moment of ammonium addition in the day-night cycle on the storage compound productivity are described. Storage compound productivity proved maximal if ammonium was supplied at the start of the dark period rather than the light period, irrespective of the volume exchange ratio. Increasing the volume exchange ratio from 33 to 50 percent per cycle induced a decrease in storage compound production if ammonium was supplied in the light whereas the storage compound productivity was comparable when ammonium was supplied in the dark. The latter indicates a shift of cell division processes to the light period at increasing volume exchange ratio, although ammonium uptake completely occurred in the dark period.

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4.1. INTRODUCTION

PHOTOSYNTHETIC microalgae can store energy and carbon in the form of starch (or other polymers of glucose) and triacylglycerides under growth-limiting conditions [93] [85]. These storage compounds can be consumed when growth and maintenance requirements exceed available extracellular energy and carbon [25]. From an industrial point of view, storage compounds can be used as a precursor for biofuels [87] [72]. Microalgae are excellent producers of these storage compounds and theoretically outcompete terrestrial plants in terms of areal storage compound productivity [20]. The water demand of freshwater microalgae cultivation will however be a serious obstacle for large-scale storage compound production [82]. Sustainable large scale microalgae cultivation will therefore most likely involve the use of marine microalgae [103].

Previously, we have described a cultivation strategy to enrich and sustain a community of storage compound producing freshwater algae based on the ecophysiological role of storage compounds [76] (or Chapter 3). This strategy enriches microalgae with high levels of internal storage compounds, which make the overall process economically more interesting [9]. The methodology used focusses on a functionality rather than on a specific strain of microalgae species. Theoretically, the approach should enrich highly productive microalgae in any environment. Here, this methodology is evaluated under marine conditions. In the described method the moment of supply of the limiting nutrient inorganic nitrogen and the volume exchange ratio (VER) are essential parameters. The influence of those parameters on the functional characteristics of the system was therefore investigated. When inorganic nitrogen is present in the dark period, storage compounds are consumed to take up and metabolize the available inorganic nitrogen [50]. The production of storage compounds in the light period is therefore mandatory as long as inorganic nitrogen is solely available in the dark period. If inorganic nitrogen is available in the light period, fixed carbon can directly be used to build new biomass. Storage compounds are a logical sink for fixed carbon when inorganic nitrogen is absent in the light period [49].

At a fixed cycle length, the VER dictates the minimal growth rate of the algae in order to prevent wash-out from the system. Increasing the VER increases this minimal growth rate. This implies that, in the same time, more inorganic nitrogen should be consumed per algae at increasing VER. If the amount of inorganic nitrogen to be consumed in the dark period increases, storage compound consumption in the dark period will also increase (assuming a fixed yield of storage compounds consumed per nitrogen consumed). Increasing the VER will therefore likely increase the minimal amount of storage compounds to be produced in the light, as long as inorganic nitrogen is exclusively available in the dark period. On the other hand, if the VER is increased and more inorganic nitrogen per algae is added in the light period, less storage compounds are expected, since a larger part of the fixed carbon can be used for biomass synthesis.

In this study two hypotheses are tested. First, it is hypothesized that supplying inorganic nitrogen in the dark period will result in cultivation of an algae community with a higher storage compound production capacity than supplying inorganic nitrogen in the light period. Second, it is hypothesized that increasing the VER will enhance storage compound production if inorganic nitrogen is supplied in the dark period and decrease storage compound production if inorganic nitrogen is supplied in the light period, respec-

tively.

4.2. MATERIALS AND METHODS

Operating Conditions

TWO non-sterile 1,5 L bioreactor (Applikon, Schiedam, The Netherlands) with a diameter of 11 cm and height of 17 cm were operated in sequencing batch mode with the following operational parameters: light period 8 h, dark period 16 h, cycle length 24 h, liquid volume 1200 mL, temperature 28 ± 1 °C, stirrer speed 200 rpm, gas flow reactor in and out 50 mL/min, gas recycle over reactor 700 mL/ min, gas composition 5% CO_2 in air, average light intensity at inner reactor surface 650 µmol m^{-2} s^{-1} provided by HPS lamps, pH setpoint 8.2, base 1 M NaOH, acid 0.5 M HCl. A Bio Controller ADI 1030 (Applikon, Schiedam, The Netherlands) controlled Masterflex pumps (Cole-Parmer, Vernon Hills, IL, USA) and mass flow controllers (Brooks Instruments, Ede, The Netherlands). The Bio Controller itself was controlled by a PC with MFCS_win software (Sartorius Stedim Systems, Goettingen, Germany). A modified ammonium-limited f/2 medium was used (see section 4.5). Several seawater samples collected at the Dutch coast were used as inoculum.

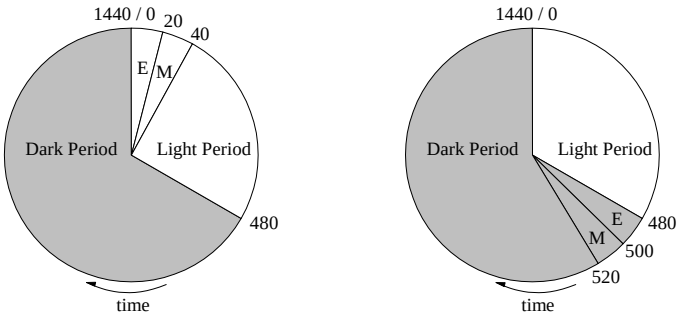

Figure 4.1: Operational cycle for reactor with effluent removal (E) and medium supply (M) at the start of the light period (Light Fed Reactor, left) or at the start of the dark period (Dark Fed Reactor, right). Numbers indicate the cumulative time in minutes from the start of the cycle.

The reactors differed in the moment of effluent and nutrient supply. In the Light Fed Reactor effluent was removed and nutrients were supplied at the start of the light period. In the Dark Fed Reactor, reactor effluent was removed and nutrients were supplied at the start of the dark phase. Figure 4.1 describes the operational cycle for both reactors. Three experiments were performed, with a VER of 16.6, 33 and 50 percent per cycle, respectively.

Sampling

Samples were taken at the transition from dark to light and from light to dark when a stable operational performance had been established. Stable operational performance was assumed when offgas CO_2, O_2 and pH showed similar profiles during multiple subsequent cycles. Besides, ammonium should be depleted before the end of the light period for the

Light Fed Reactor and before the end of the dark period for the Dark Fed Reactor. For the experiments at a VER of 33 and 50% per cycle, three cycles were measured for both reactors. For the experiment at a VER of 16.6% per cycle, one and a half cycle (one sample at the transition from dark to light and two samples at the transition from light to dark) were measured for both reactors. The cycle preceding a cycle with measurements was used to clean the reactor and remove any biofilm.

Analytical Methods

pH and DOT (dissolved oxygen tension) were continuously measured by the Bio Controller ADI 1030. Offgas CO_2 and O_2 were analysed using a Rosemount NGA offgas analyser (Emerson, USA). Ammonium was determined spectrophotometrically using Dr. Lange LCK 403 NH_4^+ cuvette tests (Hach Lange, Dusseldorf, Germany) calibrated in f/2 medium. Total dry weight was determined by centrifuging and freezedrying a known amount of sample. Ash content was determined by burning freezedried biomass for 1 hour at 550 degrees. Organic dry weight was obtained by subtracting ash amount from the total dry weight. Lipid extraction was done following the PHB extraction protocol described by [52] with the following modifications. No formaldehyde was added to the samples and myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid were used as standards. Extracted lipids were analysed by gas chromatography (model 6890N, Agilent, Lexington, MA, USA) equipped with a flame ionization detector on a HP Innowax column. Trendlines, relating peak area to lipid amount, were extrapolated for unidentified lipid peaks. Starch was measured by heating approx 5 mg freezedried biomass with 0.6 M HCl for three hours at 100 ºC. After centrifugation and filtration with a 0.45 µm pore size filter (PVDF Membrane, Millipore, Tullagreen, Ireland) the glucose monomer concentration was determined by high performance liquid chromatography, using an Aminex HPX-87H column from Bio-Rad (Hercules, CA, USA) (t=60 ºC) coupled to an ultraviolet and a refractive index detector. All glucose monomers were assumed to be formed by degradation of starch molecules. Residual biomass was calculated by subtracting the amount of lipids and starch from the organic dry weight. The residual biomass production during the light and dark period was also calculated based on the measured ammonium uptake. A yield of 10 mg residual biomass (organic dry weight) produced per mg nitrogen consumed was assumed.

PCR-DGGE

The microbial composition of the reactors at a VER of 33% per cycle was analysed using the PCR-DGGE technique. Genomic DNA was extracted using the Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The subsequent extracted DNA was used for amplifying the bacterial 16S rRNA gene using the primer pair BAC341F with a GC clamp and BAC907RM [3], eukaryotic 18S rRNA gene using the primer pair EUK1F and EUK563R with a GC clamp [30]and chloroplast 16S rRNA gene from the eukaryotes using a newly developed primer CHL21F ('5-TGGCTCAGGATGAACGCTGG-3') and CYA781RW without GC clamp. For DGGE the CHL21F-CYA781rW fragment was reamplified using the primer pair CYA359F with GC clamp and CYA781rW [81]. The temperature program for amplifying bacterial 16S-rRNA gene fragments was the same as described by [3]. For specifically amplifying the chloro-

plast 16s-rRNA gene the following program was used for the first step: starting with an initial incubation at 95 °C for 5 min. and 32 cycles of 95 °C for 30 sec., 55 °C for 40 sec., 72 °C for 40 sec. followed by an final extension of 72 °C for 30 min. The second step used a lower annealing temperature of 52 °C instead of 55 °C. The 18S and 16S rRNA gene amplicons were applied onto a 6% polyacrylamide gel with a denaturing gradient from 20 to 50% and 10 to 70% UF, respectively [89]. Individual bands were excised with a sterile razor and incubated overnight in 40 µl 10 mM tris solution water at 4 °C. Re-amplification was performed using the same primer pairs without GC clamp for 25 cycles under the same conditions as above. The purity of the PCR products was checked on an agarose gel. Subsequently, the PCR products were sequenced by Macrogen Inc. (Amsterdam, the Netherlands).

4.3. RESULTS AND DISCUSSION

Residual biomass, starch and lipid production and consumption at a VER of 33% per cycle

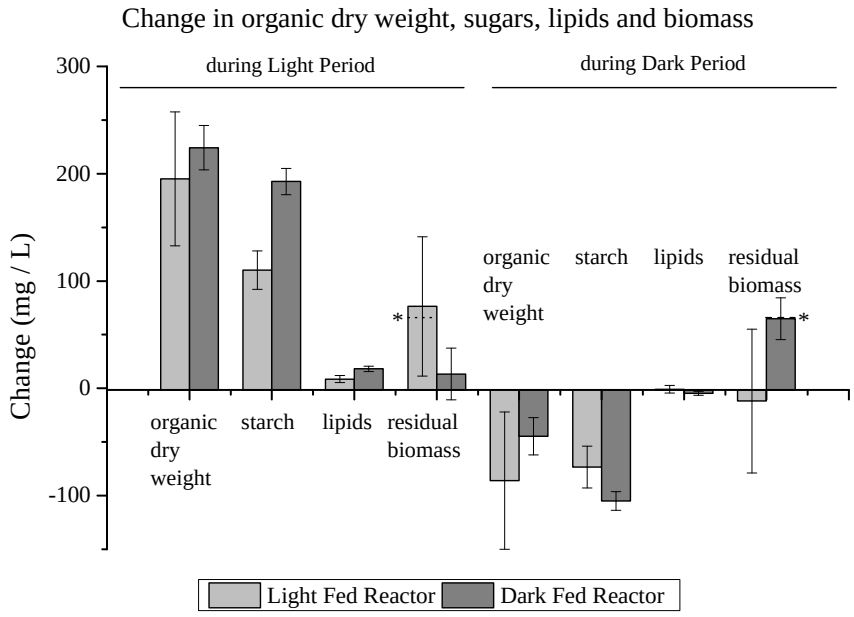

Figure 4.2: Change in organic dry weight, starch, lipids and residual biomass during light period (left panel) and dark period (right panel) for reactor receiving ammonium at the start of the light period (Light Fed Reactor, light grey) and for reactor receiving ammonium at the start of the dark period (Dark Fed Reactor, dark grey) at an volume exchange ratio of 33 percent per cycle. Asterisk symbol (*) indicates the expected biomass productions based on ammonium uptake. Error bars denote standard deviations, n = 3. Data used to construct figure 2 can be found in the supplementary online material.

The reactor fed with ammonium during the dark period produced starch and lipids

in the light period. In the dark period, with ammonium present, part of these storage compounds were consumed and residual biomass was produced (Figure 4.2). These findings are in line with the behavior observed under freshwater conditions in previous work [76]. The yield of biomass on starch during the dark period was 0.62 ± 0.19 mg X / mg starch (or 0.56 ± 0.17 mg X / mg glucose). These values are comparable to numbers reported for heterotrophic growth of algae on glucose [94]. The cyclic production and consumption of starch, lipids and residual biomass demonstrate that it is possible to obtain a storage compound producing microalgae community under marine conditions.

The second aim of this study was to investigate the influence of the moment of ammonium availability and the VER on the storage compound production by the microalgae. Figure 4.2 shows that the amount of organic dry weight produced in both reactors in the light period was comparable under both ammonium addition regimes, yet the distribution between residual biomass and storage compounds was altered. When ammonium was supplied at the start of the light period, biomass was produced. As a consequence less storage compounds were produced. Production of storage compounds after nitrogen depletion is a well-documented response in microalgae [49].

Dissolved Oxygen Tension

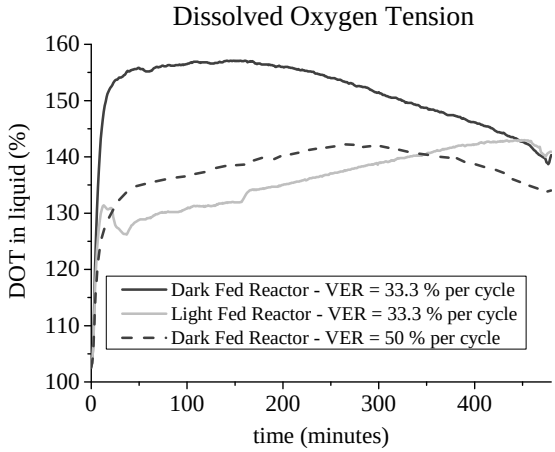

Figure 4.3: Dissolved Oxygen Tension (DOT) in the liquid in the light period for reactor receiving ammonium at the start of the light period (Light Fed Reactor, light grey) and for reactor receiving ammonium at the start of the dark period (Dark Fed Reactor) at an volume exchange ratio of 33 (dark grey solid) and 50 percent per cycle (dark grey dashed).

The Dissolved Oxygen Tension (DOT) profiles of both reactors (solid lines in Figure 4.3) show both a difference in trends as in absolute values. The absolute values are however easily influenced by any biofilm growing on the sensor membrane and should therefore be interpreted with caution. The trends do nevertheless give valuable information. The reactor receiving ammonium at the start of the dark period produced oxygen at a constant rate during the first part of the light period. This pattern suggests no increase in photosynthetic active biomass, which is in line with Figure 4.2. The decrease of the oxygen

production rate in the latter part of the light period could be due to slower storage of fixed carbon caused by rising intracellular starch levels. In the reactor receiving ammonium at the start of the light period residual biomass is produced in the light. This will lead to a rise in oxygen production and therefore in the oxygen concentration in the reactor.

