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Microalgae isolation, genetic improvement and transcriptional profiling for high-efficiency lipid production.

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Abstract

Interest in a sustainable alternative to fossil fuels has recently intensified as the effects of rising oil costs and dangers of increasing CO_2 levels are becoming more apparent. Microalgae-derived biodiesel provides a promising alternative, as theoretical calculations of areal microalgal oil production are at least 10 to 20-fold greater than any other biofuel crop. Importantly, microalgae cultivation can achieved without competing with precious arable land or rainforests and freshwater resources. At present, large-scale microalgal oil production is not economically viable, and many technical and biological barriers still need to be overcome in order to improve lipid productivity and reduce cost of production. The main objective of this thesis was to improve microalgal lipid productivity and gain a deeper understanding of the molecular mechanisms behind lipid biosynthesis.

In the first part of the present work, numerous microalgal strains were collected from coastal water in South East Queensland, Australia. After isolation of pure strains, the fastest growing algae were compared to each other using a specially developed standardised lipid induction assay. This assay combined rapid exponential growth with a nutrient starvation phase to induce lipid biosynthesis, a survival mechanism of microalgae under adverse conditions. Based on their lipid productivity and fatty acid profile, several strains, including *Nannochloropsis* sp. BR2 and several *Tetraselmis* sp,, were identified as potential feedstock cultures for biodiesel production.

As the identified cultures can be considered as undomesticated, one method of further increasing algal lipid productivity is via mutation and selection of high-lipid yielding algal strains. Instead of using a transgenic approach, this research used adaptive evolution methods, incorporating mutagenesis and high-throughput selection to select for high-lipid yielding algal strains. UV-C and different laser beams were used as mutagens, followed by a selection method encompassing flow cytometry and microplate readers to effectively select individual cells with high lipid contents, but also uncompromised growth. After several generations of mutation and selection, higher lipid accumulation potential was observed in several strains.

This research also focused on understanding the underlying mechanisms of nitrogen-starved lipid induction in *Tetraselmis* sp. M8 through various growth phases. Transcriptional profiling using RNA-Seq and quantitative real-time PCR analysis of this previously unsequenced genus, combined with physiological measurements after nutrient starvation, revealed that early lipid accumulation was predominately due to a reduced fatty acid degradation rate, while the rate of lipid biosynthesis remained unchanged. At 48 h onwards however, the expression of lipid biosynthesis genes was

significantly upregulated, indicating lipid accumulation was now an effect of active triacylglyceride (TAG) synthesis. This first report on the molecular mechanisms of lipid accumulation in *Tetraselmis* sp. identified potential bottlenecks and target genes for metabolic engineering to maximise lipid accumulation in microalgae. Apart from strain improvement, culturing and lipid induction techniques offer further scope to optimise lipid productivity. Current efforts in the development of cost-effective harvesting and algal oil extraction procedures may further position microalgae as a significant feedstock for economical biodiesel production.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer reviewed papers

Lim, D.K.Y., Sharma, K., Garg, S., Schenk, P.M. (2010) **The race for highly productive microalgae strains**, *Biofuels* 1: 835-837 – incorporated as Chapter 1

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List of Abbreviations

AA – arachidonic acid

ACCase – acetyl-CoA carboxylase

ACO – acyl-CoA oxidase

ACP – acyl carrier protein

ACSase – acyl-CoA synthetase

AGPAT – 1-acyl-sn-glycerol-3-phosphate acyltransferase

ALA – alpha-linolenic acid

ANACC – Australian National Algae Culture Collection

cDNA – complementary deoxyribonucleic acid

CFPP – cold filter plugging point

CO_2 – carbon dioxide

CSIRO - Commonwealth Scientific and Industrial Research Organisation

DEG – differentially expressed gene

DEK – differentially expressed k-mer

DERs – differentially expressed reads

DGAT – diacylglycerol acyltransferase

DHA – docosahexanoic acid

DiFFKAP – differential Kmer analysis pipeline

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DW – dry weight

ECH – enoyl-CoA hydratase

EMS – ethyl methane sulfonate

ENR – enoyl-ACP reductase

EPA – eicosapentaenoic acid

FA – fatty acid

FACS – fluorescence-assisted cell sorting

FAME – fatty acid methyl acid

FAT – Acyl-ACP thioesterase

FS – fluorescence signal

GC/MS – gas chromatography/mass spectrometry

GHG – green house gas

GK – glycerol-3-phosphate

GM – genetically modified

GO – Gene ontology

GPAT – glycerol-3-phosphate acyltransferase

HACDH – hydroxyl-CoA dehydrogenase

HD – 3-hydroxyacyl-ACP dehydratase

KAR – 3-ketoacyl-ACP reductase

KAS – 3-ketoacyl-ACP synthase

KAT – ketoacyl-CoA thiolase

LCAS – LC-PUFA acyl-Coenzyme A synthetase

LC-PUFA – long chain polyunsaturated fatty acid

LD – lethal dosage

LHC – light harvesting complex

LPAAT – lyso-phosphantidic acid acyltransferase

LPAT – lyso-phosphantidylcholine acyltransferase

MAT – malonyl-CCoA:ACP transacylase

MNU – *N*-methyl-*N*-nitrosourea

mRNA – messenger ribonucleic acid

MUFA – monounsaturated fatty acid

N – nitrogen

NCBI – National Center for Biotechnology Information

NO_{3-} - nitrate

NTG – nitrosomethylguanidine

OD – optical density

P – phosphate

PCR – polymerase chain reaction

PDAT – phospholipid:diacylglycerol acyltransferase

PDH – pyruvate dehydrogenase complex

PO_4^{3-} - phosphate

PP – phosphantidate phosphatase

PSII – photosystem II

PUFA – polyunsaturated fatty acid

qRT-PCR – quantitative reverse transcriptase polymerase chain reaction

RNA – ribonucleic acid

RNA-Seq – RNA-sequencing

ROS – reactive oxygen species

SW- seawater

TAG – triacylglyceride

TCA – tricaboxylic acid

TFA – total fatty acid

TLC – total lipid content

UV-C – ultraviolet–C

Wt – wildtype

Chapter 1: General Introduction

This chapter presents the introduction of this thesis, whose general focus is the improvement of microalgal lipid biosynthesis for the purpose of biodiesel production. The three main aims of this thesis will also be introduced, along with the background and literature review for each aim, which have been published as peer-reviewed articles attached to this paper.

Introduction

Interest in a sustainable source of biofuel has recently intensified as the demand for petroleum-based fuel and concerns about climate change continue to increase. While traditional crop-based seed plants are increasingly being used (Doan and Obbard, 2011), microalgae are now widely regarded as a promising source of biofuel due to their high lipid productivity, environmental benefits and ability to grow on non-arable land (Chisti, 2007,Malcata, 2011,Schenk et al., 2008). Theoretically, microalgae have a higher productivity per unit area which allows them to potentially produce 10 to 20 times more lipids (liter/ha) than palm oil (Ahmad et al., 2011), corn and soybean (Chisti, 2008,Gouveia and Oliveria, 2009,Hu et al., 2008). These can then be converted into biodiesel via transesterification. Nevertheless, the microalgae biodiesel industry is still in its infancy and there are still many technical and biological barriers that prevent large-scale biodiesel production. To date, the price of producing microalgal biodiesel is still more expensive than palm oil or petrodiesel (Dermirbas and Dermirbas, 2011), and there is still much room for improvement in every aspect of production in order for the price of microalgal biodiesel to be competitive. To that end, the main goal of this thesis is to **improve microalgal lipid productivity and gain a deeper understanding into the molecular mechanisms behind microalgal lipid biosynthesis**. The main aims of this PhD that will achieve this goal are:

Aim 1: Collect, isolate and screen for high lipid productivity microalgae species. The use to a suitable, high lipid productivity microalgae strain is the basis of successful microalgal lipid production. While many studies have already identified strains with potential (Araujo et al, 2011, Rodolfi et al., 2008), the use of locally isolated microalgae strains over foreign strains has its advantages. Microalgal strains suitable for lipid and biodiesel production can be obtained from local waterways using the right collection, isolation and screening techniques. The chapter for this aim will present a successful method for the collection, isolation and screening of microalgae from local South East Queensland waterways to produce strains that have the comparatively best traits for

microalgal lipid production. This aim will also identify microalgal strains most suitable for subsequent Aims 2 & 3.

Related literature reviews:

- **Lim, D. K. Y.**, Schenk, P. M. ***Microalgae selection and improvement: GM vs Non-GM.*** Submitted for publication (2014).
- **Lim, D. K. Y.**, Sharma, K., Garg, S., Schenk, P. M. ***The race for highly productive microalgae strains.*** Biofuels (2010) 1(6), 835-837.

Related Chapter: Chapter 2

Aim 2: Enhance microalgal lipid productivity via non-GM methods: Isolation of high-lipid improved strains following repeated UV-C mutagenesis and high-throughput growth selection. Non-GM methods of strain improvement do not require background genomic information or a complex transformation system in order to select for a desired trait. The mutation and selection to generate high-lipid producing mutants is desirable in situations where a wild-type strain possesses all the desirable traits for large-scale production (high growth rate, ease of harvest, robustness) except for very high-lipid content. This aim will present a mutation-selection program involving the use of induced mutagenesis and high throughput selection with the goal of producing improved *Tetraselmis suecica* strains with increased lipid productivity.

Related literature reviews:

- Lim, D. K. Y., Schenk, P. M. ***Microalgae selection and improvement: GM vs Non-GM.*** Submitted for publication (2014).

Related chapters: Chapter 3

Aim 3: Investigate the molecular mechanism of *Tetraselmis* sp. lipid production using a transcriptional profiling approach. Before genetic engineering of an organism can proceed, key related pathways and target genes must be mapped and identified. Work for this aim used next-

generation sequencing to generate the transcriptome of *Tetraselmis* sp. M8 to map key lipid-related pathways during early-stationary phase. The expression of these pathways were then further analysed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) over multiple time points to gain an understanding into lipid production in *Tetraselmis* sp. and to identify potential bottlenecks and targets for genetic engineering.

Related literature reviews:

- Lim, D. K. Y., Schenk, P. M. ***Microalgae selection and improvement: GM vs Non-GM.*** Submitted for publication (2014).
- Schuhmann, H., Lim, D. K. Y., Schenk, P. M. ***Perspectives on metabolic engineering for increase lipid contents in microalgae***. Biofuels (2012), 3(1), 71-86.

Related chapters: Chapter 4

This research project started with Aim 1, where the collection, screening and isolation of local microalgae species identified the best strains for microalgae lipid production, as well as candidate strains for the rest of the project. This was followed up by Aim 2, which enhanced the lipid productivity of the candidate strain, *Tetraselmis suecica,* using repeated-UV mutagenesis and high-throughput selection. While the objective of Aim 2 was the improvement of lipid productivity, the goal of Aim 3 was to better understand the molecular mechanisms behind lipid production in *Tetraselmis* sp., a locally isolated strain from Aim 1. Both Aim 2 and Aim 3 used different microalgae strains that shared the same genus. This was done to facilitate possible future work in the lab that would utilise information and techniques developed in Aim 3 to better understand the molecular basis behind the improvements achieved in Aim 2. *Tetraselmis seucica* was an ideal candidate for Aim 2 as it was a strain that possessed all the desirable traits for large-scale production except high lipid content. This aim would therefore present a method of improving a naturally low lipid content strain. The reason for choosing *Tetraselmis* sp. M8 for gene expression profiling (Aim 3) during lipid induction was the fact that (1) it was a local strain that had not been characterised on a molecular basis, (2) it displayed dominance under outdoor growth conditions and merit as a promising culture for large-scale production, (3) it showed high lipid productivity and it was important to identify the underlying mechanisms, (4) it provided a reference strain for the Algae Biotechnology Laboratory at UQ

Literature Review

The general background of this thesis and the literature pertaining to microalgae' improvement through strain selection, including genetically-modified and non-genetically modified methods, have been reviewed in the attached article by Lim & Schenk (Submitted), "***Microalgae selection and improvement: GM vs Non-GM"*** (Review paper 1). The article discusses the overarching impacts that selecting the right microalgal strain has on every level of microalgal biodiesel production, as well as key traits that microalgae should possess for successful biodiesel production. Suitable isolation and screening strategies are discussed, with an emphasis on isolating microalgae from local environments that are more acclimated to local conditions. Screening strategies focus on identifying key traits required for microalgal biodiesel production. Various non-GM and GM methods of improving microalgal lipid productivity, as well as their pros and cons were also discussed. Non-GM methods that were reviewed focused primarily on the various mutation-selection programs that used induced mutagenesis followed by fluorescence-activated cells sorting (FACS) to select for high performing mutants. Current GM work on microalgae was also reviewed, discussing the available genomic, transcriptomic and genetic engineering work pertaining to microalgal lipid production.

Aside from this overall literature review, more in-depth literature reviews were also published. Lim et al. (2010) "***The race for highly productive microalgae strains"*** evaluated a key paper by Huerlimann et al. (2010) "***Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production"*** (Review paper 2). The review analysed the effects of different growth media on lipid production and composition during different growth phases, as well as the methods used by Huerlimann et al. (2010) to carry out multiple strain comparisons. This review helped form a good template for Aim 1 by analysing the paper's method of identifying suitable microalgae for biodiesel production, which incorporated cell densities, growth rates, dry weight, lipid contents and fatty acid composition. Finally, "***Perspectives on metabolic engineering for increased lipid production"*** (Review paper 3) provides an in-depth review on Aim 3. It presents an overview of the triacylglyceride (TAG) metabolic pathways in microalgae, particularly the fatty acid (FA) synthesis, TAG synthesis and lipid catabolism pathways. Furthermore, it summarises the current knowledge about metabolic engineering, systems biology and genome-scale metabolic pathway modelling that form the background template for Aim 3.

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Review Paper 1: Microalgae selection and improvement: GM vs Non-GM

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Abstract

Despite being established as a sustainable feedstock for biofuel production with tremendous potential, the microalgae biofuel industry still struggles to make large-scale production economically viable. An overriding aspect in microalgae biodiesel production is strain selection, as it affects nearly all stages of production. This chapter highlights the key traits that microalgae should possess for successful biodiesel production, as well as suitable isolation and selection strategies. It also highlights the various methods that are currently available for the biological improvement of microalgae strains.

Introduction

The use of microalgae as a sustainable feedstock for biofuel production has received much recent interest in an effort to confront depleting fuel reserves, global warming and climate change. Microalgae represent a renewable source of energy as they use photosynthesis to convert CO_2, sunlight and water into energy that is stored as lipids and carbohydrates (e.g. starch). These can be converted into biofuels (biodiesel and bioethanol) with areal productivities that are significantly higher than traditional biofuel land crops, potentially without the use of precious arable land and freshwater [1, 2]. While the potential of microalgae as a sustainable energy source, particularly biodiesel has been well established, many technical and biological barriers prevent large-scale economically viable production of microalgal biodiesel. So far, microalgae cultivation facilities in

Australia (*Dunaliella salina*), USA (*Haematococcus pluvialis*), Israel (*H. pluvialis*) and China (*Spirulina*) produce high value products such as omega-3 oils and carotenoid, produce algae for their nutritional value, but not for biofuel [3]. This is because microalgae biofuel companies can currently only produce microalgae oil at a price that is more expensive than palm oil ($0.66/L) and that needs to be reduced to as low as $0.48/L to be competitive with petrodiesel [4, 5]. To achieve this, the microalgae biofuel industry must improve many technical and biological aspects of production.

One of the most important biological aspects of microalgal biodiesel production is the species being used for production. Selection of a suitable species has downstream effects on nearly every level of production, including growth conditions (pH, CO_2, light intensity, salinity), harvesting method, oil extraction and ultimately the quantity and quality of the biodiesel produced. The overarching importance and impact of the producer species has driven research into more sophisticated methods of selecting, evaluating and identifying microalgal species with suitable characteristics. Furthermore, recent years have seen tremendous interest in genetically-modified (GM) species as well as improvement of non-GM species for lipid production. This review discusses the various traits that are desirable for microalgal biofuel production with a focus on lipids as feedstock for biodiesel. It also highlights the importance of species selection and evaluation and the various GM and non-GM methods for improving lipid productivity for biofuel production.

Collection, isolation and screening of microalgae for biodiesel production

Collection and isolation of microalgae

Microalgae are found in nearly all natural waters, be it freshwater, brackish water, or marine ecosystem. Nevertheless, collection of microalgae for biodiesel production must focus on locations with the greatest likelihood of providing strains that are suitable for biodiesel production in an outdoor setting. Firstly, microalgal species should be collected from the local area, or at least an area with similar climatic and ecological conditions as in the intended production area. This is

651 because native strains are likely to be already acclimatized to local conditions and have a
652 competitive advantage over foreign species. Furthermore, the sampling should focus on the aquatic
653 environments that are exposed to fluctuating and/or occasional adverse conditions such as tidal
654 pools and estuaries. These locations naturally select for microalgae that are robust, fast-growing,
655 and have survival mechanisms (e.g. accumulation of storage lipids) to cope with changing
656 conditions [6]. This is likely to increase the chances of finding a strain that is most suitable for
657 biodiesel production.

658

659 After samples have been collected from the environment, individual microalgal strains can
660 be isolated and purified using a range of techniques. Traditional techniques such as
661 micromanipulation and serial dilution to individual cells can be time and energy intensive, but are
662 usually successful in isolating pure cultures, although they may fail to isolate rare strains. Antibiotic
663 selection and enrichment of microalgae from mixed cultures can be used to select for strains with
664 desirable traits such as a high growth rate and pH- or salinity-tolerance. Automated processes
665 involving flow cytometry and robotics have been developed for rapid isolation of microalgal strains
666 [7-9]. The use of high-throughput fluorescence assisted cell sorting (FACS) can distinguish
667 different microalgal species by relying on the species' different chlorophyll auto-fluorescence and
668 green autofluorescence properties. Microalgal cells can also be stained with sub-lethal doses of
669 lipid-staining Nile Red reagent prior to cell sorting and this can help isolate the cells with a high
670 lipid content [10]. However, high lipid containing microalgal strains (e.g. *Botryococcus braunii*)
671 often display slow growth and this may result in a low overall lipid productivity. Once isolated, a
672 pure culture should be preserved by slow propagation in stock cultures or cryopreservation to
673 prevent loss of competitiveness by genetic drift [11].

674

675 **Screening criteria**

676

677 Two of the most important criteria when screening microalgae for biodiesel production are
678 the lipid productivity (depends on growth rate and lipid contents) and composition. A fast-growing
679 highly oleaginous microalgal strain would translate directly to an overall increased productivity.
680 However many fast-growing strains have low lipid contents, but their lipid biosynthesis is highly
681 inducible and, therefore, under appropriate conditions their lipid productivity can be quite high [12].

682 Furthermore, the qualitative and quantitative composition of a species' triacylglycerides (TAG), the
683 fraction of the lipids that are suitable for biodiesel production, affects the quality of the biodiesel
684 produced and its potential to meet the biodiesel standards. The lipid content of different microalgal
685 species can vary, ranging from 10 to 30% on average [14]. To be considered potentially suitable for
686 commercial use, a microalgal strain should have a base lipid content of at least 20-30% (% of dry
687 weight, DW). In addition, its fatty acid (FA) content should consist of a mix of saturated and
688 monounsaturated short chain FAs, and as little polyunsaturated FA (PUFA) as possible [47]. More
689 importantly, these numbers should be achieved not only in the laboratory, but also in medium- to
690 large-scale outdoor operations that closely mimic an industrial production setting. Many microalgal
691 species may achieve a high lipid productivity in the laboratory, but fail to do so in the variable
692 outdoor conditions. Thus, it is important that laboratory screening is followed up by outdoor
693 evaluation to determine the suitability of a strain for biodiesel production.

694 Although most published biofuel studies have focused on a single species [11], an
695 increasing number of multi-strain comparative studies evaluating the lipid content and composition
696 in outdoor conditions is becoming available [15-17]. These studies often consist of a first round of
697 laboratory screening for comparatively assessing the growth rate, the lipid productivity and the FA
698 composition of several species prior to testing the best performers in larger scale outdoor
699 photobioreactors or raceway ponds. The use of Nile Red staining of microalgal lipids combined
700 with flow cytometry is a powerful tool in identifying the algae with a high lipid content [18]. While
701 several microalgal species so far tested, possess the suitable lipid productivity and FA composition
702 for producing biodiesel to conform to most fuel standards, no single species appears capable of
703 meeting all requirements for a top grade biodiesel. Attaining a good grade of biodiesel may require
704 mixing lipids from different species [19].

705 Another important criterion for selecting microalgae for biodiesel production is the ease of
706 harvest. Harvesting costs can contribute up to 20 to 30% of the total cultivation cost [5]. Therefore,
707 microalgal biodiesel production must use cost-effective harvesting methods such as settling and
708 flocculation to keep the cost of production of the biodiesel to a minimum [20]. Some of the
709 microalgae that have been identified as having a high lipid content have been harvested using low-
710 cost methods. Microalgae such as *Tetraselmis, Chlorella* and *Scenedesmus* settle naturally under
711 suitable conditions, while species such as *Nannochloropsis* can be harvested using various flotation
712 or flocculation techniques [21]. Nevertheless, it may be useful to specifically select a microalga that
713 is easy to harvest.

714 Screening for strains with a high tolerance to extreme environmental conditions (e.g. a high
715 pH and/or salinity) may be useful. In an outdoor setting, particularly in open ponds, contamination
716 by grazers and other undesirable microalgae can be a difficult problem. A high-tolerance microalga
717 would not only better withstand the variable environmental conditions, but its culture environment
718 could be deliberately altered to reduce the potential for contamination. A certain level of salinity
719 tolerance is necessary also for a freshwater strain because evaporation of freshwater does increase
720 salinity over time. Finally, the ease of extraction of the oil from different strains can be quite
721 different [48]. Therefore when screening for strains, the availability and cost implications of certain
722 oil extraction methods in relation to a particular strain must be taken into account. For example,
723 *Nannochloropsis* sp. is generally regarded as one of the highest TAG-accumulating algae [15, 16],
724 but its tough cell wall may require more costly pretreatments for efficient oil extraction [49], thus
725 making it more suitable as a feedstock for high-value products such as omega-3 oil and not
726 biodiesel.. Table 1 summarizes some of the desirable traits in a microalga intended for biodiesel
727 production. Although a "perfect" microalga does not exist, the species selection must consider the
728 issues relating to cultivation, harvesting and extraction.

729

730 Table 1 - Desirable traits of a microalga intended for biodiesel production.

731

Selection consideration	**Desirable traits**
Initial screening	Local strain Rapid growth High extractable oil contents High saturated fatty acids, low unsaturated fatty acids Recoverable by settling or foam flotation
Outdoor cultivation	Rapid and dominant growth Salinity tolerance High/low temperature tolerance Ability to control grazers High light tolerance

	Shear resistance
Harvesting	Cells that autoflocculates or settle at time of harvesting (this may coincide with nutrient depletion/lipid accumulation) Cells amenable to foam flotation
Extraction	Cells amenable to easy extraction High lipid recovery
Added benefits	Rapid and synchronized lipid production (high lipid inducibility) Utility of the microalgal cake after oil extraction (e.g. high protein contents for food/feed; presence of omega-3 fatty acids, antioxidants, sterols, carotenoids, astaxanthins and other pigments) for added value

732

733 **Microalgal strain improvements: Non-GM method**

734

735 No matter how robust a selection and screening process, it is rare to find an alga that meets all the
736 main criteria for biodiesel production, in particular the criteria relating to large-scale operations. For
737 example, many microalgae that are easy to harvest (e.g. *Tetraselmis, Dunaliella*) do not have as
738 high a lipid content as *Nannochloropsis,* which is difficult to harvest and rupture. Nevertheless,
739 microalgae are excellent candidates for molecular improvement, be it via GM or non-GM methods.
740 Firstly, they have short cell division times (hours to days) that reduce development time. Secondly,
741 their small size and unicellular nature excludes the need for large breeding programs and reduces
742 cost. Thirdly, ultraviolet (UV) light and chemical mutagens can be easily applied to microalgae.
743 Fourthly, microalgae can be selected and screened using traditional screening methods (e.g.
744 antibiotics) as well as automated high-throughput techniques.

745

746 The above mentioned non-GM method of mutagenesis followed by high-throughput selection are
747 commonly used for improving microalgal strains. The advantages of the non-GM methods are that
748 they require little or no knowledge of the biochemistry and genetics of the microalgal strain being
749 improved and avoid the regulatory complications associated with the use of GM strains outdoors. In
750 combination with the above noted methods, the use of Nile Red as a fluorescence probe for
751 detecting neutral lipids is common [22]. Correlations between the Nile Red fluorescence signal and

752 the TAG content have been established for some microalgal species [23]. Some of the traditional
753 improvement strategies of mutagenesis (e.g. the use of antibiotics and herbicides for selection) and
754 subsequent selection of mutants using time-consuming analyses (e.g. gas chromatography, thin
755 layer chromatography) were slow. Such studies typically achieved a yield improvement of between
756 10 to 40% and were limited mostly to two to three rounds of mutation-selection (Table 2). More
757 recently, Nile Red-staining combined with high-throughput FACS has allowed to accurately sort
758 through millions of cells and select individual cells with a high lipid content. FACS has enabled
759 isolation of cells with lipid levels of ≥60% DW in some cases without mutagenesis (Table 2).

760

761 In addition to UV light, chemical mutagens (ethyl methane sulfonate, EMS;
762 nitrosomethylguanidine, NTG; *N*-methyl-*N*-nitrosourea, MNU, have been successfully used with
763 various microalgal species (Table 2). In some of these studies, selection just for the high lipid cells
764 produced mutants with reduced growth rates [10], emphasizing the importance of a growth selection
765 step to ensure that strains maintain a high growth rate while producing a high level of lipids [24,
766 25]. A further side effect of repeated mutation-selection has been a change in the FA content. An
767 elevation of the PUFAs has been found in mutants relative to wild-types [26-29]. Rapid automated
768 screening combined with conventional mutagenesis make this non-GM improvement approach
769 attractive. These approaches combined with advances in transcriptomics could in the future help
770 reveal potential targets for genetic engineering.

771 Table 2 – Some microalgae mutation studies

Species	Mutagen	Selection method	Yield increase	Author
Phaeodactylum tricornutum	UV	survival	37-44% EPA[a]	Alonso et al. 1996
Pavlova lutheri	UV	survival	10-20% TFA[b]	Meireles et al. 2002
Nannochloropsis oculata	MNU	quizalofop	17-20% TFA[a]; 5-18% EPA[a]	Chaturvedi et al. 2004
Nannochloropsis oculata	EMS	antibiotics	14-22% TFA[a]; 12-29% EPA[a]	Chaturvedi & Fujita 2006
Haematococcus pluvialis	UV, EMS, NTG	survival	23-59% total carotenoid[c]	Kamath et al. 2008
Schizochytrium sp.	UV, NTG	selective media	35% TFA[b]	Lian et al. 2010
Tetraselmis suecica	none	FACS	400% FS	Montero et al. 2011
Nannochloropsis sp.	none	FACS	300% FS	Doan & Obbard 2011
Chlorella sorokiniana	UV	plate reader	30-40% TLC[b]	Vigeolas et al. 2012
Nannochloropsis sp.	EMS	survival-growth	13-26% TLC[d]	Anandarajah et al. 2012
Isochrysis galbana	UV	FACS	60% TLC[b]	Bougaram et al. 2012

772

773 TFA – total fatty acid

774 TLC – total lipid content

FS – fluorescence signal

b - % DW

d – mg/(L day)

Microalgal strain improvement: GM-methods

Genetically-modified microalgae are attracting a lot of interest, with a focus on developing new highly efficient strains. Unlike random mutagenesis followed by screening, developing transgenic microalgae requires a comprehensive knowledge of genomics, transcriptomics and the metabolic pathways for identifying the target genes for engineering. In addition, tools are required for gene manipulation, including selectable markers, vectors and techniques for systemic insertion in screening libraries [11]. The list of fully sequenced microalgal genomes in public databases (Phytozome, Joint Genome Institute, NCBI) continues to grow. This provides a valuable tool for annotating transcriptomic data and identifying the key genes in various metabolic pathways. While genomic data provide us with what an organism is potentially capable of doing, transcriptomics, metabolomics and proteomics reveal what pathways are currently active/suppressed with respect to a specific situation [30]. As the cost of pyrosequencing reduces, an increasing amount of transcriptomic data is becoming available. For the production of biofuels, pathways that are linked to lipid accumulation are of particular interest. These pathways have been studied in species such as *Dunaliella tertiolecta* [31], *Haematococcus pluvialis* [32], *Phaeodactylum tricornutum* [33], *Neochloris oleoabundans* [34], *Chlorella vulgaris* [35], and *Chlamydomonas reinhardtii* [36, 37]. These studies have successfully reconstructed pathways for FA, TAG, starch biosynthesis, FA β-oxidation, TAG catabolism, and starch degradation. These pathways exhibited differential expression during lipid accumulating conditions such as nutrient starvation. Genes involved in the basic metabolic pathways such as ribosome biogenesis, the peptide metabolic processes and RNA processing were upregulated during the stationary phase after nutrient depletion, suggesting an enhanced basal metabolism is required to cope with depleting nutrients [37]. On the other hand, genes related to photosynthesis were down-regulated during nutrient starvation [37]. This was followed by upregulation of lipid metabolism and membrane related genes during the lipid accumulation phase [37], pointing to possible lipid reshuffling during this stage. Examination of transcript abundance during different stages of lipid accumulation revealed multiple carbon fixation

806 pathways, suggesting that a buildup of enzyme precursors may play a more important role in lipid
807 biosynthesis than the actual enzyme levels themselves [33].

808

809 While transcriptome studies do not directly contribute to strain improvement, they identify the key
810 pathways and genes that could be the targets of genetic engineering. Genes such as ACCase (acetyl-
811 CoA carboxylase), DGAT (diacylglycerol acyltransferase) and CiS (citrate synthase) have been
812 identified this way and manipulated to increase lipid production. The overexpression of an ACCase-
813 encoding gene in the diatoms *Cyclotella cryptica* and *Navicula sapuvila* resulted in an increased
814 enzymatic activity, although no increase in lipid content was detected [38].

815

816 The silencing of a CiS-encoding gene in *C. reinhardtii* increased TAG production by 169% [39],
817 while the overexpression of DGAT2 in *P. tricornutum* increased its neutral lipid content by 35%
818 [40]. Aside from lipid-related pathways, the improvement of the microalgal photosystems has also
819 been the focus of much interest. This is because of the ~43% of the solar energy captured via
820 photosynthesis only 4-8% is converted into biomass [1]. This may be improved , for example, by
821 reducing the total light-capture antenna size to minimize the energy loss in a culture by selfshading
822 and non-photochemical quenching. This has been achieved by reducing the levels of light
823 harvesting complex (LHC) I and LHC II mRNAs and proteins [41] and also by reducing the size of
824 the photosystem II (PSII) antenna [42]. In both cases the growth rates of the transgenic algae were
825 significantly increased, with the transgenic strains achieving higher cell densities when grown in
826 large-scale bioreactors.

827

828 **Conclusion**

829

830 Advances in microalgae breeding by strain selection and improvement represent the tip of the
831 iceberg with regard to the overall effort required for making microalgae biodiesel production
832 economically viable. Compared to commercial land crops, barely any effort has gone into selection
833 and breeding of microalgal species. Similarly, compared to the petroleum industry, production of
834 algal fuels has had an extremely short developmental history. Therefore, there is much scope for
835 improving all aspects of production of algal fuels.

Chapter Summary

- Microalgal strain selection has key implications on every other aspect of production of algal biodiesel.
- Key factors for selecting microalgae are a high productivity of extractable lipids and ease of harvest.
- Laboratory screening must always be followed by larger scale outdoor testing to ensure selection of a suitable species for commercial production.
- Non-GM methods for strain improvement such as mutation-selection programs are highly effective and quite rapid.
- GM methods are focused on improving photosystems, but increasing transcriptomics studies have identified key genes for genetic modification.

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985 **Review Paper 2: The race for highly productive microalgae strains**

PRIORITY PAPER EVALUATION

The race for highly productive microalgae strains

Biofuels (2010) 1(6), 835–837

Evaluation of: Huerlimann R, de Nys R, Heimann K. Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. *Biotechnol. Bioeng.* 107(2), 245–257 (2010).

Microalgae-derived biodiesel is considered a highly promising next-generation biofuel, as current fossil fuel reserves are being depleted and combusted causing GHG emissions. Microalgae are arguably the most efficient renewable biofuel source to cover our current and future demand without competing for arable land or natural areas rich in biodiversity. Microalgae are sunlight-driven organisms that convert CO_2 to starch and a variety of lipids with applications such as biofuels, foods, animal feeds and high-value bioactive products. The critical parameters affecting species selection for biodiesel production are growth rate, lipid content, lipid productivity and fatty acid composition. Previous research papers and review articles commonly discuss growth parameters of single species, but few report a comprehensive comparison of several microalgae and provide data for lipid productivity in terms of g m^{-2} day^{-1} as done by Huerlimann *et al.* This allows for a direct comparison of microalgal lipid productivity to terrestrial production systems; thus making comparisons between volumetric and area productivity of lipids possible, which is necessary for large-scale production. Distinctively, Huerlimann *et al.* also study the effects of different growth media on lipid production and composition during different growth phases.

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Summary of methods & results

Huerlimann *et al.* have compared biomass **productivity** P_{DM} (factoring in **growth rate** and dry biomass contents over time) and **lipid productivity** (P_{DM} × **lipid content**) as well as **fatty acid composition** for four different microalgal cultures using three different media (L1, f/2 and K medium) and also at different growth phases [1]. All algae were cultured as triplicates and 1 g of wet biomass from different media and at different growth stage was sampled for biomass and fatty acid analysis. Specific growth rates of cultures were monitored by modified methods mentioned in Wood *et al.* and biomass and lipid productivities were determined based on volume and production area [2].

Amongst the four different cultures tested, *Nannochloropsis* exhibited the fastest growth rate followed by *Rhodomonas*, *Isochrysis* and *Tetraselmis*. Amongst the three different media used, L1 medium appears to have some advantages on the growth rate of *Nannochloropsis* and *Isochrysis* as it contains trace elements. K medium performed less well due to a lack of inorganic phosphate, although it appeared to be the preferred medium for *Tetraselmis*. The maximum cell densities reported for *Nannochloropsis* and *Isochrysis* were similar to those reported by Dunstan *et al.* [3], but were approximately five-times higher than the cell density reported by Renaud *et al.* [4].

The growth phase and medium played a crucial role on lipid content of microalgal species. *Nannochloropsis* had the highest overall lipid content in all three different media types. Lipid content was generally the highest during the late logarithmic and stationary growth phase. Interestingly, the highest biomass productivity

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Productivity (PDM [g m^{-2} day^{-1}]): The amount of dry mass produced by microalgae in 1 day. Calculated using the growth rate (μ [day^{-1}]), dry matter (DM [g m^{-3}]) and the diameter (d) of the carboys (used to grow the algae), PDM = DM × μ × d.

Growth rate (μ [day^{-1}]): The increase of microalgae cells over time. Calculated in Huerlimann et al. using the equation μ = LN(Ny/Nx)/(ty-tx) (Wood et al., 2005). Ny and Nx being the number of cells (N) at the start (tx) and the end (ty) of the logarithmic growth phase.

Lipid productivity (PL [g m^{-2} day^{-1}]): The amount of lipids produced by microalgae in 1 day. Calculated using lipid content (LC [%]) and productivity (PDM [g m^{-2} day^{-1}]), PL = PDM × LC/100% (g m^{-2} day^{-1}).

Lipid content (LC [%]): The weight of lipids contained in a sample of microalgae over the total dry weight of the sample.

Fatty acid composition: Here referred to fatty acid profiles obtained by gas chromatography – mass spectroscopy analysis of fatty acid methyl esters derived from triacylglycerides.

was observed for *Tetraselmis* due to its large cell size, although it showed the lowest specific growth rate. Similarly, the highest lipid productivity was also found for *Tetraselmis*. Growth phases and media also resulted in some differences for the fatty acid composition in this study. In *Tetraselmis,* palmitic acid, oleic acid, linoleic acid and α-linoleic acid comprised 85 to 90% of total lipids, while for *Rhodomonas*, palmitic acid, α-linoleic acid, stearidonic and eicosapentaenoic acid (EPA) accounted for 60 to 75% of total lipid content. The main fatty acids for *Nannochloropsis* were palmitic acid, palmitoleic acid and EPA, while *Isochrysis* contained mostly myristic, plamitic, oleic and stearidonic acid, as well as docosahexaenoic acid (DHA). When considering lipid productivity (factoring in biomass productivity and lipid contents), *Tetraselmis* was considered to be the best species for scale-up production in this study.

Discussion

Algae biofuels have become a hot topic and recent literature in this area has increased exponentially. However, one in five papers on this topic are currently review papers (35 in 2009 and 2010 alone) and a relative shortage of primary research publications is apparent. The study by Huerlimann *et al.* represents one of the first papers written with lipid productivity for biofuel production in mind [1]. In literature, lipid productivity is usually reported in mg l^{-1} day^{-1} [5,6]. Data in Huerlimann *et al.,* however, were reported in both mg l^{-1} day^{-1} and g m^{-2} day^{-1} [1]. Productivity represented in terms of g m^{-2} day^{-1} allows for aquatic productivity to be directly compared with terrestrial production systems [1], and thus appears better for evaluating a species' suitability for large-scale biofuel production. The Huerlimann *et al.* method of reporting data, incorporating cell densities, growth rates, dry weight, lipid contents and fatty acid composition, represents a good template for future biofuel research papers [1].

There have been many studies investigating the lipid contents and fatty acid profiles of various microalgae during the late logarithmic or stationary growth phase using a single medium (e.g., Martines-Fernandez *et al.* [7], Natrah *et al.*[8]; Renaud *et al.* [4] and Zhukova and Aizdaicher [9]). However, the Huerlimann *et al.* study aims not only to quantify the effects of growth media, but investigates the effects of growth media and growth phase simultaneously on both the qualitative and quantitative lipid content of microalgae [1]. Data presented in this paper reveal some differences for each microalga's nutrient requirements, but also emphasise the importance of understanding these nutritional needs in order to maximize productivity.

Aside from growth requirements, Huerlimann *et al.* reveal the effect of growth phase on both total lipid content and fatty acid composition [1]. These data combined with growth media requirements not only allow growth for maximum lipid content, but also the possibility of selecting optimal media and harvesting times to obtain a desired fatty acid, for example, DHA or EPA. To this end, the study by Huerlimann *et al.* investigated the suitability of microalgae not only as a producer of biofuel, but also of other valuable products, including biomass for animal/aquaculture feedstock, nutraceuticals and alternative lipids, such as omega-3 fatty acids [1]. Data presented by this study aids in the objective evaluation of species' suitability for biofuel production and provide insight into microalgae's potential as future biorefineries.

Something not revealed by Huerlimann *et al.* is the identity of the microalgal species used [1]. The use of the same genus, but possibly different species, as well as different culture conditions may explain the differing results between Huerlimann *et al.* [1] and other studies. Lipid productivity has been shown to be different between different microalgae species and even isolates of the same species [6,10]. As research into lipid production for biofuel production increases, Huerlimann *et al.* represents a potential benchmark study that introduces methods of obtaining and reporting data in a way that is relevant for industry-scale production [1]. Unfortunately, a standard method of culturing algae to obtain and calculate lipid productivity data does not exist, which makes direct comparison between different studies difficult. Examples of such standardized protocols are antibiotic resistance testing established by the Clinical and Laboratory Standards Institute and the International Organization of Standardization. Beal *et al.* recently published a framework to report the production of renewable diesel from algae [11].

Future perspective

Research interest into microalgal lipid production for biofuels is at an all time high, with a whole range of studies from growth optimizations [6,12] to induced mutagenesis of microalgae to improve lipid yield [13,14]. It can be envisaged that careful strain selection and improvements of microalgae for a variety of useful traits hold a lot of promise, and can be compared with efforts in conventional agricultural crop breeding.

Current bottlenecks for large-scale cultivation appear to be in harvesting/extraction processes as well as cheap and energy efficient cultivation systems. Commercial production of biodiesel from algae depends on:

- Lipid productivity in industrial scale cultivation systems;
- Production costs;
- The energy ratio of production.

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Executive summary

- Authors compare growth rate, productivity, lipid content and fatty acid composition for four different microalgae cultures at different growth phases using three different growth media.
- The four microalgae tested are *Nannochloropsis* sp., *Tetraselmis* sp., *Isochrysis* sp. and *Rhodomonas* sp. All four microalgae play an important role in the aquaculture industry as feedstock and are believed to have potential as biofuel producers.
- *Tetraselmis* sp. was found to have the best dry mass and/or lipid production profile in large-scale cultures, producing 4.8 g m^{-2} day^{-1}.
- Lipid productivity reported in terms of g m^{-2} day^{-1} makes direct comparisons with terrestrial production systems possible.
- Data reported reveal the importance of understanding a species' nutritional requirements to maximize productivity.

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Review Paper 3: Perspectives on metabolic engineering for increased lipid contents in microalgae

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REVIEW

Perspectives on metabolic engineering for increased lipid contents in microalgae

Biofuels (2012) 3(1), 71–86

Holger Schuhmann, David KY Lim & Peer M Schenk*

With increasing concerns about the world's crude oil consumption, alternative fuels based on renewable resources attract more and more attention. Microalgae have been proposed to be one of the most sustainable feedstocks for the production of lipid-based biodiesel. Naturally occurring, high-lipid producing microalgae strains can be domesticated and further genetically improved in order to redirect metabolite fluxes towards increased lipid contents. This review summarizes the current knowledge about metabolic engineering of microalgae in order to increase the cellular lipid content, with an emphasis on triacylglycerols for the production of biofuels. Additionally, it outlines the contribution of systems biology and genome-scale metabolic pathway modeling, as well as their potential impact in the future.

Our modern society currently depends on crude oil as a basis for transportation fuels and building blocks for the chemical industry. Nevertheless, the growing demand and cost of petroleum-based fuel, as well as concerns about anthropogenic climate change, sparked a search for alternative, renewable sources of transportation fuel. Photosynthetic organisms are able to convert light energy from the sun to chemical energy and store it in their biomass, which can provide a feedstock for biofuels. Methylesters of **fatty acids** (FA), the latter being building blocks of many lipids, possess properties similar to conventional diesel (reviewed by [1]), and ethanol produced by fermentation of starch and sugars can partially or totally substitute petrol [2]. Therefore, photosynthetic organisms containing high amounts of lipids and sugars/starch became a major focus of attention as potential feedstock. Today, plants producing oil- and starch-rich seeds, such as rapeseed (*Brassica napus*), soy bean (*Glycine max*), oil palm (*Elaeisguineensis*) and corn (*Zea mays*), or contain high sugar contents, such as sugar cane (*Saccharum* sp.), form the main feedstock for the production of so-called first-generation biofuels [3]. In 2008, the total global production of biofuel for transportation purposes was estimated to be at 64.5×10^9 l for ethanol and 11.8×10^9 l for biodiesel. For ethanol, this corresponds to a share of 5.46% on global gasoline-type fuels, whereas biodiesel constitutes for 1.5% of the global diesel production [4]. However, deforestation, over-use of fertilizers and pesticides and GHG emissions from crop fields have raised doubts over the sustainability of biofuel production from these crops (for examples, see [5,6]). Additionally, first-generation biofuel plants compete with food crops for arable land and fresh water, contributing to increased food prices for a growing world population. Production of second-generation biofuels, such as biodiesel from waste cooking oil and ethanol from lignocellulose, aims to circumvent these problems. However, supply of waste cooking oil is insufficient to satisfy the world's biodiesel demand, and lignocellulosics still require further optimization. Today, microalgae are considered as one of the most promising lipid sources for the production of biodiesel, giving rise to the third generation of biofuels [7].

Compared to traditional fuel crops, microalgae possess several advantages, which have been more comprehensively reviewed elsewhere [8]. For example, microalgae have much shorter life cycles (~1–10 days) than plants, which allows for multiple, or even continuous, harvests during a year. Additionally, they possess much higher light conversion rates; that is, less area is needed

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Key terms

Fatty acid: Naturally occurring carboxylic acid with a long carbon chain tail. This tail can be more than 20 carbon atoms long and contains one or multiple double bonds (unsaturated fatty acids).

Triacylglyceride: Main storage lipid for many organisms. Three fatty acids are covalently linked to the three alcohol groups of glycerol via ester bonds, forming the acyl moieties of the triacylglycerol molecule.

Calvin cycle: Metabolic pathway that incorporates inorganic carbon, that is, CO_2, into biomolecules at the expense of energy generated by photosynthesis. Rubisco, the most abundant protein on the planet, is responsible for the carboxylation of ribulose-1,5-bisphosphate, and thereby is the entry point of CO_2 into the metabolism.

Lipases: Enzymes with esterase functionality; that is, they hydrolyse ester bonds in lipids, usually triacylglyceride. The end products of this reaction are a free fatty acid and an alcohol molecule, such as a di- or mono-glyceride, or glycerol.

for the production of the same biomass as compared with traditional biodiesel crops [8,9]. Furthermore, they do not need to compete with food crops for precious arable land, since pools, tanks and bioreactors for the growth of microalgae can be placed on industrial waste land, deserts and other areas unsuitable for food production, as long as water (fresh, brackish or saline) is present [9]. Microalgae can also be grown in municipal or industrial wastewater, and some strains prefer seawater, thereby sparing precious fresh water resources [10].

Lipids & microalgae

The term 'microalgae' is not a biological, but rather a practical, description, and its scope may differ depending on the context and the author. In its widest definition, microalgae are unicellular, photosynthetic microorganisms from several more-or-less distantly related branches of the tree of life, comprising, for example, prokaryotic cyanobacteria, eukaryotic green algae, red algae and heterokonts (e.g., brown algae and diatoms) (Figure 1) [11]. Of all these taxonomic groups, green algae, diatoms and other heterokonts have attracted the most attention as potential lipid producers, providing possible feedstock for the production of biodiesel [11]. Table 1 presents a selection of some species considered for biofuel production.

This wide range of organisms provides researchers with a large number of metabolic pathways leading to a variety of different lipids. Generally, a lipid is defined as a small, hydrophobic or ambiphilic molecule that is biosynthesized either by condensing thioesters (e.g., Acyl-CoA) or isoprene units [12]. Based on their physiochemical characteristics, they are divided into polar lipids such as phospholipids and glycolipids, and neutral lipids such as mono-, di- and **tri-acylglycerides** (TAG) and carotenoids [9]. Some of the substances produced in microalgae have been intensively studied, not only as biofuel feedstock, but also as beneficial food additives and other high-value products. Some of these are already on the market or close to commercial production. For example, long-chain polyunsaturated FAs (LC-PUFA), such as eicosapentaenoic acid (20:5ω-3) and docosahexaenoic acid (22:6ω-3) are an important part of the human diet and necessary for proper brain function and the prevention of cardiovascular diseases [13]. The most important source for LC-PUFA is fish oil. However, the depleted fish stocks of our oceans are unable to satisfy the increasing demand of a growing world population, and microalgae were suggested as an alternative source for this important food supplement (reviewed by [13]). Another important class of products already commercially exploited are carotenoids, terpenoid pigments with a backbone derived from the condensation of isoprene units. β-carotene, astaxanthin, lutein and other carotenoids are used by the pharmaceutical and food industry as highly valued antioxidants and colorants, mainly obtained from microalgae, such as *Dunaliella salina*, *Chlorella* sp., *Chlamydomonas reinhardtii*, *Muriellopsis* sp. and *Haematococcus pluvialis* [14].

