



Phototrophic pigment production with microalgae

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Thesis

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Abstract

Microalgal pigments are regarded as natural alternatives for food colourants. To facilitate optimization of microalgae-based pigment production, this thesis aimed to obtain key insights in the pigment metabolism of phototrophic microalgae, with the main focus on secondary carotenoids. Different microalgal groups each possess their own set of primary pigments. Besides, a selected group of green algae (Chlorophytes) accumulate secondary pigments (secondary carotenoids) when exposed to oversaturating light conditions. In this thesis it was found for the first time that nutrient-depleted *Isochrysis* aff. *galbana* T-ISO (Haptophytes) accumulates 3-hydroxyechinenone, a precursor of astaxanthin. Besides, it was found that nitrogen-depleted *Chromochloris* (*Chlorella*) *zofingiensis* (Chlorophytes) accumulates astaxanthin, presumably synthesised via echinenone, and ketolutein. Inhibition of production of β -carotene derivatives (e.g. echinenone and astaxanthin) did not lead to increased production of primary carotenoids (e.g. lutein) or ketolutein in this species, suggesting that the regulatory mechanisms controlling the flux towards ketolutein and primary carotenoids are not affected by the decreased levels of β -carotene derivatives. Besides, optimal yields of secondary carotenoids and triacylglycerol (TAG) on light were reached with *C. zofingiensis* for a range of biomass concentrations at the moment of nitrogen depletion. This indicated that the biomass-specific photon absorption rate did not affect the amounts of energy used for secondary carotenoid and TAG production, for the range of biomass concentrations tested. It was also found that nitrogen-depleted *C. zofingiensis* resupplied with nitrogen hardly degraded astaxanthin, whereas the other major secondary metabolites were degraded rapidly. This indicated that the overall carotenoid yield on light as well as its content may possibly be improved by applying a repeated batch instead of a series of single batch cultivations, which are traditionally applied. Finally, it was discussed that the highest increases in carotenoid yield on light can be reached by optimizing strain performance (using targeted genetic engineering and/or random mutagenesis), rather than by optimizing the cultivation conditions/operation mode or reactor design.



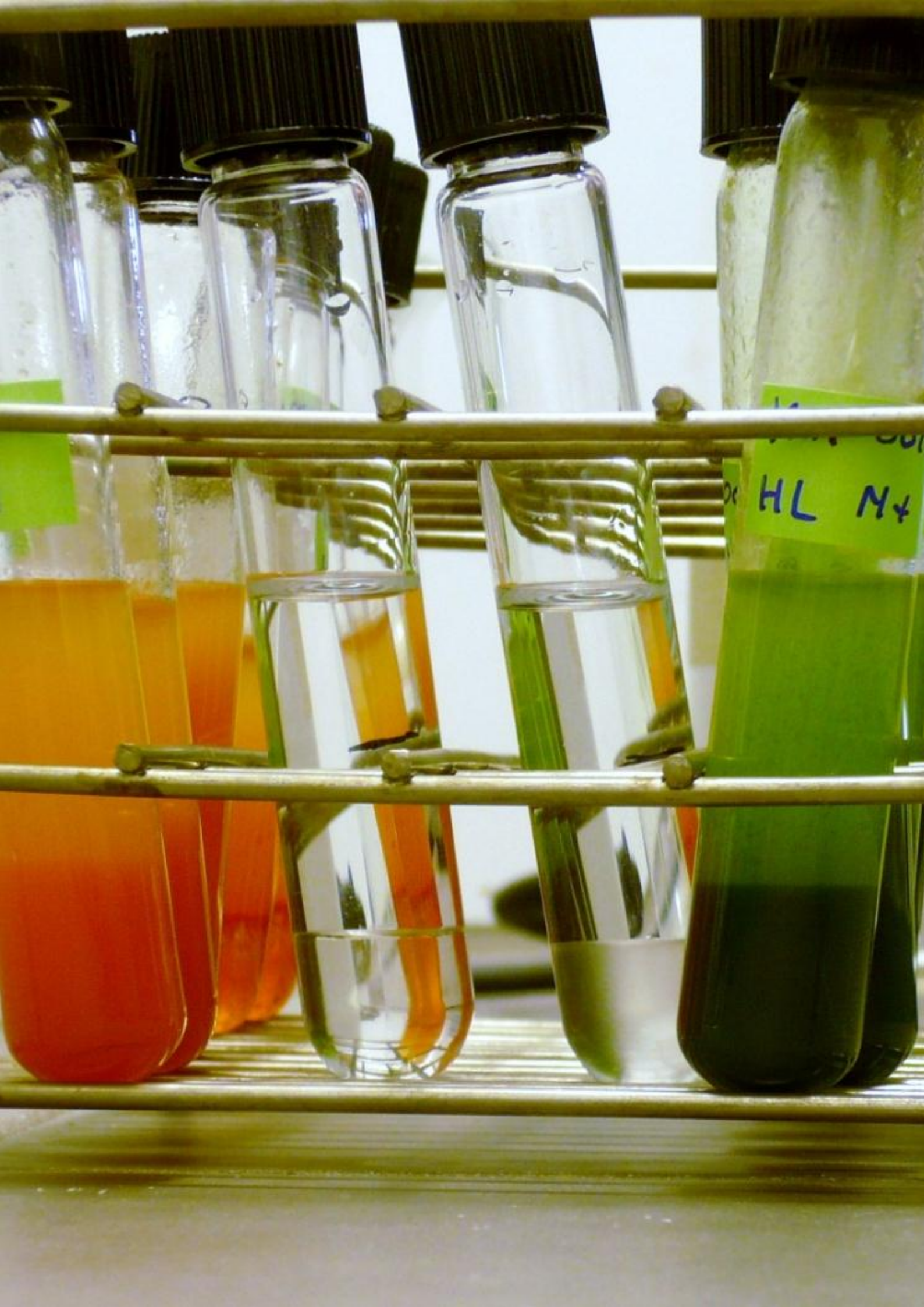
Photograph of the artwork

Pigments From Microalgae: An Ocean Of Colours

which is made of filter-dried nutrient-sufficient, nitrogen-depleted and nitrogen-resupplied *Chromochloris* (*Chlorella*) *zofingiensis* cells. The colours comprise mainly the pigments chlorophyll *a* and *b* (green), lutein (yellow), canthaxanthin (orange), ketolutein (orange) and astaxanthin (red).

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Chapter 1

Introduction and thesis outline

High lights

- The consumer demand for naturally produced pigments is rising.
- Microalgae possess a large variety of pigments and grow fast on few resources.
- For commercialization, optimizing the pigment production process is desirable.
- This requires insight in the algal pigment metabolism, as described in this thesis.

◀ IN THE PICTURE

To know very precisely which pigments and how much of each pigment is present in the orange and green coloured microalgae, the chlorophylls and carotenoids first need to be extracted. This occurs in glass tubes using a mixture of chloroform and methanol. After shaking and centrifugating the cells, the pigments end up in the chloroform (bottom) phase, separated from the cell debris.

◀ IN BEELD

Om heel precies te weten welke pigmenten en hoeveel van ieder pigment aanwezig is in de oranje en groen uitziende microalgen moeten de carotenoïden en chlorofylen eerste uit de cel gehaald worden. Dit gebeurt in reageerbuizen met behulp van chloroform en methanol. Na flink schudden en centrifugeren eindigen de pigmenten in de (onderste) chloroform fase, gescheiden van de rest van de cel.

1.1 Microalgae as a source of natural pigments

Pigments are molecules that absorb part of the visual light spectrum. The part that is not absorbed is reflected and caught by the human eye, and hence determines their colour.

Pigments are used in a wide range of products, including food and cosmetics. Although many pigments can be produced synthetically (e.g. from petroleum, organic acids and inorganic chemicals) for a lower price than by natural production, the consumer demand for naturally produced pigments is rising (BCC Research 2008, Farré et al. 2010, Kobylewski and Jacobson 2010).

Pigments of natural origin can be obtained from insects (e.g. cochineals and aphids), vegetables, fruits, petals of flowers, or from microorganisms such as microalgae (Eriksen 2008; Farré et al. 2010; Mendez et al. 2004; Piccaglia 1998; Vilchez 2011). Microalgae are a biotechnologically interesting source of pigments because they possess a large variety of such molecules, including chlorophylls (green), carotenoids (red, orange and yellow) and phycobiliproteins (red and blue). Part of these pigments can be produced in concentrations exceeding those found in higher plants by one or more orders of magnitude (**Fig. 1.1**).

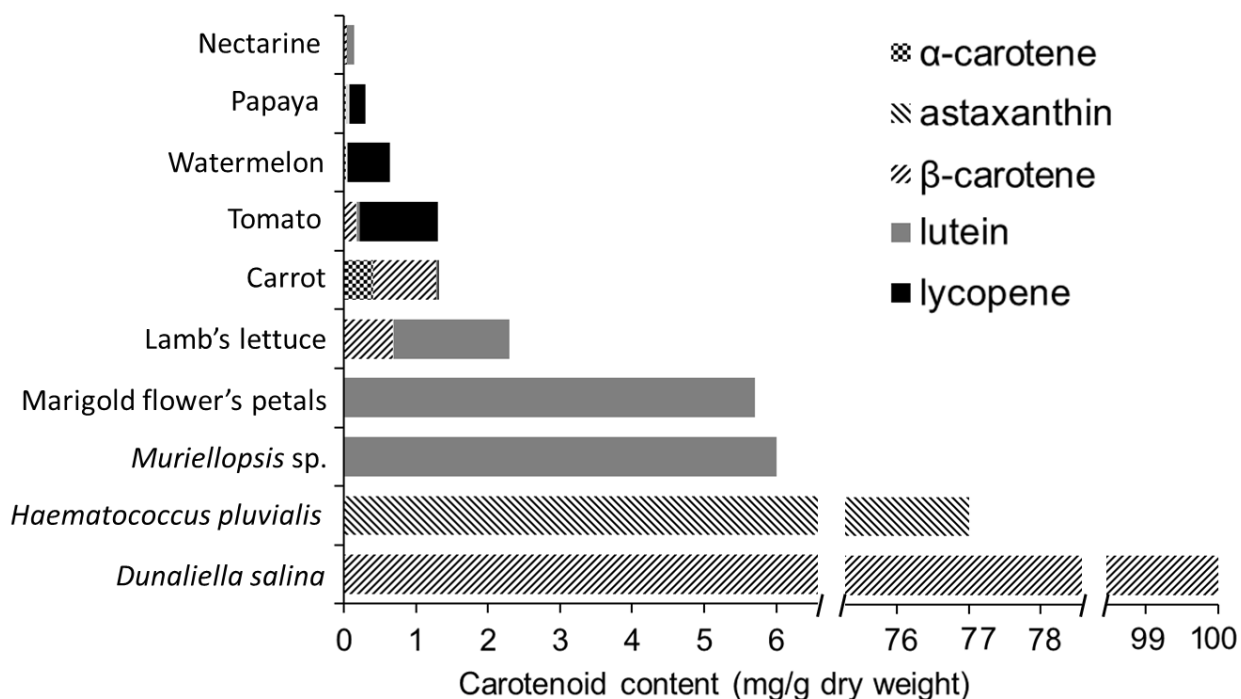


Fig. 1.1 Carotenoid contents (in mg/g dry weight) of raw carotenoid-rich fruits and vegetables, petals of marigold flowers and some microalgae (Del Campo et al. 2007; Lamers et al. 2008; NEVO 2001; Piccaglia et al. 1998)

Besides, in contrast to plants, very high areal productivities can be achieved with microalgae, which can be obtained without the use of arable land (Del Campo et al. 2001, 2007; Fernández-Sevilla et al 2010; Lamers et al. 2008). In addition, as many algal species are salt tolerant (Ben-Amotz et al. 1982), seawater can often be used for their cultivation. Using seawater is more sustainable than using freshwater and may in addition be cheaper. Furthermore, microalgae can simultaneously produce other high-value compounds, such as long-chain polyunsaturated fatty acids, vitamin E and C, and bulk compounds such as starch,

proteins and lipids (Abe et al. 1999; Borowitzka 2013). The lipid triacylglycerol (TAG) is currently of particular interest, because it is a promising alternative source for vegetable oils and next generation biofuels (Chisti 2013; Draaisma et al. 2013; Hu et al. 2008).

Despite all these advantages of using microalgae for pigment production, and despite the potential to produce a large variety of pigments, microalgae are currently exploited commercially for only three pigments, namely β -carotene (*Dunaliella salina*), astaxanthin (*Haematococcus pluvialis*) and phycocyanin (*Arthrospira platensis*). The carotenoids β -carotene and astaxanthin are claimed to have beneficial health effects, due to their anti-oxidative activity (Spolaore et al. 2006; Harari et al. 2013), which makes them particularly valuable for application in nutraceuticals. Phycocyanobilin, on the other hand, is used mainly in food applications. The reason that only those three pigments are currently exploited commercially is that the production costs of microalgal pigments are generally too high. In the cases of the aforementioned pigments that are already being commercialized, however, the market values and obtained yields are sufficiently high to lead to economically feasible microalgae-based production. However, to increase the market volumes of the mentioned carotenoids (i.e. by expanding exploitation of the food and feed markets), also their production costs need to be reduced.

The pigment production costs are mainly determined by the costs for the cultivation (e.g. resources such as CO₂, water and nutrients, labour, reactor investments and power consumption), harvesting (e.g. equipment, power consumption and labour) and downstream processing (e.g. equipment, power consumption, chemicals and labour). One of the most important factors are the investment costs for photobioreactors (Acien et al. 2012; Norsker et al. 2010), which means that an increased pigment yield on light will substantially reduce the pigment production costs. Namely, outdoor photobioreactor arrays capture a given amount of solar insolation, dependent on their geographical location and design. Consequently, the carotenoid yield on light is directly related to the financial return on investment costs.

To obtain maximal pigment yields on light, insight into optimal cultivation conditions, as well as into the pigment metabolic pathways and their regulation is indispensable. Optimal cultivation conditions were studied, for example, by Zemke et al. (2013), who assessed the key biological and engineering design parameters for production of a carotenogenic microalga (*Chromochloris zofingiensis* (used to be called *Chlorella zofingiensis* until recently (Fucíková and Lewis, 2012))) in outdoor photobioreactors. This work led to the conclusion that the biomass concentration at the start of the pigment production phase greatly influences the pigment yield on light (up to a factor four).

Fundamental discoveries regarding the pigment metabolic pathways and their regulation include the findings that in *H. pluvialis* β -carotene must be a precursor of astaxanthin and that upregulation of β -carotene ketolase, one of the most important carotenogenic genes, is dependent on *de novo* protein synthesis (Collins et al. 2011; Vidhyavathi et al. 2008). Others found that in *D. salina* β -carotene is accumulated in chloroplastidic oil bodies only, which originate partly from chloroplast membrane lipids and partly from oil bodies accumulated in the cytosol (Davidi et al. 2014). These fundamental insights are in particular of key importance for the development of metabolic engineering strategies to increase the carotenoid yield on light.

Despite these important findings, many questions remain and truly substantial increases in the pigment yield on light are not yet achieved. Therefore, the aim of the work presented in this thesis was to obtain increased insight in the pigment metabolism of phototrophic microalgae under various process conditions, with the main focus on secondary carotenoids, to facilitate optimization of phototrophic microalgae-based pigment production.

1.2 Thesis outline

To achieve this aim, first the biological constraints and opportunities of microalgal carotenoid production were assessed, based on existing knowledge. Subsequently, the carotenoid metabolism of the haptophyte *Isochrysis* aff. *galbana* T-ISO was studied, after which the focus was shifted to the carotenogenic chlorophyte *Chromochloris* (*Chlorella*) *zofingiensis*. For this latter species the carotenoid metabolism was studied in depth, optimal cultivation strategies for carotenoid production were determined and the dynamics and exploitability of its recovery from nitrogen starvation were explored. Finally, this thesis concludes with an outlook on several strain and process improvements to achieve higher carotenoid yields on light.

To enable optimization of an economically feasible microalgae-based pigment production process, an adequate understanding of the biological constraints and opportunities is important. **Chapter 2** identifies these biological constraints and opportunities, and provides an overview of the relevant underlying biological information that was known at the start of this project (in 2010), including the distribution of pigments across the most common microalgal groups, the roles of pigments in microalgae and their biosynthetic pathways.

Moreover, it is discussed that microalgal pigments can be categorized into two groups: primary and secondary pigments. Overproduction of primary pigments requires modification of the pigment biosynthesis routes. Some secondary pigments can be overproduced by optimization of the cultivation conditions (usually referred to as 'stress' conditions), while others require also modification of the pigment biosynthesis routes (e.g. by the use of inhibitors or genetic modification).

This chapter concludes with the most suitable approaches for the production of each colour that can be obtained from microalgae (red, red-orange, orange, yellow-orange, yellow, green and blue).

Subsequently, in **Chapter 3**, the carotenoid metabolism in the haptophyte *I. aff. galbana* T-ISO was studied. *I. aff. galbana* T-ISO is widely cultured for the bivalve aquaculture industry, because it produces ω -3 fatty acids that have a high nutritional value. However, this strain is also of interest because it produces high amounts of the carotenoid fucoxanthin. When cultivated under nutrient depleted conditions all enzymes are activated to produce astaxanthin, another pigment that is of interest to the aquaculture industry. Besides, under these conditions large amounts of storage lipids (TAG) are accumulated, a prerequisite for the overproduction of carotenoids. Together, this makes *I. aff. galbana* T-ISO a potential candidate for overproduction of fucoxanthin, astaxanthin or other carotenoids. Therefore, in **Chapter 3**, *I. aff. galbana* T-ISO cultures were subjected to different nutrient depletions and their growth and pigment accumulation were studied. It was found that, although the nutrient depletions

led to the production of secondary carotenoids (echinenone and 3-hydroxyechinenone), no production of astaxanthin or overproduction of other carotenoids was induced.

Because *I. aff. galbana* T-ISO was not able to overproduce secondary carotenoids, the focus was thereafter shifted to the carotenogenic chlorophyte *C. zofingiensis*. This species may be a potential alternative for mass production of astaxanthin, thereby replacing *H. pluvialis*, because it is claimed to have a higher specific growth rate and higher biomass and TAG yield on light, and a lower sensitivity to contamination in unfavourable environments (Liu et al. 2014).

However, one of the disadvantages of *C. zofingiensis* is that the maximally observed secondary carotenoid content is relatively low (0.6 % DW (w/w) (Orosa et al. 2001), compared to 4 % DW (w/w) observed for *H. pluvialis* (Boussiba et al. 1999)). Increasing this content likely requires genetic modifications. Targeted genetic modifications require insight into the algal metabolism and in particular into the carotenoid metabolism, which can be obtained by performing enzyme inhibitor studies. Besides, using enzyme inhibitors, the effects of genetic knock-outs or knockdowns can be simulated, which yields insight in the potential of such genetic modifications.

Therefore, in **Chapter 4**, *C. zofingiensis* was depleted of nitrogen, which effectively induces secondary carotenogenesis, while diphenylamine (DPA) was added, an inhibitor of β -carotene ketolase (BKT). Because BKT catalyses multiple conversions in the secondary carotenoid pathway, including the first step of secondary carotenogenesis, this approach was expected to elucidate two issues. Firstly, it was expected to indicate those conversions in the secondary carotenoid pathway that play an important role in the production of astaxanthin, and secondly, it was expected to elucidate how inhibition of the secondary carotenoid metabolism affects the primary carotenoid metabolism.

As previously mentioned, TAG deposition is assumed to be a requirement for secondary carotenoid formation, because it would serve as metabolic sink or storage vehicle (Zhekisheva et al. 2005; Rabbani et al. 1998). Besides, because secondary carotenoid and TAG accumulation are induced by the same stimuli (i.e. oversaturating light conditions), a correlation in their accumulation dynamics is assumed (Zhekisheva et al. 2002). To obtain more insight in the relation between the secondary carotenoid and TAG metabolism in *C. zofingiensis*, in **Chapter 4**, freeze-fracture scanning electron micrographs were made of nitrogen replete and nitrogen-depleted cells, to reveal the location of TAG accumulation, whereas accumulation dynamics of both compounds were studied in the following chapter.

An important aspect of the cultivation strategy that affects the secondary carotenoid and TAG yield on light could be the biomass density at the start of the nitrogen starvation (and secondary carotenoid production) phase, as suggested by Zemke et al (2013). The hypothesis for this phenomenon is that under nitrogen starvation conditions an optimum biomass-specific light absorption rate exists, resulting in maximal secondary carotenoid and TAG yields on light. At higher biomass-specific light absorption rates the yield would decrease because the excess light would be dissipated to heat or lost through photoinhibition. At lower biomass-specific light absorption rates the yield would decrease because a relatively large share of the absorbed light would be used to satisfy the maintenance requirements of the cells. The studies by

Zemke et al. were performed under outdoor conditions, which makes it difficult to isolate the effect of biomass density from the effects of other cultivation conditions, such as incident light intensity and temperature.

Therefore, **Chapter 5** studied the effect of varying biomass densities at the start of the secondary carotenoid production phase, by starving *C. zoofingensis* of nitrogen at different biomass concentrations, under well-defined and highly controlled conditions.

It was found that, in the studied range, TAG and secondary carotenoids were produced with the same yield on absorbed light with each initial biomass density, which indicated that the biomass-specific photon absorption rate did not affect the amounts of energy used for TAG and secondary carotenoid production.

Besides, it was concluded that, although secondary carotenoid and TAG accumulation are induced by the same stimuli, a causal relationship between the accumulation of secondary carotenoid and TAG does not necessarily exist and that secondary carotenoid production is most likely highly regulated.

Another important aspect of the cultivation strategy that might affect the pigment yield on light is the mode of operation. It has been suggested in literature that the areal productivity of secondary carotenoids and TAG can be increased by applying a repeated batch cultivation instead of a series of single batch cultivations (which traditionally is applied). Whether this is indeed the case depends, among others, on the recovery rate of the culture and the dynamics of TAG and secondary carotenoids during culture recovery.

Information on these matters is not available in literature and therefore **Chapter 6** explores the dynamics and exploitability of *C. zoofingensis*' recovery from nitrogen starvation. To do that, the nitrogen-depleted *C. zoofingensis* cultures described in **Chapter 5** were (after a nitrogen starvation period of two weeks) replenished with nitrate after which the physiology and rate of culture recovery were studied.

The cultures recovered quickly (within two days after nitrogen resupply). Moreover, during these two days astaxanthin was hardly degraded. Therefore, it is suggested that repeated batch cultivation may indeed result in a higher secondary carotenoid yield on light and a higher content than a series of classical single batch cultivations.

Finally, in **Chapter 7**, the maximal potential for improvement of the carotenoid yield on light is discussed for astaxanthin, β -carotene and lutein, which are the commercially most relevant microalgal carotenoids (i.e. highest market values and volumes). This is done by assessing the theoretical maximal carotenoid yields on light and comparing these with the currently obtained yields outdoors and indoors in the laboratory. This chapter concludes with presenting three cases in which it is argued how, and to what extent, the gap between current carotenoid yields and the theoretical maxima can be closed. These cases include improvements to both the cultivation process and the production strains.

All these chapters together have resulted into increased insights in the cultivation strategies to maximize the pigment yield on light, as well as into the pigment metabolic pathways and their regulation. Most importantly, the most likely biosynthesis route to astaxanthin was ascertained in *C. zoofingensis* and it was found that inhibition of secondary carotenoids does

not lead to increased production of primary carotenoids in this species. These findings are particularly relevant for the development of metabolic engineering strategies with the aim to increase the astaxanthin, ketolutein or any primary carotenoid yield on light (**Chapter 4**). Another important finding was that this species has a broad optimum in secondary carotenoid yield on light with respect to the biomass concentration at the moment of nitrogen depletion. This implies that when this strain is cultivated outdoors, a relatively high biomass concentration can be used without a loss in carotenoid yield on light (**Chapter 5**). Finally, it was found that astaxanthin, the main secondary carotenoid in this species, was hardly degraded upon nitrogen resupply, which indicates that the overall carotenoid yield on light as well as its content may possibly be improved by applying a repeated batch instead of a series of single batch cultivations (**Chapter 6**). Thus, the new insights obtained will facilitate optimization of phototrophic microalgae-based pigment production (discussed in **chapter 7**).



Chapter 2

Phototrophic pigment production with microalgae: biological constraints and opportunities

High lights

- All phototrophic microalgae contain primary pigments; some make secondary pigments.
- Process conditions exclusively induce overproduction of secondary carotenoids.
- This phenomena has been ascertained only in specific green algae (Chlorophytes)
- Overproduction of primary pigments requires modifications of metabolic pathways.

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◀ IN THE PICTURE

Nutrient-replete (dark brown) and nutrient-depleted (light brown to yellow) *Isochrysis* aff. *galbana* T-ISO cells cultivated in 24-well plates. Each well contains one milliliter of cells. Due to this very small volume the effect of many different environmental factors can be tested at once. The growth and pigment production is measured using a plate reader. Convenient and time-efficient!

◀ IN BEELD

Isochrysis aff. *galbana* T-ISO cellen met voldoende (donker bruin) of een tekort aan voedingsstoffen (licht bruin tot geel) in 24-welsplaten. Ieder welletje bevat een milliliter aan cellen. Door dit kleine volume kan het effect van heel veel verschillende groei-factoren in één run bepaald worden. De groei en pigmentproductie wordt met een apparaat automatisch afgelezen. Praktisch en efficiënt!

Abstract

There is an increasing interest in naturally produced colorants. Microalgae are a biotechnologically interesting source, because they possess a wide range of coloured pigments, including chlorophylls (green), carotenoids (red, orange and yellow) and phycobiliproteins (red and blue). However, the concentration of these pigments, under optimal growth conditions, is often too low to make microalgae-based pigment production economically feasible. In some Chlorophyta (green algae), specific process conditions, such as oversaturating light intensities or a high salt concentration, induce the overproduction of secondary carotenoids (β -carotene in *Dunaliella salina* (Dunal) Teodoresco and astaxanthin in *Haematococcus pluvialis* (Flotow)). Overproduction of all other pigments (including lutein, fucoxanthin and phycocyanin) requires modification of gene expression or enzyme activity, most probably combined with the creation of storage space outside the photosystems. The success of such modification strategies depends on adequate understanding of the metabolic pathways and the functional roles of all pigments involved. In this overview, the distribution of commercially interesting pigments across the most common microalgae groups, the roles of these pigments *in vivo*, and their biosynthesis routes are reviewed, and constraints and opportunities for overproduction of both primary and secondary pigments are presented.

2.1 Introduction

To make food attractive for consumption, colorants are often added. These colorants can be produced artificially or naturally. The demand for natural colorants is rising, because artificial food colorants are increasingly associated with a number of health issues, such as ADHD (attention deficit hyperactivity disorder) and hyperactivity (Kobylewski and Jacobson 2010; Rowe and Rowe 1994; Schab and Trinh 2004). Current numbers, as well as future predictions of pigment annual production and sales volume are scarce in the public domain. However, the numbers available show an increase of the overall pigment sales volume, and for the pigments β -carotene and astaxanthin a shift is described from chemical to natural production (BCC Research 2008; Farré et al. 2010). Markou and Nerantzis (2013) estimated an annual hypothetical pigment production of 525 kt β -carotene, 525 kt astaxanthin, 87.5 kt lutein and 2625 kt phycobilins, based on an annual biomass production of 175 Mt of which 10 % would be used for each pigment, and a pigment content of 3 % β -carotene, 3 % astaxanthin, 0.5 % lutein and 15 % phycobilins. Natural food colorants can be obtained from vegetables and fruits, or they can be produced by microorganisms such as microalgae. Microalgae are a biotechnologically interesting source of natural colorants, since they (i) possess a wide range of pigments, (ii) can grow fast, (iii) in some cases contain pigments in concentrations which can considerably exceed the concentrations found in higher plants, and (iv) in many cases can grow in marine environments (Lamers et al. 2008; Wright and Jeffrey 2006).

Microalgal pigment production requires a production strain and an accompanying production process. To choose a suitable production strain, suitable cultivation conditions and a strategy for strain improvement, knowledge is required on the various pigments present in microalgae with respect to their *in vivo* roles and the metabolic pathways involved in their syn-

Table 2.1 Examples of strains of which the pigment composition has been described in detail.

Strain	Source
Chlorophyta (green algae)	
<i>Dunaliella salina</i> (Dunal) Teodoresco	Lamers et al. 2010
<i>Haematococcus pluvialis</i> (Flotow)	Orosa et al. 2001
<i>Coelastrella striolata</i> var. <i>multistriata</i> (Trenkwalder) Kalina and Puncochárová	Abe et al. 2004
<i>Coccomyxa acidophila</i>	Casal et al. 2010
<i>Muriellopsis</i> sp.	Del Campo et al. 2000
<i>Chromochloris</i> (<i>Chlorella</i>) <i>zofingiensis</i> (Dönn)	Del Campo et al. 2000
	Ip et al. 2004
	Orosa et al. 2000
<i>Neochloris wimmeri</i> (Hilse) Archibald and Bold	Orosa et al. 2000
<i>Scenedesmus vacuolatus</i> (Shihira and Krauss)	Orosa et al. 2000
<i>Scotiellopsis oocystiformis</i> (Lund) Puncochárová and Kalina	Orosa et al. 2000
<i>Protophycus botryoides</i> (Kützinger) Klebs	Orosa et al. 2000
<i>Trentepohlia aurea</i> (Linnaeus) Martius	Mukherjee et al. 2010
<i>Trentepohlia cucullata</i> (De Wildeman)	Mukherjee et al. 2010
<i>Chlamydomonas nivalis</i> (Bauer) Wille	Remias et al. 2005
Rhodophyta (red algae)	
<i>Audouinella eugenea</i> (Skuja) Jao	Bautista and Necchi 2007
<i>Audouinella hermannii</i> (Roth) Duby	Bautista and Necchi 2007
<i>Compsopogon coeruleus</i> (Balbis ex Agardh) Montagne	Bautista and Necchi, 2007
<i>Cyanidioschyzon merolae</i> (De Luca, Taddei and Varano)	Cunningham et al. 2007
Cyanophyta (blue-green algae)	
<i>Arthrospira platensis</i> (Nordstedt) Gomont	Abd El-Baky 2003
<i>Arthrospira maxima</i> (Setchell and Gardner)	Abd El-Baky 2003
<i>Anabaena azollae</i> (Strasburger)	Venugopal et al. 2006
<i>Oscillatoria redekei</i> (van Goor)	Wyman and Fay 1986
<i>Gloeotrichia echinulata</i> (Smith) Richter	Wyman and Fay 1986
<i>Oscillatoria agardhii</i> (Gomont)	Wyman and Fay 1986
Bacillariophyta (diatoms)	
<i>Phaeodactylum tricornutum</i> (Bohlin)	Nymark et al. 2009
Dinophyta (dinoflagellates)	
<i>Gambierdiscus toxicus</i> (Adachi and Fukuyo)	Indelicato and Watson 1986
Euglenophyta	
<i>Euglena sanguinea</i> (Ehrenberg)	Grung and Liaaen-Jensen 1993
Haptophyta	
<i>Dicranema vlkianum</i> (Prauser)	Durmaz et al. 2009
<i>Isochrysis galbana</i> (T-ISO)	Mulders et al. 2013
Eustigmatophyta	
<i>Nannochloropsis oculata</i> (Droop) Hibberd	Lubian et al. 2000
<i>Nannochloropsis salina</i> (Hibberd)	Lubian et al. 2000
<i>Nannochloropsis gaditana</i> (Lubián)	Lubian et al. 2000

thesis. A large body of knowledge is available on the pigment composition of specific microalgae cultivated under defined culture conditions (see examples in **Table 2.1**), the pigments present in certain groups of microalgae (Jeffrey and Wright 2005; Schagerl et al. 2003; Schagerl and Donabau 2003; Wright and Jeffrey 2006), the roles of groups of pigments *in vivo* (Guedes et al. 2011) and on parts of pigment biosynthesis routes (Bertrand 2010; Brown et al. 1990; Cunningham and Gantt 1998; Gálová et al. 2008; Ladygin 2000; Von Wettstein et al. 1995; Takaichi 2011). However, information is scattered and a clear overview that integrates all relevant information is missing.

The aim of this work is to identify the biological constraints and opportunities for production of pigments with phototrophic microalgae (**Section 2.6-2.7**) and to give an overview of all relevant underlying biological information (**Section 2.2-2.5**). Adequate understanding of these opportunities and constraints is important for the design of an economically feasible microalgae-based pigment production process.

2.2 Microalgal pigments

Pigments present in microalgae and cyanobacteria are grouped into the chlorophylls, carotenoids and phycobiliproteins. The difference between these groups lies in their chemical structure, as shown in **Fig. 2.1**. Chlorophylls, which are defined as tetrapyrroles, consist of a large aromatic ring, the chlorin, which contains four pyrrole rings that surround a magnesium ion. To the chlorin, a hydrocarbon tail is usually attached. Carotenoids consist only of a long hydrocarbon chain, made up of eight isoprene units. Phycobiliproteins consist of two parts: a bilipigment, known as phycobilin, and a protein, which are covalently attached to each other via a cysteine amino acid. The phycobilin contains the building blocks similar to those of a chlorin, but instead of a closed ring, they form an open linear structure. Therefore, phycobilins are referred to as open tetrapyrroles.

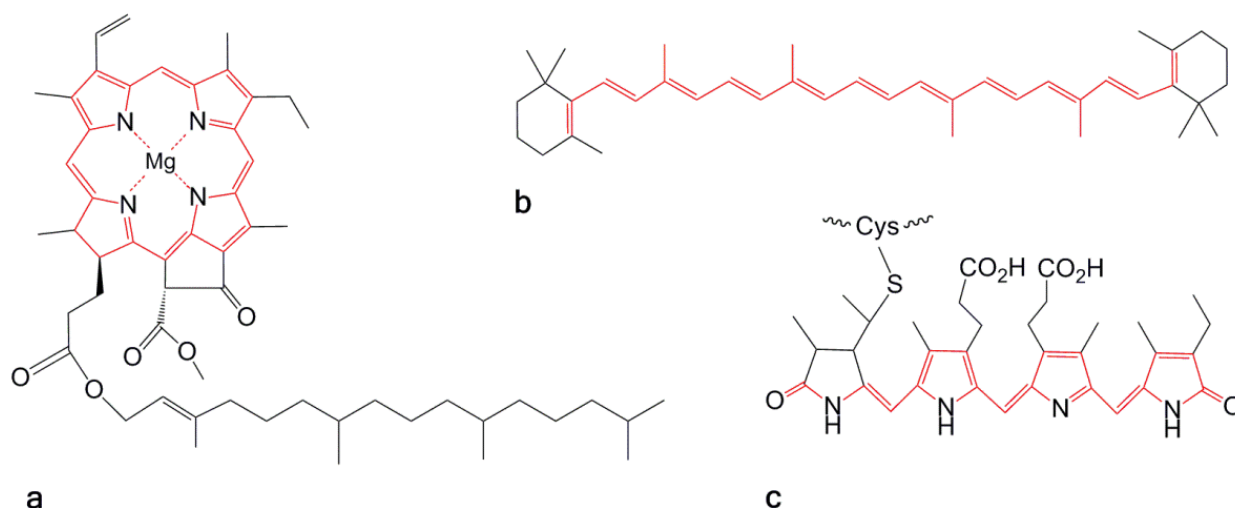


Fig. 2.1 Molecular structures of the pigments in microalgae and cyanobacteria, emphasizing (in red) the conjugated system of double bonds. **a** Chlorophyll a, representing the chlorophylls, **b** β -carotene, representing the carotenoids, **c** peptide-linked phycocyanobilin, representing the phycobiliproteins.

The similarity between the pigment groups is the presence of a large conjugated system of double bonds (indicated in red in **Fig. 2.1**). By excitation of the delocalized electrons within

these bonds, light of specific wavelengths is absorbed. Not absorbed light is reflected, giving pigments their characteristic color.

Different types of chlorophyll are found (chlorophyll *a*, *b*, *c*, *d* and *f*) (Chen et al. 2010) which are all green. In each type of chlorophyll, the molecules attached to the chlorin are slightly different. Besides, chlorophyll *c* lacks the hydrocarbon tail. These small differences cause differences in absorption spectrum and consequently in tonality: chlorophyll *a* appears more blue-green, chlorophyll *b* brilliant green, chlorophyll *c* yellow green, chlorophyll *d* brilliant/forest green and chlorophyll *f* emerald green (indicated in **Fig. 2.2** and **2.4**) (Chen et al. 2010; Roy et al. 2011). Upon loss of the magnesium ion, chlorophylls turn pale, dusky-colored (Humphrey 2004).

Carotenoids are distinguished by different end-groups; exceptionally, groups are attached to the center part of the molecule. They are divided into carotenes, which are true hydrocarbons, and xanthophylls, which additionally contain oxygen atoms. Carotenoids are yellow to red (individual colors are indicated in **Fig. 2 and 4**).

The protein-bound phycobilins known to date are phycocyanobilin (blue), phycoerythrobilin (red), phycoviolobillin (purple) and phycourobilin (yellow) (Sidler 1994). They are structurally distinguished by only small differences in the groups attached to the pyrrole rings. Nevertheless, these small structural differences have a significant effect on the length of the conjugated systems, causing the differences in color. The phycobilins most commonly found in microalgae are phycocyanobilin and phycoerythrobilin. Phycocyanobilin is found as the major component of the phycobiliproteins phycocyanin (deep blue) and allophycocyanin (light blue), whereas phycoerythrobilin is found as the major component of phycoerythrin (orange-red). Phycobiliproteins are usually found in pigment aggregates called phycobilisomes, which contain hundreds of protein-bound phycobilins (Blot et al. 2009). The close distance between the phycobilins causes interactions between the chromophores. Because the orientations of phycocyanin and allophycocyanin in the phycobilisome are different, their color shades are slightly different, as indicate above (Szalontai et al. 1994). For detailed information regarding the structure of protein-bound phycobilins and phycobilisomes, see Blot et al. (2009), Grossman et al. (1993) and Sidler (1994).

2.3 Biosynthesis of pigments

Besides light, which is the main energy source of microalgae, chemicals including water, carbon dioxide, inorganic nitrogen (ammonia or nitrate) and phosphate are required for photoautotrophic growth. **Fig. 2.2** gives a schematic overview of the pathways through which pigments are formed from these nutrients. Note that most microalgae groups contain only a part of these pathways (see **Section 2.5**).

Nitrogen and carbon are incorporated into protoporphyrin IX (**purple-green arrow**), which consists of a large aromatic ring (tetrapyrrol). The ring can be opened by oxidation reactions resulting in formation of biliverdin. Biliverdin is subsequently reduced to form the hydrophilic phycobilins (**purple arrows**), which are coupled to specific proteins (not shown). For a more detailed overview of phycobilin synthesis, see Beale (2004). In a different part of the metabolism, carbon is channeled towards geranyl geranyl-PP (**green-orange arrow**), which can subsequently be reduced to form phytyl-PP. Under release of the phosphates, phytyl-PP is

attached to a derivative of protoporphyrin IX, which has chelated a magnesium ion. This leads to formation of the chlorophylls (green arrows). As stated in **Section 2.2**, chlorophyll *c* lacks the phytol tail. It is unclear whether the tail is attached and released in subsequent steps, or not attached at all (Beale 1999). For a more detailed overview of chlorophyll synthesis, see Czarnecki and Grimm (2012) and Lohr et al. (2005).

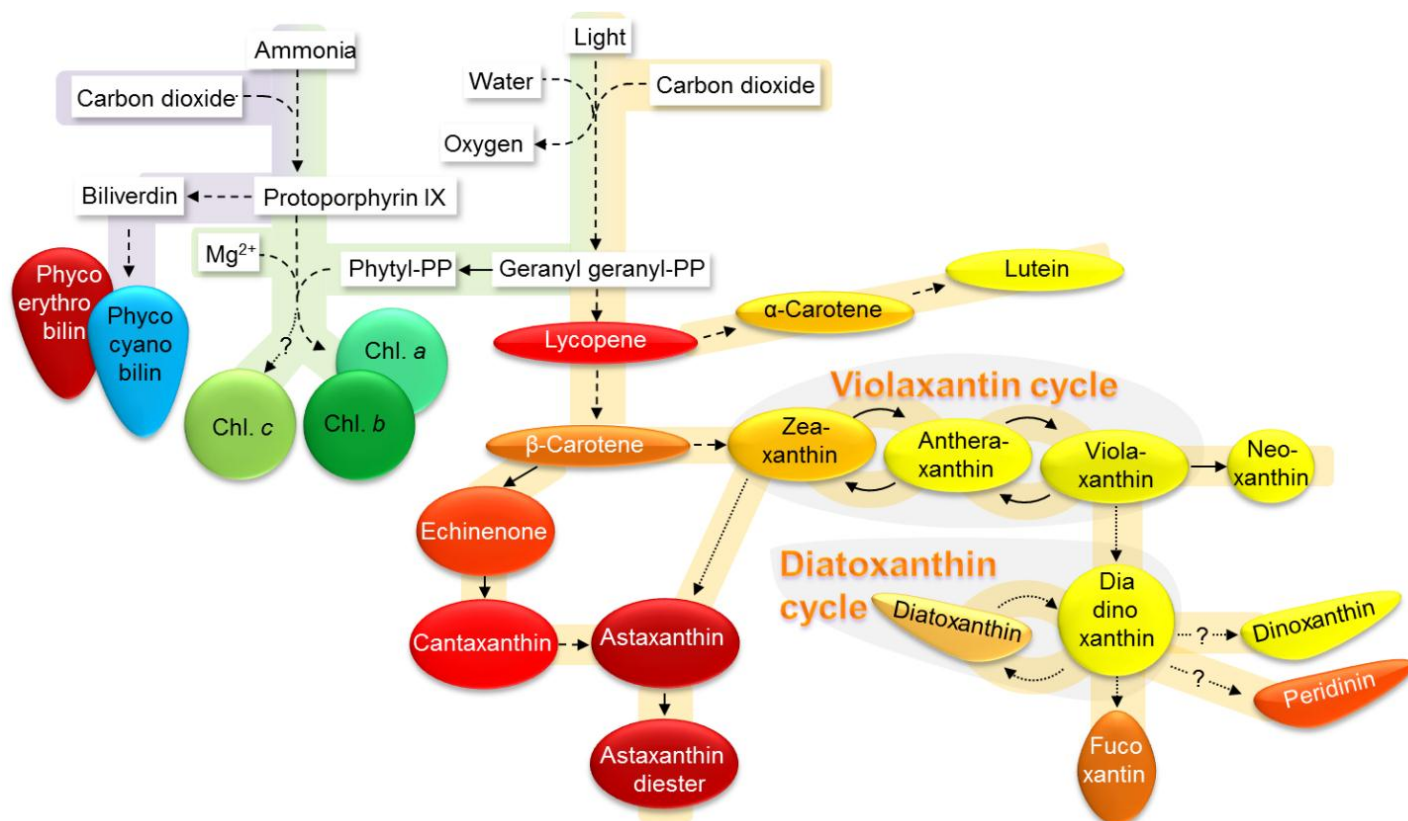


Fig. 2.2 Simplified scheme of the pigment biosynthesis pathways of cyanobacteria and microalgae. Solid arrows represent single conversion steps, dashed arrows represent lumped reactions, based on Falkowski and Raven (2007) and Kanehisa Laboratories (2011); dotted arrows represent hypothesized pathways, according to Beale (1999), Lohr and Wilhelm (1999, 2001) and Wang and Chen (2008). The arrows containing a question mark represent unresolved pathways. Pigment colours are indicated as described by Jeffrey et al. (1997) and Lee (2008). Note that the violaxanthin cycle is also referred to as xanthophyll cycle.

Apart from being a precursor of the chlorophylls, geranyl geranyl-PP is also the precursor of the carotenoids (**orange arrows**). Condensation of two molecules of geranyl-geranyl-PP followed by an isomerisation reaction and the introduction of four double bonds yields the carotenoid lycopene. After formation of lycopene, the biosynthetic pathway splits into two branches. One branch leads to the yellow lutein. In some species, including the Chlorophyte *Chlamydomonas reinhardtii* (Dangeard), lutein can be converted into loroxanthin (not shown) (Baroli et al. 2003). The other branch leads to the orange β -carotene, which itself is again a branch point. From β -carotene, one branch leads to the red astaxanthin, via two oxidation and two hydroxylation reactions. This hydrophobic carotenoid can be esterified once or twice with a fatty acid, leading to a more lipophilic astaxanthin mono- or diester. In *Haematococcus pluvialis*, for example, esters with the fatty acids C18:1 and C20:0 are predominantly found

(Goswami et al. 2010). The other branch from β -carotene leads to the orange zeaxanthin. It is proposed by Cordero et al. (2011) and Wang and Chen (2008) that in the species *Chromochloris (Chlorella) zofingiensis* zeaxanthin can be converted into astaxanthin.

Zeaxanthin is part of the violaxanthin cycle, as shown in **Fig. 2.2**. The violaxanthin cycle is also sometimes called xanthophyll cycle. To prevent confusion between the violaxanthin and the diatoxanthin cycle (described further on), which both consist of xanthophylls, the term violaxanthin cycle instead of xanthophyll cycle will be used here. Zeaxanthin is turned into antheraxanthin, and subsequently into violaxanthin, which are both yellow, by two epoxidation reactions. These reactions are induced by subsaturating light conditions, while the reverse reactions are induced by oversaturating light conditions. Violaxanthin can also be isomerized, giving rise to neoxanthin.

The metabolic routes described above are intensively studied, in contrast to the pathways leading to diatoxanthin, dinoxanthin and fucoxanthin (indicated by the **dotted lines** in **Fig. 2.2**). According to Lohr and Wilhelm (1999), violaxanthin is converted into diadinoxanthin under prolonged subsaturating light conditions. This conversion probably requires more than one conversion step. Furthermore, their results suggest that diadinoxanthin is further metabolized into fucoxanthin, which probably also requires multiple conversion steps.

Besides, according to the findings of Lohr and Wilhelm (1999), oversaturating light intensities induce the de-epoxidation of diadinoxanthin, to form diatoxanthin. This xanthophyll is epoxidated again under subsaturating light intensities. The interconversion of diadinoxanthin and diatoxanthin is termed 'the diatoxanthin cycle' (**Fig. 2.2**).

The biosynthesis pathways leading to dinoxanthin and peridinin, which are exclusively found in the peridinin-containing Dinophyta, are still unresolved (indicated by the question marks). However, it has been shown by Lohr and Wilhelm (1999) that peridinin is another derivate of zeaxanthin.

2.4 Role of pigments *in vivo*

Light can have both positive and negative effects on microalgae; for example, light not only forms the primary source of energy, driving all biochemical processes, but also causes lethal damage when present in excessively large quantities. To be able to survive the varying circumstances of subsaturating and oversaturating light conditions, photosynthetic organisms contain pigments with two distinct roles: light harvesting and photoprotection. Certain pigments can perform both roles (**Fig. 2.3**), depending on the protein subunit or sub-domain to which they are bound (Ballottari et al. 2012; Berera et al. 2012). Light harvesting pigments are subdivided into the primary light harvesting pigment and accessory light harvesting pigments, whereas photoprotective pigments are divided into three groups, which have a filtering, quenching and/or scavenging role.

Besides the distinction between the light harvesting and photoprotective role of pigments, distinction is also made between primary and secondary pigments. Primary pigments are defined as those which are functionally and structurally bound to the photosynthetic apparatus, whereas secondary pigments are those which are not functionally or structurally bound to the photosynthetic apparatus (Lemoine and Schoefs 2010). According to this definition, the primary pigments include all pigments with a light harvesting role and the

photoprotective pigments with a quenching and scavenging role, whereas the secondary pigments include only the photoprotective pigments with a filtering role.

In this section, the roles of the different pigments are further explained, and the specific pigments playing these roles in microalgae and cyanobacteria are identified.

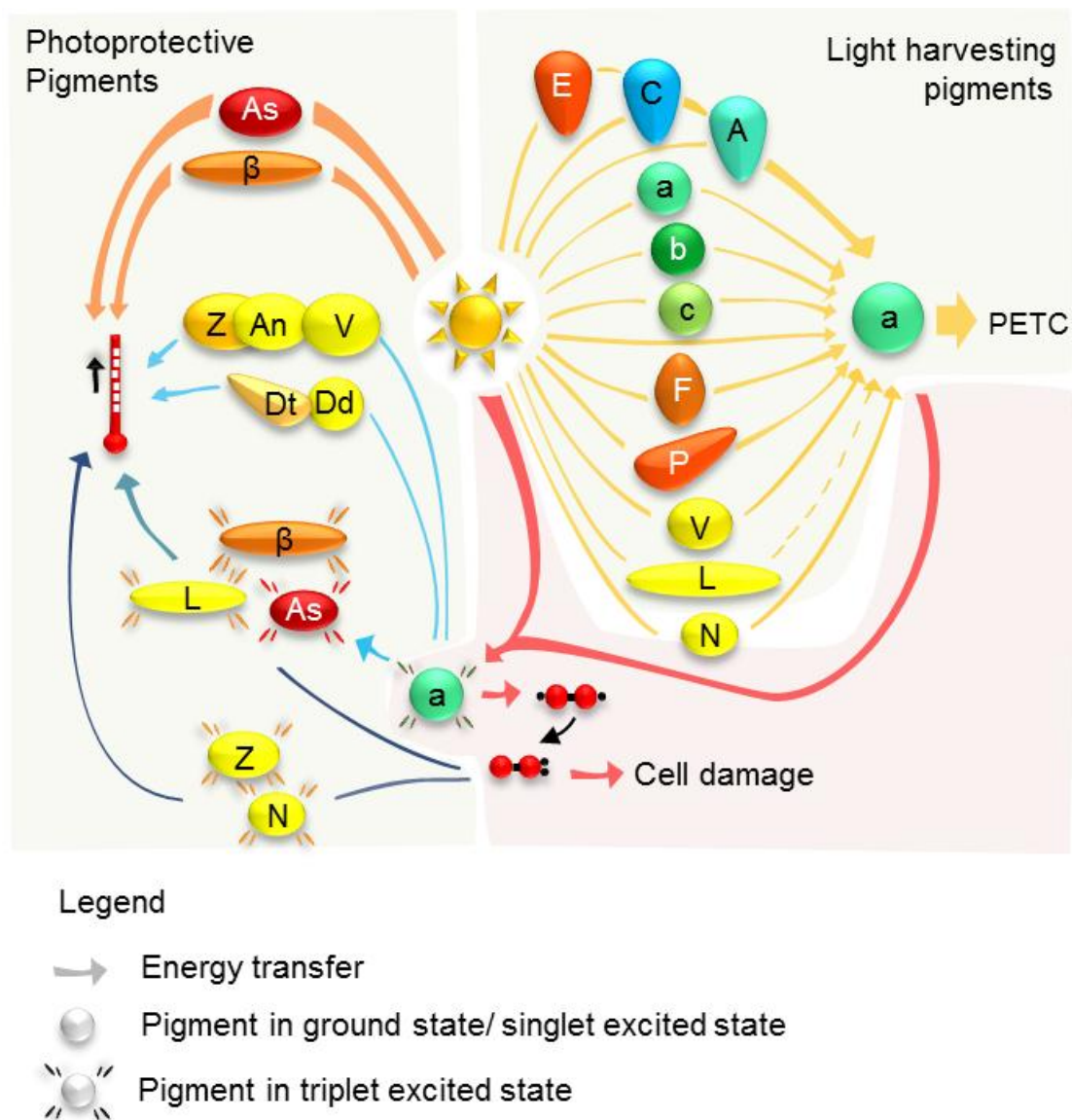


Fig. 2.3 Main roles of pigments in cyanobacteria and microalgae. Light harvesting pigments transfer light energy towards the photosynthetic electron transport chain (PETC), where it is converted into chemical energy (**thin orange arrows, directed to the right**). The cell can be damaged due to the formation of triplet excited chlorophyll *a*, which can turn molecular oxygen into reactive oxygen species (**red arrows**). Photoprotective pigments prevent cell damage by turning excess energy into heat. *E* Phycoerythrin, *C* Phycocyanin, *A* Allophycocyanin, *a* Chlorophyll *a*, *b* Chlorophyll *b*, *c* Chlorophyll *c*, *As* Astaxanthin, *β* β -carotene, *Z* zeaxanthin, *An* Antheraxanthin, *V* violaxanthin, *Dt* Diatoxanthin, *Dd* Diadinoxanthin, *L* Lutein, *N* Neoxanthin, *F* Fucoxanthin, *P* peridinin (Balashov et al. 2010; Telfer et al. 2008; Falkowski and Raven 2007; Jin et al. 2003; Lee 2008; Lohr and Wilhelm 1999; Sukenik et al. 1992).

As indicated in **Fig. 2.3**, light harvesting pigments transfer light energy towards the photosynthetic electron transport chain (PETC), where it is converted into chemical energy

(**arrows to the right**). The primary light harvesting pigment, chlorophyll *a*, is able to transfer the excitation energy directly towards the PETC, whereas the accessory light harvesting pigments transfer their excitation energy via chlorophyll *a* (Lee 2008). The accessory light harvesting pigments include all chlorophylls, all phycobiliproteins (Lee 2008), and the carotenoids neoxanthin (Dall'Osto et al. 2006), fucoxanthin, peridinin (Lohr and Wilhelm 1999) and echinenone (not shown) (Balashov et al. 2010). In addition, violaxanthin acts as an accessory pigment in some microalgae species (Suklenik et al. 1992). Lutein is a semi-photosynthetic pigment: it can transfer excitation energy towards chlorophyll *a*, but only with very low efficiency (indicated by the **dashed line between lutein and chlorophyll *a***, in Fig. 2.3) (Falkowski and Raven 2007). Light harvesting pigments are located in photosystems, with the primary light harvesting pigment located closest to the inner part of the photosystem and the accessory light harvesting pigments located closest to the outside. Accessory pigments are either interwoven with proteins into chlorophyll-containing pigment-protein complexes, or they stick out of the system, forming antennae. In order to have their excitation energy efficiently transferred, the accessory pigments need to be in very close proximity of the primary light harvesting pigment (Telfer et al. 2008).

When the energy transfer rate towards chlorophyll *a* (**arrows towards chlorophyll *a***) exceeds the energy transfer rate from chlorophyll *a* towards the PETC (**bold arrow towards the PETC**), chlorophyll *a* becomes triplet excited (**large red arrow**). Whether this will happen depends on the supply rate of energy, i.e. the light intensity perceived by the cell, and on the rate at which the PETC operates. The operation rate of the PETC on its turn depends on environmental conditions that influence the cell's metabolism, such as salinity, pH, temperature and nutrient availability, and on species specific characteristics. By itself, overexcited chlorophyll *a* is not harmful. However, in this state, it can pass its energy on to molecular oxygen (**small red arrow from overexcited chlorophyll *a***), turning it from the ground state into reactive oxygen species (ROS), such as singlet oxygen (**black arrow**). ROS are highly reactive, and therefore very toxic. They can damage all kinds of cell components including DNA, proteins and membrane lipids (**small red arrow pointing from singlet oxygen**) (Telfer et al. 2008).

This phenomenon, known as photoinhibition, is minimized by photoprotective pigments, which turn the excess amount of energy into heat (**arrows to the left**). As stated, the photoprotective pigments can be divided into three different groups, having either a filtering, quenching or scavenging role. Pigments with a filtering role prevent the formation of overexcited chlorophyll *a*, by the absorption of harmful radiation (**bold orange arrows**). They are not bound (functionally nor structurally) to the photosynthetic apparatus, which makes them secondary pigments. Instead, they are found physically separated from the photosynthetic apparatus, usually in oil droplets in the chloroplast's stroma or in the cytosol (Jin et al. 2006). Because secondary pigments include only certain carotenoids, namely astaxanthin and β -carotene, they are also referred to as secondary carotenoids (Telfer et al. 2008).

Pigments with a quenching role prevent the formation of ROS, by quenching the energy of triplet excited chlorophyll *a* (**blue arrows directing from triplet excited chlorophyll *a***), or singlet excited chlorophyll *a* (not indicated). The mechanism by which energy is quenched is referred to as non-photochemical quenching (NPQ). Similar to the accessory light harvesting

pigments, quenching pigments must be very close to chlorophyll *a* to perform their role. Astaxanthin, β -carotene and lutein perform the role of quenchers. Compared to astaxanthin and β -carotene, lutein quenches overexcited chlorophyll *a* slightly less efficient (Telfer et al. 2008). In addition, as shown in **Fig. 2.3**, the xanthophylls which belong to the violaxanthin cycle, zeaxanthin and violaxanthin, are involved in effective quenching of chlorophyll *a* (Goss and Jakob (2010); Jahns and Holzwarth 2012). Even more effective quenchers are the xanthophylls which constitute the diatoxanthin cycle, diadinoxanthin and diatoxanthin (Telfer et al. 2008). For detailed explanations of the molecular quenching mechanisms behind the violaxanthin and diatoxanthin cycle, which are still not completely understood, see Goss and Jakob (2010), Jahns and Holzwarth (2012) and Latowski et al. (2004).

Pigments with a scavenging role, astaxanthin, β -carotene, lutein and neoxanthin (Telfer et al. 2008), prevent cell damage by reacting with ROS such as singlet oxygen (**dark blue arrows pointing from singlet oxygen**). It is suggested by Jin et al. (2003) that also zeaxanthin, when interwoven within the lipid bilayer, could perform the role of scavenger, preventing damage of diacylglycerides. The protecting carotenoids perform this role most effectively when they are near the site of ROS formation, which is inside the photosynthetic apparatus, close to chlorophyll *a* (Telfer et al. 2008).

Besides the light harvesting and photoprotective roles, carotenoids also have a structural role. The shape and lipophilicity of the carotenoids makes them fit perfectly into or bridge across the lipid bilayer of membranes, thus affecting the membrane properties (Latowski et al. 2004). However, the concentration of carotenoids in the membrane is low and so far no studies have shown that their structural role *in vivo* is of physiological significance (Telfer et al. 2008).

Finally, carotenoids can have a role as electron sink or as carbon and energy storage, as discussed for astaxanthin by Lemoine and Schoefs (2010). However, these roles are still a matter of debate.

To conclude, a certain pigment can have several roles *in vivo*; the particular role it can play is connected to its location. For example, located in the thylakoids, β -carotene and astaxanthin function as primary pigments, but if they are located in oil droplets, separated from the photosynthetic apparatus, they function as secondary pigments. Furthermore, some roles are performed by a single pigment (e.g. transferring light energy into the PETC), while other roles are performed by several pigments. As will be discussed in **Section 2.6**, detailed knowledge on the roles of pigments in microalgae is essential for the development of a successful strategy to overproduce pigments.

2.5 Distribution of pigments

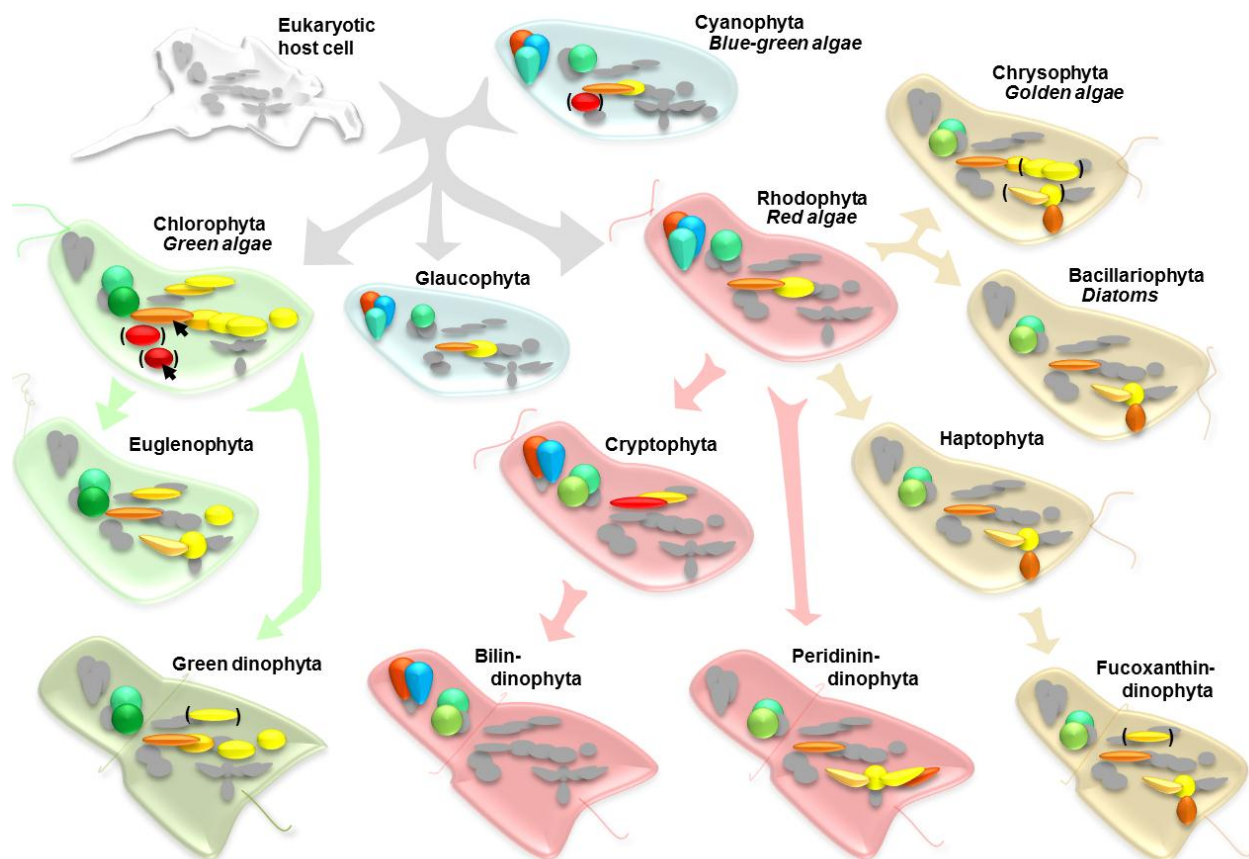
In **Section 2.3**, the biosynthetic pathways of pigments in microalgae and cyanobacteria were discussed. As stated, most microalgae groups contain only a subset of the pigments presented. The aim of this section is to indicate which pigments are present in the most common groups of microalgae and cyanobacteria. Only pigments found in significant amounts are indicated, whereas pigments present in trace amounts are excluded. First, the microalgae groups discussed in this section are placed into their evolutionary perspective.

It is generally accepted that the Chlorophyta, the Rhodophyta, and the Glaucophyta arose from endosymbiosis of Cyanophyta (which are in fact photosynthetic bacteria) and non-photosynthetic eukaryotic host cells (Vesteg et al. 2009), which is shown in **Fig. 2.4**. Further down the phylogenetic tree, the relationships between groups become more disputed. For example, it is still unclear whether the groups possessing plastids of red microalgae origin all share a common ancestor (Sanchez-Puerta and Delwiche 2008).

In particular the origin of the Dinophyta is unclear. On the one hand, all Dinophyta could be grouped together based on the similarity in morphology (one transverse and one longitudinal flagellum, and a distinct layer beneath the cell membrane). On the other hand, the Dinophyta could be divided into different groups, as shown in **Fig. 2.4**, based on the pigments they possess. Very likely, the peridinin-containing Dinophyta arose by secondary endosymbiosis of a red microalga (i.e. a red microalga, which was the product of primary endosymbiosis, was itself engulfed and retained by another free living eukaryote), while the green Dinophyta obtained their plastids via secondary replacements (i.e. replacement of algal-derived plastids by other algal-derived plastids), and the bilin- and fucoxanthin-containing Dinophyta obtained their plastids via tertiary endosymbiosis (i.e. products of secondary endosymbiosis, Cryptophyta and Haptophyta (see **Fig. 2.4**), were themselves engulfed and retained by other free living eukaryotes). However, it has also been suggested that all dinophyta arose by red microalgae endosymbiosis (Palmer 2003; Yoon et al. 2002). For more detailed information regarding the evolutionary history of microalgal plastids, see Keeling (2004), Lohr et al. (2012), Palmer (2003), Sanchez-Puerta and Delwiche (2008) and Vesteg et al. (2008).