PCR-DGGE analysis

Based on PCR-DGGE analyses of the 18S-DNA results indicated that the same microalgae *Chlamydomonas HS-5* (100 and 99% similarity for the reactor receiving ammonium at the start of the light period and dark period, respectively) was dominant in both reactors at a VER of 33% per cycle. This implies that one algal species was able to dominate under both feeding regimes, while it adapted its metabolic behavior.

Influence of the VER – ammonium fed at the start of the light period

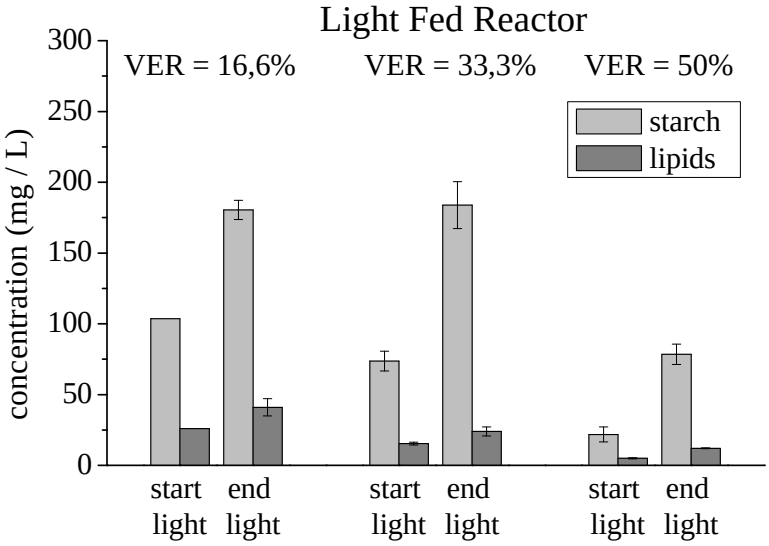

Figure 4.4: Starch (light grey) and lipid (dark grey) levels at different volume exchange ratios for reactor receiving ammonium at the start of the light period (Light Fed Reactor) at the start and end light period. The concentrations at the start of the light period are after effluent removal and medium supply. Error bars denote standard deviations, n = 3, except for VER 16.6 % - start light (n=1) and VER 16.6 % - end light (n = 2). Data used to construct Figure 4 can be found in section 4.6.

The reactor receiving ammonium at the start of the light phase showed the lowest storage compound production at the highest VER (Figure 4.4). Steady state lipid levels decreased at increasing VER. Theoretically, algae can grow unlimited in the light phase without producing storage compounds as long as ammonium is present. The observed

production of storage compounds may only occur after ammonium is depleted in the light phase. From this moment on organic carbon produced by photosynthesis cannot be used for biomass formation, but storage compounds can still be produced [49]. An increase in VER increases the minimal growth rate and the amount of ammonium available per algae at the start of the light period. The length of the time period that algae are exposed to ammonium limited conditions in the light will therefore be shorter. As a consequence, a lower storage compound productivity is expected during the light phase, which is in line with the decrease in starch production observed at a VER of 50% per cycle (56.6 ± 8.9 mg starch L^{-1} light $period^{-1}$) compared to the production at a VER of 33% per cycle (110.2 ± 18.0 mg starch L^{-1} light $period^{-1}$). Based on the proposed theory, one would expect the starch productivity at a VER of 16,6% per cycle to be higher than at a VER of 33 % per cycle. There is however a biological upper limit of the amount of starch which can be accumulated in microalgae, which will also limit productivity. Since the starch concentration at the start of the light was higher at a VER of 16.6% per cycle compared to a VER of 33%, less starch can be produced in the light period. Lipids are regarded to be secondary storage metabolites [38], the relative high lipid concentration at a VER of 16.6% per cycle is a clear indication that starch levels had reached a maximum.

Influence of the VER – ammonium fed at the start of the dark period

Similarly, in the reactor receiving ammonium in the dark an increase in VER leads to more ammonium per algae available, but in this case in the dark phase. To metabolise this ammonium more storage compounds will be consumed in the dark period and more storage compounds should be produced in the light period at increasing VER. Storage compound productivity is indeed lowest at the lowest VER and increased when the VER was increased from 16.6 (87.6 ± 10.2 mg starch L^{-1} light $period^{-1}$) to 33% per cycle (192.8 ± 12.2 mg starch L^{-1} light $period^{-1}$) (Figure 4.5). Increasing the VER from 33 to 50% per cycle should also increase storage compound production. According to Figure 4.5 this is not the case, storage compound production is slightly lowered (164.9± 15.6 mg starch L^{-1} light $period^{-1}$) at a VER of 50% per cycle. A possible explanation can be the limited time the algae have in the light period to produce starch and lipids, which was the same at all VERs.

The amount of ammonium dosed was 10 mg N / cycle at a VER of 50% per cycle and 6.6 mg N / cycle at a VER of 33% per cycle. It is interesting that at both VER's all ammonium was consumed at the end of the dark phase, while starch production was comparable. This suggests a different response to ammonium at different VER's. The DOT value of the Dark Fed Reactor at a VER of 50 volume percent per cycle (dark grey dashed line in Figure 4.3) seems to support this idea. The oxygen production rate increased during the first part of the light period. This suggests an increase in photosynthetic active biomass. Although nitrogen uptake completely occurred at night, Figure 4.2 suggests that at increasing VER some cell division processes were shifted towards the light phase. This shift could explain the lower storage compound production observed. Another hint for a different metabolic response can be found in the decrease in the yield of consumed starch per consumed nitrogen. This yield drops from 15.9 ± 1.5 mg starch / mg N at a VER of 33% per cycle to 7.7 ± 0.8 mg starch / mg N at a VER of 50% per cycle (see section 4.6 for data). Future research on the metabolic fate of consumed nitrogen can elucidate this.

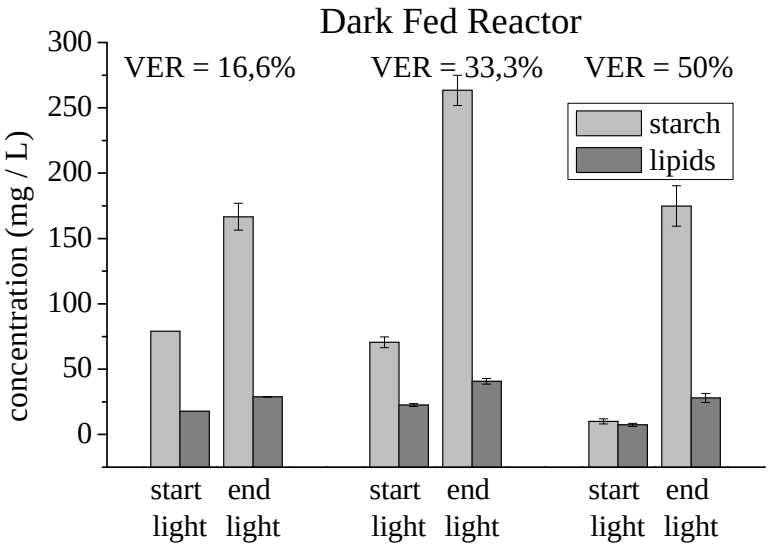

Figure 4.5: Starch (light grey) and lipid (dark grey) levels at different volume exchange ratio's for reactor receiving ammonium at the start of the dark period (Dark Fed Reactor) at the start and end light period. The concentrations at the end of the light period are before effluent removal and medium supply. Error bars denote standard deviations, n = 3, except for VER 16.6 % - start light (n=1) and VER 16.6 % - end light (n = 2). Data used to construct Figure 5 can be found in section 4.6.

Concluding on the functional characteristics of the system it can be stated that supplying ammonium in the dark enhanced storage compound productivity compared to supplying ammonium in the light, irrespective of the VER. Increasing the VER had an adverse effect on storage compound productivity if ammonium was dosed in the light. Storage compound production was lowest at 16,6% VER per cycle and comparable at 33 and 50% per cycle if nitrogen was supplied in the dark. In this study we showed it is possible to create a selective marine environment which favors storage compound producing microalgae. The method relies on non-sterile conditions and is therefore easily scalable and economically interesting. The work presented overcomes thereby two major obstacles for large scale microalgae cultivation: the large freshwater demand and the need to work under axenic conditions.

4.4. CONCLUSION

CYCLICALLY uncoupling of carbon fixation and ammonium uptake led to starch production by marine microalgae. Starch productivity was maximized when ammonium was supplied at the start of the dark period rather than the light period, irrespective of the volume exchange ratio. Increasing the volume exchange ratio from 33 to 50 percent

per cycle led to less storage compound production if ammonium was supplied in the light and to comparable storage compound production if ammonium was supplied in the dark. The latter indicates a shift of cell division processes to the light period at increasing volume exchange ratio, although ammonium uptake completely occurred in the dark period.

4.5. SUPPLEMENTARY MATERIAL - MEDIUM COMPOSITION

A modified f/2 medium was used. To ensure N limitation all nutrient and trace metal concentrations except for the N-source were multiplied by a factor three. The basis of the medium was artificial sea water, as described in Table 4.1. Major elements, as described in Table 4.2, and minor elements, as described in Table 4.3 were added to the artificial sea water.

Table 4.1: Artificial Sea Water constituents

compound	**concentration**	**concentration**
-	*g / L*	*mmol / L*
$MgCl_2 \cdot 6\,H_2O$	11.11	54.66
$CaCl_2$	1.16	10.43
$SrCl_2 \cdot 6\,H_2O$	0.042	0.158
KCl	0.70	9.32
$NaHCO_3$	0.20	2.39
KBr	0.10	0.84
H_3BO_3	0.03	0.44
NaF	0.003	0,07
NaCl	24.53	419.80
$NaSO_4$	4.09	28.82

Table 4.2: Major elements

compound	**concentration**	**concentration**
-	*mg / L*	*mmol / L*
NH_4Cl	76.41	1.43
$NaH_2PO_4 \cdot H_2O$	15.00	0.11
$Na_2SiO_3 \cdot 9\,H_2O$	90.00	0.32

Table 4.3: Minor elements

compound	**concentration**	**concentration**
-	mg/L	µmol/L
$Na_2EDTA \cdot 2\,H_2O$	13.14	35.10
$FeCL_3 \cdot 6\,H_2O$	9.49	35.10
$MnCl_2 \cdot 4\,H_2O$	0.53	2.70
$CuSO_4 \cdot 5\,H_2O$	0.03	0.12
$ZnSO_4 \cdot 7\,H_2O$	0.07	0.24
$CoCL_2 \cdot 6\,H_2O$	0.04	0.15
$NaMoO_4 \cdot 2\,H_2O$	0.02	0.09
allyl-thiourea	10.00	-

4.6. SUPPLEMENTARY MATERIAL - ADDITIONAL DATA

Table 4.4, Table 4.4, Table 4.5, Table 4.6, Table 4.7, Table 4.8 and Table 4.9 have been used to construct Figure 4.2, Figure 4.4 and Figure 4.5.

Table 4.4: Organic dry weight, starch, lipids and residual biomass for the **Light Fed Reactor** at a **SRT of 6 days** (or a VER of 16.6 volume % per cycle). Data used to construct Figure 4.4.

SRT	-	**time in cycle**	**organic dry weight**	**starch**	**lipids**	**residual biomass**
days	-	*min*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
6	end dark / start light	0 / 1440	399.7 ± 30.3	124.3	31.2	212.6 ± 30.3
6	after effluent and nutrient supply	30	283.1 ± 25.3	103.6	26.0	177.1 ± 20.2
6	end light - start dark	480	446.2 ± 22.5	180.5 ± 6.8	41.0 ± 6.1	231.6 ± 24.3
6	after effluent and nutrient supply	510	-	-	-	-
6	**harvested per cycle**	**-**	**113.2 ± 10.1**	**41.4**	**10.4**	**70.9 ± 10.1**
6	**change in light period**	**-**	**163.0 ± 33.9**	**76.9 ± 6.8**	**14.9 ± 6.1**	**54.4 ± 31.6**
6	**change in dark period**	**-**	**-106.4 ± 37.8**	**-56.2 ± 6.8**	**-9.7 ± 6.1**	**-19.0 ± 38.9**

Table 4.5: Organic dry weight, starch, lipids and residual biomass for the **Light Fed Reactor** at a **SRT of 3 days** (or a VER of 33.3 volume % per cycle). Data used to construct Figure 4.2 and Figure 4.4.

SRT	-	**time in cycle**	**organic dry weight**	**starch**	**lipids**	**residual biomass**
days	-	*min*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
3	end dark / start light	0 / 1440	372.3 ± 19.3	110.5 ± 10.5	23.0 ± 1.5	193.7 ± 22.0
3	after effluent and nutrient supply	30	218.2 ± 12.9	73.7 ± 7.0	15.4 ± 1.0	129.1 ± 14.7
3	end light - start dark	480	413.3 ± 61.0	183.9 ± 16.6	24.0 ± 3.2	205.5 ± 63.3
3	after effluent and nutrient supply	510	-	-	-	-
3	**harvested per cycle**	**-**	**109.1 ± 6.4**	**36.8 ± 3.5**	**7.7 ± 0.5**	**64.6 ± 7.3**
3	**change in light period**	**-**	**195.2 ± 62.4**	**110.2 ± 18.0**	**8.6 ± 3.3**	**76.3 ± 65.0**
3	**change in dark period**	**-**	**-86.1 ± 64.0**	**-73.4 ± 19.6**	**-0.9 ± 3.5**	**-11.8 ± 67.0**

Table 4.6: Organic dry weight, starch, lipids and residual biomass for the **Light Fed Reactor** at a **SRT of 2 days** (or a VER of 50 volume % per cycle). Data used to construct Figure 4.4.

SRT	-	**time in cycle**	**organic dry weight**	**starch**	**lipids**	**residual biomass**
days	-	*min*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
2	end dark / start light	0 / 1440	196.2 ± 28.5	43.6 ± 10.5	10.1 ± 0.8	142.5 ± 30.4
2	after effluent and nutrient supply	30	98.1 ± 14.2	21.8 ± 5.2	5.0 ± 0.4	71.2 ± 15.2
2	end light - start dark	480	239.2 ± 6.6	78.4 ± 7.2	12.1 ± 0.3	148.7 ± 9.7
2	after effluent and nutrient supply	510	-	-	-	-
2	**harvested per cycle**	**-**	**98.1 ± 14.2**	**21.8 ± 5.2**	**5.0 ± 0.4**	**71.2 ± 15.2**
2	**change in light period**	**-**	**141.1 ± 15.7**	**56.6 ± 8.9**	**7.0 ± 0.5**	**77.5 ± 18.0**
2	**change in dark period**	**-**	**-43.0 ± 29.2**	**-34.8 ± 12.7**	**-2.0 ± 0.8**	**-6.2 ± 31.9**

Table 4.7: Organic dry weight, starch, lipids and residual biomass for the **Dark Fed Reactor** at a **SRT of 6 days** (or a VER of 16.6 volume % per cycle). Data used to construct Figure 4.5.