Another microalga that has attracted much attention due to its ability to synthesize a wide range of unusual lipids is *Botyrococcus braunii*, a colony-forming green microalga that produces a variety of hydrocarbons and ether lipids comprising up to 37 carbon atoms. These substances were suggested to be one of the main original sources of fossil mineral oil [15], and can be converted to combustible products using standard petrochemical techniques [16]. However, growing *B. braunii* on a large scale has encountered problems due to the slow growth of this alga [15], and products synthesized by *B. braunii* have not entered the market so far. Due to these problems and established biodiesel production procedures from FA in plant oils [2], the main focus of research on microalgal lipids for biodiesel production so far have been on neutral storage lipids such as TAGs. Therefore, this review will provide an overview of the different metabolic engineering efforts that have been undertaken to increase the TAG content of various eukaryotic, photosynthetic organisms.

Brief overview of the TAG metabolic pathways

TAGs are usually synthesized by an organism as a way to store energy for the future and to build up a reservoir of membrane building blocks. This occurs in oil crops such as rape seed, as well as in the model organism *Arabidopsis thaliana*, which store TAGs in their seeds as an energy source for the emerging seedling before it can support itself via photosynthesis. Microalgae accumulate TAGs under stress conditions such as N and P limitation [9], when a carbon source (e.g., CO_2) and light are still available in abundance. As a result, the lipid content of a cell can reach up to 77% for some microalgal species [17]; however, a lipid content of 50% is usually considered in optimistic economic scenarios [8].

Most of our knowledge about lipid metabolism in photosynthetic organisms is derived from higher plants, especially *A. thaliana* (summarized in Box 1). However, with an increasing amount of genomic and transcriptomic data from microalgae, it was possible to

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identify algal counterparts of plant genes involved in lipid metabolism. This suggests that central metabolic pathways in lipid metabolism, as presented in Figure 2 based on data for higher plants, are conserved within photosynthetic organisms.

The energy and basic building blocks necessary for the biosynthesis of FAs and TAGs are provided by photosynthesis and the fixation of inorganic carbon in the form of CO_2 in the **Calvin cycle** [18]. Manipulation, that is, metabolic engineering of these pathways, could alter the availability of FA precursors for an organism, thereby affecting its ability to produce and store TAGs.

In photosynthetic organisms, *de novo* synthesis of FAs mainly occurs in the plastids [19]. It starts with the carboxylation of acetyl-CoA (Figure 2), which is the rate limiting step of FA biosynthesis and is catalyzed by a multi-enzyme complex called acetyl-CoA carboxylase (ACCase), yielding malonyl-CoA at the expense of one molecule of ATP (Figure 2). Malony-CoA is then transferred to an acyl carrier protein (ACP) and subsequently enters the FA elongation cycle, where in each cycle the growing acyl-ACP chain is condensed with another malonyl-CoA molecule at the expense of CO_2. Each turn of the cycle elongates the growing acyl moiety by two carbon atoms, eventually reaching 16 (palmitoyl-ACP. 16:0-ACP) or 18 (stearoyl-ACP, 18:0-ACP) carbon atoms in length, when it is released from the ACP by a thioesterase, which in some cases is preceded by a desaturation step. Alternatively, the acyl chain can be transferred to glycerol-3-phosphate and stay in the chloroplast, where it can be subjected to additional elongation or desaturation reactions [19], rendering it unavailable for the biosynthesis of TAG. Additionally, evidence was presented for a *de novo* pathway for TAG biosynthesis in the chloroplasts of *C. reinhardtii* [20], although genetic and enzymatic data is not available so far.

Once transported outside the chloroplast, FAs enter the endoplasmic reticulum as acyl-CoAs (Figure 2). Here, they can undergo a variety of modifications such as further elongation and desaturation, before becoming incorporated into a wide range of different lipids. These lipids, including TAGs, phosphatidylcholine and phosphatidylethanolamin, can exchange acyl moieties via acyltransferases. However, in TAG *de novo* synthesis, an acyl group is transferred to diacylglycerol by acyl-CoA:diacylglycerol acyltransferase (DGAT), which is unique to this pathway and regarded as a bottleneck in TAG biosynthesis (Figure 2). Finally, TAGs are transported to lipid bodies or droplets, where they are stored as a form of FA supply until needed by the cell [9,19,21,22]. Although most of our knowledge about the biosynthetic pathways of FA and TAG is derived from studying higher plants, emerging data, especially from the analysis of

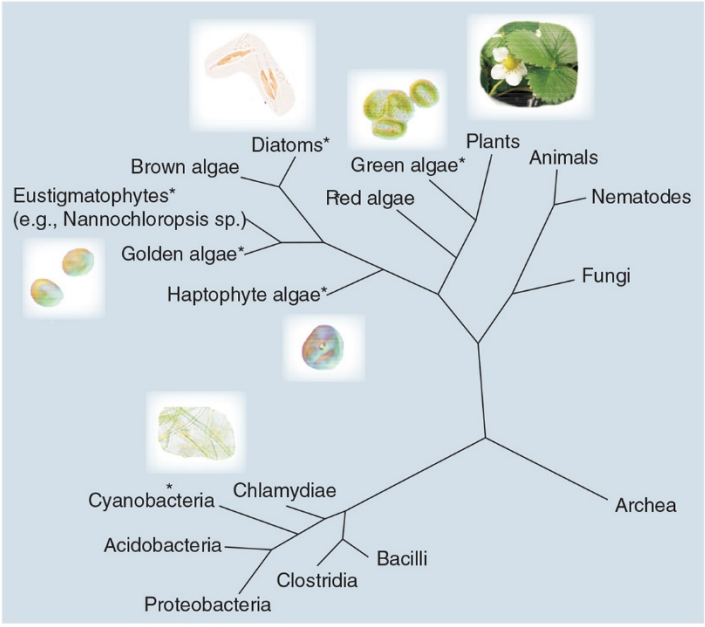

Figure 1. Schematic representation of the tree of life, with an emphasis on microalgae and their relationship to other organisms. Green algae, red algae, diatoms, golden algae, eustigmatophytes and haptophyte algae are microalgae in a stricter sense. A broader definition of microalgae inlcudes prokaryotic cyanobacteria. Microalgae according to this definition are indicated by asterisks.

sequenced genomes, suggests that the major pathways as presented in Figure 2 are present not only in green algae, but also in red algae and heterokonts, such as diatoms. This is supported by the biochemical characterization of an increasing number of lipid biosynthesis related enzymes from microalgae, particularly *C. reinhardtii*, and *Thalassiosira pseudonana* (reviewed in [21]).

Cells store TAGs mainly as energy reservoirs, building block pools and scavenger molecules against reactive oxygen species, degrading them at times when additional energy and membrane precursors are needed. Thus, targeting the TAG degradation pathway by metabolic engineering may prove to be useful in order to increase the amount of lipids the microalgal cell contains at the time of harvest. The main pathway by which organisms degrade FA, and consequently TAG, is the β-oxidation pathway. This pathway, which resides in the peroxisomes and mitochondria in mammals, is exclusively localized to the peroxisomes in plants and most fungi (Figure 2) [23].

The first step in the breakdown of TAG is the deacylation in the lipid body by the subsequent action of tri-, di- and mono-acylglycerol **lipases** [22]. Despite the importance of this step in storage lipid mobilization, the knowledge about lipases in plants (and even more so in

Table 1. Examples of microalgal species used in biofuel research, as well as fully sequenced microalgal genomes.

Organism	Genome database	Genome Size	Successful DNA transformation	Ref.
Cyanobacteria	Cyanobase [201]			[87]
Green algae				
Botyrococcus braunii				[88]
Chlamydomonas reinhardtii	Photozome [202]	112 Mb 17,114 genes	N, C	[26,89]
Chlorella protothecoides				[90]
Chlorella pyrenoidosa				[91]
Chlorella variabilis NC64A	Joint Genome Institute [203]	64 Mb 9791 genes		[27]
Chlorella vulgaris			N	[92–94]
Chlorococcum littorale				[95]
Choricystis minor				[96]
Dunaliella tertiolecta			N	[97,98]
Dunaniella salina			N	[91,99]
Micromonas pusilla	Joint Genome Institute [204]	21.9 Mb 10,575 genes		[28]
Micromonas sp. RCC299	Joint Genome Institute [205]	20.9 Mb 10,056 genes		[28]
Ostreococcus lucimarinus	Joint Genome Institute [206]	13.2 Mb 7651 genes		[29]
Ostreococcus tauri	Joint Genome Institute [207]	12.6 Mb 7892		[30]
Oocystis sp.				[100]
Tetraselmis suecica				[91]
Volvox carteri	Phytozome [208]	138 Mb 14,491 genes		[31,101]
Haptophyte algae				
Isochrysis zhangjiangensis				[102]
Isochrysis galbana				[91]
Heterokont algae (diatoms and others)				
Amphora sp.				[100]
Chaetoceros muelleri				[103]
Nannochloropsis oculata			N	[93,104]
Phaeodactylum tricornutum	Joint Genome Institute [209]	27.4 Mb 10,402 genes	N, C	[32,105,106]
Thalassiosira sp.	Joint Genome Institute [210]	32.4 Mb 11,776	N	[33,107,108]
Red algae				
Cyanidioschyzon merolae	*Cyanidioschyzon merolae* Genome Project [211]	16.5 Mb 5331 genes	N	[34,109]

For a comprehensive list of sequencing projects, including mitochondrial and plastidial genomes, see Lu *et al.* [7].
C: Successful chloroplastidial transformation; Mb: Megabases; N: Successful nucelar transformation.

algae) is sparse, with very few enzymes characterized so far [24]. Upon their release from the glycerol moiety, FAs are imported into the peroxisome where they are covalently linked to CoA by a set of acyl-CoA synthetases. The acyl group is then subjected to a series of oxidation, hydration, dehydrogenation and nucleophilic attack reactions, which shorten its carbon backbone by two atoms [23]. This cycle of reactions is repeated until the FA chain is completely degraded (Figure 2). Compared to what is known about the biosynthesis of TAGs in

Box 1. Fatty acid and triacylglycerol metabolism of photosynthetic cells.

- Most of our knowledge about fatty acid (FA) and triacylglycerol (TAG) metabolism in photosynthetic organisms is gained from work on higher plants. However, there is increasing evidence that the overall pathway, as presented here, is also present in microalgae.
- The energy and basic building blocks necessary for the biosynthesis of FAs and TAGs are provided by photosynthesis [110]. In the light-dependent reaction, the absorption of photons drives the flow of electrons from the oxygen-evolving complex to the photosystem II, to photosystem I, and eventually to $NADP^+$, creating a proton gradient that is used by the chloroplastidial ATP synthase to produce ATP. Both NADPH and ATP are used in the Calvin cycle, where inorganic carbon in the form of CO_2 is incorporated as glyceraldehyde 3-phosphate. In photosynthetic organisms, *de novo* synthesis of FAs starts with the carboxylation of acetyl-CoA in the chloroplast. Acetyl-CoA can be derived from a number of metabolic pathways, such as cytosolic and plastidial glycolysis, and the tricarboxylic acid cycle. It is catalyzed by a multi-enzyme complex named acetyl-CoA carboxylase (ACCase), yielding malonyl-CoA at the expense of one molecule of ATP. Plastidial ACCase is similar to the prokaryotic forms of ACCases and consists of four individual polypeptide chains, that is, the biotin carboxyl carrier protein, the biotin carboxylase and the α and β-subunits of carboxyltransferase [111]. Malonyl-CoA:acyl carrier-protein (ACP) transacylase then transfers the malonyl group from CoA to an acyl-carrier protein, from where it enters the FA synthesis cycle [19]. In the first step of this cycle, malonyl-ACP is condensed with acyl-CoA by 3-ketoacyl-ACP synthetase, a reaction rendered irreversible by the release of one molecule of CO_2. The resulting 3-ketoacyl-ACP is then reduced by 3-ketoacyl-ACP reductase using NADPH, dehydrated by 3-hydroxyacyl-ACP dehydratase, and finally reduced again by enoyl-ACP reductase, yielding butyryl-ACP. This molecule enters the cycle again as it is condensed with another molecule of malonyl-CoA. In each reaction cycle, the growing acyl chain is elongated by two carbon atoms, eventually reaching 16 (palmitoyl-ACP. 16:0-ACP) or 18 (stearoyl-ACP, 18:0-ACP) carbon atoms in length, when it is either released from the ACP by an acyl-ACP thioesterase, or desaturated by stearoyl desaturase and subsequently released by an acyl-ACP thioesterase. Alternatively, the acyl chain can be transferred to glycerol-3-phosphate and stay in the chloroplast, where it can be subject to additional elongation or desaturation reactions [19].
- Once transported outside the chloroplast, FA are again transferred to CoA by an acyl-CoA synthetase and enter the endoplasmic reticulum. Here, they can undergo a variety of modifications, such as further elongation and desaturation, before being transferred to glycerol-3-phosphate to yield phosphatidic acid, which is the central metabolite for the biosynthesis of glycerol lipids including TAG, phospatidylcholine and phosphatidylethanolamin. These products can exchange acyl moieties by the action of acyltransferases, thereby increasing the diversity of glycerol lipids, as well as enabling the cell to recycle FA from membranes for the production of storage lipids. In TAG biosynthesis, diacylglycerol is produced by dephosphorylating phosphatidic acid, which then becomes the substrate of acyl-CoA:diacylglycerol acyltransferase, an enzyme unique to the TAG biosynthesis pathway that forms the esther bond between the third acyl chain and the glycerol moiety. Finally, TAGs are transported to lipid bodies or droplets, where they are stored as a form of energy and FA supply until needed by the cell [9,19,21,22].
- The first step in the breakdown of TAG is their deacylation in the lipid body by the subsequent action of tri-, di-, and mono-acylglycerol lipases [22]. After being released from the glycerol moiety, FAs are imported into the peroxisome where they are covalently linked to CoA by a set of acyl-CoA synthetases, each showing a different specificity with regards to FA chain length [112]. A double bond is then introduced into the acyl group between the C2 and C3 carbons by acyl-CoA oxidase, with different acyl-CoA oxidase having different chain length preferences. The resulting 2E-Enoyl-CoA is then hydrated and subsequently dehydrogenated by a multifunctional protein that incorporates both necessary domains in one polypeptide chain [23], producing 3-ketoacyl-CoA. Finally, 3-ketoacyl-CoA thiolase catalyses the nucleophilic attack of the SH-group of another CoA molecule on the C3 atom, resulting in one molecule of acetyl-CoA and one acyl-CoA molecule shortened by two C atoms compared with the original acyl-CoA that entered the β-oxidation process. This cycle is repeated until the FA chain is completely degraded. For unsaturated FA, the process is essentially the same as outlined above for saturated FA until a 2,5-dienoyle intermediate product is reached. This then requires the subsequent actions of Δ^2, Δ^3 isomerase, $\Delta^{3,5}$, $\Delta^{2,4}$ isomerase, 2,4-dienoyl-CoA reductase and again Δ^2, Δ^3 isomerase. The action of these auxiliary enzymes yields 2E-enoyl-CoA, which can then enter the core cycle again.

microalgae, there is even less data concerning their degradation pathways in these organisms. However, the basic enzymes of the peroxisomal β-oxidation pathway (i.e., acyl-CoA oxidases, multifunctional enzymes and keto-acyl thiolases; **Figure 2**) appear to be fairly conserved among mammals, fungi and plants [23]. It therefore seems very likely that microalgae from different branches of the tree of life would have a pathway similar to the aforementioned organisms to degrade their storage lipids. Therefore, it seems safe to assume that knowledge about enzyme composition, subcellular localization and regulation gained from the study of other organisms can be transferred, to a certain extent, to microalgae too. Using sequence data from mammals, fungi and especially plants, it will be possible to identify the respective genes in microalgae.

Identification of target genes & pathways using '-omics' approaches

Genomics, transcriptomics, proteomics and metabolomics, or in short, systems biology, have become valuable tools to study microalgae at the organism level, and greatly enhanced our understanding of microalgal biology; each method with its own inherent advantages and disadvantages (reviewed by [25]). The knowledge about the presence of a gene, together with its actual sequence, strongly facilitates manipulation of the respective pathway. With the introduction of next-generation

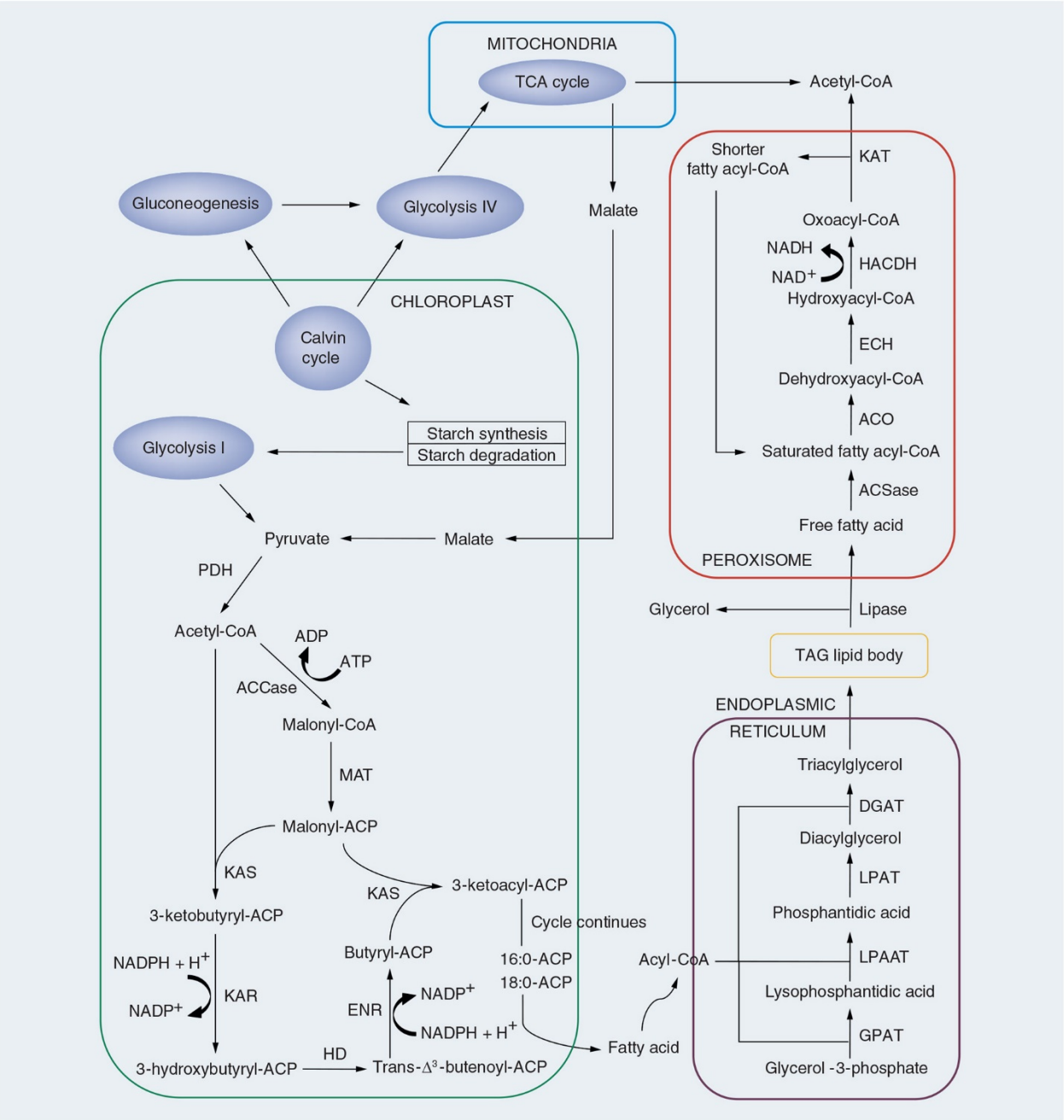

Figure 2. Overview of lipid metabolism pathways in the cell. Included are fatty acid biosynthesis in the chloroplast (green), TAG formation in the endoplasmic reticulum (purple) and fatty acid β-oxidation in the peroxisome (red). A detailed description of the pathways is presented in Box 1.

ACCase: acetyl-CoA carboxylase; ACO: Acyl-CoA oxidase; ACP: Acyl carrier protein; ACSase: Acyl-CoA synthetase; DGAT: Diacylglycerol acyltransferase; ECH: Enoyl-CoA hydratase; ENR: Enoyl-ACP reductase; GPAT: Glycerol-3-phosphate acyltransferase; HACDH: Hydroxyacyl-CoA dehydrogenase; HD: 3-hydroxyacyl-ACP dehydratase; KAR: 3-ketoacyl-ACP reductase; KAS: 3-ketoacyl-ACP synthase; KAT: Ketoacyl-CoA thiolase; LPAAT: Lyso-phosphantidic acid acyltransferase; LPAT: Lyso-phosphantidylcholine acyltransferase; MAT: Malonyl-CoA:ACP transacylase; PDH: pyruvate dehydrogenase complex; TAG: Triacylglyceride; TCA: Tricarboxylic acid.

sequencing techniques, sequencing of a whole genome became much less challenging with regards to financial resources and time, and an increasing number of microalgal genomes are being sequenced and annotated [7]. The nuclear genomes of the green algae *C. reinhardtii* [26], *Chlorella variabilis* NC64A [27], *Micromonas pusilla* [28], *Micromonas* sp. RCC299 [28], *Ostreococcus lucimarinus* [29], *Ostreococcus tauri* [30], *Volvox carteri* [31], the diatoms *Phaeodactylum tricornutum* [32], *T. pseudonana* [33] and the red alga *Cyanidioschyzon merolae* [34] are accessible in public databases (Table 1) and provide a valuable tool for the identification of lipid metabolism genes. Using sequence data from well examined organisms, such as *A. thaliana,* it is possible to identify whole lipid metabolic pathways in microalgal organisms. For example, the whole acylglycerol biosynthetic pathway of *C. reinhardtii* was reconstructed *in silico* by Riekhof *et al.* [35], and carbon acquisition and carbohydrate synthesis pathways, which feed into or compete with lipid biosynthesis, were analyzed at the genome level in *P. tricornutum* [36].

Apart from extracting whole pathways, several single genes for key lipid biosynthesis enzymes were identified in sequenced genomes and subsequently analyzed biochemically and/or phylogenetically. Examples are DGAT in *O. tauri* [37] and *T. pseudonana* [38], LC-PUFA acyl-Coenzyme A synthetase (LACS) and FA desaturases from *T. pseudonana* [39–41], a Delta12 desaturase from *C. reinhardtii* [42] and a cross-species evaluation of acyl-activating enzymes from several photosynthetic species [43].

In addition to fully sequenced genomes, the mitochondrial and plastidial genomes for even more algal species are available, providing a wealth of information for the identification of mitochondrial and plastidial encoded metabolism enzymes (see [7] for a recent review and a list of completed and ongoing microalgal genome projects).

While genomic data show us what a given organism is potentially capable of, transcriptome and proteome analyses reveal what it is actually 'preparing' and 'doing', respectively, in a specific situation. For the production of biofuels, conditions under which the organism accumulates TAG are the most interesting ones. Under N and/or P starvation, or when exposed to elevated temperatures and altered salinities, several microalgal species accumulate TAG either as an energy/membrane building block storage pool or as a protective mechanism [44,45]. With respect to metabolic engineering, obtaining information about differentially expressed genes under these conditions is necessary to understand the interplay of different metabolic pathways, and which pathways can be addressed in the engineering process for increased TAG production. Moreover, these data can assist in identification of key genes in these pathways and their respective sequences.

Despite their potential importance for lipid metabolism research, not much large-scale transcriptomics data is available for microalgae under TAG accumulation conditions, which might be due to still limited genomic sequence data. The model organism *C. reinhardtii*, which also accumulates TAG in lipid bodies when starved for N, was analysed for differentially expressed genes under N depletion conditions using 454 and Illumina next-generation sequencing techniques [46]. As expected, transcripts for genes of the β-oxidation pathway were reduced, although the increase of transcript abundance for FA synthesis was surprisingly modest. However, genes encoding enzymes of the TAG synthesis pathway in the endoplasmic reticulum, such as DGAT and phosphatidic acid dephosphatase, showed a strong increase in transcript levels, as did some genes encoding putative lipases. The authors concluded that this might be an indication for a significant role of membrane recycling during the synthesis of TAG, together with FA *de novo* synthesis. Apart from changes in lipid metabolism, genes encoding photosynthetic proteins and enzymes of gluconeogenesis were also found to be downregulated. However, transcript levels of a gene encoding one subunit of a pyruvate decarboxylase complex, which converts pyruvate into actyl-CoA that feeds into the FA synthesis cycle, were increased [46].

Key term

Catabolism: Metabolic pathway for the degradation of molecules in the cell into smaller units, thereby releasing the energy stored in the bonds of the original molecule.

In another study, the transcriptome of *Dunaliella tertiolecta* under N rich, N depletion and high salinity conditions was sequenced and annotated in order to identify genes involved in the production of starch and lipids [47]. Interestingly, not TAG but starch accumulation was observed in N starved cells, whereas an increased salinity resulted in higher lipid content. Using the sequence data obtained and comparing it to the sequenced genomes of *C. reinhardtii* and *V. carteri,* as well as using gene, genome and metabolic pathway databases, the authors were able to almost completely reconstruct the pathways for FA, TAG and starch biosynthesis, FA β-oxidation, TAG **catabolism** and starch degradation. Although no data about differentially expressed genes were presented, this study provides a valuable tool for further research on microalgal lipid metabolism [47].

Apart from these two studies, several other microalgal transcriptomic studies are available, which, although not focussed on TAG metabolism, might be useful for future lipid research and metabolic engineering. For example, the freshwater green alga *Haematococcus lacustris* accumulates the carotenoid astaxanthine under high irradiation and N depletion, and the transcriptomic changes under these conditions were addressed using microarray technology [48]. In two other studies that did not focus on lipid biosynthesis, the transcriptome of *C. reinhardtii* was analyzed under S depletion conditions, which are known to induce hydrogen production in this organism [49,50].

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Interestingly, an increase of TAG was also observed under these conditions (see below) [51]. Another green alga, *O. tauri*, was analyzed under light/dark cycles [52], while yet another study identified differentially expressed genes from the diatom *P. tricornutum* under Si starvation [53]. An expressed sequence tags database for *P. tricornutum* and *T. pseudonana* has also been established [54].

Compared to large-scale transcriptome studies, more microalgal proteome studies seem to be available. For example, several acyl-transferases, acyl-CoA synthetases and a putative structural protein (major lipid droplet protein) were identified in the lipid droplets of *C. reinhardtii* [55]. In the same organism, comparative proteomics of the chloroplast and the mitochondrion revealed the induction of several FA biosynthesis proteins under anaerobic conditions, together with other pathways metabolizing pyruvate [56]. The proteome composition under opposite conditions, that is, oxidative stress, was examined in the green algae *H. pluvialis* [57] and *O. tauri* [58], showing the induction of carbohydrate catabolism and FA biosynthesis. Since FAs, especially unsaturated FAs, can act as efficient reactive oxygen scavengers, it is possible that the main reason for the organism to produce FAs under these conditions is to eliminate reactive oxygen species in the cell. The proteome of *O. tauri* was also analyzed under N starvation conditions. Several microalgae accumulate lipids under N starvation [44], but in *O. tauri,* proteins involved in starch accumulation and FA oxidation showed an induction [59].

Although it is important to know how gene expression and protein induction change in relation to different growth conditions, knowledge about the corresponding metabolite levels is at least equally important in order to design a successful metabolic engineering strategy. Furthermore, any response of an organism to a change in condition at the transcriptome/proteome level needs to be verified by studying the respective metabolites. The metabolome of *C. reinhardtii* was analyzed under anaerobic- and S-depleted conditions, using gas chromatography coupled mass spectrometry, nuclear magnetic resonance and thin layer chromatography [51]. This study showed the large-scale rearrangement of the cell's metabolism first to accumulate starch and TAG, before entering a S depletion-induced fermentation state that led to the production of H. From a lipid research perspective, it is interesting to see that the cells retain their high TAG levels even after 120 h of S depletion [51]. Another study analyzed the changes of the metabolism of *C. reinhardtii* when exposed to low-CO_2 conditions, upon the induction of the carbon-concentrating mechanism [60]. Here, most lipid levels declined 3 h after lowering the CO_2 content. Although it seems reasonable that there is no increase in lipid content when carbon supply is too limited to provide for building blocks, the reasons for the observed decrease even after the induction of a carbon concentration mechanism, remained unclear [60].

Taken together, genomics, transcriptomics, proteomics and metabolomics prove to be powerful tools for examining cell-wide changes that occur in a microalga upon induction of FA and TAG accumulation. Besides facilitating the identification of specific genes that could be a potential target for genetic engineering (see [21] for a comprehensive review of lipid metabolism genes in microalgae), they also provide important insights in the flux of metabolites and regulation of pathways inside the microalgal cell.

Metabolic network modeling

The data obtained by the approaches described in the previous section can be fed into large-scale models analyzing the interdependence of metabolites, genes, transcripts and the respective proteins *in silico*. These metabolic network models can then be used to predict key metabolites and bottlenecks of a specific metabolic pathway under certain conditions [61,62], which then can and have to be verified *in vivo*. Although this computational approach directly addresses the very question that is important for most biofuel researchers (i.e., which conditions/backgrounds result in the highest available output of lipids), it is surprising that it has been largely ignored in recent reviews on this topic. Therefore, we will summarize the available literature on metabolic network modeling and flux balance analysis that is of special interest for the researcher of lipid metabolism in microalgae. Naturally, well-examined model organisms with relatively low genetic and metabolic complexity are more readily accessible by modeling approaches than multicellular organisms or those containing additional major pathways such as photosynthesis. For example, several genome-scale metabolic network models exist for *Saccharomyces cerevisiae*, although not all of them are able to address lipid biosynthesis and degradation in detail [63]. Including these pathways allowed identification of lipid-related reporter metabolites and key enzymes of FA biosynthesis and β-oxidation, and enabled correct prediction of the metabolite flux under varying conditions as compared with experimental data [63].

The mathematical analysis of photoautotrophic organisms requires incorporating additional pathways into the models to reflect the utilization of light as an energy source and the fixation of CO_2 as a carbon source. In a first attempt addressing the metabolism of a eukaryotic microalga, the growth of *Chlorella sorokiniana* was analyzed under autotrophic, mixotrophic, heterotrophic and cyclic autotrophic/heterotrophic (light/dark) growth conditions [64]. Using their model in combination with experimental data, the authors could show that the energy converted into ATP was highest for heterotrophic growth, but energy conversion into biomass was

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most economic under cyclic autotrophic/heterotrophic culturing conditions [64]. Additional insight into the metabolism of photosynthetic organisms came from studies of the reconstructed metabolic networks of the filamentous cyanobacterium *Spirulina platensis* [65] and the monocellular cyanobacterium *Synechocystis* sp. PCC 6803 [66], the latter making use of the sequenced genome of *Synechocystis* to include special characteristics of this cyanobacterial metabolism.

Using these approaches, it became possible to reconstruct the genome-scale metabolic network of the model algae *C. reinhardtii* with 458 metabolites and 484 metabolic reactions, which now incorporated the spatial separation of pathways and enzymes according to their subcellular localization [67]. Apart from being able to calculate and simulate metabolite fluxes, yields and growth rates under several growth conditions, this study led to the identification of one new ORF and several missing enzymes in the genome of *C. reinhardtii* [67]. Another model of the same alga was created also using genomic information, but here the transcription of almost every enzyme accounted for in the model was manually verified, leading to improvement and refinement of the gene models present in the *C. reinhardtii* database [68].

The most advanced metabolic network reconstructions for this organism incorporate photon flux, light spectral quality and refinements in the thermodynamic and bioenergetic constrains [69,70]. Furthermore, the numbers of metabolites and metabolic reactions accounted for increased to 1068 and 2190, respectively, resulting in a much more detailed picture of the cellular metabolism. This picture now includes a detailed analysis of lipid metabolism, which was lacking in previous models, and shows biosynthetic and catabolic reactions for, for example, glycerophospolipids and sterols, virtually comprising all known lipid molecules from *C. reinhardtii* [69].

Of course, data generated by *in silico* studies on microalgal lipid metabolism has to be evaluated by actual experiments. For example, it is possible to identify the rate-limiting enzymatic reactions in a metabolic pathway, and how this pathway would be affected by altering the abundance of the respective enzyme. This could lead to the potential identification of less obvious secondary bottlenecks, or how competing pathways might influence lipid yield. The next section will show examples of how shutting down these pathways does not necessarily redirect metabolic flux into the desired products.

Additionally, analysis of metabolic network models can indicate gaps in the existing models, therefore aiding with the identification of enzymes or even whole pathways. Also, the effect of the introduction of previously unknown pathways into an organism on its metabolism can be examined, and how metabolic flux has to be redirected to provide good growth and high amounts of the desired product. Finally, it is possible to test the effect of several environmental parameters such as nutrient level, nutrient composition, light regime and temperature to fine-tune lipid production without the need of lengthy and often costly experiments.

Taken together, a promising future for *in silico* research of lipid metabolism in microalgae has emerged. Further refinement of the existing models (see **Table 2** for a summary of all metabolic network modeling studies mentioned here) with data derived from transcriptomics, proteomics and metabolomics approaches will enable scientists to identify the key parameters to manipulate in order to obtain high-quality biofuel feedstocks.

Metabolic engineering by genetic modification of microalgae

So far, efforts to increase the lipid content of microalgae have been mainly focused on the optimization of growth and induction conditions, such as temperature, light, salinity and nutrient content/depletion (reviewed in, for example, [9,45,71]), and reports about genetic modifications of microalgae to alter either lipid quantity or quality (i.e., composition) are still sparse (see **Table 3** for a summary). The main reason is probably the lack of a generally applicable transformation protocol for microalgae, which is reflected by the much larger number of reports describing the transformation of *C. reinhardtii* than any other organism. Since microalgae are such a diverse group or organisms **(Figure 1)**, it is not guaranteed that a method that works for one species can be applied to another one. For example, some species, such as *D. salina*, do not possess a rigid cell wall, whereas diatoms often have a very rigid silicate shell. This directly affects the method of gene transfer into the cell (see [72] for a review of several transformation methods). Another problem is the limited range of available markers. Although auxotrophy markers are available for some species such as *C. reinhardtii*, stable transformation of other species still has to rely on co-transformed genes conferring resistance to antibiotics. However, some substances routinely used in the transformation of plants, such as kanamycin and hygromycin, are sensitive to increased NaCl concentrations and cannot be used for strains requiring sea water. Also, heterologous gene expression (i.e., the expression of genes not originating from the organisms) in microalgae suffers from the lack of available promoter sequences to control expression, and the possibility of codon usage bias. This, however, will change with an increased number of fully sequenced and annotated microalgal genomes. In summary, any protocol for the genetic transformation of a new microalgal strain (not necessarily a new species) has to be carefully modified to meet and overcome its specific requirements and limitations.

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Table 2. Metabolic network modeling studies relevant for the development of models of lipid metabolism in algae.

Species	Study	Ref.
Synechocystis sp.	Stoichiometric model of photosynthesis and elementary building blocks	[66]
Spirulina platensis	Metabolic network (biomass and growth-associated exopolysaccharide production)	[65]
Saccharomyces cerevisiae	Genome-scale metabolic network model	[63]
Chlorella sorokiniana	Energetic and metabolic network under different growth conditions	[64]
Chlamydomonas reinhardtii	Genome-scale metabolic network with spatial separation of pathways and enzymes	[67]
Chlamydomonas reinhardtii	Genome-scale metabolic network analysis integrated with transcript verification	[68]
Chlamydomonas reinhardtii	Metabolic network including detailed analysis of lipid metabolism	[69]
Chlamydomonas reinhardtii	Metabolic network incorporating bioenergetic processes	[70]

Despite the obstacles described above, genetic modification is already one of the main tools to study metabolic pathways in microalgae, and is strongly contributing to our knowledge about their biology. Metabolic engineering by genetic modification is expected to be one of the main steps that will lead to sustainable and economically viable biofuels from algae [7,9,44,45].

Improving energy & carbon uptake

Since the building blocks and energy that drive the biosynthesis of lipids are derived from photosynthesis and carbon fixation, it seems reasonable to improve the ability of a cell for carbon and light uptake. Some work has been done on the light-harvesting complex (LHC) of *C. reinhardtii*. Usually, photosynthetic organisms are able to adapt to changing light regimes, increasing the light-capturing antenna size in low-light conditions, and decreasing it when light is present in abundance. However, if the absorbed irradiation exceeds the photosynthetic capacity of the cell, LHC proteins also participate in dissipating this potentially dangerous surplus of energy into chlorophyll fluorescence and nonphotochemical quenching. This protects the cell from damage, but 'wastes' energy at the same time. In a dense culture, this also leads to a shading effect, since cells at the surface absorb the light without using it for biomass production and cells deeper in the culture experiencing suboptimal light conditions. To increase light penetration into the microalgal culture, Mussgnug *et al.* used **RNAi technology** to decrease the LHCII protein level in *C. reinhardtii* cells. This approach led to a light-green culture, which showed improved growth rates under high-light conditions [73]. Similarly, better solar conversion rates and photosynthetic performance were observed in a *C. reinhardtii* DNA insertional mutant that was also characterized by reduced antenna size [74]. Besides improving light absorption abilities of microalgae, targeting the carbon assimilation mechanism might also result in increased lipid productivity. The possibility to manipulate carbon uptake was shown by enabling *P. tricornutum* to grow light-independently on glucose by

Table 3. Overview of metabolic engineering efforts in microalgae.

Species	Gene	Technique	Note	Ref.
Chlorella pyrenoidosa		UV mutagenesis	Increased growth, protein and polyunsaturated fatty acids	[86]
Chlamydomonas reinhardtii	Light-harvesting complex	RNAi, DNA insertion mutant	Reduced light-harvesting complex antenna size	[73,74]
	Major lipid droplet protein	RNAi	Increase lipid globule size	[55]
	ADP-glucose pyrophosphorylase	DNA insertion mutant	Lipid content increase	[81–83]
	Isoamylase	DNA insertion mutant	Increased lipid and starch production	[84]
Cyclotella cryptica	ACCase	Overexpression	Increased enzyme activity, no change in lipid content	[77]
Navicula sapuvila	ACCase	Overexpression	Increased enzyme activity, no change in lipid content	[77]
Parietochloris incisa	Δ-5 desaturase	Mutagenesis	Increased saturated fatty acids	[79]
Phaeodoactylum tricornutum	Glucose transporter	Gene introduction	Trophic conversion to heterotroph	[75]
	Acyl-ACP thioesterase	Overexpression	Increased saturated fatty acids	[78]

ACCase: Acetyl-CoA carboxylase; ACP: Acyl carrier-protein.

introducing glucose-transporter genes from various organisms [75]. However, for economically viable biotechnology applications, it will probably be more useful to address either CO_2 concentration mechanisms [18] or the enzyme catalyzing its fixation, Rubisco [76]. Unfortunately, so far no reports about successful engineering are available.

Improving FA & TAG biosynthesis

The most obvious approach to increase lipid production is the manipulation of FA and TAG biosynthesis pathways; for example, by overexpressing key enzymes such as ACCase and DGAT [19]. Its feasibility has been successfully demonstrated in other organisms such as bacteria, higher plants and fungi (see Courchesne *et al.* for a list of overexpressed FA/TAG metabolism genes [45]), but the genetic engineering of FA/TAG biosynthesis in microalgae is still in its infancy. The first attempt was the overexpression of an ACCase-gene from the diatom *Cyclotella cryptica* in the same organism as well as another diatom, *Navicula sapuvila* [77]. However, the successful insertion of additional copies of the gene into the diatom genome did not result in a higher lipid content of the cell, despite increased enzyme activities [13].

Apart from the quantity of FA, the quality (i.e., length and saturation state) of FA, also affects the ability of a microalgal strain to produce high-quality biodiesel feedstock. To improve the yield of C12 (lauric acid) and C14 (myristic acid) saturated FA from *P. tricornutum*, C12- and C14-biased acyl-ACP thioesterases from *Umbellularia californica* and *Cinnamomum camphora*, respectively, were overexpressed in this organism [78]. In this study, the yield of lauric acid increased to a weight percentage of 6.2% as compared with barely detectable amounts in the wild-type (WT), whereas the yield of myristic acid doubled to 12%. Interestingly, the variance in C12 and C14 accumulation between different transformants correlated with the differences in transcript abundance, indicating the importance of a strong promoter for the expression of these acyl-ACP thioesterases [78].

Manipulating the number and positions of double bonds in (poly)unsaturated FAs is not only interesting for biodiesel production, but also for the production of high-value FAs as nutritional additives [13]. For example, a reduction in the number of double bonds was achieved in the fresh water green alga *Parietochloris incisa* [79]. After chemical mutagenesis, a mutant strain was identified that was defective in a Δ^5 desaturase gene, resulting in drastically decreased levels of arachidonic acid and increased levels of the precursor FA dihomo-γ-linolenic acid.

Reducing FA & TAG catabolism

Our lack of data concerning β-oxidation in microalgae is reflected in the lack of reports about metabolic engineering of this metabolic pathway in these organisms. Despite the lack of experimental evidence, it seems to be unlikely that this pathway can be shut down completely, since cells rely on it as an energy and carbon resource under certain conditions (see [80] and references herein for examples from higher plants). Therefore, downregulation of β-oxidation-related genes by RNAi technology might prove to be more successful than a complete knock-out.

Apart from β-oxidation, FA and TAG catabolism also rely on the susceptibility of TAG for degradation. It was shown by Moellering and Benning. that downregulation of the gene encoding major lipid droplet protein in *C. reinhardtii* increased lipid droplet size, thus altering the accessibility of the droplet surface for lipases [55]. However, no increase in TAG content was observed in this study.

Key term

RNAi technology: RNAi describes how RNA molecules can regulate the expression of genes in a cell. Genes can be artificially downregulated by introducing or stimulating the production of double-stranded RNA molecules into the cell. Since the level of downregulation can vary, this technique is attractive to examine pathways that cannot be shut down ('knocked out') completely without causing severe effects on a cell.

Inhibiting competing pathways

C. reinhardtii strains carrying mutations in the genes encoding ADP–glucose pyrophosphorylase or isoamylase, respectively, are defective in accumulation of starch under N depletion conditions and were thoroughly analyzed for their potential to accumulate TAGs under these conditions [81–84]. All strains were found to have a higher TAG content as compared with the WT strain, indicating that shuttling metabolites from starch to FA biosynthesis might indeed result in increased lipid accumulation under N starvation [81–83]. However, when lipid accumulation was not compared with the WT strain, but to the direct progenitor strain from which the starchless mutants were derived from, the mutant lines failed to show a higher lipid content, indicating that the defect in starch accumulation might not be directly responsible for the effects observed when compared with the WT strain [85]. To make the situation even more complex, starchless mutant lines carrying a disrupted isoamylase encoding gene showed over-accumulation of starch and TAG when complemented with a genomic WT copy of the respective gene [84]. In conclusion, a careful selection of the reference point is necessary when assessing mutant strains, and the downregulation of a competing pathway might not necessarily result in shuttling metabolites into the desired one. On the other hand, the feasibility of reducing starch synthesis in order to increase lipid accumulation was demonstrated in a starchless mutant of *Chlorella pyrenoidosa* [86]. These apparently contradicting results indicate that careful analysis of a cell by metabolic network modeling, as discussed above, might be necessary to determine if, and when, shutting down putative competing pathways will result in higher lipid yields.

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In summary, no report exists so far that demonstrates the successful metabolic engineering of a microalga by genetic methods (including mutagenesis) into a high-yield feedstock for biofuel production. However, studies using the model organism *C. reinhardtii* show that it is indeed possible to direct the cellular metabolism into higher lipid production, although the exact reasons for the increased lipid accumulation observed are yet to be identified. Nevertheless, the lipid composition of several algae has been successfully modified.

Future perspective

Metabolic engineering of microalgae towards increased lipid contents is still in its infancy. Although overexpression of single genes was utilized in other organisms to increase lipid yields, success in microalgae is rather limited so far. One has to keep in mind, though, that the toolkits that have been available for other organisms for decades (e.g., sequenced genomes, transformation techniques, promoters to drive gene [over-]expression, selection marker and many more) are still under development, especially for non-model organisms. Furthermore, it is questionable if a single-gene overexpression/deletion will be sufficient to rearrange the whole metabolism of a cell, or whether a multigene and/or manipulation of a regulatory gene approach is more feasible [45]. In the future, a successful, rational metabolic engineering strategy could be a 'from *in silico* to *in vivo*' process. Genome-scale models incorporating vast amounts of transcriptomic, proteomic and metabolomic data will be used to identify bottlenecks that limit product yield. These could be an enzymatic reaction in the biosynthesis, metabolite supply, excessive degradation or a combination of various factors. Then, any manipulation strategy could be tested *in silico* to verify its feasibility and to identify secondary bottlenecks that would have to be addressed too. Finally, the organisms will be manipulated using genetic transformation, mutagenesis and breeding techniques, as it is already common for high-performance field crops. Apart from creating strains that can be used for the industrial production of biofuels, this approach will also greatly enhance our general understanding of the biology of microalgae. However, increasing the lipid content of microalgal cells is only one step on the road to ecologically sustainable and economically viable biofuels. The critical parameter is not lipid content per cell, but the lipid productivity (overall lipid yield per area or volume and time) [8]. Therefore, a successful strain should also exhibit excellent growth, at least under the specific cultivation conditions. Under these conditions, which include, for example, temperature, light regime, nutrient requirement, salinity and a sterile environment, its overall fitness should not be impaired too much by the mutation(s). On the other hand, a decreased fitness under noncultivation conditions could be a desirable side effect to prevent the microalgal strain from spreading into natural ecosystems and causing environmental damage. This is especially true for genetically modified organisms, which face public opposition in many areas of the world.

Furthermore, genetic stability of the high-performance strain has to be guaranteed in order to constantly obtain high lipid yields. If cultures are subjected to multiple growth/harvesting cycles without re-inoculation from stock cultures, the performance of the strain could be decreased by genetic drift. The decrease could be even more pronounced if the introduced mutation slightly reduces the fitness of the strain; that is, there will be a selective pressure favoring any mutation counteracting the desired one. Re-inoculation from algal stock cultures, which are kept under non- or slow-growing conditions, or re-breeding from parent strains for sexually reproducing strains, could be a solution for this problem. However, the fact that the introduced mutation is actually known greatly facilitates monitoring of an engineered strain by standard molecular biology techniques such as, for example, DNA sequencing.