The phycobiliproteins phycoerythrin, phycocyanin and allophycocyanin are found in Cyanophyta, Rhodophyta (Wright and Jeffrey 2006) and Glaucophyta (Lee 2008). In these groups, the phycobiliproteins are located in phycobilisomes, on the outer surface of the thylakoid membrane. Besides, phycoerythrin and phycocyanin are also found in Cryptophyta and bilin-containing Dinophyta. In those groups, the phycobiliproteins are located within the thylakoid lumen, in an unresolved ultrastructure (as soluble heterodimers, tethered to the membranes, or otherwise possibly arranged into some type of antenna complex) (Mirkovic et al. 2009; Jeffrey and Wright 2005).

Chlorophyll *a*, which is the primary light harvesting pigment, is found in all photosynthetic organisms. In contrast, chlorophyll *b* is exclusively found in the Chlorophyta and their descendants. Similarly, chlorophyll *c* is exclusively found in the descendants of the Rhodophyta (Jeffrey and Wright 2005).



Legend

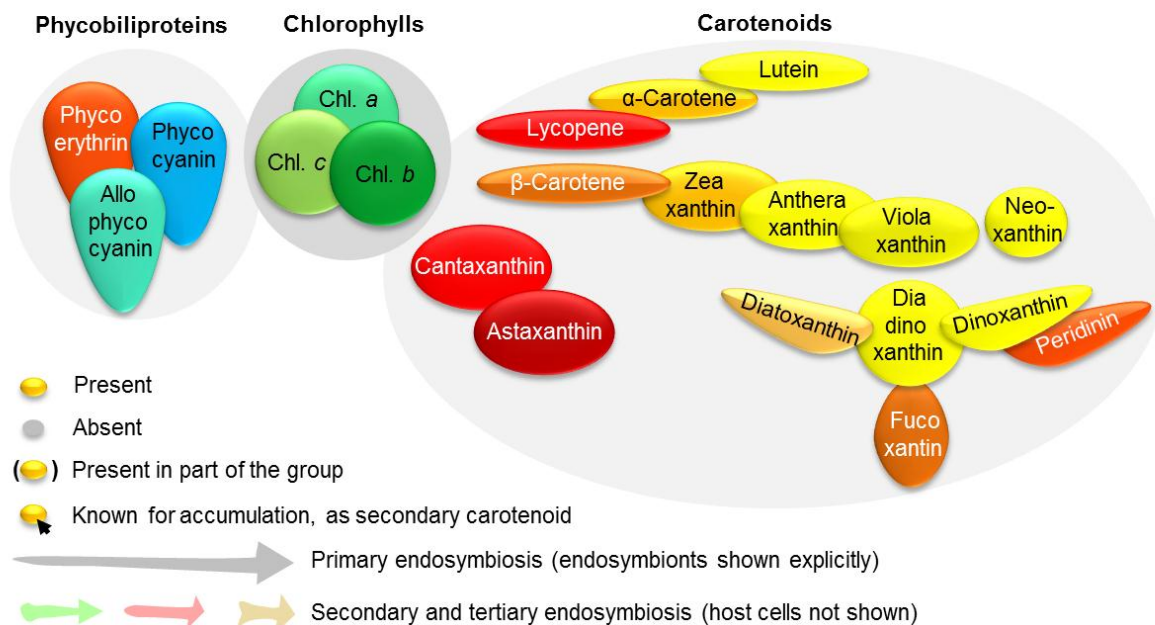


Fig. 2.4 Distribution of photosynthetically important or commercially applied pigments, across the most common microalgal groups. (The Chlorarachniophyta, descendants from the Chlorophyta, and the Apicomplexa are not included.) Only pigments present in significant amounts are shown (i.e. trace pigments are excluded). The microalgal groups are drawn in their evolutionary perspective based on the hypothesis that Green dinophyta, and Bilin- and Fucoxanthin-containing dinophyta obtained their plastids via secondary replacements and tertiary endosymbiosis, respectively (Jeffrey and Wright 2005; Lee 2008; Takaichi 2011; Vesteg 2009). Pigment colours are indicated as described by Jeffrey et al. (1997) and Lee (2008).

The carotenoid which is most abundantly used in foods and supplements, β -carotene, is also frequently found in the cyanobacteria and microalgae world. Except for the Cryptophyta and the bilin-containing Dinophyta, this pigment is found in all groups which are included in **Fig. 2.4**. Although β -carotene is widely distributed, overproduction as a secondary pigment is observed only in a select number of Chlorophyta.

In contrast, astaxanthin is exclusively found in the Chlorophyta. Similar to β -carotene, a select number of Chlorophyta is able to overproduce this pigment. The precursor of astaxanthin, canthaxanthin, is found in some Chlorophyta, as well as in some Cyanophyta.

Similarly, lutein is exclusively found in Chlorophyta and in some green Dinophyta. In part of the Chlorophyta, this yellow pigment can be converted into loroxanthin (Jeffrey and Wright 2005).

Fucoxanthin is found in the Haptophyta, the fucoxanthin-containing Dinophyta, the Chrysophyta and in the Bacillariophyta. The latter two belong to a group referred to as the Heterokontophyta.

Peridinin, complexed with chlorophyll *a*, is found exclusively in peridinin-containing Dinophyta (Schulte et al. 2010). In addition, dinoxanthin is found in these Dinophyta.

Although zeaxanthin is present in the Cyanophyta and the Rhodophyta, these phycobilisome-containing organisms do not display the violaxanthin or diatoxanthin cycle (Falkowski and Raven 2007). In contrast, most of the groups descending from the Rhodophyta do perform either violaxanthin or diatoxanthin cycling. For instance, zeaxanthin is found in the Chrysophyta, which further contain antheraxanthin and violaxanthin, or diadinoxanthin and diatoxanthin (Jeffrey and Wright 2005). Besides, Lohr and Wilhelm (1999) found microalgae that display both the violaxanthin cycle and the diatoxanthin cycle, when they are exposed to prolonged subsaturating light conditions. They intensively studied *Phaeodactylum tricornutum*, which belongs to the Bacillariophyta, but they found similar results for species belonging to the Haptophyta and the Dinophyta. Furthermore, the violaxanthin cycle is possessed by the Chlorophyta (Wright and Jeffrey 2006) and most probably by the green Dinophyta, as they possess both zeaxanthin and violaxanthin.

As stated, only pigments found in significant amounts are indicated as present in **Fig. 2.4**. This implies that untraceable intermediates such as lycopene, which could only be detected as a trace pigment in Cyanophytes (Jeffrey et al. 1997), are not included. However, the presence of the downstream metabolites such as lutein and β -carotene indicate that lycopene must be present in all presented groups except the bilin-containing Dinophyta. Apparently, once formed, lycopene is immediately converted.

In conclusion, the Chlorophyta possess the largest variety of pigments. Moreover, it is the only group which is known to contain species that overproduce secondary carotenoids as a response to specific culture conditions. Possibilities to overproduce these, and other pigments, will be discussed further in **Section 2.6**.

2.6 Pigment production: biological constraints and opportunities

Section 2.5 dealt with the pigment distribution across different groups of microalgae and cyanobacteria. Except for the chlorophylls, which are usually present in a concentration of 1-2 % g/g dry weight (Li et al. 2008; Wang and Chen 2008), most other pigments are, under optimal growth conditions, present in a concentration which is too low (i.e. below 0.5 % g/g dry weight) to make microalgae-based production economically competitive with chemical production. To make microalgae-based pigment production economically feasible, the cellular content needs to be increased.

The cellular content of a desired pigment can be increased using two different approaches: (i) by applying specific culture conditions, which is highly explored and exploited (Jin et al. 2006; Lamers et al. 2010; Lamers et al. 2012) or (ii) by intervention in the cell metabolism, which is still unexplored due to the lack of a sufficiently developed genetic engineering toolbox (Beer et al. 2009). In this section, both approaches are discussed and the pigments which can be overproduced by each of these approaches are indicated.

The first approach to obtain an increased pigment content, through the use of specific culture conditions, can be divided into two categories: (i) applying subsaturating light conditions, which results in a minor increase of primary pigments, and (ii) applying adverse growth conditions (also referred to as 'stress conditions'), which result in a major increase of secondary pigments. Both categories are discussed below.

When cells are grown under subsaturating light conditions for a prolonged time period, they acclimate to that light regime by increasing the amount of photosystems. As a consequence, the cellular content of primary pigments, which are associated to the photosystem, increases as well. However, this phenomenon, which is referred to as photoacclimation, leads only to minor increases of the content of primary pigments (Telfer et al. 2008) (typically to concentrations below 0.5 % g/g dry weight). These minor increases, which in literature are often termed 'accumulation' (e.g. accumulation of lutein in *Chromochloris (Chlorella) zofingiensis* (Del Campo et al. 2004)), should not be confused with overproduction (typically to concentrations above 0.5 %, up to 10 % g/g dry weight), which is used to describe major increases of the cellular pigment content as described in the next paragraph.

Under adverse growth conditions, such as a high salt concentration, oversaturating light intensity or nitrogen limitation, secondary carotenoids that have a light filtering role (β -carotene and astaxanthin, **Fig. 2.3**), can be transported from their site of synthesis in the thylakoids to oil droplets located in the chloroplast's stroma or the cytosol, where they accumulate (Jin et al. 2006). In contrast to photoacclimation, which is universally observed in microalgae, accumulation of secondary carotenoids is observed exclusively in a limited number of Chlorophytes. The most well-known examples, which are well explored and exploited, are *Haematococcus pluvialis* (astaxanthin) and *Dunaliella salina* (β -carotene). These strains can overproduce secondary carotenoids up to 7.7-10 % of the cell's dry weight (Lamers et al. 2008). Besides these known microalgae species, nature might supply interesting not yet discovered strains with high pigment concentrations. For instance in extreme environments, which evoke natural anti-oxidative responses.

The second approach to obtain an increased pigment content, by intervening in the cell's metabolism, can be divided into two categories: (i) up- or down-regulation of pigment biosynthetic enzymes and (ii) formation of a metabolic sink. Below, both categories are discussed.

Up- and down-regulation of pigment biosynthetic enzymes both aim to increase the flux towards a desired pigment by manipulating the pigment metabolism. To achieve this, all enzymes need to be overproduced that exert control over the flux towards the desired pigment, leaving all other metabolite concentrations and most other fluxes unchanged. However, as discussed by Fell (1997), not all enzymes on the pathway need to be overexpressed to the same degree: enzymes in the final linear sequence need to be overexpressed to a much higher degree than the enzymes in preceding branches. This method could be used, for example, to overproduce the primary pigment lutein. In that case, the enzymes which control the flux from lycopene to lutein (i.e. enzymes in the final linear sequence towards lutein, **Fig. 2.2**) need to be overexpressed most. To prevent a decreased flux towards β -carotene and its derivatives (**Fig. 2.2**), the enzymes that control the flux from geranyl geranyl-PP into lycopene may need to be overexpressed as well. Finally, it may be needed to overexpress the flux controlling enzymes leading to geranyl geranyl-PP as well, to retain sufficient flux towards the chlorophylls (**Fig. 2.2**).

Besides overproducing a single pigment, the method described above may be used to increase the content of multiple pigments simultaneously. In *Arabidopsis*, overexpression of 1-deoxy-D-xylulose-5-phosphate synthase, an enzyme which catalyses one of the early steps leading to geranyl geranyl-PP, resulted in an increase of chlorophylls and carotenoids up to 150 % (Estevez et al. 2001). Alternatively, flux controlling enzymes further down the pathway could be overexpressed, such as the ones converting geranyl geranyl-PP into lycopene (**Fig. 2.2**). It is suggested by Jin et al. (2006) that the enzyme which catalyses the first steps of this conversion (phytoene synthase) may control the flux towards the carotenoids, since this enzyme was found to be rate-limiting in ripening tomato fruits (Bramley et al. 1992; Fraser et al. 1994), in canola seeds (Shewmaker et al. 1999), and in marigold flowers (Moehs et al. 2001). In contrast, Lemoine and Schoefs (2010) suggest that the subsequent reactions (i.e. the conversion of phytoene into ζ -carotene), catalysed by phytoene desaturase, are among the rate-limiting steps of the pathway, and that upregulation of this enzyme may lead to an increased flux towards the carotenoids. However, as discussed by Kacser (1995), the flux towards a certain metabolite is in general not controlled by a single enzyme, but instead, shared among multiple enzymes. Even if a single enzyme would have significant control over the flux, overexpression of this enzyme would probably result in a control shift towards other enzymes. First of all, this implies that not a single enzyme, but multiple enzymes need to be overexpressed in order to overproduce a specific pigment or pigments. Besides, rather than to focus on a rate limiting step, one should focus on the shared control of the flux towards a metabolite of interest.

Similar to the up-regulation of pigment biosynthetic enzymes, the down-regulation of enzymes (e.g. by adding enzyme inhibitors, by RNA silencing, or by creating a gene knock-out) aims to increase the flux towards a desired pigment. In contrast to up-regulation, down-regulation aims to increase the desired flux by decreasing the flux towards side branches. For example, an enhanced flux towards lutein could be generated by blocking the branch leading towards β -carotene. However, since the derivatives of β -carotene are mainly primary pigments

(Fig. 2.2 and 2.3), which are of major importance for functioning of the cell, this could result into growth inhibition.

Two other bottlenecks which might limit the overproduction of primary pigments are crowding and feedback inhibition. When up- or down-regulation of pigment biosynthetic enzymes would lead to an enforced overproduction of primary pigments, the limited amount of space within the chloroplasts lumen may cause interference of the overproduced molecules with the photosynthetic system (crowding). Hence, overproduction of primary pigments, by up- or down-regulation of their biosynthetic enzymes, may lead to a disrupted cell functioning and consequently to growth inhibition.

The only pigments for which this problem may be less severe are the phycobilins that are located in antennae. Since the antennae are found at the stroma side of the the thylakoid membrane, sufficient space is available for them to extend. However, the extension will most probably affect the light harvesting efficiency, due to self-shading of phycobiliproteins.

Another bottleneck which may limit pigment overproduction are regulatory mechanisms which aim to keep metabolite concentrations constant (homeostasis), such as feedback inhibition. By means of negative feedback mechanisms, which may act on one or multiple pigment biosynthetic enzymes, accumulated pigment molecules may inhibit further overproduction. A way to circumvent feedback inhibition is to overexpress flux controlling enzymes that are feedback-resistant. This approach was used in the heterotrophic bacterium *Corynebacterium glutamicum*, in which overexpression of a feedback-resistant kinase resulted in lysine accumulation and secretion up to 38 mM. In comparison, overexpression of the feedback-sensitive enzyme did not result in accumulation or secretion of lysine (Cremer et al. 1991).

Similar to the method described above, the formation of a metabolic sink aims to increase the flux towards a desired pigment. Instead of pushing, the sink pulls the metabolism into a certain direction, by transporting a metabolite away from the site of formation. Thereby, this approach overcomes the possible limitations described for up- or down-regulation of pigment biosynthetic enzymes (feedback inhibition and crowding). In fact, the formation of a metabolic sink may be essential for carotenoid overproduction. Namely, in both *Dunaliella salina* and *Haematococcus pluvialis*, inhibition of lipid accumulation, which serves as a metabolic sink for β -carotene and astaxanthin, abolished carotenoid accumulation (Rabbani et al. 1998; Zhekisheva et al. 2005).

As only a select number of Chlorophytes naturally accumulate pigments in oil droplets, genetic engineering may be applied to induce the formation of storage space outside the photosystem in other species. In cauliflowers, a spontaneous mutation in the *orange (Or)* gene led to the formation of large membranous chromoplasts, in which high levels of β -carotene accumulated (Li and van Eck 2007). Introduction of the *Or* gene in potato led to the formation of similar structures in which high levels of β -carotene accumulated, for which genetic modifications in the carotenoid biosynthesis pathway were thus not required (Farré et al. 2010). Introduction of the *Or* gene, or genes with a similar function, into microalgal cells may supply extra storage space for secondary carotenoids, leading to overproduction in strains that normally do not overproduce secondary carotenoids.

The main bottleneck which might limit overproduction of pigments in additionally formed storage space is the transport of pigments out of the photosystem. Although many microalgae strains accumulate oil droplets upon adverse growth conditions (e.g. *Chromochloris* (*Chlorella*) *zofingiensis* (Liu et al. 2011), *Chlorella vulgaris* (Beijerinck) (Mutlu et al. 2011), *Isochrysis galbana* (T-Iso) (Mairet et al. 2011) and *Neochloris oleoabundans* (Chantanachat and Bold) (Santos et al. 2012)), only a limited number of Chlorophytes couple oil accumulation with pigment overproduction. This suggests that the overproduction of pigments requires a transport mechanism which is absent in most species.

In both *Dunaliella salina* and *Haematococcus pluvialis* it is suggested that β -carotene is the pigment which is transported out of the photosynthetic apparatus (Lemoine and Schoefs 2010). In *Haematococcus pluvialis*, which is known for its massive astaxanthin production, the subsequent conversion from β -carotene into astaxanthin is suggested to take place in the cytoplasm (Jin et al. 2006; Lemoine and Schoefs 2010). Thus, when the introduction of extra storage space into other microalgae is successful, it may be that this space will be solely occupied by β -carotene in case β -carotene is the only pigment which can be transported out of the photosystem, and when β -carotene converting enzymes are absent in the cytoplasm. To enable overproduction of pigments other than β -carotene in such storage space, specific enzymes may be targeted towards this storage space or towards the cytoplasm to convert β -carotene into derivatives. This approach was recently applied in carrot plants, in which lutein and β -carotene are commonly found, but in which derivatives of β -carotene, such as canthaxanthin and astaxanthin (**Fig. 2.2**), are rarely found. The synthesis of these ketocarotenoids was achieved by introduction and up-regulation of the enzymes which catalyze the conversion steps from β -carotene into astaxanthin. The enzymes were targeted to the plastids, where accumulation of β -carotene occurs, by fusing them with a targeting peptide (from Rubisco). As a result, 70 % of the β -carotene was converted into ketocarotenoids (Jayaraj et al. 2008). A similar approach may be used in microalgae, aiming at accumulation of for example echinenone in *Dunaliella salina*.

As stated in **Section 2.2**, phycobiliproteins consist of two parts: a phycobilin and a protein. Consequently, overproduction of phycobiliproteins requires an increased flux towards both parts. However, it would be interesting to test the hypothesis that upregulation of the protein content alone may be sufficient to generate an increased flux towards the phycobilin. In that way, the protein would act as metabolic sink for the phycobilins.

The pigments discussed above may be produced in a continuous, or in a two-step process. In a continuous process, biomass production and pigment overproduction take place simultaneously, while in a two-step process, biomass production and pigment overproduction take place separately (i.e. a growth phase is followed by a pigment production phase). The degree of growth inhibition that coincides with pigment overproduction determines to a large extent the best production strategy. If pigment overproduction leads to minor growth inhibition, continuous production may be the best production strategy. However, if pigment overproduction leads to severe growth inhibition, a two-step process is required. In that case, the overproduction should be inducible (i.e. it should not take place during the growth phase), for example by a nutrient limitation.

To conclude, specific process conditions, such as oversaturating light intensities or a high salt concentration, exclusively induce the overproduction of secondary carotenoids (β -carotene in *Dunaliella salina* and astaxanthin in *Haematococcus pluvialis*). Overproduction of all other pigments present in microalgae requires modification of the expression of their biosynthetic enzymes, most probably combined with the creation of storage space outside the photosystem. Since pigment overproduction may lead to severe growth inhibition, a two-step production process might prove to be most successful.

2.7 Conclusions and prospects

Microalgae produce a wide range of colored pigments. Overproduction of some pigments may be attained by applying specific process conditions, while for others metabolic engineering will be necessary. **Table 2.2** gives for each colour the approach to be used together with a preferable microalgal species.

Table 2.2 Methods to produce or overproduce natural pigments, using microalgae or cyanobacteria. See text for details.

Color	Pigment	Production organism		Production strategy	
		Group	Strain	Process conditions	Metabolic engineering
Red	Astaxanthin	Chlorophyta	<i>Chromochloris</i> (<i>Chlorella</i>) <i>zofingiensis</i> <i>Haematococcus pluvialis</i>	✓	
Red-orange	Echinenone	Chlorophyta	<i>Dunaliella salina</i>		✓
Orange	β -Carotene	Chlorophyta	<i>Dunaliella salina</i>	✓	
Yellow-orange	Zeaxanthin	Chlorophyta	<i>Dunaliella salina</i>		✓
Yellow	Lutein	Chlorophyta	<i>Chlorella sorokiniana</i> <i>Chlamydomonas reinhardtii</i>		✓
Green	Chlorophyll	Chlorophyta	<i>Chlorella sorokiniana</i>	✓	
Blue	Phycocyanobilin	Cyanophyta	<i>Synechocystis</i> sp. PCC6803		✓
		Rhodophyta	<i>Cyanidioschyzon merolae</i>		

The red pigment astaxanthin is overproduced by a select number of Chlorophytes (**Fig. 2.4**) as a response to adverse growth conditions. For example, by *Chromochloris* (*Chlorella*) *zofingiensis* and *Haematococcus pluvialis*.

The orange pigment β -carotene is also overproduced by a select number of Chlorophytes, including *Dunaliella salina*, as a response to adverse growth conditions. The astaxanthin and β -carotene contents may be increased further by metabolic engineering, for example by overexpressing flux controlling enzymes, or by introducing extra storage space.

The red-orange echinenone or the yellow-orange zeaxanthin may be overproduced by *Dunaliella salina* as well, but this does require metabolic engineering, as indicated in **Table 2.2**. β -carotene, which accumulates in chloroplast-localized oil droplets under adverse growth conditions, needs to be converted into either echinenone or zeaxanthin, which may be

achieved by targeting specific β -carotene converting enzymes into the oil droplets or their membranes.

The yellow lutein may be overproduced by *Chlorella sorokiniana* (Shihira and Krauss) or *Chlamydomonas reinhardtii*, which will require metabolic engineering as well for both (**Table 2.2**). Most probably, in addition to the overexpressing of specific enzymes, additional storage space (outside the photosystem) needs to be created. *Chlorella sorokiniana* produces lutein (as a primary pigment) in a relatively high concentration (up to 0.42 % g/g dry weight (Matsukawa et al. 2000)) and has a high maximum specific growth rate (0.24 h^{-1} (Matsukawa et al. 2000)). Therefore this species might be the first target strain for genetic modification. On the other hand, *Chlamydomonas reinhardtii*, which is capable of producing lutein (as all Chlorophyta, **Fig. 2.4**), but not to a high level, has been sequenced and genetically engineered in the past (Cordero et al. 2011) and may for that reason be a good target strain.

The green chlorophylls may not need to be overproduced in order to make an algae-based production process economically feasible. Thus, the fast growing *Chlorella sorokiniana*, in which the total amount of chlorophylls (chlorophyll *a* and *b*) was measured to be 4.5 % g/g dry weight (Cuaresma et al. 2011), may be used as a production organism, grown under optimal growth conditions.

The blue phycocyanobilin may be overproduced by strains that belong to the Cyanophyta or the Rhodophyta, which will require metabolic engineering (**Table 2.2**). Example species, of which the genome has been sequenced, are *Synechocystis* sp. strain PCC6803 (Cyanophyta) and *Cyanidioschyzon merolae* (Rhodophyta).

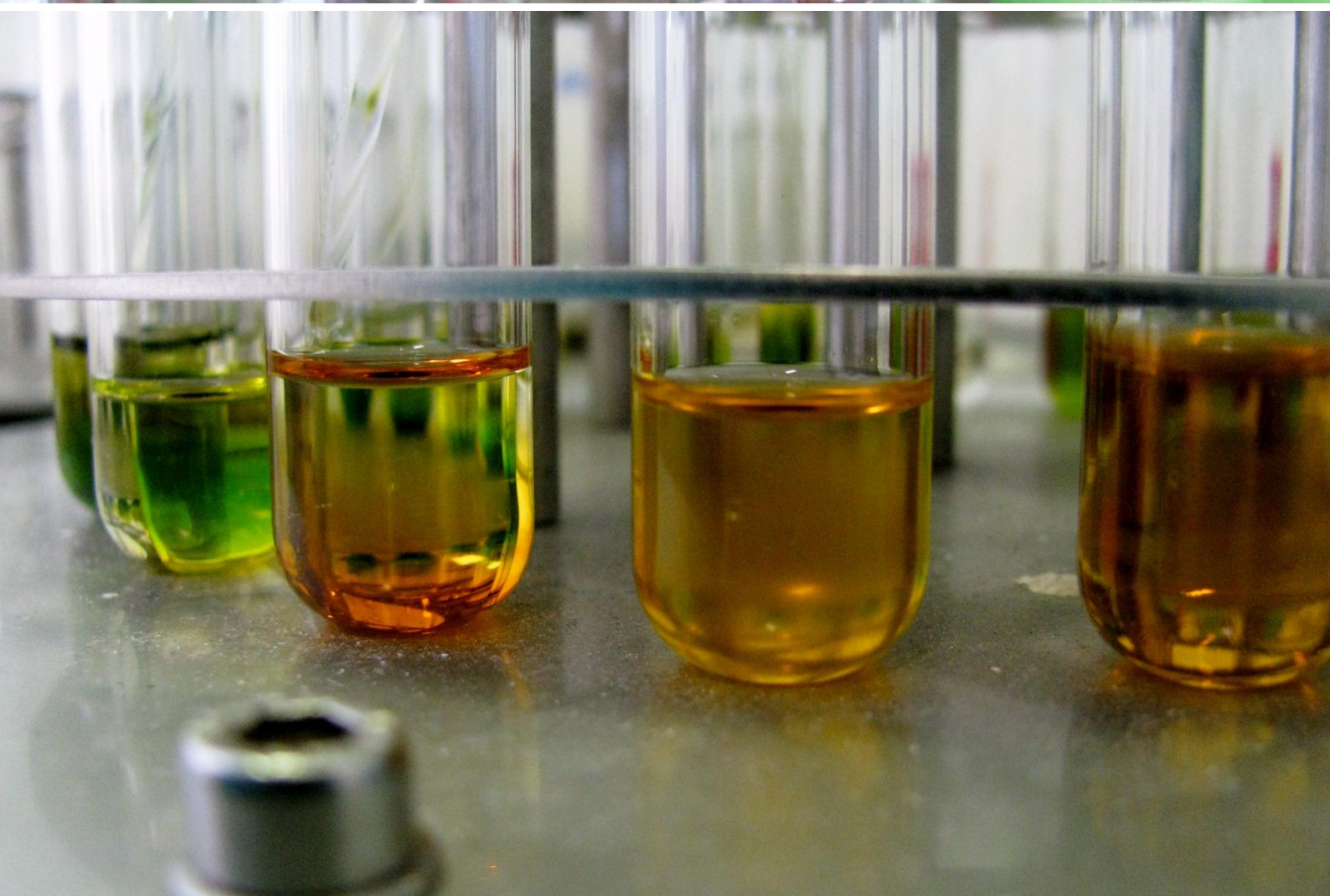
The approaches to overproduce primary pigments have in common that modifications of the metabolic pathways are required. Due to lack of knowledge on regulatory mechanisms of the cell, which generally aim for constant metabolite concentrations (homeostasis), the result of metabolic pathway modifications are largely unpredictable. To increase the predictability, and thereby the effectiveness of pathway modifications, additional research is needed which focuses on regulatory processes involved in pigment biosynthesis. In addition, a toolbox needs to be developed for stable metabolic engineering of the microalgae of choice.

Abbreviations

ADHD	Attention deficit hyperactivity disorder
NPQ	Non-photochemical quenching
PETC	Photosynthetic electron transport chain
ROS	Reactive oxygen species

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Chapter 3

Growth and pigment accumulation in nutrient-depleted *Isochrysis* aff. *galbana* T-ISO

High lights

- Nutrient-depleted *I. aff. galbana* produced echinenone and 3-hydroxyechinenone.
- The most 3-hydroxyechinenone was produced in nitrogen-depleted cultures.
- More severe limitations resulted in higher cellular 3-hydroxyechinenone contents.
- All enzymes to produce astaxanthin were active, but this carotenoid was not found.

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◀ IN THE PICTURE

Liquid chromatography, performed in silica-filled plastic columns, to separate the green chlorophylls from the yellow, orange and red carotenoids and colourless triglycides. The lipids are subsequently transesterified to form fatty acids, which are analysed by gas chromatography. The appearance of these beautifully looking pigments make this procedure a pleasure to perform!

◀ IN BEELD

Vloeistofchromatografie in met silica gevulde plastic kolommetjes om de groene chlorofylen te scheiden van de gele, oranje en rode carotenoïden en kleurloze oliën. De olie wordt omgezet in vetzuren en vervolgens geanalyseerd d.m.v. gaschromatografie. De verschijning van dit prachtige kleuren-ensemble maakt het uitvoeren van deze procedure meer dan de moeite waard!

Abstract

The effect of three different nutrient depletions (nitrogen, sulphur and magnesium) on the growth and pigment accumulation of the haptophyte *Isochrysis* aff. *galbana* (clone T-ISO) has been studied. Pigments were quantified based on RP-UHPLC-PDA-MSn analysis. All nutrient depletions led to reduced maximal biomass concentrations. Besides, all nutrient depleted cultures accumulated 3-hydroxyechinenone. To our knowledge, this is the first time that 3-hydroxyechinenone has been found in *Isochrysis* aff. *galbana* T-ISO. The most 3-hydroxyechinenone, as well as the most echinenone and diatoxanthin, were found in the nitrogen limited culture in which a more severe limitation resulted in higher cellular contents. Similar to accumulation of diatoxanthin, accumulation of 3-hydroxy-echinenone and echinenone may be part of a global (stress) response mechanism to oversaturating light conditions.

3.1 Introduction

Carotenoids such as fucoxanthin and astaxanthin derived from microalgae or plants have received much attention due to their potential health benefits (Nishino et al. 2002; Vilchez et al. 2011). For example, in animals, these pigments are reported to have anti-obesity, anti-diabetic, anti-cancer and anti-oxidative activity (Lemoine and Schoefs 2010; Maeda et al. 2008; Miyashita et al. 2011).

Naturally occurring carotenoids can be obtained from algae and higher plants. Microalgae possess a wide range of carotenoids in concentrations that can considerably exceed the concentrations found in higher plants. Besides, microalgae can be cultivated at a higher photosynthetic efficiency and higher growth rates than plants (Lamers et al. 2008; Wright and Jeffery 2006). Together, this makes microalgae an interesting source of natural carotenoids.

When microalgae grow under optimal growth conditions, these carotenoids are usually present in low concentrations (below 0.5 % g/g dry weight). Much higher concentrations (up to 10 % g/g dry weight) can be reached in some chlorophytes (green algae) when these are cultivated under adverse growth conditions, such as high light intensity, extreme temperatures or nutrient starvation (Ben-Amotz 1996; Ben-Amotz and Avron 1983; Ben-Amotz et al. 1982; Kang et al. 2007, Lamers et al. 2012). Under all these conditions, the algae experience light stress, which induces overproduction of carotenoids that have a photoprotective role *in vivo*. Most explored and exploited are the overproduction of β -carotene by *Dunaliella salina*, and astaxanthin by *Haematococcus pluvialis* (Ben-Amotz 1995; Borowitzka 1999; Olaizola 2000).

Under optimal growth conditions, the main pigments of the haptophyte *Isochrysis* aff. *galbana* T-ISO are the light harvesting pigments chlorophyll *a* and *c*, and fucoxanthin. Besides, as in all haptophytes, diadinoxanthin is present in a minor concentration (Jeffrey and Wright 2005; Liu and Lin 2001; Zapata and Garrido 1997). Diadinoxanthin can be converted into diatoxanthin under oversaturating light conditions (Lohr and Wilhelm 1999), which, for example, may result from a nutrient depletion. Both diadinoxanthin and diatoxanthin are photoprotective pigments, mediating effective quenching of excessive light energy (Goss and Jakob 2010; Telfer et al. 2008). All pigments present in *Isochrysis* aff. *galbana* T-ISO under optimal growth conditions are referred to as primary pigments, because they are functionally or structurally bound to the photosynthetic system (in the chloroplast).

One of the proposed prerequisites for carotenoid overproduction in microalgae is the formation of oil droplets. By storing the overproduced carotenoids, these oil droplets serve as a metabolic sink (Li and van Eck 2007; Rabbani et al. 1998; Zhekišheva et al. 2005). For example, in *Dunaliella salina* oil droplets accumulate in the chloroplast stroma, while in *Haematococcus pluvialis* they accumulate in the cytosol (Jin et al. 2006).

Lipid accumulation has also been observed in *Isochrysis aff. galbana* T-ISO. According to Liu and Lin (2001), this alga accumulates one or multiple oil droplets, as big as 0.5 to 3 μm , upon increasing salinity or sodium acetate addition (in comparison, the cells have a diameter of $\sim 8 \mu\text{m}$ at that stage). They reported that these lipid bodies were produced in the thylakoid space of the chloroplast and accumulated in the cytosol. Also Mairet et al. (2011) and Breuer et al. (2012) reported lipid accumulation in *Isochrysis aff. galbana* T-ISO, as a response to nitrogen depletion.

Thus, *Isochrysis aff. galbana* T-ISO can synthesise photoprotective pigments and under adverse growth conditions it is also able to produce oil droplets that may function as a metabolic sink. This makes *Isochrysis aff. galbana* T-ISO a potential candidate for overproduction of photoprotective pigments under adverse growth conditions.

Nitrogen, sulphur and magnesium are three of the main nutrients of *Isochrysis aff. galbana* T-ISO. Magnesium is incorporated mainly into chlorophyll, whereas nitrogen, which is also a constituent of chlorophyll, is incorporated mainly into proteins and nucleotides. Sulphur is also incorporated mainly into proteins (Lodish et al. 2004).

Although *Isochrysis aff. galbana* T-ISO may be a potential candidate for pigment overproduction, so far no studies have been performed on the effect of nutrient deprivation (an effective inducer of pigment accumulation in particular chlorophytes, as stated before) on the specific pigment composition of *Isochrysis aff. galbana* T-ISO. The studies performed so far on nutrient availability in this alga looked only at the effects of nitrogen depletion on total amounts of chlorophyll and carotenoids (such as Sukenik and Wahnnon 1991).

Here, we describe the effect of nitrogen, sulphur and magnesium depletions on the growth and pigment accumulation of *Isochrysis aff. galbana* T-ISO.

3.2 Materials and methods

3.2.1 Materials

Echinenone (98 %) and 3-hydroxyechinenone (99 %) were purchased from Carotenature (Lupsingen, Switzerland). Fucoxanthin (99 %) was obtained from Santa Cruz Biochemicals (Santa Cruz, CA, USA). Chlorophyll *a* (90.0 %) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Methanol (99.8 %), acetonitrile (99.97 %), ethyl acetate (99.9 %) and chloroform (99.9 %) were obtained from Biosolve (Valkenswaard, The Netherlands). Dichloromethane (99.8 %), acetone (99.8 %), n-hexane (98 %), formic acid (98.0 %), sodium chloride (99.0 %), β,β -carotene (97 %) and butylated hydroxytoluene (BHT) (99 %) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Demineralized water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA).

3.2.2 Cultivation

Isochrysis sp. CCAP 927/14 was obtained from the Culture Collection of Algae and Protozoa, Oban, UK and grown in (replete) modified F/2 medium (**Table 3.1**) (modified from Guillard and Ryther 1962). Media with a reduced or zero concentration of a nutrient, from now on both referred to as depleted medium, were prepared similarly, with the exception that the respective nutrient was added in lower amounts or omitted (**Table 3.1**). To retain a total osmolality of 1.38 Osm, the depleted media contained additional NaCl or KCl.

Table 3.1 Composition of the different modified F/2 media. Dots indicate amounts that equal replete medium.

Compound	Concentration ($\mu\text{mol L}^{-1}$)						
	Replete	Low ^a N	No N	Low ^b S	No S	Low ^b Mg	No Mg
NaCl	$419.21 \cdot 10^3$	$449.61 \cdot 10^3$	$451.21 \cdot 10^3$	$452.97 \cdot 10^3$	$453.01 \cdot 10^3$	$491.50 \cdot 10^3$	$491.56 \cdot 10^3$
NaNO ₃	$32.00 \cdot 10^3$	$1.60 \cdot 10^3$	0.00
Na ₂ SO ₄	$22.53 \cdot 10^3$.	.	$0.03 \cdot 10^3$	0.00	.	.
K ₂ SO ₄	$4.88 \cdot 10^3$.	.	$0.01 \cdot 10^3$	0.00	.	.
KCl	0.00	.	.	$7.31 \cdot 10^3$	$7.32 \cdot 10^3$.	.
NaH ₂ PO ₄ ·2H ₂ O	$2.50 \cdot 10^3$
MgCl ₂ ·6H ₂ O	$48.23 \cdot 10^3$	$0.04 \cdot 10^3$	0.00
CaCl ₂ ·2H ₂ O	$3.61 \cdot 10^3$
NaHCO ₃	$10.00 \cdot 10^3$
HEPES	$100.00 \cdot 10^3$
EDTA ferric sodium salt	27.79
Na ₂ EDTA·2H ₂ O	30.01
CuSO ₄ ·5H ₂ O	0.04
Na ₂ MoO ₄ ·2H ₂ O	0.03
ZnSO ₄ ·7H ₂ O	0.08
CoCl ₂ ·6H ₂ O	0.08
MnCl ₂ ·4H ₂ O	0.91
Vitamins							
Thiamine	200.00
Biotine	1.00
Cyanocobalamine	1.00

^a5 % of replete medium. ^b1 % of replete medium.

Cultures were pre-cultivated in 250-mL shake flasks, containing 100 mL replete medium, that were placed in a culture chamber at 25 °C, a light intensity of 20-40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a 16/8 h day/night cycle, continuously shaken at 150 rpm and at atmospheric CO₂ pressure.

Single experiments were performed in which cells were first washed twice with replete medium (control) or one of the depleted media as described in **Table 1**. Subsequently, the cells were inoculated in 250-mL shake flasks containing 120 mL of either the replete or one of the depleted media, at a cell concentration of $\sim 2 \cdot 10^5$ cells mL⁻¹. Cells were continuously shaken at 175 rpm and 25 °C, under continuous light from white fluorescent tubes (Sylvania CF-LE 55W/840, Germany) with an intensity of 32-41 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The headspace consisted

of an air/CO₂ ratio of 97.2/2.8 %. Every two to three days, a sample of 5 mL was taken for optical density measurements, cell counts and pigment extraction and quantification.

3.2.3 Biomass determination

Cell growth was monitored by determining the optical density in duplicate. For this, 1 ml of sample was transferred to a 24-wells microplate. Subsequently, the absorbance was measured at 750 nm using a BioTek EL800 microplate reader. As a comparison, the absorbance levels measured with this microplate reader are typically ~5 times lower than those measured with a regular spectrophotometer.

Cell numbers were determined with a Beckman Coulter Multisizer 3 as described by Kliphuis et al. (2011).

3.2.4 Pigment extraction

Pigment extractions were performed using methanol/chloroform as solvent as described by Lamers et al. (2010), with the exception that after sampling the cells were centrifuged for 10 min at 800×g. Dried pigment extracts were resolved in acetone : methanol 7 : 3 (v/v) containing 0.10 % (w/v) BHT and supplemented to 75 % (v/v) acetonitrile for LC analysis.

3.2.5 Separation and identification of pigments

Separation and identification of carotenoids and porphyrins was performed using a Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with pump, degasser, auto sampler, photo diode array (PDA) detector and coupled *in-line* to a LTQ-Velos dual-pressure ion trap mass spectrometer equipped with a H-ESI probe (Thermo Scientific). Samples (3.0 µL) were injected on a Aquity UPLC Shield C18 BEH column (2.1 × 150 mm, 1.7 µm particle size; Waters, Milford, MA, USA) fitted to a Vanguard pre-column (2.1 × 5 mm, 1.7 µm particle size; Waters). The eluents were (A) 50 % (v/v) acetonitrile in demineralized water, (B) acetonitrile and (C) ethyl acetate, which all contained 0.10 % (v/v) formic acid. The flow rate was maintained at 300 µL min⁻¹. The program was started from 50 % A / 50 % B and then as follows: to 7.5 min – linear gradient to 100 % B; to 15.0 min – isocratic at 100 % B; to 20.0 min – linear gradient to 100 % C; to 24.0 min – isocratic at 100 % C. After 25 min, the eluent composition returned to its initial composition in 5 min, followed by an equilibration phase of 5 min. Detection wavelengths for UV-Vis were set at 450 nm (carotenoids and chlorophyll c) and 660 nm (porphyrins) with a 1 nm wavelength step and 9 nm filter bandwidth.

All mass spectrometric data was recorded in positive ion mode. Nitrogen was used as both sheath gas (30 arbitrary units) and auxiliary gas (5 arbitrary units). Most settings were optimized via automatic tuning using 'Tune Plus' (Xcalibur 2.10, Thermo Scientific). The system was tuned using direct inflow of 290 µL min⁻¹ 50 % A / 50 % B with 10 µL min⁻¹ direct injection of a mixture of fucoxanthin, chlorophyll *a*, echinenone and 3-hydroxyechinenone standards (all approximately 1 µg mL⁻¹) in acetonitrile : ethyl acetate 3 : 1 (v/v). The heated ESI probe was set at 400 °C, the ion transfer tube was set at 300 °C, and the source voltage was 3.5 kV. Data was recorded over the *m/z* range 500 - 700 (0 - 18.0 min) and 500 - 1000 (18.0 - 25.0 min).

Dynamic data-dependent MS² fragmentation was performed on the most intense parent ion (*n* = 1), subsequently on the second most intense parent (*n* = 2), etc.. A repeat count of two

MS² spectra per parent ion with a maximum of $n = 25$ or within a time frame of 5.0 s were used as settings. The collision induced dissociation (CID) was set to 35 %. Data acquisition and reprocessing were performed with Xcalibur 2.10 (Thermo Scientific).

3.2.6 Quantification of carotenoids and porphyrins

Fucoxanthin and chlorophyll *a* standards were first dissolved in ethyl acetate, and subsequently diluted with 3 volumes of 100 % and 90 % (v/v) aqueous acetone, respectively. Echinenone and 3-hydroxyechinenone were first dissolved in dichloromethane, and subsequently diluted with 3 volumes of *n*-hexane. Standard concentrations were measured prior to UHPLC analysis, using their respective absorption coefficients ($A_{1\text{cm}}^{1\%}$): 1660 L g⁻¹ cm⁻¹ (443 nm, 100 % acetone) for fucoxanthin, 887 L g⁻¹ cm⁻¹ (664 nm, 90 % (v/v) aqueous acetone) for chlorophyll *a*, 2160 L g⁻¹ cm⁻¹ (458 nm, 100 % *n*-hexane) for echinenone, and 2277 L g⁻¹ cm⁻¹ (465 nm, 100 % *n*-hexane) for 3-hydroxyechinenone (Britton 1995a; Roy et al. 2011). PDA calibration was performed using six different concentrations of the standards injected in duplicate. For this calibration, the response of the all-*trans* and *cis* form of the carotenoid standards was considered equal for quantification. The detector was found to be linear for all standards between 0.05 and 39.00 µg mL⁻¹ with a minimum R² of 0.99. Diadinoxanthin and diatoxanthin were expressed via fucoxanthin using the fucoxanthin calibration curve, and the responses were corrected using the $A_{1\text{cm}}^{1\%}$ of 2240 (448 nm) and 2720 L g⁻¹ cm⁻¹ (453 nm), respectively (Roy et al. 2011). Peak areas for chlorophyll *c*₁ and *c*₂ were calculated at 630 nm and converted to concentrations using a chlorophyll *a* calibration curve with correction of the responses using the $A_{1\text{cm}}^{1\%}$ of 448 (631 nm) and 404 L g⁻¹ cm⁻¹ (631 nm), respectively (Jeffrey and Humphrey 1975). Deconvolution of fucoxanthin and chlorophyll *c*₁ was performed through estimation of the chlorophyll *c*₁ fraction of the total peak area at 450 nm. The latter was done through conversion of the measured chlorophyll *c*₁ peak area at 630 nm into the corresponding peak area at 450 nm, using absorption coefficients of 448 (631 nm) (Jeffrey and Humphrey 1975) and 3180 L g⁻¹ cm⁻¹ (443 nm) (Roy et al. 2011). Adequacy of this conversion was supported by the linearity (R² = 0.95) observed between a similar conversion and the directly measured peak areas at 450 nm of the non-coeluting chlorophyll *c*₂ ($A_{1\text{cm}}^{1\%} = 404$ L g⁻¹ cm⁻¹ (631 nm) (Jeffrey and Humphrey 1975) and 3740 L g⁻¹ cm⁻¹ (444 nm) (Roy et al. 2011), over all samples tested.

3.3 Results and discussion

To study the effect of nitrogen, sulphur and magnesium depletions on the growth and pigment accumulation of *Isochrysis* aff. *galbana* T-ISO, two run-out experiments were performed for each nutrient, starting with either zero or a low nutrient concentration. The low nutrient concentrations were chosen in such a way that these cultures would reach nutrient depletion at an optical density far below the maximal optical density that could be reached in the replete medium (control), which was based on the putative elemental compositions described by Fabregas and Herrero (1986), Falkowski and Raven (2007) and Ho et al. (2003). Pigment contents were analysed four times during cultivation.

3.3.1 Growth

Starting from an OD₇₅₀ of approximately 0.2, the control reached a final OD₇₅₀ of 0.5. The nitrogen, sulphur and magnesium depleted cultures reached an optical density far below 0.5

(Fig. 3.1), which confirms that the shortage of these nutrients limited the maximal biomass concentration.

The cultures without sulphur, nitrogen, or magnesium increased in OD_{750} with 0.05, 0.09 and 0.19, respectively. These increases might be explained by assuming that during pre-cultivation, when nutrients were in abundance, the cells consumed more nutrients than needed for growth (luxury consumption), or that photosynthesis and carbon fixation continued in absence of these nutrients. The latter has been described for *Isochrysis* aff. *galbana* T-ISO by Breuer et al. (2012), who observed in nitrogen depleted conditions an increase in biomass of 40 % and accumulation of triacylglycerol.

In conclusion, for all depleted cultures, starvation was attained for the culture starting with zero, as well as for the culture starting with a low nutrient concentration.

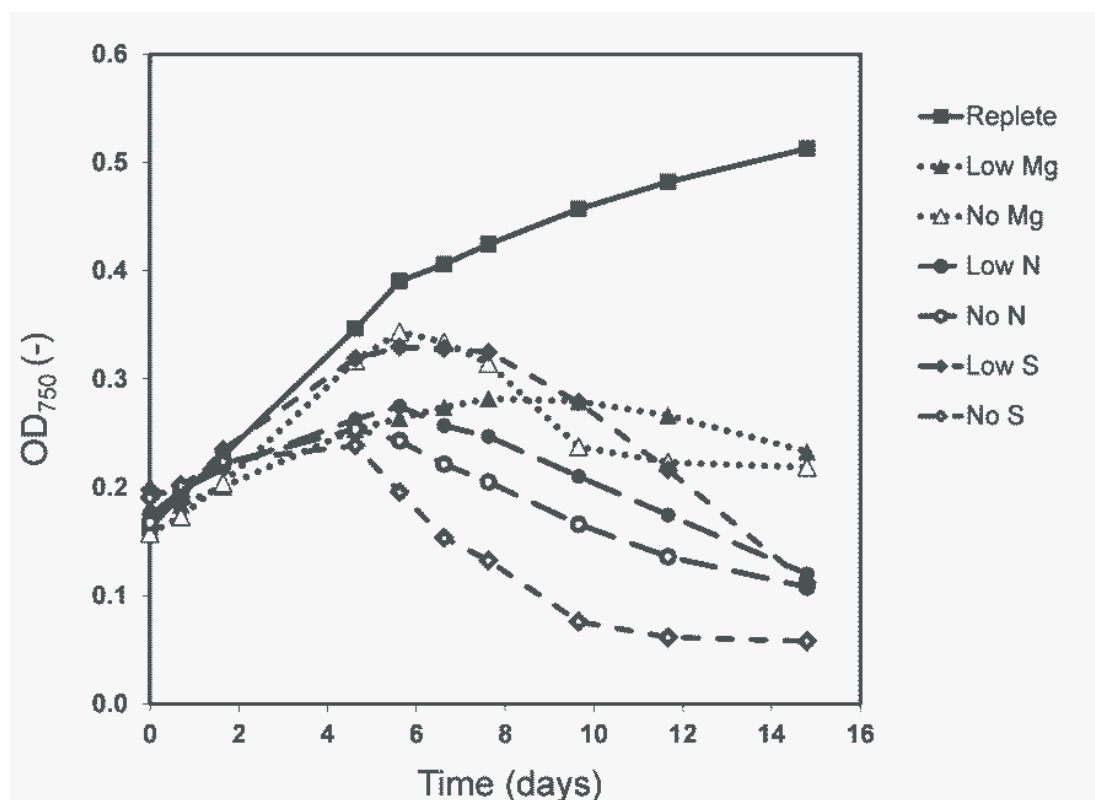


Fig. 3.1 Growth curves of *Isochrysis* aff. *galbana* T-ISO, grown under three single nutrient depletions. Absolute deviations from the mean of duplicate OD_{750} measurements were all less than 5 %.

3.3.2 Pigment identification

The chromatogram of a representative *Isochrysis* aff. *galbana* T-ISO extract is presented in Fig. 3.2, and peaks are annotated in Table 3.2. Fucoxanthin, 3-hydroxyechinenone, echinenone and chlorophyll *a* had matching retention times, absorption spectra, parent masses and fragmentation patterns as their respective standards. Fucoxanthin geometrical isomers (**peak 4 and 5**) were identified by small hypsochromic shifts (2 to 6 nm) in their absorption maximum and the presence of a *cis*-peak at approximately 133 nm from the absorption maximum (in accordance with Haugan et al. (1992)). In addition, MS^2 fragmentation of the sodium adduct of all-*trans* and *cis*-fucoxanthins all showed clear individual and combined losses of the esterified acetic acid (NL 60 Da) group, the 5,6-epoxy- β -ring (NL 154 Da), and the in-polyene toluene

elimination (NL 92 Da), which supports the assignment of *cis*-fucoxanthin to peak 4 and 5 (Airs and Llewellyn 2006). Often, all-*trans* fucoxanthin is reported to be the only naturally occurring fucoxanthin form, as for example in brown algae (Haugan and Liaaen-Jensen 1994).

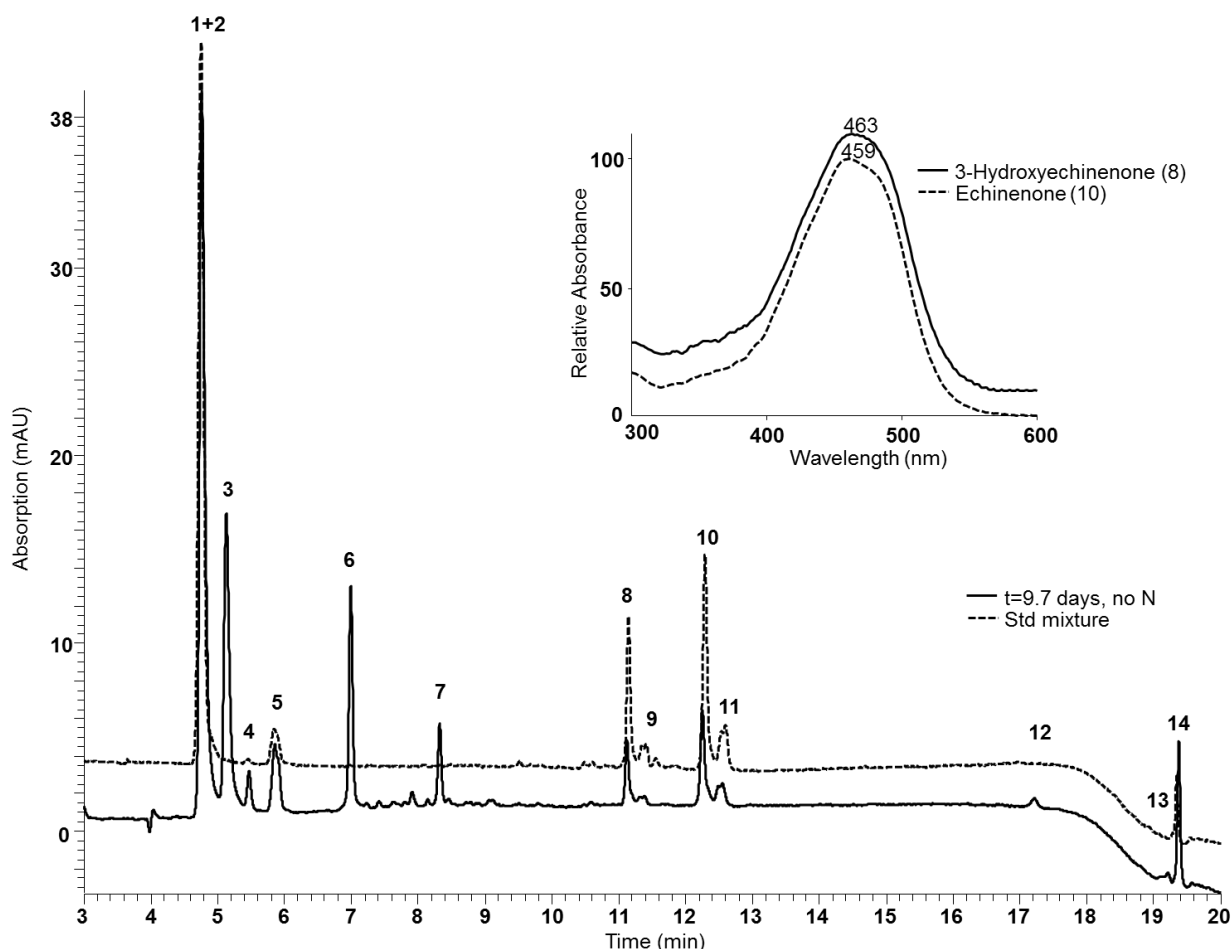


Fig. 3.2 Overlay of a representative RP-UHPLC chromatogram (450 nm) of nitrogen depleted *Isochrysis aff. galbana* T-ISO (no nitrogen, at $t=9.7$ days), and a standard mixture. Insert: absorption spectra of 3-hydroxyechinenone (**peak 8**) and echinenone (**peak 10**).

For *Isochrysis aff. galbana* T-ISO, we assumed that all-*trans* fucoxanthin was also the only fucoxanthin isomer present, although this has not been explicitly reported. Isomerisation of fucoxanthin in both samples and standards was thus assumed to be due to sample processing, as reported earlier (Haugan and Liaaen-Jensen 1992). Chlorophyll c_1 , chlorophyll c_2 , diadinoxanthin, and diatoxanthin had matching absorption spectra, parent masses and fragment ions as found in literature (Roy et al. 2011).

The parent mass of chlorophyll a appeared at m/z 871, showing a similar mass and fragmentation as pheophytin a . This implied that the magnesium ion is ejected in-source and replaced by two protons. Present in trace amounts, peak 13 was identified as a chlorophyll a allomer, which is a well-known auto-oxidation product (Walker et al. 2003).

Table 3.2 Compounds tentatively assigned in the extracts of *Isochrysis aff. galbana* T-ISO by UHPLC/ESI-MS

Peak No.	Compound	t _r (min)	λ _{max} (nm)	Ionization type	Quasi-molecular ion (m/z)	MS ² neutral loss in Da (% rel. intensity)	Ref
1	All- <i>trans</i> fucoxanthin	4.76	447, (465)	[M+Na] ⁺	681.3	[M+Na-18] ⁺ (50), [M+Na-60] ⁺ (40), [M+Na-18-60] ⁺ (80), [M+Na-92] ⁺ (30), [M+Na-154] ⁺ (100), [M+Na-154-18] ⁺ (30), [M+Na-154-60] ⁺ (20), [M+Na-154-60-18] ⁺ (25)	1, 2
2	Chlorophyll <i>c</i> ₁	4.80	447, 631 ^a	[M] ⁺⁺	611.3	[M-18] ⁺⁺ (100), [M-44] ⁺⁺ (40), [M-60] ⁺⁺ (70), [M-76] ⁺⁺ (15), [M-77] ⁺⁺ (15)	2
3	Chlorophyll <i>c</i> ₂	5.13	450, 582, 631	[M] ⁺⁺	609.2	[M-18] ⁺⁺ (100), [M-44] ⁺⁺ (30), [M-60] ⁺⁺ (40), [M-76] ⁺⁺ (10), [M-77] ⁺⁺ (10)	2
4	<i>Cis</i> -fucoxanthin	5.47	445, (460)	[M+Na] ⁺	681.4	Idem 1	1, 5
5	<i>Cis</i> -fucoxanthin	5.86	332, 442, (460)	[M+Na] ⁺	681.4	Idem 1	1, 5
6	All- <i>trans</i> Diadinoxanthin	7.00	423, 447, 477	[M+H] ⁺	583.4	[M+H-18] ⁺ (100), [M+H-36] ⁺ (20), [M+H-74] ⁺ (10), [M+H-92] ⁺ (30), [M+H-362] ⁺ (15)	2
7	All- <i>trans</i> Diatoxanthin	8.32	(432), 452, 480	[M+H] ⁺	567.4	[M+H-18] ⁺ (90), [M+H-60] ⁺ (60), [M+H-74] ⁺ (50), [M+H-88] ⁺ (40), [M+H-92] ⁺ (20)	2
8	All- <i>trans</i> 3-hydroxyechinenone	11.12	463	[M+H] ⁺	567.4	[M+H-18] ⁺ (60), [M+H-56] ⁺ (50), [M+H-60] ⁺ (40), [M+H-92] ⁺ (100), [M+H-136] ⁺ (70)	3
9	<i>Cis</i> -3-hydroxyechinenones ^c	11.31-11.38	365, 457 ^b	[M+H] ⁺	567.3	[M+H] ⁺ (100), [M+H-18] ⁺ (60), [M+H-56] ⁺ (40), [M+H-60] ⁺ (50), [M+H-92] ⁺ (80), [M+H-136] ⁺ (60)	
10	All- <i>trans</i> echinenone	12.25	459	[M+H] ⁺	551.4	[M+H] ⁺ (60), [M+H-1] ⁺ (100), [M+H-18] ⁺ (15), [M+H-56] ⁺ (30), [M+H-92] ⁺ (50)	2
11	<i>Cis</i> -echinenones ^c	12.49-12.56	362, 455	[M+H] ⁺	551.4	[M+H] ⁺ (60), [M+H-1] ⁺ (100), [M+H-18] ⁺ (10), [M+H-56] ⁺ (40), [M+H-92] ⁺ (30)	
12	All- <i>trans</i> β,β-carotene ^d	17.23	454, 478	ND	ND	ND	3
13	MeO chlorophyll <i>a</i> allomer or 15 ³ -MeO-lact-chl <i>a</i>	18.87	412, 430, 660	[M] ⁺⁺	907.6/939.5	[M-278] ⁺⁺ (100), [M-278-32] ⁺⁺ (20), [M-278] ⁺⁺ (100), [M-278-32] ⁺⁺ (60)	4
14	Chlorophyll <i>a</i>	19.37	381, 413, 430, 581, 613, 662	[M-Mg+2H] ⁺	871.6	[M-Mg+2H-278] ⁺ (100), [M-Mg+2H-278-60] ⁺ (50)	2

^aMixed spectrum with fucoxanthin. ^bAbsorption spectrum determined from standard. ^cCo-elution of *cis*-carotenoids. ^dSpectrum and retention time match with chemical standard. ND: not determined. 1. Airs and Llewellyn 2006, 2. Roy et al. 2011, 3. Britton et al. 2004, 4. Walker et al. 2003, 5. Haugan et al. 1992.

The absorption spectra of 3-hydroxyechinenone was red shifted compared to echinenone (**inset Fig. 3.2**), due to participation of the hydroxy group at the C₃ position in the conjugated system of the molecule (Britton 1995a). Other hydroxyechinenones with hydroxy groups at the C₂, C_{3'}, and C_{4'} were reported to have similar absorption maxima as echinenone (Britton et al. 2004), as the hydroxy group did not interact with the conjugated system. The C_{2'} hydroxyl variant of hydroxyechinenone has not been reported to our knowledge. MS data confirmed the attachment of a hydroxy group and a more pronounced water loss compared to echinenone. The neutral loss of 136 Da for 3-hydroxyechinenone could not be assigned. *Cis* isomerisation was observed for both 3-hydroxyechinenone and echinenone with *cis* peaks found at 365 and 362 nm, respectively.

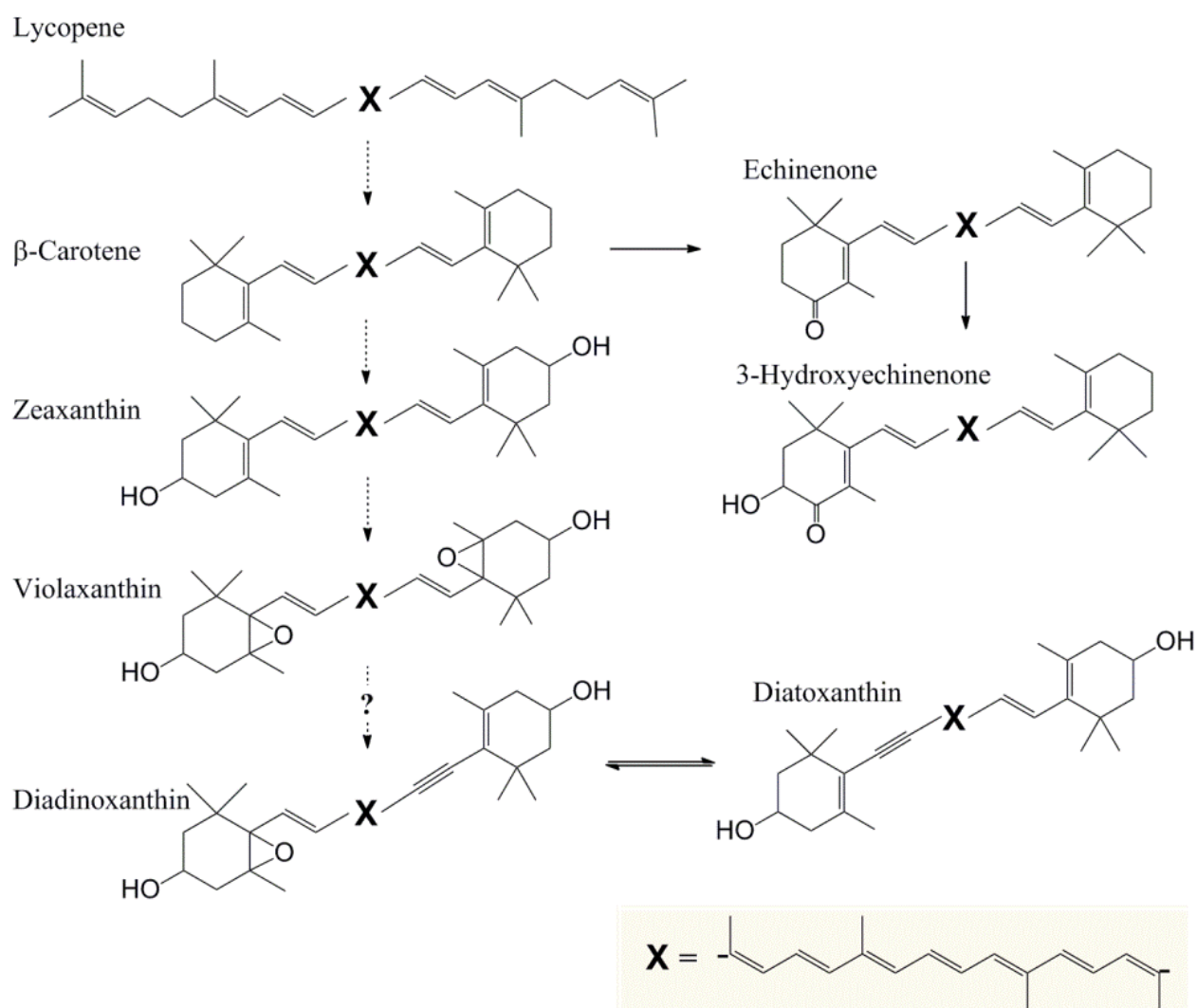


Fig. 3.3 Proposed routes of carotenoid biosynthesis (starting from lycopene) by *Isochrysis aff. galbana* T-ISO. Solid arrows represent single conversion steps, dashed arrows represent lumped reactions and the question mark indicates a putative (not proven) pathway (Kanehisa Laboratories 2011; Lemoine and Schoefs 2010; Lohr and Wilhelm 1999, 2001).