SRT	-	**time in cycle**	**organic dry weight**	**starch**	**lipids**	**residual biomass**
days	-	*min*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
6	end dark / start light	0 / 1440	281.9 ± 32.4	79.1	17.7	161.7 ± 32.4
6	after effluent and nutrient supply	30	-	-	-	-
6	end light - start dark	480	363.5 ± 27.6	166.7 ± 10.2	28.9 ± 0.2	180.7 ± 29.4
6	after effluent and nutrient supply	510	302.9 ± 23.0	138.9 ± 8.5	24.1 ± 0.2	150.6 ± 24.5
6	**harvested per cycle**	**-**	**121.2 ± 9.2**	**55.6 ± 3.4**	**9.6 ± 0.1**	**60.2 ± 9.8**
6	**change in light period**	**-**	**81.6 ± 42.5**	**87.6 ± 10.2**	**11.2 ± 0.2**	**19.0 ± 43.7**
6	**change in dark period**	**-**	**-21.0 ± 39.7**	**-59.8 ± 8.5**	**-6.4 ± 0.2**	**11.1 ± 40.6**

Table 4.8: Organic dry weight, starch, lipids and residual biomass for the **Dark Fed Reactor** at a **SRT of 3 days** (or a VER of 33 volume % per cycle). Data used to construct Figure 4.2 and Figure 4.5.

SRT	-	**time in cycle**	**organic dry weight**	**starch**	**lipids**	**residual biomass**
days	-	*min*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
3	end dark / start light	0 / 1440	314. 5 ± 14.2	70.6 ± 4.0	22.5 ± 1.1	221.4 ± 14.8
3	after effluent and nutrient supply	30	-	-	-	-
3	end light - start dark	480	538.8 ± 14.9	263.4 ± 11.5	40.7 ± 2.3	234.7 ± 19.0
3	after effluent and nutrient supply	510	359.2 ± 9.9	175.6 ± 7.7	27.1 ± 1.5	156.5 ± 12.6
3	**harvested per cycle**	**-**	**179.6 ± 5.0**	**87.8 ± 3.8**	**13.6 ± 0.8**	**78.2 ± 6.3**
3	**change in light period**	**-**	**224.3 ± 20.6**	**192.8 ± 12.2**	**18.2 ± 2.5**	**13.3 ± 24.1**
3	**change in dark period**	**-**	**-44.7 ± 17.3**	**-105.0 ± 8.7**	**-4.6 ± 1.8**	**65.0 ± 19.5**

Table 4.9: Organic dry weight, starch, lipids and residual biomass for the **Dark Fed Reactor** at a **SRT of 2 days** (or a VER of 50 volume % per cycle). Data used to construct Figure 4.5.

SRT	-	**time in cycle**	**organic dry weight**	**starch**	**lipids**	**residual biomass**
days	-	*min*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
2	end dark / start light	0 / 1440	102.1 ± 54.1	10.0 ± 2.0	7.4 ± 1.2	84.6 ± 54.1
2	after effluent and nutrient supply	30	-	-	-	-
2	end light - start dark	480	344.5 ± 79.6	174.9 ± 15.5	28.0 ± 3.5	141.6 ± 81.2
2	after effluent and nutrient supply	510	172.2 ± 39.8	87.4 ± 7.7	14.0 ± 1.7	70.8 ± 40.6
2	**harvested per cycle**	**-**	**172.2 ± 39.8**	**87.4 ± 7.7**	**14.0 ± 1.7**	**70.8 ± 40.6**
2	**change in light period**	**-**	**242.4 ± 96.2**	**164.9 ± 15.6**	**20.6 ± 3.7**	**57.0 ± 97.6**
2	**change in dark period**	**-**	**-70.2 ± 67.1**	**-77.4 ± 8.0**	**-2.1 ± 13.8**	**13.8 ± 67.7**

5

INFLUENCE OF SILICATE ON ENRICHMENT OF HIGHLY PRODUCTIVE MICROALGAE FROM A MIXED CULTURE

Peter R. MOOIJ, Lisanne D. DE JONGH, Mark C.M. VAN LOOSDRECHT and Robbert KLEEREBEZEM

Microalgae have the potential to supply a biobased society with essential feed stocks like sugar and lipids. Besides being productive, strains used for this purpose should grow fast, be resistant to predators and have good harvestability properties. Diatoms, a class of siliceous algae, have these and other preferred characteristics. In this paper we describe the enrichment of microalgae in sequencing batch reactors with and without supply of silicate. Both reactors were operated with a light-dark cycle. To maximise storage compound production carbon fixation and nitrogen uptake were uncoupled by limiting the availability of nitrate to the dark phase. After 10 cycles a stable culture was established in both reactors. The diatom Nitzschia sp. dominated the silicate rich reactor, the green algae Chlamydomonas sp. dominated the silicate depleted reactor. The remaining 27 cycles of the experiment the microalgal community structure did not change, indicating a highly stable system. Although the dominant microalgae was highly dependent on the presence of silicate, the performance of both microalgal enrichments was similar. Polymers of glucose

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were stored during the nitrogen limited light period. On organic matter dry weight basis the sugar content of the biomass increased during the light period from 17 ± 4 % to 53 ± 4 % for the silicate limited culture and from 14 ± 4 % to 43 ± 4 % (w/w) for the silicate excess culture. These results show that storage compound production can be achieved under various conditions, as long as a selective environment is maintained.

5.1. INTRODUCTION

MICROALGAE are efficient producers of triacylglycerides [49] and starch [72], and are proposed to supply these as feed stock for food, fuels and chemicals to a biobased economy [107]. Nowadays, microalgal research mainly focuses on pure culture applications. Production of bulk compounds, such as triacylglycerides, at an industrial scale is however troublesome in an axenic microalgal culture [59] [95] [95]. Previously, we described an ecology-based enrichment and cultivation method which allows for stable storage compound production under non-axenic conditions [76] [77]. The basis of this approach is uncoupling of carbon fixation in the light and nitrogen uptake in the dark by limiting the presence of an essential growth nutrient (such as nitrate) to the dark phase. Limiting the presence of nitrate to the dark period provides a competitive advantage for storage compound producing microalgae. Microalgae that efficiently convert CO_2 into storage compounds in the light phase can take up the nitrate in the dark phase for biomass production; resulting in an enrichment culture consisting of efficient storage producing microalgae [76] (or Chapter 3).

Despite their overwhelming abundance in nature, diatoms are largely overlooked in microalgal biofuel and biomass production research. Diatoms possess however various characteristics which make them interesting candidates for large scale storage compound production [47]. These include the capacity to accumulate large amount of lipids when exposed to silicate limitation, good resistance to predators and good harvestability.

The presence of diatoms in nature is to a large extent regulated by the amount of available silicate [106]. At non-limiting silicate levels diatoms are effective competitors for limiting nutrients and are able to effectively utilize nutrient pulses [69]. At external silicate concentrations exceeding 2 mM diatoms typically represent more than 70% of the phytoplankton community [36]. With increasing silicate to phosphorus ratios diatom abundance increases in competition experiments between diatoms and nonsiliceous algae [98].

The first objective of this research was to investigate if selective enrichment of storage compound producing diatoms can be established in an open system with a surplus of silicate in the medium. We expect the enriched microalgae community to differ in a system with and without silicate supply. The functionality of storage compound production is nonetheless expected to be present under both conditions, as it is a consequence of the uncoupling of carbon fixation and nitrogen uptake.

5.2. MATERIALS AND METHODS

Operating Conditions

Two 1.5 L bioreactors (Applikon, Schiedam, The Netherlands) with a diameter of 11 cm and height of 17 cm were operated in a sequenced batch mode with operational parameters as in (Table 5.1). The system was operated under non-axenic conditions. The medium composition for both reactors is described in Table 5.2.

Table 5.1: Operationel parameters of both systems

parameter	unit	value
light period	h	16
dark period	h	8
cycle length	h	24
solid retention time	h	41
liquid volume	L	1.2
temperature	°C	28
stirrer speed	rpm	200
gas flow reactor in and out	NmL / min	50
gas recycle over reactor	NmL / min	1200
gas composition	% CO_2 in air	5
average light intensity at inner reactor surface	µmol m^{-2} s^{-1}	650
light source	-	HPS lamps
PH setpoint	-	7.5
base	-	1.0M NaOH
acid	-	0.5 M HCl

Table 5.2: Medium composition of both systems

compound	amount	unit
$NaNO_3$	1.43	mM
H_3BO_3	1.66	mM
$CaCl_2$	1.07	mM
$MgSO_4 \cdot 6\,H_2O$	0.64	mM
K_2PO_4	0.23	mM
$FeCL_3 \cdot 6\,H_2O$	0.11	mM
$Na_2EDTA \cdot 2\,H_2O$	0.11	mM
$MnCl_2 \cdot 4\,H_2O$	7.71	µM
$CuSO_4 \cdot 5\,H_2O$	0.03	µM
$ZnSO_4 \cdot 7\,H_2O$	0.69	µM
$CoCL_2 \cdot 6\,H_2O$	0.43	µM
$NaMoO_4 \cdot 2\,H_2O$	0.77	µM
$NaHSeO_3 \cdot 2\,H_2O$	0.10	µM
$NaVO_3$	0.01	µM
allyl-thiourea	10.0	mg / L

11.42 mM Na_2SiO_3 was supplied to the silicate excess reactor and withheld from the silicate deplete reactor. Nitrate was supplied as the sole nitrogen source at the beginning of the dark period and was designed to be the limiting factor for algae growth. A solid retention time of 41 h and a cycle length of 24 h (Table 5.1) imply that every cycle 59% (700 mL) of the culture was harvested and replaced with medium as in Table 5.2. 14 mg NO_3-N was therefore dosed at the start of the dark period of every cycle. A mixture of several freshwater samples collected from the upper layer of canals, ponds and lakes in the vicinity of Delft, the Netherlands were used as inoculum. Figure 5.1 describes the

operational cycle for both reactors.

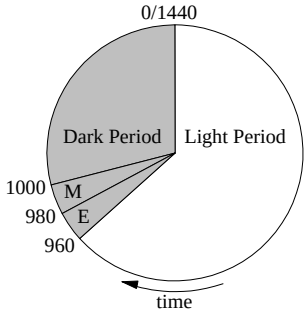

Figure 5.1: Operational cycle for reactor with effluent removal (E) and medium supply (M) at the start of dark period. Numbers indicate the cumulative time in minutes from the start of the cycle.

A Bio Controller ADI 1030 (Applikon, Schiedam, The Netherlands) controlled Masterflex pumps (Cole-Parmer, Vernon Hills, IL, USA) and mass flow controllers (Brooks Instruments, Ede, The Netherlands). The Bio Controller itself was controlled by a PC with MFCS_win software (Sartorius Stedim Systems, Goettingen, Germany).

Analytical Methods

Samples were taken at the transition from dark to light and from light to dark. NO_3^- was determined spectrophotometrically using Dr. Lange LCK 339 NO_3^- cuvette tests (Hach Lange, Dusseldorf, Germany). Silicate was determined spectrophotometrically using Dr. Lange LCW 028 SiO_2 cuvette tests (Hach Lange, Dusseldorf, Germany). Measurements of organic dry weight, lipids, glucose-polymers and analysis of the microalgal community structure were done as described in [75] (or see Chapter 4) with the following modification. Glucose-polymers were heated with 0.9 M HCl instead of 0.6 M HCl. Species succession was quantified by taking pictures using a Leica DM500B light microscope (Leica Microsystems, Wetzlar, Germany) using a 200 times magnification. These pictures were used to count and sort at least 300 microalgal cells per sample.

5.3. RESULTS AND DISCUSSION

Microalgal community structure

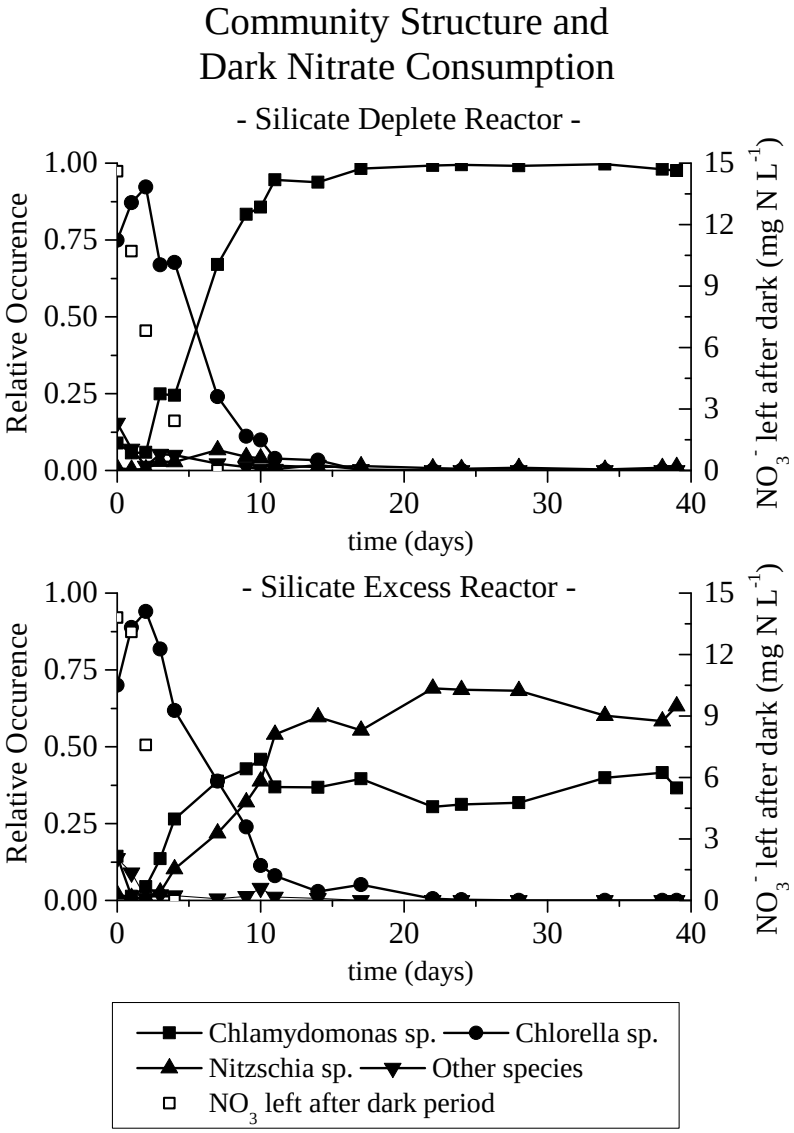

Figure 5.2: Community structure (black lines, left y-axis) and nitrate left over after the dark phase (black open squares, right y-axis) in time for the silicate deplete (top) and silicate excess (bottom) reactor.

The first aim of this study was to investigate the influence of silicate presence on the selection of storage compound producing microalgae. In absence of silicate the operational conditions imposed resulted in the enrichment of the green algae *Chlamydomonas sp.* (*Lobochlamys segnis,* Table 5.3). With silicate in excess a co-culture of the diatom *Nitzschia sp.* (accounting for 60% of the population) and the green algae *Chlamydomonas* was established (Figure 5.2). Apparently two microalgae could coexist under the given conditions, although only one resource, nitrate, was designed to be limited. A possible explanation for the occurrence of a co-culture could be a different metabolic response to the pulse-wise addition of nitrate at the start of the dark period. Diatoms are experts in nutrient uptake and storage [69]. Nitrate storage up to a intracellular concentration of 273 mM has been reported [56]. This would allow diatoms to divide during periods without external nitrate, such as during the light period in this experiment. Nitrate storage in green algae is less documented, possibly limiting the period suitable for cell division to the dark period for green algae. This metabolic difference could possibly explain the observed co-culture.