Although, to our knowledge, there is no industrial-scale oil production from algae so far, the demand is proposed to be huge. This can only be met by microalgae grown in large-scale and low-cost growth facilities, which currently are under development. Furthermore, harvesting of microalgae and subsequent lipid extraction has to be efficient and simple. Here, high-lipid content microalgae that have been additionally genetically engineered for the secretion of lipids could be a promising alternative to sequential harvesting/extraction methods. Given the successes of lipid metabolic engineering in plant crops, one can be optimistic that increased knowledge about microalgae, refined metabolic network models and improved computing resources will lead to rational engineering of microalgae for biofuel production and other valuable compounds.

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Executive summary

Background
- Microalgae are expected to become an important feedstock for the biofuel industry.

Lipids & microalgae
- Microalgae are a phylogenetically diverse group of photosynthetic, unicellular organisms producing a variety of different lipids.

Brief overview of the triacylglyceride metabolic pathways
- Fatty acid and triacylglycerol metabolism in microalgae is probably similar to higher plants, although data are still limited.

Identification of target genes & pathways using -omics approaches
- Systems biology approaches play an increasingly important role in the analysis of microalgae as lipid producers.

Metabolic network modeling
- Metabolic network modeling including lipid metabolism recently emerged for the model alga *Chlamydomonas reinhardtii.*

Metabolic engineering by genetic modification of microalgae
- Manipulation of the microalgal light harvesting apparatus can improve light penetration and cell density in cultures.
- Genetic manipulation in order to modulate fatty acid and triacylglycerol biosynthesis pathways had only limited success so far.
- Slowing down the competing starch biosynthesis pathway could result in higher lipid production of microalgae, although interpretation of the data is more difficult than expected.

Future perspective
- Future metabolic engineering of microalgae for lipid synthesis will combine *in silico* analysis of the organism's metabolism and multigene/multipathway manipulation strategies, including key regulatory genes, instead of single gene approaches.

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Chapter 2: Collection, isolation and screening for high lipid productivity microalgae species

Selecting the right microalgal strains is one of the most important factors in microalgal lipid production. A potential candidate must possess traits such as high lipid accumulation capacity, the right FA composition, fast growth rate and ease of harvest, especially under outdoor medium- to large-scale settings to be considered a viable feedstock. While it is possible to obtain strains that meet these criteria from microalgae collections, such as CSIRO's Australian National Algae Culture Collection, isolating strains from local waterways may prove advantageous, as they would already be acclimated to local environments. This chapter, published as "**Isolation and evaluation of oil-producing microalgae from Australian subtropical coastal waters and brackish waters**" (Research Paper 1), presents a successful method in collecting and isolating microalgal strains with high-lipid productivity from local waterways. It then compares these local strains with other strains from the Algae Biotechnology Laboratory's Microalgae Collection at The University of Queensland to identify the best strains for microalgae lipid and biodiesel production, as well as strains most suitable for Aims 2 & 3.

Keypoints

- Collection of microalgae was focused on locations that experienced fluctuating conditions (nutrients, temperature, salinity, light) such as tidal rock pools and river mouths.
- Nile-red staining was used as a preliminary screening method to identify strains with high-lipid content. This was followed by a more detailed comparison that, by using a standard protocol, looked at a strain's growth rates, lipid content and lipid composition when under nutrient-deplete conditions.
- New isolates *Chlorella* sp. BR2 and *Tetraselmis* sp. M8, as well as *Nannochloropsis* sp. BR2, and *Dunaliella salina* from the algae collection were identified as suitable candidates for a multi-product algae-crop.
- *Tetraselmis* sp. M8 was tested in a mid-scale 1000 L-outdoor setting to reveal high-lipid productivity and ideal FA composition for biodiesel production. Its ability to lose its flagella and sink during nutrient starvation made it easy to harvest.
- *Tetraselmis suecica* was selected as a suitable species for Aim 2's goal of improving lipid productivity via mutation-selection, as it demonstrated high growth rate, but relatively low lipid productivity.

- The methods used for the collection, isolation and screening of local microalgae successfully yielded strains *Tetraselmis* sp. M8 and *Chlorella* sp. BR2 with growth characteristics, lipid content and composition that are suitable for both biodiesel and omega-3 production.

Research Paper 1: Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters

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Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters

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Abstract

Microalgae have been widely reported as a promising source of biofuels, mainly based on their high areal productivity of biomass and lipids as triacylglycerides and the possibility for cultivation on non-arable land. The isolation and selection of suitable strains that are robust and display high growth and lipid accumulation rates is an important prerequisite for their successful cultivation as a bioenergy source, a process that can be compared to the initial selection and domestication of agricultural crops. We developed standard protocols for the isolation and cultivation for a range of marine and brackish microalgae. By comparing growth rates and lipid productivity, we assessed the potential of subtropical coastal and brackish microalgae for the production of biodiesel and other oil-based bioproducts. This study identified *Nannochloropsis* sp., *Dunaniella salina* and new isolates of *Chlorella* sp. and *Tetraselmis* sp. as suitable candidates for a multiple-product algae crop. We conclude that subtropical coastal microalgae display a variety of fatty acid profiles that offer a wide scope for several oil-based bioproducts, including biodiesel and omega-3 fatty acids. A biorefinery approach for microalgae would make economical production more feasible but challenges remain for efficient harvesting and extraction processes for some species.

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Introduction

Interest in a renewable source of biofuels has recently intensified due to the increasing cost of petroleum-based fuel and the dangers of rising atmospheric CO_2 levels. Among the various candidates for biofuel crops, photosynthetic microalgae have the advantage that they have high growth rates and can be cultured on non-arable land [1,2,3].

At present, microalgae are commercially grown at scale for fatty acid-derived nutraceuticals and as feed and food supply. Significant interest in microalgae for oil production is based on their ability to efficiently convert solar energy into triacylglycerides (TAGs), which can be converted to biodiesel via transesterification reactions [1,4,5]. Oleaginous microalgae are capable of accumulating 20–50% of their dry cell weight as TAGs and potentially have a productivity superior to terrestrial crops used as first generation biofuel feedstock [6]. Theoretical calculations of microalgal oil production (liter/ha) are 10 to 100-fold greater than traditional biodiesel crops such as palm oil [7], corn and soybeans [6,8,9], although large-scale commercial algal oil production has yet to be established. Another major advantage of microalgae over higher plants as a fuel source is their environmental benefits. Despite having to grow in an aquatic medium, microalgae production may require less water than terrestrial oleaginous crops and can make use of saline, brackish, and/or coastal seawater [10,11]. This allows the production of microalgae without competing for valuable natural resources such as arable land, biodiverse landscapes and freshwater. Furthermore, a microalgae-based biofuel industry has tremendous potential to capture CO_2. In high efficiency, large microalgae cultivation systems, the potential capture efficiency of CO_2 can be as high as 99% [12], effectively capturing 1.8 kg of CO_2 per kg of dry biomass [13]. Although CO_2 captured this way into biodiesel will eventually be released upon combustion, this would displace the emission of fossil CO_2 and the remaining biomass (e.g. ~70% of dry weight) can be fed into downstream carbon sequestration processes. For example, sequestering carbon into hard C-chips (Agri-char) via pyrolysis can be used to improve soil fertility, mitigating climate change by reintroducing durable carbon back into the soil [14], although it is debatable how long this carbon will actually stay in the soil.

Aside from biodiesel production, microalgae are gaining a reputation as "biofactories" due to the varied composition of their biomass. Akin to today's petroleum refinery, which produces a range of fuels and derivative products, a well-managed and equipped microalgal biorefinery can produce biodiesel and other value-add products such as protein, carbohydrates and a range of fatty acids (FAs). High value omega-3 fatty acids (ω-3) such as

eicosapentaenoic (EPA), docosahexanoic (DHA), alpha-linolenic acid (ALA) and arachidonic (AA) are not desirable FAs for biodiesel production. Nevertheless, these ω-3 polyunsaturated fatty acids (PUFAs) are highly valued in human nutrition and therapeutics [15] and are linked to a wide range of cardio and circulatory benefits [16]. *Ω*-3 fatty acids also play an important role in aquaculture, increasing growth performance and reducing mortality in the shellfish industry [17,18,19]. This ability to produce value-adding products in addition to biodiesel is important to reduce production cost and make large-scale production viable.

The inherent advantages of a microalgal fuel source are unfortunately offset by current limitations to economically produce it on a large-scale. For example, the cost for obtaining dry biomass, large hexane requirements and limited hexane recycling capacity are currently hindering economic viability. It was estimated that the current cost of producing 1 tonne of microalgal biomass with an average 55% ($w/w_{DryWeight}$) oil content needs to be reduced by 10-fold in order to be competitive with petroleum diesel [8]. Furthermore, despite estimates that suggest microalgal oil production (US$9–$25/gallon in ponds, $15–$40 in photobioreactors) could be cheaper than the current price of oil [20], companies commercially producing microalgae have not been able to achieve the predicted yields and production costs. Typical lipid yields of 10 g $m^{-2}d^{-1}$ (Skye Thomas-Hall, personal communication) are still short of achieving the current best case scenarios of 103 to 134 g $m^{-2}d^{-1}$ [21]. The industry is still in its infancy, although recent research and development efforts by large oil companies (e.g. Exxon, BP, Chevron and Shell) would certainly increase production capacity and decrease production costs.

As large variations (10–50%) in lipid content exist between different species of microalgae [22,23], it is necessary to identify strains with high lipid content and suitable lipid composition. The need for high-yielding microalgae is straightforward, as this directly translates to an overall increase in production, although lipid production during normal growth needs to be distinguished from lipid accumulation in response to adverse conditions (e.g. nutrient starvation). Lipid composition is equally important, as quantitative and qualitative differences in the TAG content of a given species will affect the quality of biodiesel and its ability to meet fuel standards. Fuels with high cetane number fatty acids (e.g. myristic acid, palmitic acid, stearic acid) are desirable [24], as higher cetane fuels have better combustion quality and the right cetane number of biodiesel is required to meet an engine's cetane rating [25]. Microalgal lipids are mostly polyunsaturated, which have a low cetane number and are more prone to oxidation. This can create storage problems and are thus preferred to be at a minimum level for biodiesel production. Nevertheless, polyunsaturated fatty acids lower the cold filter plugging point (CFPP) of fuel and are crucial in colder climates to enable the biodiesel to perform at lower temperatures [3]. With these factors in mind, an "ideal composition" of fatty acids would consist of a mix of saturated and monounsaturated short chain fatty acids in order to have a very low oxidative potential whilst retaining a good CFPP rating and cetane number.

To date, research efforts have focused on lipid production of individual species, usually investigating the effects different growth conditions have on lipid production and content [26,27,28,29,30]. Unfortunately, direct comparisons of results between studies are unreliable, given the different growth conditions and experimental parameters of each species and also the different methods used for lipid extraction. There is growing interest to compare lipid content and FA composition of multiple microalgae species [11,31,32,33,34,35]. Several studies have revealed algae genera such as *Tetraselmis*, *Nannochloropsis* and *Isochrysis* to have highest high lipid content, particularly under nutrient-deprived conditions [11,31].

Nutrient deprivation is regarded as an efficient way to stimulate lipid production in microalgae in several microalgae species [11,29,36,37], especially saturated and monosaturated FAs [6,38,39]. Unfortunately, lipid accumulation is often associated with a reduction in biomass, which reduces overall lipid accumulation. A batch culture strategy can be adopted to obtain maximal biomass productivity as well as induction of lipid accumulation through nutrient deprivation. Although a common research practice, only Rodolfi et al. [11] have published lipid profiles of multiple microalgae species in a batch culture setting.

The target of our work was to identify the most effective microalgal TAG producers for biodiesel production using a basic batch culture strategy. Most studies utilize experimental designs that include aeration of media volumes of 1 L to 10 L in order identify microalgae strains with high lipid content [31,32,33,36,40]. To provide a direct comparison between different species, this study evaluated eleven microalgae strains collected from local Australian coastal waterways and other collections that originate in various places in the world. Strains were first characterized by microscopy and partial 18S ribosomal RNA sequencing and total fatty acid methyl ester (FAME) contents were then analyzed via GC/MS, which quantifies the fatty acids in triacylglycerides in each strain, thus providing the most accurate representation of the substrate available for biodiesel production. Using growth rate, FAME productivity and FA composition as criteria, this study identified several algae strains to be suitable for biodiesel, including *Tetraselmis* sp. and *Nannochloropsis* sp. as highly versatile candidate strains for a multiple-product algal biorefinery.

Materials and Methods

Microalgae strain collection and isolation

Microalgae were collected as 10 mL water samples from coastal rock pools, freshwater lakes and brackish (tidal) riverways. After initial cultivation of the mixed cultures with F medium [41] pure cultures were isolated by performing serial dilutions and the use of a micromanipulator (Leica DMIL with Micromanipulator). Strains *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2 originated from the same water sample and were collected from the Brisbane river (27°31′21″S 153°0′32″E; high tide at 10 am in August 2007 on a sunny day). Strain *Tetraselmis* sp. M8 was collected in an intertidal rock pool at Maroochydore (26°39′39″S 153°6′18″E; 12 pm on 6 August 2009). Additional, microalgae strains used in this study were obtained from the Australian National Algae Culture Collection (ANACC, CSIRO) and Queensland Sea Scallops Trading Pty Ltd (Bundaberg, Australia) (Table 1). All primary stock cultures were maintained aerobically in 100 mL Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent white light (120 µmol photons $m^{-2}s^{-1}$). All cultures except *Chlorella* sp. were grown in seawater complemented with F medium [41]. *Chlorella* sp. was cultured in freshwater complemented with F medium. Primary stock cultures were sub-cultured every 3 weeks to minimize bacterial growth. Non-sterile cultures were used and maintained, as difficulties in maintaining axenic cultures in real production would arise and axenic cultures had been reported to have low biomass productivity, most likely because algae-associated bacteria may assist in nutrient recycling [42]. However, all microalgae cultures were checked during cell counting to ensure that no contamination with other microalgae occurred.

Table 1. Sources and 18S rRNA sequence accessions of microalgae strains used in this study.

Species	Genbank Accession	Location of Origin
Tetraselmis sp. M8	JQ423158	Maroochydore, Qld, Australia
Tetraselmis chui	JQ423150	East Lagoon, Galveston, TX, USA
Tetraselmis suecica	JQ423151	Brest, France
Nannochloropsis sp. BR2	JQ423160	Brisbane River, Brisbane, Australia
Dunaliella salina	JQ423154	Alice Springs, NT, Australia
Chaetoceros calcitrans	JQ423152	Unknown
Chaetoceros. muelleri	JQ423153	Oceanic Institute, Hawaii, USA
Pavlova salina	JQ423155	Sargasso Sea
Pavlova lutheri	JQ423159	Unknown location, UK
Isochrysis galbana	JQ423157	Unknown location, UK
Chlorella sp. BR2	JQ423156	Brisbane River, Brisbane, Australia

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Standard protocol for batch culture growth analysis, lipid induction phase and sampling for lipid analysis

A standard protocol was designed to allow direct comparisons of growth rates and lipid productivity between cultures. To standardize inoculum cell densities, cultures were first grown to late logarithmic phase in F medium. Late-log phase of each culture was determined when daily cell count of the pre-culture revealed a less than 20% increase in cell density. A total of 1 mL of pre-culture in late-log phase was used as inoculum (7 to 9 hours after start of light cycle) for 20 mL seawater (SW) complemented with F medium in 100 mL Erlenmeyer flasks. A minimum of three parallel cultures were grown in conditions as described above. Cell counts were performed on days 0, 2, 4, 6 and 7 post inoculation using a haemocytometer. After day 7, nutrient deprivation to stimulate lipid production was achieved by removal of previous medium by centrifugation (1,200×g, 5 min) and replacement with only SW (without F medium). Cultures were then grown for another 48 h before 4 mL of wet biomass from each replicate was harvested for lipid analyses.

Fatty Acid Methyl Ester (FAME) analyses

Algae cultures (4 mL each) were centrifuged at 16,000× g for 3 min. The supernatant was discarded and lipids present in the algal pellet were hydrolyzed and methyl-esterified by shaking (1,200 rpm) with 300 µL of a 2% H_2SO_4/methanol solution for 2 h at 80°C; 50 µg of heneicosanoic acid (Sigma, USA) was added as internal standard to the pellet prior to the reaction. A total of 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane was then added and the mixture was vortexed for 20 s. Phase separation was performed by centrifugation at 16,000× g for 3 min. A total of 1 µL of the hexane layer was injected splitless into an Agilent 6890 gas chromatograph coupled to a 5975 MSD mass spectrometer. A DB-Wax column (Agilent, 122–7032) was used with running conditions as described for Agilent's RTL DBWax method (Application note: 5988–5871EN). FAMEs were quantified by taking the ratio of the integral of each FAME's total ion current peak to that of the internal standard (50 µg). The molecular mass of each FAME was also factored into the equation. Identification of FAME was based on mass spectral profiles, comparison to standards, and expected retention time from Agilent's RTL DBWax method (Application note: 5988–5871EN).

DNA isolation and sequencing

Genomic DNA was isolated from all algal species via a phenol-chloroform method [43] on a pellet obtained by centrifugation of 10 mL of algal culture at the late-log phase. DNA amplification from genomic DNA containing a partial 18S ribosomal RNA region was performed by PCR using the following primers: Forward: 5′-GCGGTAATTCCAGCTCCAATAGC–3′ and Reverse: 5′-GACCATACTCCCCCCGGAACC-3′. Briefly, DNA was denatured at 94°C for 5 min and amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. There was a final extension period at 72°C for 10 min prior to a 4°C hold. The PCR product was isolated using a Gel PCR Clean-Up Kit (Qiagen). For sequencing reactions, 25 ng of PCR product was used as template with 10 pmol of the above primers in separate reactions in a final volume of 12 µL. The samples were then sent to the Australian Genome Research Facility in Brisbane for sequencing. All new data has been deposited in GenBank (Table 1).

Identification of microalgae and phylogenetic analysis

Nucleotide sequences were obtained from the NCBI database based on the BLAST results of each algae sequenced in this study. When sequences from multiple isolates of a species were available, two nucleotide sequences were chosen: (i) highest max score sequence, (ii) highest max score sequence with identified genus and species. Strains *Tetraselmis* sp. M8, *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2 were isolated by the authors and other strains were obtained from the Australian National Algae Culture Collection (ANACC), CSIRO and Queensland Sea Scallops Trading Pty Ltd (QSST), Bundaberg (Table 1). In total, 22 sequences from the NCBI database and eleven sequences from algae in this study were aligned with the MAFTT [44]. The resulting alignment was then manually inspected for quality and the end gaps trimmed. Phylogenetic analyses of the sequences was performed with PhyML 3.0 [45] using the ML method. Default settings were used, with the exception that 100 bootstraps were used in a nonparametric bootstrap analysis instead of an approximate likelihood ratio test as this is the more commonly used method in recent reports.

Analytical methods

Measurement of nitrate and phosphate levels in the photo-bioreactor was performed using colorimetric assays (API, Aquar-

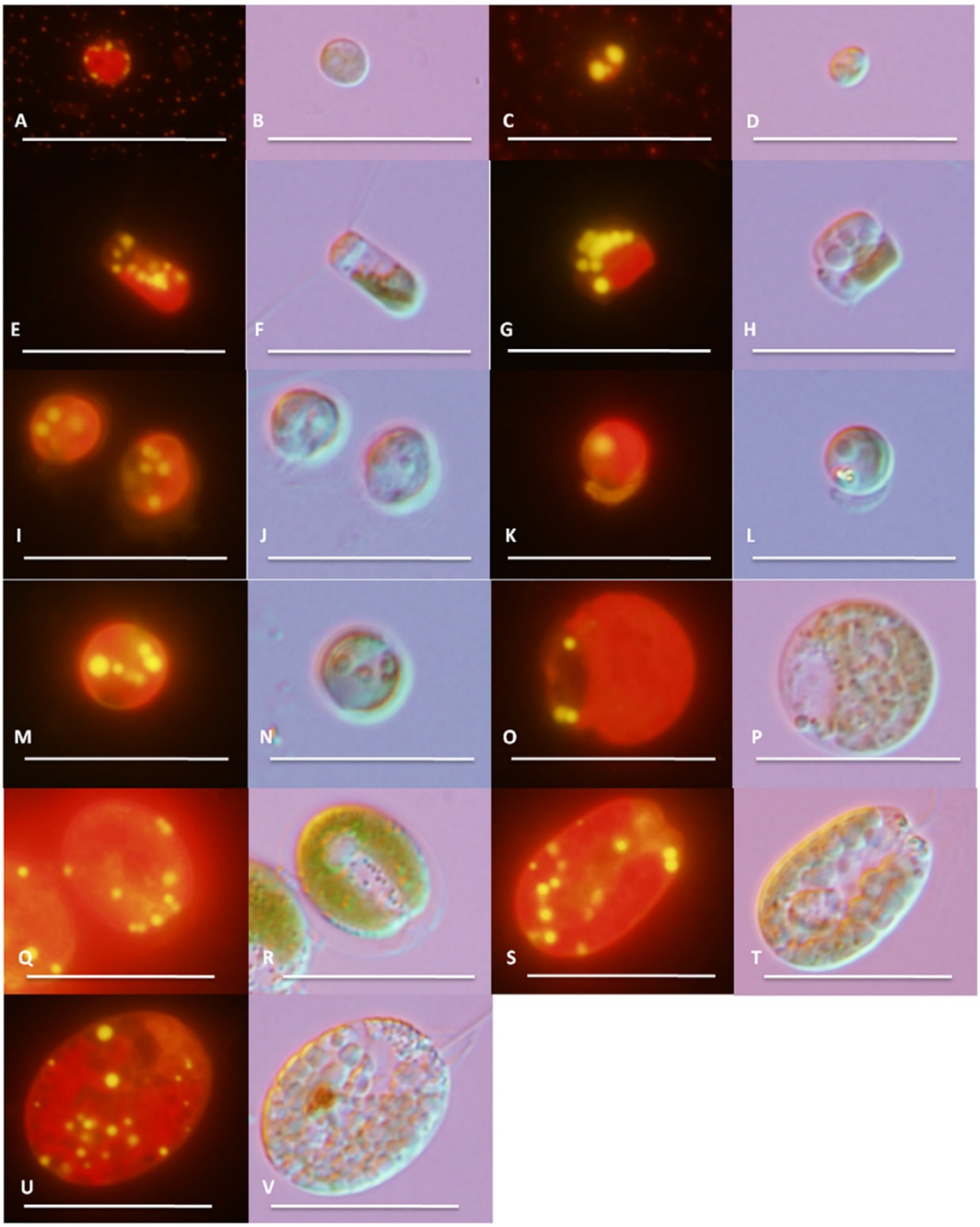

Figure 1. Epifluorescent (A, C, E, G, I, K, M, O, Q, S, U) and differential interference contrast (B, D, F, H, J, L, N, P, R, T, V) images of eleven microalgae used in this study. *Chlorella* sp. BR2 (A, B), *Nannochloropsis* sp. BR2 (C, D), *Chaetoceros muelleri* (E, F), *Chaetoceros calcitrans* (G, H), *Pavlova lutheri* (I, J), *Pavlova salina* (K, L), *Isochrysis* sp. (M, N), *Dunaliella salina* (O, P), *Tetraselmis chui* (Q, R), *Tetraselmis* sp. M8 (S, T) and *Tetraselmis suecica* (U, V). All images were taken at 100x magnification. Bars represent 20 μm.
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ium Pharmaceuticals and Nutrafin, respectively). Growth rate, doubling time and lipid productivity were calculated as follows. The average growth rate was calculated using the equation $\mu = \mathrm{Ln}(N_y/N_x)/(t_y\text{-}t_x)$ with Ny and Nx being the number of cells at the start (t_x) and end (t_y) of the growth phase (7 days). Average doubling time (T_{Ave}) was calculated using the equation $T = (t_y\text{-}t_x)/\log_2 (N_y/N_x)$ over the growth period of 7 days. The specific growth rate (μ_{Max}) was calculated between the 2 days of maximum slope on the average cell density x-axis time plot [31,46]. Lipid productivity (μg mL^{-1} day^{-1}) was calculated as total lipid content (μg/mL) over the duration of the entire batch culture (laboratory cultures – 9 days, outdoor culture – 12 days).

Microscopic analyses

After a lipid induction phase, microalgae cells were stained with 2 μg/mL Nile red (dissolved in acetone; Sigma, USA) for 15 minutes and photographed using a fluorescent Olympus BX61 microscope and an Olympus DP10 digital camera. Differential interference contrast (DIC) and epifluorescent (excitation: 510–550 nm, emission: 590 nm) images were obtained at 1000× magnification with oil immersion.

Mid-scale outdoor cultivation

In order to evaluate the growth performance and lipid productivity of microalgae in a medium-scale outdoor setting, *Tetraselmis* sp. was selected and tested in a 1000 L outdoor photobioreactor built by The University of Queensland's Algae Biotechnology Laboratory (www.algaebiotech.org) between 20th May 2011 to 1st June 2011 (sunny conditions 22°C–26.5°C). An initial cell density of 1.3×10^6/mL was cultured in SW + F/2 medium for 10 days (pH 8.8; maintained by the addition of CO_2) followed by 2 days of nutrient starvation (nitrogen measurements were 0 mg/L on day 10). Cell counts were conducted on days 0, 2, 4, 6, 7, 10, 11 and 12 and cultures were checked to ensure that no contamination with other microalgae occurred. To facilitate comparison with laboratory protocols, growth parameters were determined within the first 7 days of culture. At day 10, 4 mL of culture was sampled for lipid analysis.

Statistical analysis

Data for growth rates and lipid productivity was statistically analyzed by one-way analysis of variance (ANOVA) with different microalgae species as the source of variance and growth rate or lipid productivity as dependant variables. This was followed by Bonferroni's multiple comparisons test where appropriate.

Results

Strain collection, isolation and morphological and phylogenetic characterization of candidate microalgal biofuel strains

Over 200 water samples were collected from diverse aquatic habitats from subtropical regions in Queensland, Australia. These included samples from rock pools in coastal areas at the Sunshine Coast, Moreton Bay, Heron Island, Gold Coast and North Stradbroke Island, as well as freshwater samples from Somerset Dam, Wivenhoe Dam and brackish samples from tidal rivers, including the Brisbane and Logan rivers. Additional microalgae strains were obtained from culture collections at ANACC, CSIRO, and two local isolates from QSST, Bundaberg. Visual microscopy (Figure 1) confirmed the isolation of uniclonal cultures. Morphological comparisons to other described microalgae suggested that these strains belonged to the genera *Tetraselmis*, *Chlorella*, *Nannochloropsis*, *Dunaniella*, *Chaetoceros*, *Pavlova* and *Isochrysis*.

Nile red staining and growth analysis (Table 2, Figures 1) revealed eleven candidate strains that met the criteria required for biodiesel production (i.e. easy cultivation with no special nutrient requirements, fast growth rate, seawater-strength (35 ppt) salinity tolerance and high lipid production). One promising freshwater culture (*Chlorella* sp. BR2) was also included. Under nutrient-deprived conditions, lipids produced by microalgal cells were observed as bright yellow globules when stained with Nile red and viewed under epifluorescent light (Figure 1).

To specify the identity of the microalgae strains used in our experiments, a partial 18S region of the ribosomal RNA gene was amplified by PCR and sequenced. The obtained sequences were then compared to existing sequences in the NCBI database by the BLAST algorithm (for Genbank accession numbers see Table 1). Homology (sequence identity) searches confirmed a close relationship of the isolated candidate strains *Chlorella* sp. BR2, *Nannochloropsis* sp. BR2 and *Tetraselmis* sp. M8 with other members of the genera *Chlorella* and *Tetraselmis*. *Chlorella* sp. BR2 had a sequence identity of 99% with *Chlorella* sp. Y9, (Genbank Acc. No. JF950558) and *Chlorella vulgaris* CCAP 211/79 (Acc. No. FR865883). *Tetraselmis* sp. M8 shared a sequence identity of 99% with *Tetraselmis suecica* (CS-187) and *Tetraselmis chui* (CS-26). To characterize the diversity of the 11 microalgae strains and their relationship to other microalgae, the obtained sequences from this study were phylogenetically analyzed. The obtained maximum likelyhood phylogenetic tree (Figure 2) depicts the placement of each microalgae strain used in this study with chosen BLAST results.

BLAST 18S rRNA sequence comparison of eleven strains from this study to each other and the NCBI database (Figure 2) confirmed the taxonomic classification (suggested by microscopic studies or CSIRO/QSST) in all species based on the maximum score, while revealing high similarity within a species.

Comparison of growth rates, doubling times and cell densities of microalgae strains

To determine and compare growth rates, doubling times and cell densities, all microalgae strains were grown as three side-by-side cultures. After inoculation, an initial lag phase was observed in most cultures, except *Chorella* sp. BR2, *C. calcitrans*, *C. muelleri* and *I. galbana*, where exponential growth was observed immediately upon inoculation (Figures 3–4). Exponential growth in all cultures occurred till day 7 but for *D. salina*, *P. lutheri*, *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2, a lag phase was observed on day 4. *D. salina* culture remained in lag phase till day 7, while *P. lutheri*, *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2 resumed growth after day 6.

The highest average growth rate (μ_{ave}) was found for *P. lutheri* (0.48 μL^{-1}) and *P. salina* (0.45 μL^{-1}) (Table 2), that were significantly ($p<0.05$) higher to all other species that had a μ_{ave} of 0.34 μL^{-1}. Specific growth rates (μ_{exp}), were also compared with ANOVA, revealing that *T. chui* had the highest μ_{exp} at 1.03 μL^{-1}, followed by *Tetraselmis* sp. M8 (0.93 μL^{-1}) and *P. salina* (0.88 μL^{-1}). The fastest doubling times that were significantly different to the others were found for *P. lutheri* (1.45 days) and *Tetraselmis* sp. M8 (outdoor) (1.48 days) (Figure 3), while other microalgae strains had an average doubling time of 2.06 days. Maximum growth occurred during day 0 to day 4.

FAME productivity and fatty acid composition

GC/MS analysis revealed *Nannochloropsis* sp. (6.24 μg mL^{-1} day^{-1}) to be the highest FAME producer (ANOVA, $P<0.05$ in all cases), followed by *D. salina* (4.78 μg mL^{-1} day^{-1}; ANOVA, $P<0.05$ in all cases except *Chlorella* sp. BR2, 3.9 μg mL^{-1} day^{-1}) (Table 3; Figure 5). On the other hand, *T. chui* (1.5 μg mL^{-1} day^{-1}) and *T. suecica* (1.49 μg mL^{-1} day^{-1}) were the lowest FAME producers. The FA profile of *Nannochloropsis* sp. BR2, *C. calcitrans* and *C. muelleri* consisted predominantly of C16, C16:1 and C20:5 (>70% in total), while *Chaetoceros* strains produced C14 (10.5–11.6%). *Tetraselmis* sp. M8 contained most notably C18:3 (28.9%) and C16 (22.5%), as well as C18:2s (11.7%). *D. salina* and *Chlorella* sp. BR2's FA profile consisted mostly (nearly 90%) of C16, C18 and their unsaturated derivatives. In *T. chui* and *T. suecica*, C16 (35–37%), unsaturated C18s (37–43%) and unsaturated C20s (8–12%) were the main FAs. *I. galbana*'s FA profile was spread across C14 (19%), C16 (16%), C18:1 (22%), C20:3 (22%) and C20:6

Table 2. Growth rate analysis of eleven microalgae strains during growth phase (7 days) of batch culture.

Species	μ_{Ave}	μ_{Exp}	Day of μ_{Exp}	DT_{Ave} [days]	Cell density$_{Max}$ [x10^{6}cells mL^{-1}]	Dry weight (g L^{-1})
Nannochloropsis sp. BR2	0.32	0.62$^{c, d}$	2–4	2.18^{c}	48.4	0.53
Tetraselmis sp. M8	0.35	0.93$^{a, b}$	2–4	2.00^{c}	2.07	0.75
T. chui	0.35	1.03^{a}	2–4	1.98^{c}	1.56	0.42
T. suecica	0.37	0.5^{d}	0–2	1.85$^{b, c}$	1.52	0.73
D. salina	0.30	0.76$^{a, b, c, d}$	2–4	2.31^{c}	2.14	0.37
C. calcitrans[1]	0.34	0.59$^{c, d}$	0–2	2.03^{c}	4.71	n/a
C. muelleri[1]	0.35	0.71$^{a, b, c, d}$	0–2	1.94$^{b, c}$	4.65	0.50
I. galbana[1]	0.35	0.61$^{b, c, d}$	0–2	1.96$^{b, c}$	4.45	0.45
P. lutheri[1]	0.48^{a}	0.76$^{a, b, c, d}$	0–2	1.45^{a}	3.95	0.45
P. salina	0.45^{a}	0.88$^{a, b, c}$	2–4	1.54$^{a, b}$	5.47	1.68
Chlorella sp. BR2	0.34	0.86$^{a, b, c}$	0–2	2.06^{c}	13.8	0.59
Tetraselmis sp.M8[3]	0.47	0.48	6–7	1.45	1.61	0.58

[1]Value represents mean of two replicate samples.
[2]Different letter superscripts down a column indicate significant difference at 95% level (ANOVA, Bonferroni's test; $P<0.05$).
[3]Mid-scale outdoor culture.
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(12%). Approximately 44% of *P. salina*'s FAs consist of C14 and C16 FAs, with C20:5 and C22:6 FAs accounting for another 26%. *P. lutheri*'s FA profile consisted largely of C16 (25%), C16:1 (29%), C20:5 (22%) and C14 (11%).

On average, saturated FAs accounted for 40% of the total FAs in this study, consisting mostly of C16 (27.2%), C14 (7.2%) and C18 (6%). Similar amounts (37.4%) of FAs were polyunsaturated and included EPA C20:5 (9.6%), ALA C18:3 (10.4%) and DHA C22:6 (3.9%). Monounsaturated FAs accounted for 21% of the total FAs, consisting mostly of C16:1 (11.7%) and C18:1 (8.3%). *P. salina* was found to have the highest saturated FA (53%), *C. calcitrans* the highest monounsaturated FA (40%), and *D.* salina the highest polyunsaturated FA content (60%). C16 was found to be a major FA (17–37%) in all the strains tested, particularly in *T. chui*, *T. suecica* and *Nannochloropsis* sp. BR2. C16:1 FAs were predominantly found in *C. calcitrans*, *C. muelleri* and *Nannochloropsis* sp. BR2, while highest C14 content was found in *P. salina* and *I. galbana*. *I. galbana* also had the highest content of C18:1 FAs, while C18:3 FAs were predominantly found in *D. salina*, *Chlorella* sp. BR2 and *Tetraselmis* sp. M8. *Nannochloropsis* sp. BR2 and *P. lutheri* both had the highest content of EPA C20:5 FAs while DHA C22:6 was predominantly found in *P. salina*. *D. salina* was the only strain found to produce C16:4. It should be noted that due to the small culture volumes in this study certain fatty acids may have remained undetectable.

Outdoor scale-up

The highest lipid productivity for the microalgae strains tested in this study, was measured for *Nannochloropsis* sp. BR2 (Figure 5). However, based on its versatility and resourcefulness of fatty acids, its short doubling times, its ease of handling, and its potentially better lipid extraction efficiency, *Tetraselmis* sp. M8 was identified as a suitable candidate for large-scale cultivation whose FAME profiles would also meet the criteria for a future microalgae biorefinery. To compare laboratory cultivation with larger outdoor cultivation, *Tetraselmis* sp. M8 culture was grown in a 1000 L closed photobioreactor that was inoculated with 20 L of saturated culture. This mid-scale outdoor culture achieved a cell density of 1.6×10^6 cells mL^{-1} on day 7, eventually arriving at 2.3×10^6 cells mL^{-1} on day 10. Maximum growth rate was found between day 4 and 6 (Table 2) and was similar to average growth rates (0.47 μL^{-1} and 0.5 μL^{-1}, respectively). The culture entered stationary phase during starvation (after day 10), and cell count did not increase. The mid-scale, outdoor cultivation of *Tetraselmis* sp. M8 achieved a FAME productivity of 4.8 μL mL^{-1} day^{-1}, consisting mostly of C16 (20.8%), C18 (10.1%) and C18 unsaturated fatty acids (44.6%).

Discussion

In a microalgae-based oil industry, high oil productivity is crucial to achieving commercial feasibility. While growth conditions (e.g. solar radiation and temperature) and culture management are important, the suitable microorganism is fundamental to produce the desired quality and quantity of oil. A suitable microalgae strain must have high lipid productivity, either by possessing a high basal lipid content and/or be inducible to accumulate significant amounts of lipids. The selected strain should also be easily harvested, amenable to efficient oil extraction and flexible enough to adapt to changing physio-chemical conditions in an outdoor environment [11]. Thus, a locally isolated strain would likely adapt better to local changing environmental conditions and provide a more stable and productive culture.

Sampling at local waterways focused on inter-tidal rock pools, where the microclimate alters frequently between optimal growth conditions and unfavorable conditions (e.g. low nutrients, micro-oxic conditions, anaerobiosis, low/high light or dry, hot or cold conditions or rapid changes in salinity). Sampling at such locations was considered advantageous because suboptimal conditions would require the algae there to accumulate photo-assimilates such as starch or lipids that have important storage functions in order to survive, thereby increasing the chances of obtaining high lipid content strains [3]. This was followed by an isolation process targeted to select for high growth rate microalgae strains that could be induced to accumulate lipids under nutrient-deprived conditions. Isolation of uni-clonal microalgae strains by serial dilution and plating in F-supplemented medium was designed to

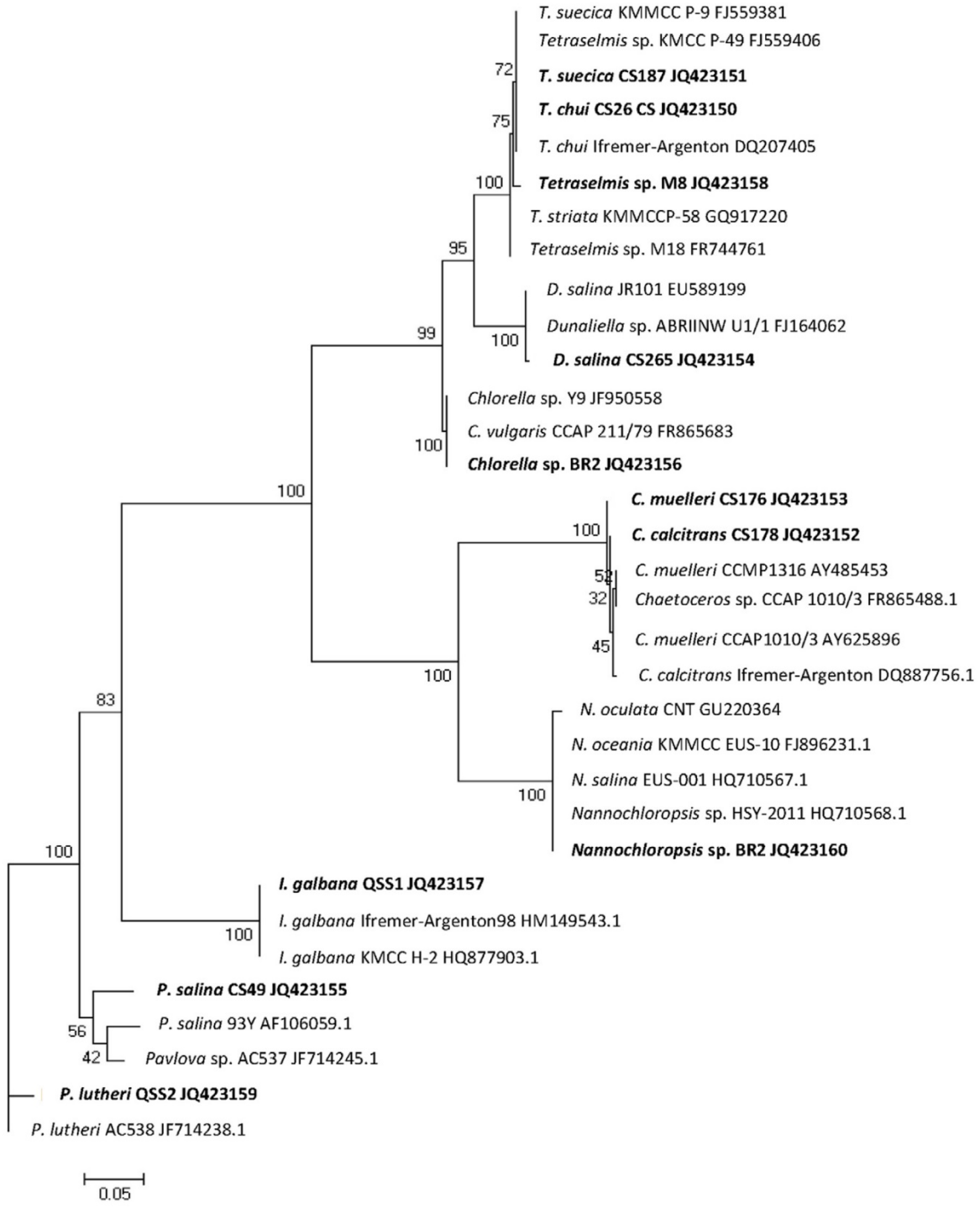

Figure 2. Maximum likelihood phylogenetic tree of 18S rRNA gene sequences from microalgae used in this study. Selected sequences from the NCBI database were also included (see Methods for selection criteria). Microalgae analyzed in this study are shown in bold. Numbers represent the results of 100 bootstrap replicates.
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select strains which grew well in F/2 medium, a common nutrient mix used for microalgae culture [31,32,40,41]. Serial dilutions would also select for fast growing strains, which would inevitably dominate a culture. Special attention must be given to ensure that a single fast growing strain does not dominate other potentially high lipid content strains but that may have a slower growth rate. After 48 hours of nutrient deprivation, Nile red staining of the isolated uni-clonal cultures revealed several strains with substantial

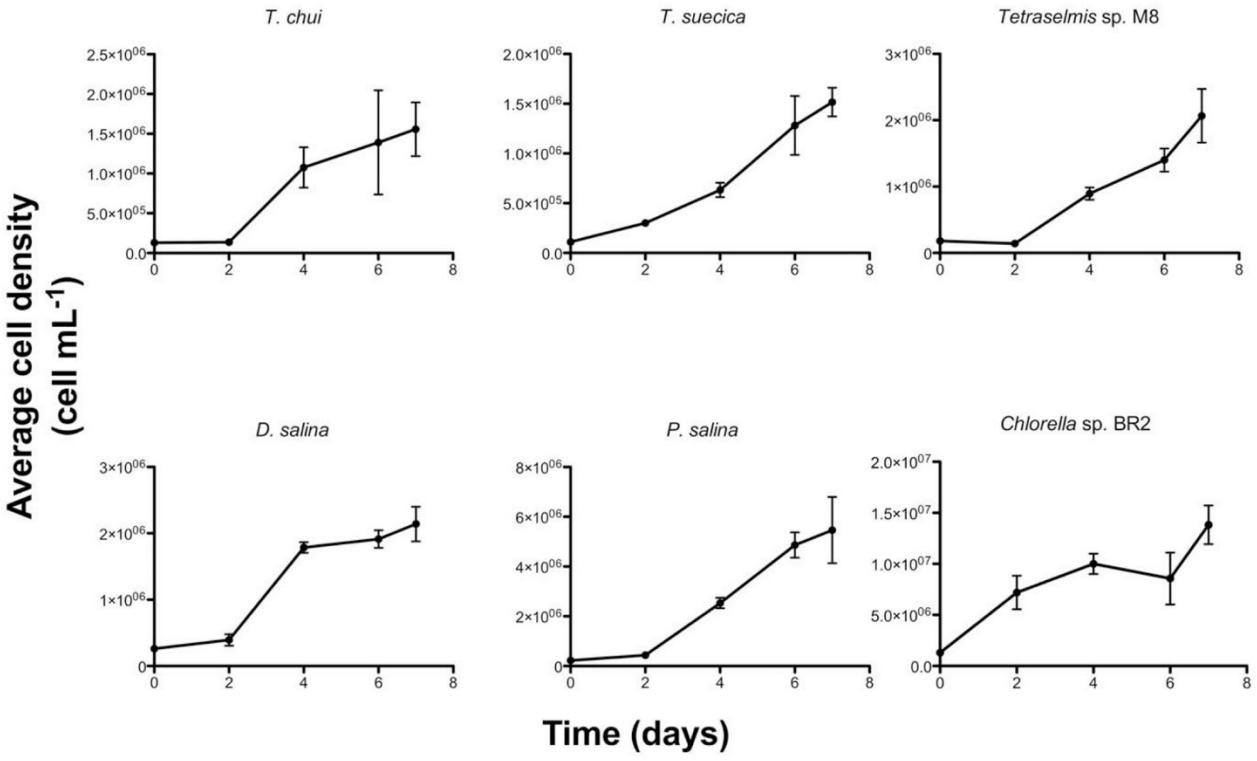

Figure 3. Growth curves of different microalgae in this study. *T. chui*, *T. suecica*, *Tetraselmis* sp. M8, *D. salina*, *P. salina* and *Chlorella* sp. BR2. Shown are average cell densities ± SD from three biological replicates.
doi:10.1371/journal.pone.0040751.g003

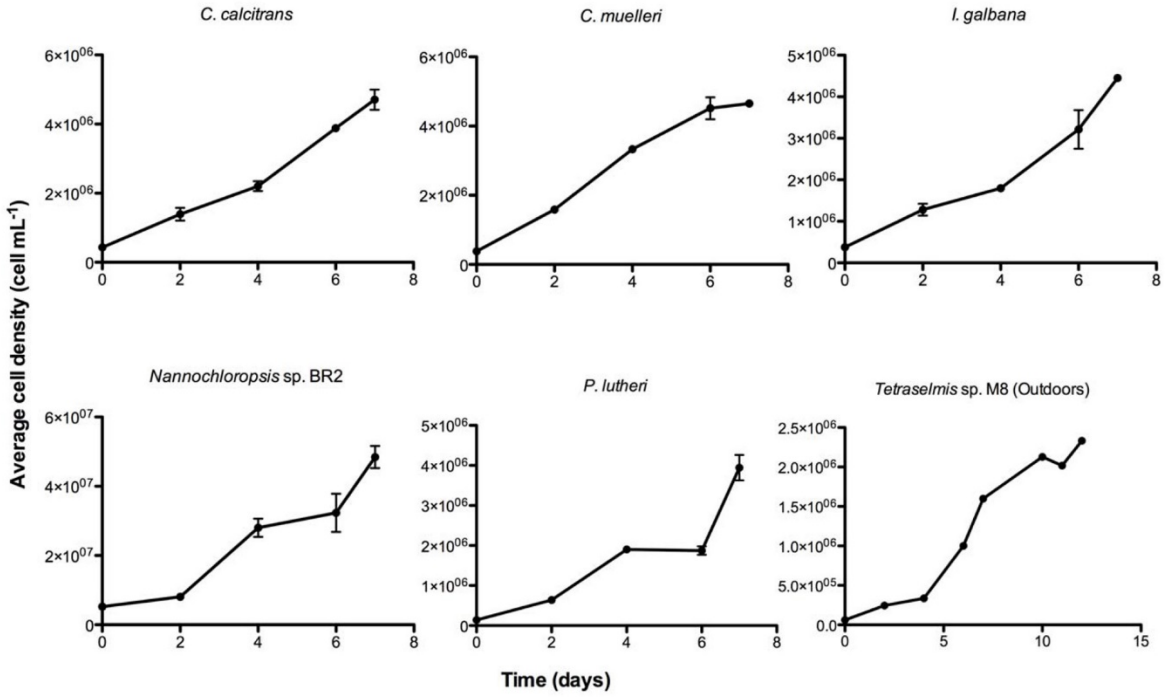

Figure 4. Growth curves of different microalgae in this study. *C. calcitrans*, *C. muelleri*, *I. galbana*, *Nannochloropsis* sp. BR2, *Chlorella* sp. BR2, *P. lutheri* & *Tetraselmis* sp. M8 (Outdoors). Shown are average cell densities ± SD from two biological replicates (3 replicates for *Nannochloropsis* sp. BR2 & 1 for *Tetraselmis* sp. M8 (Outdoors)).
doi:10.1371/journal.pone.0040751.g004

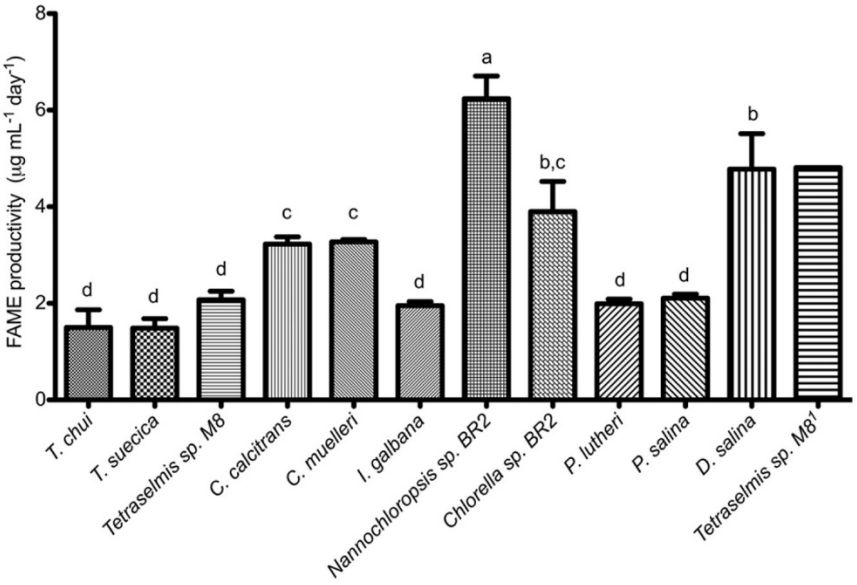

Figure 5. FAME levels of microalgae strains grown in batch culture (7 days growth + 2 days starvation by replacement of medium with seawater). Values shown are the averages of three biological replicates ± SD (except *Tetraselmis* sp.[1]). Different superscripts indicate significant difference at 95% level (ANOVA, Bonferroni's test; $P<0.05$). [1]Mid-scale outdoors culture.
doi:10.1371/journal.pone.0040751.g005

lipid producing potential. An inherent problem with using Nile red staining was that differences in cell wall structure between species do not allow for equal staining and prevented accurate comparison of lipid productivity between species. For this reason some species with thick cell walls (e.g. some other *Nannochloropsis* species) that were not included in the subsequent analysis may still have a strong potential as future microalgae crops.