To our knowledge, this is the first time that 3-hydroxyechinenone was found in *Isochrysis aff. galbana* T-ISO. Although one could suggest that this component could be an extraction artefact, previous reports on usage of the method of Lamers et al. (2010) did not show (auto-) oxidative conversion of ββ-carotene to neither echinenone nor a hydroxyechinenone.

Therefore, the presence of 3-hydroxyechinenone in the extract is considered to originate from biosynthesis by *Isochrysis* aff. *galbana* T-ISO.

Based on these identifications, a proposal for the carotenoid biosynthesis routes in *Isochrysis* aff. *galbana* T-ISO may be postulated (Fig. 3.3), assuming that echinenone and 3-hydroxyechinenone are derivatives of β -carotene, as described for the chlorophyte *Haematococcus pluvialis* (Lemoine and Schoefs 2010) and assuming that diadinoxanthin and diatoxanthin are derivatives of violaxanthin, which was suggested, but not proven, by Lohr and Wilhelm (1999, 2001). Lohr and Wilhelm (1999, 2001) also suggested, but did not prove, that fucoxanthin is a derivative of violaxanthin (not shown in Fig. 3.3).

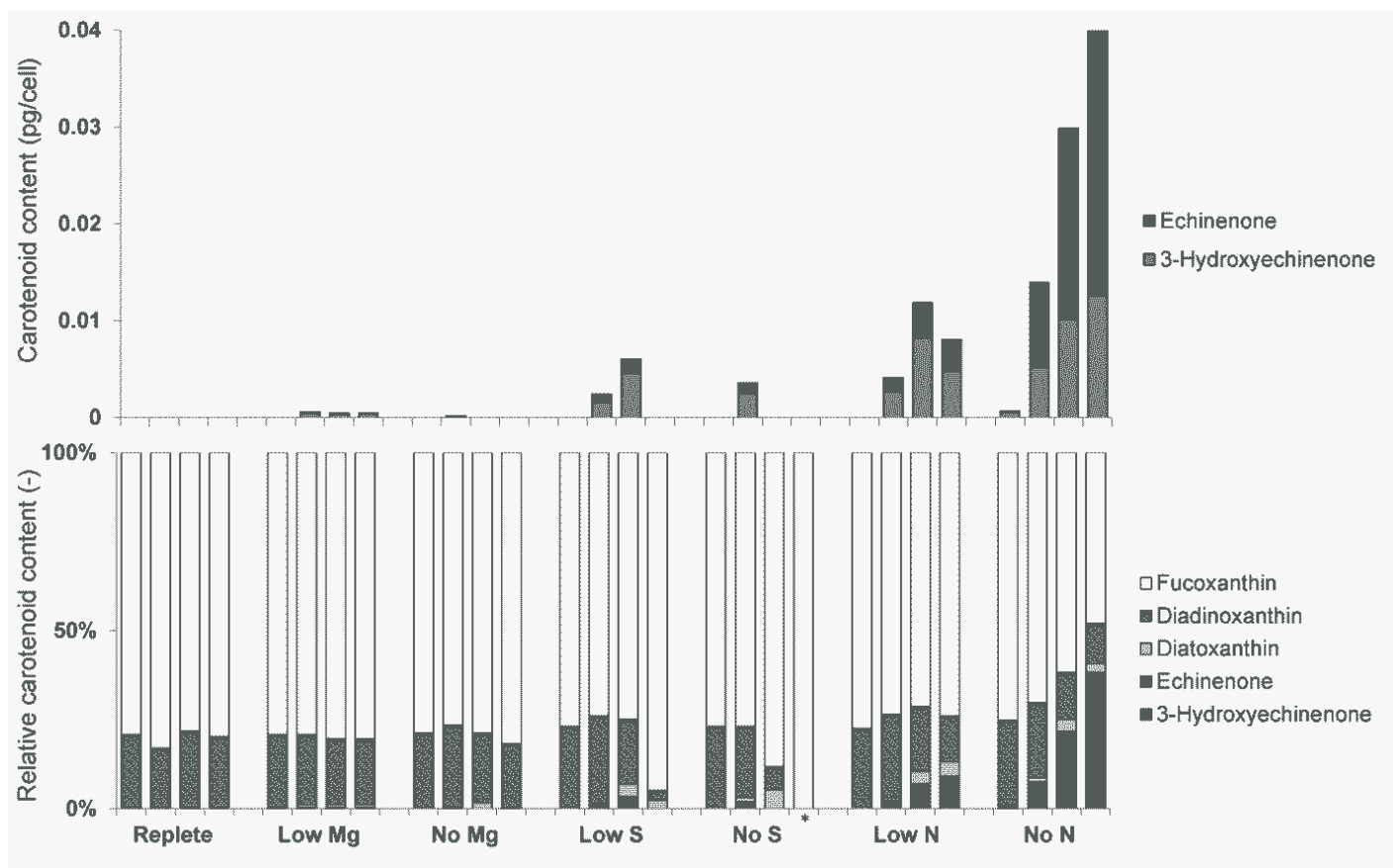


Fig. 3.4 Absolute echinenone and 3-hydroxyechinenone contents, expressed in pg cell^{-1} (top), and relative fucoxanthin, diatoxanthin, diadinoxanthin, echinenone and 3-hydroxyechinenone contents, expressed as percentages of the total carotenoid pool (bottom), in *Isochrysis* aff. *galbana* T-ISO resulting from different nutrient depletions. Columns one to four of each nutrient depletion represent samples taken after 0.7, 4.6, 9.7 and 14.8 days, respectively. Carotenoids found below quantification limit are not shown. *Culture contained mainly cell debris.

3.3.3 Pigment accumulation

The main pigments found in the control were the light harvesting pigments chlorophyll *a*, *c*₁ and *c*₂, and fucoxanthin. Cells from the control also contained the pigments diadinoxanthin and diatoxanthin, in minor and trace amounts (below quantification limit), respectively.

The ratio of total carotenoids to total chlorophylls was steady in all cultures. Only when growth was highly reduced (i.e. at the end of the sulphur and nitrogen depleted cultures), this ratio

increased from 0.43:1 to 0.91:1, which is a common observation in microalgae (Flynn et al. 1993; Ip et al. 2004; Sukenik and Wahnou 1991).

Upon nutrient depletion, diatoxanthin started to exceed the trace amounts. The highest diatoxanthin amounts were found in the cultures that were limited by sulphur and nitrogen. Considering that these nutrient depletions inevitably had led to oversaturating light conditions, these results were in agreement with Lohr and Wilhelm (1999), who reported that prolonged oversaturating light conditions induce the de-epoxidation of diadinoxanthin into diatoxanthin.

Whereas 3-hydroxyechinenone was found in all treated cultures, echinenone was found only in the nitrogen and sulphur depleted cultures (**Fig. 3.4**). The contents of echinenone and 3-hydroxyechinenone were highest in the nitrogen and sulphur depleted cultures. In the nitrogen depleted culture, a more severe limitation led to higher levels of these pigments. This was not the case in the sulphur depleted cultures, which may be explained by the observed rapid lysis of these cells, resulting in cultures containing a lot of cell debris. Although also the magnesium depletions resulted in growth reduction, accumulation of 3-hydroxyechinenone in these cultures was very low. Since 3-hydroxyechinenone was found in all treated cultures, this metabolite may well be part of a global (stress) response mechanism to nutrient-depletion-induced oversaturating light conditions.

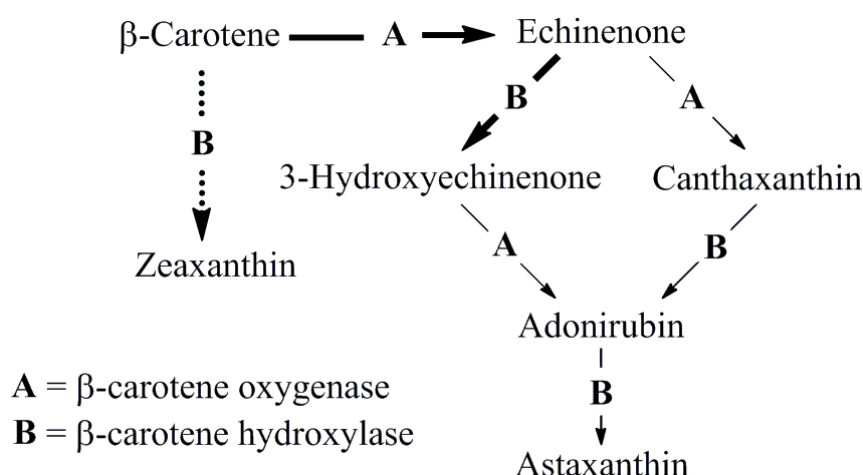


Fig. 3.5 Proposed routes of zeaxanthin and astaxanthin biosynthesis in *Haematococcus pluvialis*, including enzymes involved. **Solid arrows** represent single conversion steps, **dashed arrows** represent lumped reactions (Lemoine and Schoefs 2010). **Bold arrows** represent routes proposed to be present in *Isochrysis aff. galbana* T-ISO.

In *Haematococcus pluvialis*, a chlorophyte which is known for the overproduction of astaxanthin under oversaturating light conditions (up to 7 % g/g dry weight (Kang et al. 2007)), echinenone and 3-hydroxyechinenone are intermediates of the astaxanthin biosynthetic pathway (**Fig. 3.5**). The astaxanthin, which is accumulated in lipid globules located in the cytosol, performs a photoprotective role through the absorption of oversaturating amounts of light, which prevents cell damage (Lemoine and Schoefs 2010). In *Haematococcus pluvialis*, the conversions of β-carotene into echinenone and 3-hydroxyechinenone are catalysed by β-carotene oxygenase and β-carotene hydroxylase, respectively. These enzymes are also responsible for the subsequent conversions of 3-hydroxyechinenone into astaxanthin (via

adonirubin), the conversion of echinenone into astaxanthin via canthaxanthin and adonirubin, and the conversion of β -carotene into zeaxanthin (**Fig. 3.5**). Although the identification of both echinenone and 3-hydroxyechinenone in *Isochrysis* aff. *galbana* T-ISO suggests the presences of all enzymes needed for astaxanthin production (**Fig. 3.5**), no astaxanthin was detected in any of the treated cultures. The reason for the absence of this photoprotective pigment remains elusive, but may possibly explain why initial nutrient depleted growth rates of *Isochrysis* aff. *galbana* T-ISO are much lower compared to those of the astaxanthin accumulating *Haematococcus pluvialis* and *Chromochloris (Chlorella) zofingiensis* (Breuer et al. (2012); Grewe and Griehl (2008)).

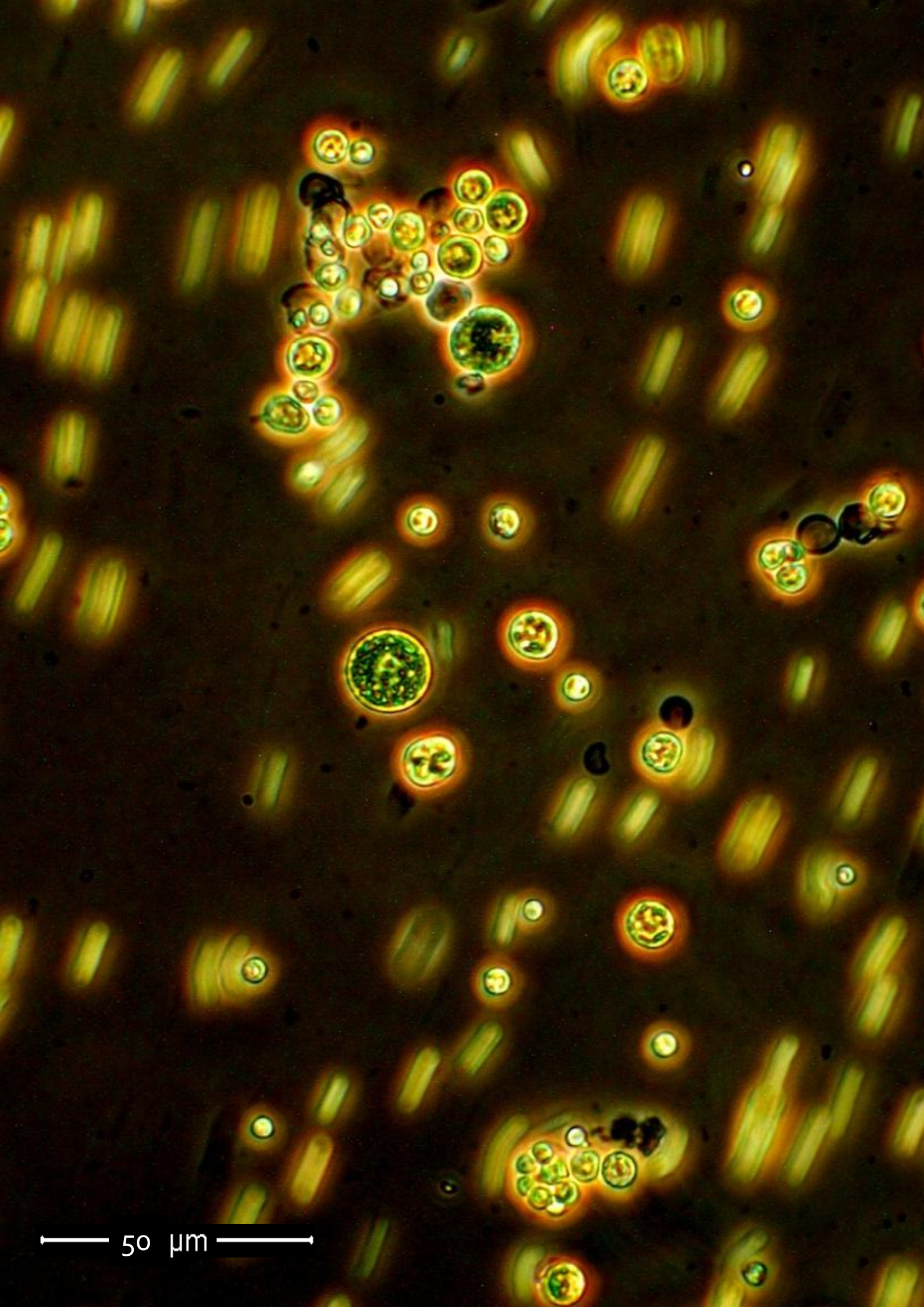
Both Breuer et al. (2012) and Mairet et al. (2011) reported lipid accumulation (triacylglycerol) by *Isochrysis* aff. *galbana* T-ISO, as a response to nitrogen starvation. As stated in the introduction, accumulated lipid globules can function as a metabolic sink for carotenoids, resulting in overproduction of secondary carotenoids (as is the case in e.g. *Haematococcus pluvialis* (Lemoine and Schoefs 2010)). We expect that, due to the low amounts of echinenone and 3-hydroxyechinenone, it will be hard to evaluate whether these carotenoids found in our nutrient starved cultures accumulated in lipid globules or elsewhere in the cell. The observed low amounts do suggest however that oil-droplet-mediated sequestration, if occurring at all, was not very effective in inducing overproduction of echinenone and 3-hydroxyechinenone to the same extent as observed for β -carotene in *D. salina* and astaxanthin in *H. pluvialis*.

3.4 Conclusion

All nutrient depleted cultures accumulated 3-hydroxyechinenone. This is the first time that this metabolite was identified in *Isochrysis* aff. *galbana* T-ISO. The most 3-hydroxyechinenone, as well as the most echinenone and diatoxanthin, were found in the nitrogen limited culture in which a more severe limitation resulted in higher cellular contents. Similar to the accumulation of diatoxanthin (Lohr and Wilhelm 1999), accumulation of 3-hydroxyechinenone and echinenone may be part of a global (stress) response mechanism to oversaturating light conditions.

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Chapter 4

Nitrogen-depleted *Chromochloris zofingiensis* produces astaxanthin, ketolutein and their fatty acid esters: a carotenoid metabolism study

High lights

- N-depleted *C. zofingiensis* produced astaxanthin, ketolutein and canthaxanthin.
- The astaxanthin was presumably synthesised via echinenone, not via zeaxanthin.
- Inhibition of β -carotene ketolase led to reduced production of β -carotene derivatives.
- But it did not lead to changes in ketolutein production or degradation of primary pigments.

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* Both authors contributed equally

Nitrogen-depleted Chlorella zofingiensis produces astaxanthin, ketolutein and their fatty acid esters: a carotenoid metabolism study

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◀ IN THE PICTURE

Chromochloris (Chlorella) zofingiensis cells visualised by an optical (light) microscope (400x enlarged). Most cells move with a high speed (blurred); others lay still (sharp). Although these microalgae form a clone of single cells, holding the same DNA, their sizes are different. The small ones are about 3 μm , the big ones are about 10 μm . This variation is caused by the differences in growth phase!

◀ IN BEELD

Chromochloris zofingiensis cellen gefotografeerd door een lichtmicroscop (400x vergroot). De meeste cellen razen in hoog tempo voorbij (wazig); anderen liggen stil (scherp). Hoewel ze allemaal eencellig zijn, een kloon met hetzelfde erfelijke materiaal, variëren ze wel in grootte. De kleintjes zijn zo'n 3 μm , de grote zo'n 10 μm . Dit komt doordat ze niet allemaal in dezelfde groeifase zitten!

Abstract

Natural carotenoids such as astaxanthin, β -carotene (in this chapter referred to as β,β -carotene) and lutein are pigments with a high market value. We studied the effects of nitrogen depletion on the carotenoid metabolism of *Chromochloris (Chlorella) zofingiensis* (Chlorophyta) and the subsequent treatment with diphenylamine (DPA), an inhibitor of the biosynthesis of secondary ketocarotenoids. Pigments were identified and quantified based on reversed phase ultra-high performance liquid chromatography photo diode array tandem mass spectrometry (RP-UHPLC-PDA-MSⁿ). Nitrogen depletion (without DPA) resulted in a degradation of chlorophylls and primary carotenoids and an accumulation of astaxanthin, ketolutein, canthaxanthin, adonixanthin, and β,β -carotene. The DPA treatment decreased the overall production of β,β -carotene derivatives (sum of astaxanthin, canthaxanthin, echinenone and adonixanthin), however, the production of ketolutein and degradation of primary carotenoids were not modified. This suggests that the regulatory mechanisms controlling the flux towards ketolutein and primary carotenoids were not affected by the decreased levels of β,β -carotene derivatives. In addition, DPA increased production of the individual carotenoids adonixanthin and echinenone. Insight into the regulation of microalgal carotenoid biosynthesis as demonstrated in this paper is essential when a large-scale carotenoid production process is to be optimized or a recombinant *C. zofingiensis* strain is to be designed with the intention of excessively producing primary or secondary carotenoids.

4.1 Introduction

Natural carotenoids such as astaxanthin, β -carotene (in this chapter referred to as β,β -carotene) and lutein are pigments with a high market value. It has been claimed that these carotenoids beneficially affect health due to their anti-oxidative activity (Spolaore et al. 2006). Possible production organisms of naturally derived carotenoids that have gained significant attention are microalgae, because they contain an extensive number of various carotenoids, part of which can be produced in concentrations exceeding those ascertained in higher plants by one or more orders of magnitude. Nevertheless, economically feasible microalgal carotenoid production will require process and/or strain optimization (Mulders et al. 2014a).

Microalgal carotenoids can be categorized into primary and secondary carotenoids. Primary carotenoids are functionally bound to the photosystems inside the chloroplast, whereas secondary carotenoids are not bound to the photosystems. An even more significant difference is their concentration under various growth conditions. Under prolonged limited-light growth conditions, secondary carotenoids are generally absent or present only in extremely low concentrations, and primary carotenoids are generally present in their maximal concentration (typically, below 0.5 % dry weight (DW) (w/w)) (Mulders et al. 2014a). Under adverse growth conditions (referred to as 'stress' conditions), primary carotenoids are generally degraded and, in certain green algae, secondary carotenoids are produced in excessive amounts (up to 10 % DW (w/w)) (Lamers et al. 2008). As a consequence of these different responses, overproduction of the primary and secondary carotenoids requires different approaches. Secondary carotenoid overproduction necessitates optimization of the

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process (stress) conditions, which may be enhanced by additional genetic engineering. Although subsaturating light conditions result in minor increases in primary pigment concentrations, actual overproduction of primary carotenoids requires modification of the carotenoid biosynthesis routes, i.e. with genetic engineering (Mulders et al. 2014a). Both approaches share that insight into pigment metabolic regulation is crucial.

Chromochloris (Chlorella) zofingiensis is one of the most important carotenogenic green algae in relationship to biotechnological applications (Solovchenko 2013). In this species, lycopene, which can be converted into β,ϵ -carotene (α -carotene) or β,β -carotene (**Fig. 4.1**), is the precursor of all primary and secondary carotenoids. Hydroxylation of α -carotene yields lutein, the most abundant primary carotenoid of *C. zofingiensis* (Del Campo et al. 2004). Hydroxylation of β,β -carotene provides zeaxanthin, which can be epoxidised twice, resulting in violaxanthin which can then be isomerised, leading to neoxanthin. Under adverse growth conditions, *C. zofingiensis* can accumulate multiple secondary carotenoids including astaxanthin, canthaxanthin, and adonixanthin (Bar et al. 1995; Orosa et al. 2000). Presumably, β,β -carotene is transferred first to the cytosol where it is subsequently converted into astaxanthin (**Fig. 4.1**). This conversion requires two oxygenation and two hydroxylation reactions. Oxygenations are catalysed by β,β -carotene ketolase (BKT), and hydroxylations are catalysed by a β,β -carotene hydroxylase (CHYb). Esterification of astaxanthin results in monoesters and diesters which appear to accumulate in triacylglyceride (TAG) oil bodies (Solovchenko 2013) (**Fig. 4.1**). It has been postulated that, in *C. zofingiensis*, zeaxanthin can also be a precursor of astaxanthin through conversion into adonixanthin (**Fig. 4.1**). However, this pathway has not yet been fully resolved (Huang et al. 2006; Wang and Chen 2008). Furthermore, the accumulation of canthaxanthin in a relatively high concentration has led to the speculation that this carotenoid is an end product rather than a precursor of astaxanthin (Huang et al. 2006). This speculation requires further confirmation. Finally, a recent discovery by Bauch (2011) suggests that BKT converts the primary carotenoid lutein into the secondary carotenoid ketolutein under adverse growth conditions. As this ascertainment is contradictory with all other available literature, this requires confirmation. Thus, whereas primary carotenoid biosynthesis pathways in *C. zofingiensis* are thoroughly investigated, those for secondary carotenoids require further elucidation. Furthermore, the metabolic regulation of primary and secondary carotenoid biosynthesis is extensively under-investigated which is, as stated, of utmost importance when designing a metabolically engineered carotenoid production strain or optimizing a large-scale carotenoid production process. The aim of this paper was to obtain additional insight into the pathways and regulation of carotenoid metabolism of *C. zofingiensis*.

This was accomplished by depleting *C. zofingiensis* of nitrogen, which effectively induces secondary carotenogenesis, and subsequently adding diphenylamine (DPA), an inhibitor of BKT (Harker and Young 1995; Fan et al. 1995; Wang and Chen 2008). Since BKT catalyses multiple secondary carotenoid conversions (**Fig. 4.1**), inhibition was expected to lead to insight into the pathway of astaxanthin biosynthesis as it did, for example, in the green alga *Haematococcus pluvialis* (Collins et al. 2011; Fan et al. 1995; Harker and Young 1995; Zhekiševa 2005). Additionally, as primary and secondary carotenoids of *C. zofingiensis* possess a common metabolic precursor, and BKT catalyses the first step of secondary carotenogenesis, inhibition with DPA was expected to alter the primary pigment metabolism

(e.g. lutein biosynthesis) and reveal insights in carotenoid metabolic regulation. To ensure that the entire collection of carotenoids of *C. zofingiensis* would be revealed, carotenoids were identified employing reversed phase liquid chromatography and additional mass spectrometry.

4.2 Materials and methods

4.2.1 Materials

(*rac.*)-Adonirubin (95 %), (3*S*,3'*R*)-adonixanthin (99 %), (*rac./meso*)-astaxanthin (97 %), (*rac./meso*)-astaxanthin monopalmitate (97 %), (*rac./meso*)-astaxanthin dipalmitate (98 %), canthaxanthin (98 %), echinenone (98 %), (*rac.*)-3-hydroxyechinenone (97 %), lutein (96 %) and (3*R*,3'*R*)-zeaxanthin (97 %) (all w/w) were purchased from CaroteNature (Lupsingen, Switzerland). Violaxanthin (98 %), lycopene (92 %), cryptoxanthin (β -cryptoxanthin) (98 %), antheraxanthin (97 %), α -cryptoxanthin (98 %), and β,ϵ -carotene (α -carotene) (88 %) (all w/w) were purchased in approximately 1 mg mL⁻¹ ethanol or acetone solutions from DHI laboratory products (Hørsholm, Denmark). Chlorophyll *a* (90.0 % w/w) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Methanol (99.8 %), acetonitrile (99.97 %), ethyl acetate (99.9 %) and chloroform (stabilized with 0.5-1.5 % (w/v) ethanol, purity after correction for stabilizer 99.9 %) were obtained from Biosolve (Valkenswaard, the Netherlands). Dichloromethane (99.8 %), acetone (99.8 %), n-hexane (98 %), formic acid (98.0 %), sodium chloride (99.0 % w/w), β,β -carotene (97 % w/w) and butylated hydroxytoluene (BHT) (99 % w/w) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Demineralized water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA).

4.2.2 Cultivation

Chromochloris zofingiensis was obtained as *Chlorella zofingiensis* UTEX B32 from the University of Texas Culture Collection of Algae (UTEX) and cultivated in (replete) modified M-8 medium (Table 4.1) (modified from Mandalam and Palsson 1998). Nitrogen-depleted medium was similarly prepared with the exception that KNO₃ was substituted with KCl (at a concentration of 29.67 mM) to retain a total osmolarity of 0.323 Osm. It was assumed that the organic pH buffer Hepes, which contains two nitrogen atoms, was not consumed by *C. zofingiensis*.

Table 4.1 Composition of (replete) modified M-8 medium

Compound	Concentration ($\mu\text{mol L}^{-1}$)	Compound	Concentration ($\mu\text{mol L}^{-1}$)
Hepes	100.00·10 ³	MnCl ₂ ·4H ₂ O	65.59
KNO ₃	29.67·10 ³	ZnSO ₄ ·7H ₂ O	11.13
NaHCO ₃	10.00·10 ³	CuSO ₄ ·5H ₂ O	7.33
Na ₂ HPO ₄ ·2H ₂ O	6.90·10 ³	H ₃ BO ₃	1.00
MgSO ₄ ·7H ₂ O	1.62·10 ³	Vitamins	($\mu\text{g L}^{-1}$)
Na ₂ EDTA·2H ₂ O	447.94	Thiamine	200.00
NaFeEDTA	277.85	Biotine	1.00
CaCl ₂ ·2H ₂ O	90.00	Cyanocobalamine	1.00

Cultures were pre-cultivated in 250-mL shake flasks containing 100 mL (replete) medium that were situated in a culture chamber at 28 °C in continuous light emanating from white fluorescent tubes with a light intensity of 231-255 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and continuously shaken at 100 rpm with a head space containing an air/CO₂ ratio of 95/5 % (v/v).

To achieve nitrogen starvation, cells were washed with a nitrogen-depleted medium. Washing was performed by centrifugation of cells at 700 rpm (94×g) for 10 min and subsequent resuspension of cell pellet in 25 mL of nitrogen-depleted medium. Following two washing occurrences, cells were inoculated in 250-mL shake flasks containing 150 mL of nitrogen-depleted medium at a dry weight cell concentration of 1.7 g L⁻¹. Cells were placed into a culture chamber in the same conditions as described for the pre-cultivation. Every two days, beginning at day zero, a sample was taken for cell dry weight measurements and pigment extraction and quantification. Due to increasing biomass concentrations, sample volumes decreased over time. The extracted sample volumes were: 16 mL, 10 mL, 10 mL, 7 mL, 5.5 mL, 5.5 mL, 5.5 mL, 5.5 mL (at day 0, 2, 4, 6, 8, 10, 12 and 14, respectively). A 10 mM DPA stock solution (in dimethyl sulfoxide (DMSO)) was prepared and retained in the dark to prevent DPA degradation. Beginning in day two, 1 mL DPA stock solution was added to each culture immediately following sampling. This resulted in concentration increases of 60 μM DPA for the DPA treated cultures. To the control cultures, only 1 mL of DMSO (without DPA) was added.

To obtain nitrogen-replete and nitrogen-depleted *C. zofingiensis* cells for Freeze Fracture Scanning Electron Micrographs, *C. zofingiensis* cells were pre-cultured in a shake flask under a low incident light intensity (20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and diluted 10 times in nitrogen-depleted medium to achieve a concentration of ~0.3 g/L. This shake flask, containing a volume of 110 mL, was incubated under a low incident light intensity (20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Cells achieved nitrogen depletion after approximately one week. Two weeks later, samples were taken from the nitrogen-replete pre-culture and from the nitrogen depleted culture for Freeze Fracture Scanning Electron Microscopy and dry weight, pigment, and triacylglyceride (TAG) analysis.

4.2.3 Biomass determination

Cell dry weight measurements were performed in duplicate by filtering and drying the biomass as described by Kliphuis et al. (2011).

4.2.4 Freeze Fracture Scanning Electron Microscopy

A small droplet containing nitrogen-replete or nitrogen-depleted *C. zofingiensis* cells was placed on copper hollow rivets and promptly frozen in liquid ethane. The rivets were situated in a cryo-sample holder in liquid nitrogen and thereafter transferred to the cryo-preparation system (MED 020/ VCT 100, Leica, Vienna, Austria) onto the sample stage at -93 °C and at 1.3×10^{-6} Torr. Thereafter, the samples were fractured and freeze dried for 5 min and subsequently sputter coated with a layer of 10 nm Tungsten at -93 °C. The samples were cryo-shielded and transferred into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, The Netherlands) onto the sample stage at -120 °C at 1.6×10^{-6} Torr. The analysis was performed at a working distance of 4-4.5 mm with SE detection at 2 kV and 6.3 pA. Contrast and sharpness of digital images were optimized with Photoshop CS5.

4.2.5 TAG extraction, identification and quantification

All lipophilic components were obtained by a sequence of mechanical cell disruption and solvent based (methanol/chloroform) lipid extraction as described by Breuer et al. (2013a). The TAG fraction was obtained using a solid phase extraction (SPE) column as described by Breuer et al. (2012) with 10 mL 7 : 1 (v/v) hexane : diethylether as eluent. Solvents were evaporated and fatty acids of TAG were transesterified to fatty acid methyl esters (FAMES). FAMES were identified and quantified utilizing GC-FID as described by Breuer et al. (2012).

4.2.6 Pigment extraction

Pigment extracts were obtained by a sequence of mechanical cell disruption and solvent based (methanol/chloroform) pigment extraction as described by Lamers et al. (2010) with the exception that, after sampling, the cells were centrifuged for 5 min at 2500 rpm (1204×g). The cell pellet was transferred to a bead beating tube and centrifuged again for 5 min at 2500 rpm (1204×g). Supernatant was discarded, and cells were stored at -80 °C. The cells were subsequently freeze dried. One mL of methanol/chloroform was added to the bead beating tube where after the cells were physically disrupted by employing a Precellys® 24 bead beater (Bertin Technology, Montigny-le-Bretonneux, France). To inhibit cell heating, 60 s of disruption at 2500 rpm (1204×g) was followed by 5 min of cooling on ice. For each sample, three series of disruption/cooling cycles were performed. After extraction, the dried chloroform fraction was resolved in acetone : methanol 7 : 3 (v/v) containing 0.10 % (w/v) BHT and supplemented to 75 % (v/v) acetonitrile for LC analysis.

4.2.7 Separation, identification and quantification of pigments

Separation and identification of carotenoids and porphyrins were performed on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, degasser, auto sampler, and photo diode array (PDA) detector, coupled *in-line* to a LTQ-VelosPro dual-pressure ion trap mass spectrometer equipped with a H-ESI probe (Thermo Scientific). Samples (5.0 µL) were quantitatively injected on an Aquity UPLC Shield C18 BEH column (2.1 × 150 mm, 1.7 µm particle size; Waters, Milford, MA, USA) fitted to a Vanguard pre-column (2.1 × 5 mm, 1.7 µm particle size; Waters). The eluents were (A) 50 % (v/v) acetonitrile in demineralized water, (B) acetonitrile and (C) ethyl acetate which all contained 0.10 % (v/v) formic acid. The flow rate was maintained at 300 µL min⁻¹. The program was initiated from 25 % A / 75% B and then as follows: to 15 min – linear gradient to 100 % B; to 22.5 min – isocratic at 100 % B; to 29.5 min – linear gradient to 87.5 % B/ 12.5 % C; to 31.5 min – linear gradient to 70 % B / 30 % C; to 41.5 min – linear gradient to 100 % C; to 42.5 min - isocratic at 100 % C. After 42.5 min, the eluent composition reverted to its initial composition in 7.5 min followed by an equilibration phase of 2.5 min. Detection wavelengths for UV-Vis were adjusted to 450 nm (carotenoids) and 660 nm (porphyrins) with a 1 nm wavelength step and 9 nm filter bandwidth.

All mass spectrometric data was recorded as described by Mulders et al. (2013) with the exception that data was recorded over the *m/z* range 500 - 620 (0.0 - 17.0 min), 500 - 900 (17.0 - 30.0 min) and 1000 - 1250 (30.0 - 42.5 min). Data acquisition and reprocessing were performed with Xcalibur 2.10 (Thermo Scientific). The standards employed for identification of carotenoids and their corresponding absorption coefficients are depicted in **Table 4.2**. The criteria for compound identification included identical retention time, UV-Vis absorption and

spectral fine structure, and parent mass as well as MS² fragmentation pattern with one of the standards. Compounds without a matching standard were identified with comparison to literature data (Britton 1995a; Egeland et al. 2011). Unknown compounds were not annotated in **Fig. 4.2** and **Table 4.3** for the sake of clarity. Chlorophyll *b*, in high concentrations, exhibited fronting in this LC system.

Table 4.2 Absorption coefficients used for quantification of carotenoids found in *C. zoefingiensis*

Carotenoid	A _{1cm} ^{1%} (L g ⁻¹ cm ⁻¹)	Ref
9'- <i>cis</i> -Neoxanthin	2330	Egeland et al. (2011)
Violaxanthin	2450	Egeland et al. (2011)
Neochrome	2270	Egeland et al. (2011)
Astaxanthin (free, monoester and diester)	2060	Egeland et al. (2011)
Ketolutein (free, monoester and diester)	2500	Britton (1995a)
Adonixanthin	2500	Britton (1995a)
Lutein	2550	Egeland et al. (2011)
Lutein-like carotenoid	2500	Britton (1995a)
Canthaxanthin	2200	Egeland et al. (2011)
Echinenone	2160	Egeland et al. (2011)
β,β-Carotene	2590	Egeland et al. (2011)

For quantification, lutein, canthaxanthin, and chlorophyll *a* were exploited. Approximately 1 mg of lutein and canthaxanthin were initially dissolved in dichloromethane and subsequently diluted with 4 volumes of ethanol (lutein) or *n*-hexane (canthaxanthin). Further dilution in ethanol or *n*-hexane respectively was performed in order to measure within the linear range of the spectrophotometer. Therefore, the content of dichloromethane was lower than 0.5 % (v/v) upon determination of the concentrations of lutein and canthaxanthin. Chlorophyll *a* was first dissolved in ethyl acetate and subsequently diluted with 3 volumes of 90 % (v/v) aqueous acetone. Standard concentrations were measured prior to UHPLC analysis, employing their respective absorption coefficients (A_{1cm}^{1%}): 2550 L g⁻¹ cm⁻¹ (445 nm, 100 % ethanol) for lutein; 2200 L g⁻¹ cm⁻¹ (469 nm 100 % cyclohexane) for canthaxanthin; and 887 L g⁻¹ cm⁻¹ (664 nm, 90 % (v/v) aqueous acetone) for chlorophyll *a* (Egeland et al. 2011). PDA calibration was performed utilizing five different concentrations of the standards injected in duplicate. For this calibration, the response of the all-*trans* and *cis* structure of the carotenoid standards was considered equal for quantification. The detector was ascertained as linear for lutein and canthaxanthin between 0.07 and 8.20 µg mL⁻¹ with a minimum R² of 0.998. For chlorophyll *a*, the detector was linear between 0.23 and 23.38 µg mL⁻¹ with an R² of 0.978.

Carotenoids were quantified as lutein equivalents employing the lutein calibration curve. The responses were corrected using the A_{1cm}^{1%} illustrated in **Table 4.2** with the exception of canthaxanthin. An absorption coefficient of 2500 L g⁻¹ cm⁻¹ was utilized for unknown carotenoids (e.g. lutein-like components) and carotenoids of which the absorption coefficient was not documented (e.g. ketolutein). Fatty acid esterified astaxanthin and ketolutein were assumed to possess similar molar extinction coefficients as their non-esterified analogues, and were expressed as equivalents of their respective non-esterified analogues in mole L⁻¹ culture volume throughout this work. In **Fig. 4.9**, esterified carotenoids were expressed as % g/g DW,

without accounting for the molecular weight of the fatty acid ester. Chlorophyll *b* ($A_{1\text{cm}}^{1\%} 514 \text{ L g}^{-1} \text{ cm}^{-1}$, 647 nm, 90 % (v/v) aqueous acetone) and derivatives were expressed via chlorophyll *a* using the chlorophyll *a* calibration curve. The detector response was corrected for chlorophyll *b* using the ratio of the absorption coefficients of chlorophyll *a* and *b*.

4.3 Results and Discussion

To obtain additional insight into the pathways and regulation of the carotenoid metabolism of *C. zofingiensis*, cells were nitrogen depleted to induce secondary carotenogenesis and subsequently DPA-treated to inhibit ketocarotenoids biosynthesis. 60 μM DPA was introduced to nitrogen-depleted cultures every two days beginning on day two. Every two days, cell dry weights and pigment compositions were analysed. Below, chlorophylls and carotenoids ascertained in nitrogen-replete and nitrogen-depleted *C. zofingiensis* cells are identified and the degradation and accumulation kinetics of the overall biomass and the annotated pigments upon nitrogen depletion and DPA treatment are discussed (**Fig. 4.2 - 4.7** and **Fig. A.4.1**). To properly represent net accumulation/degradation of pigments, pigment kinetics are depicted in moles per litre culture volume. For a reference, pigment contents on a dry weight basis are depicted in **Fig. A.4.2**. Conclusions concerning the carotenoid metabolism are summarized graphically in **Fig. 4.8**. Finally, oil droplet formation in nitrogen-depleted *C. zofingiensis* cells is elaborated to support the presumed location of carotenoid accumulation.

4.3.1 Pigment identification

A profound variation in the carotenoid profile of extracts between the nitrogen-replete and nitrogen-depleted *C. zofingiensis* cells was observed (**Fig. 4.2**). The key findings were the emergence of ketolutein and ketolutein esters upon nitrogen depletion, confirming the finding by Bauch (2011). For both chromatograms, peaks are annotated in **Table 4.3**.

The annotation of ketolutein (β,ϵ -end groups) was performed by comparison with adonixanthin (β,β -end groups). Although the molecular weight of both carotenoids is comparable, pronounced differences in visible absorption spectra and MS^2 spectra were found (**Fig. 4.3A - C**). The spectral fine structure of ketolutein had two absorption maxima, where adonixanthin had one maximum. Furthermore, the spectral fine structure of ketolutein excluded the possibilities of having a carotenoid epoxide or a carotenoid with an allenic group, or both (e.g. diadinoxanthin). The 8 nm hypsochromic shift of ketolutein, compared to adonixanthin, indicated the presence of a smaller conjugated system. This hypsochromic shift can be attributed to the presence of an ϵ -end group. A similar hypsochromic shift has also been documented for zeaxanthin (β,β -end groups, $\lambda_{\text{max}}(\text{II}) = 450 \text{ nm}$ in hexane) and lutein (β,ϵ -end groups, $\lambda_{\text{max}}(\text{II}) = 445 \text{ nm}$ in hexane) (Egeland et al. 2011). Similar observations were made when comparing this for the group of the ketoluteins, (comprising of four reported stereoisomers fritschiellaxanthin, 4-ketolutein F, α -doradexanthin and 4-ketolutein D) to adonixanthin (Britton et al. 2004).

Table 4.3 Compounds tentatively assigned in the extracts of *C. zofingiensis* by UHPLC-PDAESI-MSⁿ.

Peak No.	Compound	Rt. (min)	λ_{\max} (nm)	Band ratio (% III:II) ^a	Ionization type	Parent ion (m/z)	MS ² product ions (assignment, % rel. intensity) ^b	Ref ^c
1	9'- <i>cis</i> -Neoxanthin	2.91	416, <u>438</u> , 467	82	[M] ^{••} /[M+H] ⁺	600.5/601.4	582.5(100), 567.4(30), 508.4(M-92, 30), 507.4(M-92, 30), 383.4(50), 255.3(50), 221.2(50) ^c	1
2	Violaxanthin	3.75	418, <u>440</u> , 470	82	[M] ^{••} /[M+H] ⁺	600.5/601.4	583.4(100), 565.5(20), 508.4(M-92, 30), 491.4(M-18-92, 15), 415.3(30), 319.3(20), 265.3(40), 221.2(20) ^c	Std, 1
3	Astaxanthin	4.05	<u>474</u>	0	[M+H] ⁺	597.5	579.5(100), 561.5(40), 503.4(M-92,30), 491.4(M-106, 20), 379.3(35), 277.3(20), 287.2(25)	Std, 1
4	(<i>cis</i>)-Neochrome-like	4.51	404, <u>421</u> , <u>447</u>	58-65	[M] ^{••}	600.4	582.4(100), 568.5(60), 520.4(40), 508.4(M-92,70), 507.4(90), 476.4(50), 415.3(50), 221.1(65)	1
5	Antheraxanthin	5.00	N.D.	N.D.	[M] ^{••}	584.5	N.D.	1
6	Ketolutein	5.00	<u>454</u> , 468	0	[M] ^{••}	582.5	564.4(20), 563.5(15), 490.4(M-92, 20), 472.3(M-92-18, 15), 444.3(M-138, 100), 352.3(M-92-138, 60)	2 ^e
7	Adonixanthin	5.20	<u>460</u>	0	[M] ^{••} /[M+H] ⁺	582.5/583.5	565.5(40), 564.5(30), 549.5(10), 491.4(M-92, 40), 490.4(M-92, 100), 473.4 (20), 472.4(40) ^c	Std, 2
8	Lutein	6.36	424, <u>447</u> , 473	47	[M] ^{••}	568.5	550.4(5), 519.1(10), 476.3(M-92, 100), 338.2(75)	Std, 1
9a	<i>cis</i> -Lutein-like	6.58	422, <u>442</u> , 469	29	[M] ^{••}	568.5	550.4(10), 476.4(100), 430.4(10), 338.3(50)	1,3
9b	<i>cis</i> -Lutein-like or zeaxanthin-like	6.58	(443), <u>450</u> , 468	N.D.	[M] ^{••}	568.5	476.4(100), 338.30(20)	1,3
10	<i>cis</i> -Lutein	6.87	422, <u>441</u> , 469	50	[M] ^{••}	568.5	550.5(5), 476.4(100), 458.3(M-18-92, 10), 430.4(5), 338.3(40)	1,3
11	Canthaxanthin	7.13	<u>473</u>	0	[M+H] ⁺	565.5	547.4(60), 473.4(M-92, 20), 459.4(M-106, 20), 427.3(M-138, 15), 413.4(20), 363.3(100), 361.3(30), 349.2(20), 269.3(20), 203.1(70)	Std, 1
12	<i>Cis</i> -Lutein-like	7.31	332, 419, <u>441</u> , 468	30	[M] ^{••}	568.5	550.5(20), 476.4(100), 458.3(M-18-92, 10), 430.3(15), 338.3(70)	1,3
13	<i>cis</i> -Canthaxanthin	7.74	<u>466</u>	0	[M+H] ⁺	565.5	N.D.	1
14	<i>cis</i> -Canthaxanthin	8.05	370, <u>464</u>	0	[M+H] ⁺	565.5	N.D.	1
15	Echinenone	13.39	<u>459</u>	0	[M] ^{••}	550.7	550.7(100), 494.5(5), 458.4(M-92, 30), 427.4(10), 402.4(10), 335.3(10)	Std, 1
16	Chlorophyll <i>b</i>	15.15	456, 595, 645	N.A.	N.D.	N.D.	N.D.	1
17	Chlorophyll <i>b</i> -like	17.13	432, 457, 647	N.A.	N.D.	N.D.	N.D.	1
18	Astaxanthin ME C18:1	19.42	<u>473</u>	0	[M+Na] ⁺	883.8	839.7(M-H ₂ O, 20), 791.7(M-92, 100), 777.7(M-106, 30), 601.4(M-FA, 40), 491.4(B+FA, 15)	4,5
19	β , β -Carotene	19.19	475 ^e	N.D.	[M] ^{••}	536.5	444.4(M-92, 100)	Std, 1

Table 4.3 Continued

20	Astaxanthin ME C16:0	19.60	<u>473</u>	0		[M+Na] ⁺	857.8	813.6(M-H ₂ O, 20), 765.5(M-92, 100), 751.5(M-106, 30), 601.3(M-FA, 40), 465.3(M+FA, 10)	Std, 4, 5
21	Chlorophyll <i>a</i>	20.06	382, 413, 429, 580, 616, 661	N.A.		[M-Mg+2H] ⁺	871.7	593.4(M-Phytyl, 100), 533.4(M-Phytyl-60, 60)	Std, 1
22	Ketolutein ME C18:1	22.39	<u>451</u> , 468	0		[M+Na] ⁺	869.7	851.7(M-H ₂ O, 35), 777.7(M-92, 100), 763.7(M-106, 30), 587.5(M-FA, 15), 491.4(B+FA, 15)	2, 5
23	Ketolutein ME C16:0	22.64	<u>451</u> , 468	0		[M+Na] ⁺	843.7	825.7(M-H ₂ O, 40), 751.6(M-92, 100), 737.6(M-92, 30), 587.4(M-FA, 20), 465.4(B+FA, 10)	2, 5
24	Astaxanthin ME C18:0	23.23	334, <u>468</u>	0		[M+Na] ⁺	885.8	867.7(10), 841.7(30), 793.7(100), 779.6(30), 601.4(30), 493.4(15)	4, 5
25	Ketolutein ME	27.26	455, <u>468</u>	0		N.D.	N.D.	N.D.	4, 5
26	Astaxanthin DE C18:1/C18:3	33.29	<u>475</u>	0		[M+Na] ⁺	1143.9	1051.8(M-92, 100), 1037.9(M-106, 30), 865.7(M-FA, 40), 861.6(M-FA, 40)	4, 5
27	Astaxanthin DE C16:0/C18:3	33.44	<u>473</u>	0		[M+Na] ⁺	1117.9	1025.8(M-92, 100), 1011.8(M-92, 30), 861.6(M-FA, 40), 839.7(M-FA, 35)	4, 5
28	Astaxanthin DE C18:1/C18:2	34.13	<u>470</u>	0		[M+Na] ⁺	1146.0	1053.9(M-92, 100), 1039.9(M-106, 30), 865.7(M-FA, 40), 863.7(M-FA, 50)	4, 5
29	Astaxanthin DE C16:0/C18:2	34.24	<u>468</u>	0		[M+Na] ⁺	1120.0	1027.9(M-92, 100), 1013.9(M-106, 30), 863.7(M-FA, 50), 839.7(M-FA, 35)	4, 5
30	Astaxanthin DE C18:1/C18:1	34.86	<u>475</u>	0		[M+Na] ⁺	1148.0	1055.9(M-92, 100), 1041.9(M-106, 30), 865.7(M-FA, 80)	4, 5
31	Astaxanthin DE C16:0/C18:1	34.96	<u>476</u>	0		[M+Na] ⁺	1122.1	1029.9(M-92, 100), 1015.9(M-106, 30), 865.7(M-FA, 50), 839.7(M-FA, 50)	4, 5
32	Astaxanthin DE C16:0/C16:0	35.06	<u>474</u>	0		[M+Na] ⁺	1096.1	1033.9(M-92, 100), 989.9(M-106, 30), 839.8(M-FA, 80)	4, 5
33	<i>cis</i> of peak 30 - 32	35.19-35.38	<u>462</u>	0		[M+Na] ⁺	Idem	Idem	4, 5
34	Astaxanthin C18:0/C18:1	35.52	<u>474</u>	0		[M+Na] ⁺	1150.1	1057.9(M-92, 100), 1043.9(M-106, 30), 867.8(M-FA, 50), 865.8(M-FA, 40)	4, 5
35	Astaxanthin C16:0/C18:0	35.62	<u>474</u>	0		[M+Na] ⁺	1124.1	1031.9(M-92, 100), 1017.9(M-106, 30), 867.7(M-FA, 40), 839.7(M-FA, 40)	4, 5
36	Ketolutein DE's	35.96-36.28	<u>454</u>	0		N.D.	N.D.	N.D.	2

N.D.: not determined. N.A.: not applicable. ^aWhen no fine structure, no peak heights II and III nor a minimum between them can be defined, the band ratio was annotated as 0 %. ^bM-92 in-polyene toluene elimination, M-106 in-polyene xylene elimination, M-FA neutral loss of fatty acid, B+FA benzonium fragment with fatty acid. ^cFragments of both [M]⁺ and [M+H]⁺ occurred in the MS² spectrum due to the isolation width of the ion trap. ^dThe MS² spectrum of α-doradexanthin was closest to our finding of the ketoluteins reported in this reference ^eIncomplete spectrum due to

Additional evidence for an ϵ -end group was ascertained in the MS^2 fragmentation spectra of ketolutein and adonixanthin. It has been reported that the C6-C7 bond adjacent to a hydroxylated ϵ -end group is more prone to fragmentation than that adjacent to a hydroxylated β -end group in the case of electron impact mass spectrometry (Enzell et al. 1969). Extrapolating this for ketolutein in combination with the milder CID fragmentation, observation of at least one fragment with a neutral loss of 138 Da was expected. In the MS^2 spectrum of ketolutein, two such neutral losses were found as the two primary fragments: one direct loss at m/z 444.3 and a loss combined with an in-chain polyene elimination of toluene (92 Da) at m/z 352.3 (**Fig. 4.3B**). For adonixanthin, the neutral loss of 138 was not found, and only (combinations of) water and toluene losses were ascertained (**Fig. 4.3C**). Similar observations were made with fragmentation of lutein and zeaxanthin (Enzell et al 1969; Britton 1995b). From a biological point of view, it seems likely that the ketolutein found represents fritschiellaxanthin, one the four reported stereoisomers of ketolutein. However, the exact R/S configuration was not determined by CD / NMR. Furthermore, no chemical standards were at our disposal to confirm this stereochemistry. Therefore, throughout this work, the generic annotation ketolutein is employed.

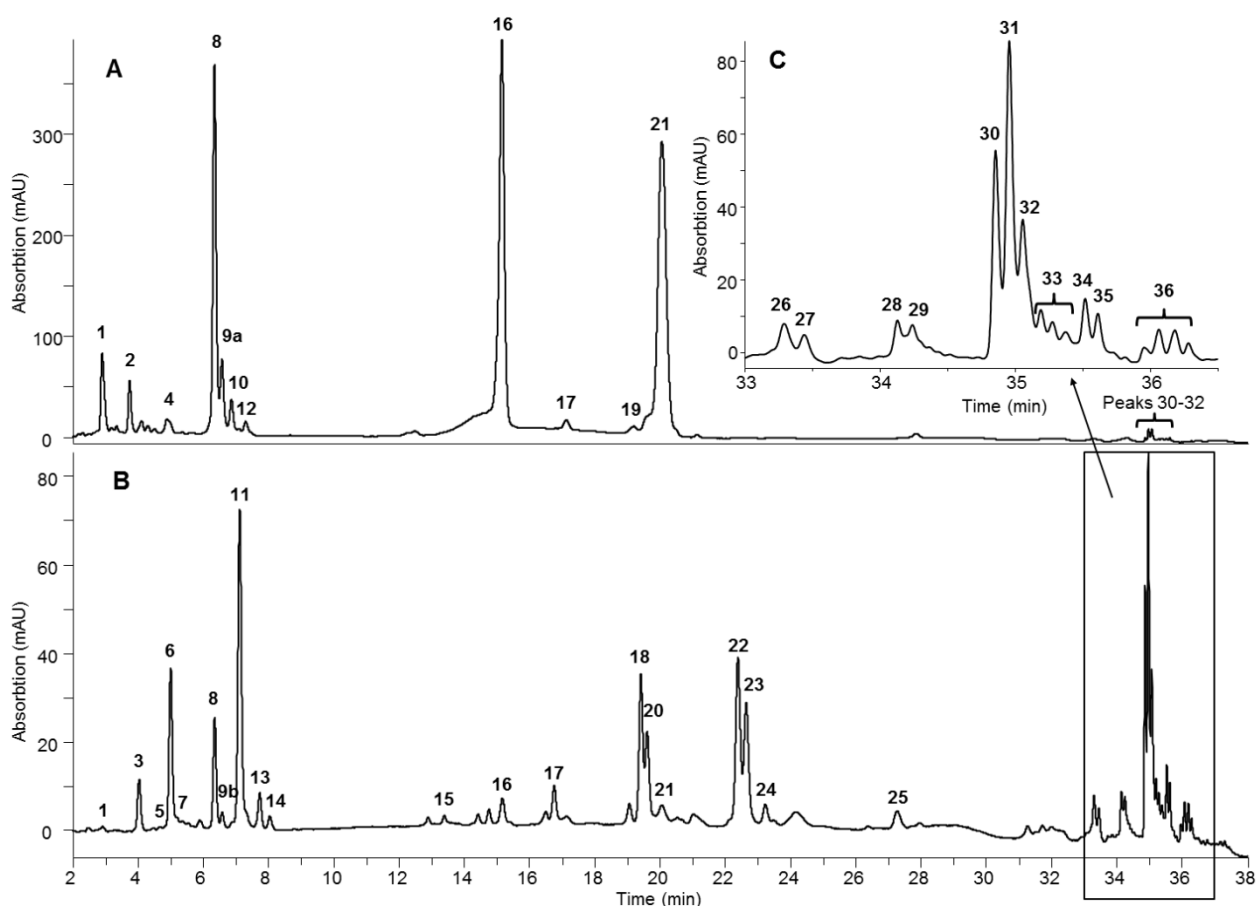


Fig. 4.2 Representative RP-UHPLC chromatograms (450+660 nm) of *C. zoefingiensis* extract. Nitrogen-replete cells (**A**), nitrogen-depleted cells (**B**), enlargement of chromatogram of nitrogen-depleted cells RT 33.0 to 36.5 min (**C**).

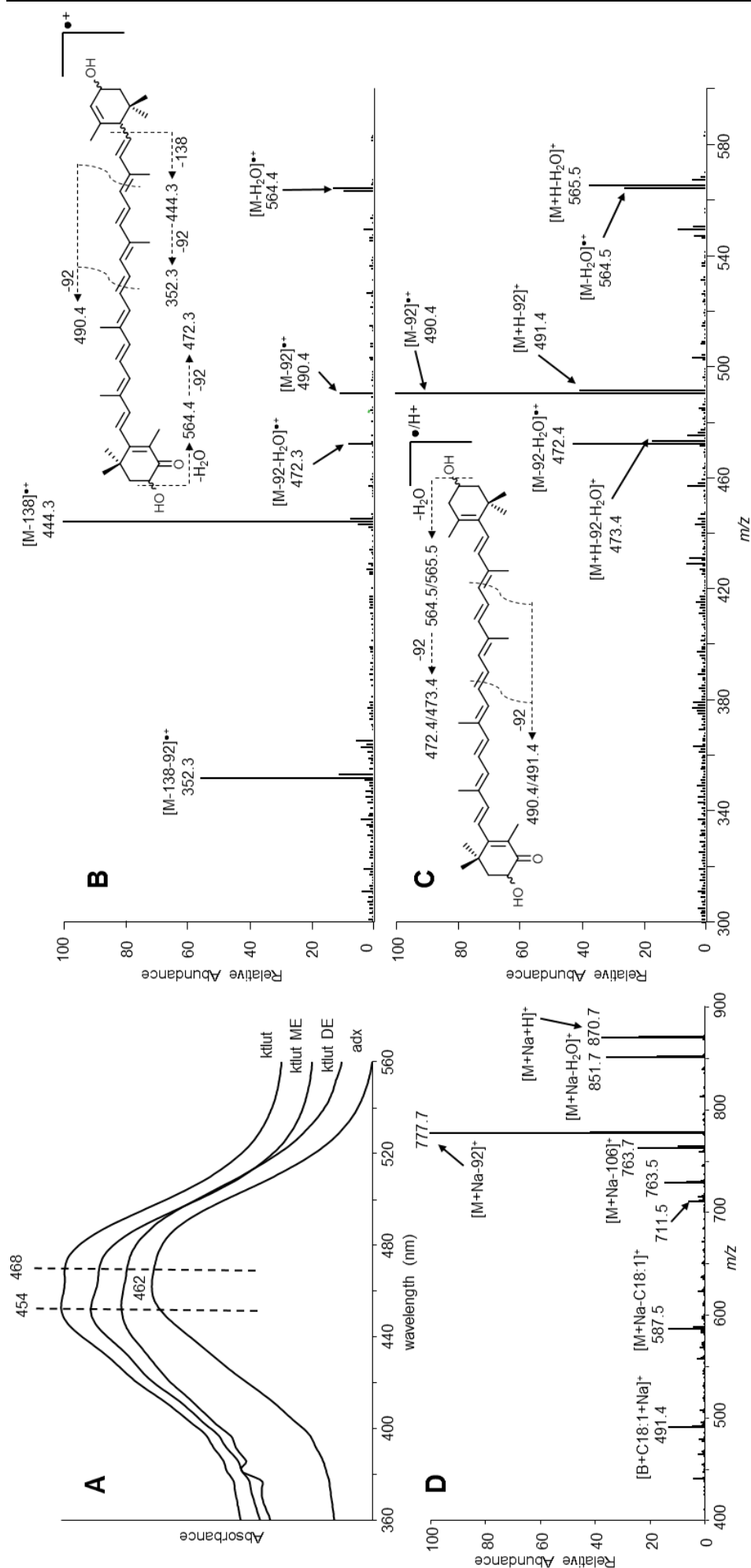


Fig. 4-3 Visible absorption spectra of the three different forms of ketolutein and adonixanthin (A). Comparison and fragment annotation of the MS² fragmentation of ketolutein $[M]^{+}$ parent (peak 6) (B), and adonixanthin $[M]^{+}$ ($[M+H]^{+}$) parent (peak 7) (C). Annotation of the C18:1 esterified fatty acid from ketolutein ME C18:1 (peak 22) by MS² fragmentation of its $[M+Na]^{+}$ parent (D). KtLut ketolutein, KtLut ME ketolutein monoester, KtLut DE ketolutein diester, adx adonixanthin; other abbreviations, see footnote Table 4.3

The visible absorption spectra of ketolutein and peaks 22, 23, 25 and 36 were identical, indicating that ketolutein was also evident in its esterified form to one or two fatty acids (**Fig. 4.3A**). Sodiated $[M+Na]^+$ fragment spectra of the carotenoid esters were employed to confirm this as they exhibited more efficient ionization and less complex mass spectra (Frassanito et al. 2008; Weesepeol et al. 2013). In **Fig. 4.3D**, the MS^2 fragment spectrum of ketolutein ME C18:1 $[M+Na]^+$ is depicted. The identification of this ester was performed similar to astaxanthin monoesters and diesters. In the case of monoesters, the fragment at m/z 587.5 was used to identify the attached fatty acid and to also confirm the molecular mass of ketolutein (e.g. $587.5 + H_2O - Na = 582.5$ Da). The visible spectra of peak group 36 exhibited similar spectral fine structures, indicating that the backbone was ketolutein. Identification of the fatty acids in these ketolutein diesters could not be performed due to lack of fragmentation data. Still, because astaxanthin mono- and diesters were predominantly esterified with C16:0 and C18:1 fatty acids, the assumption could be made that this was also the case for ketolutein diesters. The fatty acid esterification was not exclusively with C16:0 and C18:1; lower abundances were also discovered for the C18:0, C18:2 and C18:3 fatty acid esters for astaxanthin mono- and diesters.

Identification of the other primary and secondary pigments, e.g. canthaxanthin, adonixanthin, β,β -carotene, lutein, violaxanthin, and chlorophyll *a* and *b* were in accordance with previous reports of *C. zoofingensis* (Bar et al. 1995; Del Campo et al. 2004; Orosa et al. 2001; Wang and Chen 2008). *g'-cis*-Neoxanthin, *cis*-neochrome-like, and the *cis*-lutein-like compounds were not previously documented in *C. zoofingensis* which were identified empirically by comparison to reference data. From **Fig. 4.1** also adonirubin, 3-hydroxyechinenone, lycopene, cryptoxanthin, α -cryptoxanthin, and β,ϵ -carotene were expected to be present. However, upon comparison with standards, they were not ascertained in our analysis. The presence of zeaxanthin could not be determining with certainty, although Wang and co-workers (2008) detected a substantial amount of this carotenoid. Zeaxanthin standards has similar retention time as peaks 9a/9b, but the UV-Vis and MS^2 spectra did not match. This might be explained by coelution.

4.3.2 Optimizing DPA concentration and addition frequency

Although DPA is exploited in multiple studies to obtain insight into microalgae carotenoid accumulation (Fan et al. 1995; Harker and Young 1995; Wang and Chen 2008), numbers regarding DPA degradation kinetics are minimal. To gain insight in the DPA degradation rate under the experimental conditions described in this work and to locate the most optimal DPA concentration with respect to the inhibition of carotenogenesis, a range of DPA concentrations was added to nitrogen-depleted *C. zoofingensis*, and the effect on pigment accumulation (carotenoids and chlorophylls) was examined. In individual experiments, DPA was added once after two days of nitrogen starvation in the following concentrations: 0 (control), 10, 20, 30, 60, 100 or 200 μM . The most significant variation in pigment concentrations between the control and the DPA-treated culture was ascertained in the culture to which 60 μM DPA was added. In addition, the results suggested that DPA was inactivated after approximately three days following the addition (results not shown). Therefore, in the experiment described in the current paper, DPA was added every two days, resulting each time in a concentration increase of 60 μM , in order to maintain an effective DPA concentration for two weeks.