The dominance of *Chlorella luteoviridis* (Table 5.3) increased in both reactors during the first days of the experiment (Figure 5.2). After dominating both systems for around 95% on day 3, *Chlorella* numbers steeply declined in the next days. A possible explanation lies in the different conditions before and after day 5 of operation. Nutrients were dosed at the start of every dark phase in both reactors. During the first days of the experiment the limiting nutrient nitrate was not fully consumed in the dark phase (Figure 5.2). The presence of nitrate in the light phase favoured nutrient uptake and cell division over storage compound production which apparently facilitated *Chlorella* enrichment during this transition period. From day 5 onwards the nitrate was limited during the entire light phase. From this moment on algae were not able to directly divide in the light phase. The decline of *Chlorella sp.* in the enrichment culture coincided with the onset of nitrogen limitation during the light period.

Apparently, *Chlamydomonas* and *Nitzschia* outcompete *Chlorella* if carbon fixation and nitrate uptake are uncoupled. In previous work *Chlorella luteoviridis* dominated the system throughout the experiment [76] (or Chapter 3). Operational differences between the previous and current work, such as the nitrogen source used and the solid retention time, could explain the disappearance of *Chlorella luteoviridis* under the conditions applied in this experiment.

Storage compound productivity in the day/night cycle

The second aim of this study concerned the functional characteristics of both systems. In both reactors large amounts of glucose polymers were produced, increasing from 17 ± 4 % to 53 ± 5 % and from 14 ± 4 to 43 ± 4 % on organic matter basis during the light period for the silicate depleted and excess reactor, respectively (Figure 5.3). Diatoms are known to produce chrysolaminarin, a β-1,3-D-glucan, under nitrogen limitation [42]. Green algae produce starch under nitrogen-limited conditions [72]. Both of these storage compounds will be measured as glucose monomers using our analytical methods. Lipid levels showed the same trends but were always significantly lower. The lipid fraction increased from 6 ± 1 % to 7 ± 2 % and from 8 ± 2 % to 10 ± 2 % on organic matter basis during the light period for the silicate depleted and excess reactor, respectively (data not

Table 5.3: Identity of species according to microscopic observation and PCR-DGGE analysis. Only the main species present are depicted.

reactor	species determined by microscope	species determined by PCR-DGGE	RNA gene used	Identity (%)
silicate excess	*Chlamydomonas sp.*	*Lobochlamys segnis KMMCC 1045*	18S	100
	Chlorella sp.	*Chlorella luteoviridis CCAP 211/5B*	18S	96
		Chlorella sorokiniana chloroplast	16S	99
	Nitzschia sp.	*Nitzschia frustulum* chloroplast	16S	100
silicate deplete	*Chlamydomonas sp.*	*Lobochlamys segnis* KMMCC 1045	18S	99
	Chlorella sp.	*Chlorella luteoviridis CCAP 211/5B*	18S	98
		Chlorella sorokiniana chloroplast	16S	99
	Nitzschia sp.	*Nitzschia frustulum chloroplast*	16S	99

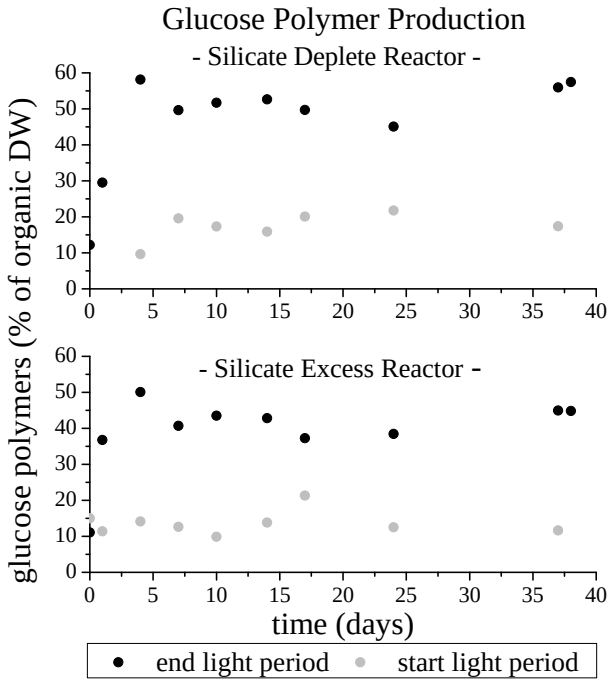

Figure 5.3: fraction of glucose polymers on organic dry weight basis at the start (grey dots) and at the end (dark dots) of the light period in time for the silicate deplete (top) and silicate excess (bottom) reactor.

shown). Comparing productivity values to literature is difficult in the microalgae field. Studies differ in light input, reactor design, reactor operation, type of limitation and other operational parameters. For pure cultures glucose content values ranging from 41 to 62.1 % of total dry solids are reported [33] [85] [13]. The values obtained for enrichment cultures are in the same range, but are reported on organic matter basis. Due to their siliceous cell wall, the ash content of diatoms account for up to 50% of the total dry solids.

Comparing productivity between algae classes on total dry solid basis should therefore be done with caution [47].

Stability

From day 10 onwards the storage compound production and the microalgae population were stable in both reactors for the remaining 27 days. With around 60% of the reactor volume harvested every day, these 27 days correspond to 30+ generations of microalgae. Gas and liquid flows leaving and entering the reactors were not sterilized and the reactors were manually cleaned, opening the reactors fully, every 3-4 days. Despite these disturbances, the systems were both in terms of functional performance and microbial community structure very stable, emphasizing the robustness of the approach used.

5.4. CONCLUSION

IN the work presented we show that uncoupling of carbon fixation in the light and nitrogen uptake in the dark under silicate excess conditions enriched a diatom dominated, glucose polymer producing microalgae community. In absence of silicate a green algae dominated community was obtained. Both communities showed the same characteristic of producing high amounts of storage compounds in the light period and stable community structure in time. These results indicate that the proposed method will in any environment enrich a storage compound producing algae which thrives in this specific environment. As a consequence, it allows stable storage compound production in open, and therefore cheap, cultivation systems.

6

ENRICHMENT CULTURE FOR STORAGE COMPOUND PRODUCING DIATOMS

Peter R. MOOIJ, Chris M. VERMEER, Mark C.M. VAN LOOSDRECHT and Robbert KLEEREBEZEM

Diatoms have beneficial characteristics for the large-scale production of microalgal storage compounds. These include a relative easy solid-liquid separation after cultivation, increased resistance to predators and the possibility to synthesize lipids in silicate limitation conditions. In this paper we describe the enrichment of diatoms from a mixed inoculum in a system with a light-dark cycle and pulse-wise addition of the limiting nutrient NH_4^+. Both supplying the NH_4^+ pulse at the start of the light or dark period enriched for Nitzschia palea from a mixed culture. The selective environment resulted in stable communities that did not change during 27 cycles of steady state culturing. The metabolic behaviour of the culture was however highly influenced by the moment of NH_4^+ addition. Sugar polymers and lipids increased during the light period from 16 ± 4 % to 27 ± 3 % and from 8 ± 2 % to 9 ± 1 % of the organic dry weight if NH_4^+ was supplied at the start of the light period. If NH_4^+ was supplied at the beginning of the dark period, sugar polymers and lipids increased from 2 ± 0 % to 51 ± 3 % and from 3 ± 3 % to 17 ± 1 % of the organic dry weight in the light period. Subjecting the obtained cultures to prolonged periods of nitrogen or silicate limitation induced different metabolic responses. Whereas cell numbers increased fourfold under nitrogen limitation, cell division immediately ceased under silicate limitation. The

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preferred storage compound under nitrogen limitation were polymers of glucose, while lipids were predominantly produced under silicate limitation. These results underline that diatoms are suitable lipid producers and that both diatom dominance and storage compound production can be obtained and sustained in open systems under selective cultivation conditions. This makes these findings easily transferable to large scale systems.

6.1. INTRODUCTION

MICROALGAE are excellent producers of low value compounds as starch and triacylglycerides, which can be converted to gaseous or liquid biofuels [48] [49] [78]. Production of starch and triacylglycerides in microalgae is promoted by growth-limiting conditions, such as the absence of an essential nutrient [49]. Consumption of these storage compounds enables microalgae to endure conditions with external energy supply limitation (e.g. during dark periods).

The term microalgae covers several microalgal classes including green microalgae, cyanobacteria, diatoms and microalgal lineages. Diatoms distinguish themselves from other microalgal lineages by their siliceous cell wall. This cell wall provides various characteristics that are beneficial for microalgal cultivation [47]. Firstly, the cell wall of diatoms offers protection against predators [45]. Secondly, diatoms have a relatively high specific density, simplifying the solid-liquid separation after microalgae cultivation. Thirdly, the need for silicate introduces another element which can be limited to induce storage compound production. In the majority of studies nitrogen limitation has been used to trigger the production of starch and lipids. Nitrogen limitation has however adverse effects on cellular fitness, since nitrogen containing compounds are essential to virtually all cellular metabolic processes [102]. Silicate is an essential element for diatoms to synthesize new cell walls. In the absence of silicate cell division is therefore halted, while other cellular processes are largely unaffected [47]. This potentially makes silicate limitation an interesting trigger for storage compound production.

Despite the wide-range use of pure cultures in research towards phototrophic lipid production we believe that for large scale production of low-value bulk products such as lipids or carbohydrates, enrichment culture techniques need to be used to enable cost-effective process operation [77] (or Chapter 2). Enrichment cultures are based on the creation of a selective environment that provides a competitive advantage for the desired group of microorganisms. In previous work we introduced an enrichment and cultivation method to select for green microalgae with a high storage compound productivity by uncoupling carbon fixation in the light and nutrient uptake in the dark [76] (or Chapter 3). The moment of the supply of the limiting nutrient ammonium proved to have a substantial effect on the metabolic processes of green marine microalgae [75] (or Chapter 4). If the presence of ammonium was limited to the dark phase, storage compounds were produced in the light phase. If ammonium was however present in the light period, biomass was the main product in the light period.

The first aim of this study was to investigate if an enrichment culture dominated by diatoms can be established from a natural inoculum. The second aim of this study was to investigate if the influence of the moment of inorganic nitrogen supply had a similar effect on the metabolic behaviour of a culture of diatoms as previously observed with green microalgae. The third and final aim of this study is to investigate the metabolic response of the enrichment culture in terms of storage compounds productivity when exposed to prolonged periods of silicate or nitrogen limitation.

6.2. MATERIALS AND METHODS

Operating Conditions in Cyclic Systems

Two 1.5 L bioreactors (Applikon, Schiedam, The Netherlands) with a diameter of 11 cm and height of 17 cm were operated for 40 days under non-axenic conditions in a sequenced batch mode with operational parameters as in Table 6.1. The medium composition for both reactors is described in Table 6.2. Ammonium was supplied as nitrogen source and was designed to be the limiting factor for algae growth. The reactors differed in the moment of effluent removal and nutrient supply. In one reactor effluent was removed and nutrients were supplied at the start of the light period. In the other reactor effluent was removed and nutrients were supplied at the start of the dark phase. Figure 6.1 describes the operational cycle for both reactors. A mixture of several freshwater samples collected from the upper layer of canals, ponds and lakes in the vicinity of Delft, the Netherlands was used as inoculum. A Bio Controller ADI 1030 (Applikon, Schiedam, The Netherlands) controlled Masterflex pumps (Cole-Parmer, Vernon Hills, IL, USA) and mass flow controllers (Brooks Instruments, Ede, The Netherlands). The Bio Controller itself was controlled by a PC with MFCS win software (Sartorius Stedim Systems, Goettingen, Germany).

Table 6.1: operational parameters for the enrichment culture reactors

parameter	unit	value
light period	h	8
dark period	h	16
cycle length	h	24
solid retention time	h	38.4
liquid volume	L	1.2
temperature	°C	25
stirrer speed	rpm	200
gas flow reactor in and out	NmL / min	50
gas recycle over reactor	NmL / min	1200
gas composition	% CO_2 in air	5
average light intensity at inner reactor surface	µmol m^{-2} s^{-1}	650
light source	-	HPS lamps
PH setpoint	-	8.0
base	-	1.0M NaOH
acid	-	0.5 M HCl

Table 6.2: Medium composition for the enrichment culture reactors

compound	amount	unit
$NaNO_3$	1.43	mM
H_3BO_3	1.66	mM
$CaCl_2$	1.07	mM
$MgSO_4 \cdot 6\,H_2O$	0.64	mM
K_2PO_4	0.14	mM
$FeCL_3 \cdot 6\,H_2O$	0.11	mM
Na_2SiO_3	5.7	mM
$Na_2EDTA \cdot 2\,H_2O$	0.23	mM
$MnCl_2 \cdot 4\,H_2O$	2.70	µM
$CuSO_4 \cdot 5\,H_2O$	0.01	µM
$ZnSO_4 \cdot 7\,H_2O$	0.24	µM
$CoCL_2 \cdot 6\,H_2O$	0.15	µM
$NaMoO_4 \cdot 2\,H_2O$	0.27	µM
$NaHSeO_3 \cdot 2\,H_2O$	0.04	µM
$NaVO_3$	0.003	µM
allyl-thiourea	10.0	mg / L

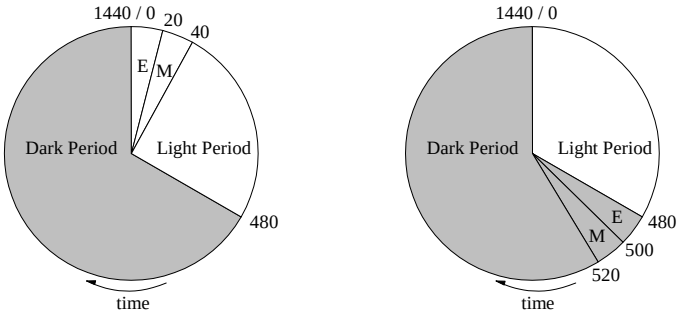

Figure 6.1: operational cycle for enrichment reactors with effluent removal (E) and medium supply (M) at the start of the light period (left) or start of the dark period (right). Numbers indicate the time in minutes from the start of the cycle.

Operating Conditions in Batch

Effluent from the cyclic systems was used as starting culture for the batch experiments. In the case of nitrogen-limited batch experiments the effluent was complemented with medium as in Table 6.2 lacking NH_4Cl. In the case of silicate-limited batch experiments the effluent was centrifuged at 4000 rpm for 5 minutes, resuspended in medium as in Table 6.2 lacking Na_2SiO_3, centrifuged once more and finally resuspended in medium as in Table 6.2 lacking Na_2SiO_3. Light was continuously present and no effluent was removed nor medium was supplied in the batch experiments. All other parameters were as described above. Batch experiments were conducted with cultures from both cyclic

systems. As these cultures showed comparable results, only data obtained using the reactor with effluent withdrawal and nutrient supply at the start of the night are reported.

Sampling

Samples were taken at the transition from dark to light and from light to dark in the cyclic systems and on regular time intervals in the batch experiments. Three cycles were measured in the cyclic systems, except for the light to dark transition for the reactor withdrawing effluent and receiving nutrients at the start of the night, for which two measurements were conducted. As significant numbers of cells started to lyse lipids after 50 hours of silicate limitation in the batch experiments, sampling was stopped after this time.