A standard protocol was established to identify the top FAME-producing microalgae strains by comparing the growth rates, FAME productivity and composition of the 11 microalgae strains in this study. Growth rate and FAME productivity data was then compared with other literature (Table 4). It is crucial that any comparison must take into consideration the different growth conditions, culture system and lipid analysis methods (available in Table S1). Both average growth rate (μ_{ave}) and specific growth rate (μ_{exp}) of the 11 analyzed microalgae strains were calculated from cell count growth curves (Figures 3–4). Overall, μ_{ave} found in the present study were similar or higher than μ_{ave} published by [36] and [34], aside from [32] which had nearly twice the μ_{ave} (Table 4). The specific growth rate (μ_{exp}) of microalgae is more widely reported in the literature, although many studies only present growth in biomass productivity [11,30,33,35,47]. Comparison with available literature revealed the present study's overall μ_{exp} to be higher than most, with the exception of microalgae from three publications [40,48,49]. The overall high growth rates of this study were observed despite a lack of culture conditions such as air bubbling, CO_2 supplementation and longer photoperiods available in other studies (Table 4; Supplementary Table S1). This could be a result of the increased nutrient availability from the F media in comparison with other studies that utilize F/2 media [31,34,36]. Increase in nutrient availability, particularly nitrogen has been documented to increase growth rate [29,30,50], particularly when the nitrogen source in F/2 media, KNO_3 is low (0.75 mM). A previous study on *Nannochloropsis* discovered light intensity to only have a slight effect on growth rates [47], especially during low cell densities (Skye Thomas-Hall, personal communication) and growth rate discrepancies may be due to differences in prior culture history [51]. Ultimately, *T. chui* and *Tetraselmis* sp. M8 were found to have the highest μ_{exp}. *Tetraselmis* strains were also the fastest growers in two other studies, [31] and [34]. The growth rate of *Nannochloropsis* sp. in this study was below average, contrary to findings by Huerlimann et al. [31]. FAME analysis by GC/MS revealed *Nannochloropsis* sp. BR2 to be the highest TAG producer, followed by *D. salina* and *Chlorella* sp. BR2. These three strains have been found to also be high lipid producers in other studies. Rodolfi et al. [11] compared the lipid productivity of 30 microalgae strains and found *Nannochloropsis oculata* and *Chlorella* amongst the best producers of lipids, both indoors and outdoors. Likewise, Huerlimann et al. [31] investigated the lipid content of five tropical microalgae and discovered *Nannochloropsis* sp. to be the highest lipid producer. A strain of *Chlorella* was similarly found to be a high lipid producer in an evaluation of ten microalgae strains for oil production [33]. Surprisingly, *Isochrysis* sp., a high lipid producing strain in other studies, [34] and [35], was found to have one of the lowest lipid production rates in this study. Likewise, *Tetraselmis* strains, top lipid producers in other studies, [31] and [11], produced the least amounts of lipids in this study.

Variations in species strains, growth conditions, experimental design and lipid extraction/analysis methods make quantitative comparisons of lipid productivity and FA content between studies very difficult (Supplementary Table S1). Nevertheless, when compared with Patil et al [35], who similarly analyzed FAME productivity by GC/MS, the total FAME/dry weight (%) of *Nannochloropsis* sp. BR2 and *Tetraselmis* sp. M8 was found to be higher, while *I. galbana* produced the same amount of FAME/dry weight. However, GC/MS obtained FAME productivity of this study was found to be lower than other sources (except for [37])(Table 4) that utilized solvent and gravimetric methods to measure total lipids. This was expected as solvent and gravimetric methods would include FFAs, TAGs and other lipid classes such as polar lipids (e.g. phospholipids and glycolipids) [6], wax esters [52], isoprenoid-type lipids, [53], sterols, hydrocarbons and

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Table 3. Fatty acid composition in percentage of total FAME of different subtropical Australian microalgae strains after batch culture (7 days growth +2 days starvation).

	Nannochloropsis sp.	*T. chui*	*T. suecica*	*Tetraselmis* sp. M8	*D. salina*	*C. calcitrans*	*C. muelleri*	*Isochrysis* sp.	*P. lutheri*	*P. salina*	*Chlorella* sp.	*Tetraselmis* sp. M8
Fatty acid	**BR2**										**BR2**	**outdoor**
C12	0.2	0.1	0.1	-	0.1	-	-	-	-	0.2	0.5	0.8
C14	3.5	0.9	0.9	0.4	0.6	10.5	11.6	19.2	11.4	19.4	0.9	4.2
C15	0.4	0.1	0.2	-	-	-	-	-	-	-	0.2	0.5
C16	33.0	37.3	35.2	22.5	24.7	23.3	26.2	16.4	25.0	24.8	30.9	20.8
C16:1	26.8	2.5	2.3	1.1	2.9	34.1	29.7	2.0	19.1	3.6	4.4	1.3
C16:2	0.4	-	-	5.0	2.5	1.5	2.7	0.9	3.1	-	3.4	-
C16:3	-	0.2	-	-	2.9	4.0	5.5	-	-	-	7.8	0.1
C16:4	-	-	-	-	11.6	-	-	-	-	-	-	-
C17	0.4	0.1	-	4.5	-	1.6	1.8	-	-	-	0.4	2.5
C18	3.0	9.0	8.8	3.0	5.8	5.1	4.5	4.4	4.8	8.3	9.7	10.1
C18:1	6.0	13.8	15.3	9.1	5.6	5.8	1.7	21.7	1.3	2.0	9.2	13.6
C18:2	0.9	8.8	19.7	11.7	7.6	0.1	0.2	0.7	-	1.1	7.9	7.0
C18:3	0.4	15.1	8.8	28.9	33.8	0.0	0.4	3.1	0.1	1.3	22.8	11.1
C18:4	-	-	-	-	-	-	-	-	-	6.1	-	12.7
C20	0.2	0.5	0.5	-	0.1	-	-	-	-	0.4	0.9	-
C20:1	-	1.8	2.1	-	0.1	-	-	5.9	0.1	-	0.8	4.6
C20:4	5.9	2.6	3.3	3.4	-	0.9	1.4	13.9	6.1	-	0.1	0.1
C20:5	18.8	7.2	2.9	10.6	1.2	12.7	14.0	0.0	21.8	16.1	-	10.6
C22	-	-	-	-	-	-	-	-	-	-	-	-
C22:4	-	-	-	-	-	-	-	-	-	6.3	-	-
C22:6	-	-	-	-	0.4	0.3	0.4	11.8	7.3	10.5	-	-
Total saturated (%)	40.7	47.9	45.6	30.4	31.4	40.5	44.0	39.9	41.1	53.0	43.6	38.9
Total monounsaturated (%)	32.8	18.2	19.7	10.2	8.6	40.0	31.4	29.6	20.5	5.5	14.4	19.5
Total polyunsaturated (%)	26.5	34.0	34.7	59.5	60.0	19.5	24.6	30.5	38.3	41.4	42.0	41.7
Total FAMEs ($\mu g\ mL^{-1}$)	56.1	13.5	13.4	18.7	43.0	29.0	29.5	17.6	17.9	19.0	31.4	57.7
Total FAME/dry weight (%)	10.6	3.2	10.8	2.5	11.4	-	5.9	3.9	4.0	1.2	5.3	9.9

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Table 4. Comparison of FAME productivity (μg mL^{-1} day^{-1}) of present study microalgae with lipid productivity of microalgae species from other references.

Species	Lipid productivity [μg mL^{-1} day^{-1}]	References
***Nannochloropsis* sp. BR2**	6.2	This study[GCMS, AG]
Nannochloropsis sp.	4.6	Huerlimann et al. (2010)[12h]
Nannochloropsis sp.	48.0	Rodolfi et al. (2009)[24h, CO2]
Nannochloropsis sp.	37.6	Rodolfi et al. (2009)[24h, CO2]
Nannochloropsis sp.	60.9	Rodolfi et al. (2009)[24h, CO2]
Nannochloropsis oculata	10.0	Converti et al. (2009)[24h, CO2]
***Tetraselmis* sp. M8**	2.1	This study[GCMS, AG]
***Tetraselmis* sp. M8 (outdoor)**	4.8	This study[GCMS]
Tetraselmis sp.	18.6	Huerlimann et al. (2010)[12h]
Tetraselmis sp.	43.4	Rodolfi et al. (2009)[24h, C02]
Tetraselmis sp.	10.7	Patil et al. (2007)[GCMS, 24h, CO2]
Tetraselmis chui	1.5	This study[GCMS, AG]
Tetraselmis chui	27.0	Rodolfi et al. (2009)[24h, CO2]
Tetraselmis suecica	1.5	This study[GCMS, AG]
Tetraselmis suecica	36.4	Rodolfi et al. (2009)[24h, CO2]
Dunaliella salina	4.8	This study[GCMS, AG]
Dunaliella salina	33.5	Takagi et al. (2006)
Chaetoceros muelleri	3.3	This study[GCMS, AG]
Chaetoceros muelleri	21.8	Rodolfi et al. (2009)[24h, CO2]
Chaetoceros calcitrans	3.2	This study[GCMS, AG]
Chaetoceros calcitrans	17.6	Rodolfi et al. (2009)[24h, CO2]
Chaetoceros sp.	16.8	Renaud et al. (2002)* [12h]
Isochrysis galbana	2.0	This study[GCMS, AG]
Isochrysis sp.	24.9	Renaud et al. (2002)* [12h]
Isochrysis sp.	12.7	Huerlimann et al. (2010)[12h]
Isochrysis sp.	37.7	Rodolfi et al. (2009)[24h, CO2]
I. galbana	12.4	Patil et al. (2007)[GCMS, 24h, CO2]
Pavlova lutheri	2.0	This study[GCMS, AG]
Pavlova lutheri	50.2	Rodolfi et al. (2009)[24h, CO2]
Pavlova salina	2.1	This study[GCMS, AG]
Pavlova salina	49.4	Rodolfi et al. (2009)[24h, CO2]
Pavlova sp.	21.7	Patil et al. (2007)[GCMS, 24h, CO2]
***Chlorella* sp.**	3.9	This study[GCMS, AG]
Chlorella sp.	7.1	Chen et al. (2010)[AG]
Chlorella sp.	20.0	Converti et al. (2009)[24h, CO2]
Chlorella sp.	42.1	Rondolfi et al. (2009)[24h, CO2]
Chlorella sorokiana	44.7	Rondolfi et al. (2009)[24h, CO2]
Chlorella sorokiana	1.0	Illman et al. (2000)[24h, CO2]
Chlorella vulgaris	5.3	Illman et al. (2000)[24h, CO2]

*Calculated total lipid content (μg mL^{-1}).
[GCMS]Values obtained by GC/MS.
[24h]Cultures grown with 24 h light and air.
[12h]Cultures grown with 12h light and air.
[CO2]Cultures grown with air supplemented with CO_2.
[AG]Cultures grown with agitation.
For a full comparison of culturing conditions see Table S1.
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pigments. Furthermore, different growth conditions in other studies such as growth enrichment with carbon dioxide [48,54], increased photoperiods and light intensity [55], different media volumes and larger initial inoculum would explain for the increased lipid productivity in other studies. This is most evident in the study by Rodolfi et al. [11], where similar strains of *P. salina*

CS-49 and *C. calcitrans* CS-178 were studied under different conditions to reveal significantly different results. It should be noted that the conditions of the current experimental design were not meant to achieve maximum lipid production but to determine the best lipid producing candidates under standard "unoptimized lab conditions", which were *Nannochloropsis* sp. BR2, *D. salina* and *Chlorella* sp. BR2. Higher confidence in the data may be obtained by growing cultures completely independently (i.e. experiments carried out separately at different times with a different culture). Subsequent studies may focus on the comparison of best strains under fully optimized and/or large-scale commercial conditions. In our study, *Tetraselmis* sp M8 was chosen for a scale-up study based on its fast growth rates, culture dominance and ease of harvesting by settling. A comparison of the indoor laboratory conditions to mid-scale (1000 L) outdoor conditions showed that lipid productivity more than doubled under these conditions. Although further long-term studies will be required, these preliminary findings demonstrate the potential for optimization and emphasize that outdoor and large-scale conditions differ strongly from laboratory conditions.

Suitable candidates for biodiesel production require not only high lipid productivity, but also suitable FA content. Recommended FAs for good biodiesel properties include C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 [3,56]. In this study, analyses of FA profiles revealed *Nannochloropsis* sp. BR2, *Chlorella* sp. BR2 and *Chaetoceros* strains (*C. calcitrans* and *C. muelleri*) to be the best candidates (Table 3). In addition to having the highest lipid productivity, the recommended FAs for biodiesel accounted for 73.6% of the total FAs in *Nannochloropsis* sp. BR2, in particular C16 (33%) and C16:1 (26.8%). Huerlimann et al. [31] reported a similar FA composition of *Nannochloropsis* sp. following nutrient deprivation, while Patil et al. [35] also reported *Nannochloropsis* sp. to have the highest C16 and C16:1 content. *Chlorella* sp. BR2 presented slightly lower lipid productivity although having more desired FAs for biodiesel (81.4%). It also had a higher C18 (9.7%) and unsaturated C18 content (39.9%) if compared to *Nannochloropsis* sp. BR2 or the *Chaetoceros* strains; making it more desirable for the production of biodiesel with a higher cold filter plugging point (CFFP) for better performance at low temperatures [3]. Both *C. calcitrans* and *C. muelleri* are good candidates despite only having mediocre lipid productivity due to high levels of C14 FAs (10.5% and 11.6% respectively) and recommended FAs for biodiesel (78.9% and 74.5% respectively). The FA content of *C. calcitrans* was observed in accordance to Lee et al. [34] during low nitrogen conditions, which caused an increase in saturated FAs like C16. *D. salina* was not considered a suitable candidate for biodiesel despite its high lipid productivity due to high levels of PUFAs (C16:4 – 11.6%. C18:3 – 33.8%). Low levels of PUFAs, as evident in *Nannochloropsis* sp. and *C. calcitrans* are desired for biodiesel production as it reduces the need for treatments such as catalytic hydrogenation. *Nannochloropsis* sp. BR2, *C. calcitrans* and *C. muelleri* also exhibited C20:5 (EPA) (18.8%, 12.7% and 14% respectively) that would allow for a biorefinery approach to biodiesel production. It should be noted, however, that microalgal biodiesel is likely to be first used as a drop-in fuel in the future which would allow to achieve blends with the desired fuel properties from most microalgae species.

Commercially feasible production of microalgal biodiesel would require a biorefinery approach to produce biodiesel as well as other value-added products such ω-3 FAs and protein-rich biomass. Microalgae possess the potential to produce high amounts of ω-3 FAs such as EPA (C20:5) and DHA (C22:6) that are used as dietary supplements. The best candidates for EPA and DHA production in this study were found to be *Nannochloropsis* sp. BR2 and the *Pavlova* strains (*P. salina* and *P. lutheri*). Overall, *Nannochloropsis* sp. BR2 produced the highest amounts of ω-3 FAs on account of its high overall lipid and EPA content (18.8%). *P. lutheri* exhibited the highest proportional content of EPA (21.8%), while *Isochrysis* sp. had the highest DHA content (11.8%). The ω-3 FA contents of *Nannochloropsis* sp. and the *Pavlova* strains were comparable to previously published values [31,35,57].

The use of a nutrient starvation phase to improve TAG productivity (particular C16:0 and C16:1) for biodiesel production was successful as C16 and C16:1 FAs were found to be the predominant FAs in the present study. During nutrient limiting conditions, unsaturated FAs are consumed as an energy source and saturated FAs are accumulated [58]. The increase of the % of saturated and monounsaturated FAs during starvation have been well documented in literature for several other species [34,59,60]. While this may prove useful for biodiesel production, the reduction in PUFAs is a problem for ω-3 FA production that has been documented [31,34]. Nevertheless, EPA and DHA contents have been reported to remain consistent despite changes in nutrient level for *T. tetrathele* [40], which may explain the high levels of PUFA observed in *Tetraselmis* sp.

In a 1000 L-outdoor setting, *Tetraselmis* sp. M8 was found to have an increased μ_{Ave} despite a longer lag phase. Cell density achieved by outdoor grown *Tetraselmis* sp. M8 was similar to other large-scale cultures of *Tetraselmis* [61]. FAME productivity and composition were also analyzed, which revealed a near tripling of FAME productivity as well as altered FA composition. High amounts of C16:2, C18:2, C18:3 previously detected in laboratory-grown *Tetraselmis* sp. M8 was found reduced, while higher amounts of recommended FA for biodiesel (particularly C14, C18 & C18:1) were present. The increase in FAME productivity and desirable FA composition of *Tetraselmis* sp. M8 in a mid-scale setting demonstrates that the microalgae isolation and selection technique used in this study can lead to the identification of microalgae strains with potential for large-scale cultivation. Additional factors to be considered for large-scale production include harvesting and oil extraction properties of different microalgae. For example, we noticed that our *Tetraselmis* strains may lose their flagella during stress conditions, resulting in rapid settling that allows easy harvesting/dewatering. Small microalgae, such as *Nannochloropsis* sp., on the other hand may instead be harvested by froth flotation or other techniques, but our results indicate that Nile red staining and lipid extraction may be compromised by thick cell walls in this strain.

Supporting Information

Table S1 Comparison of FAME productivity (μg mL^{-1} $day^{-}1$) of present study microalgae with lipid productivity of microalgae species from other references (including a full comparison of culturing conditions).
(PDF)

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Author Contributions

Conceived and designed the experiments: DKYL SG MT ESBZ SRTH YL PMS. Performed the experiments: DKYL SG MT ESBZ SRTH. Analyzed the data: DKYL SG MT ESBZ SRTH HS YL. Contributed reagents/materials/analysis tools: MT HS PMS. Wrote the paper: DKYL HS MT YL PMS.

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Chapter 3: Improving microalgae via non-GM methods: mutagenesis and high-throughput selection of high-lipid productivity improved strains

Following Chapter 2's evaluation of all available strains in the Algae Biotechnology Laboratory's Microalgae Collection, *Tetraselmis suecica* was identified as an ideal candidate for non-GM strain improvement in this chapter. The use of non-GM methods for microalgae strain improvement is very attractive as it requires very little biochemical or genetic information, and avoids the regulatory hurdles of using GM strains outdoors. Furthermore, the ability to increase a strain's lipid content is particular useful when a wild-type (undomesticated) strain possesses all the desirable traits for large-scale production (high growth rate, ease of harvest, robustness) except for very high lipid content. This made *T. suecica* an ideal strain for this purpose, as it possessed all the aforementioned characteristics with the exception of a high lipid content. This chapter, presented in Research paper 2 "**Isolation of high-lipid *Tetraselmis suecica* strains following repeated UV-C mutagenesis and high-throughput growth selection"** presents the optimisation and development of a mutation-selection program aimed at improving the lipid content of *Tetraselmis suecica*.

Keypoints

- Rounds 1 to 3 of the mutation-selection program consisted of mutagenesis followed by lipid selection by FACS, and then growth selection in 96 well-plates. Additional lipid selection steps using plate reader technology was introduced in rounds 4 & 5 to increase selection pressure.
- Two different UV-C dosages (50% & >98% lethal dosage) were used to generate two separate *T. suecica* improved strain-lines. After five rounds of mutation-selection, both improved strains had significantly higher lipid contents (114-123% more) when compared to the original wild-type.
- The growth rates of improved strains did not decrease and were not significantly different from wild-type.
- GC/MS analysis revealed that improved *T. suecica* strains accumulated less saturated and monounsaturated FA, but more polyunsaturated FA.
- Comparisons with wild-type control cultures were done 36 divisions after the last mutagenesis step and were therefore considered to have a stable genetic make-up.
- Up to now it is not clear whether strain improvements were due to stable mutations or rather adaptation of strains (e.g. by epigenetics) to the selection pressures.

Research Paper 2: Isolation of High Lipid Tetraselmis suecica Strains Following Repeated UV-C Mutagenesis, FACS, and High-Throughput Growth Selection

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Highlights

- UV-C mutagenesis and high throughput selection technology has been combined to improve lipid productivity in *Tetraselmis suecica*.
- Both 50% and >98% lethal dosage successfully produced improved strains with approximately 100% increase in lipid accumulation.
- Growth rates of improved strains remained unchanged.

Abstract

Mutagenesis and selection of microalgae can be used for accelerated breeding of elite strains, providing a significant advantage over genetic engineering as prior biochemical and genetic information is not required. UV-C-induced mutagenesis combined with fluorescence-activated cell sorting (FACS) and microtiter plate reader cell density screening was used to produce *Tetraselmis suecica* strains with increased lipid contents without compromising on cell growth. After five rounds of mutation-selection, two dosages of UV-C (50% and >98% lethality) yielded two improved strains (M5 and M24) that produced significantly more neutral lipids (increases of 114%

and 123%, respectively). This study highlights that repeated UV-C mutagenesis and high-throughput selection for cell growth can be a viable combined approach to improve lipid productivity in microalgae. These maybe used as elite strains for future breeding programs and as potential feedstock for biodiesel production.

Introduction

As fossil fuel resources diminish and cause environmental damage, there is a rapidly-growing global demand for lipids, particularly triacylglycerides (TAGs) for the biofuel, aquaculture and pharmaceutical industry. The need to develop sustainable lipid sources is now widely apparent. Traditional crop-based plants are increasingly being used for oil and biofuel production, but these cannot reasonably meet the growing demand [1]. Photosynthetic microalgae have repeatedly been proposed as a more viable lipid source due to their high productivity, environmental benefits and ability to produce different kinds of oils [2-4]. Theoretically, microalgae can produce 10 to 20 times more lipids than oil palms [5], corn and soybean [6-8] while achieving CO_2 capture efficiencies of up to 99% [9]. Furthermore, the production of microalgal biomass can be carried out without competing for valuable resources, such as arable land, biodiverse landscapes (e.g. rainforests) and freshwater [5]. However, the industry is still in its infancy and the cost of microalgal lipid production is still too high to achieve full commercialization of microalgal lipid feedstocks. Having the ideal algal strain with elevated lipid content, high growth rate and robust environmental tolerance remains one of the most important factors to improve algae economics [10,11]. While many studies have focused on species selection and characterization to identify strains that contain two seemingly antagonistic traits: fast growth and high lipid content, genetic and metabolic engineering provide opportunities to create potential elite strains that meet these requirements. At present, induced mutagenesis provides a significant advantage over genetic engineering, as little biochemical or genetic information regarding the chosen organisms is needed [12,13]. This approach requires relatively little technical manipulation. Improved non-transgenic microorganisms can be bred by incorporating mutagenesis and high-throughput selection, including microalgal strains with enhanced lipid performance.

Mutation studies involving ultraviolet (UV), ethyl methane sulfonate (EMS) and nitrosomethylguanidine (NTG) have been performed on a range of microalgal species (*Phaeodactylum tricornutum*, *Pavlova lutheri*, *Nannochloropsis oculata*, *Haematococcus pluvialis*,

Schizochytrium sp., *Chlorella sorokiana*, *Scenedesmus obliquus, Isochrysis galbana, Dunaliella salina*) to produce mutants that exhibited increased/modified lipid content and growth rate [14-23]. Many of these studies, however, rely upon time-consuming techniques like metabolite antibiotic response to select for mutants and lipid extraction techniques to analyze their lipid content. Lately, the combination of lipophilic dyes (e.g. Nile red) and high-throughput technologies such as microplate readers and flow cytometry provided a powerful tool to isolate potential mutants from a complex population based on specific fluorescence cell properties. A study using the Nile red fluorochrome in conjunction with microplate reader technology has been shown to be able to select *C. sorokiniana* and *S. obliquus* mutants with elevated lipid contents [21]. Automated fluorescence-assisted cell sorting (FACS) can be more efficient than a microplate reader-based selection, as much larger populations of single cells can be handled, resulting in the recovery of a high number of candidate cells with the desired lipid content. The use of FACS in combination with mutagenesis has been described in mutation-selection studies that produced high-lipid content strains of *I. galbana* [23] and carotenoid-hyperproducing *D. salina* strains [22]. Even without mutagenesis, the use of FACS has been successful in generating higher lipid content strains of *Nannochloropsis* sp. and *T. suecica* through selection alone [1,24]. While most studies involve only one screening step, the present study combines a mutation-selection approach using UV-C-induced mutagenesis [19-21,23] with FACS [1,24] and microplate reader screening [21] to mutate, identify and isolate *T. suecica* cells with a higher lipid content without reduced growth rate.

Tetraselmis suecica is a flagellate green microalga commonly used as aquaculture feedstock, and that is also considered a good candidate for biofuel production. This species is known to have a high lipid content as well as being robust enough to tolerate a range of environmental conditions [25,26]. Moreover, *Tetraselmis* cells have recently been shown to lose their flagella during stressful conditions, quickly settling and thus reducing harvesting/dewatering cost [27]. The growth characteristics of the *T. suecica* strain, used in the present study, have been previously described, displaying one of the highest comparative microalgal growth rates, although with a slightly lower total fatty acid content compared to other strains tested [28]. Therefore, this microalga was chosen as a suitable candidate to generate an improved strain with fast growth and high-lipid content properties.

Material and Methods

1196

1197 **Microalgae culturing conditions**

1198

1199 The parent culture of wild-type *Tetraselmis suecica* (wt) was originally collected by
1200 Queensland Sea Scallops (Bundaberg, Australia; [28]) and cultured in autoclaved artificial seawater
1201 with Guillard F medium [29] and maintained aerobically in 100 mL Erlenmeyer flasks with
1202 constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent
1203 white light (120 µmol photons $m^{-2}s^{-1}$).

1204

1205 **Mutagenesis**

1206

1207 The mutation program used UV-C (100-280 nm) as the mutagenic agent. First, the
1208 appropriate dosage of UV-C treatment was determined in order to obtain an equivalent of the rate of
1209 non-lethal mutations. To achieve this, kill curves were produced by two methods, to establish 50%
1210 (LD50) and >98% lethal dosage.

1211

1212 Mutagenesis was achieved by placing a 2 cm-deep culture of *T. suecica* (1 x 10^6 cells/mL; early
1213 starvation phase) in a 140 mm-Petri dish under the lamps of a Bio-Rad GS Gene Linker UV
1214 Chamber and exposure to 15, 25, 50 and 100 mJ of UV-C. Cultures were then left in the dark for 24
1215 h to prevent DNA repair by photo-reactivation. The first kill curve was obtained by plating 200 µL
1216 aliquots of 1/1000-diluted mutagenized cells onto a 1% agar plate containing F medium in artificial
1217 seawater, which were then allowed to grow for 3 weeks before algal colonies from control and UV-
1218 exposed cells were counted. The second kill curve was carried out in 96 well-plates inoculated with
1219 mutagenized cells after serial dilution down to 1 cell per well [22]. Wells with surviving microalgae
1220 that multiplied were then counted after 3 weeks of growth. Both, agar plates and 96 well-plates
1221 were grown under fluorescent white lights (50 µmol photons $m^{-2}s^{-1}$; 16:8 h light:dark
1222 photoperiod) and maintained at 24°C. Further stages of this study used UV-C dosages of 25 mJ
1223 and 100 mJ to induce mutagenesis, as these provided a survival rate of 50% and <2%, respectively.

1224

FACS and lipid quantification by Nile red fluorescence

Nile red (Sigma Inc.) was used to stain lipids for (i) FACS and (ii) quantification of lipid contents via a 96 well-microplate reader. First, the appropriate amount of Nile red working solution required to produce the best lipid staining, while maintaining a high FACS survival rate, had to be determined. Cell samples in starvation phase (1 mL at 0.8-1 x 10^6 cells/mL) were treated with 1, 2 and 3 µL of a working solution of Nile red in acetone or dimethyl sulfoxide (both 1 mg/mL). Samples were then gently mixed and incubated in the dark for 10 min. Single cells were sorted using a BD FACSVantage SE (Becton Dickinson) cell sorter with a 485 nm argon laser and 100 µm nozzle into 96 well-plates using F in seawater medium. Cell fluorescence was measured at 585 nm for yellow-gold fluorescence, indicative of neutral lipid content. Approximately 10,000 cells were analyzed, with dot plots of yellow-gold fluorescence (PE-A) vs. forward light scatter (FSC-A, cell size). Cell sorting regions were positioned to include cells presenting increased fluorescence and size compared to the general population of cells. FACS survival rates were then determined after 2 weeks of growth.

To enable quantification of neutral lipid contents of *T. suecica* cells in a microtiter plate reader, the ability of Nile red (in acetone working solution) to stain neutral lipids without killing the cells was first established. Two populations of microalgal cells (10^6 cells/mL): (i) cells in late starvation phase and (ii) cells in exponential growth phase were mixed to produce a population of 0, 25, 50, 75 and 100% of starved cells, as a proxy for increasing lipid content within a given volume. A total of 1 mL of these samples was then stained with 2 µL of a working solution of Nile red in acetone (1 mg/mL). Samples were then gently mixed and incubated in the dark for 10 min. A total of 100 µL from each sample was then loaded into a 96 well-microtiter plate (Sarsted) in triplicates. Yellow-gold fluorescence was measured on a POLARstar OPTIMA (BMG Labtech) plate reader using fluorescence intensity mode. Gain was set at 3000, with excitation and emission wavelengths of 485 nm and 590 nm, respectively. These settings were used for further fluorescence intensity measurements in this study.

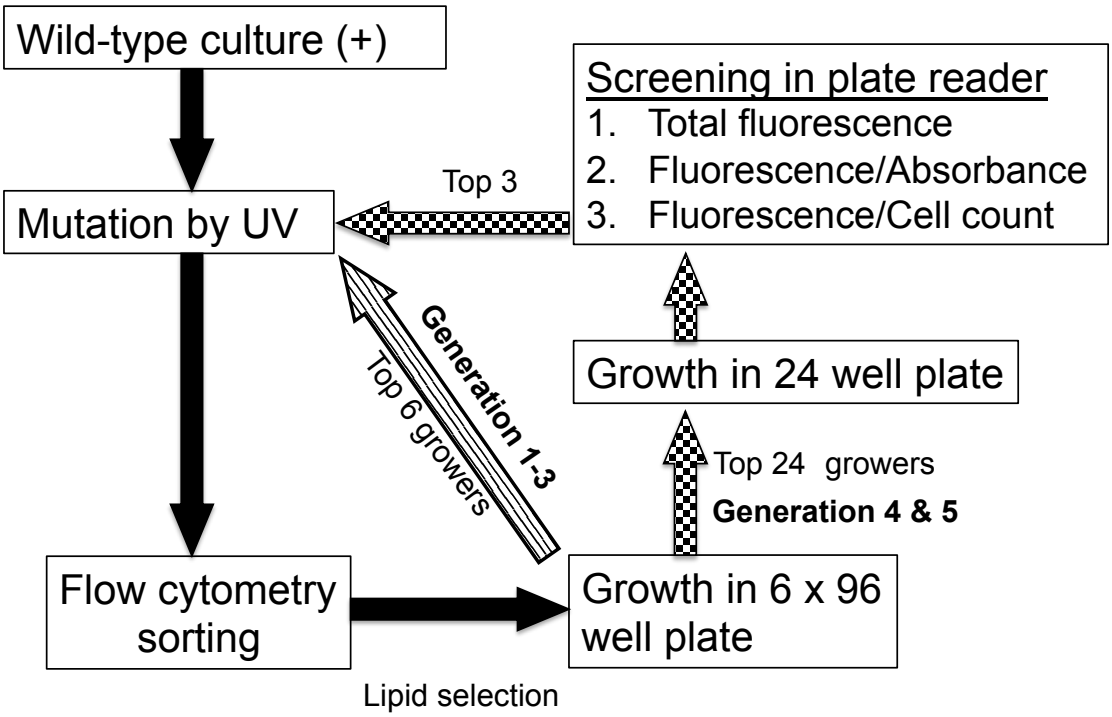


Figure 1. Mutation-selection cycle for the production of *T. suecica* cells for improved lipid production. Cycles 1-3: the top 6 clones with the fastest growth after each sorting run were selected for subsequent mutagenesis. Cycle 4 & 5: the top 24 fastest growers were scaled up and their lipid production performance evaluated. The top 3 lipid producing cultures were selected for subsequent cycles.

Mutation-selection cycles

Mutation and selection cycles are summarized in Fig. 1. Cultures were grown until late log phase and then mutagenized according to the optimized method at 25 mJ and 100 mJ. Mutagenized cultures were then left in the dark for 24 h and then cultured for 2 weeks to allow for culture recovery (addition of fresh F medium every 7 days). Two days prior to FACS, nutrient deprivation to stimulate lipid production was achieved by removal of previous medium by centrifugation (1,200 x g, 5 min) and replacement with only seawater (without F medium). FACS was then carried out according to the optimized methodology, with mutagenized single cells sorted into 96 well-plates. Plates were incubated at 24°C under a 16:8 h light:dark photoperiod of fluorescent white lights (50 µmol photons $m^{-2}s^{-1}$). For generations (cycles of mutagenesis and selection rounds) 1, 2 and 3, the plates after FACS were monitored daily, and the first 6 wells that showed visual signs of algal

growth were selected and scaled up for subsequent rounds of mutagenesis and cell sorting. For generations 4 and 5, the top 24 wells from the 96 well-plates after FACS that showed visual signs of growth were scaled up (1:10 dilution) in a 24 well-plate. After 7 days of growth at 24°C under a 16:8 h light:dark photoperiod of fluorescent white light (50 µmol photons $m^{-2}s^{-1}$), the absorbance value (450 nm) and fluorescence intensity of the wells were measured. Cell counts were performed on the top 12 wells that showed the highest fluorescence/absorbance ratio using a haemocytometer. The top 3 clones that showed the highest fluorescence/cell count ratio were selected and scaled up for subsequent rounds of mutagenesis. In all cycles of the program, selected strains were allowed to grow for 3 to 4 weeks to ensure a genetically stable population before mutagenesis.

Standard protocol for culture growth analysis, lipid induction phase, sampling for fluorescence and lipid analysis

A standard protocol was designed to allow direct comparison of growth rates, fluorescence intensity and fatty acid (FA) profile between selected strains and wt based on a modified method by Lim et al. [28]. Briefly, a total of 5 mL of selected strain or wt culture in late log phase was used as inoculum (8 h after start of the light cycle) for 50 mL artificial seawater complemented with F medium in 100 mL Erlenmeyer flasks, and grown under constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light:dark photoperiod of fluorescent white light (120 µmol photons $m^{-2}s^{-1}$). After day 7, nutrient deprivation to stimulate lipid production was achieved by centrifugation (1200 x g, 5 min) and replacement with only seawater (without F medium). Cultures were then grown for another 5 days post starvation. Cell counts were performed on days 0, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12 & 13, while fluorescence intensities were measured on days 0, 2, 3, 4, 5, 6 post starvation. The fatty acid methyl ester (FAME) contents of the cultures were analyzed on day 4 post starvation.

Fatty Acid Methyl Ester (FAME) analysis

For FAME analyses, 4 mL of algal culture was collected from each replicate and centrifuged at 16,000 x g for 3 min. After the supernatant was discarded, lipids in the algal pellet were

hydrolyzed and methyl-esterified by shaking (1,200 rpm) with 300 µL of a 2% H_2SO_4/methanol solution for 2 h at 80°C. Prior to the reaction, 50 mg of heneicosanoic acid (Sigma, USA) was added as internal standard to the pellet. A total of 300 µL of 0.9% (w/v) NaCl and 300 µL of hexane was then added followed by mixing for 20 s. Subsequently, phase separation was performed by centrifugation at 16,000 x g for 3 min. A total of 1 mL of the hexane layer was then injected splitless into an Agilent 6890 Gas Chromatograph coupled to a 5975 MSD Mass Spectrometer. A DB-Wax column (Agilent, 122–7032) was used with running conditions as described for Agilent's RTL DBWax method (Application note: 5988–5871EN). Quantification of FAMEs was carried out by taking the ratio of the integral of each FAME's total ion current peak to that of the internal standard (50 mg), with the molecular mass of each FAME also factored into the equation. FAMEs were then identified based on mass spectral profiles, in comparison to standards and expected retention times from Agilent's RTL DBWax method (Application note: 5988–5871EN).

Analytical methods

Growth rates and doubling times were calculated from day 0 to day 7 to measure the growth rate during growth phase, day 0 to day 10 to measure the overall growth rate, and from day 7 to day 10 to measure the growth rate during starvation phase.

Calculations for growth rate and doubling time are based on the following equations:

Growth rate $\mu = Ln(Ny/Nx)/(ty-tx)$

Mean doubling time $TAve = (ty-tx)/ \log2 (Ny/Nx)$

with Ny and Nx being the number of cells from the selected days of analysis.

Results

Mutagenesis survival rate

The appropriate dosage of UV-C exposure to obtain a 50% and <2% survival rate was determined by treating microalgal cells with a range of UV-C dosages (0-100 mJ), followed by growth on agar- (solid media) and in 96 well-plates (liquid media). Survival rate (Figure 2a) was found to be dosage-dependent, with the survival rate decreasing as UV-C dosage increased. The growth method was also had an effect of post-UV-C exposure survival rates. When grown on agar plates, the LD50 was found to be at 16 mJ, while <2% survival rate was found to be at 63 mJ. In liquid medium the 50% survival rate and <2% survival rate was at 26 mJ and 92 mJ, respectively (Figure 2a) and this dosage was used during the subsequent experiments.

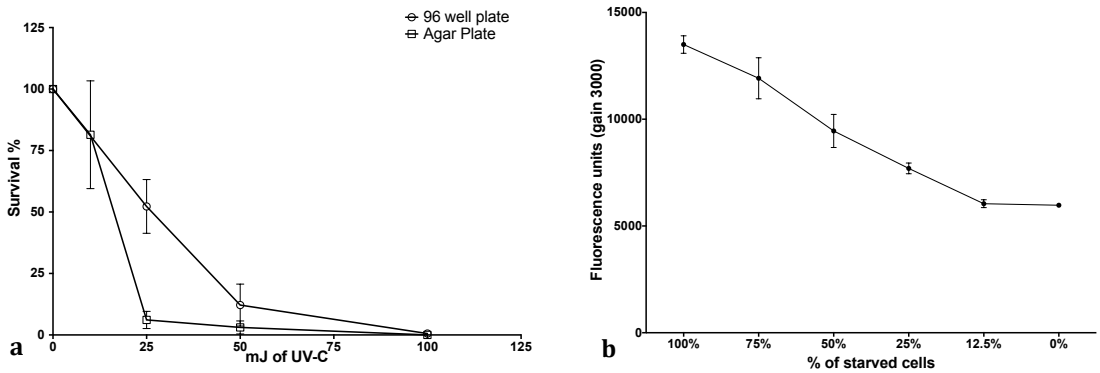


Figure 2. Optimization of UV-C lethal dosage and Nile red staining. (a) Survival rate of *T. suecica* in agar plates and 96 well-plates after exposure to varying UV-C dosages. The 10 mJ survival rates in 96 well-plates were not measured. (b) Fluorescence units of mixed starved & unstarved *T. suecica* population demonstrating the ability of Nile red staining in acetone to determine varying levels of neutral lipids in *T. suecica.* Data represent mean ± SEM from three independent replicates.

Optimization of Nile red staining for Fluorescence-Activated Cell Sorting (FACS)

The use of appropriate Nile red staining solution was required to ensure maximum fluorescence while maintaining a high recovery of viable clones. While the increase in staining solution did not affect fluorescence intensities or clone recovery, it was found that cells stained with Nile red dissolved in acetone produced fluorescence intensities markedly higher than cells stained with Nile red in DMSO (Figure 3). Unstained populations achieved a recovery rate of 78% viable cells, while both DMSO and acetone solvents achieved 40-50% recovery. Therefore for this study, the addition of 1 µL Nile red in acetone working solution was chosen for FACS. The ability of Nile red staining in acetone to determine varying levels of neutral lipids in *T. suecica* was also established (Figure 3). This study also found that 2 mg/mL of Nile red was suitable to detect populations of *T. suecica* with >12.5% of cells containing neutral lipids, demonstrating a strong linear correlation (r^2= 0.98, n=6) between the percentage of starved cells and fluorescence intensity.

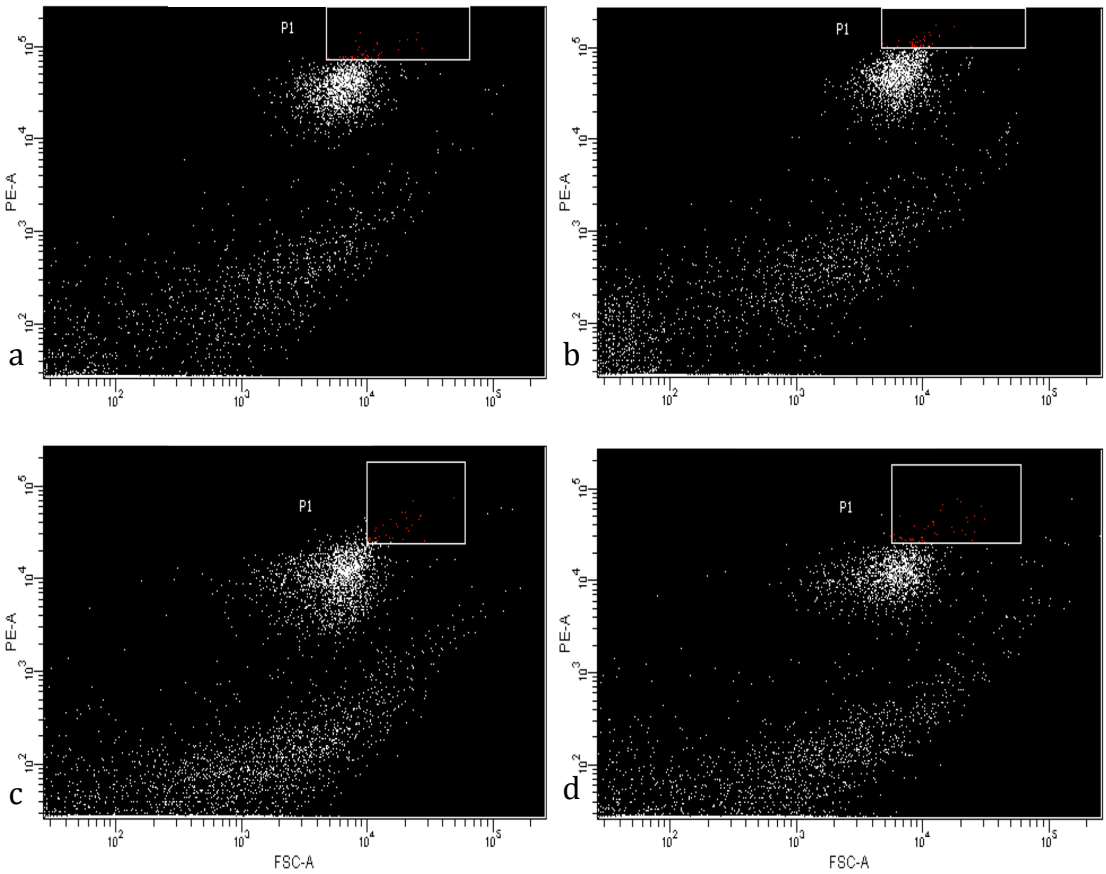
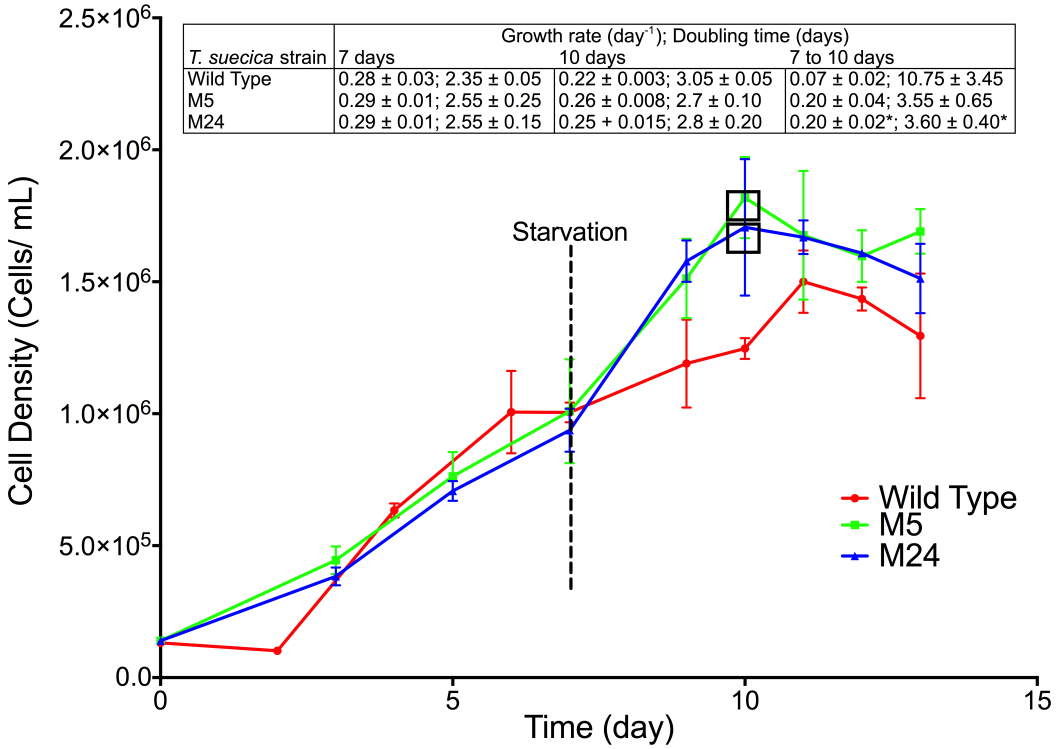


Figure 3. Two-dimensional dot plots of *T. suecica* stained with varying volumes of Nile red in acetone or DMSO working solution: (a) 1 µL Nile red-acetone, (b) 2 µL Nile red-acetone, (c) 1 µL Nile red-DMSO, (d) 2 µL Nile red-DMSO. Selected region of population P1 is an example of what was gated for cell sorting.