4.3.3 Biomass production

During the entire cultivation period, the control and the DPA-treated cultures exhibited no significant variation in biomass production. Following a steep initial increase, biomass production levelled off around day eight for all cultures. Both the treated and the control cultures increased from 1.7 to 4.6 g L⁻¹ in biomass concentration (**Fig. 4.4**). As the medium was nitrogen-depleted from the beginning of cultivation, no additional nitrogen-containing cell material such as DNA and protein could be formed. Photosynthesis and carbon fixation still continued in the absence of nitrate. Therefore, it appears plausible that the ascertained biomass increase is due to carbon-rich storage compounds such as TAG. This has been described for nitrogen-depleted *C. zofingiensis* by Breuer et al. (2012) who observed a biomass increase of 580 % DW and TAG accumulation of up to 40 % (w/w) DW. The absence of a significant variation in biomass production between the control and the treated cultures demonstrated that DPA did not affect the overall production of secondary storage compounds or carbon fixation.

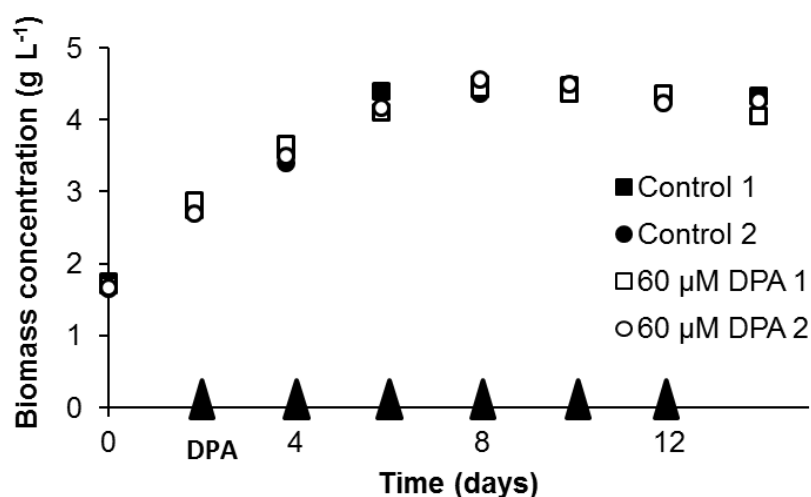


Fig. 4.4 Time courses of dry weight biomass concentration of nitrogen-depleted *C. zofingiensis* exposed to no DPA (control) or exposed to repeated additions of DPA resulting each time in a concentration increase of 60 μM. **Triangles** indicate DPA additions. Absolute deviations from the mean of duplicate dry weight measurements were all less than 4 %.

4.3.4 Degradation of chlorophylls and primary carotenoids

The chlorophylls and primary carotenoids found in the treated culture were identical to those ascertained in the control, specifically, chlorophyll *a* and *b*, lutein, 9'*cis*-neoxanthin, and violaxanthin. The cellular contents of these pigments all decreased over time with no significant difference between the control and the treated culture (**Fig. 4.5**). This demonstrates that DPA did not affect the overall degradation of primary pigments.

Under nitrogen-replete conditions, the primary pigments comprised more than 99.9 % of the total pigment content of *C. zofingiensis* (**Fig. 4.5 and 4.6, t₀**). These pigments are located in the photosystems of the chloroplast, and all are capable of harvesting light energy (Dall'Osto et al. 2006; Falkowski and Raven 2007; Mulders et al. 2014a; Sukenik et al. 1992) which is converted into chemical energy. Active degradation of these individual pigments may be a strategy of the cell to manage the oversaturating light conditions that resulted from the nitrogen depletion, as was, for example, observed in *Isochrysis galbana* (Mulders et al. 2013).

However, the equiproportional decrease of chlorophyll *a*, *b*, and *g'*-*cis*-neoxanthin lends support to the assumption that entire photosystems were degraded rather than specific light harvesting pigments.

Remarkably, in both the control and the treated culture, violaxanthin was degraded at a significantly higher rate than the other primary pigments (Fig. 4.5). Violaxanthin is a component of the violaxanthin cycle and can be converted into zeaxanthin under oversaturating light conditions via antheraxanthin, which is part of a photoprotective mechanism (Goss and Jakob 2010; Jahns and Holzwarth 2012). However, as no additional zeaxanthin or antheraxanthin was discovered in either the control or the treated culture, it is questionable whether this degradation cycle was genuinely operational in our experiments. It may be that violaxanthin, antheraxanthin and zeaxanthin were all degraded.

In addition, it is remarkable that, in both the control and in the treated culture, lutein was degraded at a lower rate compared to the other primary pigments.

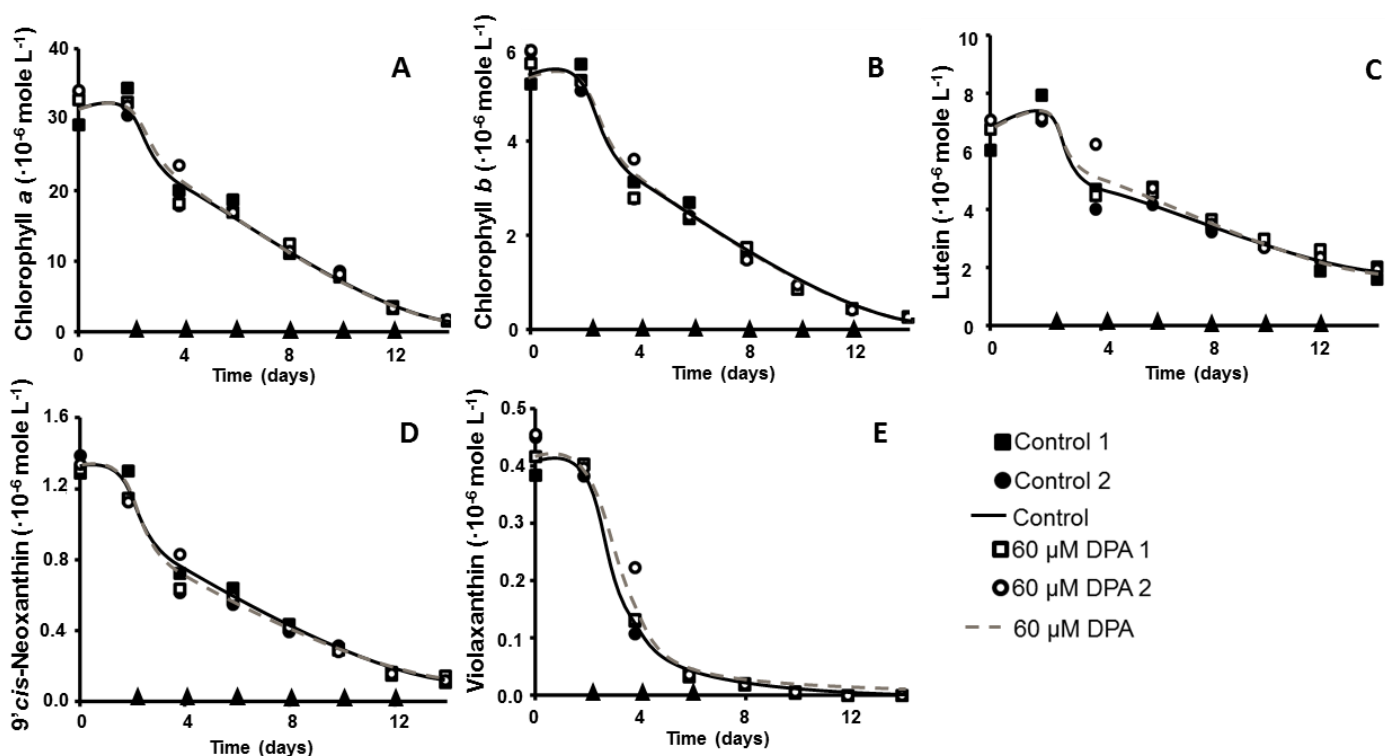


Fig. 4.5 Time courses of chlorophyll *a* (A), chlorophyll *b* (B), lutein (C), *g'*-*cis*-neoxanthin (D) and violaxanthin (E) in moles per litre culture volume of nitrogen-depleted *C. zofigiensis* exposed to no DPA (control) or exposed to repeated additions of DPA resulting each time in a concentration increase of 60 μ M. **Triangles** indicate DPA additions. **Lines** are for visual guidance.

4.3.5 Accumulation of secondary carotenoids

The secondary carotenoids ascertained in the treated culture were also the same as those discovered in the control, specifically, astaxanthin, ketolutein, canthaxanthin, adonixanthin, echinenone, and β , β -carotene. For the majority of these carotenoids, the cellular content was significantly different between the control and the treated culture (Fig. 4.6), indicating that under nitrogen-deplete conditions DPA had a significant effect on the formation of secondary carotenoids.

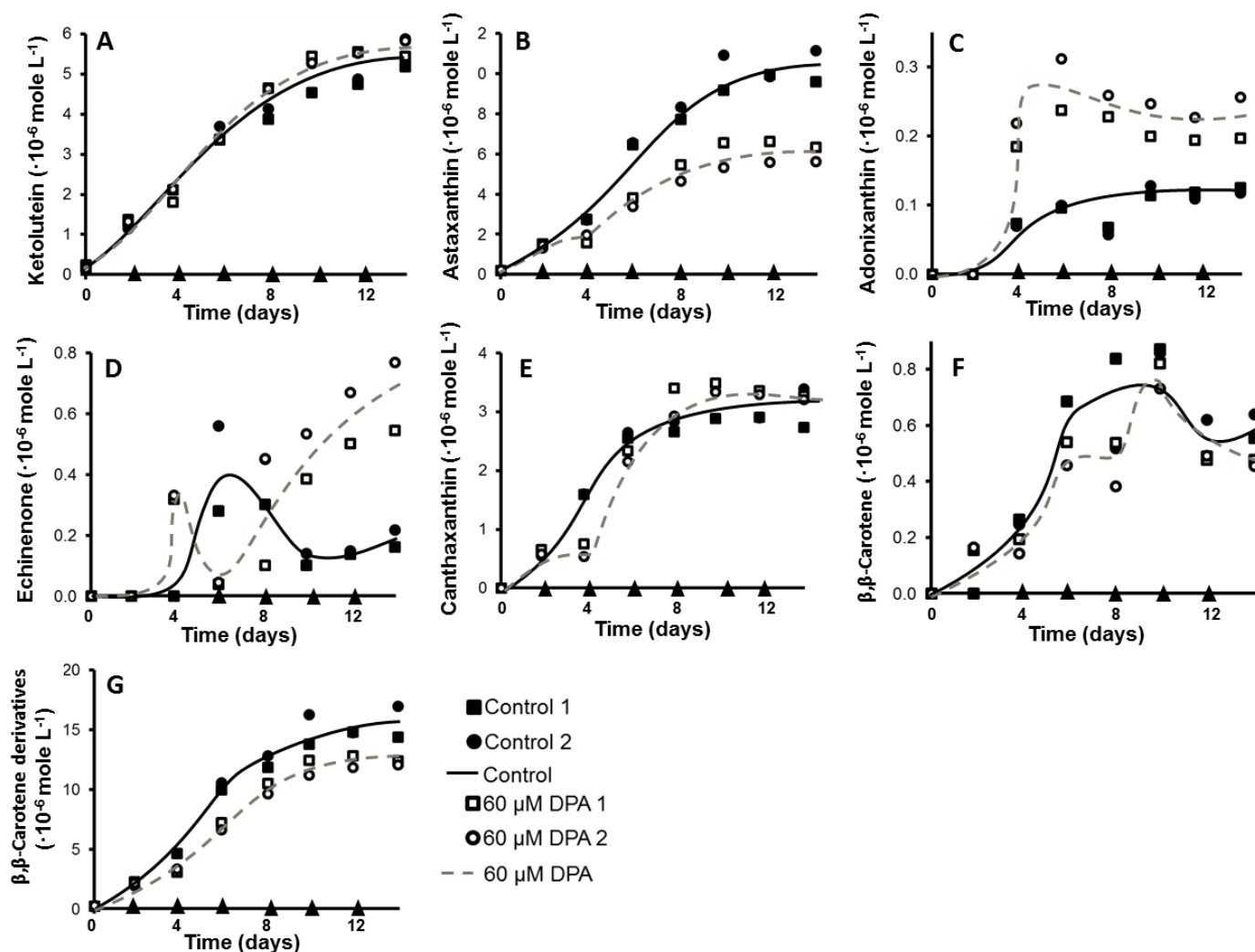


Fig. 4.6 Concentrations of total ketolutein (sum of free, mono- and diesters) (A), total astaxanthin (sum of free, mono- and diesters) (B), adonixanthin (C) echinenone (D), canthaxanthin (E), β,β -carotene (F) and all β,β -carotene derivatives (sum of total astaxanthin, adonixanthin, echinenone and canthaxanthin) (G) in moles per litre culture volume in nitrogen-depleted *C. zofingiensis* exposed to no DPA (control) or exposed to repeated additions of DPA resulting each time in a concentration increase of 60 μM . **Triangles** indicate DPA additions. **Lines** are for visual guidance.

Ketolutein. Ketolutein was the second most abundant secondary carotenoid in nitrogen-depleted *C. zofingiensis*. The cellular content increased gradually from zero to the final content with no significant variations between the control and the treated culture (Fig. 4.6A). In all cultures, ketolutein was ascertained primarily as monoester, to a more moderate extent in the free form (unesterified), and to a very minimal extent as diester (esterified with C16:0 and C18:1 fatty acids).

The absence of a significant difference in ketolutein concentration between the control and the treated culture indicated that DPA did not affect the overall formation of ketolutein. Ketolutein most plausibly resulted from lutein oxygenation which requires one enzymatic conversion catalysed by a ketolase. Expression of the BKT gene (from *H. pluvialis*) in *Chlamydomonas reinhardtii* which naturally synthesises β,β -carotene, zeaxanthin, and lutein but no β -end group ketocarotenoids such as echinenone and adonixanthin, resulted in synthesis of ketolutein, whereas no β -end group ketocarotenoids were synthesised (Vila et al.

2012). This supports the premise that ketolutein emerged from lutein via oxidation by BKT. It is remarkable that the lutein to ketolutein conversion by BKT was not affected by DPA. This may be explained by assuming that the enzyme that catalysed ketolutein biosynthesis was insensitive to DPA, either because it was a completely different ketolase (encoded by a different yet-unidentified gene), or because BKT may have been embedded in a multiprotein complex causing its insensitivity to DPA.

Ketolutein was found esterified with similar fatty acids as astaxanthin. Surprisingly, ketolutein was found mainly as monoester, whereas astaxanthin was detected primarily as diester, despite the fact that they both possess two hydroxyl groups. This might suggest that either the β -end group is more easily acylated than the ϵ -end group or that the adjacent carbonyl is a prerequisite for acylation.

Astaxanthin. Astaxanthin was the most abundant secondary carotenoid in nitrogen-depleted *C. zoefingensis*. In both the control and the treated cultures, its content increased gradually. From the moment that DPA was added, the increase in the treated culture was half as high as in the control culture (**Fig. 4.6B**), indicating that DPA significantly inhibited production of astaxanthin.

Theoretically, adonixanthin and adonirubin may both be precursors of astaxanthin (**Fig. 4.1**). Since adonixanthin was detected, the pathway to this carotenoid must have been present. Because adonixanthin was found in a very low concentration and only in the free form, it may be speculated that the pathway from adonixanthin to astaxanthin was existing as well. It remains ambiguous whether adonirubin was a precursor of astaxanthin as well since this carotenoid was lacking in nitrogen depleted *C. zoefingensis*.

Adonixanthin. In both the control and the treated cultures, the cellular content of adonixanthin increased gradually. From the moment that DPA was added, the increase in the treated culture was approximately twice as high as in the control culture (**Fig. 4.6C**). This finding supports the speculation that, in nitrogen depleted *C. zoefingensis*, adonixanthin was converted into astaxanthin.

Adonixanthin may be formed from echinenone via 3-hydroxyechinenone or 3'-hydroxyechinenone, as in *H. pluvialis* (Lemoine and Schoefs 2010), or from zeaxanthin, as proposed by Wang and Chen (2008) and Huang et al. (2006). This latter hypothesis was based on reduced zeaxanthin degradation in carotenogenic cultures exposed to DPA. As mentioned previously, no substantial amounts of zeaxanthin could be detected in any of the cultures. Additionally, we did not discover reduced degradation of zeaxanthin precursors (i.e. all other primary carotenoids) in the DPA-treated cultures. Therefore, our results do not support the hypothesis that, in nitrogen-depleted *C. zoefingensis*, adonixanthin is formed from zeaxanthin. In contrast, echinenone attained a cellular content that was approximately seven times as high in the treated cultures as in the control (**Fig. 4.6D**). This supports the hypothesis that echinenone is a possible precursor of adonixanthin.

Canthaxanthin. In both the control and the treated cultures, canthaxanthin was ascertained in relatively high cellular contents (comparable to esterified ketolutein contents). In both cultures, the cellular content increased gradually. In the treated cultures,

canthaxanthin was detected only on day four in significantly lesser amounts compared to the control culture (**Fig. 4.6E**). This indicates that canthaxanthin formation was inhibited for only two days which was compensated with increased canthaxanthin production the two days thereafter. It remains unclear what mechanism caused the recovery from DPA inhibition.

Huang et al. (2006) also discovered relatively high contents of canthaxanthin and, as stated, speculated that canthaxanthin is an end product rather than a precursor of astaxanthin. Our data do not provide further insights with regard to this speculation.

β,β -carotene. In both the control and the treated cultures, β,β -carotene was ascertained at low cellular contents (comparable to echinenone, adonixanthin, and free astaxanthin contents), with no significant variation between the control and the treated culture (**Fig. 4.6F**). The low cellular β,β -carotene content indicates that this carotenoid was promptly converted into echinenone and, moreover, that a high β,β -carotene content was not required for induction of secondary carotenogenesis.

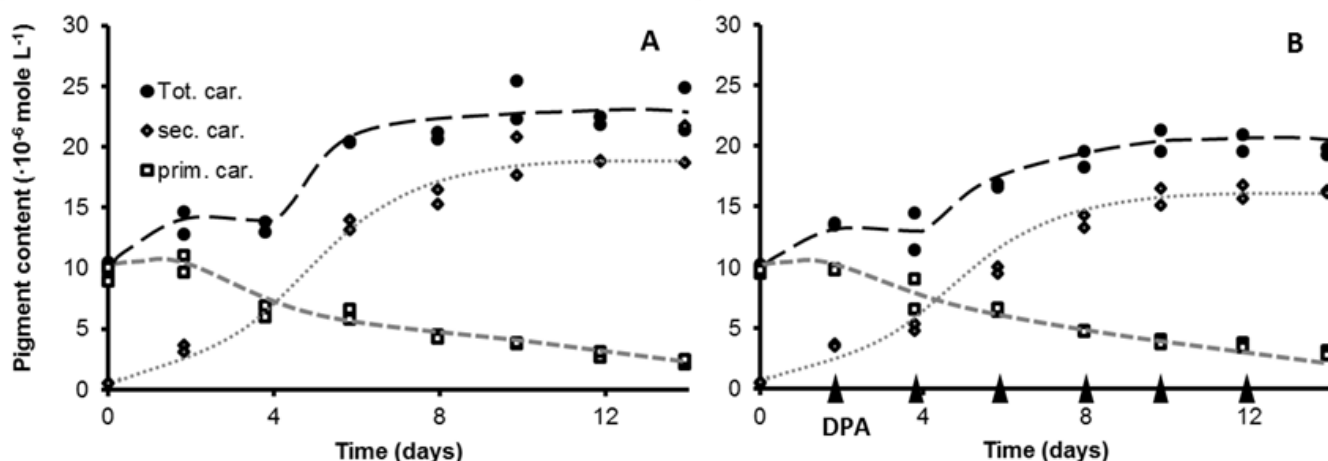


Fig. 4.7 Primary, secondary and total carotenoid contents of nitrogen-depleted *C. zofingiensis* exposed to no DPA (**A**) or exposed to repeated additions of DPA (**B**). **Primary carotenoids** include lutein, neoxanthin and violaxanthin. **Secondary carotenoids** include astaxanthin (sum of free, mono- and diesters), ketolutein (sum of free and mono- and diesters), canthaxanthin, adonixanthin, echinenone and β,β -carotene. **Total carotenoids** include both primary and secondary carotenoids. **Lines** are for visual guidance.

4.3.6 Novel insights in the regulation of carotenogenesis in *C. zofingiensis*

In both the DPA-treated culture and the control, accumulation of secondary carotenoids occurred simultaneously with a decrease in primary carotenoids (**grey lines, Fig. 4.7**). This lends support to the supposition that, under nitrogen-deplete conditions, secondary carotenoids were formed from primary carotenoids. However, the overall carotenoid content increased (**black lines, Fig. 4.7**), which indicates that, in the DPA-treated culture and in the control, at least part of the secondary carotenoids were synthesized *de novo*. Since the consumed quantity of lutein equalled approximately the produced amount of ketolutein, it may be assumed that, instead of degrading into other products, lutein was completely converted into ketolutein. Since lutein constituted the major fraction of primary carotenoids (>80 %), this implies that the remaining secondary carotenoids (echinenone, canthaxanthin,

adonixanthin and astaxanthin) must have been almost completely formed *de novo* in both the control and the DPA-treated cultures.

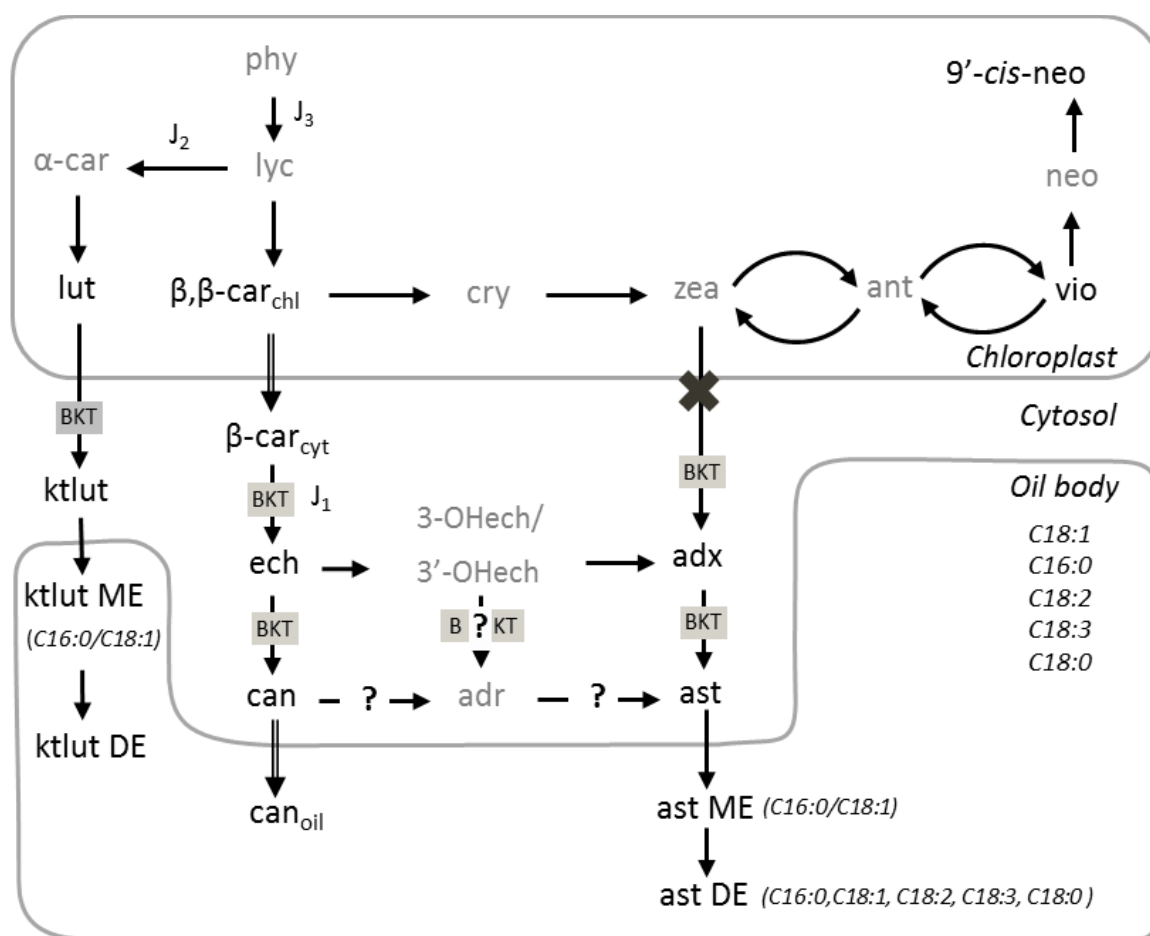


Fig. 4.8 Metabolic network model of carotenoids in nitrogen-depleted *C. zofigiensis*, summarizing obtained results. **Cross** indicates hypothesized pathway that is not present in nitrogen-depleted *C. zofigiensis*. **Question marks** indicate unresolved pathways. **Double** arrows indicate dislocations. **J_{ii}** indicate fluxes through specific pathways. **Black** carotenoids were found. **Grey** carotenoids were not found. Dislocations of lutein, ketolutein and astaxanthin are not explicitly indicated. Note that biosynthesis of ketolutein may as well take place in the chloroplast. See text for other details and assumptions. **3-OHech** 3-hydroxyechinenone, **3'-OHech** 3'-hydroxyechinenone, **9-cis-neo** 9-cis-neoxanthin, **α-car** α-carotene, **adr** adonirubin, **adx** adonixanthin, **ant** antheraxanthin, **ast** astaxanthin, **ast ME** astaxanthin monoester, **ast DE** astaxanthin diester, **β,β-car_{chl}** β,β-carotene (chloroplast), **β,β-car_{cyt}** β,β-carotene (cytosol), **cry** cryptoxanthin, **can** canthaxanthin (cytosol), **can_{oil}** canthaxanthin (oil body), **ech** echinenone, **ktlut** ketolutein, **ktlut ME** ketolutein monoester, **ktlut DE** ketolutein diester, **lut** lutein, **lyc** lycopene, **neo** neoxanthin, **phy** phytoene, **vio** violaxanthin, **zea** zeaxanthin (Kanehisa Laboratories 2012; Lemoine and Schoefs 2010; Vila et al. 2012; Wang and Chen 2008).

The overall cellular content of echinenone, canthaxanthin, adonixanthin, and astaxanthin was significantly different between the DPA-treated cultures and the controls. The overall content was reduced particularly during the first four days after the initial DPA addition (between day 2 and 6; **Fig. 4.6G**), indicating that, during this time, the flux through J_1 was significantly diminished (**Fig. 4.8**). In contrast, the overall cellular contents of lutein and ketolutein were not significantly affected (**Fig. 4.5C and 4.6A**) nor were the contents of other

primary carotenoids (**Fig. 4.5D - E**) which resulted in an overall decrease of carotenoid production (**Fig. 4.7A** versus **4.7B**). Thus, although DPA reduced the flux through J_1 , the flux through J_2 and the degradation of primary pigments remained unaffected, which implies a decreased flux through J_3 (**Fig. 4.8**). The decreased flux through J_3 can be explained by assuming that a regulatory mechanism was present which prevented increased production of ketolutein or primary carotenoids. It remains unclear what mechanism caused the recovery from DPA inhibition after day six.

In this work, BKT activity was inhibited employing an enzyme inhibitor which mimicked downregulation of BKT by genetic engineering. Our findings suggest that downregulation of the BKT enzyme of *C. zofingiensis* through genetic engineering and subsequent induction of secondary carotenogenesis (e.g. by nitrogen depletion) will not lead to excessive production of primary pigments such as lutein.

4.3.7 Oil body formation

Secondary carotenoids and their esters are generally assumed to accumulate in TAG oil bodies (Solovchenko 2013). To gain insight into oil body formation in nitrogen-depleted *C. zofingiensis*, freeze fracture scanning electron micrographs were created, and TAG and secondary carotenoid compositions of nitrogen-replete and nitrogen-depleted *C. zofingiensis* cells were analysed (no DPA added) (**Fig. 4.9**). The nitrogen-replete cells contained virtually no secondary carotenoids (less than 0.001 % (g/g DW)), whereas this concentration in the nitrogen-depleted cells was 0.1 % (g/g DW) (comparable with the nitrogen-depleted cells on day five of the control culture).

In the nitrogen-depleted culture, the TAG fatty acids C18:1, C16:0, C18:2, C18:3 and C18:0 were detected in the highest concentrations (**Fig. 4.9H**). The replete and depleted cells contained 1 % and 12 % (g/g DW) TAG fatty acids, respectively (**Fig. 4.9F**). In the replete cells, a moderate number of tiny oil bodies were ascertained (less than 100 nm in diameter) (**Fig. 4.9C**) whereas, in the depleted cells, multiple larger oil bodies were discovered throughout the cell in various sizes (up to 600 nm in length). These oil bodies remained separate despite their close proximity (**Fig. 4.9D - E**). The existence of multiple separate oil bodies suggests that these oil bodies were formed independently. In both the replete and depleted culture, oil bodies were found exclusively in the cytosol and, therefore, not in the chloroplasts (**Fig. 4.9A - E**). If oil bodies had been apparent in the chloroplast, they would have been visible as demonstrated in *Dunaliella salina* (Lamers et al. 2010) on similarly obtained micrographs.

Since the fatty acids detected most abundantly in TAG were identical to those discovered esterified to astaxanthin and ketolutein, these fatty acids may have been cleaved by lipases from *de novo* synthesised TAG, as was speculated for the astaxanthin esterified fatty acids in *H. pluvialis* (Solovchenko 2013). Otherwise, newly formed fatty acids may have been directly esterified to secondary carotenoids before forming TAG, as speculated by Lemoine and Schoefs (2010).

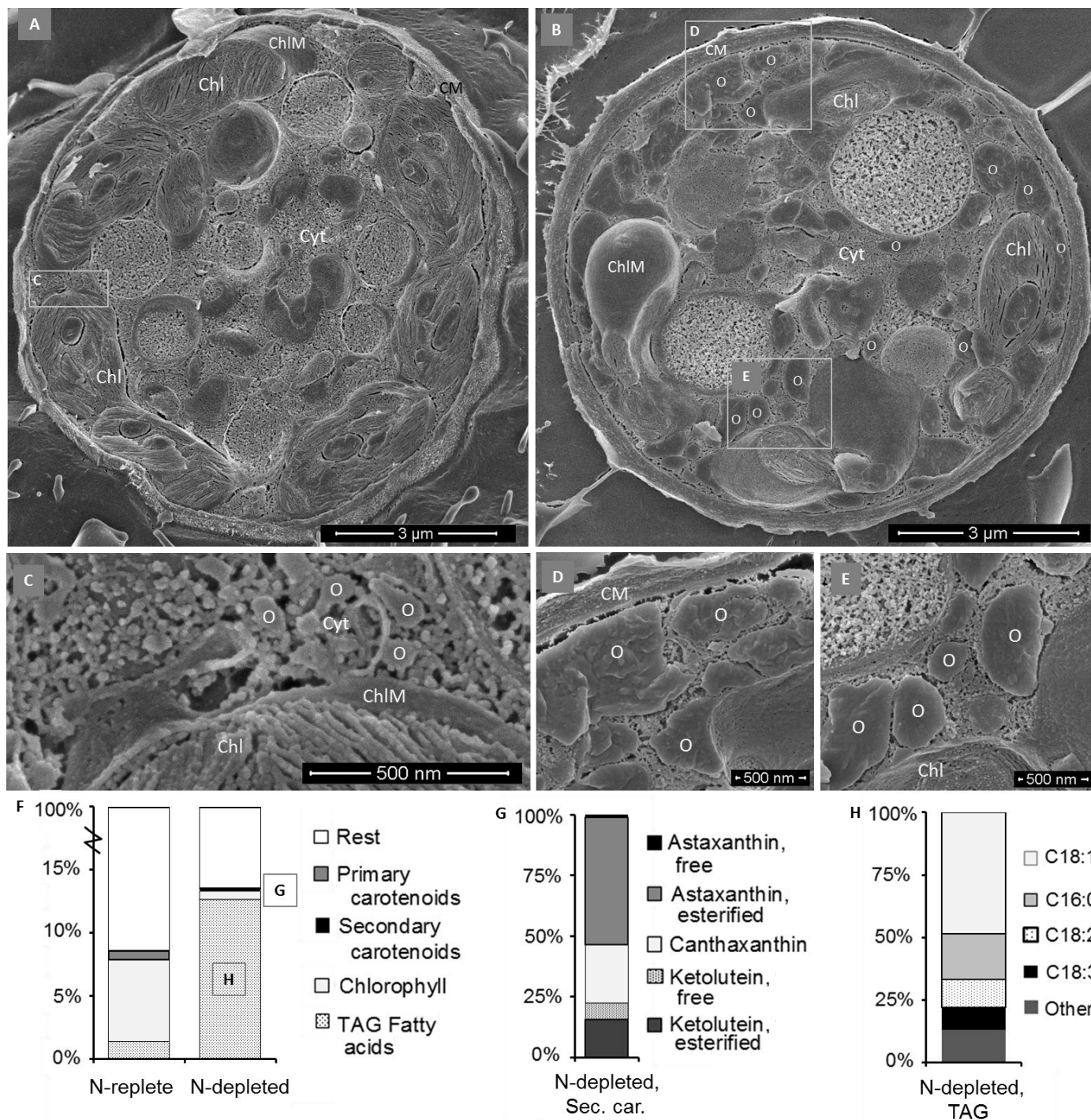


Fig. 4.9 Freeze fracture scanning electron micrographs and pigment and triacylglyceride (TAG) composition of nitrogen-replete and nitrogen-depleted *C. zofigiensis* cells. **A:** whole nitrogen-replete cell. **B:** whole nitrogen-depleted cell **C:** enlargement of cytoplasm located oil droplets in nitrogen-replete cell. **D** and **E:** enlargement of cytoplasm located oil droplets in nitrogen-depleted cells. **F:** cell composition (mass percentages of DW), including pigments and TAG fatty acids, of the nitrogen-replete and nitrogen-depleted cells. **G:** abundance of individual secondary carotenoids as percentage of total secondary carotenoids in the depleted cells. **H:** abundance of individual TAG fatty acids as percentage of total TAG fatty acids in the depleted cells. **Other** includes C12:0, C14:0, C16:1, C16:2, C16:3, C16:4, C18:0, C18:4 and C20:1. **Cyt** Cytosol, **Chl** Chloroplast, **ChlM** Chloroplast membrane, **CM** Cell membrane, **O** Oilbody.

Similar as in *C. zoofingiensis*, in *H. pluvialis* oil bodies are located in the cytosol (Grünewald et al. 2001; Lemoine and Schoefs 2010). In this species, astaxanthin as well as β,β -carotene was ascertained in oil bodies located in the cytosol (Collins et al. 2011), indicating that the conversion from β,β -carotene to astaxanthin occurs in the cytosol. Grünewald et al. (2001) demonstrated evidence of BKT in *H. pluvialis* in the chloroplast as well as in the oil bodies, however, BKT appeared to be active only on the surface of the oil bodies. Considering the many similarities between *C. zoofingiensis* and *H. pluvialis* (i.e. TAG oil bodies location, the fatty acids present in TAG and esterified to secondary carotenoids, and enzymes involved in secondary carotenogenesis), it may be speculated that, in *C. zoofingiensis*, the secondary carotenoids were also synthesised on the surface of the oil bodies and subsequently accumulated in the oil bodies. However, ketolutein may be synthesised in the chloroplast as well after which it may be transferred, esterified, and accumulated in oil bodies located in the cytosol. Both speculations need to be confirmed with more in-depth research on enzyme activity location of BKT and esterases.

4.4 Conclusion

In conclusion, besides esterified and free astaxanthin, canthaxanthin, echinenone and adonixanthin, *C. zoofingiensis* overproduces the secondary carotenoid ketolutein and its fatty acid esters. Whereas ketolutein must have been synthesised from pre-formed lutein, other secondary carotenoids were almost completely synthesised *de novo*. Moreover, although DPA inhibited the overall production of all other ketocarotenoids, it did not affect the production of ketolutein and its fatty acid esters or the metabolism of primary carotenoids such as lutein.

Acknowledgements

We gratefully thank Tiny Franssen-Verheijen of Wageningen Electron Microscopy Centre for her help with the Cryo-SEM. This work was supported by FeyeCon D&I and by grants from Rijksdienst voor Ondernemend Nederland (Project no. FND09014).

Appendices

4.A Overview of all pigment concentrations per reactor volume

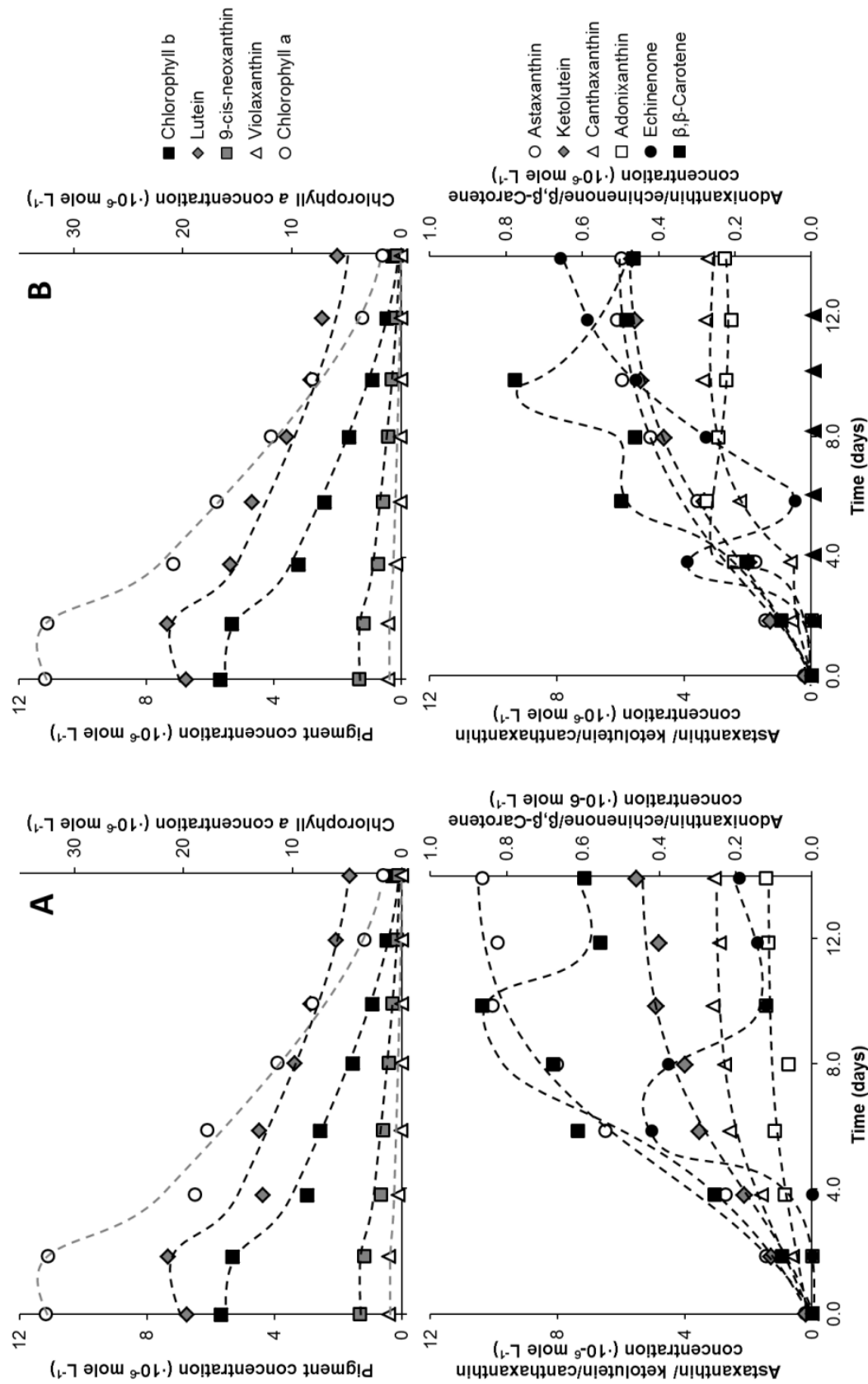


Fig. A.4.1 Time courses of chlorophyll a, chlorophyll b, lutein, 9-cis-neoxanthin, violaxanthin, total astaxanthin (sum of free, mono- and diesters), total ketolutein (sum of free, mono- and diesters), canthaxanthin, adonixanthin, echinenone and β , β -carotene in moles per litre culture volume of nitrogen-depleted *C. zoofingensis* exposed to no DPA (control) (A) and exposed to repeated additions of DPA resulting each time in a concentration increase of 60 μM (B). **Data points** represent averages of biological duplicates. For separate data points see Fig. 5 and 6. **Solid triangles** in B indicate DPA additions. **Lines** are for visual guidance.

4.B Pigment contents on a dry weight basis

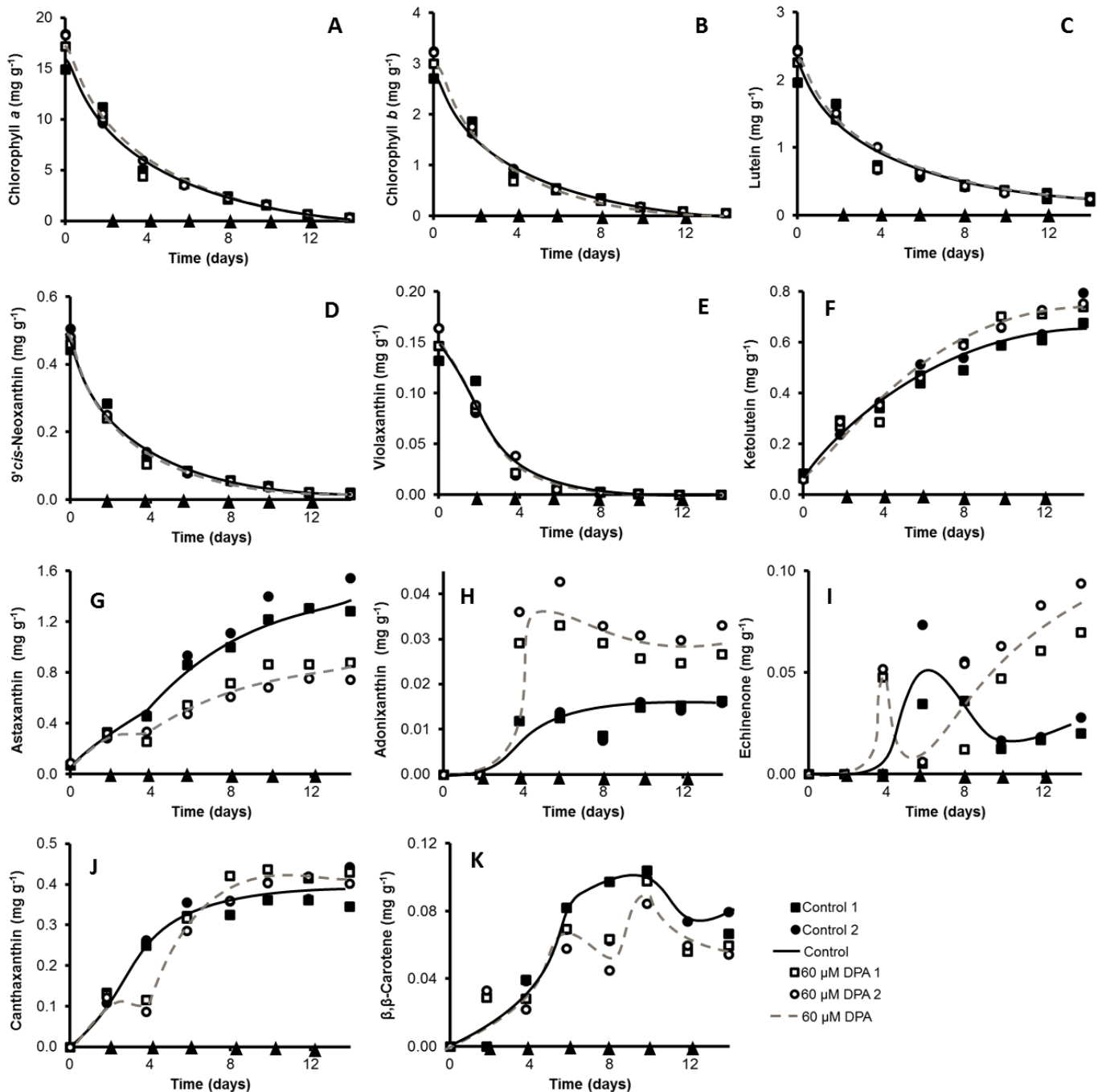


Fig. A.4.2 Contents (in mg/g DW) of chlorophyll a (A), chlorophyll b (B), lutein (C), 9'-cis-neoxanthin (D), violaxanthin (E), total ketolutein (sum of free, mono- and diesters) (F), total astaxanthin (sum of free, mono- and diesters) (G), adonixanthin (H), echinenone (I), canthaxanthin (J) and β,β-carotene (K) of nitrogen-depleted *C. zofingiensis* exposed to no DPA (control) and exposed to repeated additions of DPA resulting each time in a concentration increase of 60 μM. **Triangles** indicate DPA additions. **Lines** are for visual guidance.



Chapter 5

Effect of biomass concentration on secondary carotenoids and triacylglycerol (TAG) accumulation in nitrogen-depleted *Chromochloris zofingiensis*

High lights

- Nitrogen-depleted *C. zofingiensis* produced TAG and secondary carotenoids.
- Yields on absorbed light were equal for a range of initial biomass concentrations.
- Secondary carotenoid yields on light were reported for the first time (max. 2.75 mg mol_{ph}⁻¹).
- Maximal TAG yield (320 mg mol_{ph}⁻¹) is the highest reported for microalgae.

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Algal Research 6, pp.6-18

◀ IN THE PICTURE

In this photobioreactor microalgae can be cultivated in a well-defined and highly controlled way. That is very important when thorough research is to be conducted. For example, the temperature, CO₂ supply, pH and incident light intensity are controlled by several sensors which are connected to a computer. The light sensor (in the front) measures the outgoing light intensity *on line*.

◀ IN BEELD

In deze fotobioreactor kunnen microalgen op een goed gedefinieerde en zeer gecontroleerde manier gekweekt worden. Dat is zeer belangrijk voor het uitvoeren van nauwgezet onderzoek! De temperatuur, CO₂-toevoer, pH en ingaande lichtintensiteit worden beheerst d.m.v. verschillende sensoren en een computersysteem. De lichtsensor (op de voorgrond) meet continu de uitgaande lichtintensiteit.

Abstract

The effect of biomass-specific photon absorption rate on triacylglycerol (TAG) and secondary carotenoid yield was studied. *Chromochloris (Chlorella) zofingiensis* (Chlorophyta) was cultivated batch-wise with sufficient initial nitrogen to produce 2.5, 3.4 and 4.1 g L⁻¹ prior to nitrogen depletion, which resulted in biomass-specific photon absorption rates of 4.7, 3.5 and 2.9 $\mu\text{mol}_{\text{ph}} \text{g dry weight}^{-1} \text{s}^{-1}$, respectively. During nitrogen starvation, the TAG as well as secondary carotenoid yield on light did not differ between the cultures, which indicates that in the studied range the biomass-specific photon absorption rate did not affect the amounts of energy used for TAG and secondary carotenoid production. The maximal TAG yield on light (320 mg mol_{ph}⁻¹) was higher than values reported before for microalgae. Besides, for the first time the secondary carotenoid yield on light was reported (maximally 2.75 mg mol_{ph}⁻¹).

5.1 Introduction

One of the requirements for economically feasible microalgae-based triacylglycerol (TAG) production as feedstock for biofuel is co-production of higher value products (Wijffels et al. 2010). Furthermore, maximal areal productivities need to be obtained. Because the areal light supply rate limits the areal productivity of TAG and co-products, the yields of these metabolites on light need to be maximized. This work focused on the yield of TAG and secondary carotenoids on light, with the latter as higher value co-product. The microalga *Chromochloris (Chlorella) zofingiensis* was selected, because Breuer et al. (2012) previously identified this species as a potentially efficient TAG producer, while others highlighted its potential to produce carotenoids (Del Campo et al. 2004; Mulders et al. 2014b; Orosa et al. 2001; Rise et al. 1994; Solovchenko 2013). Thus, *C. zofingiensis* is a candidate for economically feasible TAG and secondary carotenoid production.

TAG and secondary carotenoids are produced under nitrogen-starved conditions. Under nitrogen starvation conditions excess electrons derived from photosynthesis are incorporated in TAG and secondary carotenoids, which prevents photo-oxidative cell damage, and results in storage of energy and carbon (Hu et al. 2008; Lemoine and Schoefs 2010; Mulders et al. 2014a). In addition to this role as an electron sink, secondary carotenoids can function as a sunscreen, protecting the cells by absorbing part of the excess irradiation (Lamers et al. 2008; Mulders et al. 2014a).

Previous outdoor studies showed that the biomass concentration at the moment of nitrogen depletion is an important parameter for the TAG yield on light (Feng et al. 2011; Zemke et al. 2013). In a batch cultivation a higher initial nitrogen concentration in the medium results in a higher biomass concentration at the moment of nitrogen starvation and, at the same incident light intensity, in a lower biomass-specific light absorption rate.

It is hypothesised that under nitrogen starvation a lower biomass-specific light absorption rate results in a higher TAG and secondary carotenoid yield on light, and thus into a higher areal productivity, because less absorbed light is dissipated to heat or lost through photoinhibition. However, if the biomass-specific light absorption rate is too low it is expected to lead to a lower TAG and secondary carotenoid yield on light because the absorbed light is mainly used to satisfy the maintenance requirements of the cells (Zemke et al. 2010).

Feng et al. (2011) and Zemke et al. (2013) observed under outdoor conditions that a lower supply rate of light per amount of biomass led to a higher areal TAG productivity. However, although in both studies the supplied amount of light per amount of biomass was measured, the absorbed amount of light per amount of biomass was not determined, and thus the relation between biomass-specific light absorption rate and TAG yield could not be defined. In addition, the relation between biomass-specific light absorption rate and secondary carotenoid yield on absorbed light in microalgae has not been reported. Under a high biomass-specific photon supply rate, not all supplied light may be absorbed by the culture. However, the algal cell metabolism is affected by the absorbed photons only, not by the total of supplied photons. Therefore, in addition to the insights provided by the previous studies of Feng and Zemke, knowledge of the TAG and secondary carotenoid yields on absorbed light is required to obtain insight in the mechanism of TAG and secondary carotenoid accumulation, which is important to optimize a large scale TAG and secondary carotenoid production process.

The aim of this paper is to measure the effect of biomass-specific light absorption rate on the yield of TAG and secondary carotenoids on absorbed light in *C. zofingiensis*. For this, *C. zofingiensis* was cultivated batch-wise, at different initial nitrogen concentrations leading to different biomass concentrations at the start of the nitrogen starvation phase. Thus, different biomass-specific light absorption rates were obtained at the start of nitrogen starvation.

5.2 Materials and methods

5.2.1 Culture, medium and pre-cultivation

Chromochloris zofingiensis was obtained as *Chlorella zofingiensis* UTEX B32 from the University of Texas Culture Collection of Algae (UTEX). Prior to the secondary carotenoids and TAG accumulation experiments, cultures were maintained as described in Mulders et al. (2014b). The replete (control) cultivation medium used in the experiment (modified M-8 medium (Mandalam and Palsson 1998)) consisted of KNO_3 142.4 mM; NaHCO_3 6.5 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 3.25 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.18 mM; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 13.8 mM; NaFeEDTA 0.56 mM; $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ 0.9 mM; H_3BO_3 2.0 μM ; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.13 mM; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 22.3 μM ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 15 μM ; Biotin 2 $\mu\text{g L}^{-1}$; vitamin B1 0.4 mg L^{-1} ; and vitamin B12 2 $\mu\text{g L}^{-1}$. The medium was adjusted to pH 7.0 with HCl and the medium was filter sterilized prior to use. Nitrogen-poor media were prepared similarly with the exception that part of the KNO_3 was substituted by KCl (to retain a total osmolarity of 0.189 Osm). Low-nitrogen medium contained KNO_3 9.28 mM, and KCl 61.96 mM; intermediate-nitrogen medium contained KNO_3 16.35 mM and KCl 54.86 mM; and high-nitrogen medium contained KNO_3 21.41 mM and KCl 49.89 mM. These nitrogen concentrations were chosen in such a way that a biomass concentration of 2.5, 3.4 and 4.1 g L^{-1} could be reached prior to nitrogen depletion. Hereafter, these cultures will be referred to as low biomass, intermediate biomass and high biomass cultures, respectively.

5.2.2 Batch nitrogen starvation experiments

Nitrogen starvation experiments were performed in a batch-wise operated, aseptic, heat-sterilized, flat-panel, airlift-loop photobioreactor (Labfors, Infors HT, 2010) (Fig. 5.1). The maximal liquid volume in the reactor was 1880 mL and the light path (reactor depth) was 20.7 mm.

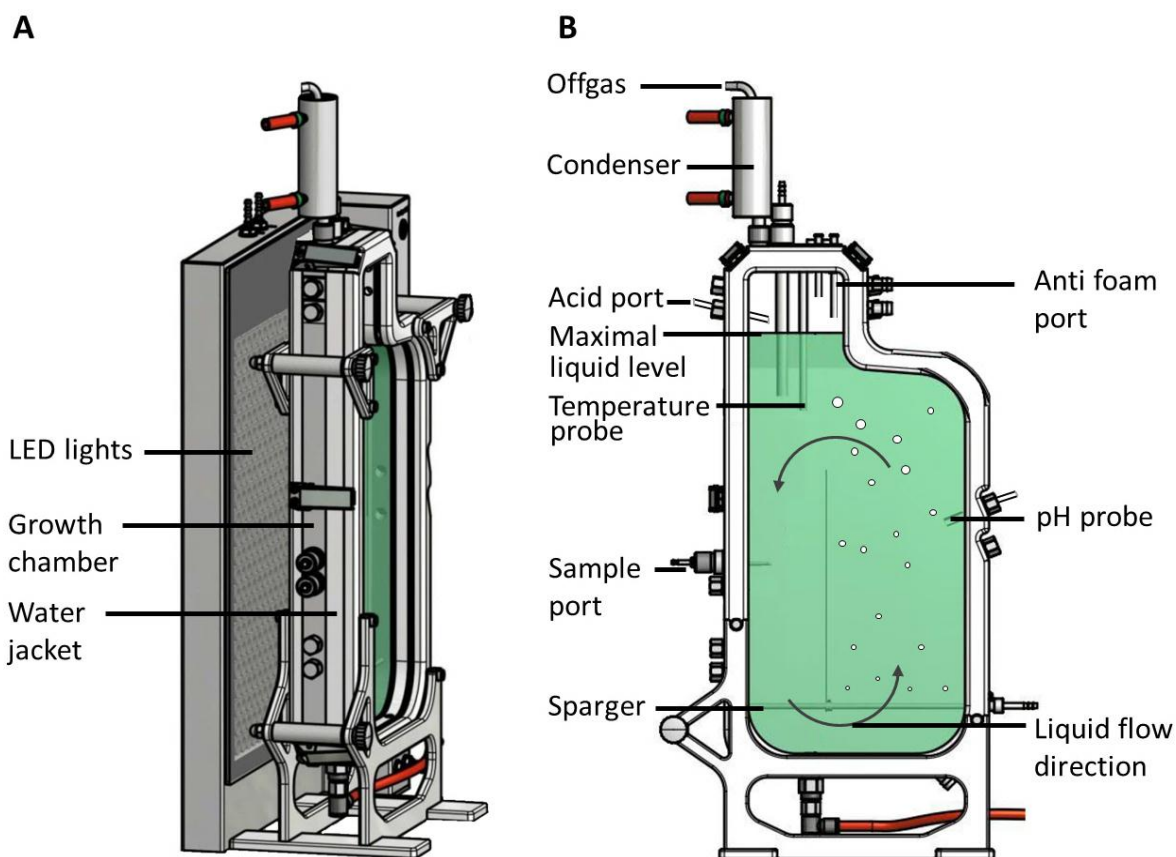


Fig. 5.1 Labfors photobioreactor used for the experiments, (A) side view and (B) front view. Growth chamber depth is 20.7 mm and maximal liquid level is 1880 mL. Adapted from Labfors 5 Lux LED Flat Panel manual.

All cultures were continuously illuminated (24 h per day). Illumination was provided on the culture side of the reactor (**Fig. 5.1a**) using 260 high power LED lights with a warm white spectrum (**Fig. A.5.1**). The incident light intensity (PFD_{in}) was determined as the average of 48 measurements spread over the entire surface of the inside of the front glass panel of the reactor. These measurements were performed using a LI 250 light meter (Li-cor). Aeration and mixing were provided by sparging a mixture of filtered CO_2 and CO_2 -stripped (using sodalime pellets), humidified, filtered air in a ratio of 2:98 (v/v) at 1.5 L/min. A pH of 7 was maintained by on-demand addition of 1M HCl. A temperature of 25 °C was maintained using a water jacket on the backside of the reactor (**Fig. 5.1a**), which was connected to an external cryostat. Heat sterilized 1 % (v/v) antifoam (Antifoam B, J.T. Baker) was added to the reactor manually when foam was visible (less than 15 mL per culture throughout the entire experiment).

The reactor was inoculated at a biomass dry weight concentration between 0.15 and 0.30 g L⁻¹, and at an incident light intensity of 63 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Until a biomass dry weight concentration of 2.2 g L⁻¹ was reached, the incident light intensity was increased step-wise to a final intensity of 245 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This took typically 7 days. 0.5, 1, and 2 days later (low biomass, intermediate biomass and high biomass cultures, respectively), nitrogen was depleted from the culture medium. This moment was considered the start of the experiment and is referred to as $t = 0$. Daily, a sample was taken directly from the reactor (6–15 ml sample volume) and analysed for dry weight concentration, pigments and TAG. In the intermediate

biomass culture, at $t = 1.07$ days, the reactor stopped for 15 hours (including air/CO₂ flow and light supply). This time interval was removed from the graphs.

5.2.3 Biomass-specific photon absorption rate

During the cultivations, the outgoing photon flux density ($PFD_{out,pbr}$) was determined as the average of 12 measurements spread over the surface of the outside of the back glass panel of the reactor. This value was corrected for light loss due to the water jacket, the glass plates and medium (PFD_{blank}). PFD_{blank} was determined as the average of 44 measurements spread over the entire surface of the outside of the back glass panel of the reactor which contained (transparent) medium. The absorbed photon flux density (PFD_{abs}) was calculated as described by Santos et al. 2013, using **eq. 1**

$$PFD_{abs} = PFD_{in} - \frac{PFD_{out,pbr}}{PFD_{blank}/PFD_{in}} \quad (1)$$

with PFD_{abs} , PFD_{out} , $PFD_{out, pbr}$, PFD_{blank} and PFD_{in} in $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The photon absorption rate per amount of biomass ($I_{abs,x}$) was subsequently calculated using **eq. 2**

$$I_{abs,x} = \frac{PFD_{abs}}{C_x \times 1000 \times l} \quad (2)$$

with $I_{abs,x}$ in $\mu\text{mol}_{ph} \text{g}_{DW}^{-1} \text{s}^{-1}$, C_x the dry weight biomass concentration in g L^{-1} and l the reactor chamber depth (light path) in m.

5.2.4 Time-specific and time-averaged productivity and yield on absorbed light

A distinction is made between the time-specific and time-averaged productivity on absorbed light of the products (TAG, secondary carotenoids or total biomass). For each product, the concentration versus time was plotted. A second order polynomial was used to fit a curve through these data, unless the data were obviously linear, in which case a linear fit was used. The time-specific productivity was estimated by calculating the first derivative of the fitted function. The time-averaged productivity was calculated as the average productivity over the period from time 0 to t (**eq. 3**)

$$P_{av} = \frac{C_p(t) - C_p(0)}{t} \quad (3)$$

with P_{av} being the time-averaged productivity in $\text{mg L}^{-1} \text{day}^{-1}$, $C_p(t)$ the product concentration at time t in mg L^{-1} , $C_p(0)$ the initial product concentration in mg L^{-1} and t the time in days.

Time-specific and time-averaged product yields on absorbed light (mg mol_{ph}^{-1}) were determined by dividing the productivity ($\text{mg L}^{-1} \text{day}^{-1}$) by the volumetric photon absorption rate of the evaluated period ($\text{mol}_{ph} \text{L}^{-1} \text{day}^{-1}$).

5.2.5 Dry weight concentration

Cell dry weight measurements were performed in triplicate by filtering and drying of the biomass as described by Kliphuis and Klok et al. (2012).

5.2.6 TAG extraction, identification and quantification

All lipophilic components were extracted as described by Breuer et al. (2013a). The TAG fraction was obtained using a solid phase extraction (SPE) column as described by Breuer et al.

(2012), with 10 ml 7 : 1 (v/v) hexane : diethylether as eluent. Solvents were evaporated and fatty acids of TAG were transesterified to fatty acid methyl esters (FAMES). FAMES were identified and quantified using GC-FID as described by Breuer et al. (2013a).

5.2.7 Pigment extraction, identification and quantification

Pigment extractions were performed using methanol/chloroform as a solvent as described by Lamers et al. (2010), with the exceptions as described in Mulders et al. (2014d). Pigment identification and quantification were performed using RP-UHPLC-PDA-MSⁿ analysis as described in Mulders et al. (2014d).

5.3 Results and discussion

The effect of biomass-specific photon absorption rate on TAG and secondary carotenoid yields on absorbed light was studied. *C. zofingiensis* was cultivated batch-wise with sufficient initial nitrogen to obtain a dry biomass concentration of 2.5 ± 0.05 , 3.4 ± 0.06 and 4.1 ± 0.06 (mean \pm standard deviation) prior to nitrogen depletion. In a control experiment, *C. zofingiensis* cells were grown with sufficient nutrients to produce 30 g L^{-1} biomass. Daily, the biomass-specific light absorption rate, cell dry weight, TAG and pigment concentrations were measured. The start of the nitrogen starvation phase corresponds to $t = 0$ for each depleted culture in **Section 5.3.1 to 5.3.3**.

5.3.1 Biomass production

The biomass dry weight concentration of the replete culture (control) increased linearly, reaching 12 g L^{-1} (**Fig. 5.2B-D**). The replete culture had a constant yield of biomass on absorbed light and a constant biomass productivity, equal to $0.75 \text{ g mol}_{\text{ph}}^{-1}$ and $0.75 \text{ g L}^{-1} \text{ day}^{-1}$, respectively. The biomass yield on absorbed light of *C. zofingiensis*, under the examined (low) light conditions, was similar to other green microalgae (Kliphuis, Klok et al. 2012).

In the low, medium and high biomass cultures, the amounts of biomass produced at the moment of nitrogen depletion were 2.5 ± 0.05 , 3.4 ± 0.06 and 4.1 ± 0.06 (mean \pm standard deviation), respectively (**Fig. 5.2A**). For the medium and high biomass concentration this was lower than expected (20 and 30 %, respectively), based on the inoculated biomass and initial nitrogen concentration in the medium. During the whole pre-cultivation period (from inoculation until nitrogen depletion) these cultures consumed more nitrogen per biomass compared to the low biomass culture. This difference in biomass-specific nitrogen consumption under different initial nitrogen concentrations was also observed by Wang and Lan (2011). Due to the difference in nitrogen consumption not only the biomass concentration at the moment of nitrogen depletion was different, but also the biomass composition must have been different.

In the depleted cultures, the biomass yield on absorbed light and biomass productivities were equal to that of the control until day four. Thereafter, they deviated significantly (**Fig. 5.2B-D**).