Analytical Methods

Measurements of organic dry weight, lipids, glucose-polymers and analysis of the microalgal community structure were done as described in [75] (or Chapter 4) with the following modifications. Glucose polymers were heated with 0.9 M HCl instead of 0.6 M HCl. NH_4^+ trends were obtained using an NH_4^+-probe (Vernier, USA), absolute NH_4^+ measurements were done as described in [75] (or Chapter 4). Silicate was determined spectrophotometrically using Dr. Lange LCW 028 SiO_2 cuvette tests (Hach Lange, Dusseldorf, Germany). Pictures were taken using a Leica DM500B light microscope (Leica Microsystems, Wetzlar, Germany) equipped with fluorescence filtercube A (excitation: UV, excitation filter: BP 340-380, dichromatic mirror: 400, suppression filter: LP 425). 1 µmol BODIPY 505/515 (Invitrogen D3921, Life Technologies, Grand Island, USA) in DMSO (1 mg/ml) was used to colour lipids in 1 mL of microalgal cells [22]. Biomass is in this work defined as the organic dry weight minus lipids and minus carbohydrate polymers and comprises therefore of DNA, protein, structural elements and other cell constituents.

6.3. RESULTS AND DISCUSSION

TWO reactor systems were inoculated with a large diversity of microalgae and operated under a cyclic day/night regime with effluent removal and nutrient supply at the start of the light or at the start of the dark period. NH_4^+ was in both systems the limiting nutrient for microalgal growth. The microalgal enrichment and its functional characteristics were investigated over time.

Microalgal enrichment

Microscopic observation revealed that the enrichments in both systems were dominated (>95% of the culture) by a diatom from the genus *Nitzschia* after 13 days of enrichment. Sequencing of the most visible band after 18sRNA PCR-DGGE of the enrichment cultures yielded a 100% match with *Nitzschia palea*. For the remaining 27 cycles of the experiment, corresponding to 38 generations, the microalgal communities remained stable. Liquid and gas flows entering and leaving the system were not sterilised, every day 62.5 of the reactor volume was harvested. The bioreactors were dis- and reassembled twice per week to remove biofilm. The dominance of *Nitzschia palea* was not affected by any of these procedures.

In previous research a stable co-culture of the green algae *Chlamydomonas sp.* and the diatom *Nitzschia sp.* was obtained (see Chapter 5). The main differences in enrichment conditions between this previous work and the work presented in this paper concern the pH (7.5 in previous work, 8 for this experiment), the nitrogen source (NO_3^- in previous work, NH_4^+ in this experiment) and the length of the light period (16h in previous work, 8 in present work). Silicate was present in excess in both experiments. Although it is not possible to pinpoint the exact reason explaining the co-culture from previous work and the monoculture enriched in this experiment, the pH is likely of great influence, as lowering the pH from 8 to 7 increased the number of green algae substantially in the enrichment cultures (data not shown).

Although the two systems differed in the moment of addition of the limiting nutrient NH_4^+, the same diatom strain became dominant. This might be explained by the pulse-wise addition of the limiting nutrient NH_4^+ in both systems. Diatoms are excellent competitors for nutrient pulses and have the possibility to store NO_3^- and NH_4^+ intracellularly [69] [70]. This feature would suggest that the NH_4^+ uptake rate is the actual critical factor determining the competition. If nitrogen storage can both be obtained in the light and in the dark it may explain why the same species became dominant under both regimes.

Off-gas oxygen and NH_4^+ profiles

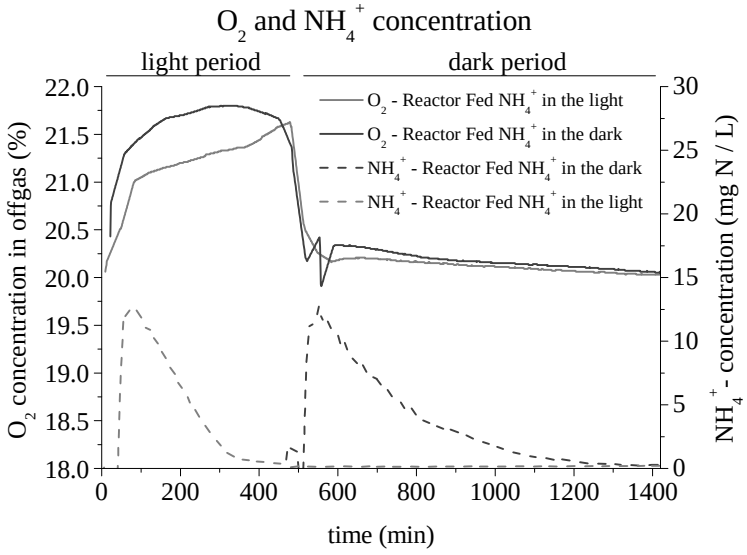

Figure 6.2: Oxygen concentration in the off-gas (solid lines, left-y-axis) and NH_4^+ concentration in the liquid (dashed lines, right y-axis) for reactor receiving NH_4^+ in the light period (light grey) or in the dark period (dark grey).

Although the moment of N-supply did not influence the microalgae population, the functional characteristics of the system were clearly different. Figure 6.2 shows the

oxygen concentration in the off-gas and the NH_4^+-concentration in both reactors. In both reactors NH_4^+ was consumed in the phase it was supplied in. The O_2 profile in the off-gas had a different shape in both reactors. The O_2 profile in the off-gas in the light phase of the reactor receiving NH_4^+ in the dark stayed relatively constant between t= 100 and t = 400 min, indicating photosynthesis was taking place at a relatively constant rate. The O_2 profile in the off-gas in the light phase of the reactor receiving NH_4^+ in the light increased continuously in the light period, indicating an increase in the rate of photosynthesis. This can be linked to the formation of new biomass in this period. The O_2 profiles in the off-gas are in line with measurements done in reactor systems with NH_4^+ supply at the start of the light period or dark period under marine conditions [75].

Glucose polymers, lipids, biomass and cell number in the light-dark cycle

Biomass measurements in the light period (Figure 6.3) showed an increase in biomass for the reactor receiving NH_4^+ in the light period and a slightly decreasing biomass concentration for the reactor receiving NH_4^+ in the dark period. This is in line with the O_2 patterns in the off-gas in Figure 6.2.

Figure 6.3 shows a different metabolic behaviour if NH_4^+ is dosed at the beginning of the light or dark period. If NH_4^+ is dosed at the beginning of the dark period (right section of Figure 6.3) large amounts of glucose and lipids were produced in the light period. These were consumed in the dark period to produce new biomass. Biomass was the main sink for photosynthesis in the light period for the reactor receiving NH_4^+ in the light period (left section of Figure 6.3). Glucose polymers and lipids were also produced in this system in the light period, but production was substantially lower compared to the system with NH_4^+ addition at the start of the dark period. This behaviour is in line with previous research [75].

A comparison between the change in biomass (Figure 6.3) and cell number (Figure 6.4) shows that the increase in cell number is maximal in the phase without biomass production. The cell numbers increased mainly in the dark period for the reactor receiving NH_4^+ at the beginning of the light period and in the light period for the reactor receiving NH_4^+ at the beginning of the dark period. *Nitzschia palea* was therefore clearly able to uncouple cell division and ammonium uptake. Whereas in most algal lineages cell division mainly occurs in the dark period, cell division patterns in diatoms differ considerably and are to a large extent affected by nutrient pulses [35]. Pulsed addition of NH_4^+ to a pure culture of *T. weisfloggi* led to a main division burst 18 hours after this pulse, regardless of the moment of the pulse in the light-dark cycle [35] The presented cell number data indicates that also *Nitzschia palea* sees a major division burst considerable time after nitrogen addition.

Cellular change in glucose polymers, lipids and biomass

Storage compound production was significantly higher if NH_4^+ was dosed in the dark period rather than in the light period. This may be partly due to the fact that 62.5 volume percent of the reactor receiving NH_4^+ in the light was replaced with medium at the start of the light period. As a consequence, the biomass concentration and cell number was lower at the start of the light period compared to the reactor receivingNH_4^+ in the dark. This makes a volumetric comparison of storage compound production in the light period likely biased towards the reactor receiving NH_4^+ in the dark period. Figure 6.5 shows

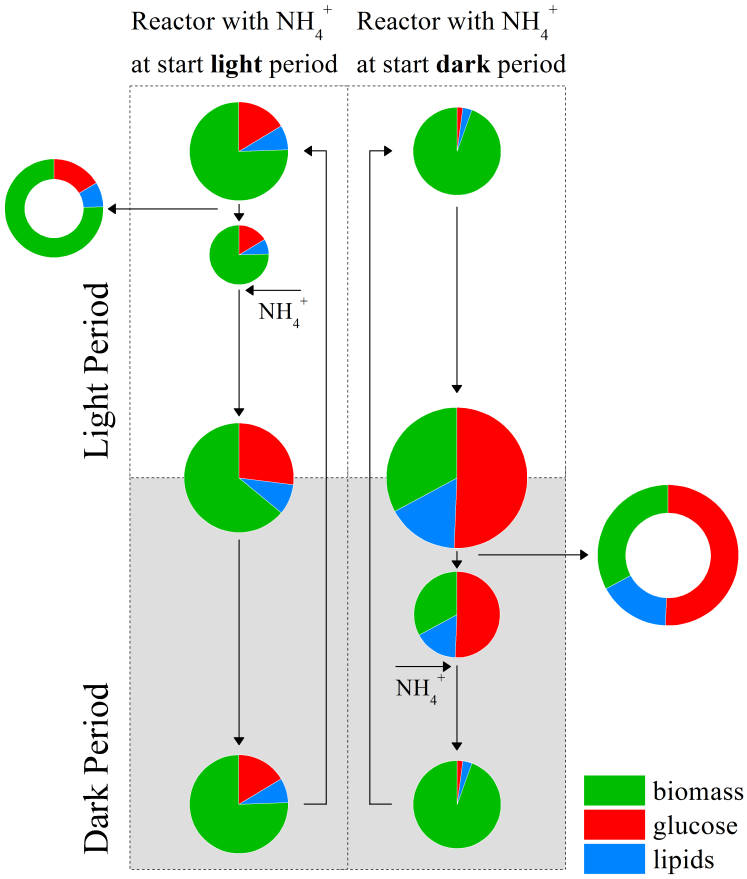

Figure 6.3: Biomass (green), glucose polymers (red) and lipids (blue) in the light-dark cycle for the reactor receiving NH_4^+ at the start of the light period (left part) or dark period (right part). Pie-area is linearly related to total amounts, which are an average of three cycles. Data used to construct Figure 6.3 can be found in section 6.5.

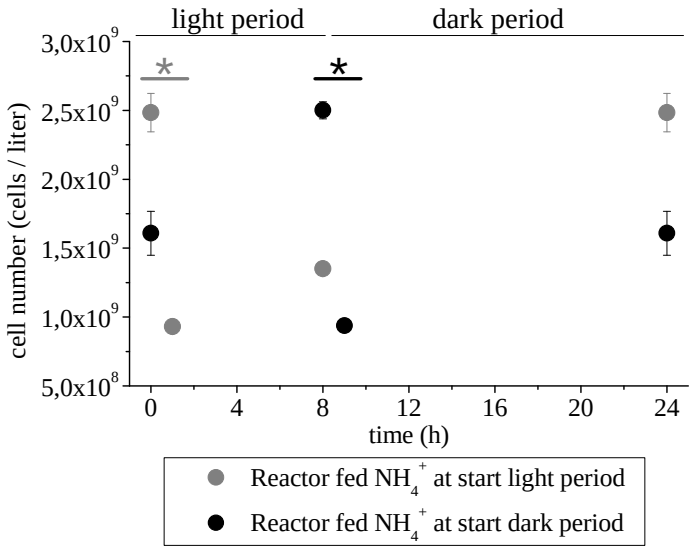

Figure 6.4: Cell number in the light-dark cycle for the reactor receiving NH_4^+ at the start of the light period (grey dots) or dark period (black dots). Error bars denote standard deviations. Asterisk (*) symbols denote moment of effluent removal and nutrient supply.

therefore the cell composition at the start and the end of the light period. The amount of storage compounds after the light phase was also on cellular basis significantly higher if NH_4^+ was supplied at the beginning of the dark period (Figure 6.5).

Prolonged periods of nitrogen or silicate limitation

The enriched cultures were subjected to prolonged periods of nitrogen- or silicate limitation. As the cultures responded comparable, only results obtained with the culture enriched by supplying NH_4^+ at the start of the dark period are presented. Table 6.3 shows the cell number and the glucose polymer and lipid concentration under nitrogen- and silicate-limitation.

Table 6.3: Cell number, glucose polymers and lipids in time under nitrogen and silicate limitation. Additional information can be found in section 6.5.

	nitrogen limitation			silicate limitation		
time limited	**cellnumber**	**glucose**	**lipids**	**cellnumber**	**glucose**	**lipids**
h	*cells / L*	*mg / L*	*mg /L*	*cells / L*	*mg / L*	*mg /L*
0	$8{,}33\times10^8$	2,1	7,8	$8{,}25\times10^8$	18,7	1,9
24	$2{,}42\times10^9$	500,1	148,8	$7{,}63\times10^8$	7,5	68,7
48	$3{,}73\times10^9$	481,3	205,1	$6{,}93\times10^8$	31,4	55,5
72	$3{,}79\times10^9$	405,9	219,7	-	-	-

Limiting either nitrogen or silicate induced major different metabolic responses. If

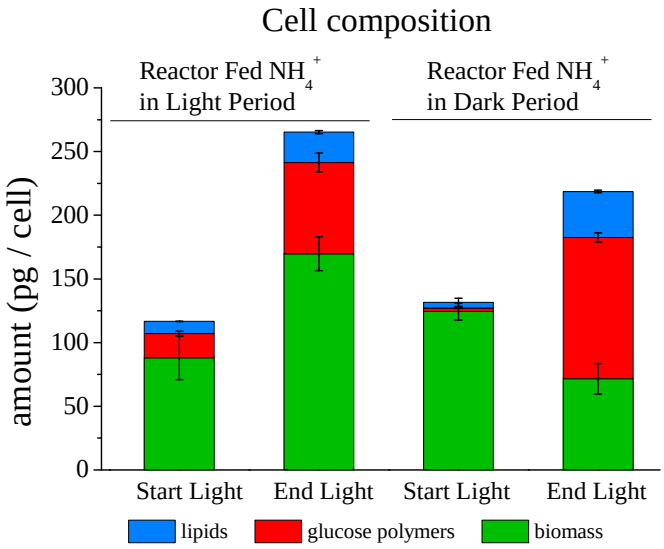

Figure 6.5: biomass (green), glucose polymers (red) and lipids (blue) per cell at the start and at the end of the light period for the reactor receiving NH_4^+ in the light (left) and dark period (right), respectively. Error bars denote standard deviations.

nitrogen was limited cell numbers increased a factor 4 in 50 hours, whereas cell numbers remained constant if silicate was the limiting compound. The increase in cell number under nitrogen limitation indicates that intracellular organic nitrogen concentrations of diatoms can be decreased under prolonged periods of nitrogen starvation. As a consequence the amount of biomass per cell declined under nitrogen limitation and increased under silicate limitation (see additional data in section 6.5).