Growth rates and cell density

After the 5th cycle of mutation-selection, two strains with one of the best fluorescence/cell count ratio (one from each UV dosage), were chosen for further analyses : (i) M5 (originating from 25 mJ UV-C (50% survival rate) mutagenesis) and (ii) M24 (100 mJ UV-C (<2% survival rate)) were compared with wt *T. suecica* to determine their growth and lipid production performance. During the first 7 days of growth, all cultures exhibited similar growth rates and cell densities. After starvation was induced, both M5 (0.2 day^{-1}; P=0.06) and M24 (0.2 day^{-1}; P<0.05) exhibited higher growth rates than wt. The strains also achieved significantly higher (P<0.05) cell densities than wt (1.25 x 10^6 cells/mL) on day 10, and reached 1.82 x 10^6 cells/mL and 1.71 x 10^6 cells/mL, respectively. Overall growth rates ($\mu_{10\ days}$) of the selected strains were also found to be slightly higher than wt, although not at a significant rate (Figure 4).

T. suecica strain	Growth rate (day^{-1}); Doubling time (days)		
	7 days	10 days	7 to 10 days
Wild Type	0.28 ± 0.03; 2.35 ± 0.05	0.22 ± 0.003; 3.05 ± 0.05	0.07 ± 0.02; 10.75 ± 3.45
M5	0.29 ± 0.01; 2.55 ± 0.25	0.26 ± 0.008; 2.7 ± 0.10	0.20 ± 0.04; 3.55 ± 0.65
M24	0.29 ± 0.01; 2.55 ± 0.15	0.25 + 0.015; 2.8 ± 0.20	0.20 ± 0.02*; 3.60 ± 0.40*



1387

1388 **Figure 4.** Cell density of *T. suecica* selected strains and wild-type over the span of 13 days with
1389 induced starvation on day 7. Boxed data points indicate significant differences from wild-type
1390 (P<0.05). Inserted table: Growth rates and doubling time for the first 7 days, first 10 days and from
1391 day 7 to day 10. Asterisks indicate significant differences from wild-type (P<0.05). Data represent
1392 mean ± SEM from two independent replicates.

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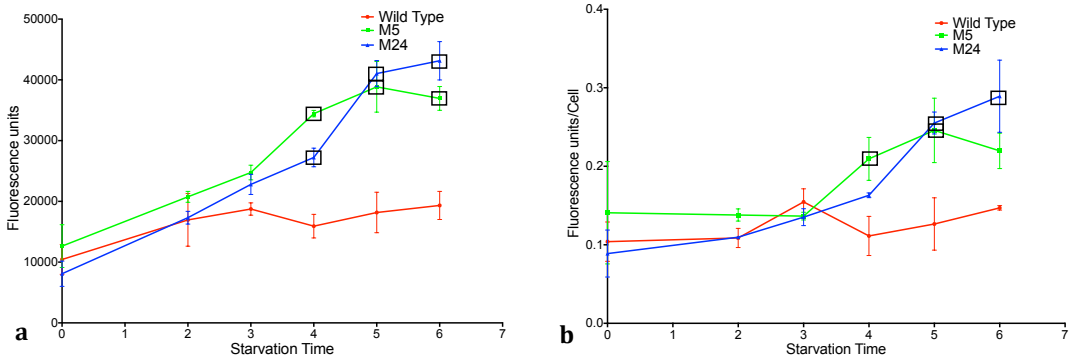

Figure 5. Lipid accumulation in *T. suecica* wild-type and selected strains (M5 & M24) during nutrient starvation phase measured by Nile red-stained fluorescence units at 485/590. (a) Total fluorescence units measured represent total lipid accumulated per mL of culture (b) Total fluorescence/cell represent total lipid accumulated per cell. Boxed data points indicate significant differences from wild-type (P<0.05), data represent mean ± SEM from two independent replicates.

Neutral lipid content

After 7 days of growth, nutrient deplete conditions were used to induce lipid production, and the neutral lipid content of selected *T. suecica* strains and wt were determined. Based on Nile Red fluorescence, the total neutral lipid content of wt increased until day 3 and then plateaued, while the total neutral lipid content of M5 and M24 continued to increase. From day 4 onwards, total neutral lipid production of the selected strains was significantly higher than wt (P<0.05), with M5 and M24 achieving maximum total neutral lipids on day 5 (114% increase from wt) and day 6 (123% increase from wt), respectively (Figure 5a). On a per cell basis, wt cells accumulated lipids from day 2 to day 3 and then stopped, while selected strains cells continued to accumulate lipids until day 6. M5 cells had 80-90% more neutral lipids than wt on day 4 and 5 (P<0.05), while M24 cells contained 96%-100% more neutral lipids than wt on day 5 and 6 (P<0.05; Figure 5b).

GC/MS analyses revealed an overall reduction in monounsaturated FAs (MUFAs; $P<0.05$) in the selected strains on day 5 (Figure 6). This trend was observed for C16, C16:1, C18, C18:1, C20 and C20:1 FAs, where significant reductions were accompanied by significant increases in polyunsaturated FAs (PUFAs), such as C16:4, C18:4 and C20:5. The selected strains also exhibited an increase in C14 and a decrease in C18:2, although this was only significant ($P<0.05$) in the M24 strain.

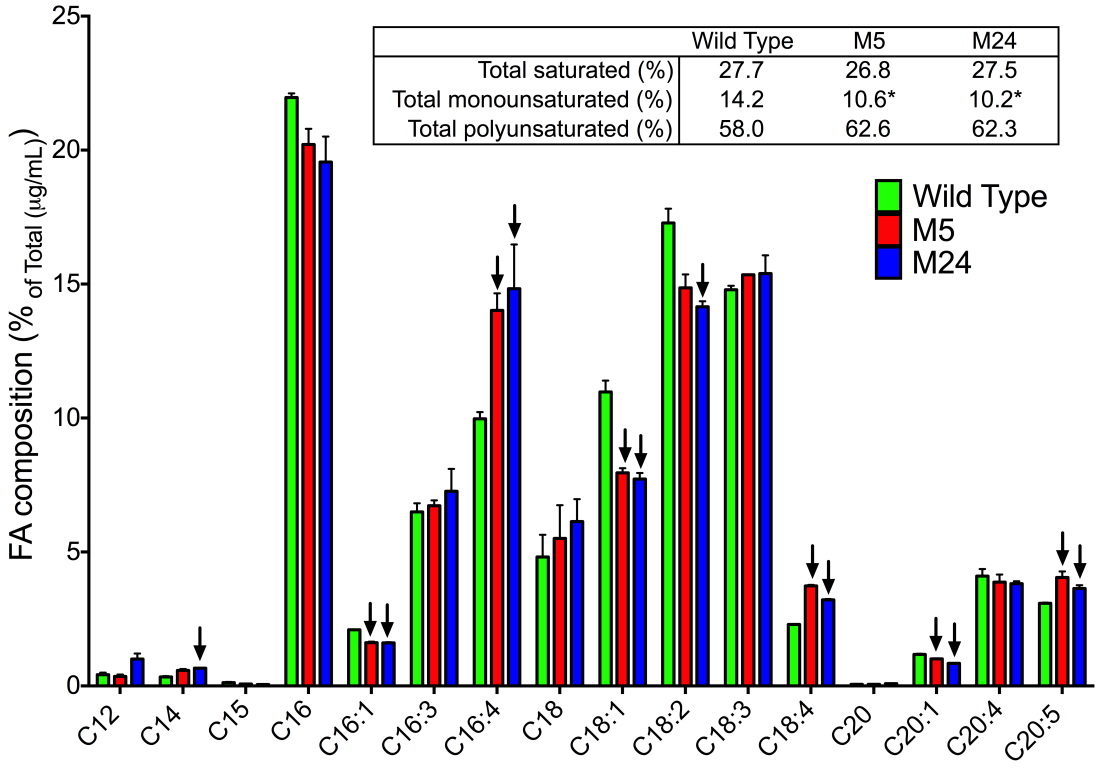

Figure 6. Fatty acid composition in percentage of total FAME of wild-type and selected *T. suecica* strains. Inserted table: Percentage of saturated, monounsaturated and polyunsaturated FA. Arrows and asterisk indicate significant differences from wild-type ($P<0.05$). Data represent mean ± SEM from two independent replicates.

Discussion

This study describes a combined and repeated mutation-selection method designed to increase the neutral lipid content of *T. suecica* without compromising its growth rate. It also includes the optimization of key steps within the mutation-selection cycle, such as the appropriate

UV-C dosage and Nile red concentration. UV-C has been successfully used to generate microalgae mutants [19, 21], and was selected as a mutagenic agent because it was practical and safer than chemical mutagens such as EMS and MTG. As chemical mutagens are more effective than UV radiation, UV-C mutagenesis requires higher dosages (with lower survival rates) [30]. However, although the frequency of mutants increases among survivors at high dosages, so does damage to the genetic background which reduces clone recovery and fitness. Therefore, two different UV-C dosages were chosen to either increase mutation probability (>98% lethal dosage) or increase recovery of a high number of clones (50% lethal dosage) with less genetic background damage, a survival rating similarly applied in other microalgae mutation studies [19,22]. The recovery of UV-C-exposed cells in liquid media was more effective, as survival rates in 96 well-plates were higher compared to solid media agar plates. The poor growth of other flagellate microalgae on solid media was also demonstrated for *Pavlova lutheri* as well as other filamentous and flagellate organisms, primarily due to the dehydration of the medium [31,32].

The use of Nile red as a fluorescence probe for neutral lipid detection and quantification in microalgae has been well documented [1,21,22,24,23,33,34]; a strong correlation between lipid content and fluorescence intensity has already been established [34]. The addition of solvents such as acetone and DMSO has been shown to improve the transition of the dye into lipids, although their efficacy varies between species, depending on the characteristics of the individual algae species [34]. The optimization stage of this study revealed acetone as a better stain carrier than DMSO for staining *T. suecica* cells that displayed higher fluorescence intensities during FACS. Other flow cytometry studies involving *D. salina* [33], *Nannochloropsis* sp. [1] and *I. galbana* [23] also utilized acetone as a carrier. The percentage of viable cells post-sorting achieved in this study (40-50%) was lower than the 80% reported by Montero et al. [24], which was achieved by seawater as sheath fluid, but still higher than the 20-30% reported by other studies involving the sorting of other phytoplankton flagellates [35]. The staining efficacy of DMSO has been shown to be better than acetone at a higher volume/volume [34], but would prove toxic and reduce FACS clone recovery. Therefore when at similar volumes, acetone produced a higher fluorescence intensity than DMSO and was the preferred solvent in this study. This study has also established a correlation between Nile red fluorescence intensity between percentage of starved cell (a proxy of total lipid content within a volume). In *T. suecica*, correlations between Nile red fluorescence signal and TAG content [24], as well as neutral lipids estimated by gravimetry, have been established [36]. Other studies have also found a strong correlation between fluorescence signal and total lipid content

[34,37], thus confirming the use of Nile red fluorescence to quantify lipid content in this study to allow for sorting of hyperlipidic *T. suecica* strains.

The overall aim of the mutation-selection program was to develop a strain that had a high lipid content while maintaining its high growth rate. For generations 1 to 3, lipid selection was achieved during flow cytometry, while growth rate selection was carried out post-sorting during the grow-up phase of the sorted cells. While other studies pooled their sorted cells [1,24], individual cells were sorted into individual 96 well-plate wells to facilitate the selection of the top six fastest growing individuals that would be carried into the next cycle of selection. The growth screening step was introduced to maintain the growth rate of the selected strains, as there have been studies that indicated reduced growth rates in strains isolated for high lipid content [22,8,38,21]. To confirm that the selected individuals still maintained their high lipid content, an additional screening step similar to that of Vigeolas et al. [21] was introduced in generations 4 and 5. A total of 24 instead of six of the fastest growing strains were selected and scaled up in 24 well-plates before their Nile red fluorescence intensity and absorbance values were measured to obtain fluorescence/absorbance. As absorbance values do not account for cell viability and can be misleading, cell numbers were then used to confirm the top six performers with the highest fluorescence/absorbance. The final mutation-selection program therefore now incorporates FACS with a fast growth selection step, followed by a high lipid per cell step.

After five cycles of mutation-selection that yielded two improved strains: M5 and M24, a standard protocol to compare the growth rates, lipid content and FA content was performed. Growth rate comparisons found the overall growth rates of improved strains to be slightly higher than wt, with significant increases occurring during the starvation period. Improved strains also achieved a significantly higher maximum cell density compared to wt. While the growth rates achieved in this study were expected to be lower than reported by Montero et al. [24] due to the lack of CO_2 aeration, FACS-isolated *T. suecica* cells in that study exhibited lower growth rates than the original wt. Reduced growth and cell density were also reported in a mutant study involving another flagellate, *D. salina,* that did not incorporate a growth selection step [22], although no reduction in growth rates was reported for *I. galbana* after two rounds of mutation-selection [23]. Other

mutation studies that reported maintained or increased growth rates in mutants were found to have incorporated a growth selection step as well [15-17,21].

When comparing neutral lipid productivities, selected *T. suecica* cultures in the current study exhibited a 114-123% increase in total fluorescence compared to wt, and a 90%-100% increase on a fluorescence per cell basis (but the cultures did not show any significant differences during nutrient replete conditions on day 0; Figure 5). These results were much higher than other UV mutation studies without FACS or Nile red fluorescence screening, which reported only a 8-35% increase in FA content [14,18,19]. Nevertheless, the yield improvement of this study was more similar to studies involving microplate reader screening and FACS. Vigeolas et al. [21] screened UV-mutated cells based on Nile red fluorescence using a 96 well-plate reader to develop *C. sorokiana* and *S. obliquus* strains with 50-300% increase in fluorescence units per cell, corresponding to similar increases in TAG content per cell, while Bougaran et al. [23] combined UV mutagenesis and FACS in an *I. galbana* mutation-selection procedure that increased lipid productivity by 80%. It is interesting to note that in FACS studies without mutagenesis, a *T. suecica* strain with up to 4-fold increase in fluorescence signal was obtained after two rounds of sorting [24], while a *Nannochloropsis* sp. strain with a 3-fold increase in total lipid content was selected after three rounds of sorting [17]. While significantly higher than wt, the neutral lipid content of both improved *T. suecica* strains in the present study was not significantly different from each other (Figure 5). This indicates that both 50% and >98% lethal dosage can be used to produce viable mutants with selectable traits. Nevertheless, a 50% lethal dosage was preferred as the recovery of clones was easier and less time-consuming.

As cell sizes between selected cells and wt cells were not significantly different during FACS analyses, it is expected that, similar to a per cell basis (Figure 5b), lipid contents per dry weight would also be increased (although this was not directly measured here). Although selected cells were not found to be significantly smaller, it appears that both, a more rapid growth after N depletion (Figure 4) as well as an increased cellular lipid fluorescence (Figure 5b), contributed to an increased overall lipid fluorescence in the selected cells (Figure 5a). This is in alignment with the selection protocol (Figure 1) that selected for both of these traits. GC-MS data only showed a slight increase of total fatty acid contents in the selected strains (30.7 and 29.7 µg/mL for M5 and M24, respectively) compared to the wt (26.8 µg/mL), raising the question whether lipids other than fatty

acids may have contributed to the higher lipid fluorescence in the selected strains. The comparison of FA profiles between improved strains and wt *T. suecica* revealed a decrease in MUFAs such C16:1 and C18:1, accompanied by increases in PUFAs such as C16:4, C18:4 and C20:5 (Figure 6). While certain studies report unaltered FA composition in their mutants [1,17,21], there have been studies that have also reported elevated PUFAs. The increase of PUFA in mutants has been documented in *P. lutheri* mutants irradiated by UV [19] and *Nannochloropsis* sp. irradiated by EMS and *N*-methyl-*N*-nitrosourea [15,16]. Furthermore, Chaturvedi & Fujita (2006) and Chaturvedi et al. (2004) also reported increases in C14 and reductions in C18:1. This decrease in the proportion of saturated FAs and MUFAs in comparison to an increase in PUFAs suggests that the mutants preferentially store lipids in the form of PUFAs. This, along with an overall increase in neutral lipids detected by elevated Nile red fluorescence, point towards mutations occurring in the gene coding for ACCase enzyme and the coding sequences of key desaturases(s) genes. Changes in the ACCase enzyme, considered to be the rate-limiting step in FA biosynthesis [39], would increase the substrate pool of TAG production, leading to more short-chain FAs (e.g. C12, C14, C16) that become precursors for MUFAs and PUFAs, as well as an increase in overall TAG accumulation [16]. Variations in PUFA contents between wt and selected strains also point towards mutations in key desaturase genes, which could explain the shift towards PUFA production [16]. Another explanation towards increased PUFA production in selected strains is the antioxidant effect of PUFAs against reactive oxygen species (ROS) generated during mutagenesis [40]. The antioxidant function of PUFAs in marine microorganisms, particularly eicosapentaenoic acid (EPA), has been reviewed and points towards their stabilizing function against oxidation by ROS. This is achieved by increased PUFA presence as membrane phospholipids to function as shield molecules [41]. Therefore, during the course of multiple rounds of mutation and selection, selected strains with increased PUFA production would have increased survivability towards UV-C radiation. Future studies should also investigate whether carbon partitioning is altered in the selected strains and whether the increased lipid content may result from a decrease of starch reserves as was found for other strains with elevated lipid contents [42].

Considering the average doubling time of 2.55 days of the selected strains, the fluorescence values obtained during the comparison to wt were obtained more than 24 cell divisions after the final selection cycle step, and 36 divisions after UV radiation in cycle 5. This supports the idea of a stable genetic makeup of the obtained putative mutants that produce increased neutral lipid content. Although the nature of these putative mutations are beyond the scope of this study, the observed

improvements could be a result of adaptation (e.g. by epigenetics), and not DNA mutations. It cannot be ruled out that strains adapted to, instead of mutated to UV-C exposure. Therefore, the lipid content of these strains should be evaluated again in the future, particularly after long-term storage without selection pressure for high lipid content. At that point, unchanged lipid content values in comparison to wt would indicate a stable genetic mutation, while epigenetic change would cause these strains to adapt back a more wt phenotype. Unlike plant crops that undergo very few reproductive cycles between harvests (typically just one), bred elite microorganisms are constantly at high risk to revert back to faster growing wt-like strains. At present, the risks of this occurring for the microalgae in this study appear reduced, as the selected strains did not compromise on their ability to grow.

Acknowledgements

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Chapter 4: Understanding the molecular mechanism of *Tetraselmis* sp. lipid production: a new transcriptional profiling approach

Another way to improve the lipid content of microalgae strains is via genetic engineering. Successful genetic manipulation of an organism first requires a comprehensive understanding of its genomic and transcriptomic background. With regards to lipid content improvement, key lipid-related pathways such as FA synthesis, TAG synthesis and β-oxidation pathways must first be mapped out, and key bottlenecks and committing steps identified. While genes can be identified via genomic sequencing, global comparative transcriptomic analysis can provide the expression profile of each gene within lipid-related pathways, allowing for a better understanding of the molecular mechanisms behind lipid production. This chapter, presented in Research Paper 3 "**The transcriptome of *Tetraselmis* during nitrogen starvation reveals two-stage lipid accumulation.**" analysed the physiological and transcriptional changes related to lipid accumulation in *Tetraselmis* sp. M8 as it transitions from growth phase into nitrogen starvation phase. An Illumina Mi-Seq sequencing platform was used to generate the transcriptome of *Tetraselmis* sp. M8 and to reveal global transcriptomic changes during early stationary phase. This mapped out key lipid-related pathways and served as a platform for qRT-PCR analyses that investigated the expression profile of these pathways as *Tetraselmis* sp. M8 transition from growth phase to starvation phase.

Key findings

- Physiological observations revealed a distinct early-stationary phase (0-48 h) and a stationary phase (48 h onwards) in *Tetraselmis* sp. M8. Significant lipid accumulation could be detected as early as 16 h after exhaustion of exogenous nitrogen, but the rate of accumulation significantly increased from 48 h onwards and was accompanied by an arrest in cell division.
- Illumina Mi-Seq sequencing of control and nitrogen-starved samples generated approximately 36,000,000 reads per sample, with 593 unique genes identified as differentially expressed by The Differential Kmer Analysis Pipeline (DiffKAP). The expression of lipid-related pathways by DiffKAP analysis was confirmed by qRT-PCR, which was also performed in a time course at 16, 24, 32, 48, 72 h after nitrogen deprivation.
- Lipid accumulation during early-stationary phase was found to be a result of reduced lipid catabolism, as expression of committing steps in the β-oxidation pathway was found to be

1721 significantly down-regulated, while expression of lipid biosynthesis pathways remained at
1722 basal levels.

1723 • As cells transitioned into stationary phase, lipid biosynthesis genes were found to be
1724 significantly up-regulated, indicating that lipid accumulation was a result of active lipid
1725 synthesis at that stage.

1726 • A circadian effect on gene expression was observed in only the FA synthesis pathway, while
1727 the TAG synthesis and β-oxidation pathway was unaffected.

1728 • Genes encoding for ACCase, PP, ACSase and ECH are potential bottlenecks of lipid
1729 biosynthesis in *Tetraselmis* sp.

1730

Research Paper 3: The transcriptome of *Tetraselmis* during nitrogen starvation reveals two-stage lipid accumulation

Submitted to *Plant Physiology* (2014), currently under review

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Abstract

Comparative transcriptomic analysis provides insight into the molecular mechanism of lipid metabolism and can reveal potential genetic engineering targets in microalgae. Using RNA-Seq, transcriptome analysis on the previously unsequenced genus *Tetraselmis* was performed with the oleaginous strain *Tetraselmis* sp. M8. RNA-Seq was carried out at 24 h after exhaustion of exogenous nitrogen to reveal molecular changes during early stationary phase and to map out key lipid-related pathways. Further gene expression profiling by quantitative real-time PCR at 16, 24, 36, 48 and 72 h revealed a distinct shift in the expression of the fatty acid (FA) and triacylglyceride (TAG) biosynthesis pathways, as well as the β-oxidation pathway, when cells transitioned from log phase into early-stationary and stationary phase. During early-stationary phase, observed lipid accumulation could be linked to reduced expression of lipid catabolism genes, while lipid biosynthesis genes were maintained at basal levels. During stationary phase however, genes involved in lipid biosynthesis were up-regulated, indicating that *Tetraselmis* sp. shifts from reduced lipid consumption to active lipid production during this period, as reflected in the increase in the rate of lipid accumulation. This process appeared to be independent from *DGAT* gene expression, a key gene for lipid accumulation identified in other microalgae.

Introduction

Currently, microalgae are considered as one of the most promising feedstocks for biofuel production. Under the appropriate conditions (e.g. nutrient deprivation), oleaginous microalgae can be induced to accumulate neutral lipids or triacylglycerides (Hu et al., 2008), which can be converted into biodiesel via transesterification. Theoretically, microalgae can produce 10 to 20 times more lipids than oil palms (Ahmad et al., 2011), and in a manner without competing for precious arable land, biodiverse landscapes (e.g. rainforests) and freshwater resources. Despite their potential, the high cost of large-scale production still needs to be reduced in order for microalgal biofuel to achieve its full commercialization potential and wide-scale use. Currently, algal strain development remains one of the most important aspects of microalgae-for-biofuel development. Research efforts are continuously advancing bioprospecting (Nascimento et al., 2013), selective breeding (Zayadan et al., 2014) and genetic engineering (Gimpel et al., 2013) of microalgae in an effort to maximize growth and lipid accumulation of the highest performing strains. Importantly, several lipid induction techniques have been identified in microalgae (Rodolfi et al., 2009; Sharma et al., 2012). Microalgae typically reduce cell division during adverse conditions, such as nutrient starvation or UV radiation, but are still able to accumulate starch or lipids during photosynthesis as a survival mechanism (Timmins et al., 2009; Wang et al., 2009; Sharma et al., 2014).

Metabolic engineering via genetic modification or modulation of cultivation techniques provides a promising area for increased lipid accumulation. This can be greatly assisted by comprehensive genomic, transcriptomic, proteomic and metabolomic knowledge. For example, key lipid-related pathways must be mapped out, and important bottleneck enzymes and their genes identified as targets for manipulation. To that effect, global transcriptional profiling of microalgal cells during lipid accumulation enables the identification of the underlying transcriptional networks. Even without pre-existing reference genomes, comparative transcriptional analyses have been used in microalgae to successfully map pathways and observe changes during induced lipid accumulation (Rismani-Yazdi et al., 2011; Radakovits et al., 2012; Sun et al., 2013). In most studies, the focus has been on metabolic pathway reconstruction and gene discovery at a single time-point, usually 48 to 96 h into starvation phase when lipid accumulation is at its peak (Guarnieri et al., 2011; Rismani-Yazdi et al., 2012; Sun et al., 2013). While this approach successfully allowed for the reconstruction of fatty acid (FA), triacylglyceride (TAG), β-oxidation and other metabolic pathways, the limited scope of these studies restricts our understanding how the expression of these pathways change,

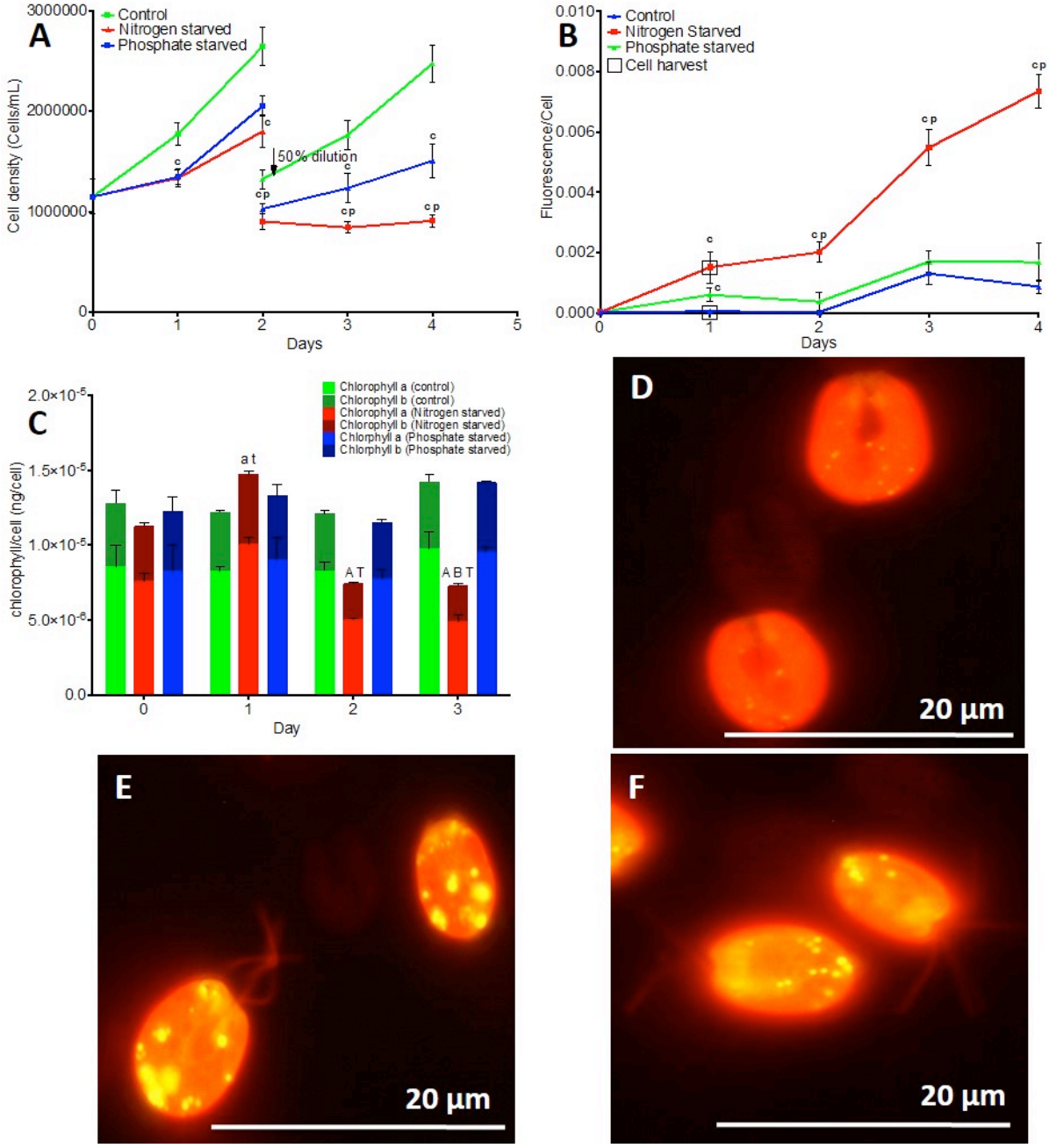
1791 particularly during early stationary phase as cells transition from growth phase into starvation
1792 phase. The few studies that have monitored the transcriptional profile of microalgae at various
1793 growth stages have observed more transcriptional changes during early-stationary phase compared
1794 to stationary phase. These changes occur particularly in photosynthesis, carbon and lipid synthesis
1795 pathways, and can be linked to physiological changes (e.g. reduced cell division & increased lipid
1796 synthesis) observed during that phase (Valenzuela et al., 2012; Lv et al., 2013).

1797 The appropriate lipid induction conditions and time point of RNA sampling are crucial in obtaining
1798 distinct expression profiles between control and treatments cultures. Nitrogen depletion is a
1799 commonly used method to induce lipid accumulation in microalgae (Hu et al., 2008; Rodolfi et al.,
1800 2009; Miller et al., 2010).

1801 The flagellate green microalga *Tetraselmis* sp. is widely mentioned in the literature, but very little
1802 sequence information is available on this genus in public databases. *Tetraselmis* sp. M8
1803 (Chlorodendrophyceae) presents a good model organism, based on its reported ability to accumulate
1804 high lipid content as well as its robustness to tolerate a range of environmental conditions (Chini
1805 Zitelli et al., 2006; Rodolfi et al., 2009). Recently it has been shown that *Tetraselmis* cells lose their
1806 flagella during stressful conditions, resulting in rapid settling, a feature that can significantly reduce
1807 harvesting/dewatering costs and provide an avenue for commercial production (Lim et al., 2012).
1808 The growth characteristics of *Tetraselmis* sp. M8 strain and its lipid accumulation capability and
1809 composition were previously found suitable, in principle, for biodiesel production under both
1810 laboratory and outdoor cultivation conditions (Lim et al., 2012).

1811 Even in the absence of a fully sequenced and annotated genome, transcriptomic analysis by
1812 microarrays or RNA-Seq can provide a powerful tool to improve our understanding of the
1813 underlying physiological networks that allow microalgae to respond to environmental changes
1814 (Nguyen et al., 2008; Rismani-Yazdi et al., 2012; Valenzuela et al., 2012; Lv et al., 2013; Sun et al.,
1815 2013). The primary objective of the present study was to gain insights into the lipid accumulation
1816 mechanism of the genus *Tetraselmis*, particularly the expression of genes in the FA synthesis, TAG
1817 synthesis and β-oxidation oxidation pathways, as cells transition from growth phase into stationary
1818 phase. Physiological observations such as growth, lipid accumulation and FA profiles were linked
1819 to transcriptional data obtained first by global transcriptomic sequencing, followed by quantitative
1820 reverse transcriptase PCR (qRT-PCR) time-course analysis of each of the aforementioned
1821 pathways.

1822



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1824

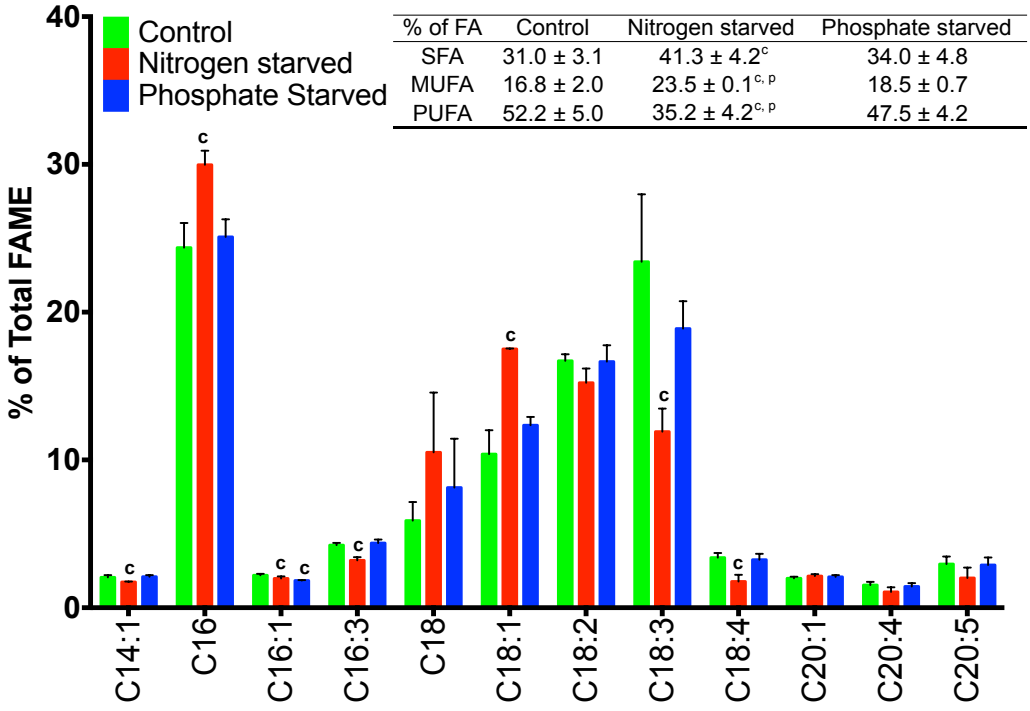
1825 **Figure 1. Growth, lipid accumulation and chlorophyll content of *Tetraselmis* sp. M8 under**
1826 **nitrogen & phosphate-starved conditions**. **A.** Cell density growth curve of *Tetraselmis* sp. M8,
1827 cultured semi-continuously with dilutions every 2 days, showed reduced cell growth in nutrient
1828 deplete cultures. **B.** Characterization of lipid accumulation via Nile red fluorescence (shown as
1829 intensity per cell number) displayed increased lipid accumulation under nitrogen-starved conditions.
1830 Boxed data points indicate time and treatments at which cells were harvested for RNA sequencing.
1831 "c" and "p" indicate significant differences from control and phosphate-starved treatments,
1832 respectively (Student's T-test, $P<0.05$). **C.** Characterization of chlorophyll content (a, b and total)

per cell number showed reduced chlorophyll content after 48 h of nitrogen starvation. “a” indicates significant differences of chlorophyll a content to control. “A” indicates significant differences of chlorophyll a content to both control and phosphate-starved treatment. “B” indicates significant differences of chlorophyll b content to both control and phosphate-starved treatments. “T” indicates significant differences of total chlorophyll content to both control and phosphate-starved treatments. **D-F.** Epifluorescent images of *Tetraselmis* sp. M8 at day 1 of nutrient deprivation experiments, using control (D), nitrogen-starved (E), and phosphate-starved (F) conditions, reveal increased lipid accumulation in nitrogen-starved cells. All images were taken at 400x magnification.

Results

Nitrogen rather than phosphate deprivation leads to lipid biosynthesis in *Tetraselmis*

Nutrient deprivation is a well established method for inducing lipid biosynthesis in microalgae and it had to be determined whether nitrogen or phosphorus starvation had the strongest effect in *Tetraselmis* sp. M8. Semi-continuous cultures of *Tetraselmis* sp. M8 were established and allowed to grow in parallel for three feeding cycles to adapt the cultures to exponential growth with regular nutrient supply. Nitrogen and phosphorus deprivation treatments were then initiated by feeding with nitrogen- and phosphate-deficient F/2 medium, respectively. Physiological parameters such as cell density, lipid accumulation and chlorophyll content were monitored for the duration of the experiment using replicates from three separately-grown cultures for each treatment (Figure 1). The mock-treated control cultures displayed the highest cell density before and after dilution on day 2. Both nitrogen and phosphate-starved cultures were found to have significantly reduced cell accumulation from day 1 onwards (Figure 1A, $P<0.05$). Phosphate-starved cultures had reduced growth rates when compared to control cultures but accumulated cells throughout the experiment, while nitrogen-starved cultures only underwent cell divisions until day 2. Lipid accumulation was observed via measurement of Nile red fluorescence per cell number of the cultures (Figure 1B). Nitrogen-starved cultures showed significantly higher Nile red fluorescence than the other treatments from day 1 onwards ($P<0.05$), with a marked increase particularly after day 2 of starvation. However, Nile red fluorescence for phosphate-starved cultures was only significantly higher than the controls on day 1, and had similar fluorescence levels on subsequent days, suggesting that N, rather than P starvation had the strongest effect on lipid accumulation in *Tetraselmis* sp. M8. Microscopic analysis of day 1-cultures also confirmed that nitrogen-deprived cells (Figure 1D) had the most and largest lipids bodies, followed by phosphate-deprived (Figure 1E) and control cells (Figure 1F), which showed very small lipid bodies.



% of FA	Control	Nitrogen starved	Phosphate starved
SFA	31.0 ± 3.1	41.3 ± 4.2^{c}	34.0 ± 4.8
MUFA	16.8 ± 2.0	23.5 ± 0.1$^{c, p}$	18.5 ± 0.7
PUFA	52.2 ± 5.0	35.2 ± 4.2$^{c, p}$	47.5 ± 4.2

Figure 2. Fatty acid profile of control and nutrient deficient culture at day 1 of the RNA-Seq experiment. Inserted table: Percentage of saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA). "c" indicates significant differences between nitrogen-starved and control cultures. (Student's T-test; $P<0.05$).

GC/MS analyses were performed on day 1 after treatments to determine FA composition. Nitrogen-starved cultures exhibited significantly higher saturated (SFA) and monounsaturated FAs (MUFAs) composition compared to controls, while polyunsaturated FAs (PUFAs) were significantly lower ($P<0.05$, Figure 2). C16 and C18:1 FAs showed the most significant increases, 5.6 % and 7.1% respectively, while C18:3 decreased the most, 11.5%. It was also found that while there was a general increase in SFAs and MUFAs, there were significant decreases in C14:1 and C16:1 FAs, indicating that *Tetraselmis* sp. M8 could be storing lipids in the form of C16 and C18:1. Chlorophyll content was also measured under the different nutrient deprivation treatments (Figure 1c). Compared to other treatments, N starvation led to significant increases in chlorophyll a on day 1, but was followed by significant decreases on day 2 and an overall decrease in chlorophyll content (a & b) on day 3.

RNA-Seq of *Tetraselmis* sp. cells reveals distinct sequences compared to other known microalgal sequences

To determine which genes would be required for N deprivation-induced lipid accumulation in *Tetraselmis* sp. M8, cell harvesting for RNA sequencing was performed on day 1 control and nitrogen-starved cultures (replicates from three separately-grown cultures each). This time point was selected because it was the earliest time point to exhibit significant lipid accumulation. The concentration of cDNA was normalized and then pooled for each treatment. Sequencing using the Illumina Mi-Seq platform of the cDNA libraries produced approximately 36,000,000 reads per treatment, at an average length of 151 bp per read. Initially, Tophat/Cufflinks was used to assemble the RNA-Seq data using the closest available genome, *Chlamydomonas reinhardtii*. However, the analysis was unsuccessful with less than 0.02% reads mapped and so this approach was abandoned. The Differential Kmer Analysis Pipeline (DiffKAP) approach was then used to identify Differentially Expressed Reads (DERs) between control and N-starved treatments (see Materials and Methods for details). A total of 990,249 DERs were identified as higher expressed in the controls (also considered as down-regulated in N-deprived samples), while 1,046,741 DERs were identified as higher expressed in N-deprived cultures. These DERs were then annotated by BLAST-matching reads to Swissprot. A total of 195,291 DERs that were higher expressed in controls and 24,400 that were higher expressed in N-deprived samples could be annotated. This revealed a total of 593 unique genes that were differentially expressed between treatments (Supplementary Table 1). It should be noted that this only represents 10.68% of total DERs due to stringent BLAST criteria, as well as low similarity to other available sequences. Out of those, the majority of DERs matched to *Arabidopsis* (18%), while the closest-related alga, *Chlamydomonas reinhardtii,* only had a 3.6% match.

Function of differentially expressed genes during N-deprivation of *Tetraselmis*

To assign functions to differentially expressed genes, , annotated DERs in both treatments were assigned with GO terms using the Blast2Go platform, with their distribution presented in Figure 3 (full dataset in Supplementary Table 2). The distribution of DERs from nitrogen-deprived treatments was found to be distinctively different from those of control samples. In the N-starved treatment, carbohydrate metabolic processing (45%) and nucleotide binding (42%) accounted for the largest percentage of DERs, followed by DERs coding for catabolic processes, generation of

precursor metabolites and response to stress (~20-30%). In comparison, the functional categories that accounted for the largest percentage of DERs in control sequences were plastid, thylakoid, generation of precursor metabolites and protein complex (~70-90%), followed by cellular protein modification process, carbohydrate metabolic process, catabolic process and response to abiotic stimulus (~10-33%). DERs linked to lipid metabolic process were found in both control and N-starved treatments at 7% and 11%, respectively, while DERs linked to lipid particle were found at 0.9% and 0.1%, respectively.

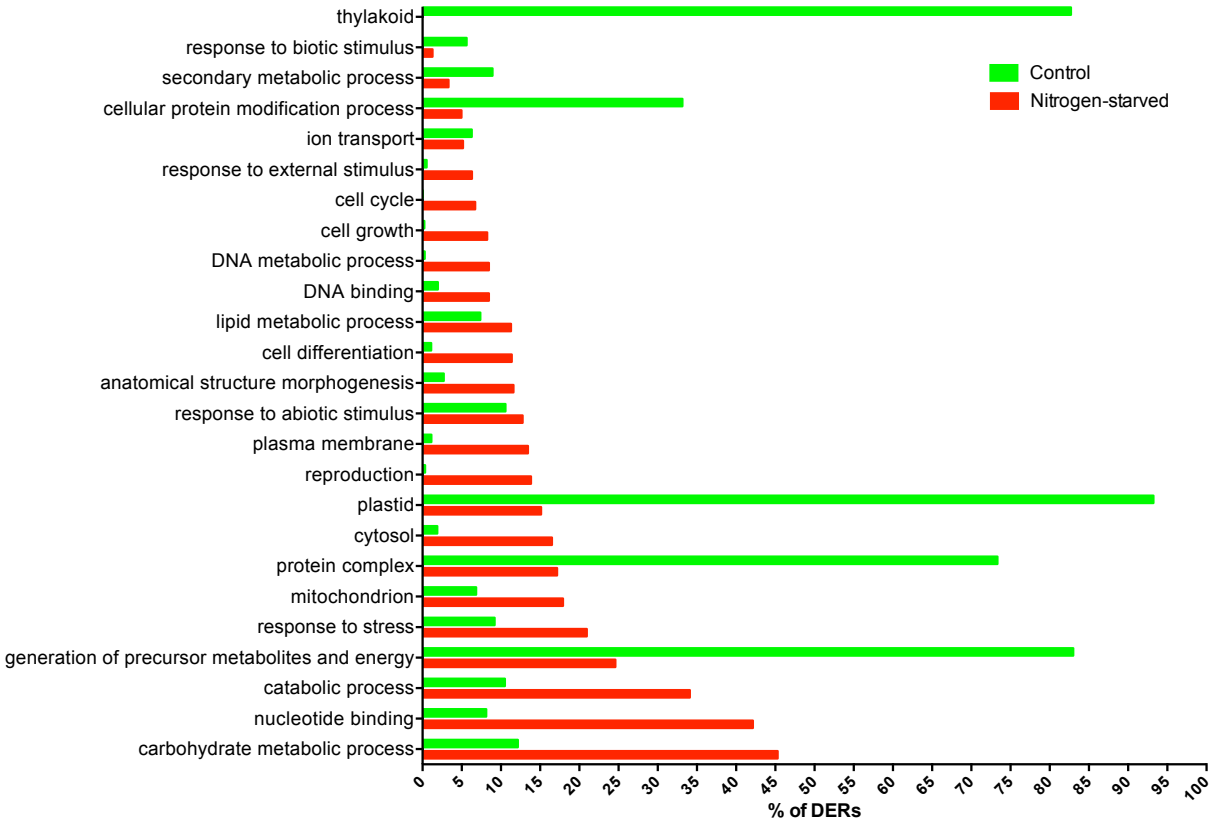


Figure 3. Distribution of Gene ontology (GO) terms assigned to annotated differentially expressed reads (DERs) in Control and Nitrogen-starved treatments. Data are shown as a percent of total annotated DERs from each treatment. Only GO terms containing more than 5% of DERs are included in this figure.