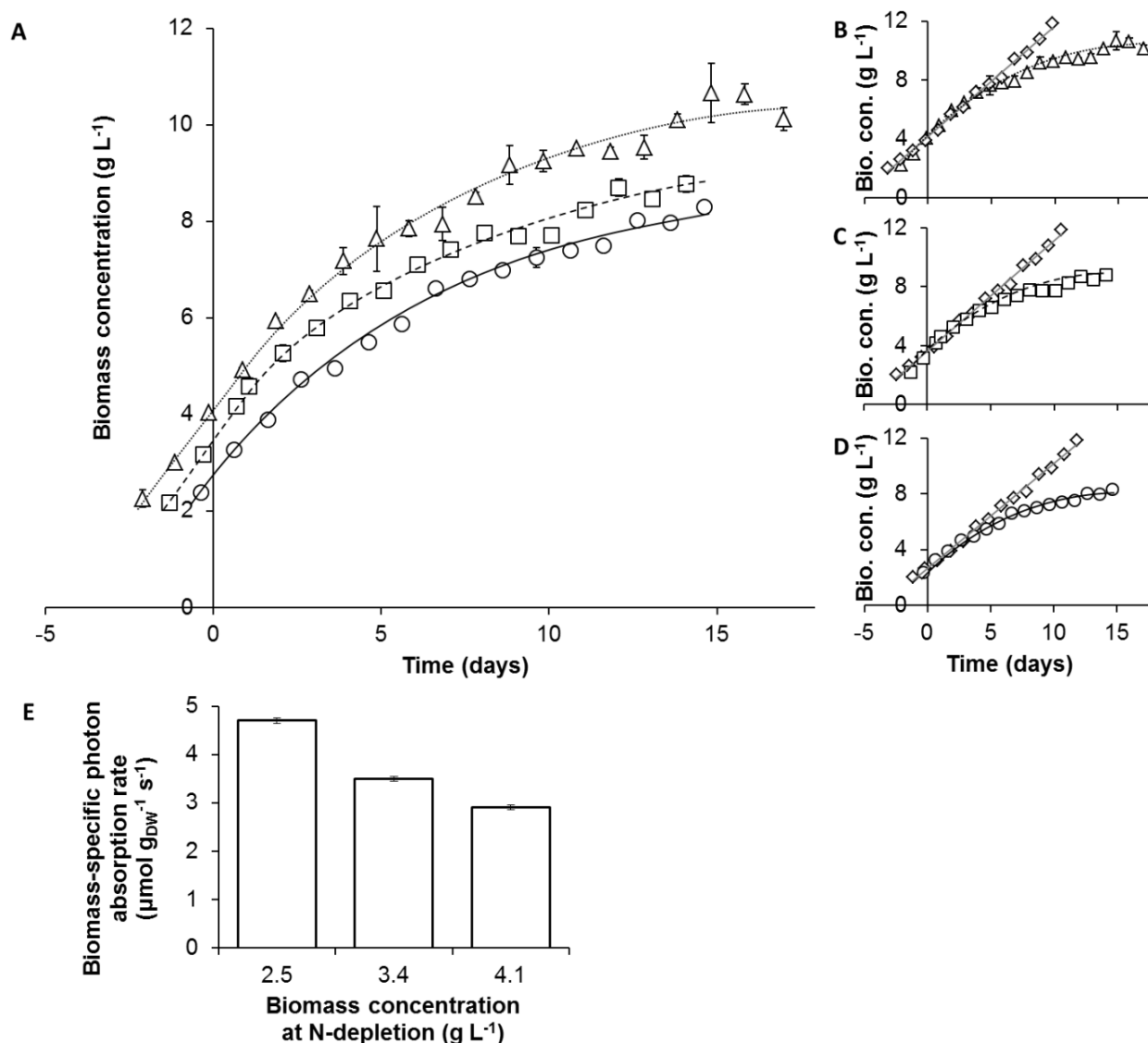


Fig. 5.2 Time courses of dry weight biomass concentration of *C. zofigiensis* (A) for different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○), 3.4 g L⁻¹ (□) and 4.1 g L⁻¹ (Δ), (B) for 4.1 g L⁻¹ (Δ) and replete culture (◇), (C) for 3.4 g L⁻¹ (Δ) and replete culture (◇) and (D) for 2.5 g L⁻¹ (Δ) and replete culture (◇). In N-depleted cultures, nitrogen was depleted at $t = 0$. (E) Biomass-specific photon absorption rate, at the moment of nitrogen depletion, for the three nitrogen-depleted *C. zofigiensis* cultures. Error bars represent standard deviations of triplicate measurements.

5.3.2 Biomass-specific photon absorption rate

In the replete and each depleted culture all incident light was absorbed (>99 %) during the whole cultivation period. Thus, each culture absorbed the same, constant amount of light per culture volume per time (i.e. 1.0 mol_{ph} L⁻¹ day⁻¹). At the point of nitrogen depletion, the biomass concentration of the high biomass culture was 1.6 times higher than that of the low biomass culture (4.1 vs. 2.5 g L⁻¹). Consequently, at the moment of nitrogen depletion, the biomass-specific photon absorption rate of the high biomass culture was 1.6 times lower than that of the low biomass culture (Fig. 5.2E).

In conclusion, each nitrogen-depleted culture had the same, constant volumetric photon absorption rate, but a significantly different biomass-specific photon absorption rate at the start of the nitrogen starvation phase.

5.3.3 Pigment and TAG accumulation: mechanistic insights

5.3.3.1 Primary pigments

In the replete culture, the primary (light harvesting) pigments chlorophyll *a*, chlorophyll *b*, lutein and, in lower amounts, *g*-*cis*-neoxanthin and violaxanthin were detected. As primary biomass contains primary pigments, an increasing biomass concentration led to an increasing primary pigment concentration per reactor volume (**Fig. 5.3A**). Besides, cellular pigment contents (on a dry weight basis) increased due to adaptation to a decreasing light availability (photoacclimation) (**Fig. A.5.2A**).

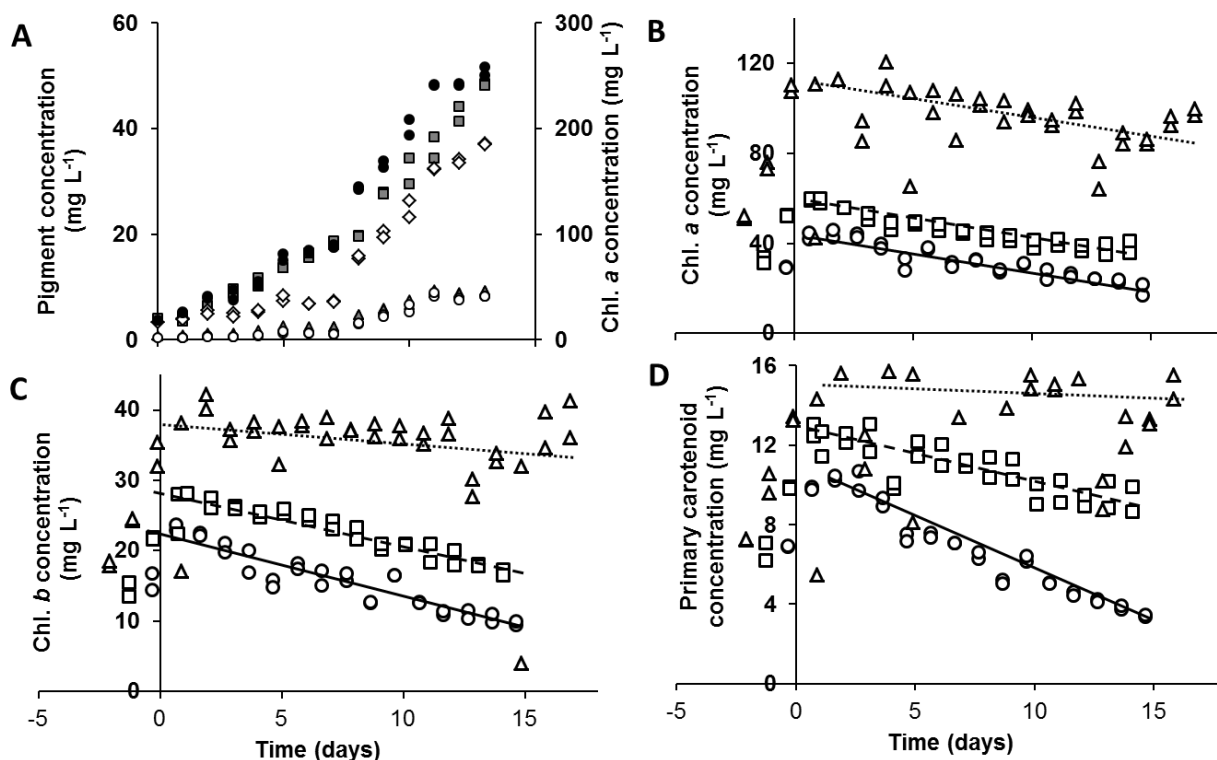


Fig. 5.3 Time courses of (A) primary pigment concentrations in nitrogen-replete *C. zoofingensis* culture (control) (chlorophyll *a* (●), chlorophyll *b* (■), lutein (◇), *g*-*cis*-neoxanthin (Δ) and violaxanthin (○)). (B) Chlorophyll *a*, (C) chlorophyll *b* and (D) total primary carotenoid (sum of lutein, *g*-*cis*-neoxanthin and violaxanthin) concentration in nitrogen-deplete *C. zoofingensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○), 3.4 g L⁻¹ (□) and 4.1 g L⁻¹ (Δ). In N-deplete cultures, nitrogen was depleted at *t* = 0.

Also in the depleted cultures, due to photoacclimation, cellular contents of primary pigments increased prior to nitrogen depletion (**Fig. A.5.2B-F**). Therefore, besides in biomass-specific photon absorption rate, the depleted cultures differed also in primary pigment content per amount of biomass at the moment of nitrogen depletion (i.e. they were adapted to different light regimes).

During the nitrogen starvation phase, all primary pigments were actively degraded. For chlorophyll *b*, lutein and *g*-*cis*-neoxanthin (all accessory light harvesting pigments), a higher photon absorption rate per amount of biomass resulted into higher degradation rates (**Fig. 5.3** and **Fig. A.5.3**). This observation is in agreement with Zemke et al. (2013) and Wang and Chen. (2013a), who reported higher degradation rates of total pigments in outdoor *C. zoofingensis*

cultures and higher degradation rates of total chlorophyll in outdoor *Haematococcus pluvialis* cultures with lower biomass concentrations (i.e. higher photon absorption rates per amount of biomass). Such active degradation is a well-known effect of nitrogen starvation and may be considered as a strategy of the cells to cope with the oversaturating light conditions resulting from the shortage of nitrogen (Falkowski and LaRoche 1991; Turpin 1991). A higher photon absorption rate per amount of biomass leads to a higher production rate of excessive electrons per amount of biomass, and induces more damage per photosystem (Aro et al. 1993; Osmond et al. 1997). Consequently, the higher photon absorption rate per amount of biomass must have led to the faster degradation of primary pigments (Melis 1999; Takahashi and Murata 2008).

5.3.3.2 Secondary carotenoids

In the replete culture, no substantial amounts of secondary carotenoids were produced (**Table 5.1** and **Fig. 5.4A-D**).

In the depleted cultures, the secondary carotenoids astaxanthin, canthaxanthin and ketolutein were produced up to cellular contents of 2.4, 1.3 and 0.8 mg g⁻¹ DW, respectively (**Table 5.1** and **Fig. A.5.4**). The first three to five days of nitrogen depletion secondary carotenoid concentrations increased exponentially, which was followed by a linear increase (**Fig. 5.4A-D**). Consequently, the yields of secondary carotenoids on light were initially zero and, in a period of three to five days, they reached their maximum values which remained constant thereafter (**Fig. 5.4A-D**). Because in each depleted culture the light was absorbed with a constant volumetric photon absorption rate, of 1 mol_{ph} L⁻¹ day⁻¹, the yield of secondary carotenoids on absorbed light was proportional to the volumetric productivity. The maximal yield on absorbed light and productivity of total secondary carotenoids were 2.75 mg mol_{ph}⁻¹ and 2.75 mg L⁻¹ day⁻¹, respectively.

In each depleted culture the total secondary carotenoid yield on absorbed light was almost equal, regardless of the biomass concentration at the moment of nitrogen depletion (**Fig. 5.4A**). Thus, the different biomass-specific photon absorption rates did not result in different yields of secondary carotenoids on absorbed light. Rather, the photons absorbed per culture volume were turned into secondary carotenoids with the same yield, irrespective of the biomass concentration at the moment of nitrogen depletion.

In contrast to the equal yields of secondary carotenoids on absorbed light and equal volumetric productivities, secondary carotenoid contents (on a dry weight basis) were different for each depleted culture. The highest secondary carotenoid contents were obtained in the culture with the lowest biomass concentration at the moment of nitrogen depletion (**Table 5.1** and **Fig. A.5.4**). The highest total secondary carotenoid content of *C. zoofingiensis* found in this work (4.5 mg g⁻¹ DW) was slightly lower, but in the same order of magnitude as those found by others (6 mg g⁻¹ DW (Orosa et al. 2001)) for this alga, but substantially lower than obtained with *H. pluvialis*, which can produce secondary carotenoids (mainly astaxanthin) up to 40 mg g⁻¹ DW (Boussiba et al. 1999). Whereas secondary carotenoid contents are frequently reported in literature, secondary carotenoid yields on light were not reported before. Therefore, the yields of secondary carotenoids on light obtained with *C. zoofingiensis* cannot be compared with other microalgae.

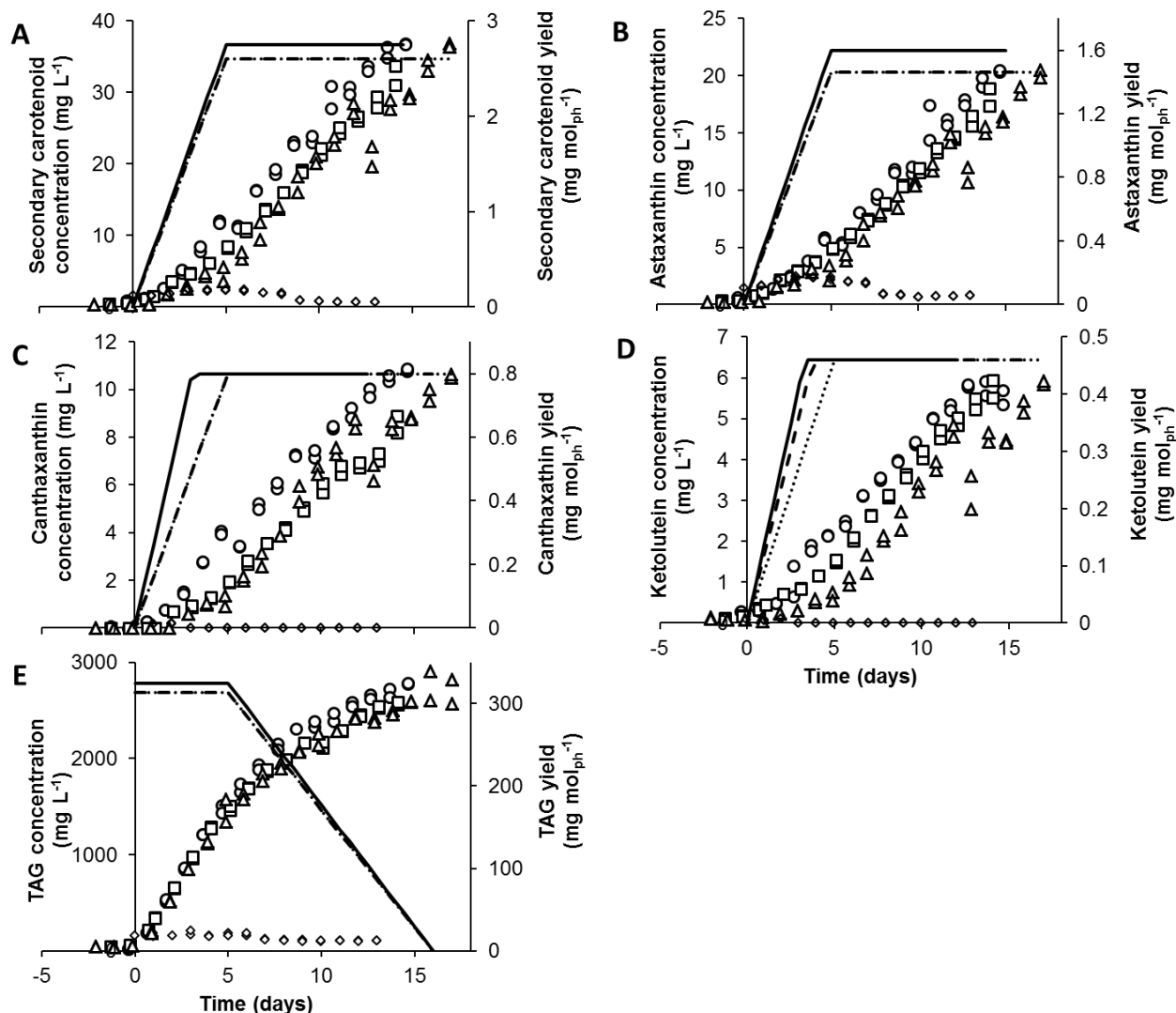


Fig. 5.4 Time courses of concentrations (**symbols**) and time-specific yields on absorbed light (**lines**) of secondary carotenoid and TAG in nitrogen-deplete *C. zofingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L^{-1} (\circ and solid line), 3.4 g L^{-1} (\square and dashed line) and 4.1 g L^{-1} (Δ and dotted line). (**A**) Total secondary carotenoids, (**B**) total astaxanthin (free, mono- and diesters), (**C**) canthaxanthin, (**D**) total ketolutein (free, mono- and diesters), and (**E**) total TAG. In addition, each figure contains the concentrations of nitrogen-replete *C. zofingiensis* culture (\diamond). Time-specific yields were estimated by calculating the first derivative of a fit through the concentration versus time data (see **Materials and Methods, Section 5.2.4**). In N-deplete cultures, nitrogen was depleted at $t = 0$; in N-replete culture $t = 0$ at 2 g L^{-1} .

The dynamics of secondary carotenoid accumulation (initially low and eventually maximal yield of secondary carotenoids on light) is in agreement with observations by Bar et al. (1995) and has also been generally observed in other carotenogenic microalgae, for example for astaxanthin accumulation in nitrogen starved *H. pluvialis* (Wang and Chen 2013a; Zhekisheva et al. 2002) and for β -carotene accumulation in nitrogen starved *Dunaliella salina* (Lamers et al. 2012). Different possible factors may have caused the low initial secondary carotenoid yield, such as the involved enzymes and the initial TAG productivity. Since canthaxanthin and ketolutein production requires activity of β -carotene ketolase, and astaxanthin production requires additional activity of β -carotene hydroxylase, it may be hypothesised that in *C.*

zofingiensis the initial secondary carotenoid yield on light was limited by one or both of these enzymes. According to Li et al. (2010c), the astaxanthin production in *H. pluvialis* is mainly under control of β -carotene hydroxylase. Others proposed that enzymes further up the carotenogenic pathway may be rate limiting, such as phytoene synthase (Jin et al. 2006) or phytoene desaturase (Lemoine and Schoefs 2010), which convert geranylgeranyl diphosphate into lycopene. In **Section 5.3.3.4** the initial TAG productivity is dismissed as possible factor causing the low initial secondary carotenoid yield.

Table 5.1 Contents of secondary carotenoids and TAG of *C. zofingiensis* for nitrogen-replete cells and nitrogen-depleted cells with different biomass concentrations at nitrogen depletion, 5 and 14 days after nitrogen was depleted.^a

Biomass concentration at N-depletion (g L ⁻¹)	Astaxanthin content (mg g ⁻¹)	Canthaxanthin content (mg g ⁻¹)	Keto-lutein content (mg g ⁻¹)	Total secondary carotenoid content (mg g ⁻¹)	TAG content (mg g ⁻¹)
N-replete	< 0.05	0.0	0.0	< 0.05	< 30
<i>5 days after N-depletion</i>					
2.5	1.0	0.6	0.4	2.0	270
3.4	0.7	0.3	0.2	1.2	220
4.1	0.5	0.2	0.1	0.8	195
<i>14 days after N-depletion</i>					
2.5	2.4	1.3	0.8	4.5	335
3.4	2.0	1.0	0.6	3.6	290
4.1	1.5	0.8	0.4	2.7	250

^aData represent the mean of duplicate measurements. The absolute deviations from the mean were always less than 0.15 mg g⁻¹ for secondary carotenoids and always less than 5 mg g⁻¹ for TAG.

5.3.3.3 TAG

In the replete culture, no substantial amount of TAG was produced (**Table 5.1** and **Fig. 5.4E**).

In the depleted cultures, TAG was produced up to a cellular content of 335 mg g⁻¹ DW (**Fig. A.5.5** and **Table 5.1**). In all depleted cultures, TAG concentrations increased linearly in the first five days of nitrogen depletion, after which the concentration-increase declined progressively (**Fig. 5.4E**). Consequently, in all depleted cultures TAG was produced at a maximal yield on absorbed light during the first five days, which was followed by a decrease to zero (**Fig. 5.4E**). The maximal TAG yield on absorbed light and TAG productivity were 0.32 g mol_{ph}⁻¹ and 0.32 g L⁻¹ day⁻¹, respectively. At day five, when the TAG yield was still maximal and the secondary carotenoid yield just became maximal, the TAG content had reached ~77 % of its maximal value, whereas the secondary carotenoid content had reached only ~37 % of its maximal values (**Table 5.1**). In each depleted culture the TAG yield on absorbed light was almost equal, regardless of the biomass concentration at the moment of nitrogen depletion (**Fig. 5.4E**).

Similar to the secondary carotenoids contents, TAG contents (on a dry weight basis) were different in each depleted culture. The highest TAG content was obtained in the culture with the lowest biomass concentration (**Table 5.1** and **Fig. A.5.5**). The highest obtained TAG content (335 mg g⁻¹ DW) was slightly lower, but in the same order of magnitude, as previously found with nitrogen-depleted *C. zofingiensis* (400 mg g⁻¹ DW) and also similar to contents obtained with other TAG producing microalgae (Breuer et al. 2012).

TAG was composed mainly of C18:1, C16:0, C18:2 and C18:3 fatty acids (representing 83 ± 1 % of total TAG). The yields on absorbed light of the two major fatty acids, C18:1 and C16:0, were not affected by the biomass concentration at the moment of nitrogen depletion (**Fig. A.5.6A-B**). In contrast, the minor fatty acids, C18:2 and C18:3, were produced with a higher yield on light in the low biomass culture (with 55 % and 25 %, respectively, compared to the high biomass culture) (**Fig. A.5.6C-D**). These latter changes did not have a substantial impact on the total TAG yield on light (**Fig. 5.4E**).

Thus, the different biomass-specific photon absorption rates did not result into a different TAG yield on absorbed light. Rather, the photons absorbed per culture volume were turned into TAG with the same yield irrespective of the biomass concentration at the moment of nitrogen depletion, similarly to what was found for secondary carotenoids.

Table 5.2 Comparison of maximal time-averaged TAG yield on absorbed light found in this study with those reported for several other microalgae.

Species	TAG yield on light ($\text{g mol}_{\text{ph}}^{-1}$)	Reference
<i>Chromochloris (Chlorella) zofingiensis</i>	0.32	This study
<i>Neochloris oleoabundans</i>	0.27 ^a	Santos et al. (2013)
<i>Nannochloropsis</i> sp.	0.27 ^{a,b}	Rodolfi et al. (2009)
<i>Scenedesmus obliquus</i>	0.24 ^a	Breuer et al. (2013b)
<i>Phaeodactylum tricornutum</i>	0.21 ^a	Dillschneider et al. (2013)
<i>Nannochloropsis</i> sp.	0.19 ^{a,b}	Bondioli et al. (2012)
<i>Chlorella vulgaris</i>	0.16 ^a	Münkel et al. (2013)

^a Calculated based on the assumption that 90 % (w/w) of totally produced lipids were TAG.

^b Calculated from outdoor measurements based on the assumptions that photons in the photosynthetic active radiation (PAR) spectrum have an energy content of $0.217 \text{ MJ mol}^{-1}$ and 42 % (J/J) of the irradiance has a wavelength within the PAR spectrum.

Outdoor observations by Feng et al. (2011) and Zemke et al. (2013) show an increased areal lipid productivity upon decreasing biomass-specific light supply rates in nitrogen-depleted *C. zofingiensis*, which appear to be in disagreement with our findings. However, these works were performed under biomass-specific photon supply rates that were up to eight times higher than in the current work (i.e., average incident light intensities were 1.4 times higher and biomass concentrations were up to five times lower). Consequently, in the work by Feng et al. (2011) and Zemke et al. (2013), the cultures with the lowest biomass concentrations at the start of the nitrogen starvation phase may have absorbed only part of the incident light, resulting in low yields on incident light. In addition, in these cultures relatively more energy may have been dissipated to heat or have caused photoinhibition, also resulting into low yields on absorbed light and, consequently, into low areal productivities. Thus, under very high biomass-specific photon supply rates, as applied by Feng et al. (2011) and Zemke et al. (2013), a decrease in biomass-specific photon supply rates may indeed lead to an increase in lipid yield on absorbed light. However, in the highest biomass cultures of Zemke et al. (2013), a further increase in biomass concentration did not further increase the areal lipid productivity. In other words, under lower biomass-specific photon supply rates, as applied in the current work, a decrease in biomass-specific photon supply rate did not lead to an increase in lipid yield on absorbed light. Such a plateau in lipid yield on absorbed light corresponds with our results and with results

obtained with other microalgae (e.g., *Chlorella vulgaris* (Münkel et al., 2013), *Nannochloropsis oculata* (Su et al., 2011), *Phaeodactylum tricornutum* (Dillschneider et al., 2013) and *Scenedesmus dimorphus* (Wang et al., 2013b)).

Compared to our results, Feng et al. (2011) and Zemke et al. (2013) reported substantially lower maximal TAG yields on incident light with *C. zofingiensis* (approximately 1.2 and 2.6 times lower, respectively) in their cultures with maximal areal productivities. These lower yields may be explained by the very high incident light intensities reported at noon (up to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$), which may have led to elevated photoinhibition. Besides, TAG may have been degraded during the night, for example to supply energy for maintenance. Finally, fluctuating temperatures during night and day may have inhibited higher TAG yields. The TAG yield on absorbed light reported in this work was also higher than values reported for other microalgae (Table 5.2).

5.3.3.4 Relation between secondary carotenoid and TAG metabolism

Astaxanthin was detected primarily as diester, whereas ketolutein was primarily detected as monoester. They were acylated primarily with C16:0 and C18:1 fatty acids and in lower amounts with C18:0, C18:2 and C18:3 fatty acids, which were also the five main fatty acids detected in TAG. Therefore, it has been proposed that astaxanthin and ketolutein esters are formed in close relationship with TAG formation, and subsequently accumulate in TAG oil droplets (Mulders et al. 2014b), as was observed for astaxanthin and β -carotene in *H. pluvialis* and *D. salina*, respectively (Zhekisheva et al. 2002; Rabbani et al. 1998). Accordingly, it may be speculated that the initial yield of secondary carotenoids on light is limited by the initial TAG production rate.

However, in each depleted culture, the final secondary carotenoid to TAG ratio was much higher than the initial ratio (Fig. 5.5A), which suggests that the low initial yield of secondary carotenoids on light (the first five days (Fig. 5.4A)) was most likely not due to saturation of TAG oil droplets with secondary carotenoids.

Additionally, TAG was produced at a much higher rate than astaxanthin and ketolutein esters (100 and 1000 times, respectively) (Fig. 5.5B-C). Nevertheless, free astaxanthin and ketolutein accumulated (Fig. 5.5D-E). This suggests the existence of a regulatory mechanism which ensured that the majority of fatty acids was used for TAG formation and only a small fraction for acylation of either astaxanthin or ketolutein.

Secondary carotenoid and TAG accumulation are known to be induced by the same stimuli (i.e. oversaturating light conditions), leading to a correlation in their accumulation dynamics (Zhekisheva et al. 2002). Consequently, a causal relationship in which TAG deposition drives secondary carotenoid accumulation has been postulated by, for example, Rabbani et al. (1998) thereby identifying TAG biosynthesis as a potential target for upregulation to ultimately enhance secondary carotenoid productivity. However, our data show that this causal relationship does not necessarily exist, which has also been shown by others (Lamers et al. 2010; Lamers et al. 2012). Accordingly, the initial yield of secondary carotenoids on light is likely not limited by the initial TAG production rate. If this is indeed the case, enhancement of the initial TAG production rate (e.g., by genetic engineering) will probably not lead to an increase in initial secondary carotenoid yield on light.

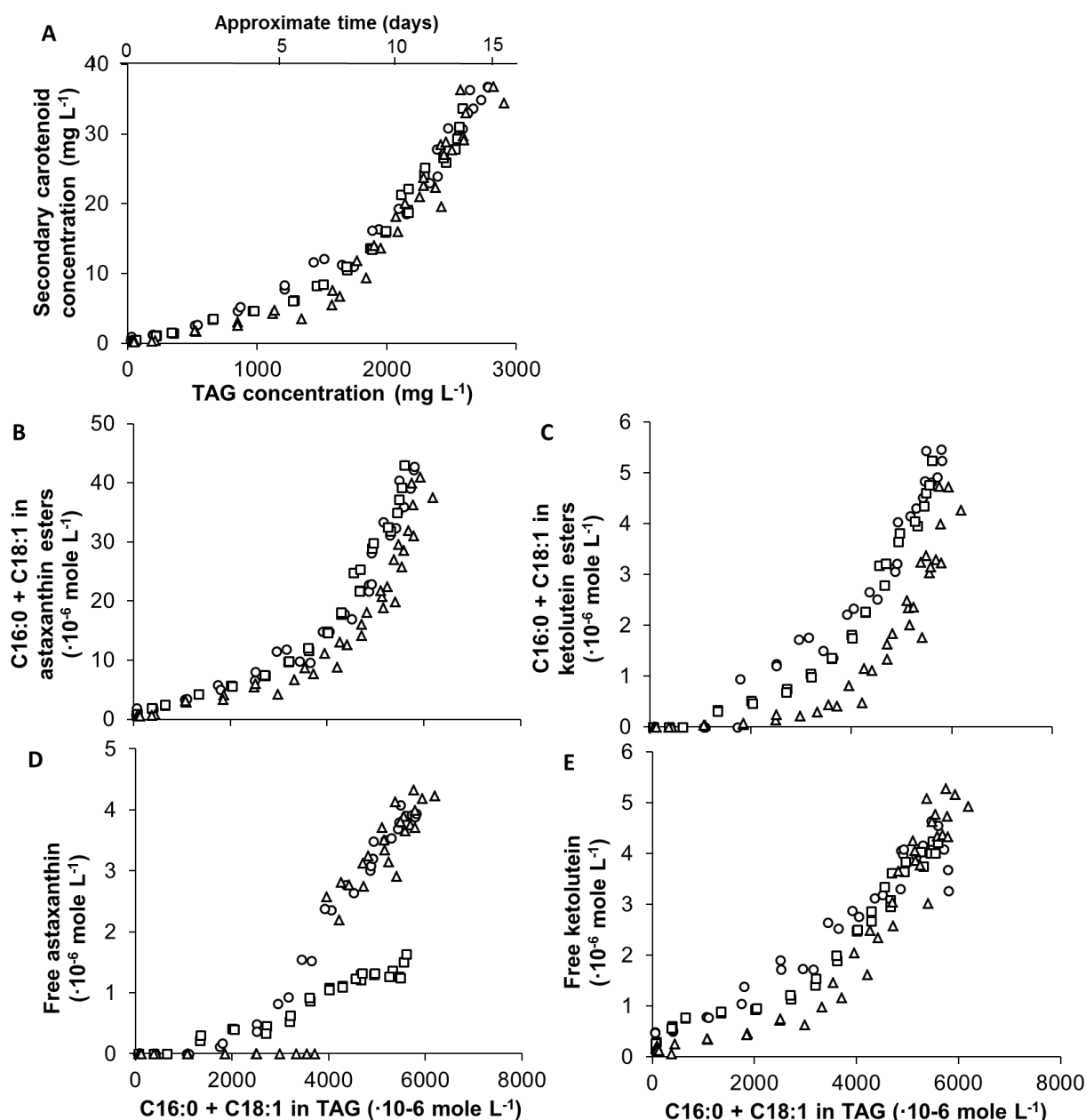


Fig. 5.5 TAG and secondary carotenoid accumulation in nitrogen-deplete *C. zoefingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○), 3.4 g L⁻¹ (□) and 4.1 g L⁻¹ (Δ). **(A)** Total secondary carotenoid versus total TAG concentration. Upper y-axis indicates the average time of the data points of the three depleted cultures. **(B)** C16:0 and C18:1 fatty acids in astaxanthin esters versus TAG C16:0 and C18:1 fatty acids, **(C)** C16:0 and C18:1 fatty acids in ketolutein esters versus TAG C16:0 and C18:1 fatty acids, **(D)** free astaxanthin versus TAG C16:0 and C18:1 fatty acids and **(E)** free ketolutein versus TAG C16:0 and C18:1 fatty acids.

5.3.4 Pigment and TAG accumulation: time-averaged yields

In **Sections 5.3.1** to **5.3.3**, time-specific (instantaneous) TAG and secondary carotenoid yields on absorbed light were presented from the start of the nitrogen starvation phase. In this section, time-averaged TAG and secondary carotenoid yields are presented from the start of the biomass production phase. Thus in this paragraph, the start of the biomass production phase refers to $t = 0$.

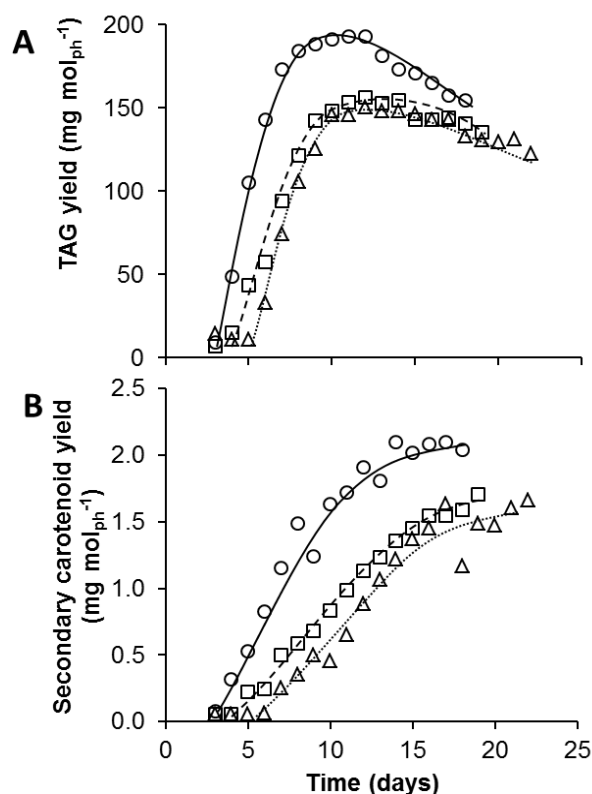


Fig. 5.6 Time-averaged yields on absorbed light, from the start of the biomass production phase ($t = 0$), for nitrogen-depleted *C. zofingiensis* cultures with different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○), 3.4 g L⁻¹ (□) and 4.1 g L⁻¹ (Δ). (A) For total TAG and (B) for total secondary carotenoids. A biomass yield on absorbed light of 0.75 g mol_{ph}⁻¹ was presumed during the biomass production phase. Yields were averaged over the period between $t = 0$ and each time point (see **Materials and Methods, Section 5.2.4**). Data represent the mean of duplicate measurements. The absolute deviations from the mean were always less than 5 %.

With a biomass yield of 0.75 g per mol absorbed photons (the biomass yield on absorbed light observed for the replete (control) culture), it would have taken 3.3 days to produce 2.5 g L⁻¹ DW (the biomass concentration of the low biomass culture at the start of the nitrogen starvation phase). For the intermediate and high biomass cultures, the biomass production phase would have taken respectively 4.3 and 5.1 days. During this initial biomass production phase, absorbed photons were used only to produce biomass, and thus not to accumulate secondary compounds, and hence the yields of these secondary metabolites on light were zero during these periods. After nitrogen depletion, each culture produced TAG and secondary carotenoids with yields on light that were independent of the biomass concentration at the moment of nitrogen starvation (Fig 5.4). Thus, the culture with the lowest biomass concentration at the moment of nitrogen depletion reached the nitrogen starvation phase fastest, having consumed the least amount of photons. Consequently, this culture has the highest time-averaged yields of TAG and secondary carotenoid on absorbed light when the whole cultivation period is considered (from the moment of inoculation until the moment of harvesting) (Fig 5.6).

5.4 Conclusions

During the nitrogen starvation phase both TAG and secondary carotenoids were produced with yields on absorbed light that were equal for each biomass-specific photon absorption rate. This indicates that, in the studied range, the biomass-specific photon absorption rate did not affect the amounts of energy used for TAG and secondary carotenoid production.

Consequently, the culture with the lowest biomass concentration at nitrogen depletion obtained the highest TAG and secondary carotenoid yield on light when also the biomass

production phase was taken into account, and this lowest biomass culture reached the highest TAG and secondary carotenoid contents (335 and 4.5 mg/g, respectively).

Acknowledgements

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Appendices

5.A Spectral distribution provided to the culture

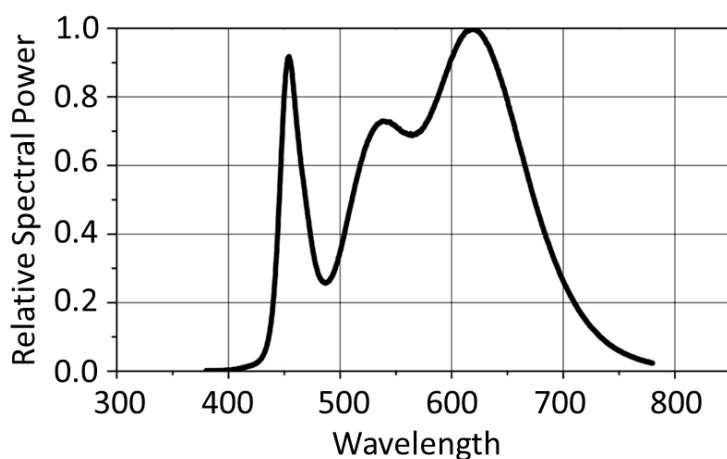


Fig. A.5.1 Relative spectral distribution of the (warm white) spectrum provided to the cultures.

5.B Additional pigment contents and pigment concentrations

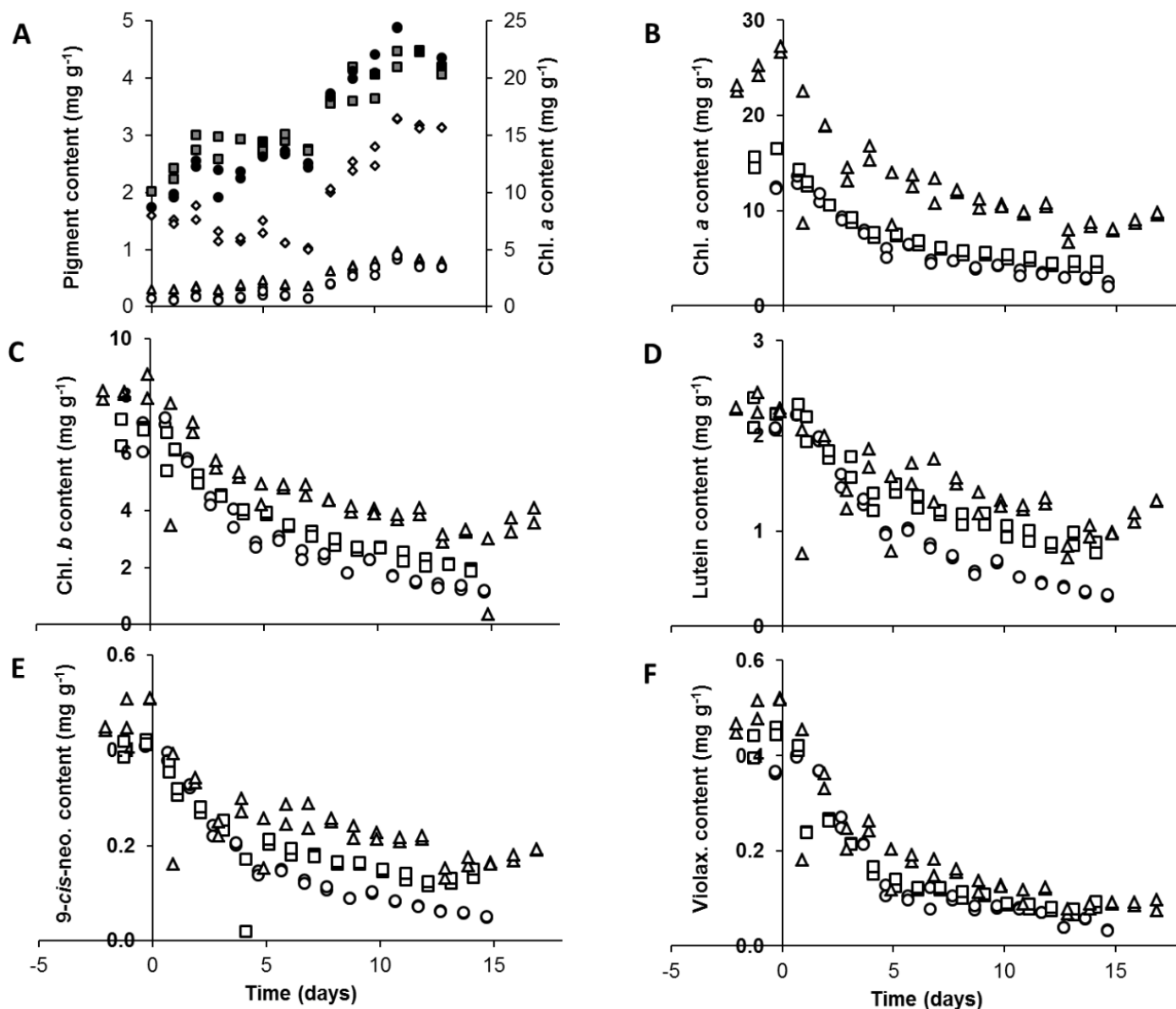


Fig. A.5.2 Time courses of (A) primary pigment contents in nitrogen-replete *C. zofingiensis* culture (control) (chlorophyll a (●), chlorophyll b (■), lutein (◇), 9-cis-neoxanthin (Δ) and violaxanthin (○)). (B) Chlorophyll a, (C) chlorophyll b and (D) lutein, (E) 9-cis-neoxanthin and (F) violaxanthin contents in nitrogen-deplete *C. zofingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○), 3.4 g L⁻¹ (□) and 4.1 g L⁻¹ (Δ). In N-deplete cultures, nitrogen was depleted at $t = 0$.

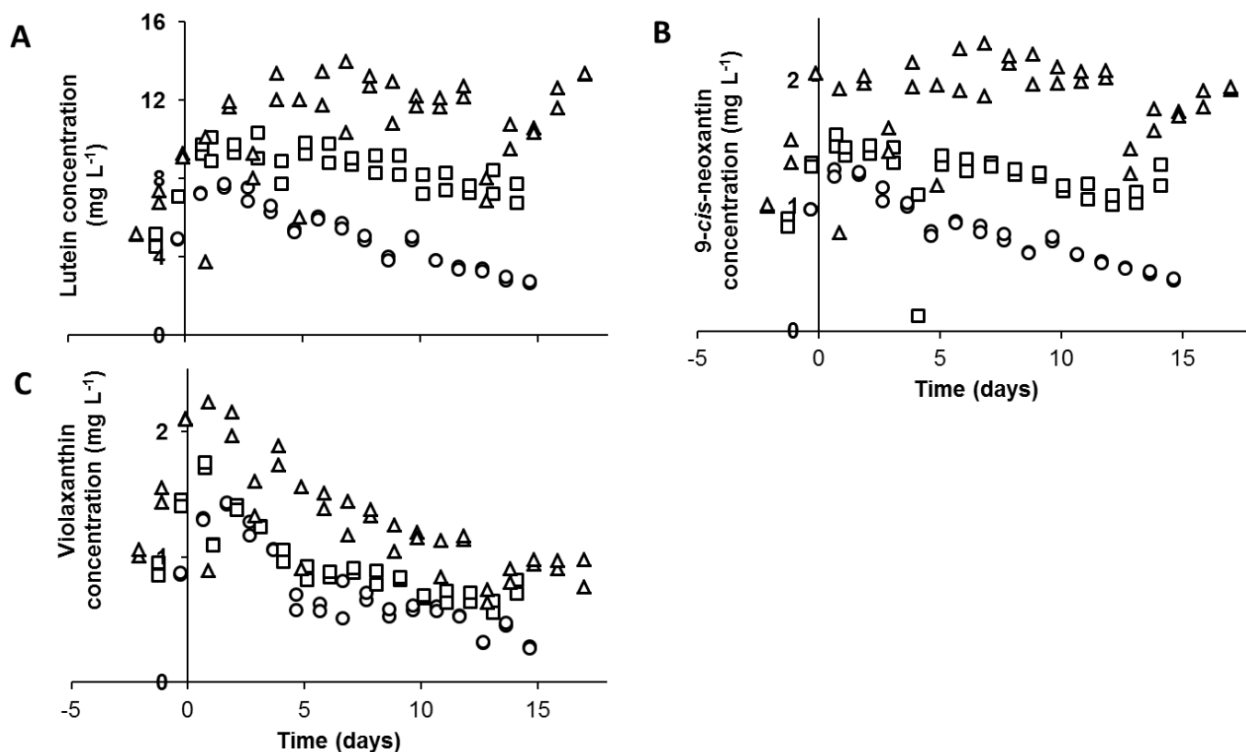


Fig. A.5.3 Time courses of primary carotenoid concentrations in nitrogen-deplete *C. zoefingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L^{-1} (\circ), 3.4 g L^{-1} (\square) and 4.1 g L^{-1} (Δ). (A) lutein, (B) 9-cis-neoxanthin and (C) violaxanthin. Nitrogen was depleted at $t = 0$.

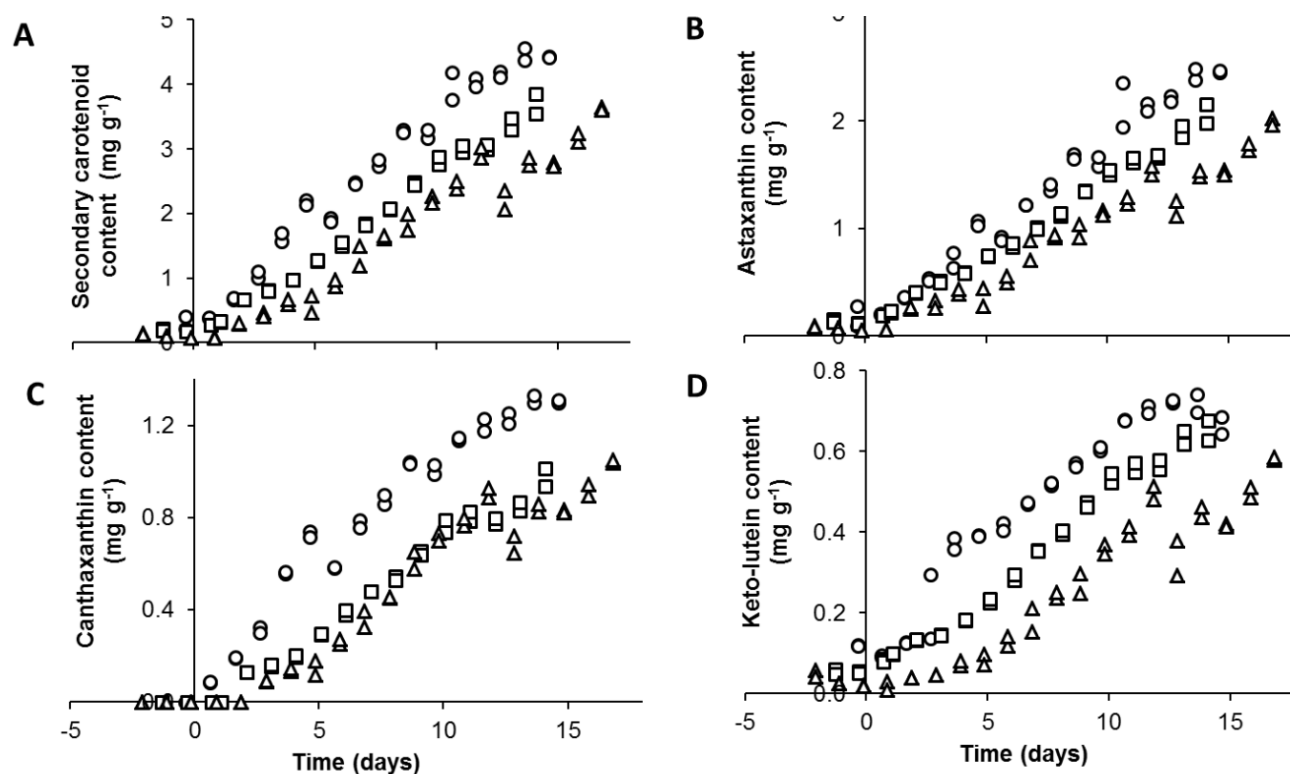


Fig. A.5.4 Time courses of secondary pigment contents in nitrogen-deplete *C. zoefingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L^{-1} (\circ), 3.4 g L^{-1} (\square) and 4.1 g L^{-1} (Δ). (A) Total secondary carotenoids, (B) total astaxanthin (free, mono- and diesters) (C) canthaxanthin and (D) total keto-lutein (free, mono- and diesters). Nitrogen was depleted at $t = 0$.

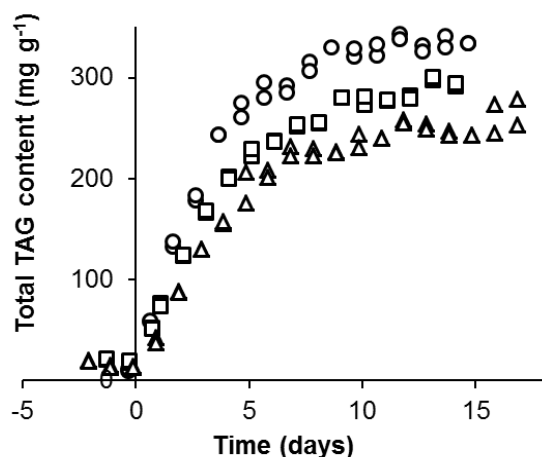


Fig. A.5.5 Time courses of TAG contents of nitrogen-deplete *C. zoofingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○), 3.4 g L⁻¹ (□) and 4.1 g L⁻¹ (Δ).

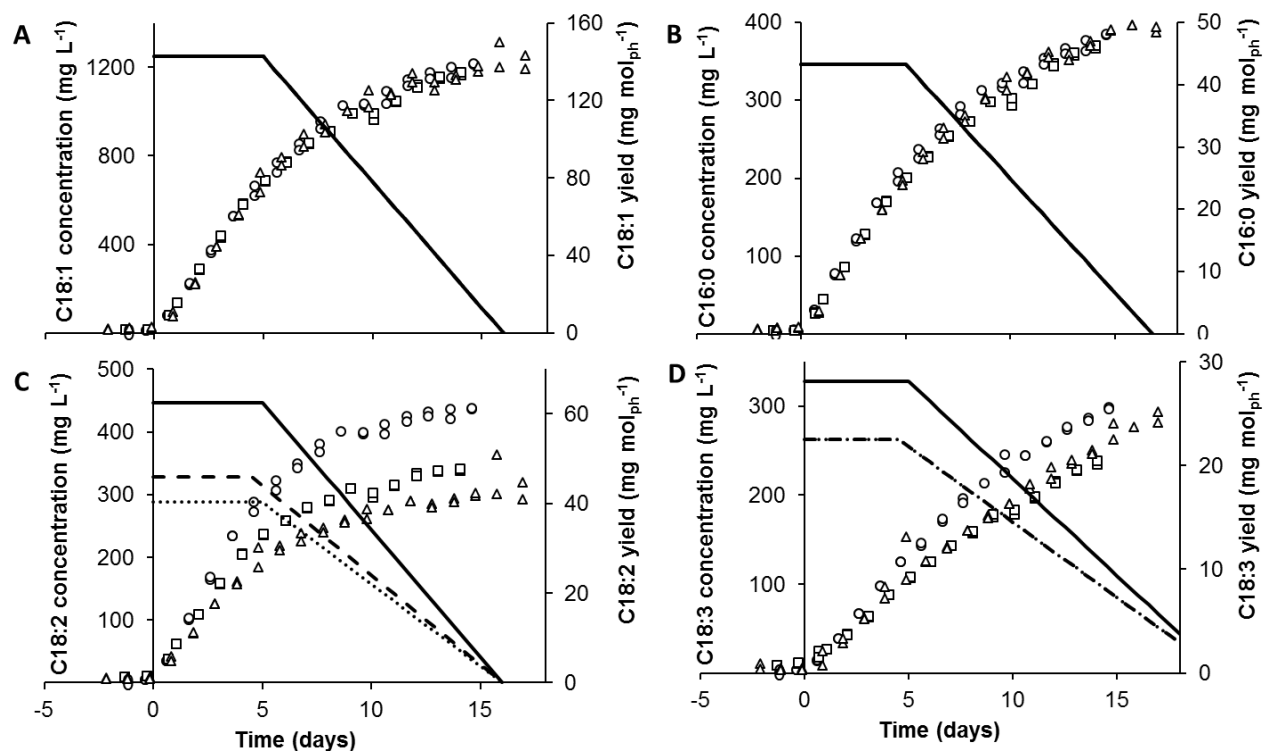


Fig. A.5.6 Time courses of time-specific TAG fatty acid concentrations (**symbols**) and time-specific yields on absorbed light (**lines**) in nitrogen-deplete *C. zoofingiensis* cultures for different biomass concentrations at nitrogen depletion: 2.5 g L⁻¹ (○ and solid line), 3.4 g L⁻¹ (□ and dashed line) and 4.1 g L⁻¹ (Δ and dotted line). **(A)** C18:1 TAG fatty acid, **(B)** C16:0 TAG fatty acid **(C)** C18:2 TAG fatty acid and **(D)** C18:3 TAG fatty acid. **Time-specific yields** were estimated by calculating the first derivative of a fit through the concentration versus time data (see **Materials and Methods, Section 5.2.4**). Nitrogen was depleted at $t = 0$.



— 2 μm —

Chapter 6

Dynamics of biomass composition and growth during recovery of nitrogen-starved *Chromochloris zofingiensis*

High lights

- Nitrogen-depleted *C. zofingiensis* consumed resupplied nitrogen very quickly.
- Primary pigments, polar (membrane) lipids and proteins were rapidly resynthesised.
- Triacylglycerol, starch, ketolutein and canthaxanthin were rapidly degraded.
- Astaxanthin was only degraded upon full recovery, 2 days after nitrogen resupply.

This chapter has been published (online first) as:

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Dynamics of biomass composition and growth during recovery of nitrogen-starved Chromochloris zofingiensis

Applied microbiology and biotechnology, DOI: 10.1007/s00253-014-6181-x

◀ IN THE PICTURE

Electron micrograph of nitrogen-depleted *Chromochloris zofingiensis*, beautifully showing the three dimensional structure of the cell. The nucleus, in the centre, is the head quarter. The chloroplasts, sticking out, capture light energy. Due to the nitrogen depletion, the cell has accumulated oil globules (mainly at the side), presumably containing secondary carotenoids. In one word: amazing!

◀ IN BEELD

Electronenmicroscopiefoto van stikstof-gelimiteerde *Chromochloris zofingiensis*, die de 3D-structuur van de cel laat zien. De cel-kern, in het midden, is het hoofdkwartier van de cel. De uitstekende chloroplasten vangen lichtenergie op. Door het tekort aan stikstof heeft de cel olie opgehoopt (aan de rand), waar vermoedelijk de secundaire carotenoiden in opgeslagen zitten. Wonderbaarlijk!

Abstract

The effect of nitrogen replenishment on the kinetics of secondary carotenoids, TAG and primary cell components was studied in nitrogen-starved *Chromochloris (Chlorella) zofingiensis* (Chlorophyta), an oleaginous and carotenogenic microalga. Nitrogen resupplied after a period of starvation was initially consumed at a more than four times higher rate than in an equivalent nitrogen-replete culture. Simultaneously, chlorophylls, primary carotenoids, polar (membrane) lipids and proteins were rapidly produced. After two days, the contents of these primary metabolites, as well as the nitrogen consumption rate and the overall biomass production rate, had returned to values equivalent to those of cells grown under nitrogen-replete conditions, indicating that culture recovery required two days. Nitrogen resupply was immediately followed by rapid degradation of triacylglycerol (TAG) and starch, suggesting that these metabolites served as carbon and energy source for the recovery process. Also, the secondary carotenoids canthaxanthin and ketolutein were rapidly degraded upon nitrogen resupply, whereas degradation of astaxanthin, the main secondary carotenoid, started only when the cells were fully recovered, two days after nitrogen resupply. This is the first time that such culture recovery has been described in detail and, moreover, that astaxanthin was found to be not immediately degraded after nitrogen resupply. The observed rapid recovery of *C. zofingiensis* and the delay in astaxanthin degradation suggest that a repeated batch cultivation may result in a higher secondary carotenoid productivity than a series of classical single batch cultivations.

6.1 Introduction

Natural carotenoids, such as astaxanthin, β -carotene and lutein, and triacylglycerol (TAG) are important biotechnological products. Natural carotenoids are claimed to have beneficial health effects due to their anti-oxidative activity and are therefore used in the cosmetic, food and feed industry (Spolaore et al. 2006). TAG is a promising feedstock for the generation of biofuels, thereby replacing petroleum-derived transport fuels, and a possible replacer for vegetable oils (Chisti 2013; Draaisma et al. 2013; Hu et al. 2008). Secondary carotenoids and TAG are produced in excess by specific microalgae. However, the production costs are still too high to make microalgae-based production economically competitive with synthetic production (carotenoids) or production by higher plants (TAG and most carotenoids). One of the requirements for economically feasible TAG and carotenoid production is that maximal areal productivities are obtained.

In a selection of green algae, secondary carotenoids and TAG are produced under adverse growth conditions, such as a high light intensity, extreme temperatures or a nitrogen starvation (Ben-Amotz 1996; Ben-Amotz and Avron 1983; Hu et al. 2008; Lamers et al. 2010; Lamers et al. 2012; Mulders et al. 2014a). Under nitrogen starvation conditions, excess electrons derived from photosynthesis are incorporated in TAG and secondary carotenoids, which prevents photo-oxidative cell damage. Besides, carbon and energy are stored (Hu et al. 2008; Lemoine and Schoefs, 2010; Mulders et al. 2014a), which may be mobilized during culture growth resumption upon nitrogen resupply (Solovchenko 2013). In addition, secondary

carotenoids can function as sunscreen, protecting the cells by absorbing part of the excess irradiation (Lamers et al. 2008; Mulders et al. 2014a).

Besides secondary carotenoid and TAG accumulation, nitrogen starvation also affects the cellular content of other essential macromolecules, such as photosynthetic pigments (chlorophylls and primary carotenoids) (Falkowski and LaRoche, 1991; Mulders et al. 2014c; Rhiel et al. 1985), proteins (Turpin 1991; Pribyl et al. 2013), carbohydrates, such as starch (Breuer et al. 2014; Turpin 1991; Zhu et al. 2014), and polar (membrane) lipids (Goncalves et al. 2013; Xiao et al. 2013). In addition, nitrogen starvation leads to a significant decrease in photosynthetic rate (Klok et al. 2013a; Klok et al. 2013b). Consequently, nitrogen-starved and nitrogen-sufficient grown microalgae deviate substantially in cellular composition and physiological state.

Traditionally, secondary carotenoids and TAG are produced in a two phase process. A (nitrogen-sufficient) biomass production phase is followed by a nitrogen-depleted phase in which the secondary metabolites are accumulated. Subsequently, all biomass is harvested. Alternatively, secondary carotenoids and TAG may be produced using a repeated batch cultivation, which has been suggested as a potentially more productive approach (Feng et al. 2011; Hsieh and Wu 2009). This semi-continuous production process starts the same as a two-phase batch cultivation, but instead of that all biomass is harvested at the end of the accumulation phase, only part of the biomass is harvested. The remaining biomass is resupplied with a limited amount of nitrogen resulting initially in culture growth resumption. During growth resumption the biomass returns presumably towards a macromolecular composition equivalent to biomass grown under nitrogen-sufficient conditions (referred to as recovery). Subsequently, the biomass is again nitrogen depleted, which results in repeated secondary carotenoid and TAG accumulation.

Whether a repeated batch cultivation may indeed result into higher overall TAG and secondary carotenoid productivities than a classical two-phase process depends on multiple factors, including the recovery rate of the culture (i.e. the nitrogen consumption rate and the production rate of primary cell components required to resume photosynthesis) and the dynamics of TAG and secondary carotenoids upon nitrogen resupply. Therefore, insight in the physiological changes occurring during the recovery of a nitrogen-replenished microalga is important and may enable optimization of a TAG and secondary carotenoid production process.

So far, only some TAG-rich microalgae (*Parachlorella kessleri*, *Chlorella vulgaris* and *Chlamydomonas reinhardtii*) have been nitrogen replenished, after which the dynamics of TAG, total lipids, starch, chlorophyll and/or total dry weight concentrations have been reported (Fernandes et al. 2013; Pribyl et al. 2013; Siaut et al. 2011). These studies revealed rapid TAG and starch degradation after nitrogen resupply, which coincided with rapid chlorophyll and total biomass production. However, other parameters required to draw conclusions on the recovery rate of the culture, such as the nitrogen-uptake rate and protein production rate, were not determined. Moreover, because the investigated species were not carotenogenic, the dynamics of secondary carotenoids after nitrogen resupply have not yet been studied.

An oleaginous and carotenogenic microalga potentially suitable for the production of TAG and/or secondary carotenoids is *Chromochloris (Chlorella) zofingiensis*. This species was

previously identified as an efficient TAG producer (Breuer et al. 2012; Mulders et al. 2014c) and it produces several secondary carotenoids, including astaxanthin, canthaxanthin and ketolutein (Del Campo et al. 2004; Mulders et al. 2014d; Orosa et al. 2000; Orosa et al. 2001; Rise et al. 1994).

The aim of this paper is to study the dynamics of TAG, secondary carotenoids and primary cell components in nitrogen-resupplied *C. zofingiensis* to obtain more insight in the physiology and rate of culture recovery.

6.2 Materials and methods

6.2.1 Culture and nitrogen-starved pre-cultivation

Chromochloris zofingiensis was obtained as *Chlorella zofingiensis* UTEX B32 from the University of Texas Culture Collection of Algae (UTEX). *C. zofingiensis* cultures were nitrogen-starved in a batch-wise operated, 1.75 L flat-panel, airlift-loop photobioreactor with a light path (reactor depth) of 20.7 mm (Labfors, Infors HT, 2010) as described in Mulders et al. (2014c), under the conditions as described in Mulders et al. (2014c). In short: continuous illumination with $245 \mu\text{mol m}^{-2} \text{s}^{-1}$ incident light, an air to CO_2 ratio of 98:2 (v/v) sparged at 1.5 L/min, pH maintained at 7, temperature maintained at 25 °C and antifoam addition upon visible foaming. Off gas was analysed online by mass spectrometry.

In a previous study, *C. zofingiensis* cultures were nitrogen starved at three different biomass concentrations (2.5, 3.4 and 4.1 g L^{-1}), to investigate how TAG and secondary carotenoid accumulation are affected by the biomass concentration at the moment of nitrogen depletion (Mulders et al. 2014c). After 2 weeks of nitrogen depletion these cultures were used in this work to study the recovery process upon nitrogen replenishment.