Glucose polymers were the main photosynthetic products under nitrogen limitation but were virtually absent under silicate limitation. Volumetric lipid production was highest under nitrogen limitation, but the cellular lipid content established was higher under silicate-limitation (90 pg /cell versus 62 pg / cell after 24 hours of silicate or nitrogen limitation, respectively, (see additional data in section 6.5).

Microscopic pictures of silicate limited diatoms show lipid droplets inside the cells increasing in time (Figure 6.6). The observed behaviour under silicate limitation, cessation of cell division and lipids as preferred storage compound are in line with literature [47] [88] [105]. Chemical methods to stop cell division, in the presence of silicate, have been shown to also induce lipid production [47].

The preferred storage compound was dependant on the type of limitation, with lipids as preferred storage compound under silicate limitation and glucose polymers under nitrogen limitation. If the goal is to produce lipids and if CO_2 and light are abundantly available, such as in the experiments described here, limiting nitrogen will yield more lipids than limiting silicate. In large scale systems light and CO_2 are normally limiting the process. In these conditions any carbon and energy spent on production of glucose

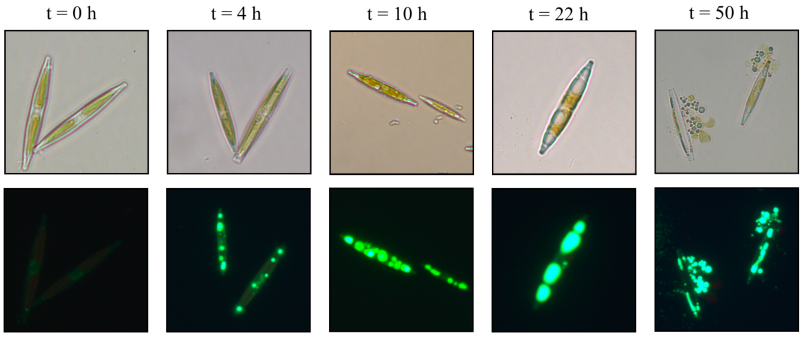

Figure 6.6: Lipid production under silicate limitation in time. Pictures taken with bright field (top row) and fluorescence microscopy (bottom row) after staining lipid droplets in cells with Bodipy 505/515 [22]. Length of one diatom cell is approx. 35 µm.

polymers lowers the lipid productivity and silicate limitation is therefore preferable.

These results indicate that a two-stage system, consisting of a silicate-rich enrichment step and a silicate-free accumulation step, can be used to obtain lipid production over glucose polymer production in a phototrophic enrichment culture.

In this study we show it was possible to enrich a microalgal culture fully dominated by the diatom *Nitzschia palea* from a natural inoculum. The metabolic properties in the light-dark cycle were strongly influenced by the moment of nitrogen addition. If NH_4^+ was supplied in the dark phase, lipids and sugars were produced in the light period. If NH_4^+ was present in the light period, biomass was the main product. The lipid: glucose storage polymer ratio upon nutrient limitation was highly influenced by the type of limitation, with polymers of glucose as major storage compound under nitrogen limitation, whereas silicate limitation led to production of preferably lipids. The above results indicate that the cell composition of diatom is heavily dependent on the moment of nutrient addition if cultivated cyclically and on the type of limitation if cultivated in batch. The data obtained can help to adapt the cultivation environment in such a way that the desired cell composition is reached. This, combined with the advantageous characteristics of diatoms, such as an increased resistance to predators, a relatively easy solid-liquid separation and easy cell lysis, will help in creating diatom-based microalgal production processes.

6.4. CONCLUSION

PULSE-WISE addition of the limiting compound NH_4^+ enriched a phototrophic culture fully dominated by the diatom *Nitzschia palea* from a mixed inoculum, irrespective if the NH_4^+ pulse was given at the beginning of the light or the dark period. The products from photosynthesis were highly influenced by the moment of NH_4^+-addition, with biomass or storage compounds as the main products if NH_4^+ was dosed at the beginning of the light and dark period, respectively. The culture obtained produced mainly glucose polymers under prolonged periods of nitrogen limitation and mainly lipids under prolonged periods of silicate limitation.

6.5. SUPPLEMENTARY MATERIAL - ADDITIONAL DATA

Table 6.4: Glucose polymers, lipids and biomass concentrations for the reactor receiving NH_4^+ at the start of the light period or dark period. Data used to construct Figure 6.3

		reactor receiving NH_4^+ at start light period			reactor receiving NH_4^+ at start dark period		
moment	**time**	**glucose**	**lipids**	**biomass**	**glucose**	**lipids**	**biomass**
-	*h*	*mg / L*	*mg / L*	*mg / L*	*mg / L*	*mg / L*	*mg / L*
end dark / start light	24 / 0	47,8 ± 7,1	23,6 ± 0,9	220,0 ± 53,5	4,4 ± 0,5	7,2 ± 5,2	199,6 ± 13,0
after effluent	0.5	17,9 ± 2,6	8,8 ± 0,3	82,5 ± 20,1	-	-	-
end light / start dark	8	96,7 ± 9,5	32,1 ± 1,5	229,0 ± 15,8	277,2 ± 2,0	90,3 ± 2,3	179,5 ± 34,5
after effluent	8.5	-	-	-	104,0 ± 0,8	33,9 ± 0,9	67,3 ± 12,9

Table 6.5: Volumetric and cellular glucose polymers, lipids and biomass concentration in time under prolonged periods of nitrogen limitation. Data used to construct Table 6.3

	Nitrogen limitation						
time limited	**cellnumber**	**glucose**	**lipids**	**biomass**	**glucose**	**lipids**	**biomass**
h	*cells / L*	*mg / L*	*mg / L*	*mg / L*	*pg / cell*	*pg / cell*	*pg / cell*
0	$8{,}3\times10^8$	2,1	7,8	108,0	2,5	9,4	129,7
24	$2{,}4\times10^9$	500,1	148,8	62,1	206,9	61,6	25,7
48	$3{,}7\times10^9$	481,3	205,1	158,9	129,0	55,0	42,6
72	$3{,}8\times10^9$	405,9	219,7	104,9	107,0	57,9	27,6

Table 6.6: Volumetric and cellular glucose polymers, lipids and biomass concentration in time under prolonged periods of silicate limitation. Data used to construct Table 6.3

	Silicate limitation						
time limited	**cellnumber**	**glucose**	**lipids**	**biomass**	**glucose**	**lipids**	**biomass**
h	*cells / L*	*mg / L*	*mg / L*	*mg / L*	*pg / cell*	*pg / cell*	*pg / cell*
0	$8{,}3\times10^8$	18,7	1,9	115,2	22,7	2,4	139,7
24	$7{,}6\times10^8$	7,5	68,7	277,1	9,8	90,2	363,4
48	$6{,}9\times10^8$	31,4	55,5	206,1	45,3	80,0	297,3

7

GENERAL CONCLUSION AND OUTLOOK

Peter R. MOOIJ

7.1. INTRODUCTION

THIS thesis deals with selective environments in microalgal cultivation. Based on ecological principles a selective environment for the cultivation of carbohydrate producing microalgae was identified. This selective environment uncoupled carbon fixation in the light from nutrient uptake in the dark by limiting the presence of the essential growth nutrient nitrogen (in the form of NH_4^+ or NO_3^-) to the dark period. As consumption of NH_4^+ and NO_3^- requires carbon skeletons and energy, dark nitrogen assimilation can only take place at the expense of storage compounds (in the form of carbohydrates or lipids) produced in the light period. Microalgae are therefore only able to sustain themselves under these conditions by producing storage compounds in the light period. This methodology was evaluated under various cultivation conditions in this thesis.

7.2. INFLUENCE OF THE MOMENT OF NITROGEN-ADDITION

AN overview of the steady state conversions of the various microalgal enrichment cultures as described in this thesis is provided in Table 7.1. In order to assess the influence of the moment of nitrogen addition on the steady state conversions of the enrichment cultures, several key parameters from Table 7.1 are discussed in this section.

The distribution of the fixed CO_2 over biomass, lipids and carbohydrate-polymers was strongly influenced by the moment of nitrogen addition to the system. Comparing the systems receiving NH_4^+ at the start of the light period and the systems receiving NH_4^+ at the start of the light period (Chapter 4 and Chapter 6 in Table 7.1) shows that the majority of the fixed CO_2 is used for lipid and carbohydrate synthesis if NH_4^+ is dosed at the beginning of the dark phase. Substantial amounts of biomass are produced if NH_4^+ is supplied at the start of the light phase. Limiting the presence of an essential nutrient to the dark phase shifts the biomass production processes partly to the dark period, and a larger fraction of the photosynthetic products will hence be used for storage compound formation in the light period. As a consequence, the storage compound content after the light period is higher if the limiting nutrient is dosed at the beginning of the dark period rather than at the beginning of the light period. Storage compound levels, of which carbohydrates were the main constituent, of around 50-70% on organic matter base were achieved in the enrichment cultures after every light period in steady state. An increased storage compound content is beneficial for downstream processing and the economics of microalgal cultivation [24]. If the aim is to obtain microalgae rich in storage compounds after the light period, the limiting nutrient should be dosed at the beginning of the dark period.

The volumetric CO_2-fixation rate was comparable or higher if NH_4^+ was dosed at the beginning of the dark, rather than the beginning of the light period. It should however be noted that in the system with nitrogen dosed at the start of the light period substantial fractions of the reactor volume (60% in the case of Chapter 6 and 33% in the case of Chapter 4) were harvested at the beginning of the light period. This lowered the microalgal concentration at the start of the light period and thereby the average volumetric productivity. To facilitate the comparison between supplying nitrogen at the beginning of the light period or at the beginning of the dark period a biomass specific CO_2-fixation

Table 7.1: Key figures for enriched microalgal cultures in steady state. Enrichment conditions and additional data can be found in Appendix A

Chapter	N dosed at start	Dominant Alga after enrichment	Carbon partitioning in Carbohydrates	Lipids	Biomass	Storage After Light Phase	CO_2-fixation rate	Lipid : Carbohydrate Production Ratio	Yield C / N in Dark Phase
-	-	-	*mg C mg C fixed^{-1}*			*% of VSS*	*mg C L^{-1} h^{-1}*	*mg lipids mg carbohydrates^{-1}*	*mg C mg N^{-1}*
3	dark	*C.luteoviridis*, green	0.62	0.06	0.32	60	21.9	0.05	-4.1
4	dark	*C.reinhardtii*, marine green	0.78	0.14	0.08	56	12.3	0.10	-6.9
4	light	*C.reinhardtii*, marine green	0.47	0.07	0.46	50	11.7	0.08	-
5	dark	*C.reinhardtii*, green	0.55	0.05	0.39	54	18.7	0.05	-4.9
5	dark	*N.frustulum*, diatom	0.44	0.13	0.43	49	18.8	0.16	-4.4
6	dark	*N.palea*, diatom	0.68	0.39	-0.07	67	20.0	0.30	-4.8
6	light	*N.palea*, diatom	0.24	0.13	0.63	36	16.4	0.30	-

rate would be preferable. The downside of such an approach is the relatively high error in the biomass determination , which would propagate in a biomass specific CO_2-fixation rate. Biomass was in this thesis calculated by subtracting ash, glucose polymers and lipid from the total dry solids. Errors in measurements of any those compounds propogate therefore and lead to relatively large errors in the reported biomass concentration. A direct biomass measurement, based on proteins, DNA or chlorophyll, would solve this issue and is recommended for future work.

Table 7.1 furthermore indicates that the average CO_2-fixation rate was comparable for all fresh water experiments. Although the the light- and CO_2-input were comparable in the experiments under marine and freshwater conditions, the CO_2-fixation rate was significantly lower under marine conditions.

7.3. CARBOHYDRATES AS PREFERRED STORAGE COMPOUND

IN all experiments polymers of glucose were the dominant storage compounds. The diatom enrichments had a higher lipid:carbohydrate production ratio than the green microalgal dominated enrichments (Table 7.1), but also here carbohydrates were the dominant storage compounds produced. Limiting the presence of nitrogen to the dark period is therefore a successful strategy if carbohydrate productivity is the main objective. Up to 78% of fixed carbon in the light period was stored in the form of carbohydrates under the proper cultivation conditions.

Carbohydrates are the building blocks for many bioprocesses [72] or can be converted to methane containing biogas. Lipid production is however preferable for liquid biofuel production, and lipids are therefore the preferred microalgal storage compound. A better understanding of the metabolic switch between carbohydrate and lipid production is needed for rational design of selective environments for lipid production. Understanding the ecological reasons why microalgae produce lipids under certain conditions will prove crucial in this respect.

7.4. POSSIBLE REASONS FOR CARBOHYDRATE PRODUCTION

SEVERAL possible explanations for the observed preferred production of carbohydrates over lipids can be formulated. These are discussed in the sections below.

TIMESCALE OF THE NITROGEN-DEPRIVED CONDITIONS

A prolonged absence of nitrogen is the most widely used strategy to induce lipid production [49]. Lipid production experiments under nitrogen limitation typically cover days to weeks. Also in these experiments carbohydrate polymers are produced in the first hours of nitrogen deprivation after which lipid production, either due to de novo fatty acid synthesis or due to a conversion of sugar polymers, is reported [68] [51]. As carbohydrate production precedes lipids production, lipids are widely regarded secondary storage compounds. In this light it can be argued that all to the experiments conducted in this thesis selected, by the relative short light period in the absence of nitrogen, for carbohydrate producing microalgae. Increasing the nitrogen deprived light period would likely yield lipid producing microalgae. From a productivity and process point of view prolonged periods of nitrogen absence are however undesirable, as these have detrimental effects on

cellular fitness and carbon fixation productivity [102]. Direct production of lipids would therefore be a preferred outcome of the imposed operating conditions. Possible strategies to achieve this are discussed later on in this chapter.

NITROGEN UPTAKE IN THE DARK PHASE

Another factor possibly favouring carbohydrate over lipid production relates to the pulse-wise addition of nitrogen in all experiments. Carbohydrate-polymers are of value in the supply of energy and carbon skeletons during nitrogen uptake [102]. As nitrogen was dosed as a pulse at the start of the dark period, the nitrogen uptake rate was an important selection criteria. This could explain the observed carbohydrate production in the light period. To eliminate this selection pressure nitrogen should constantly be present in the dark phase. These conditions can be obtained by setting an appropriate nitrogen dosage per microalgae in the dark period or by constructing a control loop to maintain a minimal nitrogen concentration in the dark phase. Under these conditions, lipid production would prove beneficial over carbohydrate production, if all cell division processes would take place in the dark period (Appendix B). It remains however to be tested if the presence of nitrogen, in itself, would trigger microalgae to execute all division processes in the dark.

Spill-over of nitrogen to the light period should be minimised in such a system, as this would favour fast-growing over storage compound producing microalgae. In this respect systems with immobilised biomass, either as biofilm or in dense aggregates, could facilitate the change between a nitrogen-limited light period and a nitrogen-rich dark period, as the hydraulic and solid retention time are easily uncoupled in these systems.