To identify and construct the lipid biosynthesis and degradation (including β-oxidation and lipases) pathways in *Tetraselmis* sp., genes coding for key enzymes in these pathways were identified by BLAST-searching the sequenced cDNA library uploaded onto TAGDB, using reference genes. In

addition, following the Illumina Mi-Seq transcriptomics experiment, a subsequent independent time course experiment was performed to investigate the expression of these pathways in more detail at 16, 24, 32, 48 and 72 h after nitrogen starvation (samples from three separately-grown and treated cultures, each). Monitoring of the physiological parameters revealed cell accumulation in the N-starved cultures, similar to the RNA-seq experiment (Figure 4). As previously observed, cell numbers in N-starved cultures increased only until 48 h, and then subsequently stopped dividing, with significant differences between treatments occurring from 32 h onwards (Figure 4a, $P<0.05$). In comparison, cell numbers in control cultures tripled between 0 and 48 h, and continued to increase after dilution and feeding. Lipid accumulation in the time-course experiment exhibited similar results to that of the RNA-Seq experiment. The nitrogen-starved cultures were found to accumulate significantly more lipids as early as 16 h onwards (Figure 4b, $P<0.05$), and also exhibited another marked increase after 48 h. RNA was extracted from control and N-starved cultures at every time point, and qRT-PCR analysis performed to determine the expression of the various pathways. Primers for qRT-PCR experiments were based on conserved regions, using RNA-Seq data, in an effort to capture most gene family members.

Figures 5, 6 and 7 show the reconstructed pathways for FA synthesis, TAG synthesis and lipid degradation based on the identified enzymes, using combined DiffKAP and qRT-PCR results. Based on the DiffKAP results, the entire FA synthesis pathway, except for malonyl-CoA:ACP transacylase (MAT), was found to be down-regulated when compared to controls. The expression of genes coding for the entire TAG synthesis pathway remained unchanged. In the lipid degradation pathway, genes encoding acyl-CoA synthetase (ACSase) was down-regulated, and acyl-CoA oxidase (ACO) was up-regulated (Figure 7). Similarly, qRT-PCR data found the entire FA synthesis pathway to be significantly down-regulated, except for genes encoding 3-ketoacyl-ACP reductase (KAR) and enoyl-ACP reductase (ENR) whose expression remained unchanged. Transcript abundances of genes encoding acetyl-CoA carboxylase (ACCase), ketoacyl-ACP synthase (KAS) and Malonyl-CoA:ACP transacylase (MAT) were found to be 4- to 5-fold lower ($P<0.05$) in the N-starved treatments. The expression level of 3-ketoacyl-ACP reductase (KAR)-encoding gene(s) was lower as well. qRT-PCR analysis of the genes in the TAG synthesis pathway confirmed the DiffKAP findings, except for diacylglycerol acyltransferase (DGAT)-encoding gene(s) exhibiting reduced expression levels (2-fold, $P<0.05$). In the lipid degradation pathway, ACSase expression levels were significantly lower in nitrogen-starved cells by 2-fold, also confirming DiffKAP results. Furthermore, although this was not seen in the DiffKAP results, qRT-PCR analysis revealed enoyl-CoA hydratase (ECH) expression levels to be 10-fold lower ($P<0.05$) than in control cultures. The ACO-encoding gene(s) was also found to be up-regulated, although not at a significant level.

Overall, qRT-PCR analysis confirmed the DiffKAP findings, with no contrasting results being found.

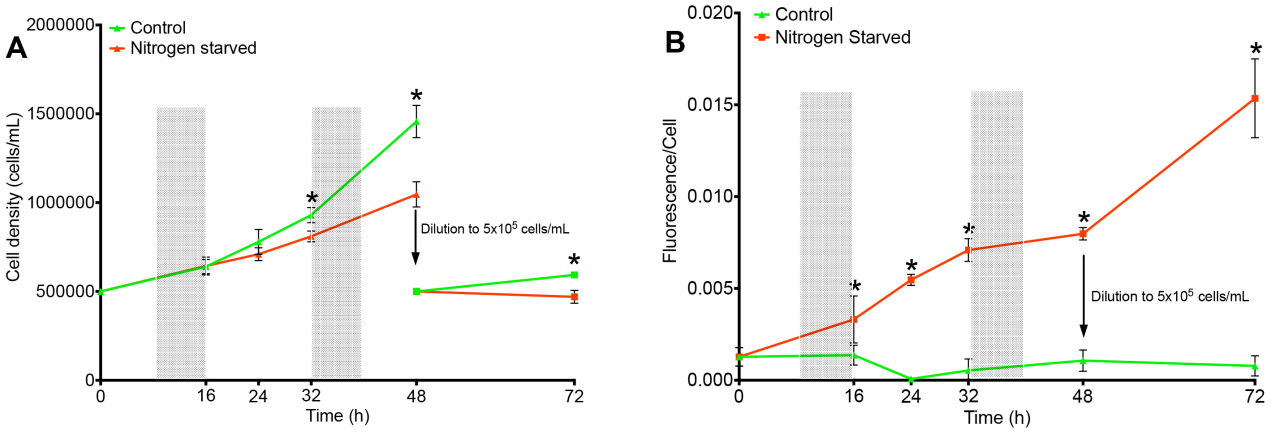


Figure 4. Growth and lipid accumulation of *Tetraselmis* sp. M8 in a time-course experiment using control and nitrogen-starved cultures. Grey shaded areas indicate time in dark cycle. A. Growth curve of *Tetraselmis* sp. M8 cultures with significant differences (*) in cell density after 32 h (Student's T-test; $P<0.05$). B. Characterization of lipid accumulation via Nile red fluorescence per cell number with nitrogen-starved cultures displaying significantly higher Nile red fluorescence from 16 h onwards (Student's T-test; $P<0.05$).

Despite earlier signs of cellular lipid accumulation, FA and TAG synthesis genes were only upregulated at 48 h after N deprivation

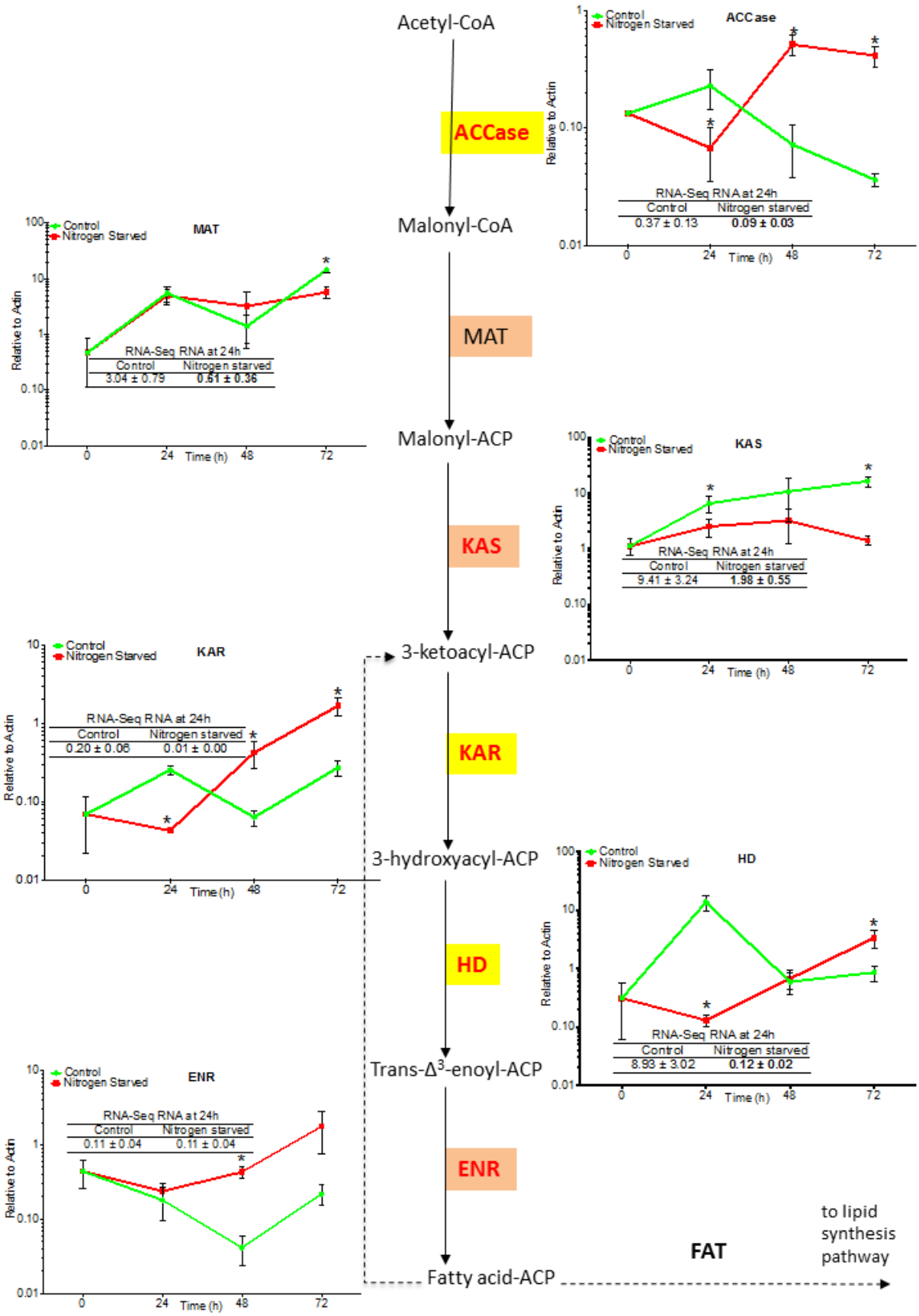
Irrespective of experimental approach taken, gene expression levels in the FA synthesis pathway of nitrogen-starved cells were mostly down-regulated or unchanged in the first 24 h, but were then upregulated significantly from 48 h onwards (Figure 5). In the first 24 h, the expression levels of genes encoding ACCase, KAR and 3-hydroxyacyl-ACP dehydratase (HD) (FA synthesis) in control cells were 3-, 5.9- and 113-fold lower, respectively, ($P<0.05$) than in control cells, but were then significantly up-regulated 3- to 11-fold higher ($P<0.05$) than control cells at 48 h and 72 h. For the MAT-encoding gene(s), expression levels between treatments were similar until 72 h, when it was down-regulated by 2.5-fold ($P<0.05$). The expression level of ENR-encoding gene(s) was similar between the treatments until 48 h onwards when this gene was found to be significantly down-regulated in control cells (8 fold, $P<0.05$). In the nitrogen deprivation treatment, the KAS-encoding gene(s) was consistently down-regulated throughout the experiment. In the TAG synthesis

pathway (Figure 6), gene expression remained largely unchanged between control and treated cells, particularly in the first 24 h. After 48 h onwards, upregulation of the PP-encoding gene(s) in nitrogen-starved cells was observed (P<0.05), followed by the upregulation of the gene(s) encoding GK at 72 h (4-fold, P<0.05). No differences in expression were observed between control and treatment for genes encoding glycerol-3-phosphate O-acyltransferase (GPAT) and 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT) throughout the entire experiment. DGAT-encoding gene(s) was found to be significantly down-regulated (P<0.05) at 48 h and 72 h after nitrogen starvation. In the lipid degradation pathway (Figure 7), only gene(s) encoding TAG lipase was observed to be down-regulated (4.6-fold, P<0.05) in N-starved cells, while other genes remained unchanged between treatments. Changes in gene expression can be observed from 48 h onwards, with genes encoding ACSase being up-regulated (5-fold, P<0.05) in nitrogen-starved cellsand ECH being down-regulated (83-fold, P<0.05) at 72 h.

In summary, as FA and TAG synthesis genes were mostly down-regulated at 24 h after N deprivation (Figures 5 and 6), the observed increased lipid accumulation of cells harvested at this time (Figures 1, 4B) is likely to be attributed to a reduced rate of FA degradation by beta oxidation (Figure 7). Cells at a later stage then clearly show upregulation of FA and TAG synthesis which coincides with further increases in lipid fluorescence.

Circadian effect

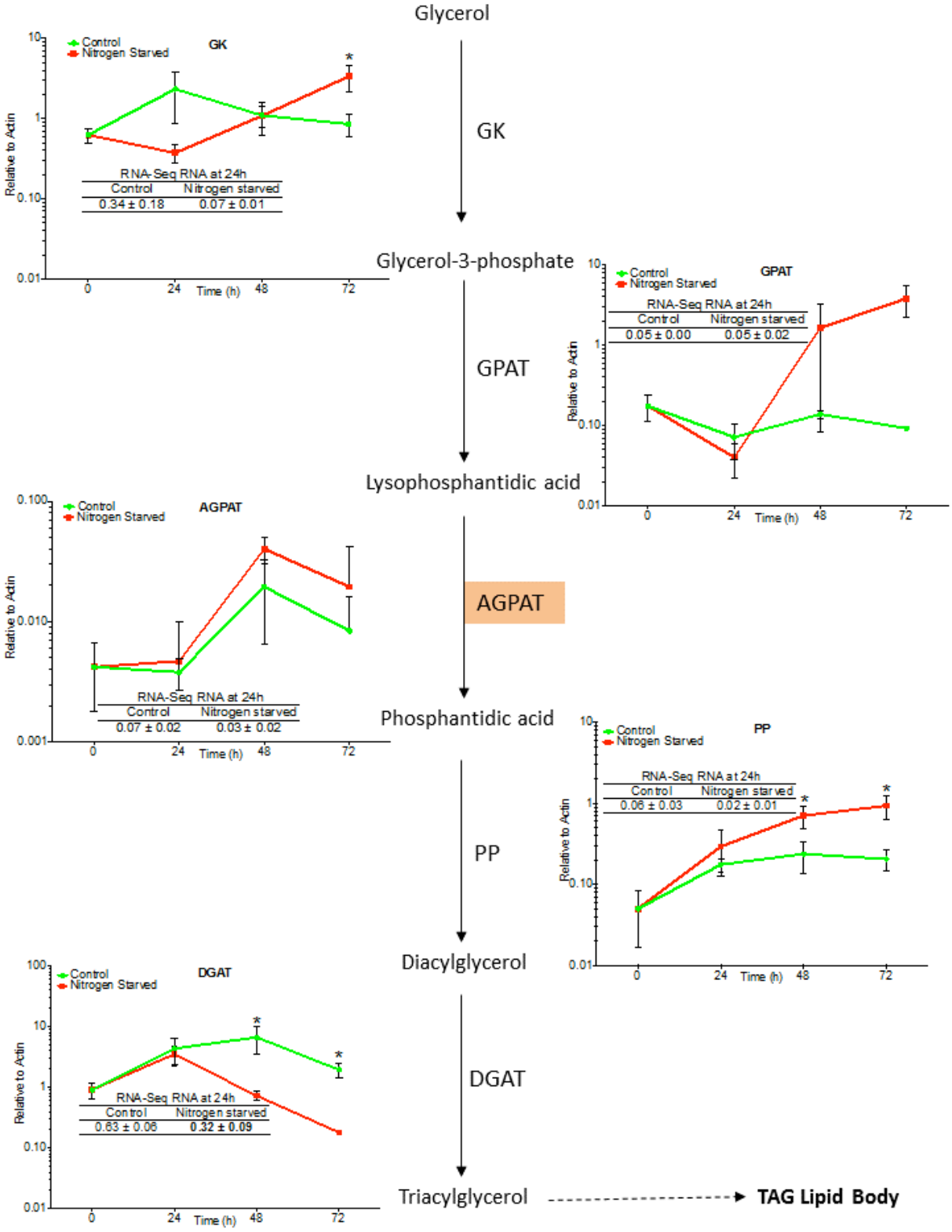
The expression levels of the pathways were also investigated at 16 h (at the end of the dark cycle) and at 32 h (at the end of the light cycle) to determine the effects of the circadian rhythm on gene expressions (Figure 8). The complete set of graphs for all genes including the 16 h and 32 h time points is available in Supplementary Figures 1-3. In genes that encode ACCase, KAR, HD and glycerol kinase (GK) (Figure 6, highlighted yellow), a circadian effect was observed in both control and nitrogen-starved treatments, whereby expression was significantly up-regulated after and before the dark cycle. In genes encoding MAT, 3-ketoacyl-ACP synthase (KAS), ENR and 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT) (Figure 6, highlighted orange) however, the circadian effect was only observed in the control treatment, while gene expression in N-starved cells remained consistent throughout the 16 h and 32 h time points. Furthermore, while all the genes affected by the circadian cycle presented a diurnal expression, ENR- and GK-encoding genes showed increased expression only towards the end of the light cycle at 32 h. Lastly, the circadian rhythm had no measurable effect on genes involved in lipid catabolism.



2023

2024 **Figure 5. Fatty acid synthesis pathway and changes in gene expression under nitrogen**
2025 **deprivation.** Genes in red font were down-regulated in nitrogen-starved treatment according to

DiffKAP analysis. Inserted tables show qRT-PCR analysis of the RNA-seq RNA, with bold numbers indicating significant differences (Student's T-test; $P<0.05$). Graphs show qRT-PCR expression analysis of genes at 0, 24, 48 and 72 h of the time course experiment, with asterisks (*) indicating significant differences (Student's T-test; $P<0.05$). Genes affected by the circadian rhythm were highlighted yellow (both control and nitrogen-starved affected) and orange (only control affected). Acetyl-CoA carboxylase (ACCase); Malonyl-CoA:ACP transacylase (MAT); 3-ketoacyl-ACP synthase (KAS); 3-ketoacyl-ACP reductase (KAR); 3-hydroxyacyl-ACP dehydratase (HD); Enoyl-ACP reductase (ENR); FAT (Acyl-ACP thioesterase).



2034

2035 **Figure 6. Triacylglyceride synthesis pathway and changes in gene expression under nitrogen**
2036 **deprivation.** Genes in red font were found down-regulated in DiffKAP analysis of RNA-seq RNA.

Inserted tables show qRT-PCR analysis of the RNA-seq RNA, with bold numbers indicating significant differences (Student's T-test; $P<0.05$). Inserted graphs show qRT-PCR expression analysis of genes at 0, 24, 48 and 72 h of the time course experiment, with asterisks (*) indicating significant differences (Student's T-test; $P<0.05$). Genes affected by the circadian rhythm were highlighted yellow (both control and nitrogen-starved affected) and orange (only control affected). Glycerol kinase (GK); Glycerol-3-phosphate O-acyltransferase (GPAT); 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT); Phosphatidate phosphatase (PP); Diacylglycerol O-acyltransferase (DGAT); Triacylglyceride (TAG) lipase.

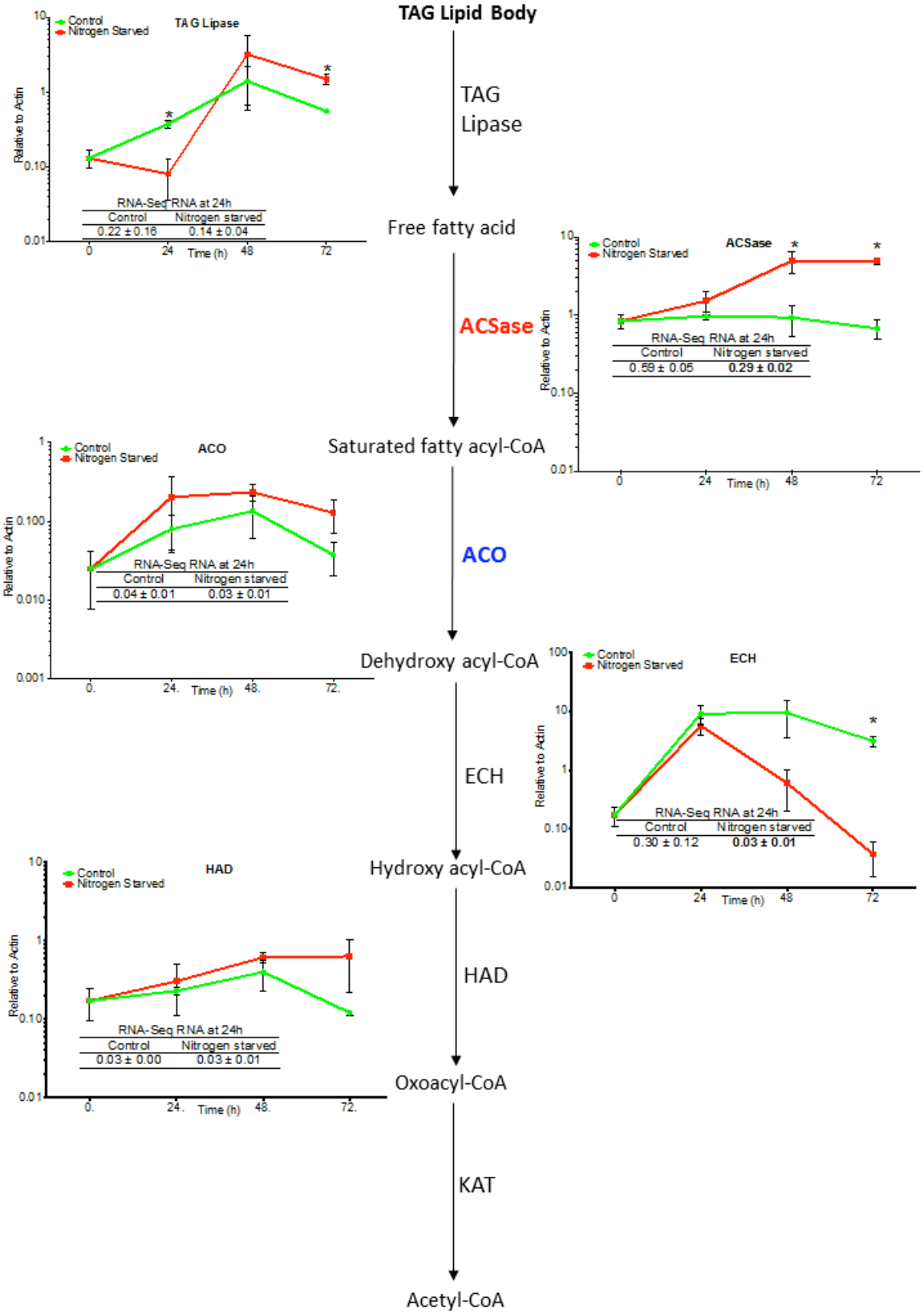


Figure 7. Lipid degradation pathway and changes in gene expression under nitrogen deprivation. Genes in red font were found down-regulated in DiffKAP analysis of RNA-seq RNA.

Inserted tables show qRT-PCR analysis of the RNA-Seq RNA, with bold numbers indicating significant differences (Student's T-test; $P<0.05$). Inserted graphs show qRT-PCR expression analysis of genes at 0, 24, 48 and 72 h of the time course experiment, with asterisks (*) indicating significant differences (Student's T-test; $P<0.05$). Acyl-CoA synthetase (ACSase); Acyl-CoA oxidase (ACO); Enoyl-CoA hydratase (ECH); Hydroxyacyl- CoA dehydrogenase (HAD); Ketoacyl- CoA thiolase (KAT).

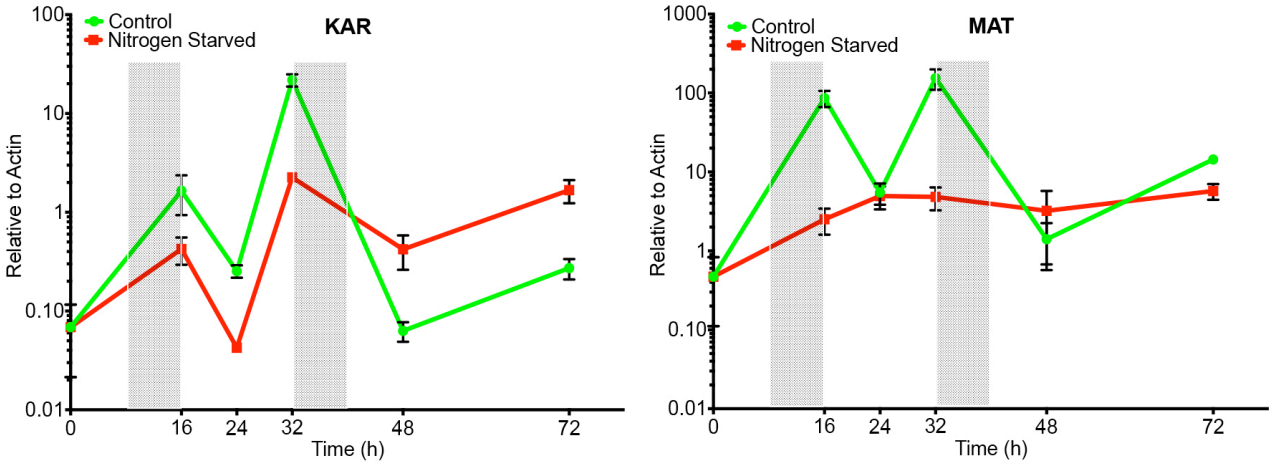


Figure 8. qRT-PCR expression analysis of circadian genes at 0, 16, 24, 32, 48 and 72 h after nitrogen depletion. Grey shaded areas indicated dark cycle in the first 48 h. Relative transcript abundance of KAR-encoding gene(s) was found to increase just after and right before the dark cycle in both control and nitrogen-starved treatments, while only control cells exhibited a circadian response in MAT-encoding gene(s).

Discussion

Oleaginous microalgae such as *Tetraselmis* sp. have the potential to accumulate large quantities of lipids during nutrient deplete conditions. To understand the expression profiles of lipid-related pathways in *Tetraselmis* sp. during the early stages of lipid accumulation, this study first determined the best nutrient conditions to stimulate lipid production and timepoint for RNA sampling. The neutral lipid content of *Tetraselmis* sp. was quantified by measuring its Nile red fluorescence. Nile red is commonly used as a fluorescence probe for neutral lipid detection in microalgae (Mendoza et al., 2008; Chen et al., 2009; Doan and Obbard, 2011; Montero et al., 2011; Bougaran et al., 2012; Mendoza et al., 2012; Vigeolas et al., 2012), with a strong correlation

between fluorescence signal and total neutral lipid content well established (de la Jara et al., 2003; Chen et al., 2009). RNA was extracted at 24 h after treatments and was followed by Illumina Mi-Seq transcriptomic analysis and qRT-PCR on control and nitrogen-starved cultures. This then enabled the assembly of the TAG synthesis, lipid synthesis and lipid catabolism pathways for *Tetraselmis* sp., including the differential expression of genes in those pathways, along with other major gene groups between the two treatments. Data from this first round of "RNA-Seq" analysis was followed by a second "detailed time course" experiment that analyzed the differential expression of the aforementioned pathways at 16, 24, 32, 48 and 72 h by qRT-PCR. Primers were designed in conserved regions based on reads with significant matches to the corresponding genes, which allowed transcript quantification for several differentially expressed gene family member simultaneously. However, it should be mentioned that this does not include reads obtained from transcripts that were not differentially expressed.

Pysiological response of *Tetraselmis* sp. to nutrient depletion

Physiological observations (Figure 1) of *Tetraselmis* sp. M8 during nutrient deprivation revealed that nitrogen and not phosphate depletion induced lipid accumulation. This is similar to many other microalgae (Rodolfi et al., 2009; Miller et al., 2010; Rismani-Yazdi et al., 2012; Valenzuela et al., 2012) and shows that the transition to starvation phase, detected via a significant increase in lipid accumulation compared to control, was as early as 16 h after exhaustion of exogenous nitrogen. Furthermore, N-deprived cultures were found to undergo just one doubling period within the first 48 h, after which cell growth ceased and the rate of lipid accumulation significantly increased. This sudden halt in growth and increase in lipid content after 1 doubling period also coincides with a significant decrease in chlorophyll *a* content (Figure 1C), and may indicate the depletion of internal nitrogen stores and the transition from early-starvation to starvation phase. Changes in these physiological parameters during the transition from early-stationary to stationary phase within the first 48 h have also been observed in *Botryospharella sudeticus* (Sun et al., 2013), *Phaeodactylum tricornutum* (Valenzuela et al., 2012) and *Neochloris oleoabundans* (Rismani-Yazdi et al., 2012).

Functional category analysis of *Tetraselmis* sp. transcriptome during early-stationary phase

RNA sequencing of control and nitrogen-starved treatments were performed on RNA sampled 24 h after nitrogen depletion using an Illumina Mi-Seq platform. This was followed by DiffKAP analysis (Figure 9), which revealed reads that were differentially expressed between the two different sets of transcriptomes. These DERs were then annotated by BLAST-matching to Swissprot and the annotated DERs were assigned with GO terms to determine the functional categories that were differentially expressed (Figure 3). In the control cultures, GO terms linked to plastid, thylakoid, generation of precursor metabolites and protein complex were each assigned more than 70% of DERs, indicating down-regulation of genes linked to these pathways in N-starved cultures that had less than 25% of DERs assigned to similar terms. This finding, when taken into account the high abundance of DERs linked to carbohydrate metabolic process, nucleotide binding, catabolic process and stress response in nitrogen-starved cultures, suggests a shift in carbon flux away from photosynthesis as the cells respond to unfavorable growth conditions and transition into stationary phase. These changes in transcript abundance can be linked to the reduction in chlorophyll content 24 h later, as thylakoids are degraded and not replaced. Other microalgae transcriptome studies have documented down-regulation of photosynthesis-related genes during nitrogen deprivation, and suggest that light harvesting proteins may be in excess, and that this response is linked to the recycling of nitrogen-rich proteins, but with no immediate effect on photosynthesis capacity (Valenzuela et al., 2012; Lv et al., 2013; Sun et al., 2013). This response, along with the observed reduction of transcripts linked to protein complex suggests that *Tetraselmis* sp. cells begin to convert sugar and change nitrogen allocation during early-stationary phase.

While the reduction in protein synthesis support the observations of reduced cell accumulation, it is interesting to find that transcripts linked to cell growth, cell cycle and reproduction were more abundant in the transcriptome from nitrogen-deprived cells. The latter group may include negative regulators of cell growth and reproduction, as cell density in starved cultures did not increase (Figure 1A). Transcripts linked to lipid particle and lipid metabolic processes were also found to be higher in nitrogen-deplete cultures, which was reflected in the larger lipid particles observed by microscopic analyses (Figure 1E).

Lipid accumulation during early-stationary phase of *Tetraselmis* sp. possibly due to reduced β-oxidation.

To further analyze key pathways linked directly to lipid accumulation in *Tetraselmis* sp., individual genes within FA synthesis, TAG synthesis and lipid catabolism were identified and

linked to associated DERs from the DiffKAP analysis (Figure 6-8). This was followed by qRT-PCR analysis of these pathways to confirm the DiffKAP analysis as well as a time-course analysis of these genes. At 24 h after exhaustion of exogenous nitrogen, the entire FA synthesis pathway was down-regulated. Expression of both TAG synthesis and lipid catabolism pathways was unchanged, with only DGAT- and ACSase-encoding genes down-regulated in their respective pathway. These results were confirmed in the follow up time-course experiment, which had similar expression profiles at 24 h post nitrogen depletion.

In *Tetraselmis* sp., although FA synthesis is down-regulated, the down-regulation of genes (TAG lipase, ACSase) at the committing steps, as well as at the ECH-gene of the β-oxidation pathway may indicate that the observed increase in lipid accumulation at this time point is a result of reduced lipid degradation, rather than increased lipid synthesis. This observation has also been observed in *Nannochloropsis gaditana*, where the lack of up-regulation amongst lipid biosynthesis genes despite increased lipid production has been attributed to sufficiently abundant existing lipid production machinery carried over from growth phase, coupled with a shift in carbon flux away from carbohydrate synthesis (Radakovits et al., 2012). There is evidence supporting this in *Tetraselmis* sp., as the maintenance of basal levels lipid production coupled with the decrease of lipid catabolism would result in an overall increase in lipid production. Also similar to *N. gaditana* and *P. tricornutum,* the observed down-regulation of genes encoding fructose-1,6-biphosphate and fructose-1,6-biphosphate aldolase (Supplementary Table 1), key regulatory enzymes of carbon metabolism (Calvin cycle and gluconeogenesis) in *Tetraselmis* sp., suggests a possible shift in the carbon flux away from carbohydrate synthesis to lipid synthesis. This could contribute to the increase of lipid production as carbon is being "pushed" into FA synthesis and not being "pulled" by increased FA synthesis genes (Radakovits et al., 2012; Valenzuela et al., 2012; Yang et al., 2013). This diversion of carbon towards lipid metabolism is further supported by the observed reduction in starch synthase genes (Supplementary Table 1). Overall, results in this study suggest that the observed lipid accumulation of *Tetraselmis* sp. at 24 h is the result of a reduction in lipid catabolism, coupled with a possible shift in carbon flux towards lipid synthesis.

High lipid accumulation during stationary phase of *Tetraselmis* sp. due to active FA synthesis

The expression of lipid-related genes of *Tetraselmis* sp. M8 at 48 h and 72 h after nitrogen deprivation was analyzed in the time course experiment. The aim was to investigate the increase in

rate of lipid accumulation, as well as determine if the lack of expression in FA synthesis and TAG synthesis pathways were consistent throughout the entire stationary phase. The expression of the entire FA pathway in nitrogen-deprived cultures was significantly higher than the control cultures after 24 h (Figure 6). The committing steps of TAG synthesis (GK- and GPAT-encoding genes) and the phosphatidate phosphatase (PP)-gene were also found to be up-regulated in nitrogen-deprived cultures (Figure 7). Interestingly, both TAG lipase and ACSase-encoding genes were similarly upregulated in nitrogen-starved treatments, with only the ECH-gene being down-regulated at 72 h. These observations suggest that lipid accumulation after 24 h has switched from a result of decrease in catabolism to an increase in FA and TAG synthesis activity. *Tetraselmis* sp. M8 cells would be actively producing more lipids as opposed to just consuming less, which would explain the increase in the rate of lipid accumulation. The lack of a clear significant increase in many TAG synthesis genes may suggest the TAG assembly pathway on a whole, may be more post-transcriptional controlled in *Tetraselmis* sp., particularly for DGAT which exhibited reduced expression during nitrogen-starvation. Post-transcriptional control of DGAT has been reported in proteomics studies of *Chlorella vulgaris* (Guarnieri et al., 2011) and *Brassica napus* (Nykiforuk et al., 2002), and has also been suggested for *Neochloris oleoabundans* (Rismani-Yazdi et al., 2012). The evidence for post-transcriptional control of DGAT is further supported by the lack of the phospholipid:diacylglycerol acyltransferase (PDAT) gene within the *Tetraselmis* sp. transcriptome comprising more than 73,000,000 reads. This indicates that that *Tetraselmis* sp. may lack the acyl-CoA-independent mechanism for TAG biosynthesis that has been found in certain microalgae (e.g. *Dunaliella tertiolecta*; Rismani-Yazdi et al., 2011), and thus relies solely on the TAG synthesis pathway for lipid production. Further proteomic and metabolic studies have to be performed to confirm this. The up-regulation of genes (e.g. for TAG-lipase and ACSase) within the β-oxidation pathway during starvation phase is not completely unexpected, and has previously been observed in *P. tricornutum* in association with changing membrane dynamics to cope with nutrient depletion (Valenzuela et al., 2012). The ECH-encoding gene however, was found to be progressively down-regulated after 24 h, and could present a potential bottleneck in the pathway. Nevertheless, the increased rate in lipid accumulation observed after 24 h, coupled with the up-regulation of the FA synthesis pathway is indicative that *Tetraselmis* sp. has transitioned from a state of reduced lipid consumption during early-stationary phase to a state of active lipid production during stationary phase.

Circadian effect on expression of lipid-related pathways

Gene expression of *Tetraselmis* sp. lipid pathways was analyzed at 16 h (start of light cycle) and at 32 h (light/dark cycle transition) after nitrogen deprivation to identify lipid-related genes that were affected by the circadian rhythm (Figure 9). These time points were removed from Figures 6-8 graphs to rule out the circadian influence on the overall expression analyses. Within the FA pathway, GK- and AGPAT-encoding genes were found to be affected by the circadian cycle, while genes in the lipid catabolism pathway were unaffected. Furthermore, the majority of these genes (except for those encoding ENR and GK) were found to have a divergent expression, exhibiting a spike in expression just at the start and end of the light cycle. This was different from those in *P. tricornutum* (Chauton et al., 2013), where most of the FA synthesis genes showed increased expression only at the onset of the light cycle, while β-oxidation genes showed increased expression towards the end. This could suggest that FA synthesis in *Tetraselmis* sp. could be linked to photosynthesis and the changing influx of carbon, while TAG and β-oxidation is not. Furthermore, nitrogen-starvation appears to have an overriding effect on certain genes such as those encoding MAT, KAS, ENR and AGPAT, where a spike in expression at the start or end of the light cycle was no longer observed in comparison with control cultures.

Nitrogen-starvation improves FA profile of *Tetraselmis* sp. for potential biodiesel production

A main reason *Tetraselmis* sp. presents a suitable feedstock for biodiesel production is its suitable FA composition (Lim et al., 2012). Under nitrogen-deplete condition, the FA profile of *Tetraselmis* sp. M8 was found to increase in its proportion of saturated (C16) and monounsaturated FA (C18:1), and to decrease in polyunsaturated FA (C16:3, C18:3, C18:3,; Figure 2). Several genes encoding enzymes involved in FA desaturation were identified by DiffKAP to have decreased expression in nitrogen-deprived cultures (Supplementary Table 1), such as genes encoding omega-6 FA desaturase, palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, lipid desaturase ADS3.2 and delta-9 acyl-lipid desaturase. Desaturases such as delta-9 acyl- lipid desaturase have been found in other microalgae that have a similar reduction in poly-unsaturated FA and an increase in saturated FA (C16), improving their FA content's cetane number and resulting biodiesel (Miller et al., 2010; Rismani-Yazdi et al., 2012).

DiffKAP vs qRT-PCR and experimental limitations

There are several limitations associated with this study, most of which are present due to the fact that *Tetraselmis* sp. is an unsequenced organism. The initial attempt at transcriptome assembly to *C. reinhardtii* and *Volvox carteri* genomes had a <0.02% match, and we therefore used DiffKAP to identify differentially-expressed read between the two treatments. Reads that were revealed as either highly expressed in control or nitrogen-starved treatments were then annotated to Swissprot by BLAST analyses. Limitations arose due to the high stringency of the BLAST parameters (e-value 10^{-16}), coupled with the fact that *Tetraselmis* sp. sequences were not closely related to any available genomes. This resulted in only 10.68% of DERs being annotated, leaving nearly 90% of the DiffKAP results unannotated. This could have caused certain genes in our DiffKAP analysis of the identified lipid metabolism pathways as not being differentially expressed. For example, genes encoding MAT, DGAT and ECH, which were classified as "not differentially expressed" by DiffKAP, although qRT-PCR analysis revealed these to be significantly down-regulated at 24 h in both RNA-Seq and time-course experiments. Primers for this experiment were designed based on the consensus sequence of all reads (extracted via TAGDB) that were related to a reference gene, and thus the qRT-PCR results would more likely reflect the overall expression of whole gene families, and not be limited to what was annotated. The nature of qRT-PCR analysis and the primers would also explain the expression of genes that encode ACO and ENR, whose differential expression was identified by DiffKAP, but not by qRT-PCR analysis. This is because DiffKAP would have identified individual genes as being differentially expressed, while qRT-PCR analysis revealed the gene family as being unchanged. Therefore, due to the limitations caused by the low degree of annotation in DiffKAP, results from this analysis were used primarily as an overview of gene expression in *Tetraselmis* sp. This was then followed up with qRT-PCR analysis, providing a more accurate representation of gene expression in the lipid pathways.

Another limitation in this study is the discrepancies in the feeding regime of the RNA-Seq and time-course experiments. Cultures in the time-course experiment were fed with full strength F-media and diluted to 0.5 x 10^6 cells/mL every 48 h, compared to F/2 media and diluted by half in the RNA-Seq experiment, effectively giving the cells in the time-course experiment more nutrients per cell. This was done to induce a more extreme starvation difference, and by extension a more distinct transcription profile between the two treatments as more nutrients would be available for control cultures towards the end of every 48 h feeding cycle. The extra exogenous nutrients would allow N-deprived cultures to have additional internal nutrient stores, and could exhibit a more delayed expression profile compared to the RNA-Seq experiment. This could explain the

discrepancies in the DGAT-encoding gene expression, where the significant difference observed in the RNA-seq experiment was only observed 24 h later in the time-course experiment.

Conclusions

Nitrogen-induced lipid accumulation in *Tetraselmis* sp. is not a simple case of increased FA and TAG synthesis. As this study has shown, there is a distinct early-stationary phase, characterized by reduced cell division and increased lipid accumulation, followed by a stationary phase that is characterized by a cessation of cell division and a significant increase in the rate of lipid accumulation. These physiological changes are also reflected in the expression profile at these time points, obtained through transcriptomics and qRT-PCR analyses. At 24 h after exhaustion of exogenous nitrogen, the majority of FA and TAG synthesis genes remained unchanged or down-regulated, while genes for committing steps of the β-oxidation pathway were significantly down-regulated. This implies that lipid accumulation at this phase was more of a result of decreased lipid consumption, while maintaining basal lipid production levels using cell machinery still available from growth phase. However, at 48 h after the onset of N-deprivation, the previously unchanged/downregulated FA pathway was significantly up-regulated. This shift from reduced lipid degradation to active FA production explains the increase in the rate of lipid accumulation at 48 h and 72 h. Results from this study also revealed that DGAT gene expression is not a bottleneck gene in *Tetraselmis* sp. lipid biosynthesis. Further proteomic and metabolomic work will be required to confirm the possibility of post-transcriptional control of DGAT.

Materials and Methods

Culture growth conditions – In order to detect changes in lipid-related pathways as cells transition from continuous exponential growth in log phase to stationary phase, it was important that RNA sampling was carried out on concurrently-grown control cultures that were maintained in log phase. Therefore, semi-continuous cultures of *Tetraselmis* sp. M8 were first established in order to maintain cells under constant nutrient-replete conditions and exponential growth phase before the start of each experiment. This way, cells could be maintained in constant growth phase and cell density by feeding and dilution in a constant cycle until the start of experiment. Three 1 L-master cultures were maintained by replacing half the culture (500 mL each) with autoclaved 25 PSU artificial seawater (Aquasonic) supplemented with F/2 medium (Guillard and Ryther, 1962; enriched with an additional 100 μM of phosphate) every 48 h. The cultures were grown in 1 L-Schott bottles with constant bubbling at 24°C under 16:8 light/dark photoperiod of fluorescent white lights (80 μmol photons $m^{-2}s^{-1}$). For RNA-Seq, semi-continuous cultures were maintained with a regime as above. At the start of the experiment, master cultures were mixed and distributed

to nine cultures (three cultures per treatment). Nitrogen-deprived and phosphate-deprived cultures had media replaced with nitrogen-deficient or phosphate-deficient F/2 medium to induce lipid production, while control cultures received N/P-replete medium. For the time course experiment, the semi-continuous cultures were maintained by diluting to 0.5 x10^6 cells/mL and feeding with F medium (enriched with an additional 100 μM of phosphate) every 48 h. Full strength F medium was used as larger difference in nutrient levels between treatments were expected to lead to more pronounced lipid induction. At the start of the experiment the nitrogen-starvation treatment was supplied by replacing with nitrogen deficient F-medium. In both experiments, the nitrogen and phosphate concentration of the cultures were measured daily to ensure nutrient-deplete conditions only occurred at 48 h after feeding (Supplementary Figure 4). The dilution and feeding regime was altered in the time course experiment to reduce the duration in which cultures experienced nutrient-deplete conditions at 48 h before feeding.

Physiological parameter analysis

During the course of the experiments, various physiological parameters such as cell density, Nile red fluorescence, nitrate and phosphate concentration, chlorophyll a & b and fatty acid (FA) content were measured.

Total nitrate and phosphate contents in the media were measured as described by Adarme-Vega et al. (2014) using API Aquarium pharmaceutical Nitrate NO^{3-} and Phosphate PO_4^{3-} test kits with absorbance measurements taken on a spectrophotometer (Hitachi U-2800 UV-VIS) at 545 nm and 690 nm, respectively. Cultures are considered nutrient (nitrogen and/or phosphate) starved (or deprived) when we cannot detect nitrate NO_{3-} and/or phosphate PO_4^{3-}. For chlorophyll extraction, 90% acetone and glass beads were added to a 5 mL microalgal pellet (extracted via centrifugation, 10000 x g, 7 min) and then vortexed for 3 min before being stored in the dark at 4°C for 2 h. Cellular debris was then pelleted (500 x g, 20 min) and the optical density (OD) of the acetone supernatant was measured on a spectrophotometer at 664 nm, 647 nm and 630 nm. The calculations for the concentration of chlorophyll a & b were performed as described by Franson et al. (2005).

For lipid accumulation measurements, 1 mL of culture was stained with 6 µL of Nile red in DMSO solution (250 mg/mL). Samples were then gently vortexed and incubated in the dark for 10 min. 200 µL was loaded into a 96 well-microtiter plate (Sarsted) in triplicates. Yellow-gold fluorescence was then measured on a POLARstar OPTIMA (BMG Labtech) plate reader using fluorescence intensity mode. Gain was set at 3000, with excitation and emission wavelengths of 485 nm and 590

nm selected. Specific fluorescence was obtained by dividing the Nile red fluorescence intensity by the cell number. Cell density was monitored via cell counts using a haemocytometer. FAs were analyzed using GC/MS by the Metabolomics Australia as described previously (Lim et al., 2012), with the exception that 5 mg of culture was used, instead of 4 mL culture.

Microscopic analyses

Cells were stained with Nile red (250 μg/mL) at 24 h after nitrogen and phosphorus deplete media were added to cultures. Photographs were taken using an Olympus BX60 microscope and an Olympus DP50 digital camera. Epifluroescent (excitation: 510-550 nm; emission: 590 nm) images were captured at 400x magnification.

RNA sampling and extraction

Sampling for RNA was performed on control and nitrogen-starved cultures at the desired time-points of 24 h for the RNA-Seq experiments and at 16, 24, 32, 48 and 72 h for the time-course experiment. At these time points, 10 mL of culture was collected by centrifugation (10000 x g, 7 min) from each replicate; the supernatant was discarded and the collected cell pellets immediately flash-frozen with liquid nitrogen and stored at -80°C. Just before RNA extraction, cell pellets were resuspended in lysis buffer (SV Total RNA Isolation System, Promega) and then ground using a micro pestle. Total RNA was then extracted following the manufacturer's instructions, with the exception that the incubation at 70°C was done at room temperature instead. Total RNA was not pooled but kept as respective replicates and then stored at -80°C.

cDNA library contruction & sequencing

cDNA libraries were made from replicates of the RNA-seq experiment, following the TruSEQ RNA V2 kit protocol; each replicate with their own adapters to barcode samples. cDNA products were then quantified on a Qubit ® 2.0 Fluorometer (Invitrogen) and checked for quality on a Bioanalyzer 2100 (Agilent). 151 bp paired-end sequencing of the cDNA libraries was then performed on an Illumina Mi-Seq platform using standard manufacturer protocols. Libraries of the same treatment were pooled together, with each treatment being sequenced on a separate run.