6.2.2 Batch replenish experiments

Replenish experiments were performed in the same photobioreactor and under the same conditions as described for the nitrogen-starved pre-cultivation.

The nitrogen replenish medium (modified M-8 medium (Mandalam and Palsson 1998)) consisted of KNO_3 494.5 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 3.25 mM; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.18 mM; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 13.8 mM; NaFeEDTA 0.56 mM; $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ 0.9 mM; H_3BO_3 2.0 μM ; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.13 mM; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 22.3 μM ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 15 μM ; Biotin 2 $\mu\text{g L}^{-1}$; vitamin B1 0.4 mg L^{-1} ; vitamin B12 2 $\mu\text{g L}^{-1}$. The medium pH was adjusted to pH 7.0 with HCl and filter sterilized prior to use. The nitrogen concentration of the replenish medium was chosen in such a way that, after addition, nitrogen would be in excess throughout the experiment (supporting a theoretical biomass increase of $\sim 14 \text{ g L}^{-1}$).

Replenish medium (200 mL) was added to the nitrogen-depleted cultures (1550 mL) when the biomass productivity was reduced to a value below 5 % of the maximum biomass productivity and a maximal time-averaged productivity of secondary carotenoids was reached, thereby signifying the optimal point for harvest and possible regrowth of the culture (typically 2 weeks after nitrogen depletion) (Mulders et al. 2014c). As at that point the cultures had become more or less metabolically inactive, the specific time of nitrogen resupply (i.e. one day earlier or later) was not expected to have a large effect on the physiology and rate of culture recovery. The moment of nitrogen resupply was considered the start of the experiment and is referred to as $t = 0$.

One to three times a day a sample was taken directly from the reactor. The sample volume (15-25 ml) was precisely determined to allow estimation of the remaining culture volume. The sample was analysed for dry weight concentration, nitrogen, pigment, TAG, polar acyl lipid, protein, starch and total carbohydrate contents.

6.2.3 Off gas analysis

The composition of the gas leaving the reactor was analysed using a mass spectrometer (PRIMA δ B process MS, Thermo Scientific, USA). In addition, a reference measurement was performed analysing the composition of the gas mixture entering the reactor. Thereby, the volumetric oxygen production rate (r_{O_2} , mol L⁻¹ day⁻¹) and the carbon dioxide consumption rate (r_{CO_2} , mol L⁻¹ day⁻¹) could be calculated:

$$r_{O_2} = F_{gas,out} \cdot x_{O_2,out} - F_{gas,in} \cdot x_{O_2,in} \quad (1)$$

$$r_{CO_2} = F_{gas,out} \cdot x_{CO_2,out} - F_{gas,in} \cdot x_{CO_2,in} \quad (2)$$

with $x_{O_2,out}$, $x_{O_2,in}$, $x_{CO_2,out}$ and $x_{CO_2,in}$ as the molar fraction of oxygen and carbon dioxide in the gas (-), and $F_{gas,in}$ and $F_{gas,out}$ as the total molar gas flow rates entering and leaving the reactor (mol L⁻¹ day⁻¹), respectively. Due to cellular respiration, $F_{gas,out}$ differed from $F_{g,in}$. Assuming a constant absolute N-inflow and N-outflow, $F_{gas,out}$ could be determined using eq.3.

$$F_{gas,out} = \frac{x_{N_2,in}}{x_{N_2,out}} \cdot F_{gas,in} \quad (3)$$

6.2.4 Absorbed photon flux density

During the cultivations, the outgoing photon flux density ($PFD_{out,pbr}$) was determined as the average of 12 measurements spread over the surface of the outside of the back glass panel of the reactor. This value was corrected for light loss due to the water jacket, the glass plates and medium (PFD_{blank}). PFD_{blank} was determined as the average of 44 measurements spread over the entire surface of the outside of the back glass panel of the reactor which contained (transparent) medium. The absorbed photon flux density (PFD_{abs}) was calculated as described by Santos et al. 2013, using eq. 4.

$$PFD_{abs} = PFD_{in} - \frac{PFD_{out,pbr}}{PFD_{blank}/PFD_{in}} \quad (4)$$

with PFD_{abs} , PFD_{out} , $PFD_{out,pbr}$, PFD_{blank} and PFD_{in} in $\mu\text{mol}_{ph} \text{ m}^{-2} \text{ s}^{-1}$.

6.2.5 Dry weight concentration

Cell dry weight concentrations were determined gravimetrically in triplicate by filtering and drying of the biomass (~2 mg DW) as described by Kliphuis et al. (2011).

6.2.6 Nitrogen content

To determine the nitrogen content of the biomass, sampled cells (~10 mg DW) were washed twice with MiliQ water to remove the salt and extracellular nitrogen present in the medium. Washing was performed by centrifugation of cells at $9391\times g$ for 3 minutes and subsequent resuspension of the cell pellet in 1 mL miliQ water. Washed cell pellets were stored at -20 °C until later use. Cell pellets were freeze dried (to a water content below 1 % (w/w)) and dried further overnight in a stove of 100 °C. Dry cell pellets were cooled to room temperature in a desiccator, to prevent rewetting, after which the mass of each sample was determined. The nitrogen content was determined using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience), with methionine as standard.

6.2.7 TAG and polar acyl lipid extraction, identification and quantification

TAG and polar acyl lipids were extracted from a precisely determined amount of biomass (within the range of 4-6 mg DW) with methanol/chloroform as solvent as described by Breuer et al. (2013a). TAG and polar acyl lipids were separated from each other using a solid phase extraction (SPE) column as described by Breuer et al. (2013b). After transesterification to FAMES, fatty acids from TAG and polar acyl lipids were identified and quantified using GC-FID as described by Breuer et al. (2013a). An internal standard (C15:0) was added to the samples prior to extraction, which ended up in the TAG pool. This internal standard was used for TAG fatty acid quantification and for the quantification of fatty acids from the polar acyl lipids assuming that TAG and polar acyl lipids were extracted and transesterified with a similar efficiency.

6.2.8 Pigment extraction, identification and quantification

Pigments were extracted from a precisely determined amount of biomass (~4 mg DW) with methanol/chloroform as solvent as described by Lamers et al. (2010), with the exceptions as described in Mulders et al. (2014d). Pigments identification and quantification were performed using reversed phase ultra-high performance liquid chromatography photo diode array tandem mass spectrometry (RP-UHPLC-PDA-MSⁿ) analysis as described in Mulders et al. (2014d).

6.2.9 Proteins

The protein content of a precisely determined amount of biomass (~8 mg DW) was determined using a DC protein assay (BioRad) as described by De Winter et al. (2013). Freeze dried biomass was dissolved in 1 mL of lysis buffer, containing 60 mM Tris, 2 % SDS, 10 % glycerol and 10 mM DTT. Cells were physically disrupted by employing a Precellys® 24 bead beater (Bertin Technology, France). To inhibit excessive cell heating, 60 s of disruption at 6,500 rpm was followed by 120 s of cooling on ice. For each sample, three series of disruption/cooling cycles were performed. Another mL of lysis buffer was added and samples were incubated for 30 min at 100 °C in a heating block. After incubation, samples were centrifuged for 10 min at 3500 rpm. The protein content of the supernatant was analyzed by measuring absorbance at 750 nm using a plate reader (EL800, BioTek Instruments, USA). BSA was used as a standard.

6.2.10 Starch

The starch content of a precisely determined amount of biomass (~10 mg DW) was determined using the Total Starch Kit of Megazyme (Megazyme International, Ireland, 2011), as described by De Winter et al. (2013). Freeze dried biomass was dissolved in 1 mL of 80 % (v/v) ethanol. Cells were physically disrupted by employing a Precellys® 24 bead beater (Bertin Technology, France). To inhibit cell heating, 60 s of disruption at 6,000 rpm was followed by 60 s of cooling on ice. For each sample, three series of disruption/cooling cycles were performed. Another 4 mL of 80 % (v/v) ethanol were added to the biomass and from that point the standard procedure of the Total Starch kit was followed. Absorbance was measured at 510 nm on a spectrophotometer (DU 640, Beckman, U.S.A.). Pure glucose was used as a standard.

6.2.11 Non-starch carbohydrates

The non-starch carbohydrate content was determined as the difference between the total carbohydrate content and the starch content. To determine the total carbohydrate content of the biomass, a precisely determined amount of biomass (~10 mg DW) was transferred to beat beating tubes (containing Lysing Matrix E (MP Biomedicals, Santa Ana, California, USA)) and centrifuged at $9391\times g$ for 3 minutes. Cell pellets were freeze dried and one mL of 2,5 M HCl was added to the beat beating tubes. Thereafter, cells were physically disrupted using a Precellys® 24 beat beater (Bertin technology, Montigny-le-Bretonneux, France). To prevent excessive heating of the tubes, 60 seconds of disruption, at 6000 rpm, was followed by 2 minutes of cooling in ice. For each sample, two series of disruption/cooling were performed. To hydrolyse the carbohydrates, the content of the tubes was transferred to glass tubes and another 4 mL of 2,5 M HCl were added, after which the samples were incubated at 100 °C for 3 hours while being vortexed every hour. Samples were cooled down to room temperature and neutralized by addition of 5 mL 2,5 M NaOH. The biomass was treated with a phenol solution and concentrated sulphuric acid, according to Dubois et al. (1956) and Herbert et al. (1971). The absorbance of the resulting solution was measured at 483 nm. Pure glucose was used as a standard.

6.3 Results

We studied the dynamics of TAG, secondary carotenoids and primary cell components in nitrogen-resupplied *C. zoefingiensis*. The starting points of this replenishment study were three different cultures which were nitrogen depleted at different biomass concentrations. As a result, each culture contained a different assimilated nitrogen concentration at the start of the replenishment. To each culture, an excess amount of nitrogen was added after a nitrogen starvation phase of two weeks. The moment of nitrogen resupply was considered as the start of the cultivation and is referred to as $t = 0$.

6.3.1 Total biomass, CO₂ and O₂

Immediately after nitrogen resupply ($t=0$), the cultures contained total biomass dry weight concentrations of 7.4, 7.7 and 8.7 g L⁻¹. During the first day after nitrogen resupply these concentrations decreased in each culture with 13 ± 3 %. Thereafter, biomass concentrations increased in each culture reaching a more or less constant biomass productivity of 0.66 ± 0.08 g L⁻¹ day⁻¹ (Fig. 6.1a). Prior to nitrogen resupply, each culture net produced O₂ and consumed

CO₂ (not shown). During the first two days after nitrogen resupply the cultures net consumed O₂ and produced CO₂, whereas two days after nitrogen resupply all cultures net produced O₂ and consumed CO₂ again. After those two days, volumetric CO₂ consumption and O₂ production rates decreased slightly (**Fig. 6.1b – c**).

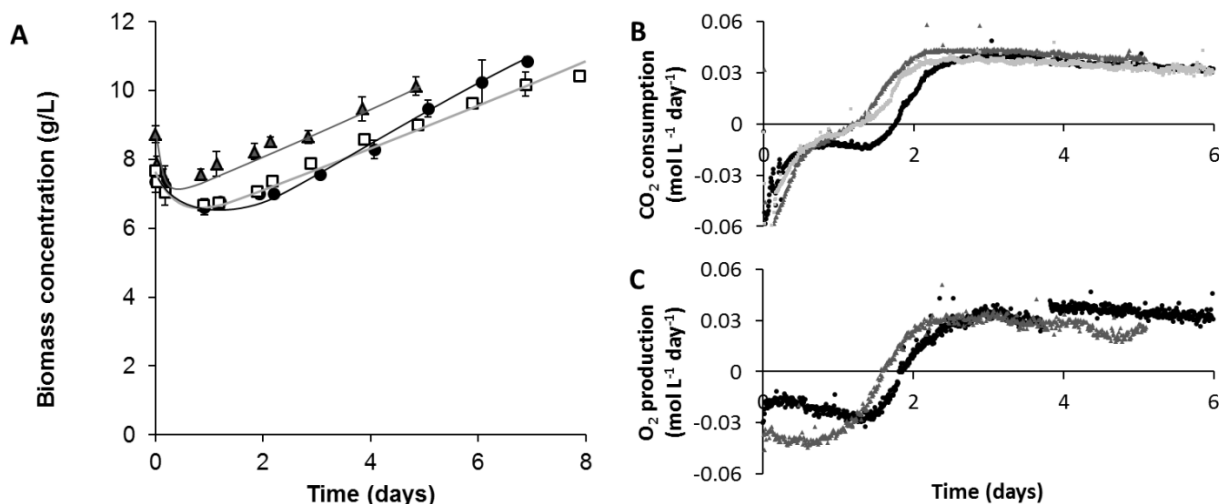


Fig. 6.1 Time courses of (A) dry weight biomass concentration, (B) CO₂ consumption rate and (C) O₂ production rate of nitrogen resupplied *C. zoefingensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L⁻¹ (●, black line), 0.18 g L⁻¹ (□, light grey) and 0.23 g L⁻¹ (▲, dark grey). **Error bars** in A represent standard deviations of triplicate dry weight measurements. **Lines** in A are for visual guidance. O₂ production rates of the 0.18 g L⁻¹ culture were not measured due to a technical failure.

6.3.2 Nitrogen consumption

The cultures, with total biomass concentrations of 7.4, 7.7 and 8.7 g L⁻¹, had assimilated nitrogen concentrations (per culture volume) of 0.12, 0.18 and 0.23 g L⁻¹, respectively, immediately after nitrogen resupply (**Fig. 6.2a**). At that point, the average assimilated nitrogen content (on a dry weight basis) was on average 2.2 ± 0.5 % DW (w/w) (**Fig. 6.2b**). In each culture, nitrogen was consumed quickly the first two days after nitrogen resupply (with 0.20 ± 0.01 g L⁻¹ day⁻¹), resulting in a fast increase in assimilated nitrogen content (reaching 7.5 ± 0.3 % DW (w/w) after two days) (**Fig. 6.2**). Two days after nitrogen resupply the nitrogen consumption rate decreased (to 0.038 ± 0.003 g L⁻¹ day⁻¹), resulting in a more or less constant assimilated nitrogen content (of 6.7 ± 0.2 % DW (w/w)) (**Fig. 6.2b**).

6.3.3 TAG

Immediately after nitrogen resupply, the TAG content was on average 33 ± 6 % DW (w/w). In each culture, TAG concentrations decreased rapidly for two days (with 1.0 ± 0.05 g L⁻¹ day⁻¹), resulting in a TAG content of 7 ± 2 % DW (w/w) (**Fig. 6.3**). As a comparison, the maximal TAG accumulation rate during the nitrogen starvation period was 0.32 g L⁻¹ day⁻¹. Remarkably, the TAG concentration decreased in each culture with approximately the same amount (2.0 ± 0.1 g L⁻¹), rather than that the same final content or concentration was reached in each culture (**Fig.**

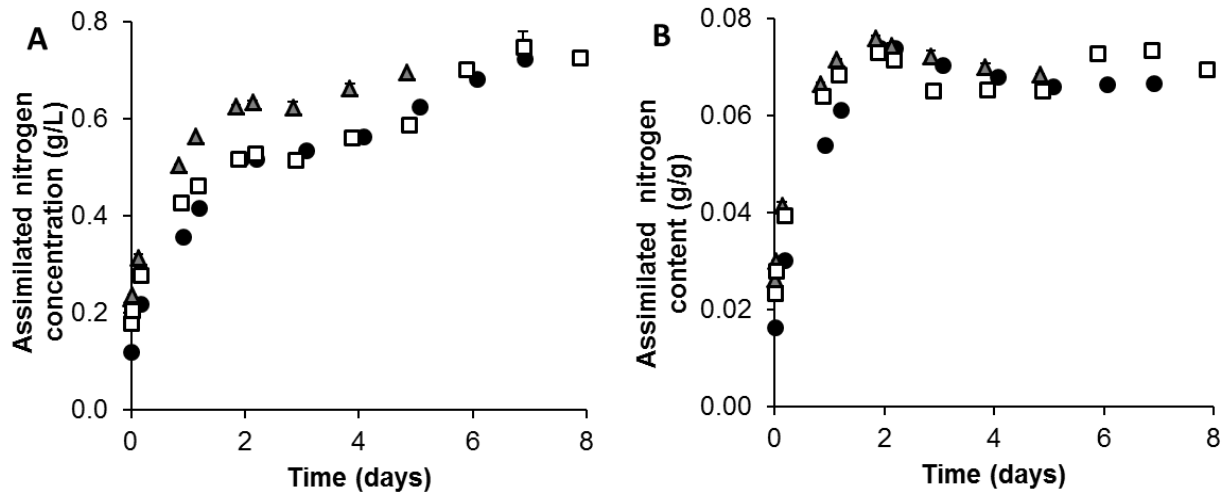


Fig. 6.2 Time courses of (A) assimilated nitrogen concentration (in the reactor) and (B) assimilated nitrogen content (on a dry weight basis) of nitrogen resupplied *C. zofigingensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L⁻¹ (●), 0.18 g L⁻¹ (□) and 0.23 g L⁻¹ (▲). Error bars represent standard deviations of triplicate assimilated nitrogen measurements.

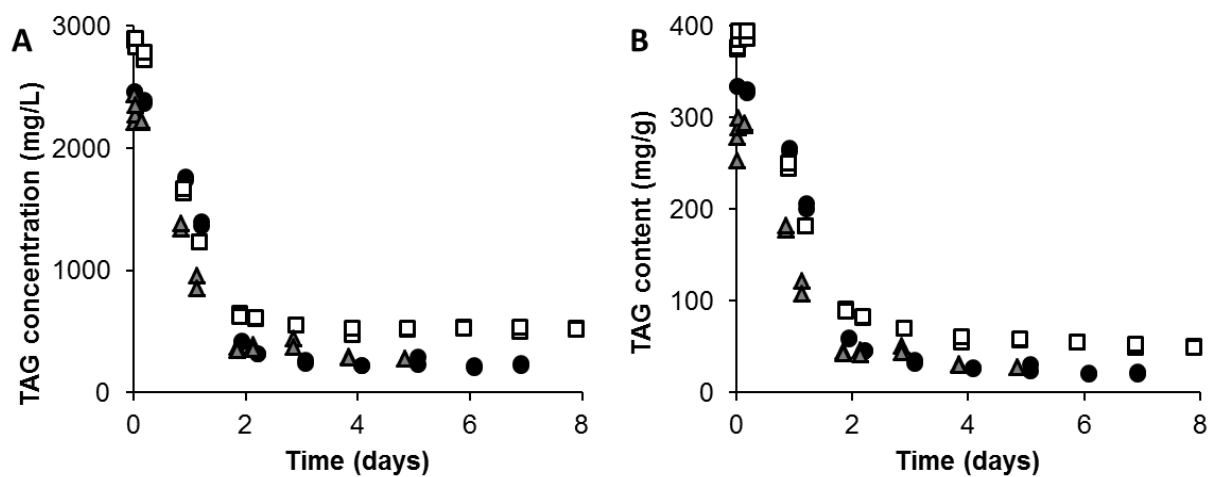


Fig. 6.3 Time courses of (A) total TAG concentration and (B) total TAG content of nitrogen resupplied *C. zofigingensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L⁻¹ (●), 0.18 g L⁻¹ (□) and 0.23 g L⁻¹ (▲). TAG concentrations were measured in duplicate; both data points are shown.

6.3). In each culture, two days after nitrogen resupply the TAG concentration as well as the TAG content remained more or less constant (Fig. 6.3).

6.3.4 Secondary carotenoids

Immediately after nitrogen resupply, the secondary carotenoids astaxanthin, canthaxanthin and ketolutein were present. At that point, contents of these carotenoids were on average 0.2 ± 0.02 , 0.1 ± 0.02 and 0.06 ± 0.003 % DW (w/w), respectively. From the moment of nitrogen resupply, canthaxanthin and ketolutein concentrations and contents decreased, resulting after six days in an average content below 0.01 % DW (w/w) for both carotenoids. In each culture, the astaxanthin concentration remained constant the first two days after nitrogen resupply

(Fig. 6.4a). After these two days the astaxanthin concentration decreased (at a rate of $0.9 \pm 0.2 \text{ g L}^{-1} \text{ day}^{-1}$), resulting also in a decrease in astaxanthin content (Fig. 6.4a - b).

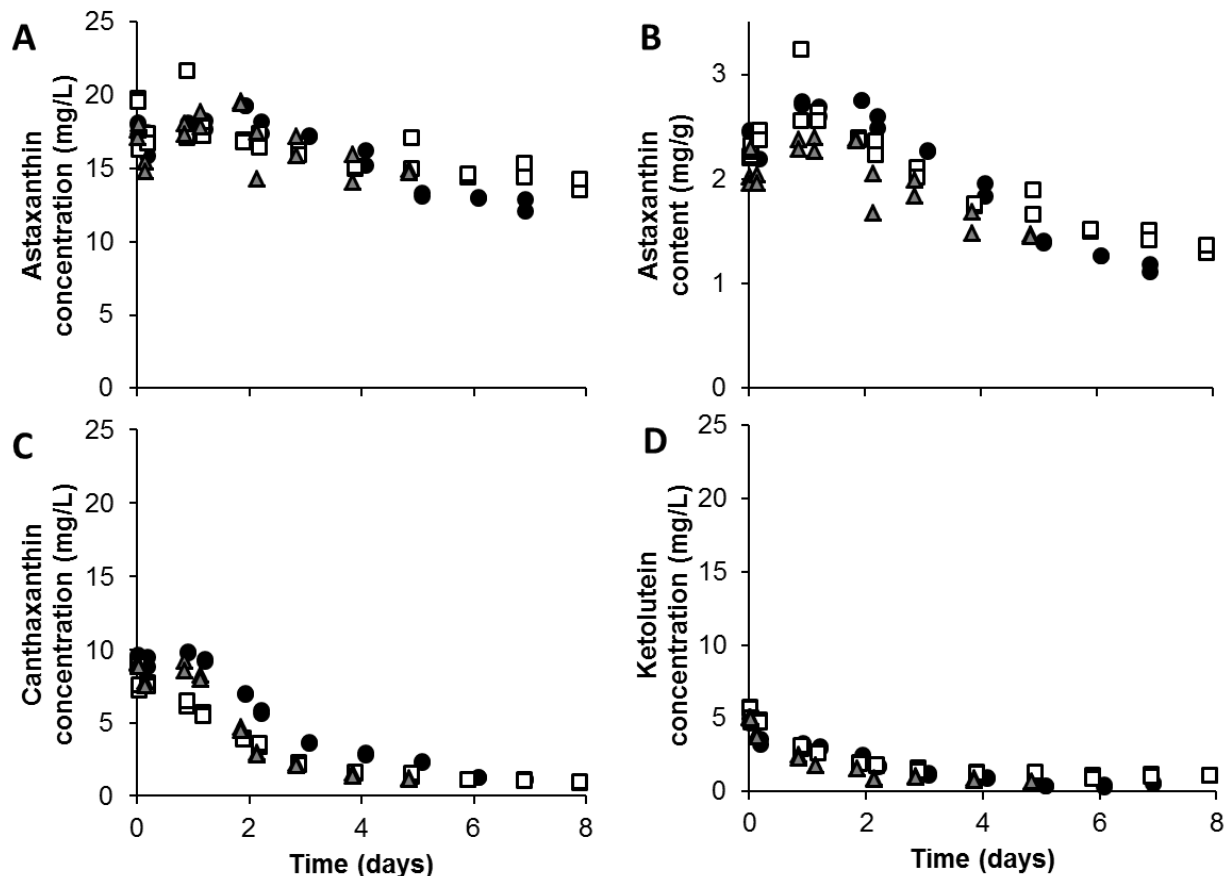


Fig. 6.4 Time courses of (A) total astaxanthin concentration (free, mono- and diesters), (B) total astaxanthin content, (C) canthaxanthin concentration and (D) total ketolutein concentration (free, mono- and diesters) of nitrogen resupplied *C. zofigiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L^{-1} (●), 0.18 g L^{-1} (□) and 0.23 g L^{-1} (▲). Pigment concentrations were measured in duplicate; both data points are shown.

6.3.5 Polar lipids

Immediately after nitrogen resupply, the polar lipid content was on average $6 \pm 0.5 \text{ \% DW}$ (w/w). In each culture, the polar lipid concentration increased after nitrogen resupply at a more or less constant rate (of $0.07 \pm 0.01 \text{ g L}^{-1} \text{ day}^{-1}$) (Fig. 6.5). This resulted after two days in a polar lipid content of $9.5 \pm 1 \text{ \% DW}$ (w/w), which thereafter remained more or less constant.

6.3.6 Primary pigments

Immediately after nitrogen resupply, the primary (light harvesting) pigments chlorophyll *a* and *b*, lutein, violaxanthin and neoxanthin were present. At that point, total chlorophyll and total primary carotenoid contents were on average $0.8 \pm 0.4 \text{ \%}$ and $0.1 \pm 0.06 \text{ \% DW}$ (w/w), respectively (Fig. A.6.1). In each culture, chlorophyll and primary carotenoid concentrations remained constant for at least five hours after nitrogen resupply (Fig. 6.6). Thereafter, in each culture, chlorophyll and primary carotenoid concentrations increased rapidly for two days, resulting in a fast increase in chlorophyll and primary carotenoid contents (Fig. A.6.1). Two days after nitrogen resupply the production of chlorophylls and primary carotenoids decreased in each culture, by at least a factor 1.5, whereas the chlorophyll and primary pigment content

still increased slightly (Fig. A.6.1). In all cultures, the ratio between primary pigments remained more or less constant.

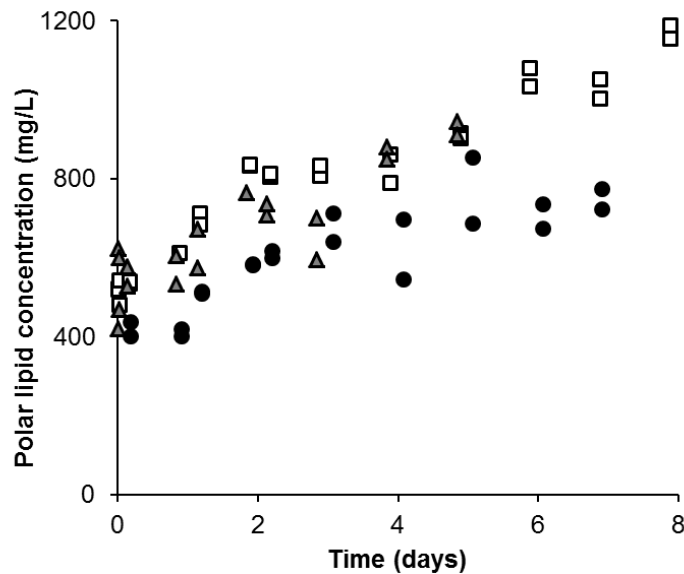


Fig. 6.5 Time course of total polar lipid concentration of nitrogen resupplied *C. zofigiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L^{-1} (●), 0.18 g L^{-1} (□) and 0.23 g L^{-1} (▲). Polar lipid concentrations were measured in duplicate; both data points are shown.

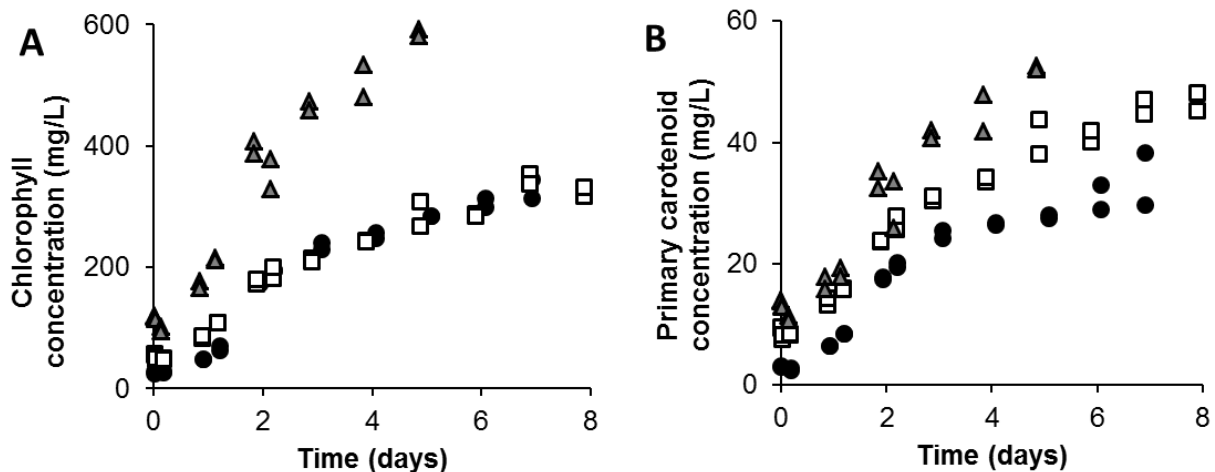


Fig. 6.6 Time courses of (A) total chlorophyll concentration (chlorophyll a and b) and (B) total primary carotenoid concentration (lutein, violaxanthin and neoxanthin) of nitrogen resupplied *C. zofigiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L^{-1} (●), 0.18 g L^{-1} (□) and 0.23 g L^{-1} (▲). Pigment concentrations were measured in duplicate; both data points are shown.

6.3.7 Proteins

The protein dynamics were measured for two cultures only. Immediately after nitrogen resupply, the protein content was on average $18 \pm 3 \text{ \% DW (w/w)}$ (Fig. 6.7b). In both cultures, protein concentrations seemed to remain constant for at least five hours after nitrogen resupply (Fig. 6.7a). Thereafter, protein concentrations increased rapidly for two days (with $0.6 \pm 0.05 \text{ g L}^{-1} \text{ day}^{-1}$), resulting in a fast increase in protein content (reaching after two days $33 \pm 0.1 \text{ \% DW (w/w)}$). In each culture, two days after nitrogen resupply the production of proteins slowed down (Fig. 6.7a), resulting in a slight decrease in protein content (Fig. 6.7b).

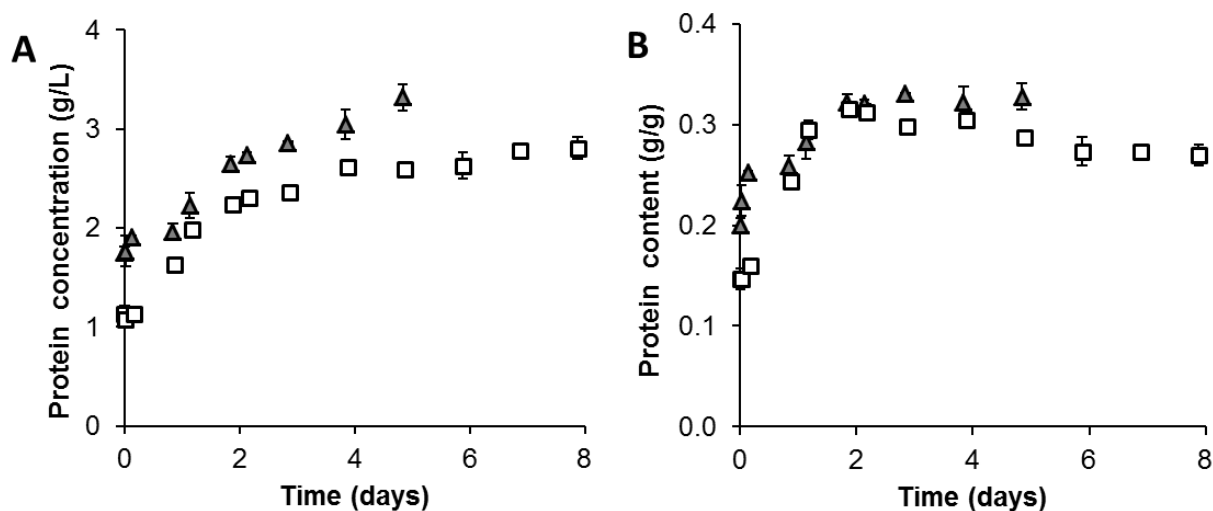


Fig. 6.7 Time courses of (A) protein concentration and (B) protein content of nitrogen resupplied *C. zoefingiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.18 g L^{-1} (□) and 0.23 g L^{-1} (▲). Error bars represent standard deviations of triplicate protein measurements.

6.3.8 Starch and non-starch carbohydrates

The dynamics of the starch and non-starch carbohydrates were measured for two cultures only. Immediately after nitrogen resupply, the starch and non-starch carbohydrate contents were on average 11 ± 2 and 20 ± 1 % DW (w/w), respectively (Fig. 6.8b). In both cultures, starch and non-starch carbohydrate concentrations decreased rapidly the first day after nitrogen resupply (with 0.7 ± 0.1 and $0.3 \pm 0.01 \text{ g L}^{-1} \text{ day}^{-1}$, respectively). Thereafter, until day four, these

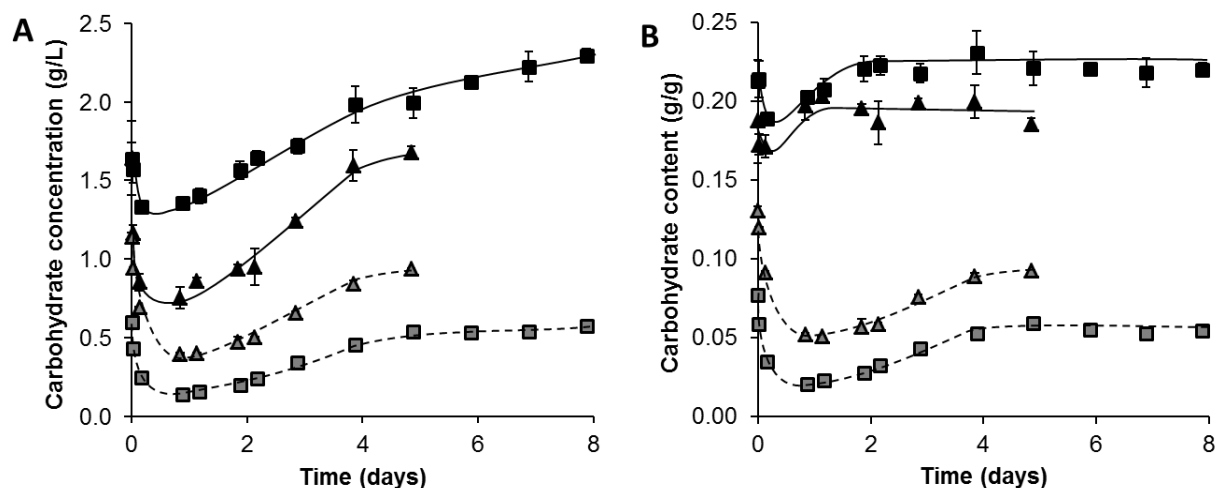


Fig. 6.8 Time courses of (A) concentrations of starch (dashed) and non-starch carbohydrates (solid line) and (B) contents of starch (dashed) and non-starch carbohydrates (solid line) of nitrogen resupplied *C. zoefingiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.18 g L^{-1} (□) and 0.23 g L^{-1} (▲). Error bars represent standard deviations of triplicate starch and non-starch carbohydrate measurements. Lines are for visual guidance.

concentrations increased again (with 0.1 ± 0.02 and $0.25 \pm 0.03 \text{ g L}^{-1} \text{ day}^{-1}$, respectively). From the fourth day after nitrogen resupply starch and non-starch carbohydrate production rates

slowed down (**Fig. 6.8a**). This resulted in a constant starch content from day four, whereas the non-starch carbohydrate content became constant from approximately day two (**Fig. 6.8b**).

6.4 Discussion

The starting points of this replenishment study were three different cultures which were nitrogen depleted at different biomass concentrations. As a result, each culture contained a different assimilated nitrogen concentration when nitrogen was resupplied (two weeks after the onset of nitrogen depletion). In addition, at that point all other biomass components were present in different concentrations as well. This probably will have caused the observed minor differences in the production and degradation rates of these compounds between the three cultures (**Fig. 6.1 – 6.8**). However, most importantly, the general trends in the dynamics of TAG, secondary carotenoids and the primary cell components were very similar in the three cultures, which alludes to the existence of a general recovery mechanism that was independent of the varying starting material tested in this study. Therefore, the section below focusses on these similarities, rather than on the differences between the cultures.

6.4.1 Recovery metabolism

In this section, the physiological changes occurring after nitrogen resupply are interpreted, which leads to the conclusion that two days after nitrogen resupply the nitrogen-starved cells seemed fully recovered.

Nitrogen was consumed very quickly the first two days after nitrogen resupply. Compared to an equivalent nitrogen-replete culture (i.e. a culture that was cultivated under the same conditions as the nitrogen-replenished cells, but which contained sufficient nitrogen throughout the cultivation), nitrogen was consumed four times more rapidly (0.20 vs. $0.045 \text{ g L}^{-1} \text{ day}^{-1}$ (**Table 6.1**)) in the nitrogen-replenished cultures. This rapid nitrogen consumption is likely linked to biochemical actions that occurred at the expression level of nitrogen assimilation mechanisms (Ahmad and Hellebust 1984; Hipkin et al. 1983).

In addition, TAG and starch were quickly degraded immediately after nitrogen replenishment. In comparison to the maximal TAG accumulation rate during the nitrogen starvation period ($0.32 \text{ g L}^{-1} \text{ day}^{-1}$ (Mulders et al. 2014c)), TAG was degraded at a more than three times higher rate ($1 \text{ g L}^{-1} \text{ day}^{-1}$).

The initial TAG and starch degradation was accompanied by net CO_2 production, net O_2 consumption, a decreasing overall dry weight concentration and a constant cell number (not shown), indicating that these storage compounds were respired, most likely to fuel the recovery processes. Besides, TAG and starch degradation likely also provided carbon required for the production of primary cell components such as proteins, primary pigments and thylakoid membranes, which were needed to recover the photosynthetic machinery.

The observed TAG respiration supports the assumption that TAG is accumulated under nitrogen-starved conditions as an energy reserve for when more favourable conditions appear. Besides, this finding is in agreement with observations in other nitrogen-starved TAG-rich microalgae (e.g. *Parachlorella kessleri*, *Chlorella vulgaris* and *Chlamydomonas reinhardtii*), which also degraded TAG very quickly upon nitrogen resupply (within one to two days, which are similar rates compared to those described in this work) (Fernandes et al. 2013; Pribyl et al. 2013; Siaut et al. 2011). It was suggested in these works that accumulated TAG, and starch, may serve as sources of energy and carbon for recovery and reproductive processes.

Table 6.1 Characteristics of a nitrogen-replete *C. zofingiensis* culture and of the nitrogen-replenished cultures immediately and two days after nitrogen resupply (average of the three replenished cultures \pm absolute deviations from the mean). The nitrogen-replete culture was cultivated under the same culture conditions as the nitrogen-replenished cultures described in this work (i.e. in the same photobioreactor, at the same pH, temperature, incident (and absorbed) irradiance and CO₂ supply rate), but with an excess of nitrogen throughout the cultivation, enabling the culture to grow from 2 to 12 g DW/L (Mulders et al. 2014c).

Parameter	Unit	N-replete	N-replenished	
			Immediately after N-resupply	Two days after N-resupply
Nitrogen consumption rate	g L ⁻¹ day ⁻¹	0.045	0.20 \pm 0.01 ^a	0.038 \pm 0.003 ^b
Biomass productivity	g L ⁻¹ day ⁻¹	0.75	-1.2 \pm 0.07 ^c	0.66 \pm 0.08 ^b
Biomass yield on light	g mol _{ph} ⁻¹	0.75	-1.2 \pm 0.07 ^c	0.66 \pm 0.08 ^b
Assimilated nitrogen content	% DW (w/w)	6	2.2 \pm 0.5	6.7 \pm 0.2
Protein content	% DW (w/w)	28 ^d	18 \pm 3	33 \pm 0.1
Chlorophyll content	% DW (w/w)	1.2 – 2.6	0.8 \pm 0.4	3.5 \pm 0.7
Primary carotenoid content	% DW (w/w)	0.2 – 0.4	0.1 \pm 0.06	0.33 \pm 0.08
TAG content	% DW (w/w)	< 1	33 \pm 6	7 \pm 2
Polar lipid content	% DW (w/w)	7	6 \pm 0.5	9.5 \pm 1
Secondary carotenoid content	% DW (w/w)	< 0.01	0.24 \pm 0.02	0.36 \pm 0.05
Primary carotenoid productivity	mg L ⁻¹ day ⁻¹	3	0 \pm 1 ^e	4.5 \pm 1 ^b
Chlorophyll productivity	mg L ⁻¹ day ⁻¹	23	0 \pm 20 ^e	44 \pm 20 ^b

^a Average during the first two days after nitrogen resupply.

^b Average during day two to day five after nitrogen resupply.

^c Average during the first day after nitrogen resupply.

^d Estimated using a nitrogen into protein conversion factor of 4.6, which was determined using several microalgal species at different growth phases (Lourenço et al. 1998).

^e Average during the first four hours after nitrogen resupply.

After a starch degradation period of one day starch was produced again. In contrast, TAG was continuously degraded during the first two days after nitrogen resupply. Apparently, although both TAG and starch are energy storage compounds which are accumulated under nitrogen-starved conditions, they have different roles in *C. zofingiensis*. It has been proposed by Zhu et al. (2014) that starch is accumulated in *C. zofingiensis* as a quick response to environmental stress, whereas TAG is accumulated for long-term energy storage. A similar division of roles has also been discussed for other microalgae, such as *Pseudochlorococcum* sp. and *C. reinhardtii* (Li et al. 2011b; Siaut et al. 2011).

From the moment of nitrogen resupply all supplied light (1 mol_{ph} L⁻¹ day⁻¹) was completely absorbed by the cultures. However, it is unlikely that immediately after nitrogen replenishment this light energy was effectively turned into chemical energy. Firstly, because at that point the primary photosynthetic machinery likely had a limited capacity to generate energy, as indicated by the low photosynthetic rate at the end of the nitrogen starvation phase (i.e. at that point the biomass productivity was reduced to a value below 5 % of the maximal biomass productivity observed under nitrogen-replete conditions) (Mulders et al. 2014c). This is confirmed by the net O₂ consumption and CO₂ production observed immediately after nitrogen resupply, which is in agreement with the data discussed by Turpin et al. (1988) and

Huppe and Turpin (1994), who explained the complex biochemical interactions between photosynthesis, nitrogen metabolism and carbon metabolism after nitrogen resupply. Secondly, immediately after nitrogen replenishment 30 % (w/w) of the total pigment content consisted of secondary pigments (astaxanthin, canthaxanthin and ketolutein). These red and orange pigments were most likely situated in cytosolic oil bodies at the outer part of the cell, as discussed by Mulders et al. (2014d). Consequently, these protective pigments undoubtedly absorbed a part of the light, reducing the ability to generate energy by photosynthesis.

During the first two days after nitrogen resupply, a physiological transition took place. After approximately five hours of nitrogen consumption, rapid production of the nitrogen-containing chlorophylls and proteins followed. The delay of chlorophyll and protein production in respect to nitrogen assimilation (**Fig. A.6.2** and **A.6.3**) can be explained by assuming that other nitrogen-containing compounds (such as RNA and amino acids) were synthesised first, or that internal storage pools of nitrate and ammonium were initially formed, as was reported for the nitrogen resupplied macroalgae *Fucus vesiculosus* and *Fucus serratus* (Young et al. 2009).

In addition, primary carotenoids (mainly lutein) were rapidly produced for two days from approximately five hours after nitrogen resupply. As a result, two days after nitrogen replenishment the protein and primary pigment contents (on a dry weight basis) were similar to the contents of an equivalent nitrogen-replete culture (**Table 6.1**). Besides, from that point the nitrogen consumption rate decreased and approached the consumption rate of an equivalent nitrogen-replete culture.

The further increase in chlorophyll and primary carotenoid content two days after nitrogen resupply, which occurred at a rate comparable to the chlorophyll and primary carotenoid production in equivalent nitrogen-replete cells, was most probably due to photoacclimation which is generally observed when cells are cultured under low irradiance levels (Falkowski 1980; Falkowski and LaRoche 1991; MacIntyre et al. 2002; Telfer et al. 2008). In addition to the changes in primary pigment contents, also the contents of polar lipids were restored during the first two days after nitrogen replenishment.

Besides, shortly after nitrogen replenishment the secondary carotenoids canthaxanthin and ketolutein were degraded. Remarkably, degradation of astaxanthin, which immediately after nitrogen replenishment made up more than 50 % (w/w) of the total amount of secondary carotenoids, started only two days after nitrogen resupply.

Altogether, these physiological changes resulted after one day into an increasing total biomass dry weight concentration and after two days the biomass approached a productivity and yield on light of an equivalent nitrogen-replete culture (**Table 6.1**). At that point the biomass production was accompanied by net CO₂ consumption and net O₂ production, indicating that also the photosynthetic capacity, and thus the ability to generate energy, was recovered two days after nitrogen resupply.

Strikingly, when two days after nitrogen replenishment TAG was hardly oxidized anymore, the TAG content was reduced to approximately 7 % DW (w/w). Cells grown under nitrogen-replete conditions contained less than 1 % (w/w) TAG (**Table 6.1**), indicating that in the nitrogen-resupplied cultures TAG break down stopped before TAG was completely degraded to baseline-levels. This is remarkable because the energy-rich TAG could theoretically have been used to enhance the specific biomass production rate (which was at that moment

approximately ten times lower than the maximally observed specific growth rate of 1 day^{-1} (Del Campo et al. 2004)). Apparently, a regulation mechanism was present that prevented further degradation of TAG.

Thus, taking into account that two days after nitrogen replenishment the nitrogen and CO_2 consumption rates, the biomass and O_2 production rates and the primary biomass composition had all returned to values similar to those of an equivalent nitrogen replete culture, we conclude that the cells fully recovered from nitrogen depletion two days after nitrogen replenishment.

6.4.2 Implications for a repeated batch production process

The maximally observed secondary carotenoid content of *C. zoefingiensis* is relatively low (0.6 % DW (w/w) (Orosa et al. 2001), compared to for example 4 % DW (w/w) observed for the green alga *Haematococcus pluvialis* (Boussiba et al. 1999)). A higher secondary carotenoid content would be ideal, as a too low content increases the down-stream processing costs of the biomass and thus the carotenoid production costs.

The low secondary carotenoid content of *C. zoefingiensis*, obtained after a single batch cultivation, may be increased by applying a repeated batch cultivation. Namely, it was shown that the astaxanthin accumulated during nitrogen starvation was not degraded during the two days following nitrogen replenishment. Therefore, a second nitrogen starvation, starting two days after nitrogen resupply, may lead to a further increase in astaxanthin content. Perhaps even higher astaxanthin contents may be reached with multiple subsequent cycles of nitrogen starvation and replenishment (i.e. when a true repeated batch is performed). Likewise, similar sequential accumulation of canthaxanthin and ketolutein, which were degraded to approximately one third of their maximal contents two days after nitrogen replenishment, may also contribute to an increased secondary carotenoid content. Besides, as canthaxanthin and ketolutein were selectively degraded the first two days after nitrogen resupply, a repeated batch may lead to a more selective (astaxanthin-rich) carotenoid profile.

In addition, due to the rapid recovery of the cells, a repeated batch cultivation may lead to an increased overall productivity of secondary carotenoids, compared to a series of single batch cultivations.

In contrast to the secondary carotenoids, accumulated TAG was almost completely degraded two days after nitrogen resupply. Although a repeated batch may still be beneficial for TAG accumulation, it will be less beneficial than for the case of astaxanthin due to this degradation of TAG.

Because light energy was not effectively converted into chemical energy during the first period after nitrogen resupply, nitrogen may best be resupplied around sunset, thereby preventing wastage of light energy. The culture will likely initiate recovery in the dark, as indicated by fast degradation of TAG by TAG-rich *C. reinhardtii* which was nitrogen replenished in the dark (Siaut et al. 2011).

Finally, although the results suggest that a repeated batch may lead to a higher overall productivity of secondary carotenoids than a series of classical single batch cultivations, with *C. zoefingiensis*, follow-up research is required to confirm this. Most importantly, secondary carotenoids dynamics during subsequent cycles of nitrogen depletions and replenishments need to be investigated.

6.5 Conclusion

In conclusion, irrespective of the assimilated nitrogen concentration at the moment of nitrogen replenishment, nitrogen was consumed at a more than four times higher rate than under equivalent nitrogen-replete conditions, leading to quick resumption of photosynthesis and biomass production. Within two days, the content of all primary macromolecular compounds, as well as the nitrogen consumption rate and the overall biomass production rate had returned to values equivalent to those of cells grown under nitrogen-replete conditions, indicating that culture recovery required two days. Whereas culture recovery coincided with degradation of TAG, ketolutein and canthaxanthin, astaxanthin was not immediately degraded. Because the cells were able to recover very quickly without immediately degrading astaxanthin, a repeated batch cultivation may result in a higher secondary carotenoid productivity than a series of classical single batch cultivations.

Acknowledgement

We gratefully thank Yannick Weesepeel of Wageningen University, Laboratory of Food Chemistry, for performing the pigment identification and quantification and helping with the Dumas analyses. This work was supported by FeyeCon D&I and by grants from Rijksdienst voor Ondernemend Nederland (Project no. FNDog014).

Appendix

6.A Additional figures

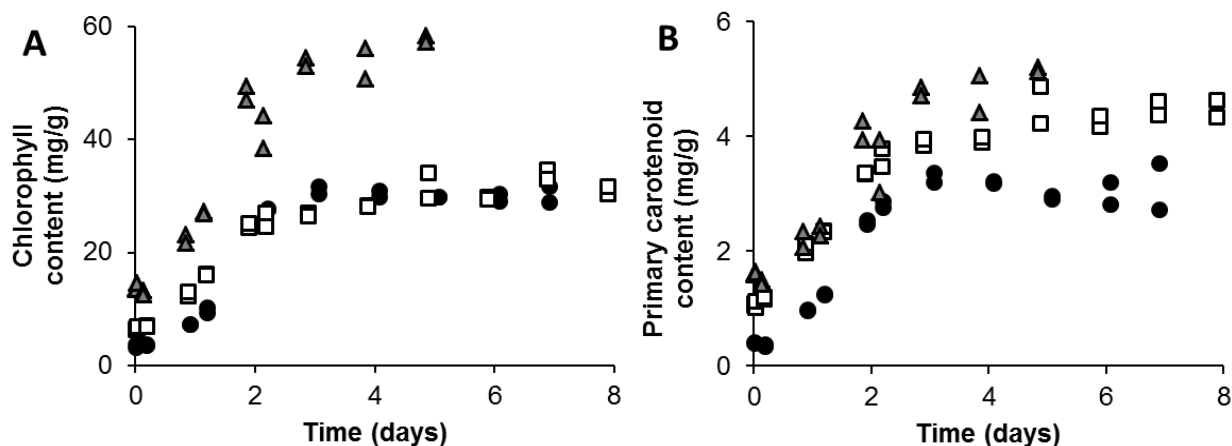


Fig. A.6.1 Time courses of (A) total chlorophyll content (chlorophyll a and b) and (B) total primary carotenoid content (lutein, violaxanthin and neoxanthin) of nitrogen resupplied *C. zoofingiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L⁻¹ (●), 0.18 g L⁻¹ (□) and 0.23 g L⁻¹ (▲).

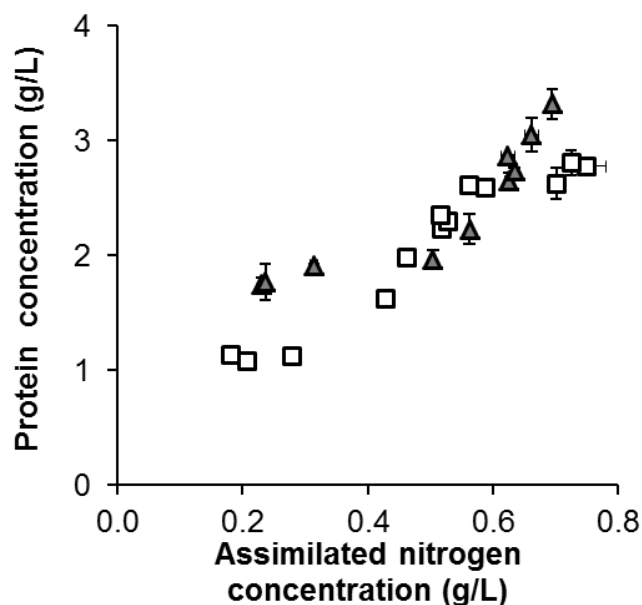


Fig. A.6.2 Protein concentration versus assimilated nitrogen concentration of nitrogen resupplied *C. zoofingiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.18 g L⁻¹ (□) and 0.23 g L⁻¹ (▲). **Error bars** represent standard deviations of triplicate protein and assimilated nitrogen measurements.

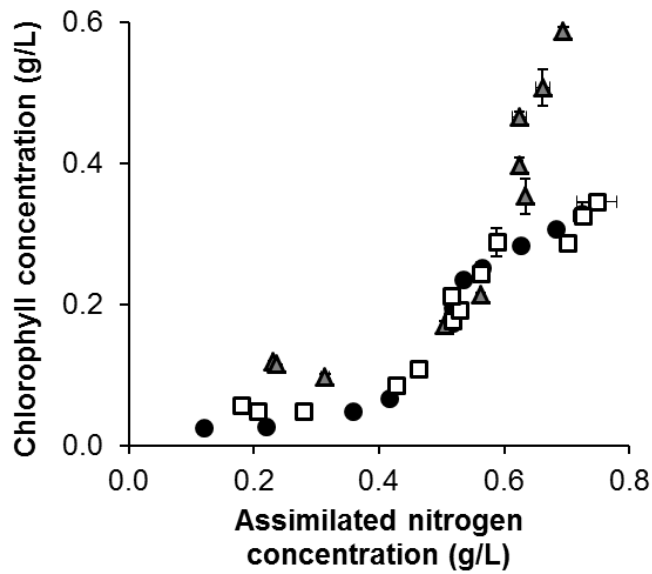
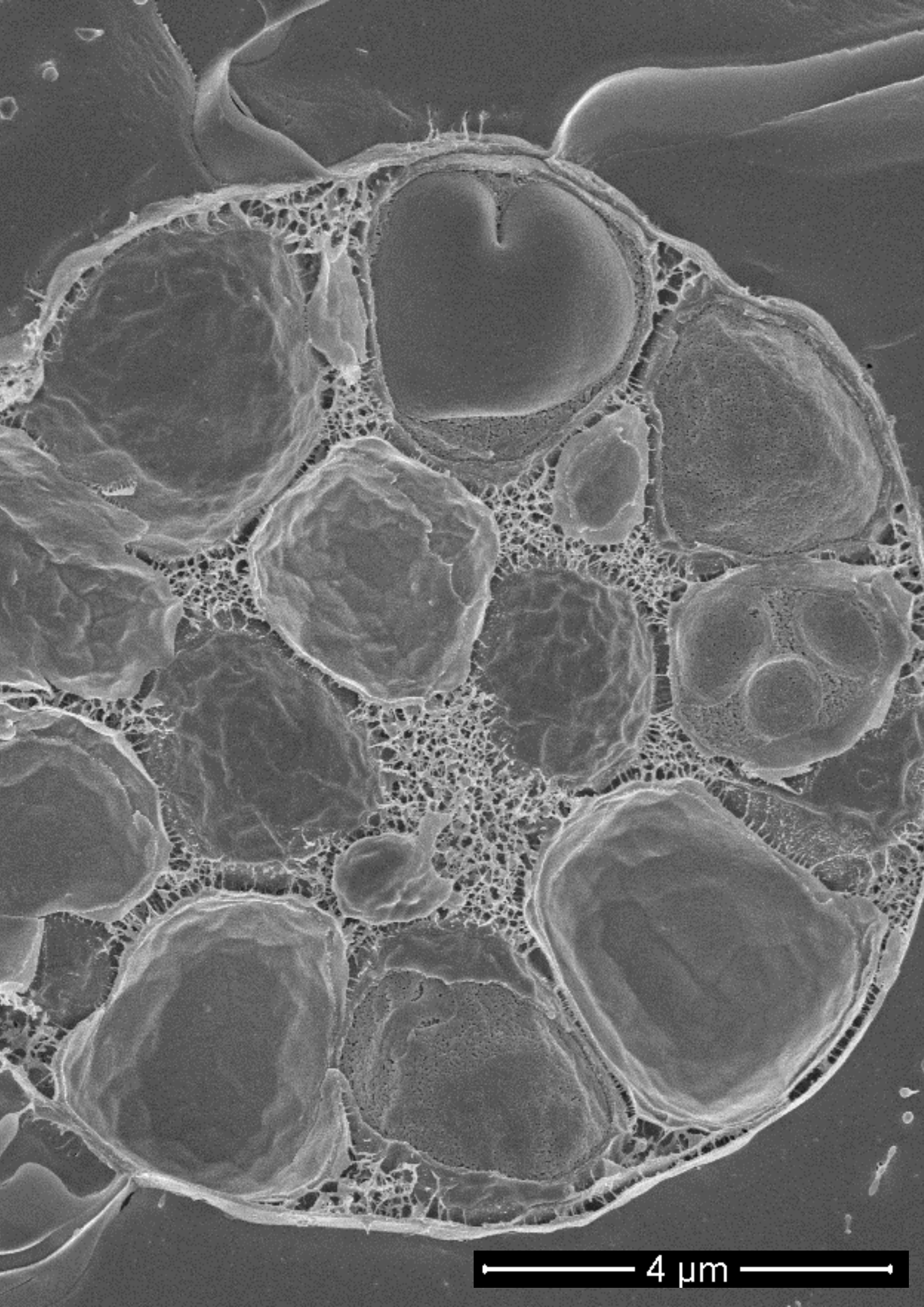


Fig. A.6.3 Total chlorophyll concentration (sum of chlorophyll a and b) versus assimilated nitrogen concentration of nitrogen resupplied *C. zoofingiensis* for cultures with different assimilated nitrogen concentrations after nitrogen resupply: 0.12 g L^{-1} (●), 0.18 g L^{-1} (□) and 0.23 g L^{-1} (▲). **Vertical error bars** represent absolute deviations from the mean of duplicate chlorophyll measurements and **horizontal error bars** represent standard deviations of triplicate nitrogen measurements.



Chapter 7

General Discussion

Microalgal carotenoid yields on light: current status and future perspectives

High lights

- Carotenoid production costs can be decreased by increasing the carotenoid yields on light.
- Optimizing the reactor/cultivation mode can lead only to minor increases in carotenoid yields.
- Optimizing strain performances can lead to major increases in carotenoid yields.
- For this, targeted genetic engineering and random mutagenesis should be explored.

◀ IN THE PICTURE

Electron micrograph of reproducing *Chromochloris zofingiensis* cells. When *C. zofingiensis* divides, one mother cell can separate into multiple daughter cells, forming one big clump. The amount of cells in such a clump can reach 64 (depending on the day/night length)! The daughter cells are kept together by a membrane. At a certain moment the membrane opens and liberates all the cells.

◀ IN BEELD

Electronenmicroscopiefoto van reproducerende *Chromochloris zofingiensis* cellen. Wanneer deze alg deelt kan een moedercel zich splitsen in meerdere dochtercellen, waarbij een grote klomp cellen gevormd wordt. Zo'n klomp kan uit wel 64 cellen bestaan (afhankelijk van de dag- en nachtlengte)! Het membraan die de cellen bij elkaar houdt gaat op een gegeven moment open, waarna de cellen vrijkomen.

Abstract

Microalgal carotenoids such as astaxanthin, β -carotene and lutein are used as nutraceuticals and hence have a high market value. To make these carotenoids applicable in a wider range of products, the production costs should be decreased. This can be done through, among others, enhancement of the areal productivity. Because the solar insolation on a certain area is a given, the areal carotenoid productivity of a microalgae production facility is directly proportional to the carotenoid yield on light. Thus, this yield on light is an important optimization target in microalgal carotenoid production. In this chapter, theoretical maximal carotenoid yields on light were estimated and compared to the currently obtained yields outdoors and in the laboratory. It was found that for both primary and secondary carotenoids there is a large gap ($> a \text{ factor } 100$) between the maximal theoretical carotenoid yields on light and those currently obtained outdoors. This gap is caused by suboptimal cultivation conditions/operation mode, a suboptimal photobioreactor design and/or by a suboptimal cell metabolism. The carotenoid yields on light obtained outdoors are only slightly lower than those obtained indoors, where the cultivation conditions are controlled and optimized. This indicates that the potential to improve the cultivation conditions/operation mode and/or reactor design is small. Strain optimization, on the other hand, may lead to higher yield increases ($\sim a \text{ factor } 4 - 7$). The primary carotenoid yield on light can be optimized by redirecting the carbon flow from excessively produced primary cell compounds (e.g. excessive proteins, membrane lipids or starch) towards primary carotenoids, whereas the secondary carotenoid yield on light can be optimized by redirecting the carbon flow from excessively produced secondary cell compounds (e.g. triacylglycerol or starch) towards secondary carotenoids. For this, both targeted genetic engineering and random mutagenesis (with subsequent selection) should be explored. Targeted genetic engineering requires further investigation of the microalgal physiology and, more specifically, of the carotenoid metabolism, and besides, it requires development of robust nuclear transformation protocols. Random mutagenesis, on the other hand, requires development of selection methods to select for mutants with a desired phenotype. In this chapter, two cases are given in which these genetic modification approaches are coupled to the aforementioned optimization targets and one case is given in which the operation mode is modified.

7.1 Introduction

Phototrophic microalgae produce a variety of different carotenoids, including astaxanthin, β -carotene and lutein. It has been claimed that these naturally derived carotenoids beneficially affect health, due to their anti-oxidative activity (Spolaore et al. 2006). Therefore, they are of particularly high value and preferably used as nutraceuticals. To make microalgal carotenoids applicable in a wider range of products, such as food and feed, the production costs should be decreased. This can be done through, among others, enhancement of the areal carotenoid productivity. In a microalgae-based carotenoid production plant, the solar insolation on a certain area is a given, and thus the areal carotenoid productivity is directly proportional to the

carotenoid yield on light. Hence, this yield on light is an important optimization target in microalgal carotenoid production.

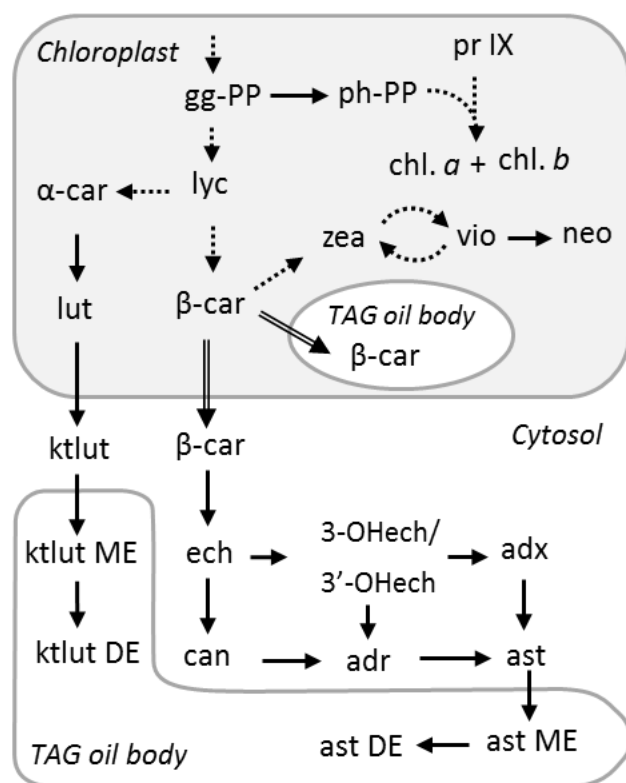


Fig. 7.1 Biosynthesis pathways of carotenoids and chlorophylls in green algae. Chloroplastidic metabolites appear in all green algae; others appear in only a small selection of green algae (e.g. *Haematococcus pluvialis* and *Chromochloris (Chlorella) zofingiensis*). Accumulation of β -carotene in chloroplastidic oil bodies has been observed in *Dunaliella salina* only (Davidi et al. 2014). **Double** arrows indicate dislocations, **solid** arrows represent single conversion steps, **dashed** arrows represent lumped reactions. Dislocations of lutein, ketolutein and astaxanthin are not explicitly indicated, because the location of biosynthesis and storage of these compounds is not clear. **3-OHech** 3-hydroxyechinenone, **3'-OHech** 3'-hydroxyechinenone, **α -car** α -carotene, **adr** adonirubin, **adx** adonixanthin, **ast** astaxanthin, **ast ME** astaxanthin monoester, **ast DE** astaxanthinn diester, **β -car** β -carotene, **can** canthaxanthin, **chl. a** chlorophyll a, **chl. b**

chlorophyll *b*, **ech** echinenone, **gg-PP** geranylgeranyl diphosphate, **ktlut** ketolutein, **ktlut ME** ketolutein monoester, **ktlut DE** ketolutein diester, **lut** lutein, **lyc** lycopene, **neo** neoxanthin, **ph-PP** phytyl diphosphate, **phy** phytoene, **pr IX** protoporphyrin IX, **vio** violaxanthin, **zea** zeaxanthin (Bauch 2011; Kanehisa Laboratories 2012; Lemoine and Schoefs 2010; Mulders et al. 2014d; Wang and Chen 2008).