THE FATE OF NITROGEN IN THE DARK PHASE

A third possible explanation for the the observed production of carbohydrates in the light phase concerns the fate of the supplied nitrogen in the dark phase. If all cell division processes would take place in the dark phase, lipids would be the preferred carbon source as the yield of biomass on lipids exceeds the yield of biomass on sugar polymers (see calculation in Appendix B). Microalgae do however not necessarily have to carry out all cell division processes in the dark in a system with the presence of nitrogen limited to the dark phase. Production of amino acids and / or proteins in the dark period and other cell division processes in the light period could be a viable strategy.

The observed yield of intracellular storage compound consumption to consume nitrogen in the dark period of 5.1 ± 1.0 mg C / mg N (Table 7.1), which is in line with literature [42], indicates that not all metabolic processes needed for cell division took place in the dark. Assuming an elemental biomass composition of $CH_{1.78}O_{0.36}N_{0.12}$ [34] 7.1 mg C is incorporated per mg N in the biomass. Consumption of carbon to yield energy for biomass production is not included in this number. A metabolic ratio of the organic carbon source to nitrogen in the dark period of 10 mg C / mg N is therefore likely. This clearly indicates that not all cell division processes take place in the dark.

Production of the amino acid glutamate from glucose polymers would require around 5 mg C / mg N (Appendix B). This would suggest that the production of amino acids, rather than complete cell division, takes place in the dark period. As production of amino-acids also consumes energy and carbon skeletons [102], this would not fundamentally alter the enrichment principle. Carbon and energy should still be stored in the N-depleted

light phase to allow amino-acid production in the N-rich dark phase.

The type of storage compounds produced in the light could however be dependant on the metabolic processes in the dark. If amino acids are produced in the dark with lipids as substrate, large amounts of reduction equivalents are produced as the degree of reduction of lipids (5.7 mol electrons / mol C) exceeds the degree of reduction of glutamate (3.6 mol electrons / mol C). The cell has to find a way to get rid of these reduction equivalents. If glucose polymers, with a degree of reduction of 4 mol electrons / mol C, are used as substrate for dark amino-acid assimilation this is much less the case. If therefore only amino acid synthesis occurs in the dark period, and if there is no added value in the production of reduction equivalents, the production of lipids in the light period does not have to be beneficial for microalgae.

7.5. DIFFERENCES BETWEEN MICROALGAL LINEAGES

DATA from Chapter 5 indicates that lipid production is higher in diatom dominated system than in green microalgal dominated system. Over and above, data presented in Chapter 6 and literature [47] shows that lipids are the preferred storage compound in silicate limited diatoms. Silicate limitation in diatoms leads to cell cycle arrest and this triggers lipid production [47]. As intracellular storage of silicate is minimal, the cell cycle is relatively fast arrested upon silicate limitation. Nitrogen, on the other hand, is stored intracellular to a great extent, both in diatoms [21] as in other microalgal classes [32]. For lipid production in diatom dominated cultures, limiting silicate to the dark phase seems therefore more efficient than limiting nitrogen. However, temporal unavailability of silicate will likely enrich green- and other non-siliceous microalgae. A double limitation of silicate and an element essential to all microalgal lineages, such as phosphate, could be an interesting environment to favour diatom dominance and obtain lipid production in an open cultivation system. Although diatoms are excellent competitors for nutrient uptake when nutrients are provided pulse wise [69] it remains to be tested if diatoms encountering two limitation (silicate and e.g. phosphate) can outcompete other microalgal lineages encountering only one limitation (phosphate).

A better understanding of the ecological reasons why silicate limitation favours lipid production could however prove much more valuable, as a better understanding could help shaping environments which generally select for lipid accumulating microalgae.

7.6. NUTRIENT LIMITATION STRATEGIES TO OBTAIN LIPID PRODUCTION

A conclusion from the above section is that limitation of other nutrients than nitrogen can be considered if a nutrient limitation approach for selecting lipid accumulating microalgae is pursued. Preferably the limiting nutrient should be essential to all microalgal lineages and should not be stored intracellular to force the microalgae to execute all growth processes in the dark period. In line with this, the cell cycle should immediately be arrested upon limitation, yet the limitation should not have a detrimental effect on overall cellular fitness. From a practical point of view a macronutrient, which can be easily dosed and monitored, would be preferable. Table 7.2 list elements which can possibly be

Table 7.2: Advantages and disadvantages of several nutrient limitation strategies

Nutrient	Advantages	Disadvantages
C	Amount of light per C possibly influences carbon allocation	Limitation of carbon will decreases rate of photosynthesis
N	Most abundant element after C, easily dosed and monitored, ample used in literature	Can be stored intracellular, leads to carbohydrate production
P	Abundant cofactor in carbohydrate synthesis, where CoA is more used in lipid metabolism	Can be stored as polyphosphate [1], essential to energy metabolism
Si	Lead to cell cycle arrest and lipid production	Only essential for diatoms
Ca	-	In large quantities only essential for coccolithophores
Mg	Essential for photosynthesis, possible enriches algae with high photosynthetic efficiency	Essential to ATP, RNA and DNA metabolism. Possible detrimental effect on photosynthesis
S	-	Difficult to dose as low amounts are needed, used to trigger carbohydrate production
Fe	-	Difficult to dose as low amounts are needed, can be stored as ferritin in diatoms [71]

limited.

For a culture of diatoms, silicate limitation in the light phase would be preferred. As stated in the above section, a double limitation of silicate and a nutrient essential for all microalgal lineages might sustain diatom dominance. For green microalgae magnesium limitation might be worthwhile to try. Magnesium is the central atom in chlorophyll and magnesium limitation could possibly select for microalgae with a relatively high photosynthetic efficiency. On the other hand, overall cellular fitness will likely decrease when magnesium is limited.

Even if lipid production is observed using a nutrient-limitation strategy, a more profound understanding of the ecological reasons for lipid production under these conditions is of paramount importance, as it will be the only way to assess the risk of a contaminant or naturally risen mutant producing carbohydrates rather than lipids to appear.

7.7. OTHER STRATEGIES TO OBTAIN LIPID PRODUCTION

THE exact role of lipids in microalgae is still under debate. Lipids are linked to energy- and carbon storage, but could also play additional roles in microalgae. An increased ecological understanding of these roles could open up approaches to enrich lipid producing microalgae, not based on the ecological role of carbon- and energy storage.

Possible ecological roles of lipid production include electron scavenging and protection of the photosystem [64]. Electrons produced in photosynthesis are not consumed

by cell division processes under adverse growth conditions. Other electron sinks have to be found to prevent over-reduction of the electron transport chain. Lipids are the most reduced carbon compounds and lipid production can therefore function as electron sink. An environment with a high light to CO_2 ratio would possible require a lot of electron scavenging and hence select lipid producing microalgae.

Membranes and other parts of the photosynthetic machinery are lipid soluble and can therefore be stored in lipid bodies during prolonged periods of nutrient starvation. An environment exploiting the protection of the photosynthetic machinery in lipid bodies could possibly consist of a nutrient limited period under normal light, followed by a nutrient limited period under high light and finally a period with normal light and nutrients. If lipids are produced in the first light period, part of the photosynthetic machinery can be stored in the second light period in these lipid bodies, which would allow a quick recovery under normal light conditions, and hence quick access to the nutrients, in the third light period.

7.8. METRICS FOR REPORTING MICROALGAL PERFORMANCE

IN the experiments presented in this thesis storage compound contents of around 60% on organic matter base were reached after every light period in steady state. These values are in line with the highest values reported in literature [13]. Although high values of internal storage compounds are important for downstream processing, these values do not tell the complete story. An obvious parameter missing is the time needed to achieve this carbohydrate content. If this would be taken in account and a volumetric rate would be reported, this value would be a function of light input, reactor design, CO_2-concentration and other operational parameters. These factors differ considerable in experiments, indicating the need to report data in units which allow comparison.

Microbial systems can be characterised in chemotrophic cultivation by biomass specific q-rates (mol product C-mol biomass^{-1} time^{-1}) and product yields (mol product mol substrate^{-1}). In the microalgal field there is an increased awareness of the need to report productivities [43] but q-rates and product yields (mol lipid mol photons^{-1}) are scarce. This can partly be explained by the fact that measuring the amount of light consumed by microalgae is not straightforward. The cylindrical reactors used in the research presented in this thesis are designed for chemotrophic cultivation and do not allow proper measurements on the amount of light energy consumed by the microalgal culture.

In the end parameters as (mol lipids mol photons^{-1}) and (mol lipids euro^{-1}) will determine the feasibility of microalgal cultivation. Research on different cultivation strategies and reactor design should in the end provide these parameters.

7.9. OTHER DESIRED CHARACTERISTICS

THE application of selective environments to obtain and sustain a characteristic is not limited to production of energetic storage compounds. Several authors have listed microalgal characteristics desirable for large-scale microalgal cultivation [11] [107]. If the ecological role of displaying a characteristic is understood and if this knowledge allows the coupling between displaying this characteristic and an increased chance of survival,

it should theoretically be possible to enrich a culture with this characteristic.

A straightforward example is the need to cultivate under marine conditions and consequently the need for marine microalgae which can be enriched under marine conditions. Creation of a selective environment for some other characteristics will however not be so straightforward. Consider for example the characteristic of having less light harvesting antenna complexes. As microalgae absorb more light energy than they actually consume under high light conditions, less antenna molecules per microalgae would allow higher biomass concentrations and lipid productivities [74] Shaping an environment in which it is beneficial for microalga to leave more light to other microalgae, might prove difficult to construct. For such characteristics, genetic engineering strategies [79] could prove more fruitful than approaches based on selective environments.

7.10. APPLICABILITY OF SELECTIVE ENVIRONMENTS FOR LARGE-SCALE MICROALGAL CULTIVATION

ONE of the challenges in large-scale microalgal cultivation is to maintain the desired species and its functionality in time [11] [14]. As discussed in Chapter 2 the use of selective environments prevents competition by unwanted microalgae and diminish the loss of the desired functionality through strain degeneration.

In the experiments described in this thesis, the microalgal culture and productivity was highly stable in time. Typical experiments ran for weeks to months and no culture instabilities were observed in this time. It should be noted that uncoupling of carbon fixation in the light and nitrogen uptake in the dark will, in theory, not prevent the growth of nitrogen-fixating cyanobacteria. In the experiments presented in this thesis nitrogen fixation cyanobacteria were never present in substantial amounts (<1% of the culture), unless the renewal rate of the system was lower than approximately 25% of the volume per 24 hours. The imposed minimal growth rate by removing >25% of the culture per day proved to be an effective strategy to diminish nitrogen-fixating cyanobacteria. The same holds true for predation by protozoa, which was virtually non-existent at volume exchange ratio's exceeding 25 volume percent per day. Bacterial competition for the N-source was in the experiments with NH_4^+ oxidation inhibited by dosing allylthiourea, as it selectively inhibits nitrifying bacteria. If nitrate is used as nitrogen source in large-scale systems, there is no reason to dose allylthiourea.

For now we can conclude that lab-scale microalgal cultivation under selective cultivation conditions led to a stable process. If this also holds true at larger scale remains to be tested.

7.11. APPLICATION OF SELECTIVE ENVIRONMENTS FOR MUTANT SCREENING

AN approach to gain insight in a certain microalgal characteristic is by creating mutants lacking or overexpressing this characteristic. Creation of mutants can relatively easy be achieved by for example exposing a culture to UV-radiation, screening of mutants is however time-consuming. Most screening-methodologies rely on pure culture cultivation of every mutant and assessing the characteristic of interest expressed by this mutant, as

done for example in [26]. Another option is to screen single cells using flow cell sorting techniques [17].

The enrichment approach described in this thesis deals with mixed cultures and the characteristic of interest is assessed by enrichment under selective process conditions. This approach can therefore also be used to screen mutants. A major advantage of mutant screening using selective environments is that selection directly takes place on the characteristic of interest. If a mutant with an increased carbohydrate productivity is to be selected the only difference with the method described in Chapter 3 is that the starting culture will consist of millions of mutants rather than a mixed natural culture. The mutant that will dominate the system after enrichment can likely yield information on carbohydrate production in microalgae.

7.12. CONCLUDING REMARKS

THE above sections provide a wealth of possible research directions involving selective environments in microalgal cultivation. This calls to mind Beijerinck's quote: '*Gelukkig zij die nu beginnen*' ('*Fortunate are those who start now*').

Although the directions of possible further research differ, a common approach is suggested for all of them. If we are interested in a certain microalgal characteristic, we should reward the microalgae for displaying this characteristic. The best reward in nature is an increased chance of survival and reproduction. The task therefore becomes to couple the desired characteristic to an increased survival potential. This will be straightforward for some characteristics and thought-provoking for others. In any case, clues for creating an environment can be deducted from the ecological role of the desired characteristic.

Microalgae have shown in history to possess the ability to induce major changes on Earth. As mankind is currently in need for a revolution in its food and energy supply chains, it is not more than logical to rely on the organisms that have an excellent track record in causing global changes. Microalgae are fully equipped to play a key role in a biobased society. It is up to science, industry and politics to realise this potential.

A

Appendix A

Table A.1: Enrichment conditions of cultures described in this thesis

Chapter	**N dosed at start**	**T**	**Media**	**Light input**	**Cycle Length**	**[N] in Media**	**Reactor Volume**	**Effluent Volume**	**Light period**	**Dark Period**	**SRT**	**N dosed**	**N source**
-	-	*°C*	-	*µmol* m^{-2} s^{-1}	*hours*	*mg N/L*	*mL*	*mL*	*hours*	*hours*	*hours*	*mg N / L / cycle*	-
3	night	25	fresh	800	24	10	1320	950	8	16	33.3	7.2	NH_4
4	night	25	marine	650	24	20	1200	400	8	16	72	6.7	NH_4
4	day	25	marine	650	24	20	1200	400	8	16	72	6.7	NH_4
5	night	25	fresh	650	24	20	1200	700	16	8	41	11.7	NO_3
5	night	25	fresh+silica	650	24	20	1200	700	16	8	41	11.7	NO_3
6	night	25	fresh+silica	650	24	20	1200	700	8	16	38.4	12.5	NH_4
6	day	25	fresh+silica	650	24	20	1200	700	8	16	38.4	12.5	NH_4

Table A.2: Properties of the enriched cultures described in this thesis in steady state

Chapter	N dosed at start	Dominant Alga	Carbon partitioning			Lipid : Carb production ratio	CO_2-fixation rate	Storage after light phase	Yield C / N in dark	Change in light period			Change in dark period		
			Carbs	Lipids	Biomass					Carbs	Lipids	Biomass	Carbs	Lipids	Biomass
-	-	-	mg C / mg C fixed			*mg lipids / mg glucose*	*mg C / L / h*	*% of VSS*	*mg C / mg N*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*	*mg/L*
3	night	*C.luteoviridis*, green	0.62	0.06	0.32	0.05	21.9	60	-4.1	272	14	100	-69	-2	41
4	night	*C.reinhardtii*, marine green	0.78	0.14	0.08	0.10	12.3	56	-6.9	192	19	13	-105	-5	65
4	day	*C.reinhardtii*, marine green	0.47	0.07	0.46	0.08	11.7	50	-	110	9	76	-73	-1	-12
5	night	*C.reinhardtii*, green	0.55	0.05	0.39	0.05	18.7	54	-4.9	413	22	209	-141	-1	40
5	night	*N.frustulum*, diatom	0.44	0.13	0.43	0.16	18.8	49	-4.4	329	53	230	-112	-10	37
6	night	*N.palea*, diatom	0.68	0.39	-0.07	0.30	20.0	67	-4.8	273	83	-20	-100	-27	133
6	day	*N.palea*, diatom	0.24	0.13	0.63	0.30	16.4	36	-	79	23	147	-49	-5	-9

B

APPENDIX B

B

THE YIELD OF BIOMASS STORED GLUCOSE-POLYMERS OR LIPIDS

IF all nutrients for growth are present under dark conditions microalgae can execute all division processes in the dark at the expense of glucose polymers or lipids stored during light conditions. This section calculates the difference in yield between producing biomass from sugar polymers or from lipids.