Sequence analysis and differential Kmer Analysis Pipeline (DiffKAP)

Standard RNA-Seq analysis relies on mapping individual short sequence reads to a reference genome or transcriptome and then applying statistical tests to identify differentially expressed genes. We attempted to apply the popular Tophat/Cufflinks (Trapnell et al., 2012) on the RNA-Seq data using an available genome of the closest species, *Chlamydomonas reinhardtii*, as reference. The analysis was unsuccessful as less than 0.02% reads were mapped. Therefore, we developed a Differential Kmer Analysis Pipeline (DiffKAP) that enabled identification of differentially expressed genes in RNA-Seq data between the two treatments without using a reference. The DiffKAP pipeline consists of six steps, as shown in Figure 9. DiffKAP uses Jellyfish (Marçais and Kingsford, 2011) to perform k-mer counting, and automatically determines an optimal k-mer size by finding the 'knee point' in the k-mer uniqueness graph (Kurtz et al., 2008). For this study, an optimal k-mer size of 17 was found to give the best balance between the specificity and sensitivity of the information content. The abundance of each k-mer was normalized by dataset size, and differentially expressed k-mers (DEKs) were determined using the following formula, where k is the query k-mer, c1 and c2 represent the normalized k-mer occurrence in datasets 1 and 2, respectively, X represents the minimum difference of the k-mer occurrence and Y is the minimum fold change of k-mer occurrence between the two datasets required to call a k-mer as differentially expressed. In this study, the minimum difference (X) used was 3, while the minimum fold change (Y) used was 2

$$(\mathrm{k} = \mathrm{DEK}) \Leftrightarrow \left(|\mathrm{c}_1 - \mathrm{c}_2| \geq \mathrm{X} \bigvee \left(\mathrm{Y} \leq \frac{\mathrm{c}_2}{\mathrm{c}_1} \bigwedge \mathrm{Y} \leq \frac{\mathrm{c}_1}{\mathrm{c}_2}\right)\right)$$

A single set of unique reads was obtained by removing duplicate reads within the datasets. Differentially expressed reads (DERs) were determined by a strict criterion to minimize false positives and defined as when all constituent k-mers in the read are differentially expressed k-mers (DEK), where r is the query read and $\mathbb{K}$ denotes all constituent k-mers in r.

$$(\mathrm{r} = \mathrm{DER}) \Leftrightarrow (\forall \mathrm{k} \in \mathbb{K} = \mathrm{DEK})$$

For each DER, the median k-mer abundance was calculated for each of the two datasets, and the ratio of median k-mer abundance (RoM) provided as a prediction of expression ratio. These reads were then categorized based on their expression ratios.

1. $RoM = 0$: Only present in nitrogen-starved
2. $0 < RoM < 0.5$: Highly induced in nitrogen-starved
3. $0.5 \leq RoM \leq 2$: Not differentially expressed
4. $2 < RoM < \infty$: Highly induced in control
5. $RoM = \infty$: Only present in control

All DERs were annotated by comparison with a user-specified protein database, e.g. Swissprot database (Boutet et al., 2007), with a user-defined e-value of 10^{-16}. The expression level of differentially-expressed genes was calculated as the median RoM of all DERs which were annotated with the same gene. The DiffKAP program is available from http://appliedbioinformatics.com.au/index.php/DiffKAP

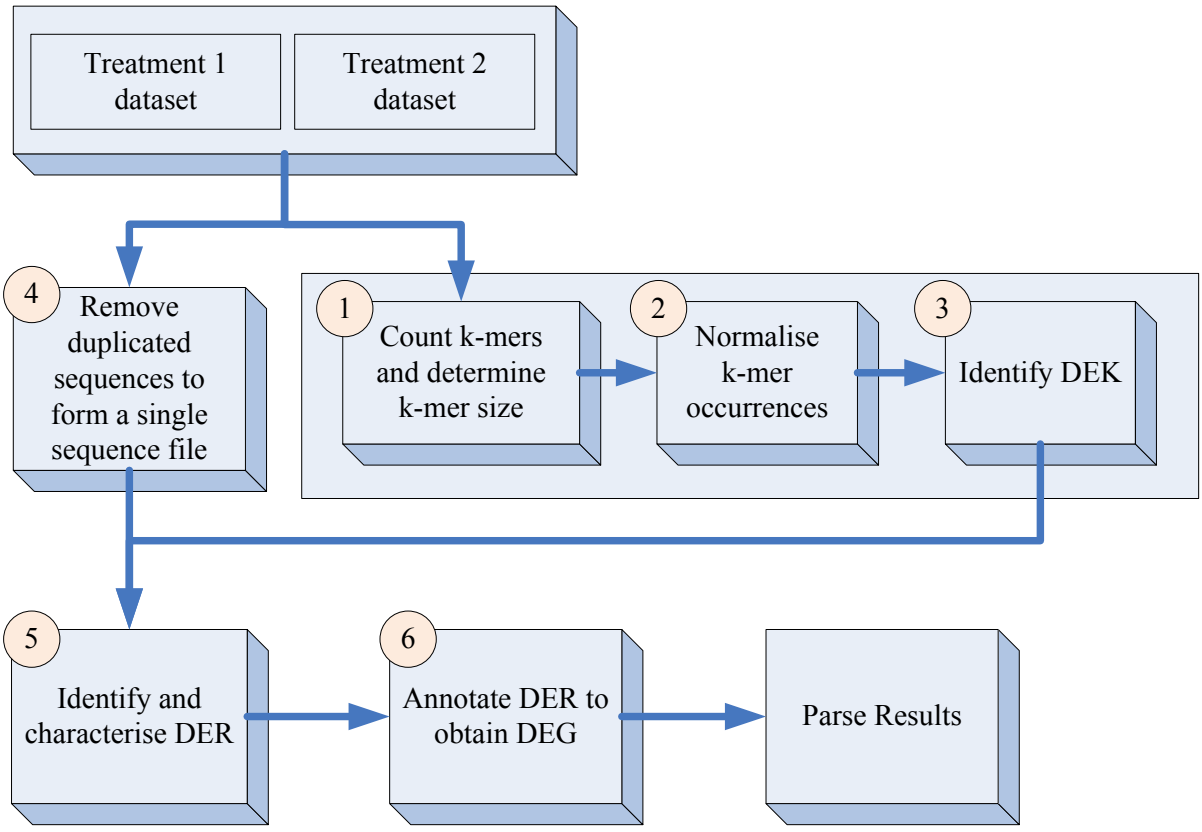


Figure 9. DiffKAP dataflow diagram

Functional annotation and pathway assignments

Successfully-annotated DERs from both treatments were fed into Blast2GO software in order to assign associated gene ontology (GO) terms with an annotation cutoff of 55, and GO

weight of 5 (Gotz et al., 2008). Genes in FA synthesis, TAG synthesis and lipid catabolism pathways were identified by BLAST matching the *Tetraselmis* sp. transcriptome that was first uploaded into TAGdb (Marshall et al., 2010) using reference sequences obtained from DiffKAP or NCBI. Similar reads from the BLAST results were also extracted from TAGdb for primer design. Automated gene assembly was not carried out to avoid the generation of potentially false contig sequences that could then be wrongly used by other studies for further assembly of other sequence data. Gene assembly for individual genes was carried out for qRT-PCR primer design (see below).

Quantitative reverse transcriptase real-time PCR

For optimal primer design, reads extracted from TAGdb were assembled to the initial reference gene using Geneious to yield a consensus sequence, which was then used as the next reference sequence for TAGdb to BLAST the transcriptome for more similar reads for assembly. This procedure was repeated until a sequence length of more than 500 bp was obtained. This sequence was then used for Primer Express to generate a primer pair. The full primer list in shown in Supplementary Table 3.

Extracted RNA from all replicates and experiments were used for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) for quantitative reverse transcriptase real-time PCR (qRT-PCR) following the manufacturer's instructions. For qRT-PCR, each reaction was performed in a final volume of 10 µL, and contained 1 µl cDNA (10 ng/µL), 1 µL of each primer (1 µM), 5 µL SYBR Green using the 7900 HT Fast Real-time PCR system (Applied Biosystems). Thermal cycling conditions consisted of 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C prior to 2 min at 25°C. Transcript levels were normalized to the expression of β-*ACTIN*.

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Supplementary table 1. Annotated Differentially Expressed Reads (DERs) between control and nitrogen-starved treatment of *Tetraselmis* sp. M8

Lipid-related pathways
fatty acid synthesis
lipid catabolism
gluconeogenesis
polyunsaturated fatty acid synthesis

Protein Name	# of annotated reads identified as differentially expressed by DIFFKAP				
	Only in -N	Upregulated in -N	Downregulated in -N	Absent in -N	Total
[NU+] prion formation protein 1	0	79	0	0	79
125 kDa kinesin-related protein	0	12	0	0	12
15-cis-phytoene desaturase	0	0	4	0	4
1-deoxy-D-xylulose-5-phosphate synthase	0	0	363	0	363
1-deoxy-D-xylulose-5-phosphate synthase 1, chloroplastic	0	0	175	0	175
1-deoxy-D-xylulose-5-phosphate synthase, chloroplastic	0	0	20	0	20
2-Cys peroxiredoxin BAS1, chloroplastic	0	0	239	0	239
2-Cys peroxiredoxin BAS1, chloroplastic (Fragment)	0	0	43	0	43
2-Cys peroxiredoxin BAS1-like, chloroplastic	0	0	1	0	1
2-dehydro-3-deoxyphosphooctonate aldolase	0	426	0	0	426
2-dehydro-3-deoxyphosphooctonate aldolase 1	0	832	0	0	832
2-dehydro-3-deoxyphosphooctonate aldolase 2	0	804	0	0	804
2-isopropylmalate synthase	0	0	5	0	5
2-isopropylmalate synthase 2, chloroplastic	0	0	4	0	4
2-isopropylmalate synthase B	0	0	8	0	8
2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	0	39	0	0	39
2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial (Fragment)	0	6	0	0	6
30S ribosomal protein S10	0	0	9	0	9
30S ribosomal protein S13, chloroplastic	0	0	6	0	6
37 kDa inner envelope membrane protein, chloroplastic	0	0	101	0	101
3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ (HD)	0	0	4	0	4
3-ketoacyl-CoA thiolase 1, peroxisomal (KAT)	0	41	0	0	41
3-ketoacyl-CoA thiolase 2, peroxisomal (KAT)	0	1	0	0	1
3-ketoacyl-CoA thiolase 5, peroxisomal (KAT)	0	2	0	0	2
3-ketoacyl-CoA thiolase, peroxisomal (KAT)	0	5	0	0	5
3-oxoacyl-[acyl-carrier-protein] reductase 5, chloroplastic (KAR)	0	0	11	0	11
3-oxoacyl-[acyl-carrier-protein] reductase, chloroplastic (KAR)	0	0	28	0	28
3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplastic (KAS)	0	0	245	0	245
3-oxoacyl-[acyl-carrier-protein] synthase II, chloroplastic (KAS)	0	0	6	0	6
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, chloroplastic	0	0	19	0	19
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, chloroplastic/chromoplastic (Fragment)	0	0	1	0	1
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, chloroplastic	0	0	217	0	217
4-hydroxy-3-methylbut-2-enyl diphosphate reductase	0	0	270	0	270
4-hydroxy-3-methylbut-2-enyl diphosphate reductase, chloroplastic	0	0	42	0	42
4-hydroxyphenylpyruvate dioxygenase	0	4	0	0	4
4-hydroxy-tetrahydrodipicolinate synthase, chloroplastic	0	0	22	0	22
50S ribosomal protein L1	0	0	58	0	58
50S ribosomal protein L11	0	0	96	0	96
50S ribosomal protein L13, chloroplastic	0	0	8	0	8
50S ribosomal protein L15	0	0	72	0	72
50S ribosomal protein L15, chloroplastic (Fragment)	0	0	9	0	9
50S ribosomal protein L27, chloroplastic	0	0	4	0	4
50S ribosomal protein L3, chloroplastic	0	0	20	0	20
50S ribosomal protein L4	0	0	11	0	11
50S ribosomal protein L4, chloroplastic	0	0	2	0	2
5'-adenylylsulfate reductase 1, chloroplastic	0	0	5	0	5
5'-adenylylsulfate reductase 3, chloroplastic	0	0	4	0	4
ABC transporter F family member 5	0	3	0	0	3
ABC transporter G family member 7	0	0	15	0	15
ABC transporter I family member 6, chloroplastic	0	0	15	0	15
Acetate kinase	0	1	0	0	1
Acetyl-CoA acetyltransferase, cytosolic 1	0	2	0	0	2
Acetyl-CoA carboxylase (ACCase)	0	0	14	0	14
Acetyl-CoA carboxylase, mitochondrial (ACCase)	0	0	3	0	3
Aconitate hydratase, mitochondrial	0	1043	0	0	1043
Actin	0	0	1	0	1
Actin-1	0	0	5	0	5
Acyl-coenzyme A oxidase 2, peroxisomal (ACO)	0	2	0	0	2
Acyl-coenzyme A oxidase, peroxisomal (ACO)	2	0	0	0	2
Adenosine 3'-phospho 5'-phosphosulfate transporter 1	0	11	0	0	11
Adenosylhomocysteinase	0	0	10	0	10
Adenosylhomocysteinase 1	0	0	60	0	60
Adenylate kinase	0	0	14	0	14
Adenylate kinase 1	0	0	10	0	10
Adenylate kinase 2, mitochondrial	0	0	6	0	6
Adenylate kinase A	0	0	65	0	65
Adenylate kinase B	0	0	3	0	3
Adenylate kinase, chloroplastic	0	0	9	0	9
Adenylosuccinate synthetase 2, chloroplastic	0	0	3	0	3

Adenylosuccinate synthetase, chloroplastic	0	0	103	0	103
Adenylyl-sulfate kinase	0	202	0	0	202
Adenylyl-sulfate kinase 1, chloroplastic	0	12	0	0	12
Adenylyl-sulfate kinase 2, chloroplastic	0	69	0	0	69
Adenylyl-sulfate kinase, chloroplastic	0	102	0	0	102
ADP,ATP carrier protein	0	0	50	0	50
ADP,ATP carrier protein 1, chloroplastic	0	3	103	0	106
ADP,ATP carrier protein 2, chloroplastic	0	17	310	0	327
Alanine--glyoxylate aminotransferase 2 homolog 1, mitochondrial	0	48	0	0	48
Alanine--glyoxylate aminotransferase 2 homolog 3, mitochondrial	0	42	0	0	42
Alanine--glyoxylate aminotransferase 2, mitochondrial	0	42	0	0	42
Alanine--tRNA ligase	0	0	3	0	3
ALBINO3-like protein 1, chloroplastic	0	0	4	0	4
Aldehyde-alcohol dehydrogenase	0	58	0	0	58
Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1	0	0	8	0	8
Aminomethyltransferase, mitochondrial	0	0	277	0	277
Ammonium transporter 1 member 2	0	57	0	0	57
Ammonium transporter 1 member 3	0	108	0	0	108
AP2-like ethylene-responsive transcription factor BBM	0	10	0	0	10
Argininosuccinate synthase	0	0	411	0	411
Aspartate aminotransferase 1	0	8	0	0	8
Aspartate aminotransferase P2, mitochondrial (Fragment)	0	14	249	0	263
Aspartate aminotransferase, chloroplastic	0	0	179	0	179
Aspartate aminotransferase, cytoplasmic	0	32	87	0	119
Aspartate aminotransferase, cytoplasmic isozyme 1	0	0	13	0	13
Aspartate aminotransferase, mitochondrial	0	0	846	0	846
ATP synthase delta chain, chloroplastic	0	0	8	0	8
ATP synthase gamma chain, chloroplastic	0	0	676	0	676
ATP synthase subunit b', chloroplastic	0	0	304	0	304
ATP-dependent Clp protease ATP-binding subunit clpA homolog	0	16	0	0	16
ATP-dependent Clp protease ATP-binding subunit ClpC	0	7	0	0	7
ATP-dependent RNA helicase dbp2	0	53	0	0	53
ATP-dependent RNA helicase DBP2	0	33	0	0	33
Aurora kinase	0	51	0	0	51
Aurora kinase A	0	9	0	0	9
Aurora kinase A-B	0	24	0	0	24
Beta-carotene 3-hydroxylase, chloroplastic (Fragment)	0	0	30	0	30
Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase	0	5	0	0	5
Cell division control protein 2 homolog	0	1	0	0	1
Cell division control protein 2 homolog C	0	137	0	0	137
Chaperone protein ClpB	0	58	0	0	58
Chaperone protein ClpC, chloroplastic	0	12	0	0	12
Chaperone protein ClpD, chloroplastic	0	8	0	0	8
Chaperone protein ClpD1, chloroplastic	0	32	0	0	32
Chaperone protein ClpD2, chloroplastic	0	1	0	0	1
Chaperonin CPN60-1, mitochondrial	0	0	6	0	6
Chaperonin CPN60-like 1, mitochondrial	0	0	4	0	4
Chloride channel protein CLC-c	0	3	0	0	3
Chlorophyll a-b binding protein 1, chloroplastic	0	0	1772	0	1772
Chlorophyll a-b binding protein 13, chloroplastic	0	0	1736	0	1736
Chlorophyll a-b binding protein 151, chloroplastic	0	0	47	0	47
Chlorophyll a-b binding protein 1A, chloroplastic	0	0	210	0	210
Chlorophyll a-b binding protein 1B, chloroplastic	0	0	1	0	1
Chlorophyll a-b binding protein 1B-21, chloroplastic	0	0	274	0	274
Chlorophyll a-b binding protein 2, chloroplastic	0	0	129	0	129
Chlorophyll a-b binding protein 215, chloroplastic	0	0	5	0	5
Chlorophyll a-b binding protein 22L, chloroplastic	0	0	43	0	43
Chlorophyll a-b binding protein 22R, chloroplastic	0	0	4	0	4
Chlorophyll a-b binding protein 25, chloroplastic	0	0	2	0	2
Chlorophyll a-b binding protein 3, chloroplastic	0	0	992	0	992
Chlorophyll a-b binding protein 36, chloroplastic	0	0	498	0	498
Chlorophyll a-b binding protein 37, chloroplastic	0	0	792	0	792
Chlorophyll a-b binding protein 3B, chloroplastic (Fragments)	0	0	418	0	418
Chlorophyll a-b binding protein 3C, chloroplastic	0	0	16	0	16
Chlorophyll a-b binding protein 4, chloroplastic	0	0	1630	0	1630
Chlorophyll a-b binding protein 48, chloroplastic	0	0	71	0	71
Chlorophyll a-b binding protein 5, chloroplastic (Fragment)	0	0	24	0	24
Chlorophyll a-b binding protein 7, chloroplastic	0	0	4	0	4
Chlorophyll a-b binding protein 8, chloroplastic	0	0	859	0	859
Chlorophyll a-b binding protein AB80, chloroplastic	0	0	37	0	37
Chlorophyll a-b binding protein AB96 (Fragment)	0	0	34	0	34
Chlorophyll a-b binding protein CP24 10A, chloroplastic	0	0	33	0	33
Chlorophyll a-b binding protein CP26, chloroplastic	0	0	7	0	7
Chlorophyll a-b binding protein CP29	0	0	2439	0	2439
Chlorophyll a-b binding protein E, chloroplastic	0	0	3	0	3
Chlorophyll a-b binding protein L1818, chloroplastic	0	0	620	0	620
Chlorophyll a-b binding protein M9, chloroplastic	0	0	329	0	329
Chlorophyll a-b binding protein of LHCII type 1 (Fragment)	0	0	11	0	11
Chlorophyll a-b binding protein of LHCII type I, chloroplastic	0	0	83950	0	83950
Chlorophyll a-b binding protein of LHCII type III, chloroplastic	0	0	1677	0	1677
Chlorophyll a-b binding protein type 1 member F3, chloroplastic	0	0	299	0	299
Chlorophyll a-b binding protein type 2 member 1A, chloroplastic	0	0	7160	0	7160
Chlorophyll a-b binding protein type 2 member 1B, chloroplastic	0	0	1817	0	1817
Chlorophyll a-b binding protein type 2 member 2 (Fragment)	0	0	935	0	935
Chlorophyll a-b binding protein type I, chloroplastic	0	0	3	0	3
Chlorophyll a-b binding protein, chloroplastic	0	0	18313	0	18313
Chlorophyll a-b binding protein, chloroplastic (Fragment)	0	0	1261	0	1261
Chlorophyll synthase, chloroplastic	0	0	43	0	43
Chlorophyllide a oxygenase, chloroplastic	0	0	756	0	756

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Chromosome-associated kinesin KIF4	1	5	0	0	6
Citrate synthase 2, peroxisomal	0	19	0	0	19
Citrate synthase 3, peroxisomal	0	15	0	0	15
Citrate synthase, glyoxysomal	0	137	0	0	137
CMP-sialic acid transporter 1	0	23	0	0	23
CMP-sialic acid transporter 3	0	27	0	0	27
CMP-sialic acid transporter 4	0	21	0	0	21
Copper methylamine oxidase	0	3	0	0	3
Coproporphyrinogen-III oxidase, aerobic	0	0	15	0	15
Coproporphyrinogen-III oxidase, chloroplastic	0	0	306	0	306
Cyanate hydratase	0	0	4	0	4
Cyanate hydratase 2	0	0	6	0	6
Cyclin-B2-1	0	5	0	0	5
Cyclin-dependent kinase A-2	0	1	0	0	1
Cyclin-dependent kinase B1-1	0	326	0	0	326
Cyclin-dependent kinase B1-2	0	45	0	0	45
Cycloartenol-C-24-methyltransferase	0	0	16	0	16
Cysteine synthase	0	0	43	0	43
Cysteine synthase, chloroplastic/chromoplastic	0	0	52	0	52
Cytochrome b6-f complex iron-sulfur subunit	0	0	444	0	444
Cytochrome b6-f complex iron-sulfur subunit 1, cyanelle	0	0	161	0	161
Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	0	0	645	0	645
Cytochrome P450 97B1, chloroplastic	0	0	11	0	11
DEAD-box ATP-dependent RNA helicase 20	0	65	0	0	65
DEAD-box ATP-dependent RNA helicase 30	0	38	0	0	38
Delta-9 acyl-lipid desaturase 1	0	0	47	0	47
Delta-aminolevulinic acid dehydratase, chloroplastic	0	0	53	0	53
Deoxycytidylate deaminase	0	4	0	0	4
Deoxyhypusine synthase	0	28	0	0	28
Deoxyuridine 5'-triphosphate nucleotidohydrolase	0	407	0	0	407
Dicarboxylate transporter 1, chloroplastic	0	0	36	0	36
Dicarboxylate transporter 2, chloroplastic	0	0	5	0	5
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mit	0	24	0	0	24
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mit	0	3	0	0	3
Dihydroxy-acid dehydratase	0	0	57	0	57
DNA excision repair protein ERCC-6-like	0	2	0	0	2
DNA mismatch repair protein MSH7	0	15	0	0	15
DNA repair and recombination protein RAD54-like (Fragment)	0	2	0	0	2
DNA repair helicase UVH6	0	38	0	0	38
DNA repair protein RAD51 homolog	0	6	0	0	6
DNA repair protein RAD51 homolog 1	0	38	0	0	38
DNA repair protein RAD51 homolog A	0	37	0	0	37
DNA repair protein RAD51 homolog B	0	52	0	0	52
DNA topoisomerase 2	0	52	0	0	52
DNA topoisomerase 2-alpha	0	20	0	0	20
Dual specificity protein kinase TTK	0	2	0	0	2
Dynamin-related protein 5A	0	27	0	0	27
Dynein gamma chain, flagellar outer arm	0	1	0	0	1
Dynein light chain 2, cytoplasmic	0	1	0	0	1
Elongation factor 3	0	128	0	0	128
Elongation factor 3A	0	44	0	0	44
Elongation factor G, chloroplastic	0	0	2	0	2
Elongation factor G, chloroplastic (Fragment)	0	0	5	0	5
Enoyl-[acyl-carrier-protein] reductase [NADH] 1, chloroplastic (ENR)	0	0	31	0	31
Enoyl-[acyl-carrier-protein] reductase [NADH] 2, chloroplastic (ENR)	0	0	98	0	98
Enoyl-[acyl-carrier-protein] reductase [NADH], chloroplastic (ENR)	0	0	107	0	107
Eukaryotic translation initiation factor 3 subunit A	0	0	3	0	3
Ferredoxin	0	0	1227	0	1227
Ferredoxin, chloroplastic	0	0	5	0	5
Ferredoxin-1	0	0	2435	0	2435
Ferredoxin-2	0	0	4	0	4
Ferredoxin--NADP reductase, chloroplastic	0	0	1175	0	1175
Ferredoxin--NADP reductase, embryo isozyme, chloroplastic	0	0	81	0	81
Formate acetyltransferase	0	59	0	0	59
Formate acetyltransferase (Fragment)	0	2255	0	0	2255
Fructose-1,6-bisphosphatase, chloroplastic	0	0	921	0	921
Fructose-bisphosphate aldolase 1, chloroplastic	0	0	1839	0	1839
Fructose-bisphosphate aldolase, chloroplastic	0	0	21	0	21
Fumarate hydratase 2, chloroplastic	0	0	3	0	3
Fumarate hydratase, mitochondrial	0	0	10	0	10
G2/mitotic-specific cyclin-1	0	1	0	0	1
Geranylgeranyl diphosphate reductase	0	0	89	0	89
Geranylgeranyl diphosphate reductase, chloroplastic	0	0	1880	0	1880
Geranylgeranyl pyrophosphate synthase homolog	0	0	125	0	125
Geranylgeranyl pyrophosphate synthase, chloroplastic	0	0	8	0	8
Geranylgeranyl pyrophosphate synthase, chloroplastic/chromoplastic	0	0	31	0	31
Glucose-1-phosphate adenylyltransferase	0	0	4	0	4
Glucose-1-phosphate adenylyltransferase large subunit 1, chloroplastic	0	0	14	0	14
Glucose-1-phosphate adenylyltransferase large subunit 2 (Fragment)	0	0	1	0	1
Glucose-1-phosphate adenylyltransferase large subunit 2, chloroplastic	0	0	75	0	75
Glucose-1-phosphate adenylyltransferase large subunit 2, chloroplastic/amyloplastic	0	0	37	0	37
Glucose-1-phosphate adenylyltransferase large subunit 3, chloroplastic	0	0	6	0	6
Glucose-1-phosphate adenylyltransferase large subunit 3, chloroplastic/amyloplastic	0	0	21	0	21
Glucose-1-phosphate adenylyltransferase large subunit, chloroplastic/amyloplastic	0	0	86	0	86
Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic	0	0	200	0	200
Glucose-1-phosphate adenylyltransferase small subunit, chloroplastic/amyloplastic	0	0	546	0	546
Glucose-6-phosphate isomerase, cytosolic	0	0	3	0	3
Glutamate synthase [NADH]	0	29	0	0	29
Glutamate synthase [NADH], amyloplastic	0	418	0	0	418

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Glutamate synthase [NADPH] large chain	0	18	0	0	18
Glutamate synthase 1 [NADH], chloroplastic	0	1536	0	0	1536
Glutamate synthase 2 [NADH], chloroplastic	0	768	0	0	768
Glutamate-1-semialdehyde 2,1-aminomutase	0	0	279	0	279
Glutamate-1-semialdehyde 2,1-aminomutase, chloroplastic	0	0	771	0	771
Glutamate--glyoxylate aminotransferase 1	0	0	63	0	63
Glutamate--glyoxylate aminotransferase 2	0	0	13	0	13
Glutamate--tRNA ligase, chloroplastic/mitochondrial	0	0	201	0	201
Glutamine synthetase	0	29	0	0	29
Glutamine synthetase cytosolic isozyme	0	127	0	0	127
Glutamine synthetase nodule isozyme	0	16	0	0	16
Glutamyl-tRNA reductase 1, chloroplastic	0	0	29	0	29
Glutamyl-tRNA reductase 2, chloroplastic	0	0	37	0	37
Glutamyl-tRNA reductase, chloroplastic	0	0	2	0	2
Glyceraldehyde-3-phosphate dehydrogenase	1	328	0	0	329
Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	0	210	0	0	210
Glyceraldehyde-3-phosphate dehydrogenase 1	0	17	0	0	17
Glyceraldehyde-3-phosphate dehydrogenase 1 (Fragment)	0	17	0	0	17
Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic	0	1	0	0	1
Glyceraldehyde-3-phosphate dehydrogenase 2	0	1	0	0	1
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	0	3	0	0	3
Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic (Fragment)	0	2	0	0	2
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic	0	0	1488	0	1488
Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic (Fragment)	0	0	518	0	518
Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic	0	0	234	0	234
Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic (Fragment)	0	0	4	0	4
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	0	836	0	0	836
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Fragment)	0	119	0	0	119
Glycine dehydrogenase [decarboxylating], mitochondrial	0	0	10	0	10
Glycine-rich protein 2	0	0	61	0	61
Glycogen phosphorylase 1	0	8	0	0	8
Granule-bound starch synthase 1, chloroplastic/amyloplastic	0	0	62	0	62
GTP-binding protein 128up	0	0	28	0	28
H/ACA ribonucleoprotein complex subunit 4	0	0	38	0	38
Heat shock 70 kDa protein	0	0	5	0	5
Heat shock 70 kDa protein 6, chloroplastic	0	0	24	0	24
Histone H2B	0	38	0	0	38
Histone H2B.3	0	33	0	0	33
Histone H2B.4	0	826	0	0	826
Histone H2B.6	0	1	0	0	1
Histone H2B.7	0	29	0	0	29
Histone H2B.9	0	21	0	0	21
Homogentisate 1,2-dioxygenase	0	20	0	0	20
Homospermidine synthase 2	0	3	0	0	3
Hydroxylamine reductase	0	0	336	0	336
Inducible nitrate reductase [NADH] 1	0	0	12	0	12
Inducible nitrate reductase [NADH] 2	0	0	9	0	9
Inner membrane ALBINO3-like protein 1, chloroplastic	0	0	1	0	1
Inner membrane ALBINO3-like protein 2, chloroplastic	0	0	3	0	3
Inosine-5'-monophosphate dehydrogenase	0	0	13	0	13
Isoamylase 1, chloroplastic	0	4	0	0	4
Isoamylase 3, chloroplastic	0	13	0	0	13
Isocitrate dehydrogenase [NADP]	0	13	0	0	13
Isoleucine--tRNA ligase	0	0	3	0	3
Katanin p60 ATPase-containing subunit A1	0	4	0	0	4
Kinesin-3	0	3	0	0	3
Kinesin-like calmodulin-binding protein	0	152	0	0	152
Kinesin-like calmodulin-binding protein homolog	0	119	0	0	119
Kinesin-related protein 4	0	1	0	0	1
Kinesin-related protein 8	0	2	0	0	2
Leucine aminopeptidase 2, chloroplastic	0	0	24	0	24
Leucine aminopeptidase 3, chloroplastic	0	0	43	0	43
Leucine aminopeptidase, chloroplastic	0	0	21	0	21
Long chain acyl-CoA synthetase 2 (ACSase)	0	0	70	0	70
Long chain acyl-CoA synthetase 4 (ACSase)	0	0	3	0	3
Magnesium-chelatase subunit ChlD	0	0	12	0	12
Magnesium-chelatase subunit ChlD, chloroplastic	0	0	77	0	77
Magnesium-chelatase subunit ChlH, chloroplastic	0	0	3383	0	3383
Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase 1, chloroplastic	0	0	2388	0	2388
Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase 2, chloroplastic	0	0	2619	0	2619
Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, chloroplastic	0	0	175	0	175
Malate dehydrogenase [NADP] 1, chloroplastic	0	0	329	0	329
Malate dehydrogenase [NADP], chloroplastic	0	0	1712	0	1712
Malate dehydrogenase 2, glyoxysomal	0	3	0	0	3
Malate dehydrogenase, chloroplastic	0	16	0	0	16
Methionine synthase	0	0	230	0	230
Methionine synthase (Fragment)	0	0	28	0	28
Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	0	17	0	0	17
Methylenetetrahydrofolate reductase 1	0	0	7	0	7
Methylenetetrahydrofolate reductase 2	0	0	3	0	3
Methylmalonate semialdehyde dehydrogenase [acylating]	0	5	0	0	5
Methylmalonate semialdehyde dehydrogenase [acylating] 2	0	4	0	0	4
Methylmalonate-semialdehyde dehydrogenase [acylating]	0	36	0	0	36
Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	0	55	0	0	55
Mitochondrial uncoupling protein 1	0	0	32	0	32
Mitogen-activated protein kinase kinase kinase 2	0	1	0	0	1
Mitotic spindle assembly checkpoint protein MAD2A	0	4	0	0	4
Molybdenum cofactor sulfurase	0	12	0	0	12
NAD(P) transhydrogenase, mitochondrial	0	168	0	0	168

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NADH-cytochrome b5 reductase 2	0	0	14	0	14
NADP-specific glutamate dehydrogenase	0	0	19	0	19
NAD-specific glutamate dehydrogenase	0	0	2	0	2
Negative regulator of genetic competence ClpC/MecB	0	25	0	0	25
Nitrate reductase [NAD(P)H]	0	0	57	0	57
Nitrate reductase [NADH]	0	0	170	0	170
Nitrate reductase [NADH] 2	0	0	17	0	17
Nitrogen regulatory protein P-II	3	0	0	0	3
Nucleolar GTP-binding protein 2	0	0	10	0	10
Nucleoside diphosphate kinase	0	0	11	18	29
Nucleoside diphosphate kinase (Fragment)	0	0	212	0	212
Nucleoside diphosphate kinase 1	0	0	36	16	52
Nucleoside diphosphate kinase 2, chloroplastic	0	0	37	7	44
Nucleoside diphosphate kinase A	0	0	65	0	65
Nucleoside diphosphate kinase B	0	0	94	0	94
Nucleoside diphosphate kinase II, chloroplastic	0	0	61	0	61
Omega-6 fatty acid desaturase, chloroplastic	0	0	671	0	671
Oxygen-evolving enhancer protein 1, chloroplastic	0	0	2182	0	2182
Oxygen-evolving enhancer protein 1-2, chloroplastic	0	0	444	0	444
Oxygen-evolving enhancer protein 2, chloroplastic	0	0	1316	0	1316
Oxygen-evolving enhancer protein 2-3, chloroplastic	0	0	30	0	30
Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, chloroplastic	0	0	555	0	555
Peptide methionine sulfoxide reductase MsrA	0	0	153	0	153
Peptide methionine sulfoxide reductase MsrA 1	0	0	59	0	59
Peptidyl-prolyl cis-trans isomerase	0	0	85	0	85
Peptidyl-prolyl cis-trans isomerase B	0	0	51	0	51
Peptidyl-prolyl cis-trans isomerase CYP20-2, chloroplastic	0	0	87	0	87
Peptidyl-prolyl cis-trans isomerase CYP38, chloroplastic	0	0	77	0	77
Peptidyl-prolyl cis-trans isomerase F, mitochondrial	0	0	43	0	43
Peptidyl-prolyl cis-trans isomerase, chloroplastic	0	0	11	0	11
Peroxiredoxin-2B	0	0	66	0	66
Peroxiredoxin-2E-2, chloroplastic	0	0	47	0	47
PFL-like enzyme TdcE	0	26	0	0	26
Phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic	0	11	0	0	11
Phosphoenolpyruvate carboxylase 1	0	10	0	0	10
Phosphoenolpyruvate carboxylase 2	0	6	0	0	6
Phosphoenolpyruvate/phosphate translocator 2, chloroplastic	0	0	1	0	1
Phosphoglycerate kinase	0	0	738	5	743
Phosphoglycerate kinase 1, chloroplastic	0	0	5	5	10
Phosphoglycerate kinase 2, chloroplastic	0	0	13	5	18
Phosphoglycerate kinase, chloroplastic	0	0	454	8	462
Phosphoglycolate phosphatase 1B, chloroplastic	0	0	46	0	46
Phosphoribosylaminoimidazole-succinocarboxamide synthase	0	10	0	0	10
Phosphoribosylaminoimidazole-succinocarboxamide synthase, chloroplastic	0	8	0	0	8
Phosphoribosylaminoimidazole-succinocarboxamide synthase, chloroplastic (Fragment)	0	31	0	0	31
Phosphoribulokinase, chloroplastic	0	0	2960	0	2960
Photosystem I reaction center subunit II	0	0	96	0	96
Photosystem I reaction center subunit II, chloroplastic	0	0	2558	0	2558
Photosystem I reaction center subunit III, chloroplastic	0	0	610	0	610
Photosystem I reaction center subunit IV	0	0	635	0	635
Photosystem I reaction center subunit IV, chloroplastic	0	0	459	0	459
Photosystem I reaction center subunit XI, chloroplastic	0	0	754	0	754
Photosystem II 10 kDa polypeptide, chloroplastic	0	0	8	0	8
Photosystem II CP43 chlorophyll apoprotein	0	0	12	0	12
Photosystem II D2 protein	0	0	3	0	3
Photosystem II reaction center PSB28 protein, chloroplastic	0	0	3	0	3
Photosystem II stability/assembly factor HCF136, chloroplastic	0	0	90	0	90
Phytoene dehydrogenase	0	0	4	0	4
Phytoene dehydrogenase, chloroplastic/chromoplastic	0	0	17	0	17
Phytoene synthase, chloroplastic	0	0	157	0	157
Plastidic ATP/ADP-transporter	0	11	159	0	170
Plastocyanin	0	0	14	0	14
Plastocyanin, chloroplastic	0	0	3052	0	3052
POC1 centriolar protein homolog B	0	2	0	0	2
Polyadenylate-binding protein 1-B	0	14	0	0	14
Polyadenylate-binding protein, cytoplasmic and nuclear	0	17	0	0	17
Potassium-transporting ATPase alpha chain 1	0	7	0	0	7
Potassium-transporting ATPase alpha chain 2	0	118	0	0	118
Presenilin-like protein At2g29900	0	38	0	0	38
Probable 125 kDa kinesin-related protein	0	35	0	0	35
Probable 1-deoxy-D-xylulose-5-phosphate synthase 2, chloroplastic	0	0	56	0	56
Probable 1-deoxy-D-xylulose-5-phosphate synthase, chloroplastic	0	0	165	0	165
Probable 30S ribosomal protein 3, chloroplastic	0	0	42	0	42
Probable 30S ribosomal protein PSRP-3	0	0	6	0	6
Probable 5'-adenylylsulfate reductase 1, chloroplastic	0	0	13	0	13
Probable acetyl-CoA acetyltransferase, cytosolic 2	0	2	0	0	2
Probable aconitate hydratase, mitochondrial	0	275	0	0	275
Probable adenylate kinase 2, chloroplastic	0	0	61	0	61
Probable ADP-ribosylation factor GTPase-activating protein AGD6	0	7	0	0	7
Probable agmatinase 2	0	45	0	0	45
Probable arabinose 5-phosphate isomerase	0	476	0	0	476
Probable ATP-dependent Clp protease ATP-binding subunit	0	5	0	0	5
Probable coproporphyrinogen-III oxidase	0	0	4	0	4
Probable cytosol aminopeptidase	0	0	1	0	1
Probable deoxyhypusine synthase	0	2	0	0	2
Probable DNA topoisomerase 2	0	7	0	0	7
Probable E3 ubiquitin-protein ligase ARI7	0	5	0	0	5
Probable E3 ubiquitin-protein ligase ARI8	0	4	0	0	4
Probable fructose-bisphosphate aldolase 1, chloroplastic	0	0	120	0	120

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Probable fructose-bisphosphate aldolase 3, chloroplastic	0	0	922	0	922
Probable glucose-1-phosphate adenylyltransferase large subunit, chloroplastic	0	0	7	0	7
Probable granule-bound starch synthase 1, chloroplastic/amyloplastic	0	0	14	0	14
Probable H/ACA ribonucleoprotein complex subunit 4	0	0	4	0	4
Probable hydroxyacid-oxoacid transhydrogenase, mitochondrial	0	3	0	0	3
Probable lipid desaturase ADS3.2, chloroplastic	0	0	182	0	182
Probable mannose-1-phosphate guanylyltransferase 1	0	0	1	0	1
Probable mannose-1-phosphate guanylyltransferase 2	0	0	18	0	18
Probable mannose-1-phosphate guanylyltransferase 3	0	0	1	0	1
Probable mediator of RNA polymerase II transcription subunit 36b	0	0	5	0	5
Probable methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	0	8	0	0	8
Probable methylenetetrahydrofolate reductase	0	0	56	0	56
Probable methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	0	110	0	0	110
Probable phenylalanine-4-hydroxylase 1	0	15	0	0	15
Probable phosphoserine aminotransferase	0	6	0	0	6
Probable pyridoxal biosynthesis protein PDX1	0	0	132	0	132
Probable pyridoxal biosynthesis protein PDX1.1	0	0	41	0	41
Probable pyridoxal biosynthesis protein PDX1.2	0	0	13	0	13
Probable pyruvate kinase, cytosolic isozyme	0	78	0	0	78
Probable rhamnose biosynthetic enzyme 1	0	140	0	0	140
Probable rhamnose biosynthetic enzyme 2	0	174	0	0	174
Probable rhamnose biosynthetic enzyme 3	0	151	0	0	151
Probable serine hydroxymethyltransferase, cytosolic	0	0	2	0	2
Probable serine/threonine-protein kinase mps1	0	2	0	0	2
Probable serine/threonine-protein kinase ndrB	0	22	0	0	22
Probable ubiquitin-conjugating enzyme E2 C	0	122	0	0	122
Probable WRKY transcription factor 3	0	178	0	0	178
Probable WRKY transcription factor 4	0	13	0	0	13
Proliferating cell nuclear antigen (Fragment)	0	128	0	0	128
Prolycopene isomerase, chloroplastic	0	0	21	0	21
Propionyl-CoA carboxylase alpha chain, mitochondrial	0	3	0	0	3
Protein henna	0	2	0	0	2
Protein LUTEIN DEFICIENT 5, chloroplastic	0	0	15	0	15
Protein TOC75-3, chloroplastic	0	0	7	0	7
Protein translocase subunit SecA, chloroplastic	0	0	75	0	75
Protein translocase subunit SECA1, chloroplastic	0	0	9	0	9
Protein transport protein Sec61 subunit alpha	0	10	0	0	10
Protochlorophyllide reductase (Fragment)	0	0	5	0	5
Protochlorophyllide reductase, chloroplastic	0	0	38	0	38
Protoporphyrinogen oxidase, chloroplastic	0	0	65	0	65
Pullulanase 1, chloroplastic	0	56	0	0	56
Putative ammonium transporter 1 member 5	0	7	0	0	7
Putative diflavin flavoprotein A 3	0	0	5	0	5
Putative glutamate synthase [NADPH]	0	399	0	0	399
Putative glycerol-3-phosphate transporter 1	0	21	0	0	21
Putative glycerol-3-phosphate transporter 3	0	5	0	0	5
Putative K(+)-stimulated pyrophosphate-energized sodium pump	0	0	10	0	10
Putative peroxiredoxin sll0755	0	0	39	0	39
Putative peroxiredoxin sll1621	0	0	61	0	61
Putative peroxiredoxin ycf42	0	0	559	0	559
Putative ribonucleoside-diphosphate reductase small chain B	0	6	0	0	6
Pyridoxal biosynthesis lyase PdxS	0	0	151	0	151
Pyridoxine biosynthesis protein PDX1	0	0	2	0	2
Pyrophosphate-energized vacuolar membrane proton pump	0	0	952	0	952
Pyrophosphate-energized vacuolar membrane proton pump 1	0	0	223	0	223
Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial	0	240	0	0	240
Pyruvate dehydrogenase E1 component subunit alpha-1, mitochondrial	0	87	0	0	87
Pyruvate dehydrogenase E1 component subunit alpha-2, mitochondrial	0	168	0	0	168
Pyruvate dehydrogenase E1 component subunit beta	0	0	5	0	5
Pyruvate dehydrogenase E1 component subunit beta-3, chloroplastic	0	0	20	0	20
Pyruvate kinase	0	92	0	0	92
Pyruvate kinase, cytosolic isozyme	0	110	0	0	110
Pyruvate, phosphate dikinase	0	22	0	0	22
Pyruvate, phosphate dikinase 1, chloroplastic	0	16	11	0	27
Pyruvate, phosphate dikinase 2	0	45	16	0	61
Pyruvate, phosphate dikinase, chloroplastic	0	68	28	0	96
Ribonucleoside-diphosphate reductase small chain	0	567	0	0	567
Ribonucleoside-diphosphate reductase small chain A	0	68	0	0	68
Ribonucleoside-diphosphate reductase small chain C	0	314	0	0	314
Ribonucleoside-diphosphate reductase subunit M2	0	151	0	0	151
Ribosome biogenesis protein nsa2	0	13	0	0	13
Ribosome biogenesis protein NSA2	0	5	0	0	5
Ribosome biogenesis protein NSA2 homolog	0	377	0	0	377
Ribosome modulation factor	2	3	0	0	5
Ribulose bisphosphate carboxylase small chains, chloroplastic	0	0	90	0	90
Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	0	0	1468	0	1468
Ribulose-phosphate 3-epimerase	0	7	0	0	7
Ribulose-phosphate 3-epimerase, cytoplasmic isoform	0	80	0	0	80
RNA-binding protein PNO1	0	39	0	0	39
RNA-binding protein pno1	0	7	0	0	7
rRNA 2'-O-methyltransferase fibrillarin	0	0	5	0	5
rRNA-processing protein FCF1 homolog	0	46	0	0	46
Sec-independent protein translocase protein TATA, chloroplastic	0	0	32	0	32
Serine hydroxymethyltransferase	0	0	61	0	61
Serine hydroxymethyltransferase 1	0	0	51	0	51
Serine hydroxymethyltransferase 2	0	0	79	0	79
Serine hydroxymethyltransferase 2, mitochondrial	0	0	89	0	89
Serine hydroxymethyltransferase, cytosolic	0	0	260	0	260
Serine hydroxymethyltransferase, mitochondrial	0	0	2243	0	2243