As discussed in previous work, the carotenoids produced by phototrophic microalgae can be categorized into primary and secondary carotenoids (Mulders et al. 2014a). As primary cell constituents, primary carotenoids are produced during growth (i.e. when cells replicate and form new cells). These primary carotenoids are produced in the chloroplast (similar to the chlorophylls, **Fig. 7.1**). Secondary carotenoids, on the other hand, are produced when culture conditions are suboptimal for growth, often referred to as stress conditions (e.g. nutrient deprivation, high light intensity or extreme temperature or pH). Secondary carotenoids accumulate, possibly esterified with fatty acids, in triacylglycerol-filled oil bodies (**Fig. 7.1**) (Mulders et al. 2014a).

Carotenoids are produced in conjunction with other cell compounds. In fact, when carotenoids are produced, the major part of the biomass consists of compounds other than carotenoids (**Fig. 7.2**). The low carotenoid fraction indicates that a large part of the photosynthetic capacity is not employed for carotenoid biosynthesis. The carotenoid yield on light would thus become substantially larger when part of the total photosynthetic capacity could be redirected towards carotenoid synthesis. Hence, there must be a substantial gap between the theoretical maximal carotenoid yield on light and the currently obtained yield on

light. However, the size of this gap and the extent to which it can be bridged in practice are unknown.

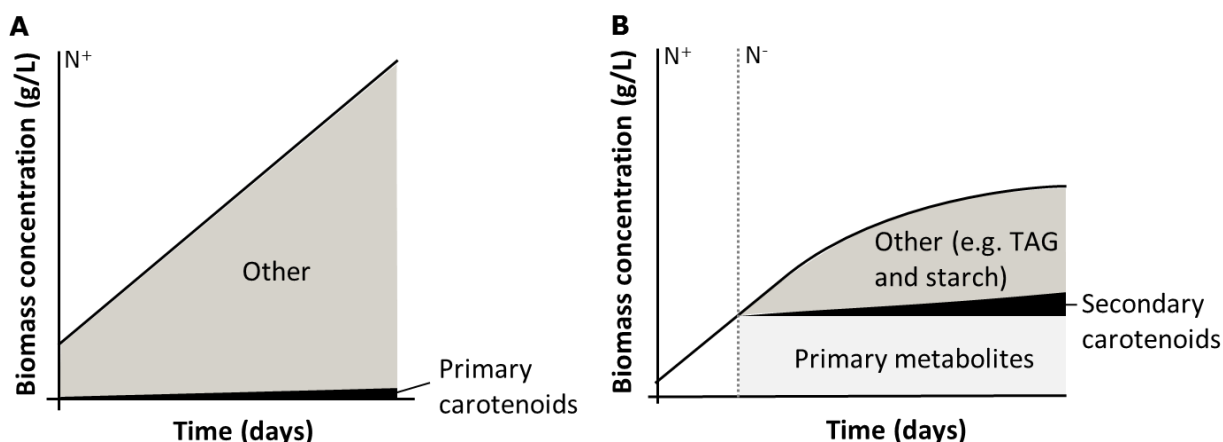


Fig. 7.2 General trends of biomass and carotenoid production **(A)** in a typical green alga (e.g. *Chromocloris* (*Chlorella*) *zofingiensis*, *Chlorella* *sorokiniana*, *Scenedesmus* *almeriensis* or *Muriellopsis* sp.) under optimal growth conditions and **(B)** in a typical carotenogenic green alga (e.g. *Haematococcus* *pluvialis* or *Dunaliella* *salina*) under nitrogen depleted conditions. Only a small fraction of the produced biomass consists of carotenoids (Cuaresma et al. 2009; Davidi et al. 2014; Del Campo et al. 2001; Han et al. 2012; Lamers et al. 2012; Mulders et al. 2014c; Sánchez et al. 2008b; Wayama et al. 2013).

Here, we assumed that the theoretical maximal carotenoid yield on light, in $\text{g}_{\text{car}} \cdot \text{mol}_{\text{ph}}^{-1}$, is proportional to the reduction state and the molecular mass of the carotenoids (see **Appendix 7A** for the calculation of the theoretical maximal carotenoid yield on light and for assumptions). The theoretical maximal yield on light of the carotenoids astaxanthin, β -carotene and lutein are 1.2, 1.0 and 1.1 $\text{g}_{\text{car}} \cdot \text{mol}_{\text{ph}}^{-1}$, respectively, when accumulated in the free form (e.g. not esterified with fatty acids) (**Table 7.1**).

When carotenoids are esterified the yield on light is somewhat lower, because part of the energy is required to synthesise the fatty acids. Namely, the theoretical maximal yield of pure astaxanthin on light of astaxanthin diesters (i.e. without accounting for the molecular weight of the fatty acid ester), esterified twice with C16:0 fatty acids, is 50 % lower than for free astaxanthin (**Table 7.1** and **Appendix 7A**).

In this chapter, theoretical maximal yields on light of both primary and secondary carotenoids are estimated and compared to the currently obtained yields outdoors and in the laboratory. Besides, three practical examples are discussed which each aim to substantially bridge the carotenoid yield gap.

7.2 Current carotenoid yields on light and theoretical maxima

By comparing the currently obtained carotenoid yields on light of actual outdoor production plants with theoretical maxima, an upper boundary for the room for improvement of these outdoor carotenoid yields can be extracted. However, carotenoid yields on light are scarcely reported in literature and the available data are largely incomplete and thus difficult to convert into carotenoid yields on light. Based on the limited data available, this section discusses the currently obtained yields on light for astaxanthin, β -carotene and lutein. These carotenoids are commercially the most relevant microalgal carotenoids (i.e. highest market values and

volumes (BCC Research 2008; Del Campo et al. 2000; Markou and Nerantzis 2013; Pulz and Gross 2004)) and are produced outdoors at semi-large to large scale (160 - 40,000 m²). These values are subsequently compared to theoretical maximal carotenoid yields on light, to estimate the theoretical room for improvement.

7.2.1 Theoretical maximal yields

Light energy and carbon dioxide are captured and converted into chemically fixed energy via a sequence of photochemical reactions (photosynthesis). Subsequently, the fixed carbon and electrons are incorporated into biomass constituents (anabolism), including carotenoids. The maximal carotenoid yield on light is reached when both the photosynthetic efficiency is maximal (i.e. heat dissipation is minimal) and the fixed carbon and electrons are optimally used for carotenoid synthesis.

7.2.2 Outdoor and indoor yields

Lutein is a primary carotenoid that is produced during growth, as a primary cell constituent. Therefore, lutein is best produced under conditions that lead to a maximal (primary) biomass yield on light (i.e. optimal growth conditions). Astaxanthin and β -carotene, on the other hand, are secondary carotenoids that accumulate under so-called stress conditions, conditions that are suboptimal for growth (i.e. nutrient deprivation, high light intensity or extreme temperature or pH). The most applied production method is a two-step process. In this approach, first biomass is produced under optimal growth conditions (inducing no or very low production of secondary carotenoids), after which stress conditions are applied that lead to secondary carotenoid accumulation.

Outdoors, maximal astaxanthin, β -carotene and lutein yields on light have been obtained with *Dunaliella salina* (8.7 mg _{β -car}·mol_{ph}⁻¹), *Haematococcus pluvialis* (6.7 mg_{ast}·mol_{ph}⁻¹) and *Scenedesmus almeriensis* (9.3 mg_{lut}·mol_{ph}⁻¹), respectively (**Table 7.1**). To better enable comparison with the theoretical yields on light, these outdoor yields on light refer to the carotenoid production phase only. Thus, in the case of astaxanthin and β -carotene, the biomass production phase is not included.

For astaxanthin and β -carotene these outdoor obtained yields are about 3 and 2 times lower, respectively, than the yields obtained indoors. The outdoor lutein yield is even 2 to 4 times higher than the yields obtained indoors, although it should be noted that the data were obtained with different species, namely with *Scenedesmus almeriensis* (outdoors) and with *Chromochloris (Chlorella) zofingiensis* and *Chlorella sorokiniana* (indoors) (**Table 7.1**). For both *D. salina* and *H. pluvialis* a large amount of indoor studies have been performed to optimize the cultivation conditions, as indicated in the reviews by Liu et al. (2014), Jin et al. (2006), Han et al. (2013), Guedes et al. (2011) and Jin and Melis (2003). It may thus be assumed that the best astaxanthin and β -carotene yields in these indoor studies have been obtained in systems operated under close-to-optimal conditions, such as optimal pH and temperature, no carbon dioxide limitations or oxygen inhibition, and an optimal light distribution. This implies that optimization of the bioreactor design and operation will most likely not further increase the obtained indoor yields and can lead only to minor increases (2 to 3 times) in outdoor yields when compared with the potential of the theoretical maxima (**Table 7.1**).

Table 7.1. Theoretical maximal yields, and maximal reported indoor and outdoor yields of β -carotene, astaxanthin and lutein on light ($\text{mg}_{\text{car}} \cdot \text{mol}_{\text{ph}}^{-1}$) during the carotenoid production phase with in between brackets the corresponding concentrations per dry weight ($\text{mg}_{\text{car}} \cdot \text{g}_{\text{DW}}^{-1}$).

	β -carotene	Astaxanthin	Lutein
Theoretical maximum	994	1170 (Free) 807 ^a (ME) 615 ^a (DE)	1073
Indoor	16 ^b (27); <i>D. salina</i>	1.3 ^d (2.4); <i>C. zoofingensis</i> 19 ^e (28); <i>H. pluvialis</i>	2.3 ^g (3.1); <i>C. zoofingensis</i> 5.0 ^h (5); <i>C. sorokiniana</i>
Outdoor	8.7 ^c (~100); <i>D. salina</i>	6.7 ^f (25); <i>H. pluvialis</i>	9.3 ⁱ (4.5); <i>S. almeriensis</i> 3.8 ^j (6); <i>Muriellopsis sp.</i>

Free unesterified, **ME** monoester, **DE** diester.

^a Esterified with C16:0 and expressed as free astaxanthin equivalent (see **Appendix 7A**).

^b Closed system, 2 L flat panel photobioreactor, turbidostat mode, N-depletion, continuous incident light intensity of $200 \mu\text{mol}_{\text{ph}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. (Time-average) yield on absorbed light was maximal after 37 hours of N-depletion (Lamers et al. 2012).

^c Open system, ~4 ha raceway ponds, Israel (Nature Beta Technologies), yearly average of $300 \text{ mg}_{\beta\text{-carotene}} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (Ben-Amotz 1995, Del Campo et al. 2007). Yield on light recalculated from areal productivity as described by Breuer et al. 2013, using an incident solar irradiance of $7200 \text{ MJ} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$ (annual irradiance of Israel; <http://www.jewishvirtuallibrary.org/jsource/Environment/Solar.html>). In this calculation a 10 % energy loss is assumed due to nocturnal respiration. The system is assumed to be operated year round.

^d Closed system, 1,8 L flat panel photobioreactor, batch, N-depletion, continuous incident light intensity of $245 \mu\text{mol}_{\text{ph}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Time-average yield on absorbed light was obtained after ~14 days (Mulders et al. 2014c).

^e Open system, 3 L circular tank, batch. Astaxanthin yield on light recalculated from Zhang et al. (2009), based on an average incident light intensity of $217 \mu\text{mol}_{\text{ph}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, a light-dark cycle of 14:10h, an astaxanthin concentration obtained after 12 days of $51.06 \text{ mg} \cdot \text{L}^{-1}$ and a culture depth of 0.05 m.

^f Open system, 100 m² raceway pond, Shenzhen China, yearly average of $170 \text{ mg}_{\text{astaxanthin}} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (measured during 2 years). This areal astaxanthin productivity was calculated from Li et al. (2011a), based on an initial astaxanthin content in DW of 0 % and, after 9 days (the reddening time), an astaxanthin content of 2.5 % and (at that point) a biomass concentration of $0.4 \text{ g} \cdot \text{L}^{-1}$, with a culture depth of 0.15 m. Yield on light recalculated from areal productivity as described by Breuer et al. 2013, using an incident solar irradiance of $5278 \text{ MJ} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$ (annual irradiance of Hong Kong; <http://web.hku.hk/~cmhui/hksolar/hksolar.htm>). In this latter calculation a 10 % energy loss is assumed due to nocturnal respiration. The system is assumed to be operated year round.

^g Closed system, 1,8 L flat panel photobioreactor, nutrient sufficient batch culture, continuous incident light intensity of $245 \mu\text{mol}_{\text{ph}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Mulders et al. 2014c).

^h Closed system, 1,6 L flat panel photobioreactor, nutrient sufficient continuous culture, continuous incident light intensity of $2,100 \mu\text{mol}_{\text{ph}} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Cuaresma et al. 2009).

ⁱ Closed system, 4,000 L tubular photobioreactor, continuous culture, yearly average of $290 \text{ mg}_{\text{lutein}} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (Sánchez et al. 2008b, Fernández-Sevilla et al. 2010). Yield on light recalculated from areal productivity as described by Breuer et al. 2013, using an incident solar irradiance of $6480 \text{ MJ} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$ (the yearly average in South Europe). In this calculation a 10 % energy loss is assumed due to nocturnal respiration. The system is assumed to be operated year round.

^j Closed system, 55 L (2.2 m²) tubular photobioreactor, $180 \text{ mg}_{\text{lutein}} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (Del Campo et al. 2001). Yield on light recalculated from areal productivity as described by Breuer et al. 2013, with an areal productivity that was obtained in southern Spain in July only (not year-round), using an incident solar irradiance of $9744 \text{ MJ} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$ (Sánchez-Lorenzo et al. 2012). In this calculation a 10 % energy loss is assumed due to nocturnal respiration.

Differences between indoor and theoretical maximal yields on light, on the other hand, are much larger. The theoretical maximal yield of respectively astaxanthin and β -carotene is about 60 times larger than obtained indoors, and for lutein this differs by a factor over 100. Assuming again that the process conditions used indoors were optimal to reach a high carotenoid yield on light, this implies that by optimizing the strain itself (e.g. by genetic engineering), instead of the cultivation process, still substantial improvements of the carotenoid yield on light may be realized.

7.3 Methods to maximize carotenoid yields on light

In this section, optimization of the primary carotenoid yield on light (e.g. lutein) is discussed (**Section 7.3.1**) followed by the secondary carotenoid yield on light (β -carotene and astaxanthin) (**Section 7.3.2**), both using strain optimization approaches. Last, optimization of the secondary carotenoid yield on light (astaxanthin) is discussed using a process operation optimization approach (**Section 7.3.3**).

7.3.1 Increasing primary carotenoid yield on light via laboratory evolution in a turbidostat

7.3.1.1 Current and desired situation

As discussed in the introduction, primary pigments, including primary carotenoids, are synthesised during growth (i.e. when cells replicate and form new cells), which occurs in concurrence with the production of other primary compounds (e.g. proteins, membrane lipids and carbohydrates such as starch). The fraction of primary carotenoids is generally low (i.e. below 0.6 % DW (w/w) (**Fig. 7.2a** and **Lutein** in **Table 7.1**). This implies that only a small fraction of the fixed carbon and energy is directed towards primary carotenoids and thus that currently obtained primary carotenoid yields on light can be improved when the primary carotenoid fraction would be increased and/or when the overall photosynthetic efficiency would be increased.

In previous work it was attempted to increase the primary carotenoid fraction in *C. zofingiensis* by redirecting the flux from secondary carotenoids towards primary carotenoids by applying nitrogen depleted conditions, an effective way to induce secondary carotenogenesis, and simultaneously adding diphenylamine (DPA), an inhibitor of β -carotene ketolase (BKT) (Mulders et al. 2014d). BKT catalyses, among others, the first step of secondary carotenogenesis (i.e. the conversion from β -carotene to echinenone (**Fig. 7.1**)) and inhibition of this conversion was thus expected to affect the primary carotenoid fraction. However, although BKT inhibition led to a decreased flux towards the secondary carotenoids, the flux towards primary carotenoids remained unchanged. This result alluded to the presence of a regulatory mechanism that prevented an increased flux towards the primary carotenoids. Because the mechanism behind this regulation remains unknown, it may be difficult to change the primary carotenoid fraction by targeted genetic manipulation, simply because we do not know what the right target is.

However, it is well-known that enhancement of the primary pigment content (including primary carotenoids) can be achieved by exploiting photoacclimation to low light conditions, a process in which the cells increase the amount of light harvesting pigments to maintain an adequate cellular light absorption rate at low irradiance or high biomass density. For example, it was found in previous work that in *C. zofingiensis* the total primary pigment content

increased from 14 to 28 g_{pig} g_{DW}⁻¹ when the biomass concentration increased at a constant (low) incident irradiance (Mulders et al. 2014c). Thus, there must be a mechanism present in microalgae that naturally induces an increased flux towards the primary pigments.

However, low light conditions do generally not only lead to an increased content of primary pigments, but also lead to a decreased specific growth rate, as observed in the same work with *C. zoofingiensis* (Mulders et al. 2014c). A decreased specific growth rate is undesired as this generally leads to a suboptimal carotenoid productivity.

Thus, a novel method is desired to obtain microalgae that combine an enhanced primary pigment content with an increased or constant specific growth rate, without the need for targeted genetic manipulations. For example, by applying random mutagenesis in the presence of a selection pressure that automatically selects for microalgae with these traits.

7.3.1.2 Approach

This can be achieved by cultivating microalgae in a turbidostat, while subjecting them periodically to ultraviolet light, causing random genetic changes. In a turbidostat (a continuous culture mode), the concentration of light-absorbing material (i.e. pigments) is kept constant. Pigment production leads to culture dilution, implying that the dilution rate is proportional to the pigment production rate, and because the specific light absorption rate is constant in a turbidostat, the dilution rate is also proportional to the pigment yield on light. When a mutant is generated with an increased pigment yield on light, the dilution rate will increase. Most likely, such a mutant will also have an increased specific growth rate and hence will overgrow the culture, while others are washed out. Thus, with this method genetically modified microalgae are generated, through random mutagenesis, and when one, or multiple, of such modifications lead to a strain with an enhanced pigment yield on light and specific growth rate, this mutant is (most likely) automatically selected. For a more thorough explanation of how this method leads to cells with an enhanced pigment yield on light, see **Appendix 7B**.

In green algae, primary pigments include both chlorophylls (chlorophyll *a* and *b*) and carotenoids (of which generally ~ 90 % (w/w) is lutein (Mulders et al. 2014c; Macías-Sánchez et al. 2010)), which all absorb light in the photosynthetic active radiation (PAR) range (400 - 700 nm). When the whole PAR range would be used to measure the turbidity, mutants would be selected with maximal overall yields of all these pigments. However, if the absorption at only a specific wavelength range of 475 to 520 nm would be used, for which lutein has a higher relative absorbance than the chlorophylls (**Fig. 7.3**), the chance increases that cells are selected for with a maximal lutein yield on light, instead of a maximal yield of both lutein and chlorophylls on light.

In brown algae (e.g. *Isochrysis galbana*) and diatoms (e.g. *Phaeodactylum tricornutum*), the primary carotenoids (mainly fucoxanthin and diadinoxanthin) have no overlapping absorbance with the chlorophylls (chlorophyll *a* and *c*) in the wavelength range of 510 to 540 nm (Bricaud et al 2004; Mulders et al. 2013; Bertrand 2010) (**Fig. A.7.1, Appendix 7C**). Therefore, brown algae and diatoms can be specifically selected for a maximal carotenoid yield on light (and thus no high chlorophyll yield on light).

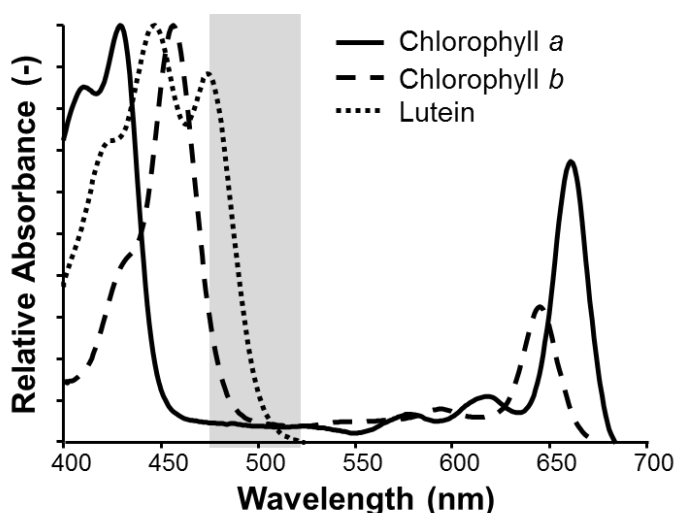


Fig. 7.3 Visible absorption spectra of chlorophyll a, chlorophyll b and lutein, the main primary pigments of green algae. **Shaded area** indicates wavelengths that are mainly absorbed by lutein. Spectra were obtained as described in Mulders et al. 2014d using standards (chlorophyll a and lutein) or *C. zoofingensis* extracts (chlorophyll b).

7.3.1.3 Maximal expected increase of carotenoid yield on light

It is difficult to predict to what extent the carotenoid yield on light can be improved with this method. However, as discussed in **Appendix 7B**, the largest enhancements of the pigment yield on light can be expected when mutations lead to reduced excessive biomass production.

Proteins, carbohydrates (e.g. starch) and membrane lipids are generally present in concentrations of 49, 25 and 12 % of dry weight (w/w) (Sánchez et al. 2008a) and it is likely that part of these components are present in excess. Specifically for microalgae that have evolved in environments with frequent scarcity of resources, excessive accumulation of storage components may occur. Assuming a 20 % excessive primary biomass production (e.g. starch), which thus can be removed, and a redirection of a very small fraction of the left energy and carbon towards primary carotenoids (causing a doubling in the flux towards carotenoids), this would result into a 2-fold and 2.5-fold increases in, respectively, the primary carotenoid yield on light and the primary carotenoid content. An increase towards the primary carotenoids by at least a factor two seems rational as it was found in previous work that the flux towards carotenoids can be doubled naturally by means of photoacclimation, as discussed in **Section 7.3.1.1** (Mulders et al. 2014c).

7.3.1.4 Possible challenges and required research

A practical advantage of this method is that the cell is approached as a black box (genetic changes are randomly made), so improved cellular properties (such as enhanced carotenoid yields on light) can be reached without knowledge of metabolic or regulatory details of the microalga. So far, random mutagenesis with subsequent selection have been proved feasible and efficient for many microorganisms, as reviewed for example by Dragosits and Mattanovich (2013). For example, an isolated mutant of *Saccharomyces cerevisiae* produced 41 % more glycerol than the parental strain it was derived from (Kutyna et al. 2012) and an isolated mutant of *Gluconobacter oxydans* had doubled its growth rate and enhanced the yield of dihydroxyacetone on glucose by 21 % (Lu et al. 2012). However, as previously discussed, a downside of this method is that it is difficult to predict to what extent the carotenoid yield on light can be improved in comparison to the wild type strain.

In addition, care should be taken with this method to mimic the actual process conditions under which the microalgae will be cultivated on large scale. Namely, selection occurs in a turbidostat under the specific set of applied conditions, whereas a totally different mutant strain may be selected when these conditions are changed. Especially the mixing and light regime will be difficult to mimic in lab-scale photobioreactors, so eventually selection in outdoor real-size photobioreactors will be necessary.

7.3.2 Carbon flow redirection from TAG and starch to secondary carotenoids

7.3.2.1 Current and desired situation

Under stress conditions (e.g. nitrogen depletion), secondary carotenoids are produced in concurrence with other secondary compounds. As mentioned previously, the fraction of secondary carotenoids is much smaller than the other produced secondary compounds, such as TAG and starch (**Fig. 7.2b**), which implies that only a small fraction of the fixed carbon and energy is directed towards secondary carotenoids. Thus, currently obtained secondary carotenoid yields on light can be increased by redirecting (part of) the fixed carbon and energy towards secondary carotenoids.

7.3.2.2 Approach

This redirection may, for instance, be achieved by reducing the activity of TAG and/or starch producing enzymes, either by targeted genetic engineering or random, genome-wide, mutagenesis (with subsequent targeted selection).

Down-regulating or inhibiting the starch synthesis has been shown to enhance TAG production in non-carotenogenic strains (Breuer et al. 2014, de Jaeger et al. 2014, Li et al. 2010a, Li et al. 2010b, Siaut et al. 2011, Wang et al. 2009, Ramazanov and Ramazanov 2006). For example, in a starchless mutant of the model alga *Chlamydomonas reinhardtii*, the volumetric TAG productivity was increased four times (Li et al. 2010a). However, as *C. reinhardtii* is originally non-oleaginous, the TAG yield on light of the mutant did not exceed the TAG yield obtained in wild-type oleaginous microalgae. Recently, a starchless mutant of the oleaginous microalga *Scenedesmus obliquus* was shown to have a 51 % increase in TAG yield on light (0.14 to 0.22 g_{TAG}·mol_{ph}⁻¹). In this mutant the photosynthetic efficiency was not affected. In other words, the mutant redirected the carbon flow from starch to TAG, while leaving the overall photosynthetic capacity unchanged (Breuer et al. 2014; de Jaeger et al. 2014).

Similarly, the β-carotene (*D. salina*) or astaxanthin (*H. pluvialis*) yield on light may be increased by reducing the TAG or starch synthesis in these carotenogenic species. However, complete inhibition of TAG synthesis should be prevented, as that has been shown to reduce secondary carotenoid accumulation (Zhekisheva et al. 2005; Rabbani et al. 1998). It has been proposed in these works that a certain minimal amount of TAG is required for secondary carotenoid formation to serve as metabolic sink or storage vehicle.

7.3.2.3 Maximal expected increase of carotenoid yield on light

The lab-scale cultivated *H. pluvialis* cells with the highest observed astaxanthin yield on light (19 mg_{ast}·mol_{ph}⁻¹, **Table 7.1**), had an astaxanthin content of about 3 % DW (w/w). It may be assumed that the amount of starch present at that point was negligible (Han et al. 2012; Wayama et al. 2013). In contrast, the amount of TAG must have been substantial. Assuming an

astaxanthin to TAG ratio of one to ten (Zhekisheva et al. 2005), a TAG content of about 30 % DW (w/w) must have been reached. If this TAG concentration would be reduced to 5 % DW (w/w), and the carbon flow would be completely redirected towards astaxanthin monoesters (namely, in *H. pluvialis* astaxanthin is mainly accumulated as monoester (Zhekisheva et al. 2002)), the astaxanthin monoester content would increase to about 28 % DW (equivalent to 20 % DW (w/w) free astaxanthin), and thus the astaxanthin yield on light would become 6.7 times as large.

Alternatively, *C. zofingiensis* may be genetically modified to become a astaxanthin production strain. Although the current yield of astaxanthin on light of *C. zofingiensis* is much lower (~15 times) than the astaxanthin yield obtained with *H. pluvialis* (**Astaxanthin in Table 7.1**), it was found in previous work that the TAG yield on light of *C. zofingiensis* is very high. More specifically, the TAG yield on light of *C. zofingiensis* was found to be about 1.5 times higher than obtained with *H. pluvialis* in the above case example (320 vs. 190 mg_{TAG}·mol_{ph}⁻¹), whereas the maximally obtained TAG content was similar (i.e. ~30 % DW (w/w) (Mulders et al. 2014c). Consequently, also the potential to produce astaxanthin is about 1.5 times higher with *C. zofingiensis* than with *H. pluvialis*. However, *C. zofingiensis* naturally produces mainly astaxanthin diesters, which reduces the maximal potential of the astaxanthin yield on light by 25 % (**Appendix 7.A**), if this would not be modified. Besides, whereas *H. pluvialis* accumulates mainly astaxanthin, as a secondary carotenoid, *C. zofingiensis* accumulates also ketolutein and canthaxanthin (up to 50 % of the total secondary carotenoids) (Mulders et al. 2014c and d), reducing the astaxanthin production potential again by 50 %, if this would not be modified. Thus, *C. zofingiensis* may indeed be a potentially more efficient astaxanthin production strain than *H. pluvialis*, but that would require some additional genetic modifications.

The β-carotene yield on light may be increased similarly. It may be assumed that when the cells have reached a β-carotene content of 10 % DW (w/w) (**β-carotene in Table 7.1**), the TAG content increased by 7 % DW (w/w), and starch content increased by roughly 30 % DW (Davidi et al. 2014; Lamers et al. 2010). When the increase in TAG content would be reduced to 2 % DW (w/w), the starch flux would completely be inhibited, and assuming that the carbon flows would be completely redirected towards β-carotene, the β-carotene yield on light would become approximately 4.5 times as large as in the wild type.

Note that in both examples the maximal potential is estimated, assuming that the whole carbon flow is redirected towards secondary carotenoids. As indicated below, this most likely involves rigorous metabolic changes, not just a single mutation. Therefore, this method has the potential to result in large increases of the carotenoid yield on light, but this aim may prove difficult to achieve.

7.3.2.4 Possible challenges and required research

In wild type *D. salina*, *H. pluvialis* and *C. zofingiensis* the flux towards secondary carotenoids is much smaller than towards starch and TAG. Besides, it was found in previous work that in *C. zofingiensis* the (low) flux towards secondary carotenoids remained constant despite that the overall photosynthetic capacity and the flux towards other compounds, such as TAG, decreased (Mulders et al. 2014c). This indicates that the flux towards secondary carotenoids is likely highly regulated and/or that the carotenogenic enzymes have a limited capacity, which is not easily modified.

The flux towards secondary carotenoids can be redirected by both targeted genetic engineering and random, genome-wide, mutagenesis (with subsequent targeted selection). Targeted genetic engineering requires development of a robust transformation toolbox and detailed knowledge of the microalgae physiology and metabolic regulation.

According to Li et al. (2010c), the astaxanthin production in *H. pluvialis* is mainly under control of β -carotene hydroxylase. Others proposed that enzymes further up the carotenogenic pathway may be rate limiting, such as phytoene synthase (Jin et al. 2006) or phytoene desaturase (Lemoine and Schoefs 2010), which convert geranylgeranyl diphosphate into lycopene (**Fig. 7.1**). However, as discussed in previous work (Mulders et al. 2014a), the flux towards a desired carotenoid (i.e. β -carotene or astaxanthin) is likely controlled by multiple enzymes and all those enzymes need to be overproduced to realize a substantial increase in carotenoid yield on light (Fell 1997).

Random, genome-wide, mutagenesis requires development of selection methods to select for mutants with a desired phenotype. Secondary carotenoids are generally formed under high light intensities to protect the cells from the otherwise damaging irradiation. Thus, by cultivating mutants, on agar or in well plates, under high light of a specific colour (e.g. blue), mutants should be selected with increased secondary carotenoid contents.

It should be noted that increasing the secondary carotenoid content inevitably leads to a decreased photosynthetic efficiency, because the light energy absorbed by secondary carotenoids is dissipated as heat and can thus not be used to generate chemical energy.

7.3.3 Increased overall secondary carotenoid yield on light by repeated batch cultivation.

7.3.3.1 Current and desired situation

As previously mentioned, the secondary carotenoids astaxanthin and β -carotene are currently produced in a two-step batch process. In this approach, first primary biomass is produced under optimal (nitrogen replete) conditions, after which secondary carotenoids are produced under stress conditions (e.g. nitrogen depletion) in a second phase. At the end of each batch, the reactor is fully harvested and inoculated with fresh biomass (**Fig. 7.5a**).

In this approach, the incident solar irradiance is only used partly for secondary carotenoid production, because another part is required for the production of biomass. The more light is relatively required for biomass production, the less can relatively be used for secondary carotenoid production, and thus, the lower the overall carotenoid yield on light becomes. Consequently, currently obtained overall secondary carotenoid yields on light can, among others, be increased by reducing the irradiance required for primary biomass production.

7.3.3.2 Approach

This may be achieved by producing secondary carotenoids in a repeated batch cultivation. In this approach only part of the reactor is harvested after carotenoid accumulation, while the remaining part of the stressed biomass is recovered and reused, for example by resupplying nitrogen to nitrogen-depleted cells. At some point the nitrogen will be depleted again after which the cells will enter a second stress phase, resuming secondary carotenoid accumulation (**Fig. 7.5b**).

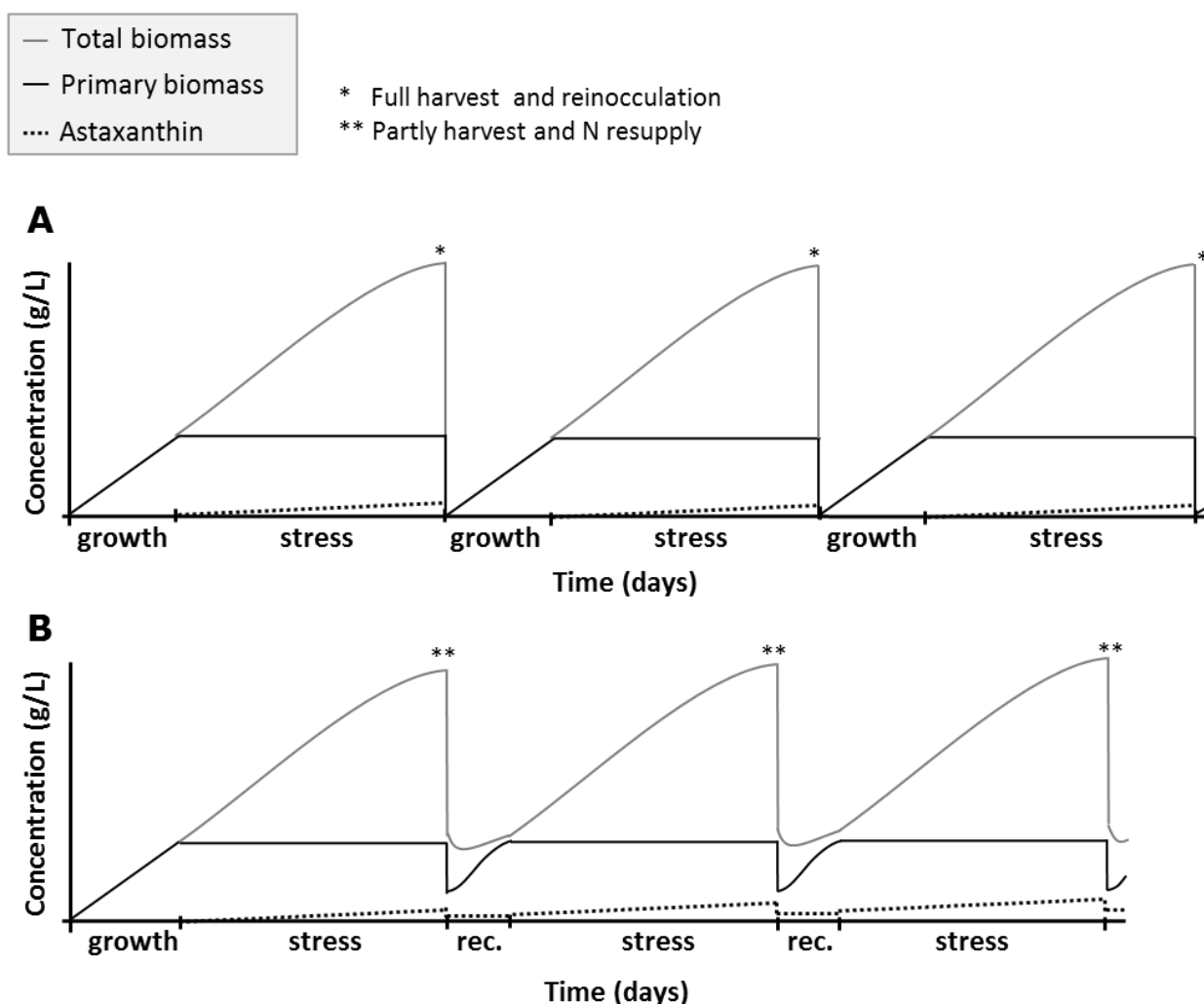


Fig. 7.5. Biomass and astaxanthin production (A) in a two-step batch approach and (B) in a repeated batch approach.

If the recovery phase in a repeated batch process requires less light energy than the biomass production phase in a classical two-step batch process, and the carotenoid yield on light during the stress phases are comparable in both processes (i.e. no carotenoid degradation during recovery), a repeated batch may result into a higher carotenoid yield on light than a classical two-step batch process. Namely, in that case, in the repeated batch relatively more light energy is used for the secondary carotenoid production, leading thus to an increased overall carotenoid yield on light.

In previous work, it was observed that nitrogen-depleted astaxanthin-rich *C. zoefingiensis* cells quickly recovered when nitrogen was resupplied. Besides, astaxanthin was not degraded during the recovery period (Mulders et al. 2014b). It may also be that during subsequent recovery periods the accumulated astaxanthin is not degraded and that during each subsequent stress phase astaxanthin is produced with the same high yields as observed during a single batch run (thus there is no product inhibition). As discussed above, this, in combination with the fast recovery, may lead to an increased overall carotenoid yield on light.

Besides, in that case, the astaxanthin content will be increased after each subsequent cycle. A higher astaxanthin content decreases the down-stream processing costs of the biomass and thus the carotenoid production costs. Thus, in addition to that a higher overall carotenoid yield

on light may be obtained with a repeated batch, also the possibly increased astaxanthin content makes a repeated batch an interesting approach to investigate.

7.3.3.3 Maximal expected increase of carotenoid yield on light

The expected fold-increase in carotenoid yield on light (f) between a two-step batch cultivation and a repeated batch cultivation is estimated in **Appendix 7D**. When it is assumed that during recovery no carotenoids are degraded, f is described by the equation below (see for other assumptions **Appendix 7D**):

$$f = \frac{n \cdot \left(\frac{C_{x0}^u \cdot d_s}{Y_{x,Ph}} + I \cdot t_s \right)}{\frac{C_{x0}^u \cdot d_s}{Y_{x,Ph}} + n \cdot (I \cdot t_s) + (n - 1) \cdot (I_r \cdot t_r)}$$

With

- I incident light intensity ($\text{mol}_{ph} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$)
- $Y_{x,Ph}$ biomass yield on photons during growth ($\text{g}_x \cdot \text{mol}_{ph}^{-1}$)
- C_{x0}^u biomass concentration at the start of stress phase ($\text{g}_x \cdot \text{m}^{-3}$)
- d_s culture depth of the batch/repeated batch reactor (m)
- t_s duration of one stress batch (days)
- t_r recovery time (days)
- n repeated number of batches that is performed in a row (-)

This equation shows that when a relatively high amount of light is required for the growth phase, in comparison to the carotenoid production phase, a repeated batch becomes more beneficial. This is mainly expressed by the parameters $C_{x0}^u \cdot d_s$, I , $Y_{x,Ph}$ and t_s .

$C_{x0}^u \cdot d_s$ is a measure for the amount of biomass that needs to be produced during the growth phase. The higher this parameter, the more light is relatively needed for the growth phase and thus the more advantageous a repeated batch becomes in comparison to a classical batch. Similarly, the lower the biomass yield on light ($Y_{x,Ph}$), the more light is relatively needed for the growth phase and thus the more advantageous a repeated batch becomes. Also, the shorter the duration of one stress batch (t_s), the less light is required for the carotenoid production phase, and the more light is needed for the growth phase and thus the more advantageous a repeated batch becomes.

The incident light intensity and the amount of biomass that needs to be produced during the growth phase are interdependent. Namely, the higher the incident light intensity, the higher the amount of biomass needs to be to prevent photoinhibition and obtain an optimal pigment yield on light during the stress phase (Zemke et al. 2013). As these parameters oppositely affect the benefit of a repeated batch, they cancel each other out to some extent.

With *H. pluvialis*, which has the highest astaxanthin yield on light determined so far (**Table 7.1**), an incident irradiance of $28 \text{ mol}_{ph} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ ($5278 \text{ MJ} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$), a biomass concentration at the start of the stress phase of $0.3 \text{ g} \cdot \text{L}^{-1}$ and a culture depth of 0.15 m , the optimal stress time was 9 days. In this time, an astaxanthin content of 2.5 \% DW (w/w) was reached (Li et al. 2011a). Assuming a biomass yield on light of $0.45 \text{ g}_x \cdot \text{mol}_{ph}^{-1}$ (which was obtained outdoors with

H. pluvialis by Olaizola (2000)¹), a recovery time of 2 days (as was observed for nitrogen stressed *C. zoofingensis* and *Parachlorella kessleri* (Fernandes et al. 2013; Mulders et al. 2014b)) no degradation of carotenoids (as observed for *C. zoofingensis* (Mulders et al. 2014b)) and a constant astaxanthin yield on light during each carotenoid production phase (no product inhibition), a repeated batch would be more beneficial than a classical batch when more than one cycle is performed (**Bold line, Fig. 7.6**). Note that a repeated batch with one cycle ($n=1$), is in fact a classical batch (**Fig. 7.5**) and thus f equals 1 (**Fig. 7.6**).

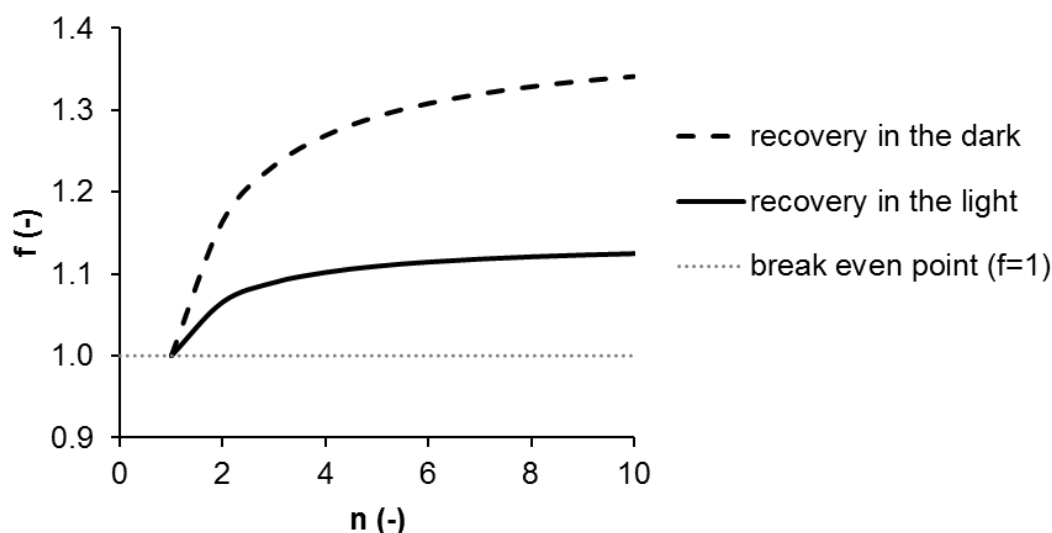


Fig. 7.6. The fold-increase in carotenoid yield on light between a two-step batch cultivation and a repeated batch cultivation (f) as function of the repeated number of batches that is performed in a row (n) when the irradiance equals $28 \text{ mol}_{ph} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$, the initial biomass concentration $0.3 \text{ g} \cdot \text{L}^{-1}$, the culture depth 0.15 m , the stress time 9 days, the biomass yield on light $0.45 \text{ g}_x \cdot \text{mol}_{ph}^{-1}$, the recovery time 2 days, no carotenoids are degraded during recovery and the astaxanthin yield on light is constant during each carotenoid production phase (no product inhibition). For other assumptions see **Appendix 7D**.

The fold increase in carotenoid yield on light increases asymptotically with the number of cycles (**Fig. 7.6**). In this case example, the maximal fold increase in carotenoid yield on light is 1.14. This asymptotic increase implies that the relative benefit of the repeated batch decreases with an increasing number of cycles. In contrast, assuming that during the recovery phase no astaxanthin is degraded, and during the carotenoid production phase the astaxanthin is accumulated with a constant yield on light, the astaxanthin content would increase each cycle with 25 mg/g DW. For example, after three cycles the astaxanthin content would be approximately 7.5 % DW (w/w), and after eight cycles a content of approximately 20 % DW (w/w) would be reached.

¹ Maximally obtained areal biomass productivity was $13 \text{ g}_{biomass} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ (in an outdoor closed system, 25,000 L tubular photobioreactor). Biomass yield on light was recalculated from this areal productivity as described by Breuer et al. 2013, using an incident solar irradiance of $6018 \text{ MJ} \cdot \text{m}^{-2} \cdot \text{year}^{-1}$ (annual irradiance of Hawaii; http://rredc.nrel.gov/solar/old_data/nsrdb/1961-1990/redbook/atlas/). A 10 % energy loss was assumed due to nocturnal respiration.

In the above case example the recovery was performed in the light, but recovery in the dark could also have been considered as an alternative option. Namely, in previous work it was found that the energy dense storage compounds TAG and starch were quickly degraded upon nitrogen resupply, which led to the hypothesis that accumulated TAG and starch may serve as sources of energy and carbon for recovery, without the requirement of external light energy (Mulders et al. 2014b). In agreement with our findings, quick degradation of TAG and starch was also found upon nitrogen resupply to nitrogen-depleted TAG-rich *Parachlorella kessleri*, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* cultures (Fernandes et al. 2013; Pribyl et al. 2013; Siaux et al. 2011). Culture recovery occurred even when performed in the dark (Pribyl et al. 2013; Siaux et al. 2011), which supports our hypothesis that the recovery processes do not require light energy.

If prior to recovery the culture would be transferred to a dark place, the light energy, which otherwise would have illuminated the recovering culture, could be used for a parallel production process instead, thereby making more efficient use of the light. In the case example above, that would imply that the maximal fold increase in carotenoid yield on light becomes 1.4, instead of 1.14 for recovery in the light (**Dashed line, Fig. 7.6**).

3.3.4. Possible challenges and required research

In the estimation above, it was assumed that the carotenoid yield on light, during the stress phase, was constant throughout the repeated batch cultivation. However, this yield may become higher or, more likely, lower each cycle, which thus needs to be investigated. In addition, no sequential repeated batch has been described in literature so far, so the whole concept needs to be investigated.

Besides, a simplified model was used in this section, which needs to be further refined with respect to certain parameter's dependencies of chosen cultivation conditions and with respect to the flexibility of the chosen cultivation approaches.

Nevertheless, the relation as described in this section gives a good first impression of the benefits of a repeated batch over a series of single batch cultivations and thus whether it is useful to further investigate the repeated batch production approach.

7.4 Conclusions

In conclusion, for both primary and secondary carotenoids there is a large gap (> a factor 100) between the maximal theoretical carotenoid yields on light and those currently obtained outdoors. This gap is caused by suboptimal cultivation conditions, a suboptimal photobioreactor design and/or by a suboptimal cell metabolism. The carotenoid yields on light obtained outdoors are only slightly lower than those obtained indoors, where the cultivation conditions are controlled and optimized. Therefore, the potential to improve the cultivation conditions/operation mode and/or reactor design seems small. In contrast, because the differences between indoor and theoretical yields are rather high (~ a factor 60 – 100), the outdoor yield may still substantially be increased by optimizing the strain performance. In practice, strain optimization may lead to an increased carotenoid yield on light, with a maximal potential of a factor four to seven (**Table 7.2**). However, such large increases require rigorous metabolic changes, shifting the whole carbon flux from TAG or starch towards secondary carotenoids, which may prove very difficult to achieve (**Table 7.2**). Finally, it can be concluded

that true strain improvements require exploration of both targeted genetic engineering and random mutagenesis (with subsequent targeted selection), which requires further investigation of associated knowledge gaps such as regulation of carotenoid metabolism, development of robust nuclear transformation protocols and development of selection methods to select for mutants with a desired phenotype.

Table 7.2. Summary of examples to increase carotenoid yields on light. Maximal expected fold-increases of carotenoid yields on light are rough estimates which are not based on empirical studies. See text for details.

	1. Directed evolution in turbidostat	2. Carbon flow redirection during stress		3. Repeated batch
Process/strain optimization	Strain	Strain		Process
Carotenoid	Lutein	Astaxanthin	β -carotene	Astaxanthin
Maximal expected fold-increase of carotenoid yield on light	>2	6.7	4.5	1.1 – 1.3
Difficulty	Moderate	Very difficult		Moderate

Appendices

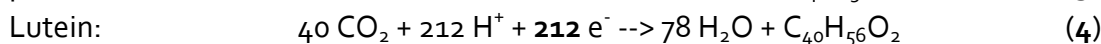
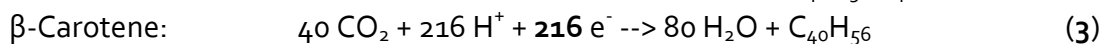
7.A Theoretical maximal carotenoid yields on light

The theoretical maximal carotenoid yield on (absorbed) light ($Y_{car,ph}$, $g_{car} \cdot mol_{ph}^{-1}$) can be calculated using **eq. 1**.

$$Y_{car,ph} = Y_{e^-,ph} \times Y_{car,e^-} \times M_{car} \quad (1)$$

with $Y_{e^-,ph}$ the electron yield on photons ($mol_{e^-} \cdot mol_{ph}^{-1}$), Y_{car,e^-} the carotenoid yield on electrons ($mol_{car} \cdot mol_{e^-}^{-1}$) and M_{car} the molecular weight of the carotenoid ($g_{car} \cdot mol_{car}^{-1}$).

In theory, 8 photons are required to generate 4 electrons. However, in practice, this seems to be at least 10 photons (Allen 2003; Bjorkman and Demmig 1987; Dubinsky et al. 1986; Evans 1987; Hogewoning et al. 2012; Kliphuis, Klok et al. 2012; Malkin and Fork 1996; Meyer zu Tittingdorf et al. 2004; Seelert et al. 2000; von Stockar et al. 1999; Tanada 1951; Vejrazka et al. 2013), so we used this ratio for our calculations. Besides, we assumed that the theoretical maximal carotenoid yield on light is proportional to the reduction state and the molecular mass of the carotenoids. Stoichiometry (**eq. 2 - 4**) shows that 204, 216 and 212 mol electrons are required to produce 1 mole of astaxanthin, β -carotene and lutein, respectively. These carotenoids have a molecular weight of 596.84 (astaxanthin), 536.87 (β -carotene) and 568.87 (lutein) $g_{car} \cdot mol_{car}^{-1}$. Thus, the maximal yield of these carotenoids on light are 1.17 (astaxanthin), 0.99 (β -carotene) and 1.07 (lutein) $g_{car} \cdot mol_{ph}^{-1}$.



Equations 5 and 6 show that 296 and 388 mol electrons are required to produce 1 mole of astaxanthin monoester and astaxanthin diester, respectively. These carotenoid esters have a molecular weight of 835.24 and 1073.65 $g_{car} \cdot mol_{car}^{-1}$, respectively. Thus, the maximal yield of these carotenoid esters on light are 1.13 (astaxanthin monoester), 1.11 (astaxanthin diester) $g_{car-est} \cdot mol_{ph}^{-1}$.

Yields on light of the carotenoid part only (excluding the fatty acid parts) is somewhat lower, because part of the energy is required to synthesise the fatty acids. The maximal yield on light of only the carotenoid part of the carotenoid esters is 0.81 (astaxanthin monoester) and 0.62 (astaxanthin diester) $g_{car} \cdot mol_{ph}^{-1}$.



Note that the ATP requirements for cellular maintenance and for biosynthesis of the carotenoids are neglected in these calculations.

7.B A How a turbidostat leads to selection of mutants with a maximal pigment yield on light

In a turbidostat, the light intensities entering and leaving the culture are set, and thereby the light absorption rate of the culture is fixed. This also implies that the amount of light absorbing material (i.e. pigments) in the culture is constant. To maintain such a constant light absorption,

the culture is automatically diluted with fresh medium when pigments are produced. Thus, in a turbidostat the volumetric pigment production rate dictates the dilution rate.

Because the pigment concentration in a turbidostat is constant, the volumetric pigment production rate is proportional to the pigment-specific pigment production rate (i.e. amount of pigment produced per amount of pigment present in the reactor, per unit of time). This latter rate, and ultimately thus also the dilution rate of the turbidostat, is dependent on the specific growth rate of the cells and on the pigment accumulation rate by the cells. Because pigment accumulation, as a result of mutations, is a temporary process (at some point the cells will stop accumulating more pigments), this will only have a transient effect on the dilution rate. Hence, it is only the specific growth rate of the cells that eventually dictates the dilution rate of the turbidostat. This implies that slowly dividing cells will be washed out of the culture, and thus the turbidostat automatically selects for cells with a high specific growth rate.

The specific growth rate of the algae (μ , $\text{mol}_x \text{mol}_x^{-1} \text{s}^{-1}$) is dependent on the biomass-specific photosynthate (e.g. NADPH and ATP) production rate (q_{ps} , $\text{mol}_{\text{photosynthate}} \text{mol}_x^{-1} \text{s}^{-1}$), the biomass-specific consumption rate of photosynthate for cellular maintenance (m_{ps} , $\text{mol}_{\text{photosynthate}} \text{mol}_x^{-1} \text{s}^{-1}$) and the biomass yield on photosynthate ($Y_{x,ps}$, $\text{mol}_x \text{mol}_{\text{photosynthate}}^{-1}$), analogous to Pirt's model (Pirt 1965):

$$\mu = (q_{ps} - m_{ps}) \cdot Y_{x,ps} \quad (7)$$

Thus, when a mutant has a higher efficiency of photosynthesis (q_{ps} increases) or a lower maintenance energy requirement (m_{ps} decreases), its specific growth rate will increase. Also, when a mutant makes less of an energy-dense biomass constituent without affecting the efficiency of photosynthesis, for instance by reducing the amount of excessive proteins, (q_{ps} stays equal and $Y_{x,ps}$ goes up), the specific growth rate will increase.

Mutations that positively influence the specific growth rate of the algae will be selected for in the turbidostat. Ultimately, a steady state will be obtained for which there is no increase of the specific growth rate possible anymore. At this maximal dilution rate the pigment yield on light is maximal because the volumetric light absorption rate and pigment concentration in the reactor are constant in a turbidostat. Moreover, in those cases where the mutation causing the higher pigment yield on light was related to a reduction of excessive biomass components, the pigment content will simultaneously be elevated.

In conclusion, with this method genetically modified microalgae are generated, through random mutagenesis, and when one, or multiple, of such modifications lead to a strain with an enhanced pigment yield on light and specific growth rate, this mutant is automatically selected.

It is difficult to predict to what extent the carotenoid yield on light can be improved with this method as natural evolution also partly selects for high specific growth rates. Not much is known with respect to the maintenance requirements of different microalgae (Kliphuis, Klok et al. 2012) and it may be expected that not much improvement is possible because natural evolution will already have put a severe downward pressure on this maintenance energy requirement. For the efficiency of photosynthesis, a larger improvement can be expected in case a wild type microalgae is chosen that is adapted to a light regime that is very different

from the one applied in the turbidostat. For instance, a microalga that has evolved in environments with low light intensities is likely to have a much lower efficiency of photosynthesis when cultivated under high light intensity than a microalga such as *Chlorella sorokiniana*, which naturally thrives at high light intensity (Morita et al. 2000). Random mutagenesis may accelerate the laboratory evolution of the naturally low-light-suited wild type into a high-light-suited strain.

Compared to the above, mutations that affect the yield of biomass on photosynthate through reduced production of excessive biomass components are more likely to result in a substantial enhancement of the carotenoid yield. Proteins, carbohydrates (e.g. starch) and membrane lipids are generally present in concentrations of 49, 25 and 12 % of dry weight (w/w) (Sánchez et al. 2008a) and it is likely that part of these components are present in excess. Specifically for microalgae that have evolved in environments with frequent scarcity of resources, excessive accumulation of storage components may occur.

7.C Weight-specific absorption spectra of chlorophyll a, chlorophyll c, fucoxanthin and diadinoxanthin

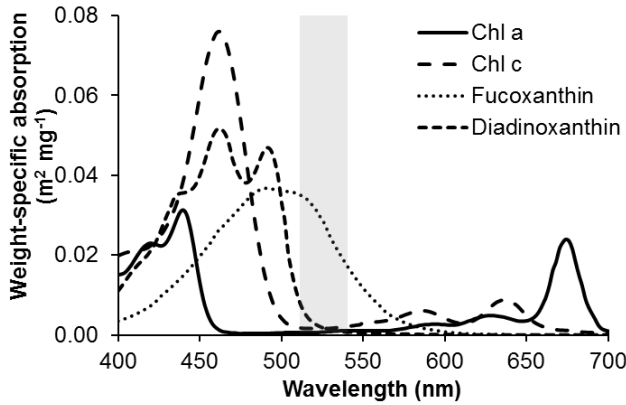


Fig. A.7.1 Weight-specific absorption spectra of chlorophyll a, chlorophyll c, fucoxanthin and diadinoxanthin, the main primary pigments of brown algae and diatoms. **Shaded area** indicates wavelengths that are only absorbed by fucoxanthin. Adapted from Bricaud et al. (2004). Spectra were obtained as described by Bricaud et al. (2004).

7.D. The fold-increase in carotenoid yield on light between a two-step batch cultivation and a repeated batch cultivation

Two-step batch

In a two-step batch, the total areal amount of photons (Ph^b , $\text{mol}_{\text{ph}} \cdot \text{m}^{-2}$) required to produce a certain amount of carotenoids during a certain stress time (t_s , days) equals the areal amount of photons required to produce the required biomass plus the areal amount of photons required for the actual carotenoid production (eq. 8).

$$Ph^b = \frac{C_{x0}^u \cdot d_g}{Y_{x,Ph}} + \frac{r_{car}^u \cdot d_s \cdot t_s}{Y_{car,Ph}} \quad (8)$$

With C_{x0}^u the biomass concentration at the start of stress phase ($\text{g}_x \cdot \text{m}^{-3}$), d_g the culture depth during the growth phase, $Y_{x,Ph}$ the biomass yield on photons during growth ($\text{g}_x \cdot \text{mol}_{\text{ph}}^{-1}$), r_{car}^u the

volumetric carotenoid productivity during stress ($g_{car} \cdot m^{-3} \cdot day^{-1}$), d_s the culture depth during the stress phase (m) and $Y_{car.Ph}$ the carotenoid yield on photons during stress ($g_{car} \cdot mol_{ph}^{-1}$). In this equation it is assumed that yields on light (and the volumetric carotenoid productivity) are constant and that there is no downtime.

The overall carotenoid yield on light equals the areal amount of carotenoids produced during t_s ($g_{car} \cdot m^{-2}$) divided by the total areal amount of photons required, during t_s , to produce these carotenoids ($mol_{ph} \cdot m^{-2}$) (**eq. 9**).

$$Y_{car.Ph}^b = \frac{r_{car}^u \cdot d_s \cdot t_s}{Ph^b} \quad (9)$$

Thus, in a classical two step batch, the overall carotenoid yield on light is given by **eq. 10**.

$$Y_{car.Ph}^b = \frac{\frac{r_{car}^u \cdot d_s \cdot t_s}{\frac{C_{x0}^u \cdot d_g}{Y_{x.Ph}} + \frac{r_{car}^u \cdot d_s \cdot t_s}{Y_{car.Ph}}}}{\frac{r_{car}^u \cdot t_s}{\frac{C_{x0}^u}{Y_{x.Ph}} + \frac{r_{car}^u \cdot t_s}{Y_{car.Ph}}}} \quad (10)$$

In the simplification (right part) of eq. 10 it is assumed that the d_g equals d_s .

Repeated batch

During a repeated batch, the total areal amount of photons (Ph^{rb} , $mol_{ph} \cdot m^{-2}$) required to produce a certain amount of carotenoids equals the areal amount of photons required to produce the starting biomass, plus the areal amount of photons required for the actual carotenoid production, plus the areal amount of photons used (or lost) during recovery. At the end of the repeated batch, the reactor is fully harvested. This will occur after the carotenoid production phase, thus prior to recovery. Thus, the total areal amount of photons required to produce a certain amount of carotenoids in a repeated batch is described by **eq. 11**.

$$Ph^{rb} = \frac{C_{x0}^u \cdot d_g}{Y_{x.Ph}} + n \cdot \left(\frac{r_{car}^u \cdot d_s \cdot t_s}{Y_{car.Ph}} \right) + (n - 1) \cdot (I_r \cdot t_r) \quad (11)$$

With n the repeated number of batches that is performed in a row, I_r the incident light intensity during recovery ($mol_{ph} \cdot m^{-2} \cdot day^{-1}$) and t_r the recovery time (days). Note that the amount of recovery periods that occur equal the repeated number of batches minus one ($n-1$).

As discussed in the main text, in principle the recovery may occur in the dark in which case I_r equals 0. Alternatively, the recovery occurs in the illuminated reactor itself, in which case I_r equals I (the incident light intensity during growth/stress, $mol_{ph} \cdot m^{-2} \cdot day^{-1}$).

The overall carotenoid yield on light equals the areal amount of carotenoids produced during the repeated batch ($g_{car} \cdot m^{-2}$) divided by the total areal amount of photons required during the repeated batch ($mol_{ph} \cdot m^{-2}$). When no carotenoids are degraded during the recovery processes, as assumed in the main text, the overall carotenoid yield on light in a repeated batch is given by **eq. 12**. The other extreme would be that all carotenoids would be degraded during recovery, in which case the overall carotenoid yield on light in a repeated batch is given by **eq. 13**.

$$Y_{car.Ph}^{rb} = \frac{n \cdot r_{car}^u \cdot d_s \cdot t_s}{P_h^{rb}} \quad (12)$$

$$Y_{car.Ph}^{rb} = \frac{((n-1) \cdot f_H + 1) \cdot r_{car}^u \cdot d_s \cdot t_s}{P_h^{rb}} \quad (13)$$

With f_H the fraction of carotenoids that is harvested after a stress period. This fraction is harvested for $(n-1)$ stress periods. After the last stress period the reactor is fully harvested. Below, only the case of no carotenoid degradation (eq. 11) has been worked out.