BIOMASS PRODUCTION FROM CARBOHYDRATES

The following procedure is followed to calculate the biomass yield on starch. Starch is used as model for carbohydrates. First, the anabolic reaction to produce 1 C-mol biomass from starch is given. The catabolic reaction to yield ATP from starch is given afterwards. Based on the ATP requirement for microalgal biomass formation from literature a metabolic yield can be calculated.

Anabolism

$0.465\ H_2O + 0.175\ CO_2 + 0.12\ H^+ - 0.12\ NH_4^+ + 1\ CH_{1.78}O_{0.36}N_{0.12} - 0.196\ C_6H_{12}O_6$

Yield of X on glucose: 0.85 C-mol X / C-mol glucose
Yield of glucose on X: 1.175 C-mol glucose / C-mol X

Catabolism

$-1\ \text{starch}(n+1) + 1\ \text{starch}(n) - 6\ O_2 + 6\ CO_2 + 14\ H_2O + 33\ ATP$

Yield of ATP on starch: 5.5 mol ATP / C-mol starch

ATP requirement for biomass formation from biopolymers

A value of 18.9 mol ATP / N-mol X has been reported [63]. Using a measured elemental biomass composition of $CH_{1.62}O_{0.41}N_{0.14}P_{0.01}S_{0.003}$ this translates to 2.65 mol ATP / C-mol X. If starch is used as C-source an additional 2.65 / 5.5 = 0.48 C-mol starch / C-mol X needs to be catabolised to obtain the energy needed for anabolism.

Metabolism

The expected metabolic yield of biomass on starch is the sum of the anabolic and catabolic yield and equals 1.175 + 0.48 = 1.65 C-mol starch / C-mol X or 0.60 C-mol X / C-mol starch.

BIOMASS PRODUCTION FROM LIPIDS

The same procedure as described for starch is followed to obtain a metabolic yield of biomass on lipids. The TAG $C_{51}H_{104}O_9$ is used as model for lipids.

Anabolism

$-0.184\ H_2O + 1.106\ H^+ - 0.12\ NH_4^+ + 1\ CH_{1.78}O_{0.36}N_{0.12} - 0.020\ C_{51}H_{104}O_9 + 0.99\ e^-$

As the degree of reduction of a C-mol TAG is higher than the degree of reduction of a C-mol biomass, electrons are produced in anabolism.

Yield of X on TAG: 1 C-mol X / C-mol TAG
Yield TAG on X: 1 C-mol TAG / C-mol X

0.99 mol electrons / C-mol X are produced, this equals 0.493 mol reduction equivalents per C-mol biomass. Assuming a P/O ratio of 2.5 mol ATP / mol reduction equivalent, this would yield: 1.23 mol ATP / C-mol X. Therefore 1.23 mol ATP / C-mol TAG is already produced in anabolism.

Catabolism
-1 TAG + 1 glycerol - 69 O_2 + 48 CO_2 + 138 H_2O + 325 ATP

Yield of ATP on TAG: 6.37 mol ATP / C-mol TAG

ATP requirement for biomass formation from biopolymers
A value of 18.9 mol ATP / N-mol X has been reported [63]. Using a measured elemental biomass composition of $CH_{1.62}O_{0.41}N_{0.14}P_{0.01}S_{0.003}$ this translates to 2.65 mol ATP / C-mol X. As ATP has already been produced in anabolism if TAG is the C-source the ATP requirement for biomass formation on TAG is actually 2.65 – 1.23 = 1.42 mol ATP / C-mol X. If TAG is C-source an additional 1.42 / 6.37 = 0.22 C-mol TAG / C-mol X is needed.

Metabolism
The expected yield on TAG becomes 1 + 0.22 = 1.22 C-mol lipids / C-mol X or 0.82 C-mol X / C-mol lipids.

The obtained metabolic yields of biomass on lipids (0.82 C-mol X / C-mol lipids) is higher than the obtained metabolic yield of biomass on starch (0.60 C-mol X / C-mol starch). Lipids are therefore, on a C-mol basis, a more preferable storage compound, if all biomass formation processes take place in dark conditions.
The density of starch granules is 1.5 g/cm3 [15], whereas the density of lipids is around 1 g / cm3 [54]. If cell volume is therefore the limiting factor in the production of storage compounds, the 50% higher density of starch would outweigh the 30% lower biomass yield.

AMINO ACIDS FROM GLUCOSE POLYMERS

Limiting nitrogen to the dark phase of a light/dark cycle could result in microalgae producing amino-acids in the dark period while production of all other cell components takes place in the light period. The yield of amino-acid production on stored glucose polymers is calculated to asses if the observed yield of consumed storage compounds / consumed nitrogen in the dark and the theoretical yield of amino acid production from glucose polymers are comparable. It is assumed that glutamate is the final product in the dark period, starch is used as model for glucose-polymers. The reaction for producing 1 mol of glutamate from starch is the following:

- 1 Starch(n+1) - 1 ADP - 4 NAD - 1 NAPDH - 1 NH_4^+ + 1 Starch(n) + 1 Glutamate + 1 CO_2 + 1 ATP + 4 NADH + 1 NADP

This gives a yield of 0.83 C-mol glutamate / C-mol starch. In the experiments in this thesis the amount of storage compounds in mg C invested per mg N consumed is used. The theoretical yield calculated in this section would expressed in these units be 5.14 mg C / mg N for glucose polymers.

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Mario (maa-rie-joo) *m* • 1.korrelkoning

Marco (mar-koo) *m* • 1. totalitarian head of kitchen

Mark (mark) *m* • 1. sympathieke leermeester met bijzonder snelle geest 2. schenker van vertrouwen in PhD's 3. verschaffer van inzicht dat de microbiële wereld praktisch onontgonnen terrein is 4. formuleur van oneindige hoeveelheid vragen uit eindige hoeveelheid data 5. potentieel Nobel prijs winnaar 6. schepper van licht anarchistische werkomgeving waarin werknemers zeer veel vrijheid genieten 7. immer bereikbaar, met name op momenten ('s nachts van 03:00-07:00, op zon- en feestdagen) dat andere mensen niet bereikbaar zijn

Matthijs (ma-tijs) *m* • 1. archetype Opel Meriva rijder 2. korte broeken liefhebber

Mervin (mer-fin) *m* • 1. goedlachse GC commandant

Miranda (mi-ran-da) *v* • 1. uitermate vriendelijke administratieve ruggengraat van EBT

Mitchell (mit-sjul) *m* • 1. kroonprins van de moleculaire techniek 2. eigenlijk leider van EBT 3. vaak jarig 4. ijzersterk duo met Ben 5. ringtone-connaisseur ◦ *~, ~, hoe vind je deze klinken dan?*

Monkey's & Typewriters (mon-kies en taip-rai-ters) *m* • 1. onduidelijke organisatie-structuur waarin vriendschap voortgezet gaat worden 2. schrik der statistici 3. opstellers van notulen tijdens essentiele evenementen

Pap en Mam (pap en mam) *m* & *v* • 1. liefdevolle scheppers van fantastische jeugd 2. stimuleren en mogelijk maken van 30 jaar leren

promotiecommissie (proo-moo-tsie coh-mi-sie) *m/v* • 1. groep hooggeleerde dames en heren, bijvoorbeeld bestaande uit Alison Smith, Jean-Philippe Steyer, Ellen van Donk, Maria Barbosa, Sef Heijnen, Jack Pronk, Mark van Loosdrecht en Robbert Kleerebezem die bereid zijn dit proefschrift ter discussie te stellen

Renco (ren-koo) *m* • 1. ombouwer van Spaans witgoed tot algenkweekapparatuur

Richard (rie-sjard) *m* • 1. niet vies (schoon) van slechte woordgrappen

Ritsert (rits-urt) *m* • 1. man die dingen kan; zoals lassen, oceanen overzeilen en kippenhokken APK gekeurd krijgen 2. avondvullend programma 3. BN'er in wording

Rob (rob) *m* • 1. aanprijzer van de Masterflex Multitool 2. stevige doorstapper 3. onderdeel van bekend duo 'Rob en Dirk'

Ron (ron) *m* • 1. meedenkende geldbeheerder

Robbert (ro-beurt) *m* • 1. uitmuntend intercontinentaal reisgezel 2. liefhebber van allerhande formulieren 3. immer bereikbare begeleider met scherpe geest 4.

de Bezem 5. pleitbezorger van het idee dat elk systeem doorgrond kan worden, mits de balansen sluiten. 6. bijzonder sympatiek figuur

Sander (san-dur) *m* • 1. sociale magneet die na het verzamelen van te veel vrienden van stad, land danwel continent wisselt 2. wars van tradities 3. bekende Chileen in wording 4. jongere / oudere broer 5. combinatie van joie de vivre en je ne sais quoi ◦ *Die krant moet NU naar mevrouw Splint ~!*

Sanne (sa-nu) *v* • 1. onverschrokken algenvangster, het wilde algenmeisje 2. chef van de jacht op het dikke stokje 2. schrijfster van originele eindverslagen

Sef (sef) *m* • 1. zeer gerespecteerd professor 2. in staat om microbiële, of andere wereldproblemen, samen te vatten en op te lossen op de achterkant van een bierviltje

Shell (sjel) *m* • 1. multinational die met angst en beven de opkomst van Edje gadeslaat

Soldaat Tamis (sol-daat taa-mis) *m* • zie Jelmer

Sjaak (sjaak) *m* • 1. godfather van BT 2. cement van het Kluyver

Steef (steef) *m* • 1. grondlegger hedendaags algenonderzoek in het Kluyver 2. illuster figuur, zorgt altijd voor leven in de brouwerij 3. wormenmenner 4. munter van de term 'Survival of the Fattest'

Susanne (suu-sa-nu) *v* • 1. verzorger van het bouwen van zonnecellen op zonnige festivals 2. mevrouw donder

Team Algae (tiem el-djie) *m/v* • 1. wisselend team met vast doel: groene wereldheerschappij

Tommaso (to-ma-soo) *m* • 1. enthusiastic person exclaiming 'Longi!' while displaying a warm smile 2. gifted trombone player

Udo (uu-doo) *m* • 1. man die liefdevol over vissen, kippen, honden, mountainbikes en andere gadgets praat 2. sympathieke brompot 3. behendig technician 4. boeker van christelijke bezinningsoorden

Vrienden (vrien-dun) *m/v* • 1. diverse groep aapachtigen waarmee allerhande activiteiten anders dan het kweken van algen, zoals bijvoorbeeld droogvallen, pizza bakken, surfen, vakantie vieren en muziek maken, te ondernemen is.

Werkplaats (werk-plaats) *m* • 1. één van de beste plekken van het Kluyver om rond te snuffelen 2. bemand door ondere andere Arno en Marcel 3. essentieel voor soepel lopende experimenten ◦ *Even de lagertjes smeren in de ~.*

Wolf (wo-luf) *m* • 1. alom gewaardeerd gebruikers-commissie lid 2. malende professor danwel hooglerende molenaar 3. samen met Georgette samenbrenger der neven en nichten 4. kan qua praten en whatsappen 'met de beste mee'

Wouter (wou-tur) *m* • 1. ouwe fijnproevert 2. Beckmann 3. fashion-icon

Yang (jang) *m* • 1. excellent driver 2. formidable roommate that is always willing to help 3. yang-jayo

Yara (yaa-raa) *v* • 1. bondgenoot van het 'jongste kind front' 2. design fenomeen

CURRICULUM VITÆ

Peter Rudolf MOOIJ

Peter Rudolf Mooij was born on April 14 1985 in Haarlem, the Netherlands. In 2004 he started his bachelor study Life Science and Technology at the Delft University of Technology in Delft, the Netherlands. In 2010 he finished the master-programme Cell Factory at the Delft University of Technology. In April 2011 he started his PhD-research on storage compound production by microalgae in the Environmental Biotechnology Section of the Delft University of Technology under the guidance of Robbert Kleerebezem and Mark C.M. van Loosdrecht.

In 2007 Peter co-founded with four friends the sustainable surf-, skate-, and music-festival MadNes on Ameland.

From 2014 on Peter is member of the Royal Dutch Academy of Sciences' Faces of Sciences program, for which he blogs about his research-activities.

List of Publications

Journal articles

5. **P.R. Mooij**, Chris M. Vermeer, Mark C.M. van Loosdrecht and Robbert Kleerebezem, *Enrichment culture for storage compound producing diatoms*, manuscript under review

4. **P.R. Mooij**, Lisanne D. de Jongh, Mark C.M. van Loosdrecht and Robbert Kleerebezem, *Influence of silicate on enrichment of highly productive microalgae from a mixed culture*, Journal of Applied Phycology (2015)

3. **P.R. Mooij**, Gerben R. Stouten, Mark C.M. van Loosdrecht and Robbert Kleerebezem, *Ecology-based selective environments as solution to contamination in microalgal cultivation*, Current Opinion in Biotechnology **33**, 46-51 (2015)

2. **P.R. Mooij**, Danny R. de Graaff, Mark C.M. van Loosdrecht and Robbert Kleerebezem, *Starch productivity in cyclically operated photobioreactors with marine microalgae—effect of ammonium addition regime and volume exchange ratio'*, Journal of Applied Phycology **27**, 1121-1126 (2015)

1. **P.R. Mooij**, Gerben R. Stouten, Jelmer Tamis, Mark C.M. van Loosdrecht and Robbert Kleerebezem, *Survival of the Fattest*, Energy Environ. Sci. **6**, 3404-3406 (2013)

Selected oral presentations

5. **P.R. Mooij**, Mark C.M. van Loosdrecht and Robbert Kleerebezem, (2014) *Survival of the fattest – A microbial community approach to select for highly productive microalgae*, Netherlands Biotechnology Congress, Ede, the Netherlands (invited)

4. **P.R. Mooij**, Mark C.M. van Loosdrecht and Robbert Kleerebezem, (2014) *A microbial community approach to select for highly productive microalgae*, Department of Plant Sciences, University of Cambridge, Cambridge, UK(invited)

3. **P.R. Mooij**, Mark C.M. van Loosdrecht and Robbert Kleerebezem, (2014) *Enrichment and long-term stability of a community of storage compound producing microalgae*, YAS 2014, Narbonne, France

2. **P.R. Mooij**, Mark C.M. van Loosdrecht and Robbert Kleerebezem, (2013) *Survival of the fattest – A microbial community approach to select for highly productive microalgae*, Civil and Environmental Engineering, CalPoly, San Luis Obispo, USA (invited)

1. **P.R. Mooij**, Mark C.M. van Loosdrecht and Robbert Kleerebezem, (2012) *Survival of the Fattest*, YAS 2012, Wageningen, France

Other publications

1. **P.R. Mooij**, Chapter in *BWM cahier Algen*, Stichting Biowetenschappen en Maatschappij, Kennislink

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