When there is not carotenoid degradation, the overall carotenoid yield on light, during a repeated batch, is given by **eq. 14**.

$$Y_{car.Ph}^{rb} = \frac{n \cdot r_{car}^u \cdot d_s \cdot t_s}{\frac{C_{x0}^u \cdot d_s}{Y_{x.Ph}} + n \cdot \left(\frac{r_{car}^u \cdot d_s \cdot t_s}{Y_{car.Ph}} \right) + (n-1) \cdot (I_r \cdot t_r)} \quad (14)$$

Comparison

The fold-increase (f) in carotenoid yield on light between a repeated batch cultivation and a two-step batch cultivation equals the ratio between the overall carotenoid yields that can be obtained with the two respective cultivation approaches. When no carotenoids are degraded during recovery, this fold-increase is given by **eq. 15**.

$$f = \frac{Y_{cp}^{rb}}{Y_{cp}^b} = \frac{\frac{n \cdot r_{car}^u \cdot d_s \cdot t_s}{\frac{C_{x0}^u \cdot d_s}{Y_{x.Ph}} + n \cdot \left(\frac{r_{car}^u \cdot d_s \cdot t_s}{Y_{car.Ph}} \right) + (n-1) \cdot (I_r \cdot t_r)}}{\frac{r_{car}^u \cdot t_s}{\frac{C_{x0}^u}{Y_{x.Ph}} + \frac{r_{car}^u \cdot t_s}{Y_{car.Ph}}}} = \frac{n \cdot d_s \cdot \left(\frac{C_{x0}^u}{Y_{x.Ph}} + \frac{r_{car}^u \cdot t_s}{Y_{car.Ph}} \right)}{\frac{C_{x0}^u \cdot d_s}{Y_{x.Ph}} + n \cdot \left(\frac{r_{car}^u \cdot d_s \cdot t_s}{Y_{car.Ph}} \right) + (n-1) \cdot (I_r \cdot t_r)} \quad (15)$$

The volumetric productivity is given by **eq. 16**.

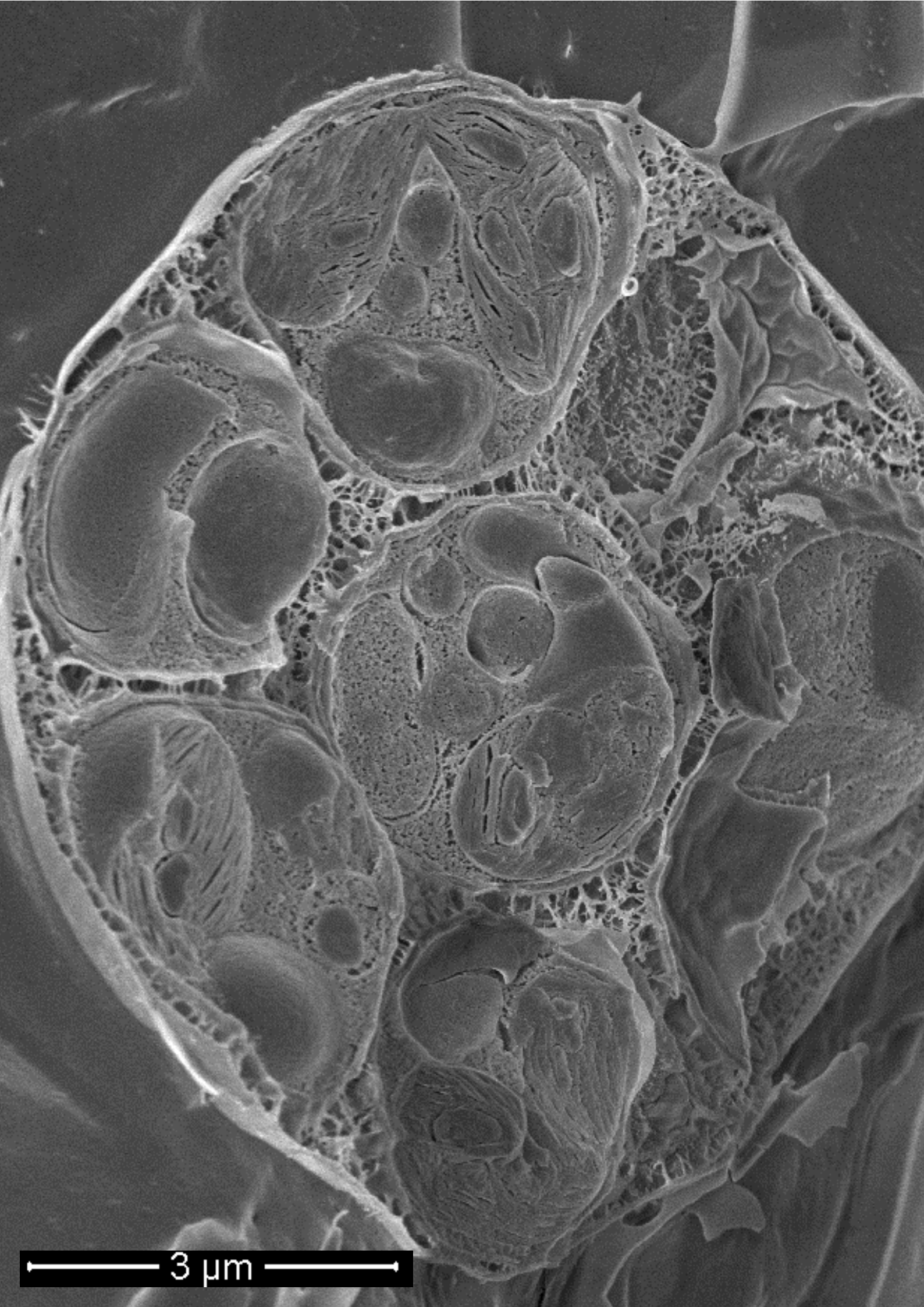
$$r_{car}^u = \frac{I \cdot Y_{car.Ph}}{d_s} \quad (16)$$

Combining eq. 15 and 16 gives **eq. 17**.

$$f = \frac{n \cdot \left(\frac{C_{x0}^u \cdot d_s}{Y_{x.Ph}} + I \cdot t_s \right)}{\frac{C_{x0}^u \cdot d_s}{Y_{x.Ph}} + n \cdot (I \cdot t_s) + (n-1) \cdot (I_r \cdot t_r)} \quad (17)$$

Assumptions:

- During stress (N-depletion), only secondary (nitrogen-free) biomass is formed (e.g. TAG, starch and secondary carotenoids).
- Carotenoid yield on light is constant.
- Biomass yield on light is constant.
- Downtimes of batch and repeated batch do not affect f .
- Culture depths during growth and stress are equal.
- No breakdown of carotenoids during recovery



— 3 μm —

References

◀ IN THE PICTURE

Electron micrograph of reproducing *Chromochloris zofingiensis* cells. The seven visible daughter cells are stunningly efficiently arranged. Comparable to the seeds in a sunflower! Each cell contains one big chloroplast (filling approximately two third of the cell!). Within the chloroplast starch granules are present. Starch is consumed during the night, when no light energy can be captured.

◀ IN BEELD

Electronenmicroscopiefoto van reproducerende *Chromochloris zofingiensis* cellen, De zeven zichtbare dochtercellen zijn verbazingwekkend efficiënt gerangschikt. Vergelijkbaar met de zaden in een zonnebloem! Iedere cel bevat één chloroplast, die zo ongeveer tweederde van de cel in beslag neemt. De zetmeelkorrels in de chloroplast vormen een energievoorraadje dat 's nachts aangesproken wordt.

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Summary

◀ IN THE PICTURE

Green *Chromochloris zofingiensis* cells turn orange when they receive too much light. The orange (and red) pigments form a protecting sun screen. Comparable to the tanning process in the human skin! The green cells (in suspension) are only for short moments fully illuminated and thus stay green, whereas the orange cells (stuck to the glass plate) receive a high amount of light all the time.

◀ IN BEELD

Groene *Chromochloris zofingiensis* cellen worden oranje wanneer ze te veel zonlicht ontvangen. De oranje (en rode) pigmenten vormen een beschermend zonneschermb. Vergelijkbaar met het bruiningsproces van onze huid! De groene cellen (in de vloeistof) worden telkens maar even belicht en blijven daardoor groen. De oranje cellen (aan de glasplaat geplakt) ontvangen continu veel licht.

Microalgae are a biotechnologically interesting and sustainable source of a wide variety of resources, including high-value compounds such as pigments, long-chain polyunsaturated fatty acids, vitamin E and C, and bulk compounds such as starch, proteins and lipids. Microalgae can be grown year round on non-arable land, using only sunlight, sea or waste water and a few added nutrients. Besides, the projected productivities that can be obtained with microalgae are much higher than those obtained with higher plants.

A good example of high-value microalgal products are pigments. Microalgae possess a large variety of pigments, including chlorophylls (green), carotenoids (red, orange and yellow) and phycobiliproteins (red and blue). Part of these pigments can be produced by microalgae in concentrations exceeding those found in higher plants by one or more orders of magnitude. Moreover, in contrast to higher plants, very high areal productivities can be achieved with microalgae.

Despite the advantages of using microalgae for pigment production, and despite the potential to produce a large variety of pigments, microalgae are currently exploited commercially for only three pigments, namely β -carotene (*Dunaliella salina*), astaxanthin (*Haematococcus pluvialis*) and phycocyanin (*Arthrospira platensis*). The carotenoids β -carotene and astaxanthin are claimed to have beneficial health effects, due to their anti-oxidative activity, which makes them particularly valuable for application in nutraceuticals. To increase their market volumes (i.e. by expanding exploitation of the food and feed markets), their production costs need to be reduced. In addition, to make microalgae-based pigment production economically feasible for other pigments than the afore mentioned ones (e.g. fucoxanthin, lycopene or lutein) the production costs of also these pigments need to be reduced.

The pigment production costs can be decreased through, among others, enhancement of the areal pigment productivity. In a microalgae-based pigment production plant, the solar insolation on a certain area is a given, and thus the areal pigment productivity is directly proportional to the pigment yield on light. Hence, this yield on light is an important optimization target in microalgal pigment production.

To obtain maximal pigment yields on light, insight into optimal cultivation conditions, as well as into the pigment metabolic pathways and their regulation is indispensable. Therefore, the aim of this thesis was to obtain increased insight in the pigment metabolism of phototrophic microalgae under various process conditions, with the main focus on secondary carotenoids, to facilitate optimization of phototrophic microalgae-based pigment production.

To achieve this aim, first the biological constraints and opportunities of microalgal carotenoid production were assessed, based on existing knowledge. Subsequently, the carotenoid metabolism of the haptophyte *Isochrysis* aff. *galbana* T-ISO was studied, after which the focus was shifted to the carotenogenic chlorophyte *Chromochloris* (*Chlorella*) *zofingiensis*. For this latter species the carotenoid metabolism was studied in depth, optimal cultivation strategies for carotenoid production were determined and the dynamics and exploitability of its recovery from nitrogen starvation were explored. Finally, this thesis concludes with an outlook on several strain and process improvements to achieve higher carotenoid yields on light.

To enable optimization of an economically feasible microalgae-based pigment production process, an adequate understanding of the biological constraints and opportunities is

important. In **Chapter 2**, these biological constraints and opportunities were identified and an overview of the relevant underlying biological information that was known at the start of this project (in 2010) was provided, including the distribution of pigments across the most common microalgal groups, the roles of pigments in microalgae and their biosynthetic pathways.

Besides, it was discussed that microalgal pigments can be categorized into two groups: primary and secondary pigments. Overproduction of primary pigments requires modification of the pigment biosynthesis routes. Some secondary pigments can be overproduced by optimization of the cultivation conditions (usually referred to as 'stress' conditions), while others require also modification of the pigment biosynthesis routes (e.g. by the use of inhibitors or genetic modification). Moreover, pigment overproduction requires most likely the presence of storage space outside the photosystems. Such storage space is in a selected group of microalgae naturally provided in the form of triacylglycerol (TAG) oil bodies.

Chapter 2 concludes with the most suitable approaches for the production of each colour that can be obtained from microalgae (red, red-orange, orange, yellow-orange, yellow, green and blue).

An effective stress condition that can induce secondary carotenoid overproduction is a nutrient depletion. In **Chapter 3**, the microalga *I. aff. galbana* T-ISO was subjected to three different nutrient depletions (nitrogen, sulphur or magnesium) and growth and pigment accumulation in the cultures was studied. Each nutrient depletion led to production of the secondary carotenoids echinenone and 3-hydroxyechinenone (both precursors of astaxanthin in *H. pluvialis*). Although the nutrient depletions must have led to accumulation of TAG oil bodies (shown in previous literature), secondary carotenoids were produced in only very low amounts. Moreover, although all enzymes were active to produce astaxanthin, this carotenoid was not detected.

Because *I. aff. galbana* T-ISO was not able to overproduce secondary carotenoids, the focus was thereafter shifted to the carotenogenic chlorophyte *C. zofingiensis*. This species may be a potential alternative for mass production of astaxanthin, thereby replacing *H. pluvialis*, because it is claimed to have a higher specific growth rate and higher biomass and TAG yield on light, and a lower sensitivity to contamination in unfavourable environments.

However, one of the disadvantages of *C. zofingiensis* is that the maximally observed secondary carotenoid content is relatively low (0.6 % DW (w/w), compared to 4 % DW (w/w) observed for *H. pluvialis*). Increasing this content likely requires genetic modifications. Targeted genetic modifications require insight into the algal metabolism and in particular into the carotenoid metabolism, which can be obtained by performing enzyme inhibitor studies.

Therefore, in **Chapter 4**, *C. zofingiensis* was depleted of nitrogen to induce secondary carotenogenesis after which diphenylamine (DPA) was added, an inhibitor of secondary ketocarotenoid biosynthesis. Subsequently, pigment production and TAG oil body formation were studied. To ensure that the entire collection of carotenoids of *C. zofingiensis* would be revealed, carotenoids were identified using reversed phase liquid chromatography and additional mass spectrometry.

Nitrogen-depleted *C. zofingiensis* (without DPA addition) accumulated astaxanthin, ketolutein, canthaxanthin, adonixanthin and β -carotene, which were synthesised at least partly *de novo*. Astaxanthin was accumulated primarily as diester, ketolutein mainly as monoester and in the free form. Both carotenoids were esterified mainly with C16:0 and C18:1 fatty acids, which were also found most abundantly in TAG oil bodies. TAG oil bodies were found exclusively in the cytosol, not in the chloroplast. These results led to the speculation that also the secondary carotenoids accumulated in the cytosol.

Nitrogen-depleted *C. zofingiensis* treated with DPA showed a decreased overall production of β -carotene derivatives (sum of astaxanthin, canthaxanthin, echinenone and adonixanthin); however, the production of ketolutein and degradation of primary carotenoids were not modified. In addition, DPA increased production of the individual carotenoids adonixanthin and echinenone. In contrast, no elevated production of zeaxanthin was found (zeaxanthin was not at all detected). These results led to the suggestion that a regulatory mechanism must have been present which prevented increased production of ketolutein and primary carotenoids. Besides, it strongly indicated that the hypothesised conversion of zeaxanthin into adonixanthin (the latter is a precursor of astaxanthin) does most likely not occur in this species.

An important aspect of the cultivation strategy that affects the secondary carotenoid and TAG yield on light may be the biomass density at the start of the nitrogen starvation (and secondary carotenoid production) phase. The hypothesis for this phenomenon is that under nitrogen starvation conditions an optimum biomass-specific light absorption rate exists, resulting in maximal secondary carotenoid and TAG yields on light. At higher biomass-specific light absorption rates the yield would decrease because the excess light would be dissipated to heat or lost through photoinhibition. At lower biomass-specific light absorption rates the yield would decrease because a relatively large share of the absorbed light would be used to satisfy the maintenance requirements of the cells.

Therefore, in **Chapter 5** the effect of varying biomass densities at the start of the secondary carotenoid production phase were studied, by starving *C. zofingiensis* of nitrogen at different biomass concentrations.

It was found that, in the studied range, TAG and secondary carotenoids were produced with the same yield on absorbed light with each initial biomass density, which indicated that the biomass-specific photon absorption rate did not affect the amounts of energy used for TAG and secondary carotenoid production.

Consequently, the culture with the lowest biomass concentration at the moment of nitrogen depletion obtained the highest TAG and secondary carotenoid yield on light when also the biomass production phase was taken into account, and this lowest biomass culture reached also the highest TAG and secondary carotenoid contents (335 and 4.5 mg/g, respectively).

Besides, it was found that the maximal TAG yield on light obtained with *C. zofingiensis* ($320 \text{ mg mol}_{\text{ph}}^{-1}$) was higher than values reported before for microalgae, which makes this alga a promising TAG production strain and also a potentially promising secondary carotenoid production strain (i.e. after application of genetic engineering strategies (which was further discussed in **Chapter 7**)). The current secondary carotenoid yield on light was reported to be

maximally $2.75 \text{ mg mol}_{\text{ph}}^{-1}$ in *C. zofingiensis*, which is about 7 times lower than the total carotenoid yield on light found in *H. pluvialis*.

Finally, it was concluded that, although secondary carotenoid and TAG accumulation are induced by the same stimuli, a causal relationship between the accumulation of secondary carotenoid and TAG does not necessarily exist and that secondary carotenoid production is most likely highly regulated and/or that the carotenogenic enzymes have a limited capacity.

Another important aspect of the cultivation strategy that might affect the pigment yield on light is the mode of operation. It has been suggested in literature that the areal productivity of secondary carotenoids and TAG can be increased by applying a repeated batch cultivation instead of a series of single batch cultivations (which traditionally is applied). Whereas in a classical batch cultivation the reactor is fully harvested at the end of the secondary carotenoid and TAG production phase, in a repeated batch, only part of the reactor is harvested while the remaining part of the secondary carotenoid and TAG-rich biomass is recovered and reused. For example by resupplying nitrogen to nitrogen-depleted cells. At some point the nitrogen will be depleted again after which the cells will enter a second stress phase, resuming secondary carotenoid accumulation.

Whether a repeated batch leads indeed to a higher areal productivity than a classical batch depends, among others, on the recovery rate of the culture and the dynamics of TAG and secondary carotenoids during culture recovery.

Information on these matters was not available in literature and therefore **Chapter 6** explored the dynamics and exploitability of *C. zofingiensis*' recovery from nitrogen starvation. To do that, the nitrogen-depleted *C. zofingiensis* cultures described in **Chapter 5** were (after a nitrogen starvation period of two weeks) replenished with nitrate after which the physiology and rate of culture recovery were studied.

Nitrogen-starved *C. zofingiensis* cultures (rich in secondary carotenoids and TAG) recovered within two days after nitrogen was resupplied. During these two days, TAG and starch were rapidly degraded, whereas astaxanthin, the main secondary carotenoid, was degraded only from the point that the cells were fully recovered. It is suggested that, due to the rapid recovery of *C. zofingiensis* and the delay in astaxanthin degradation, a repeated batch cultivation may indeed result in a higher secondary carotenoid productivity than a series of classical single batch cultivations.

Finally, in **Chapter 7**, the maximal potential for improvement of the carotenoid yield on light was discussed for astaxanthin, β -carotene and lutein, which are the commercially most relevant microalgal carotenoids (i.e. highest market values and volumes). This was done by assessing the theoretical maximal carotenoid yields on light and comparing these with the currently obtained yields outdoors and indoors in the laboratory.

It was found that for both primary and secondary carotenoids there is a large gap (> a factor 100) between the maximal theoretical carotenoid yields on light and those currently obtained outdoors. This gap is caused by suboptimal cultivation conditions/operation mode, a suboptimal photobioreactor design and/or by a suboptimal cell metabolism. Because the carotenoid yields on light obtained outdoors are only slightly lower than those obtained

indoors, where the cultivation conditions are controlled and optimized, it was concluded that the potential to improve the cultivation conditions/operation mode and/or reactor design seems small. In contrast, because the differences between indoor and theoretical yields are rather high (~ a factor 60 – 100), the outdoor yield may still substantially be increased by optimizing the strain performance.

The primary carotenoid yield on light can be optimized, among others, by redirecting the carbon flow from excessively produced primary cell compounds (e.g. excessive proteins, membrane lipids or starch) towards primary carotenoids, whereas the secondary carotenoid yield on light can be optimized, among others, by redirecting the carbon flow from excessively produced secondary cell compounds (e.g. triacylglycerol or starch) towards secondary carotenoids.

It is expected that the primary carotenoid yield on light may be increased by at least a factor 2, by exploiting directed evolution in a turbidostat, whereas the secondary carotenoid yield on light may be increased by a factor four to seven, by redirecting the carbon flux from TAG or starch towards secondary carotenoids. However, it was argued that the latter case requires rigorous metabolic changes which may prove very difficult to achieve.

Furthermore, it was concluded that true strain improvements require exploration of both targeted genetic engineering and random mutagenesis (with subsequent targeted selection). Targeted genetic engineering requires further investigation of the microalgal physiology and, more specifically, of the carotenoid metabolism, and besides, development of robust nuclear transformation protocols. Random mutagenesis, on the other hand, requires development of selection methods to select for mutants with a desired phenotype.

Finally, the exploitability of a repeated batch, instead of a series of classical single batch cultivations, of which the possibility was introduced in **Chapter 6**, was examined more thoroughly by the use of a simple model. It was concluded that a repeated batch may indeed result into a higher secondary carotenoid yield on light than the classical single batch cultivation when (i) the recovery phase in a repeated batch process requires less light energy than the biomass production phase in a classical two-step batch process, and (ii) the carotenoid yield on light during the stress phases are comparable in both processes (i.e. no carotenoid degradation during recovery).

All these chapters together have resulted into increased insights in the cultivation strategies to maximize the pigment yield on light, as well as into the pigment metabolic pathways and their regulation. Most importantly, the most likely biosynthesis route to ketolutein and astaxanthin were ascertained in *C. zoofingiensis* and it was found that inhibition of secondary carotenoids does not lead to increased production of primary carotenoids in this species. These findings are particularly relevant for the development of metabolic engineering strategies with the aim to increase the astaxanthin, ketolutein or any primary carotenoid yield on light (**Chapter 4**). Another important finding was that this species has a broad optimum in secondary carotenoid yield on light with respect to the biomass concentration at the moment of nitrogen depletion. This implies that when this strain is cultivated outdoors, a relatively high biomass concentration can be used without a loss in carotenoid yield on light (**Chapter 5**). Finally, it was found that astaxanthin, the main secondary carotenoid in this species, was hardly

degraded upon nitrogen resupply, which indicates that the overall carotenoid yield on light as well as its content may possibly be improved by applying a repeated batch instead of a series of single batch cultivations (**Chapter 6**). Thus, the new insights obtained will facilitate optimization of phototrophic microalgae-based pigment production (discussed in **Chapter 7**).



Samenvatting

◀ IN THE PICTURE

To determine very precisely the dry weight of a liter with *Chromochloris zofingiensis* cells, the algae suspension is filtered, oven-dried and subsequently weighted on a highly accurate scale. One filter contains about two and a half milligrams of microalgae. When the cells turn orange, they become more and more heavy. This is because the cells not only accumulate pigments, but also lipids.

◀ IN BEELD

Om heel precies te kunnen bepalen wat het droge gewicht is van een liter met *Chromochloris zofingiensis*, wordt een oplossing met algen over een filter gegoten, gedroogd en daarna gewogen op een zeer nauwkeurige weegschaal. Op één filter zit ± tweeënhalve milligram aan algen. Als de algen oranje kleuren worden ze steeds zwaarder, doordat ze niet alleen pigment, maar ook olie ophopen.

Pigmentproductie op licht met microalgen

Microalgen (eencellige planten) zijn een duurzame en biotechnologisch interessante bron van een scala aan producten, waaronder hoogwaardige componenten zoals kleurstoffen (pigmenten), meervoudig onverzadigde vetzuren, vitamine E en C, en laagwaardige massaproducten zoals zetmeel, eiwitten en vetten. Microalgen kunnen het hele jaar door gekweekt worden, zonder gebruik te maken van vruchtbare landbouwgrond. Om te groeien hebben ze zonlicht nodig als energiebron (ze zijn 'fototroop') en daarnaast slechts zee- of afvalwater en enkele nutriënten. De verwachte landopbrengst (opbrengst per vierkante meter) van microalgen is dan ook vele malen hoger dan die van traditionele gewassen.

Pigmenten vormen een goed voorbeeld van hoogwaardige producten die met behulp van microalgen gemaakt kunnen worden. Microalgen bezitten een grote verscheidenheid aan pigmenten, waaronder chlorofyl (groen), carotenoiden (rood, oranje en geel) en phycobili-eiwitten (rood en blauw). Microalgen kunnen een deel van deze pigmenten maken in concentraties die ver uitsteken boven de concentraties die gevonden worden in hogere planten zoals tomaten en wortels. Bovendien kunnen met microalgen veel hogere pigment-opbrengsten per vierkante meter gehaald worden.

Ondanks het feit dat microalgen zo goed zijn in het produceren van een grote verscheidenheid aan pigmenten zijn er momenteel slechts drie door microalgen geproduceerde pigmenten op de markt: β -caroteen (oranje, uit de groenalg *Dunaliella salina*), astaxanthine (rood, uit de groenalg *Haematococcus pluvialis*) en phycocyanine (blauw, uit de blauwalg *Arthrospira platensis*). Van de carotenoiden β -caroteen en astaxanthine wordt geclaimd dat ze positieve effecten hebben op de gezondheid, omdat ze kunnen fungeren als antioxidant, wat hen bijzonder waardevol maakt als bestanddeel van voedingssupplementen. Echter, om hun marktvolumes en die van andere pigmenten (zoals fucoxanthine, lycopene of luteïne) te vergroten (o.a. door hun gebruik in de levensmiddelenindustrie uit te breiden), moeten de productiekosten omlaag.

Microalgen gebruiken zonlicht niet alleen als energiebron om te groeien, maar ook om pigmenten te maken. De hoeveelheid zonlicht die buiten op een bepaald stuk landoppervlak valt ligt vast en daarmee ligt dus ook de maximale hoeveelheid zonlicht vast die, per vierkante meter, door microalgen geabsorbeerd kan worden en vervolgens kan worden omgezet in pigment(en). Hoe meer geabsorbeerd licht omgezet wordt in pigment (ofwel: hoe hoger het 'rendement'), des te hoger de productiesnelheid van pigmenten per vierkante meter landoppervlak, en dus lager de pigmentproductiekosten per vierkante meter landoppervlak. Dit rendement wordt ook wel de 'opbrengst van pigment op licht' genoemd, wat dus een belangrijk doelwit is om de productiekosten van pigmenten omlaag te brengen.

Om maximale opbrengsten van pigment op licht te krijgen, is inzicht nodig in zowel de optimale kweekcondities waaronder pigmenten gemaakt worden, als de stofwisselingsroutes die leiden tot pigmentproductie (het pigmentmetabolisme) en hun regulatie. Daarom was het doel van dit proefschrift om meer inzicht te krijgen in het pigmentmetabolisme van microalgen, met de focus op secundaire carotenoiden, om uiteindelijk het proces van fototrope pigmentproductie met microalgen te kunnen optimaliseren.

Om dit doel te bereiken zijn eerst de biologische beperkingen en mogelijkheden van pigmentproductie met microalgen op basis van bestaande kennis op een rij gezet. Vervolgens is het carotenoïdemetabolisme van de microalg *Isochrysis* aff. *galbana* T-ISO (bruinalgen) bestudeerd, waarna de focus werd verschoven naar de microalg *Chromochloris* (*Chlorella*) *zofingiensis* (groenalgen). Voor deze laatste microalg is het carotenoïdemetabolisme in detail bestudeerd, zijn optimale kweek strategieën voor de productie van carotenoïden bepaald en is het effect van stikstof toevoeging, na een lange periode van stikstoftekort, op de groei en pigmentproductie onderzocht. Tenslotte wordt dit proefschrift afgesloten met een vooruitblik om met microalgen, door middel van zowel proces- als soortverbeteringen, maximale pigmentopbrengsten op licht te behalen, gebaseerd op de kennis opgedaan in de experimentele hoofdstukken van dit proefschrift.

Om een pigmentproductieproces gebaseerd op microalgen economisch haalbaar te kunnen maken, of te kunnen optimaliseren, is een goed begrip van de biologische beperkingen en mogelijkheden van pigmentproductie met microalgen erg belangrijk. In **Hoofdstuk 2** zijn deze biologische beperkingen en mogelijkheden op een rij gezet en wordt een overzicht van de achterliggende biologische informatie gegeven dat aan de start van dit project (in 2010) bekend was. Hieronder valt bijvoorbeeld de pigmentsamenstelling van de meest voorkomende groepen microalgen, de rol van pigmenten in microalgen en de opbouw van pigmenten (de biosyntheseroutes) in microalgen.

Daarnaast wordt in **Hoofdstuk 2** besproken dat de pigmenten in microalgen ingedeeld kunnen worden in twee groepen: primaire en secundaire pigmenten. Primaire pigmenten bevinden zich in en om het fotosysteem, in de chloroplast (bladgroenkorrel). De meeste primaire pigmenten (zoals chlorofyl *a* en *b* en de carotenoïden luteïne en fucoxanthine) absorberen lichtenergie dat in het fotosysteem omgezet wordt in chemische energie, wat vervolgens gebruikt kan worden om nieuwe microalgen mee te vormen. Omdat deze nakomelingen ook primaire pigmenten bevatten, worden er dus zodoende primaire pigmenten bijgemaakt. De concentratie van primaire pigmenten in algen is echter veelal laag (lager dan 0.5 % van het drooggewicht), wat de commercialisatie bemoeilijkt. Om deze concentratie in sterke mate te verhogen is het noodzakelijk om aanpassingen te maken in de biosyntheseroutes van primaire pigmenten.

Secundaire pigmenten worden gevormd wanneer de kweekcondities suboptimaal zijn voor de voortplanting (deze condities worden meestal aangeduid met 'stress'). Bijvoorbeeld wanneer er een overschot is aan energie, in de vorm van licht, of een tekort aan essentiële nutriënten zoals stikstof, zwavel of magnesium. In beide gevallen ontstaat er een energie-onbalans waarbij meer energie de cel binnenkomt dan gebruikt kan worden voor de voortplanting. Deze onbalans leidt tot de vorming van zuurstofradicalen die met alles wat zij in de cel tegen komen kunnen reageren. Deze reacties leiden tot schade aan de cel. Om deze schade te beperken worden in sommige microalgen secundaire pigmenten opgehoopt. De lichtenergie geabsorbeerd door secundaire pigmenten wordt geheel omgezet in warmte. Omdat hierbij dus geen chemische energie ontstaat, wordt een deel van de energie-onbalans teniet gedaan en worden er minder schadelijke moleculen gevormd, wat leidt tot verminderde schade aan de cel. Kortom, het is een overlevingsstrategie van de alg!

Terwijl sommige secundaire pigmenten in extreem hoge concentraties opgehoopt kunnen worden (tot wel 10 % van het drooggewicht) door enkel het toepassen van stresscondities, vereisen andere, net als primaire pigmenten, aanpassingen aan hun biosynthese routes. Daarnaast vereist ophoping in extreem hoge concentraties, voor zowel primaire als secundaire pigmenten, zeer waarschijnlijk opbergruimte buiten het fotosysteem. In een kleine groep microalgen wordt deze opbergruimte op natuurlijke wijze voorzien in de vorm van oliedruppels. Deze oliedruppels bestaan voor een groot deel uit triacylglycerolen (TAG), moleculen die opgebouwd zijn uit glycerol en drie vetzuren.

Hoofdstuk 2 sluit af met de meest gepaste manier om iedere kleur die met microalgen gemaakt kan worden te produceren (rood, rood-oranje, oranje, oranje-geel, geel, groen en blauw).

Zoals hiervoor aangegeven kan een nutriënttekort leiden tot ophoping van secundaire pigmenten. Daarom werd in **Hoofdstuk 3** de microalg *I. aff. galbana* T-ISO gelimiteerd in de nutriënten stikstof, zwavel of magnesium, en werd vervolgens de groei en pigmentophoping in iedere gelimiteerde algencultuur bestudeerd. Ieder nutriënttekort leidde tot de productie van de secundaire carotenoïden echinenone en 3-hydroxyechinenone (wat in de alg *H. pluvialis* beide voorlopers zijn van de carotenoïde astaxanthine). Ondanks dat ieder nutriënttekort ook geleid moet hebben tot de vorming van oliedruppels (dit was aangetoond in eerdere literatuur), werden de secundaire carotenoïden slechts geproduceerd in zeer lage concentraties. Bovendien kon er geen productie van astaxanthine aangetoond worden, terwijl alle enzymen actief waren om deze carotenoïde te maken.

Omdat *I. aff. galbana* T-ISO niet in staat bleek tot verhoogde productie van secundaire carotenoïden, werd daarna de focus verschoven naar de alg *C. zofingiensis*. Deze alg zou een mogelijk goede vervanger kunnen zijn van *H. pluvialis* voor de productie van astaxanthine, omdat deze alg in vergelijking tot *H. pluvialis* een hogere specifieke groeisnelheid en een hogere biomassa- en TAG-opbrengst op licht zou hebben en onder stresscondities minder snel overgroeid zou worden door andere (micro)organismen. Een van de nadelen van *C. zofingiensis* is echter dat de maximaal geobserveerde secundaire carotenoïde concentratie in deze alg nogal laag ligt (0.6 % van het drooggewicht, terwijl in *H. pluvialis* een concentratie van 4 % gehaald is). Om deze concentratie te verhogen zijn waarschijnlijk gerichte genetische modificaties nodig die het carotenoïdemetabolisme beïnvloeden. Om dat te kunnen doen is eerst inzicht nodig in het carotenoïdemetabolisme, wat verkregen kan worden doormiddel van studies met enzymremmers.

Daarom werd in **Hoofdstuk 4** *C. zofingiensis* gelimiteerd in stikstof, om er voor te zorgen dat secundaire carotenoïden gevormd werden, waarna difenylamine (DPA) werd toegevoegd, om een aantal specifieke stappen in de biosynthese van secundaire carotenoïden te remmen. Vervolgens werd de productie van pigment en de vorming van TAG-oliedruppels bestudeerd. Om er voor te zorgen dat alle carotenoïden die door *C. zofingiensis* gemaakt worden aan het licht gebracht zouden worden, werden ze geïdentificeerd door middel van omgekeerde fase vloeistofchromatografie in combinatie met massaspectrometrie, een uiterst nauwkeurige techniek.

Stikstofgelimiteerde *C. zofingiensis* (zonder toevoeging van DPA) maakte astaxanthine, ketoluteïne, cantaxanthine, adonixanthine en β -caroteen. Deze secundaire carotenoiden werden ten minste gedeeltelijk nieuw aangemaakt (niet uit afgebroken primaire carotenoiden). Astaxanthine werd voornamelijk opgehoopt als diëster, ketoluteïne voornamelijk als mono-ester en in de vrije vorm (niet veresterd). Beide carotenoiden werden voornamelijk veresterd met de vetzuren C16:0 en C18:1, welke ook het meest gevonden werden in de TAG-oliedruppels. Deze oliedruppels werden uitsluitend in het cytoplasma (celvloeistof) gevonden, niet in het chloroplast. Deze resultaten leidden daarom tot de speculatie dat ook de secundaire carotenoiden opgehoopt werden in het cytoplasma.

Stikstofgelimiteerde *C. zofingiensis* behandeld met DPA liet een afname zien in de productie van β -caroteenderivaten (astaxanthine, ketoluteïne, cantaxanthine en adonixanthine te samen); echter, de productie van ketoluteïne en afbraak van primaire carotenoiden bleef onveranderd. Daarnaast leidde de DPA-behandeling tot verminderde productie van de individuele carotenoiden adonixanthine en echinenone, terwijl geen verhoogde productie van zeaxanthine werd waargenomen. Sterker nog, zeaxanthine werd in zijn geheel niet gevonden in *C. zofingiensis*! Deze resultaten wezen er zeer sterk op dat de veronderstelde omzetting van zeaxanthine in adonixanthine (deze laatste is een voorloper van astaxanthine) zeer waarschijnlijk niet plaatsvindt in *C. zofingiensis*. Daarnaast leidden de resultaten tot het vermoeden dat er een regulatiemechanisme in *C. zofingiensis* aanwezig moet zijn dat verhoogde productie van ketoluteïne en primaire carenoïde voorkomt.

Een belangrijk aspect van de kweekstrategie, wat mogelijk de secundaire carotenoiden- en TAG-opbrengst op licht beïnvloedt, is de biomassadichtheid op het moment dat de stikstof op is (waarna secundaire carotenoiden en TAG beginnen op te hopen). De hypothese is dat er onder stikstofgelimiteerde condities een optimale biomassaspecifieke lichtabsorptiesnelheid bestaat (een optimale hoeveelheid licht per cel) die leidt tot een maximale secundaire carotenoiden- en TAG-opbrengst op licht. Als de hoeveelheid licht per cel hoger wordt zou dit leiden tot lagere opbrengsten van carotenoiden en TAG op licht, doordat er dan relatief meer lichtenergie omgezet wordt in warmte en verloren gaat via schade aan de cel. Een lagere hoeveelheid licht per cel zou ook leiden tot lagere opbrengsten van carotenoiden en TAG op licht, doordat er in dat geval relatief meer lichtenergie gebruikt zou worden voor het rustmetabolisme (het onderhoud van de cel).

Daarom werd in **Hoofdstuk 5** *C. zofingiensis* gelimiteerd in stikstof, waarbij de biomassadichtheid op het moment dat de stikstof op raakte gevarieerd werd. Dit werd gedaan door een vaste starthoeveelheid *C. zofingiensis* te laten groeien in medium met verschillende starthoeveelheden stikstof. Vervolgens werden de carotenoiden- en TAG-opbrengst op licht gemeten.

Er werd gevonden dat, in de bestudeerde range aan biomassadichtheden (2,5, 3,4 en 4,1 g_{biomassa}/L_{reactorvolume}), secundaire carotenoiden en TAG gevormd werden met dezelfde opbrengst van deze producten op licht. Dit gaf aan dat de hoeveelheid licht per cel geen invloed had op de hoeveelheid energie dat, door de cultuur in zijn geheel, gebruikt werd voor de productie van secundaire carotenoiden en TAG.

Hierdoor had de cultuur met de laagste biomassadichtheid op het moment van stikstof-depletie de hoogste secundaire carotenoiden- en TAG-opbrengst op licht wanneer ook de productiefase van biomassa, voorafgaand aan het stikstoftekort, meegerekend werd. Daarnaast bereikte deze laagste biomassacultuur ook de hoogste TAG- en secundaire carotenoidenconcentraties (respectievelijk 335 en 4,5 mg_{product}/gram_{biomassa}).

De maximaal bereikte TAG-opbrengst op licht (320 mg_{TAG}/mol_{photon}) bleek hoger dan ooit vermeld met microalgen. Dit maakt *C. zofingiensis* een veelbelovend productieorganisme van TAG en daarnaast mogelijk ook een veelbelovend productieorganisme van secundaire carotenoiden (na toepassing van genetische modificaties (dit was verder bediscussieerd in **Hoofdstuk 7**)). Momenteel is de maximaal bereikte secundaire carotenoidenopbrengst op licht voor *C. zofingiensis* gelijk aan 2,75 mg_{secundaire carotenoiden}/mol_{photon}, wat ongeveer 7 keer lager is dan de totale secundaire carotenoidenopbrengst op licht gevonden met *H. pluvialis*.

Tenslotte werd er geconcludeerd dat er niet per se een oorzaak-gevolgrelatie hoeft te bestaan tussen de ophoping van TAG en secundaire carotenoiden, ondanks dat ophoping van deze twee componenten geïnduceerd wordt door dezelfde stressoren (een energie-onbalans).

Een ander belangrijk aspect van de kweekstrategie dat mogelijk de secundaire carotenoiden-opbrengst op licht beïnvloedt is de manier waarop de reactor gerund wordt. Traditioneel worden secundaire carotenoiden en TAG geproduceerd door middel van een serie van afzonderlijke batches. Hierbij wordt de biomassa, die verrijkt is met secundaire carotenoiden en TAG, geheel geoogst waarna opnieuw biomassa gekweekt wordt en verrijkt met secundaire carotenoiden en TAG. In de literatuur werd echter gesuggereerd dat de opbrengst van TAG en secundaire carotenoiden op licht verhoogd zou kunnen worden door, in plaats van deze traditionele manier van kweken, een herhaaldelijke (meervoudige) batch toe te passen. In een herhaaldelijke batch wordt slechts een gedeelte van de met secundaire carotenoiden- en TAG-verrijkte biomassa geoogst, terwijl het resterende gedeelte wordt hersteld en hergebruikt. Bijvoorbeeld door opnieuw stikstof toe te voegen aan cellen die al enige tijd zonder stikstof overleefd hebben. Nadat deze cellen zich dan zullen herstellen, zal op een zeker moment de stikstof weer opraken, waarna de cellen opnieuw secundaire carotenoiden en TAG zullen gaan ophopen.

Of zo'n herhaaldelijke batch inderdaad leidt tot een verhoogde opbrengst van TAG en secundaire carotenoiden op licht hangt onder meer af van de snelheid waarmee de cultuur zich herstelt en van de concentratieveranderingen van TAG en secundaire carotenoiden tijdens en na het herstel.

Omdat deze informatie nog niet beschikbaar was in de literatuur werd in **Hoofdstuk 6** aan de al in **Hoofdstuk 5** beschreven stikstofgelimiteerde *C. zofingiensis* culturen opnieuw stikstof toegevoegd, waarna de fysiologie (een groot aantal levensprocessen in de cellen) en de herstelsnelheid van de culturen bestudeerd werden.

De stikstofgelimiteerde culturen (rijk aan secundaire carotenoiden en TAG) herstelden binnen twee dagen nadat stikstof opnieuw was toegevoegd. Tijdens die twee dagen werden TAG en zetmeel zeer snel afgebroken, terwijl astaxanthine, het voornaamste secundaire carenoid in *C. zofingiensis*, slechts afgebroken werd vanaf het moment dat de cellen volledig hersteld waren. De observatie dat de cellen razendsnel herstelden en astaxanthine met enige

vertraging werd afgebroken, suggereert inderdaad dat een herhaaldelijke batchkweek zou kunnen leiden tot een verhoogde secundaire carotenoïdenopbrengst op licht.

Tenslotte werd in **Hoofdstuk 7** voor de carotenoïden astaxanthine, β -caroteen en luteïne de maximale potentie bediscussieerd om de carotenoïdenopbrengst op licht te verbeteren. Dit zijn commercieel gezien de meest relevante carotenoïden uit microalgen omdat zij de hoogste marktwaardes en -volumes hebben. Dit werd gedaan door de theoretisch maximale carotenoïdenopbrengsten op licht te berekenen en deze te vergelijken met de carotenoïdenopbrengsten die momenteel buiten en binnen in het laboratorium gehaald worden.

Voor zowel primaire en secundaire carotenoïden bleek een groot gat te zitten tussen de maximale theoretische carotenoïdenopbrengst op licht en de opbrengsten die momenteel buiten gehaald worden (> een factor 100). Dit gat kan veroorzaakt worden door suboptimale kweekcondities, een suboptimale manier waarop reactoren gerund worden, een suboptimaal reactorontwerp en/of door een suboptimaal metabolisme van de alg. Omdat er slechts een klein verschil gevonden werd tussen de carotenoïdenopbrengsten buiten en binnen, terwijl de kweekcondities binnen gecontroleerd en geoptimaliseerd zijn, werd er geconcludeerd dat de mogelijkheden beperkt zijn om de carotenoïdenopbrengsten te verbeteren door middel van optimalisatie van de kweekcondities, de manier van reactor runnen en/of het reactorontwerp. Daarentegen bleek het verschil tussen de carotenoïdenopbrengsten die binnen gehaald worden en de theoretisch haalbare opbrengsten relatief groot (~ een factor 60 – 100), waardoor buiten behaalde carotenoïdenopbrengsten mogelijk nog enorm verhoogd kunnen worden door het metabolisme van de alg te optimaliseren.

De primaire carotenoïdenopbrengst op licht kan onder andere geoptimaliseerd worden door de koolstof en energie die via fotosynthese door de cel wordt vastgelegd in de richting van de primaire carotenoïden te verschuiven, ten koste van de productie van andere primaire celcomponenten zoals eiwitten, membraanlipiden en zetmeel. Daarnaast kan de secundaire carotenoïdenopbrengst op licht geoptimaliseerd worden door de koolstofstroom te verschuiven in de richting van de secundaire carotenoïden, ten koste van de productie van andere secundaire celcomponenten zoals TAG en zetmeel.

Naar verwachting kan de primaire carotenoïdenopbrengst op licht verhoogd worden met minimaal een factor 2, door middel van kunstmatige evolutie in een turbidostaat, terwijl de secundaire carotenoïdenopbrengsten op licht mogelijk verhoogd kunnen worden met een factor 4 (β -caroteen) tot 7 (astaxanthine), door middel van genetische modificaties waarbij de koolstofstroom omgeleid wordt van TAG of zetmeel naar secundaire carotenoïden. In dit laatste geval zijn wel rigoureuze metabolische veranderingen nodig, wat het in de praktijk erg lastig zou kunnen maken om deze verbeterde opbrengsten te behalen.

Daarnaast werd geconcludeerd dat voor ware soortverbeteringen zowel gerichte genetische manipulatie als kunstmatig ongerichte evolutie (met gerichte selectie) verder verkend moeten worden. Gerichte genetische manipulatie vereist verder onderzoek van de fysiologie van de alg en in het bijzonder van het carotenoïdenmetabolisme, en daarnaast vereist het ontwikkeling van protocollen van robuuste transformatietechnieken. Daarnaast vereist kunstmatige evolutie de ontwikkeling van selectiemethodes die, na het toepassen van genetische modificaties, automatisch leiden tot algen met de gewenste eigenschappen.

Tenslotte werd het toepassen van een herhaaldelijke batch, geïntroduceerd in **Hoofdstuk 6**, verder uitgediept met behulp van een eenvoudig model. Er werd geconcludeerd dat een herhaaldelijke batch inderdaad kan leiden tot een verhoogde carotenoïdenopbrengst op licht wanneer (i) het herstel van de kweek minder lichtenergie kost dan de productie van biomassa tijdens de klassieke batch en (ii) de carotenoïdenopbrengst op licht tijdens de stressfases vergelijkbaar is in beide processen (er mag dus niet te veel afbraak van carotenoïden plaatsvinden).

Al deze hoofdstukken samen hebben geresulteerd in een toegenomen inzicht in zowel de kweekcondities die leiden tot een maximale pigmentopbrengst op licht, als in het pigmentmetabolisme en diens regulatie. In het oog springend zijn de opheldering van de meest waarschijnlijke biosynthese routes in *C. zofingiensis* naar astaxanthine en ketoluteïne en de vinding dat remming van secundaire carotenoïdenvorming niet leidt tot verhoogde productie van primaire carotenoïden in deze alg. Deze vindingen zijn in het bijzonder relevant wanneer gerichte genetische modificatiestrategieën ontwikkeld moeten worden met als doel om de opbrengst van astaxanthine, ketoluteïne of een primaire carotenoïde op licht te verhogen (**Hoofdstuk 4**). Een ander belangrijke vinding is dat deze alg een breed optimum heeft in secundaire carotenoïdenopbrengst op licht met betrekking tot de biomassadichtheid op het moment van stikstofdepletie. Dit houdt in dat als deze alg buiten gekweekt wordt een relatief hoge biomassa gebruikt kan worden zonder dat er verlies optreedt van carotenoïdenopbrengst op licht (**Hoofdstuk 5**). Tenslotte is het een belangrijke vinding dat astaxanthine, de voornaamste secundaire carotenoïde van *C. zofingiensis*, nauwelijks wordt afgebroken wanneer opnieuw stikstof wordt toegevoegd aan stikstofdeplete cellen. Deze vinding wijst er namelijk op dat zowel de carotenoïdenopbrengst op licht als de carotenoïdenconcentratie mogelijk verhoogd kan worden door het toepassen van een herhaaldelijke batch in plaats van de traditioneel toegepaste serie van enkele batches (**Hoofdstuk 6**). Zoals bediscussieert in **Hoofdstuk 7**, zullen deze nieuw verworven inzichten de optimalisatie van fototrope pigmentproductie met microalgen vereenvoudigen.



Dankwoord

◀ IN THE PICTURE

Filters with nutrient-sufficient (bright green), nitrogen-depleted (green to orange, top four rows) and nitrogen-resupplied (orange to green, bottom two rows) *Chromochloris zofingiensis*. Nitrogen-depleted *C. zofingiensis* produces red and orange pigments and degrades green pigments. When nitrogen is resupplied, green pigments are rapidly produced again, while some red pigments remain for a while.

◀ IN BEELD

Chromochloris zofingiensis met voldoende eten (helder groen), een stikstoftekort (groen tot oranje, bovenste vier rijen) en waaraan weer stikstof is toegevoegd (oranje tot donker groen, onderste twee rijen). Zonder stikstof maakt deze alg rode en oranje pigmenten en breekt groene af. Na toevoeging van stikstof worden de groene snel weer gemaakt, terwijl ook sommige rode nog even aanwezig blijven.

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Miranda, Marina, Douwe, Gerard, Mathieu, Shirley, Rouke, Richard, João, Aggi, Sina, Klaske, Petra, Nadia, Karin, Sarah, Ellen, Rupali, Emre, Giulia, Michiel, Niels-Hendrik, Kanjana, Vincent, Brenda, Edwin, Stefan, Maria B., Michel, Guiseppe, Hans R., Marjan, Marcel, Bas, Ruud, Frank, Mgeni, Janneke, Dione, Hylke, all Foodies, Fré, Eva, and many others, thank you for truly helping me, making me laugh or supporting me in *any* other way!

Labuitjecommissies

De 'werk'dag waar ik jaar na jaar intens van genoten heb (en een beetje nerveus naartoe ging) was die van het **labuitje**. Want op welke andere dag mag je zowel creatief als sportief samenwerken met en strijden tegen je collega's? Klomp/mokbeschilderen, maskersmaken, jambéspelen, fiets/step-speurtochten door (de omgeving van) Wageningen, met Duplo 'spelen', fotopuzzeltochten door Nijmegen (en in de ballenbak), karaoke, naar een ketchupfabriek, workshops mindmappen/e-mail managen/(met een clownsneus op je neus) positief denken, survivallen, cocktailsmaken, salsadansen, 'wie is de mol'-spelen, vliegersknutselen, pubquizen, bbq'en en nog véél meer. **Labuitjecommissies**, bedankt!

Buitenschoolse activiteiten

De harmonie: *Marco, Jeroen, Diane, Joost, Robert, Erwin, Tim, Sandra, Theo, Daniëlle, Bert, Rob, Thea* en *vele anderen*, dankzij jullie hoefde ik op woensdagavonden aan niets anders te denken dan aan de muziek. Bedankt, voor alle zwetsgesprekken, het 'we-gaan-er-voor'-groepsgevoel voor, tijdens en na concerten, en dat ik bij jullie altijd mezelf kon zijn!

Tartléto: *Joep, Anneke, Evi, Lennart, Mischa, Arend, Evelien, Nout* en *anderen*, bedankt voor de gezelligheid en jullie steun!

Vriendinnetjes

Beter een goede buur dat een verre vriend. Toch zijn verre vriendinnetjes van wereldbelang.

Anne, ook al zagen we elkaar niet zo vaak, bij jou kon ik alles kwijt. Dank je wel, dat ik ook jóuw beste vriendinnetje mag zijn.

Marieke, heerlijk kon ik met je kletsen aan de telefoon, over wat er allemaal op de 6^e was gebeurd. Dank je wel, voor je nieuwsgierigheid en interesse.

Floor en **Sanne**, bedankt dat ook jullie met interesse naar mijn werk wilden kijken/luisteren!

Buffi, een brief van jou uit Zürich, of een kaartje uit 'weet ik waar' en mijn hart maakte een sprongetje. Dank je wel, dat je altijd aan me denkt!

Djoyce, oude liefde roest niet. En zo is het. Fijn dat je er al zo lang bent!

Familie

Genen, geniaal!?

Mama, jij hebt mij al vroeg geleerd assertief te zijn. *Zélf bellen, zélf achteraan gaan!* Zonder jou was ik nooit zo ver gekomen. Dank je wel liefste mama!

Papa, *winners find a way*. Jij hebt mij laten zien wat het is om ergens *volledig* voor te gaan. Je bent de allerbeste en allerliefste papa van de héle wereld! En ja, ik blijf jouw kleine meisje.

Ina en **Hans**, wat fijn dat jullie er altijd zijn en dat het altijd zo fijn is met jullie! Dank jullie wel!

Roy & Petra, Wout & Sanne, Sander & Saffira, Lisette, Thomas & Zoë, dank jullie wel, dat jullie met interesse naar mijn reactor kwamen kijken! En voor de avondjes, etentjes en spelletjes die we altijd doen. Ik ben heel blij (en trots!) dat jullie mijn familie zijn.

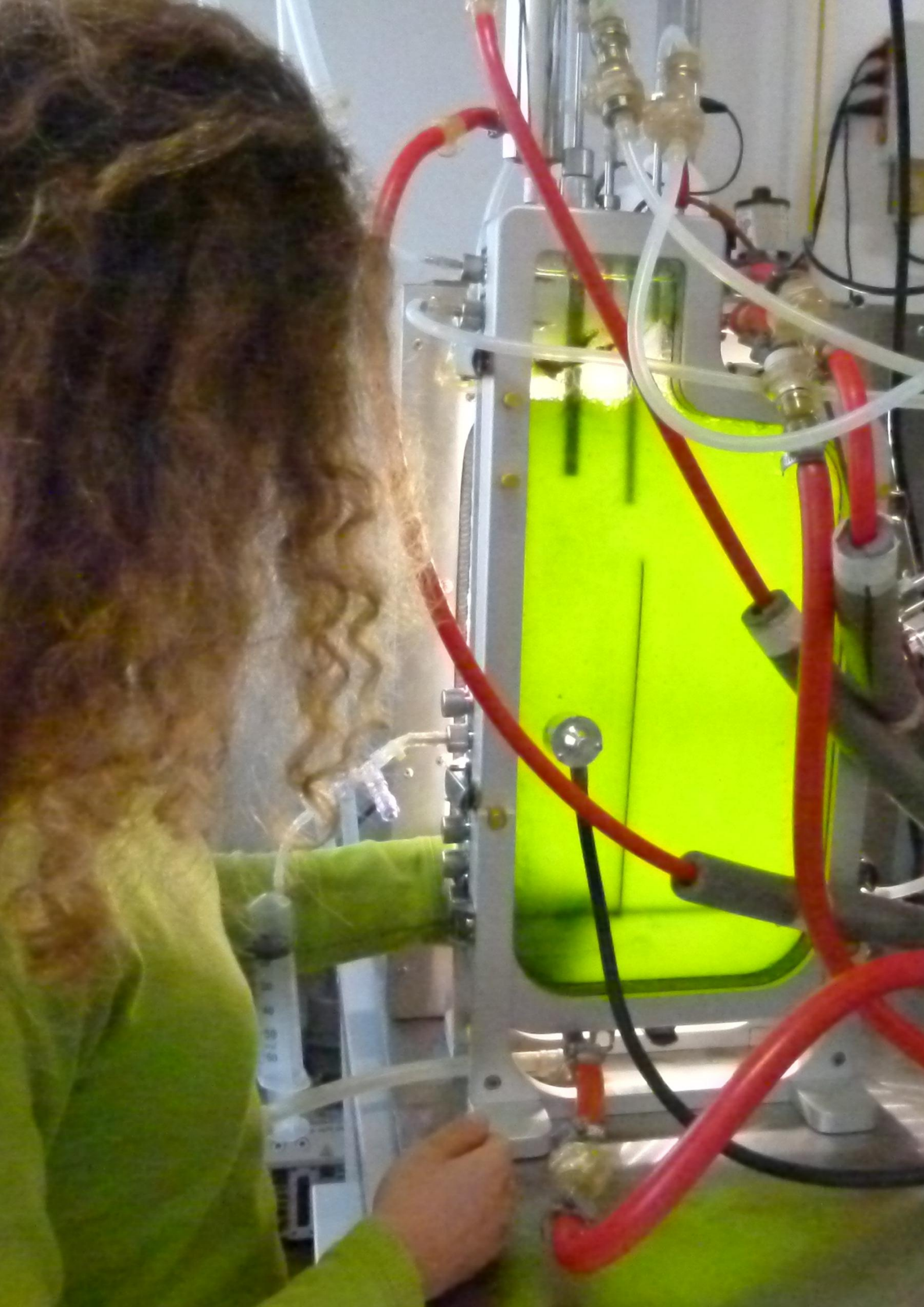
Allesomvattend

Dennis, jij was er altijd voor mij. En ik had je zo erg nodig. Je zei altijd precies de juiste dingen, vooral als ik het moeilijk had. Mijn grote kleine mannetje.

Jij liet mij inzien dat ik trots mocht zijn op mijn werk, en jouw mening was niet ongegrond. Ik vond het zo fijn dat je, meer dan eens, met me mee bent gegaan naar het lab als ik in het weekend met mijn kleintjes ging spelen. En dat je al m'n werk wilde lezen (artikelen voor me wilde opzoeken) en dat ik al mijn presentaties op jou mocht oefenen. Als jij het duidelijk vond dan wist ik dat het duidelijk wás. 'Dit wordt echt een héél mooi artikel', zei je glunderend.

Jij was het die mij naar mijn werk stuurde als ik het niet meer zag zitten, naar me luisterde als de kritiek me te zwaar werd en voor me zorgde als ik dat zelf niet meer kon. Schatje, we hebben het sámen gedaan.

Kim



The Author

◀ IN THE PICTURE

Photobioreactor containing *Chromochloris zofingiensis* cells. A mixture of air and CO₂ is pumped into the reactor via a sparger (± 7 cm above the bottom). This generates a liquid flow which keeps the cells into suspension. Besides, the CO₂ is consumed by the cells and converted, eventually, into new cells. The big syringe (left) is used to withdraw small samples from the reactor for analysis.

◀ IN BEELD

Fotobioreactor met *Chromochloris zofingiensis* cellen. Een mix van lucht en CO₂ wordt de reactor in gepompt via een gaatjesbuis (± 7 cm boven de bodem van de reactor). Dit leidt tot stroming, wat de cellen in oplossing houdt. Daarnaast voorziet de CO₂ de cellen van koolstof, waarvan nieuwe cellen gemaakt worden. Met de grote spuit (links) kan een klein volume getapt worden voor analyse.

Curriculum Vitae

Kim Johanna Maria Mulders was born in Boxmeer (Beugen), The Netherlands, on May 7 1985. She went to Elzendaal College in Boxmeer, where she obtained her HAVO diploma in 2002, followed by her VWO diploma in 2004, both with majors in *Natuur en Gezondheid* and *Natuur en Techniek*.

In 2004 she started her study Biotechnology at Wageningen University.

In her minor thesis project, at the laboratory of Bioprocess Engineering of Wageningen University, she worked on the design of a model which described the chain length distribution and kinetic characteristics of an enzymatically produced polymer. This work resulted in a peer-reviewed scientific publication (see **List of Publications**).

During her internship at the Bioengineering Research Group (laboratory of gene processing) of Instituto Superior Técnico (Lisbon Technical University), Portugal, she studied the stability of plasmid DNA employing circular dichroism.

Kim finished her studies with a major thesis at the Marine Biomedical and Biotechnology group of Harbor Branch Oceanographic Institution, Florida, USA, under the auspices of the laboratory of Bioprocess Engineering of Wageningen University. There, she worked on the transfection of sponge cells.

She graduated *Cum laude* in 2008 (BSc) and 2010 (MSc).

After completing her studies, she continued working as a PhD at the laboratory of Bioprocess Engineering of Wageningen University, on the project *Pigment production with microalgae*.

After completing the course *Bedrijfshulpverlening* (BHV), right after the start of her PhD, she participated in the first aid and BHV teams of Bioprocess Engineering of Wageningen University.

During the second year of her PhD, she co-organised the first International Young Algae-neers Symposium, a symposium that aims to connect and inspire young scientists working in the field of microalgae biotechnology.



Contact Information

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List of Publications

This thesis

Mulders KJM, Weesepeel Y, Lamers PP, Vincken J-P, Martens DE, Wijffels RH (2013). Growth and pigment accumulation in nutrient-depleted *Isochrysis* aff. *galbana* T-ISO. Journal of Applied Phycology 25:1421-1430

Mulders KJM, Lamers PP, Martens DE, Wijffels RH (2014). Phototrophic pigment production with microalgae: biological constraints and opportunities. Journal of Phycology 50:229-242

Mulders KJM, Weesepeel Y, Bodenes P, Lamers PP, Vincken J-P, Martens DE, Gruppen H, Wijffels RH (2014). Nitrogen-depleted *Chlorella zofingiensis* produces astaxanthin, ketolutein and their fatty acid esters: a carotenoid metabolism study. Journal of Applied Phycology, DOI: 10.1007/s10811-014-0333-3

Mulders KJM, Martens DE, Wijffels RH, Lamers PP (2014). Effect of biomass concentration on secondary carotenoids and triacylglycerol (TAG) accumulation in nitrogen-depleted *Chlorella zofingiensis*. Algal research 6:8-16

Mulders KJM, Lamers PP, Wijffels RH, Martens DE (2014). Dynamics of biomass composition and growth during recovery of nitrogen-starved *Chromochloris zofingiensis*. Applied microbiology and biotechnology. DOI: 10.1007/s00253-014-6181-x.

Other work

Mulders KJM, Beeftink HH (2013). Chain length distribution and kinetic characteristics of an enzymatically produced polymer. E-polymers 24:1-12

Overview of Completed Training Activities



Discipline specific activities

Courses

- *Advanced Course on Metabolomics for Microbial Systems Biology* (Delft), 18-22 October 2010
- *Bio-energy Production from Crop Plants and Algae* (Wageningen), 21-23 November 2012

National symposia

- *NPS11 "From plan to plant" (11th Annual Dutch Process Technology Symposium)* (Papendal), 24-26 October 2011 **Poster presentation**
- *NBC-14 "delivering value: bridging science and business" (14th Dutch Biotechnology Congress)* (Ede), 16-17 April 2012 **Poster presentation**

International symposia

- *9th European workshop Biotechnology of Microalgae "where we are and will go in microalgal biotechnology" (Nuthetal, Germany)*, 4-5 June 2012 **Poster presentation**
- *Young Algaeneers Symposium I* (Wageningen), 14-16 June 2012 **Poster presentation**
- *Young Algaeneers Symposium II* (Montpellier-Narbonne, France), 3-5 April 2014 **Oral presentation**

General courses

- *Advanced course Guide to Scientific Artwork* (Wageningen), 10-11 May 2010
- *VLAG PhD week* (Baarlo), 25-28 October 2010
- *Effective behaviour in your professional surroundings* (Wageningen), 3 and 24 October 2010
- *Pictures, tables, and infographics in your research* (Wageningen), 23 March 2011
- *Teaching and supervising thesis students* (Wageningen), 28 and 29 March 2011
- *Scientific writing* (Wageningen), 20 April - 8 June 2011
- *PHD competence assessment* (Wageningen), 24 May and 21 June 2011
- *Project and time management* (Wageningen), 9 and 23 November and 21 December 2011
- *Networking course* (Wageningen), 15 Februari 2012
- *Career perspectives* (Wageningen), 7, 21, 28 November and 5 December 2013

Optionals

- *Brainstorm/PhD-students days* Bioprocess Engineering Group, 1 July 2010, 6 February, 25 April and 12 December 2012, 26 April and 10 December 2013
- *International excursion to the United States (PhD-trip Bioprocess Engineering)*, 2-10 October 2010 **Poster presentation, Oral presentation**
- *Organization Young Algaeneers Symposium*, July 2011 - June 2012
- *International excursion to Spain (PhD-trip Bioprocess Engineering)*, 8-18 July 2012 **Oral presentation**
- *FeyeCon Team meetings (Weesp/Wageningen)*, 20 April, 29 June, 5 Augustus, 12 October and 9 December 2010, 10 February, 6 April, 14 June, 27 October and 16 December 2011, 24 April, 20 June, 15 October 2012, 19 June, 2013, 29 January, 27 March 2014

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All photos in this thesis were taken by Kim Mulders, except the ones on the following pages:

p. 68, 158, 174	Dennis Bast
p. 88, 108, 132	Tiny Franssen-Verheijen, Wageningen Electron Microscopy Centre
p. 176	Leo Huijssoon

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