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Serine/threonine-protein kinase 38	0	91	0	0	91
Serine/threonine-protein kinase Aurora-1	0	87	0	0	87
Serine/threonine-protein kinase Aurora-2	0	8	0	0	8
Serine/threonine-protein kinase tricorner	0	7	0	0	7
Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	0	1	0	0	1
Sodium/potassium-transporting ATPase subunit alpha	0	689	0	0	689
Sodium/potassium-transporting ATPase subunit alpha-1	0	5	0	0	5
Sodium/potassium-transporting ATPase subunit alpha-3	0	52	0	0	52
Sodium/potassium-transporting ATPase subunit alpha-4	0	8	0	0	8
Sodium/potassium-transporting ATPase subunit alpha-A	0	96	0	0	96
Sodium/potassium-transporting ATPase subunit alpha-B	0	17	0	0	17
Soluble starch synthase 1, chloroplastic/amyloplastic	0	0	3	0	3
Spermidine synthase 2	0	0	2	0	2
Starch synthase 1, chloroplastic/amyloplastic	0	0	26	0	26
Starch synthase 3, chloroplastic/amyloplastic	0	0	16	0	16
Structural maintenance of chromosomes protein 2	0	16	0	0	16
Structural maintenance of chromosomes protein 2-1	0	114	0	0	114
Structural maintenance of chromosomes protein 2-2	0	81	0	0	81
Structural maintenance of chromosomes protein 3	0	2	0	0	2
Structural maintenance of chromosomes protein 4	0	296	0	0	296
Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial	0	18	0	0	18
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	0	30	0	0	30
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	0	4	0	0	4
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	0	93	0	0	93
Sugar phosphate exchanger 2	0	34	0	0	34
SUMO-conjugating enzyme SCE1	0	3	0	0	3
Superoxide dismutase [Mn]	0	0	31	0	31
Superoxide dismutase [Mn], mitochondrial	0	0	25	0	25
TFIIH basal transcription factor complex helicase XPD subunit	0	10	0	0	10
Thermospermine synthase ACAULIS5	0	0	1	0	1
Thiamine thiazole synthase 2, chloroplastic	0	0	369	0	369
Thiamine thiazole synthase 4, chloroplastic	0	0	150	0	150
Thiamine thiazole synthase, chloroplastic	0	0	79	0	79
Thiol protease aleurain-like	0	3	0	0	3
Thioredoxin	0	0	9	0	9
Thioredoxin M-type, chloroplastic	0	0	366	0	366
Thioredoxin-1	0	0	183	0	183
Transcription factor MYB1R1	0	44	0	0	44
Transketolase	0	0	515	0	515
Transketolase 10	0	0	104	0	104
Transketolase 2	0	0	32	0	32
Transketolase 7	0	0	9	0	9
Transketolase, chloroplastic	0	0	537	0	537
Transketolase, chloroplastic (Fragment)	0	0	91	0	91
Transketolase-1, chloroplastic	0	0	192	0	192
Transketolase-2, chloroplastic	0	0	27	0	27
Triosephosphate isomerase, chloroplastic	0	0	2	0	2
Triosephosphate isomerase, cytosolic	0	0	10	0	10
Tryptophan 5-hydroxylase 1	0	5	0	0	5
Tyrosine--tRNA ligase	0	0	1	0	1
Ubiquitin-conjugating enzyme E2 20	0	5	0	0	5
Ubiquitin-conjugating enzyme E2 C	0	41	0	0	41
UDP-galactose/UDP-glucose transporter 5	0	81	0	0	81
UDP-galactose/UDP-glucose transporter 5B	0	375	0	0	375
UDP-glucose 4-epimerase	0	1	0	0	1
UDP-glucose 4-epimerase 2	0	1	0	0	1
UDP-glucose 4-epimerase 4	0	27	0	0	27
UDP-glucose 4-epimerase 5	0	49	0	0	49
UDP-glucose 4-epimerase GEPI48	0	5	0	0	5
UDP-glucose 6-dehydrogenase	0	24	0	0	24
UDP-glucose 6-dehydrogenase 1	0	87	0	0	87
UDP-glucose 6-dehydrogenase 2	0	84	0	0	84
UDP-glucose 6-dehydrogenase 3	0	60	0	0	60
UDP-glucose 6-dehydrogenase 4	0	259	0	0	259
UDP-glucose 6-dehydrogenase 5	0	187	0	0	187
UDP-N-acetylglucosamine pyrophosphorylase	0	1	0	0	1
UDP-sulfoquinovose synthase, chloroplastic	0	0	637	0	637
UMP-CMP kinase	0	75	0	0	75
Uncharacterized protein ycf45	0	65	0	0	65
Urea-proton symporter DUR3	0	1093	0	0	1093
Uroporphyrinogen decarboxylase 1, chloroplastic	0	0	48	0	48
Vacuolar cation/proton exchanger 2	0	18	0	0	18
Vacuolar cation/proton exchanger 3	0	1	0	0	1
Valine--tRNA ligase	0	0	2	0	2
WD repeat-containing protein WRAP73	0	2	0	0	2
Xanthine dehydrogenase	0	15	0	0	15
Zeta-carotene desaturase	0	0	23	0	23
Zeta-carotene desaturase, chloroplastic/chromoplastic	0	0	26	0	26

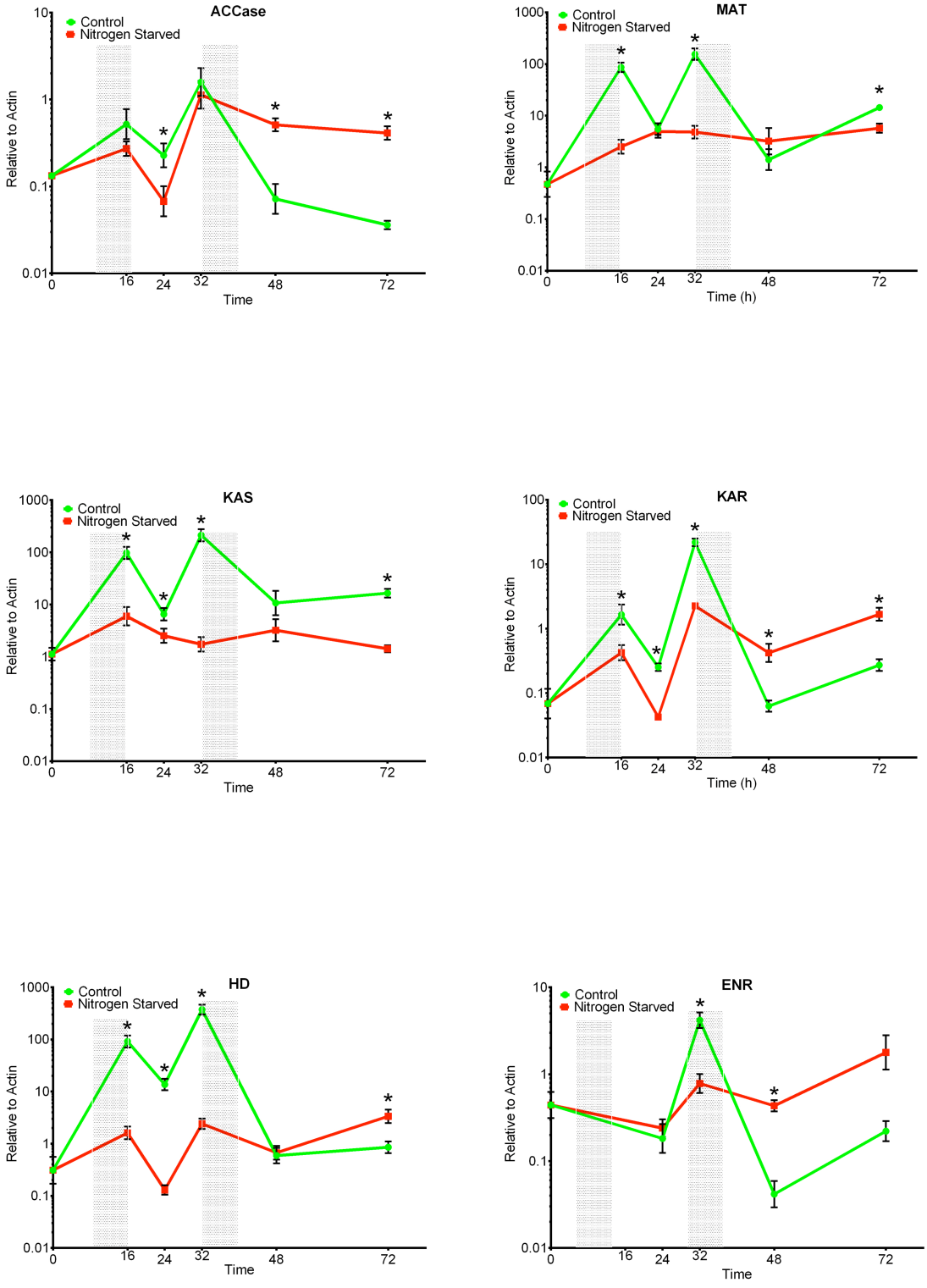
Supplementary Table 2. Distribution of annotated Differentially Expressed Reads (DERs) assigned with GO terms presented as a percentage of total annotated DERs in each treatment

GO Terms	Nitrogen-starved	Control
actin binding	0.32	0.00
anatomical structure morphogenesis	11.60	2.72
antioxidant activity	0.00	0.53
behavior	1.93	0.00
calcium ion binding	0.01	2.16
carbohydrate binding	2.17	0.00
carbohydrate metabolic process	45.29	12.16
catabolic process	34.10	10.50
cell cycle	6.71	0.03
cell death	2.36	2.98
cell differentiation	11.39	1.10
cell envelope	0.00	1.43
cell growth	8.25	0.23
cell proliferation	0.82	0.04
cell wall	0.31	1.04
cell-cell signaling	2.09	0.00
cellular homeostasis	3.14	1.69
cellular protein modification process	4.97	33.14
chromatin binding	0.42	0.03
cilium	0.10	0.00
cytoplasmic membrane-bounded vesicle	2.70	0.56
cytoskeleton organization	1.63	0.01
cytosol	16.50	1.88
DNA binding	8.47	1.96
DNA metabolic process	8.46	0.27
electron carrier activity	0.57	4.51
embryo development	3.70	0.24
endoplasmic reticulum	2.73	0.09
endosome	2.56	0.49
enzyme regulator activity	0.55	0.91
extracellular region	2.14	4.99
generation of precursor metabolites and energy	24.61	83.01
Golgi apparatus	1.94	0.77
ion channel activity	0.25	0.00
ion transport	5.17	6.27
lipid metabolic process	11.29	7.37
lipid particle	1.00	0.11
microtubule organizing center	0.60	0.01
mitochondrion	17.91	6.84
mitochondrion organization	1.48	0.15

motor activity	2.57	0.00
nuclear chromosome	0.53	0.00
nuclease activity	0.74	0.02
nucleolus	0.32	0.18
nucleoplasm	1.41	0.02
nucleotide binding	42.15	8.11
oxygen binding	0.00	0.07
peptidase activity	0.68	0.96
peroxisome	0.76	0.07
plasma membrane	13.46	1.12
plastid	15.12	93.23
protein complex	17.17	73.34
protein kinase activity	3.49	0.03
protein transport	0.50	2.88
receptor binding	0.17	0.00
regulation of gene expression, epigenetic	2.66	0.04
reproduction	13.84	0.32
response to abiotic stimulus	12.78	10.58
response to biotic stimulus	1.27	5.62
response to endogenous stimulus	0.61	2.79
response to external stimulus	6.30	0.52
response to stress	20.95	9.20
ribosome	2.68	0.73
secondary metabolic process	3.32	8.93
sequence-specific DNA binding transcription factor activity	1.07	0.01
signal transducer activity	0.03	0.00
signal transduction	0.93	2.88
thylakoid	0.01	82.71
transcription regulator activity	0.14	0.00
translation	2.99	0.39
translation factor activity, nucleic acid binding	1.03	0.01
translation regulator activity	0.06	0.00
vacuole	0.50	0.69
viral process	0.23	0.00

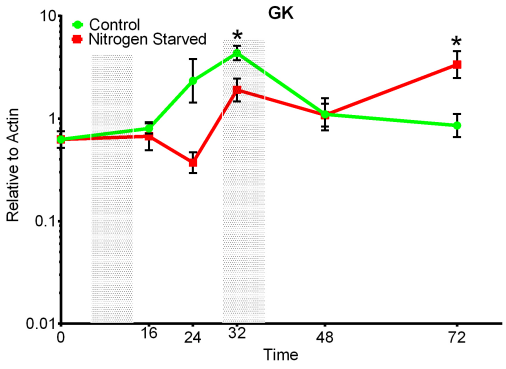
Supplementary table 3. List of primers used for qRT-PCR

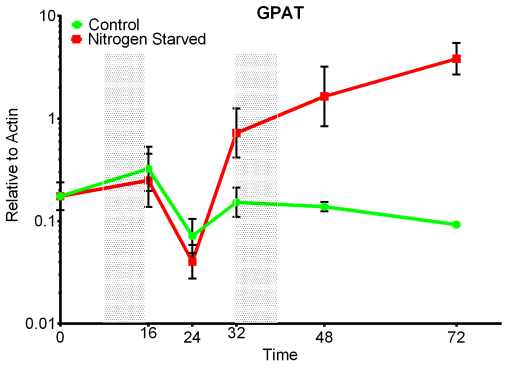
Primer Name	Forward	Reverse
Acetyl-CoA carboxylase (ACCase)	CTCTTCAACGCCAACACGAAAG	TTCCCGTGATACCCTCCGTTA
Malonyl-CoA:ACP transacylase (MAT)	AATTCTCGCCCAGCAGTTGAC	CACCTCGTAAGCCCTCTCCAG
3-ketoacyl-ACP synthase (KAS)	AGCATAACCTGATCGAGGAGCTT	GAAAGAGTTGGACATTGCTGCAGT
3-ketoacyl-ACP reductase (KAR)	CGGAGGAGATGTTAATGATGCG	ATCAACCTCACCGGCGTCTT
3-hydroxyacyl-ACP dehydratase (HD)	ACCCGCATCATGAGGCAATC	CGACACAGCAGGCAAGAACA
Enoyl-ACP reductase (ENR)	CTCCTTGACCTCAGTTGGGACA	CTCAAACGGGTCCTTAATGGAGT
Glycerol kinase (GK)	ATTGCGTCCAGCACCTCCTT	CTGGTGGGAATGACGCTGTC
Glycerol-3-phosphate O-acyltransferase (GPAT)	ATCGAGTAGTGAGCGACAAACTTT	GGGTCATCCATTATGTGCTTCTTG
1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT)	CTGGACCTTCCTCCTCGCTATC	AGAGGTGCGCTTGAACTTATCG
Phosphatidate phosphatase (PP)	TGTGGTCGGAGATCACATACGATA	CAGTAGAGCGAGAACGACACCAG
Diacylglycerol O-acyltransferase (DGAT)	ATCAGAGGAACCTGTCCCATCA	CTGCCATTTTTCACGAGCTAATG
Triacylglyceride (TAG) lipase	CTACTGTCGCATCTGGTTACCAAA	GAAATGTGAGGTCGCCGATTAG
Acyl-CoA synthetase (ACSase)	CACGTTGCTGTGCTTAATCTGC	CGAGTGCAACCCTGAGGATATG
Acyl-CoA oxidase (ACO)	GATGGTGGCTGCTTTGGACA	GGCCAAGGGAAACGAAAAGTC
Enoyl-CoA hydratase (ECH)	CGCCTCATCCATAAGCTGCTC	CCTCACCTGCAGAAGGATTGAT
Hydroxyacyl- CoA dehydrogenase (HAD)	TGTTTCTTTCCCTACAACATGGC	CAAACTCGCTCTTGATGATCTTGTC
Ketoacyl- CoA thiolase (KAT)	ACTGCGGCATCACCATCG	CAGGCTGAGGTCCCGCAT

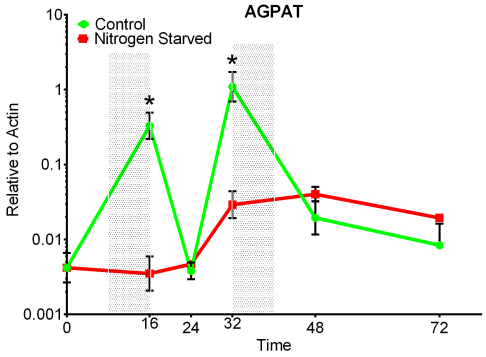


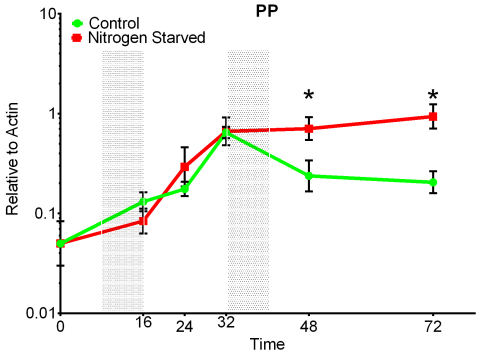
Supplementary Figure 1

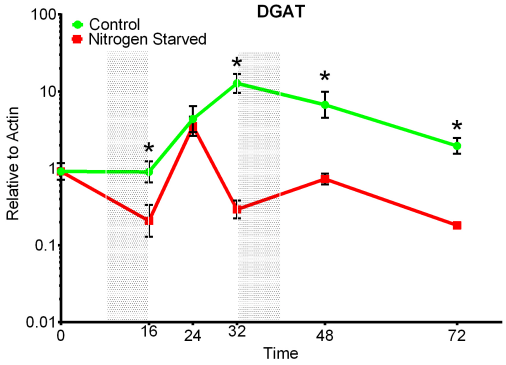
Supplementary Figure 1. Expression of genes in the FA synthesis pathway at 0, 16, 24, 32, 48 and 72 hours.





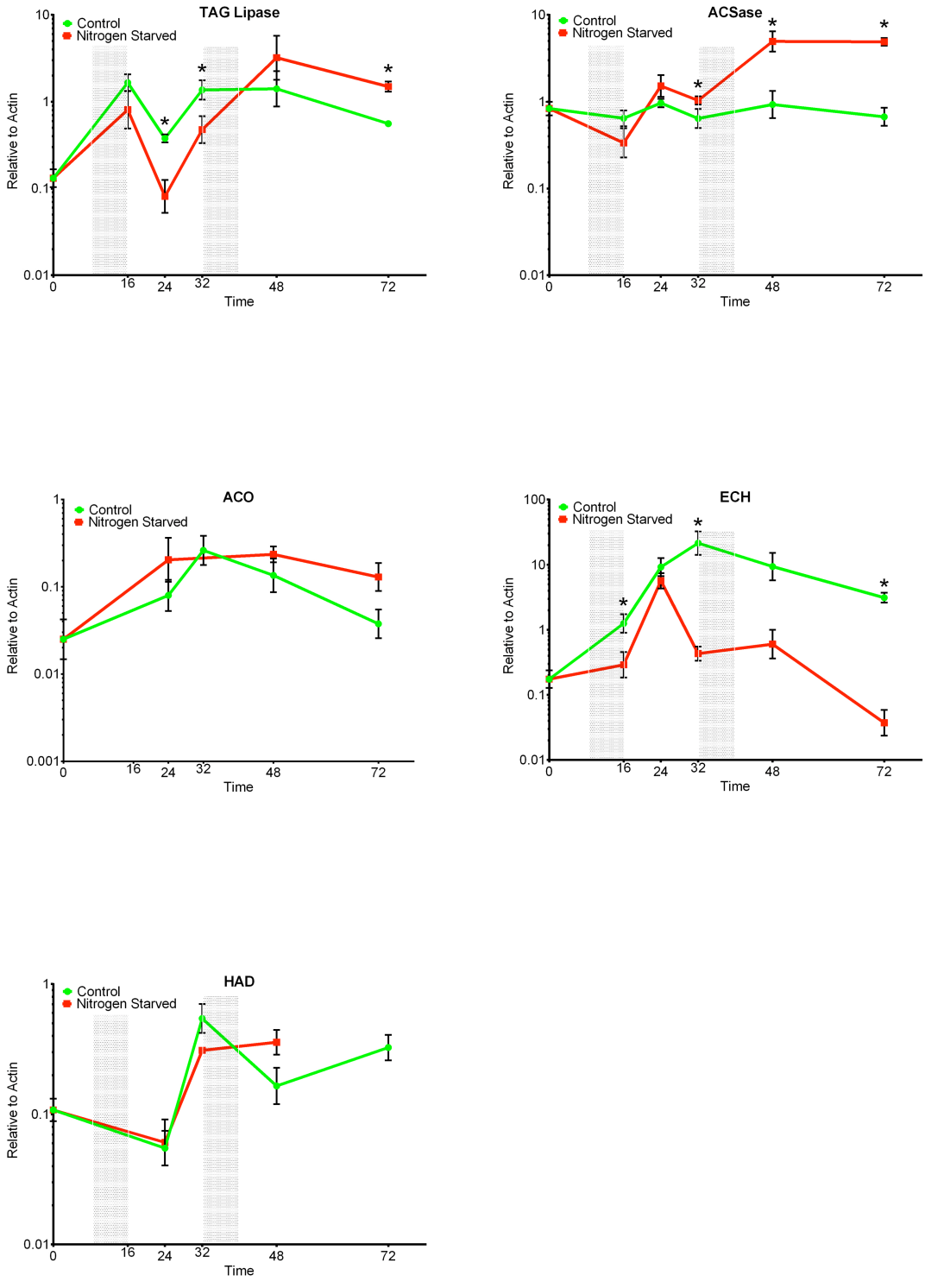






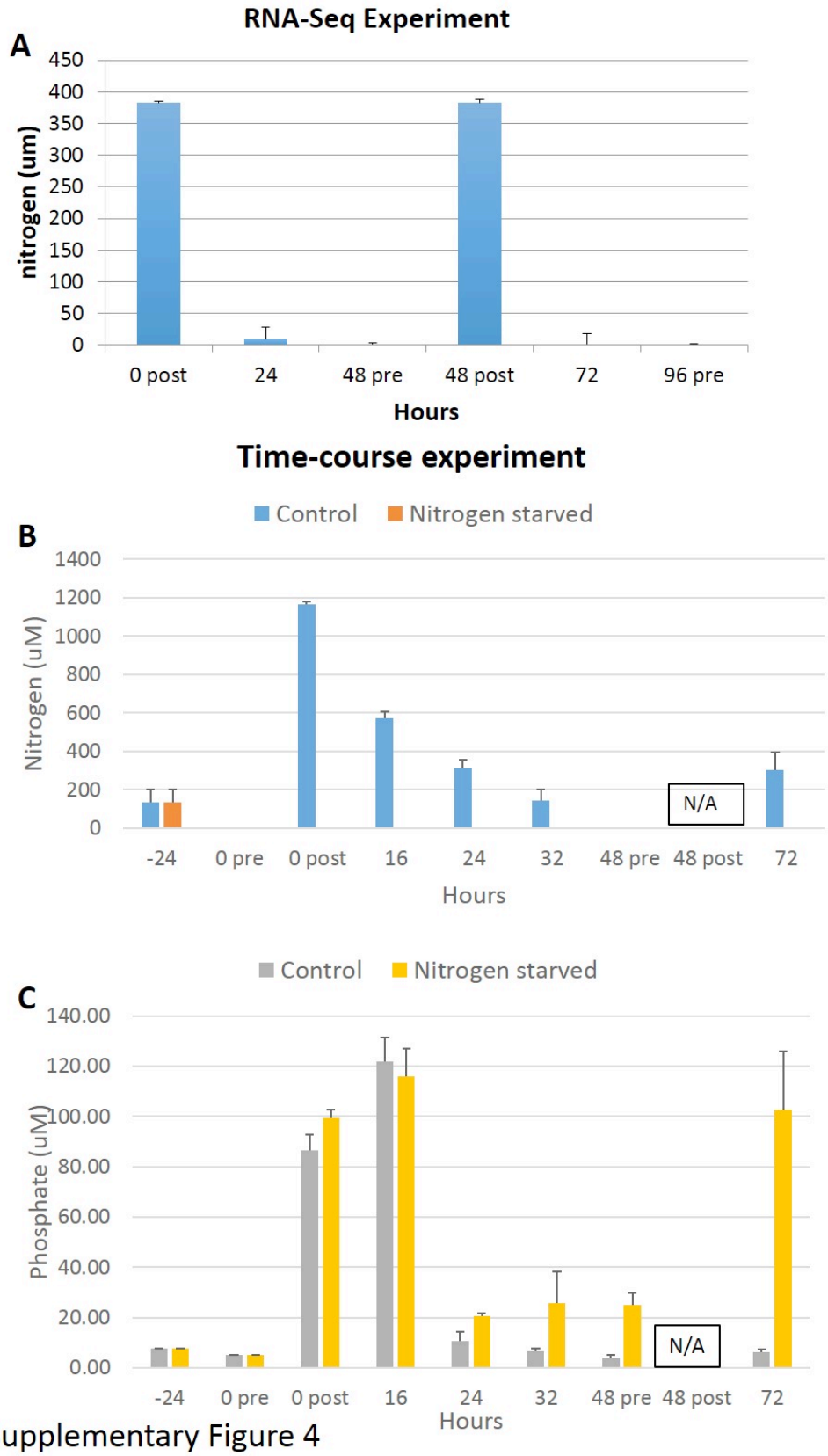
Supplementary Figure 2

Supplementary Figure 2. Expression of genes in the TAG synthesis pathway at 0, 16, 24, 32, 48 and 72 hours.



Supplementary Figure 3

Supplementary Figure 3. Expression of genes in the lipid catabolism pathway at 0, 16, 24, 32, 48 and 72 hours.



Supplementary Figure 4

Supplementary Figure 4. Nitrogen and Phosphate concentrations monitored during (A) RNA-seq experiment and (B) &(C) Time-course experiment.

Chapter 5: Concluding Discussion and Future Work

This thesis had the overall aim of identifying approaches for improving microalgal lipid productivity and gaining a deeper understanding of the molecular mechanisms behind microalgal lipid biosynthesis**.** The first aim (chapter 2) collected and identified candidate microalgal strains with high lipid productivity suitable for lipid and biodiesel production. Two strains that were isolated from local waterways, *Tetraselmis* sp. M8 and *Chlorella* sp. BR2, belong to genera that have been established as good candidates for large-scale production (Araujo et al., 2011,Huerlimann et al., 2010). This showed that strains with the ideal characteristic for large-scale lipid production can be obtained from local waterways, and thus present a possible alternative to purchasing commercial strains. It must be noted that the objective of the 'Standard Protocol' developed in Aim 1 was to compare all the strains under the same growth/starvation conditions and time period. It was never meant to determine a strains 'true' lipid producing potential, as different strains have different growth rates, and thus be at different stages of growth when the starvation period was applied on day 7. It is recognized that the applied nutrient starvation has different impacts on cells that still contain nutrient reserves vs. stationary phase. The 'Standard Protocol' was designed as an initial screen to rule out low lipid productivity strains, with more in depth analysis on the strains that show high-lipid potential to be carried out later, preferably in conditions that more closely resemble a commercial setting.

Future work regarding this aim should be focused on improving the standard protocol to more closely resemble a commercial setting. Other members of the laboratory have already begun this work. The volume of cultures were increased to 200 mL and aeration via bubbling added in the evaluation of other new strains in the laboratory (Van Thang, Unpublished). Furthermore, as commercial viability of large-scale microalgal lipid production is dependent on a biorefinery approach, other parameters such as protein, starch and carotenoid contents should also determined during strain evaluation. Another approach that can be taken to improve the collection and isolation of high-lipid strains is to identify the best sampling location and times that could yield such strains. The characterisation of collected samples from various water bodies, along with the related environmental data could reveal the natural environmental conditions which high-lipid microalgae are selected for. This information would not only help to improve the collection of high-lipid strains, but could potentially help the industry gain insight into the best conditions to select for and maintain laboratory strains' lipid productivity.

The second aim (Chapter 3) of this thesis improved the lipid productivity of *Tetraselmis suecica* via non-GM methods. The mutation-selection method that was developed successfully produced two lines of improved *T. suecica* strains, both exhibiting >110% increase in lipid content (measured by Nile red fluorescence) when compared to the wild-type strain. The two improved lines *T. suecica* M5 and *T. suecica* M24 were generated from two different lethal dosages (LD), 50% and >98% respectively. Both lethal dosages were selected to either increase recovery of clones (50% LD) or increase mutation probability (>98% LD). In the end, although both dosages yielded strains with improved lipid productivity after five rounds of mutations, no significant differences in lipid productivity or growth rates were found between them. Therefore, as the goal of mutagenesis was to induce a positive mutation, a lower LD (i.e. 50%) is recommended for future mutagenesis studies as (i) culture recovery after UV exposure was less time-consuming and (ii) this reduces the possibility of background damage.

As discussed in Chapter 3, the observed improvements in the putative mutants could be either a mutation to, or adaptation to UV-C. Therefore, after 6 months of growth under maintenance conditions during storage, the growth rates and lipid production of M5 and M24 were compared once again to wild-type. During storage, both improved strains and wild-type *T. suecica* were cultured in 100 mL filter cap tissue culture flasks (CellStar) at 25°C, under a 16:8 h light/dark photoperiod of fluorescent white light (60 μmol photons $m^{-2}s^{-1}$). Cultures were not shaken and were maintained by replacing half the culture (50 mL) with autoclaved artificial seawater (Aquasonic) supplemented with F/2 medium once every 2 weeks. These conditions aimed at minimising cell division and at avoiding high selection pressures. After the cultures were revived by gradual volume scale-up, strain comparisons were carried out in 250 mL cultures supplemented with F medium and air bubbling at 25°C, under a 16:8 h light/dark photoperiod of fluorescent white light (120 μmol photons $m^{-2}s^{-1}$). The growth rates and lipid content (Nile red fluorescence) of these cultures were measured as in Chapter 3, with starvation of cultures occurring 2 days into growth upon exhaustion of exogenous nitrogen.

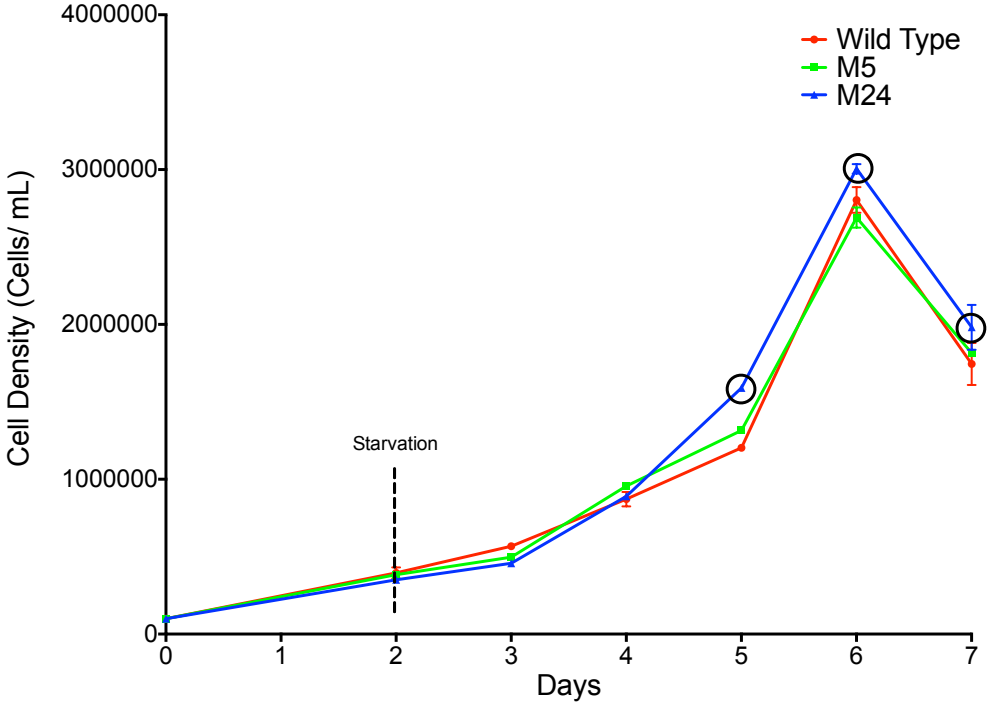


Figure 1. Cell density of *T. suecica,* M5 and M24 over the span of 7 days with nutrient starvation occurring on day 2. Circled data points indicate significant differences from wild-type ($P<0.05$).

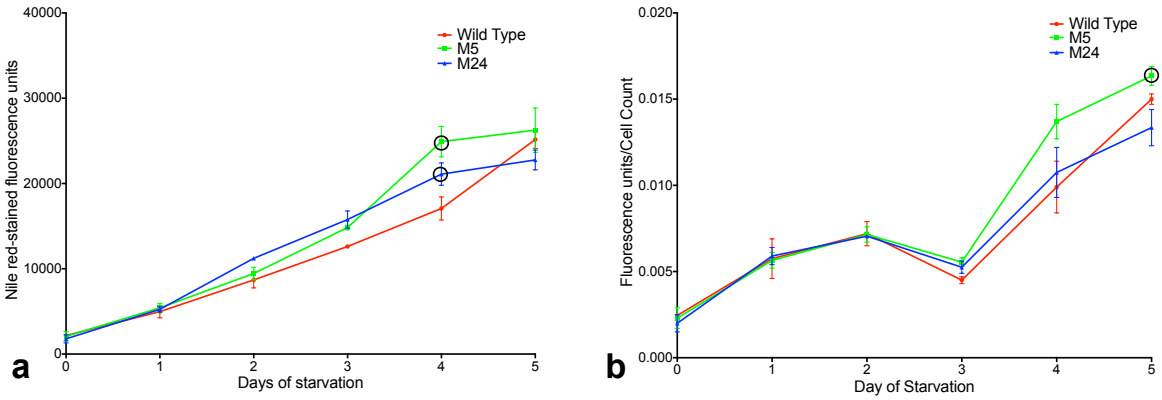


Figure 2. Lipid accumulation in *T. suecica* wild-type and selected strains (M5 & M24) during nutrient starvation phase measured by Nile red fluorescence. (a) Total fluorescence units measured represent total lipid accumulated per mL of culture (b) Total fluorescence/cell represents total lipid accumulated per cell. Circled data points indicate significant differences from wild-type ($P<0.05$), data represent mean ± SEM from two independent replicates.

Interestingly, strain comparison after 9 months in storage (6 months after comparison in Chapter 3) revealed some changes in strain performance. Firstly, while cell proliferation was similar amongst all three strains in the first comparison, results in this comparison revealed that M24 displayed higher cell densities ($P<0.05$) than wild-type five days into the experiment. Secondly and more importantly, total fluorescence values of the putative mutants were only significant higher ($P<0.05$) than wild-type on day 4 of starvation, while no significant differences in fluorescence/cell were detected. These changes seem to indicate that the lipid content of the improved strains may have reverted back to wild-type conditions, and that initial improvements in lipid content may have been a result of epigenetics (e.g. via DNA methylation), instead of a stable DNA mutation. This 9-month period after selection pressure was last applied, appears to be a sufficiently long period for the strains to re-adapt to the absence of selection pressure. DNA methylation has been reported an adaptive response to environmental stimuli in plants such as osmotic stress (Jarvis et al., 1992,Turner et al., 2001), and has been argued to be the main source of amplified fragment length polymorphism (AFLP) changes in cryopreserved microalgae (Muller et al., 2007). In fact, the methylated base m^6Da, which plays an important role in processes such as mismatch repair, transposition, replication and chromosome segregation has been detected in *T. suecica* under normal growth conditions (Jarvis et al., 1992). This implies that DNA methylation occurs naturally in *T. suecica* and presents a dominant source of epigenetic drift in a species with a high reproductive cycle. This would explain the results of other *T. suecica* and *Nannochloropsis* sp. selection studies that isolated high-lipid content strains with just high-throughput selection alone (no mutagenesis step) (Doan and Obbard, 2011,Montero et al., 2011), as the short time period between isolation and lipid content analysis would not have been sufficient for the methylation to have reverted.

The effect of epigenetics on highly reproductive organisms such as microalgae brings into question the efficacy of strain improvement in microalgae, be it via GM or non-GM methods. While the ability of such programs to generate an improved strain is no longer in doubt, the overarching effects of epigenetics implies that consistent selection pressures must be applied even on stable DNA mutants/transformants to prevent strains from reverting back to unimproved features. Nevertheless, the methods developed in this thesis for the selection of high-lipid content strains without compromising growth rate represents a useful tool that can be used to revive a strain's lipid performance.

The main objective of Aim 3 (Chapter 4) was to gain a deeper understanding of the underlying mechanism of lipid accumulation in the previously unsequenced *Tetraselmis* genus. This was achieved by using a combination of global comparative transcriptomics and qRT-PCR analysis.

Transcriptomic analysis performed 24 hours after nitrogen deprivation successfully mapped out three main lipid-related pathways (FA synthesis, TAG synthesis and β-oxidation), while physiological observations revealed a distinct pre-starvation phase from 0 to 24 h after nitrogen deprivation. The RNA-Seq analysis of *Tetraselmis* sp. M8 was not only crucial in revealing the molecular changes in key gene groups, it more importantly provided a platform from which more in-depth expression profile studies on *Tetraselmis* sp. could be performed. The follow-up qRT-PCR analsysis of *Tetraselmis* sp.'s lipid-related revealed that lipid accumulation in the first 48 hours of nitrogen deprivation was a result of significant reduction in lipid catabolism gene expression, as lipid synthesis genes were maintained at basal levels, arguably relying on cell machinery still available from cell growth (Radakovits et al., 2012). This was followed by a significant up-regulation of lipid synthesis genes at 48 hours after N-deprivation as lipid accumulation was now a result of active lipid synthesis. Furthermore, this study also showed the commonly considered bottleneck gene, *DGAT*, to be consistently down-regulated during the starvation phase, despite increased lipid accumulation being observed. While post-transcriptional control of DGAT has been previously observed (Guarnieri et al., 2011,Nykiforuk et al., 2002), the lack of a clear up-regulation in TAG pathway genes as a whole may indicate that the pathway may be more post-transcriptionally controlled in *Tetraselmis* sp. M8. Although these key findings do not conform fully to traditional microalgal lipid pathway expression profiles, they do not come as a surprise due the diversity of microalgae and their genomes. Attempts to assemble the *Tetraselmis* sp. M8 transcriptome to one of its closest sequenced relative *Chlamydomonas reinhardtii* had less than 1% match. In fact, phylogenetic analysis reveals *Tetraselmis* (class: Chlorodendrophyceae) to belong to a totally separate class to that of other microalgae that have been similarly anslysed. (e.g. *Chlamydomoas, Dunaliella, Chlorella, Phaedactylum*).

Analysis of the lipid-related pathways also successfully revealed key genes and potential bottlenecks in the lipid biosynthesis pathway of *Tetraselmis* sp. Genes encoding for the committing steps of β-oxidation (TAG lipase and ACSase) and ECH were found to play a key role in reducing lipid catabolism. This was crucial in reducing lipid consumption and thus increasing lipid accumulation during early starvation phase. Furthermore, these genes exhibited significant changes in the nitrogen-starved treatments throughout the entire experiment, and thus can be considered essential to lipid accumulation in *Tetraselmis* sp. Other potential bottleneck genes are those encoding for ACCase, KAR and ENR in the FA synthesis pathway, which exhibited significant activity during the starvation phase. ACCase and ENR in particular, are the committing and final step of FA synthesis and could therefore be rate-limiting, and have been found to be potentially rate-limiting in *Neochloris oeloabundans* as well (Rimani-Yazdi et al., 2012) Overall, the

information obtained from Chapter 4 was not limited to the lipid synthesis pathway in this study, but has also recently been used as the basis for other studies in the lab such as Adarme-Vega et al. (2014)'s (Adarme-Vega et al., 2014) gene expression study of enzymes involved in long chain-PUFA synthesis, and Ahmed, F.'s work on carotenoid gene expression (Ahmed, Unpublished).

With regards to the future work arising from this thesis, the sequencing of the *Tetreselmis* sp. M8 genome should be a priority. The current transcriptomics data could then be accurately assembled to it, and the remaining 90% of the DiffKAP data annotated to allow for a more robust analysis of *Tetraselmis* gene expression. This should not be limited to lipid metabolism, but expanded to include other key carbon metabolism pathways (e.g. starch and protein synthesis) as well, thus gaining a deeper understanding of the carbon flux during lipid accumulation. The assembly of the transcriptome would also allow for the transcriptional profiling of individual gene members, and not whole gene families as done in this study. Aside from genomic sequencing and analysis of the carbon flux within *Tetraselmis* sp. M8 during lipid accumulation, proteonomic analysis should also be done to investigate the post-transcriptoinal control of many of the genes in this study, in particular the *DGAT* gene. The genetic information gained regarding *Tetraselmis* sp. M8 in Chapter 4 could also be used in conjunction with the work done in Chapter 5. Lipid-related pathways of improved strains generated by the selection-mutation program could be analysed to reveal the genetic (if any) and transcriptional changes behind the observed improvements. This could ultimately lead to a better understanding of transcriptional and epigenetic control of physiological pathways in microalgae, and/or the identification of potential DNA engineering targets.

In conclusion, this thesis has successfully met its aims and made key contributions to knowledge. Aim 1 developed a method by which locally sourced microalgae with high lipid content and suitability for lipid production can be obtained, while showing that locally sourced strains are as competitive as purchased strains from CSIRO. Aim 2 developed a non-GM, strain improvement program that successfully improved the lipid productivity of a strain without compromising its growth rate. This program can be easily applied to maintain the lipid content of lab strains that have lost their lipid productivity after long periods in storage, or further improve the lipid content of strains that already have a good growth rate. Furthermore, aim 2 also work also raised the possibility that increased lipid productivity could be an effect of epigenetics, phenotypic plasticity and/or adaptation and not necessarily permanent genetic change. Aim 3 investigated the molecular mechanisms behind lipid production in *Tetraselmis* sp. M8 and discovered that during the 2 distinct phases of lipid accumulation, early-stationary and stationary, both had different

underlying molecular mechanisms. This thesis demonstrates that it is possible to build up a microalgal production system without prior infrastructure of established strains or protocols. The thesis therefore spans the initial steps of isolating and characterising new strains, to establishing new protocols for microalgae breeding using a non-GM high-throughput mutation/selection approach, to molecular characterisation of metabolites and gene expression profiles during N starvation-induced lipid accumulation. Although a lot more work can be done, this study has significantly advanced knowledge and may serve as an example how a research team can establish a new program in algae biotechnology by combining both classical as well as cutting-edge new methods.

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2. Huerlimann R, de Nys R, Heimann K (2010) Growth, Lipid Content, Productivity, and Fatty Acid Composition of Tropical Microalgae for Scale-Up Production. *Biotechnology and Bioengineering* 107: 245-257.

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Appendices

Research Paper 4: Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production

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MICROBIAL CELL FACTORIES

REVIEW Open Access

Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production

T. Catalina Adarme-Vega[1], David K Y Lim[1], Matthew Timmins[2], Felicitas Vernen[1], Yan Li[1,2] and Peer M Schenk[1*]

Abstract

Omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) provide significant health benefits and this has led to an increased consumption as dietary supplements. Omega-3 fatty acids EPA and DHA are found in animals, transgenic plants, fungi and many microorganisms but are typically extracted from fatty fish, putting additional pressures on global fish stocks. As primary producers, many marine microalgae are rich in EPA (C20:5) and DHA (C22:6) and present a promising source of omega-3 fatty acids. Several heterotrophic microalgae have been used as biofactories for omega-3 fatty acids commercially, but a strong interest in autotrophic microalgae has emerged in recent years as microalgae are being developed as biofuel crops. This paper provides an overview of microalgal biotechnology and production platforms for the development of omega-3 fatty acids EPA and DHA. It refers to implications in current biotechnological uses of microalgae as aquaculture feed and future biofuel crops and explores potential applications of metabolic engineering and selective breeding to accumulate large amounts of omega-3 fatty acids in autotrophic microalgae.

Keywords: Docosahexaenoic acid, DHA, Eicosapentaenoic acid, EPA, Microalgae, Omega-3 fatty acids, Polyunsaturated fatty acids

Introduction

Omega-3 (ω-3) fatty acids are polyunsaturated fatty acids (PUFAs) and essential components for the growth of higher eukaryotes [1]. Nutritionally, eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are the most important fatty acids belonging to this group of bioactive compounds. These long chain omega-3 fatty acids provide significant health benefits to the human population, particularly in reducing cardiac diseases such as arrhythmia, stroke and high blood pressure [2,3]. Additionally, they have been seen to offer beneficial effects to depression, rheumatoid arthritis and asthma [4-6].

Currently, the principal source of EPA and DHA for human consumption is marine fatty fish such as salmon, mullet and mackerel [7,8]. However, global catches have been in decline since the late 1980s and the number of overfished stocks has been increasing exponentially since the 1950s [9,10]. Furthermore, the presence of chemical contaminants (e.g. mercury) in fish oil can be harmful to consumers [11,12]. In addition, fish oil is not suitable for vegetarians and the odour makes it unattractive. There is a variety of alternative EPA and DHA sources such as bacteria, fungi, plants and microalgae that are currently being explored for commercial production. Fungi require an organic carbon source and typically long growth periods [13], plants need arable land, have longer growth times and have no enzymatic activity for producing long chain PUFAs EPA and DHA, unless genetically modified [14]. Microalgae are the initial EPA and DHA producers in the marine food chain and can naturally grow fast under a variety of autotrophic, mixotrophic and heterotrophic culture conditions with high long chain ω-3 fatty acid production potential [15]. Autotrophic and mixotrophic microalgae fix atmospheric carbon dioxide during photosynthesis, can potentially grow on non-arable land and have short harvesting times [16,17]. A comparison shows that microalgae can reach much higher EPA

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and DHA contents and productivities compared with other possible sources (Table 1). In particular heterotrophic microalgae are well established as an alternative source of DHA and are added to infant milk formula or other food [18]. Other microalgal products are used as food additives, animal feed (including aquaculture), vitamins, pigments, pharmaceutical compounds, cosmetics and potentially as a biofuel source [17,19,20]. The development of an efficient large-scale cultivation system for the commercial production of EPA and DHA would address a major global need. Here, we review the potential of autotrophic eukaryotic microalgae as biofactories for large-scale production of omega-3 fatty acids.

Microalgae in aquatic food chains: the initial omega-3 producers

Microalgae are by far the most abundant primary producers that can be found in most aquatic systems, photosynthetically converting light energy and carbon dioxide (CO_2) into biomass such as carbohydrates [44], proteins [45] and lipids [46]. Under high nutrient supply (eutrophic conditions), algae blooms commonly occur as microalgal cell density drastically increases [47]. During microalgal blooms the limitation of nutrients or light halters the increase of biomass. If nutrients, but not light, are limiting, this leads to the accumulation of photosynthetic bioproducts such as lipids and carbohydrates. These serve as storage products in order to survive the stressful growth limiting conditions, after which a large number of cells die [47,48]. Algal biomass is subsequently degraded by microorganisms, consuming large amounts of oxygen. As a result an anaerobic zone in the water is formed (Figure 1). In extreme cases, this can lead to anaerobiosis of the entire water body, causing the death of plants and animals in the waterway; interestingly this process is also believed to have been the key factor for large-scale oceanic anoxic events that led to fossil mineral oil deposition [17].

Importantly, microalgae are also the primary producers of EPA and DHA that are eventually accumulated through the various trophic levels. Changes in microalgal lipid content are carried on up the food chain (Figure 2), impacting the growth and dietary make-up of zooplankton, crustacean larvae, mollusc and some fish [49]. This subsequently affects the accumulation of EPA and DHA fatty acids in higher organisms and humans. Consequently, lipid profiles in microalgae play a vital role in maintaining the integrity of the world's aquatic food webs.

The nutritional importance of microalgae and EPA content in aquaculture

Microalgae are essential to the aquaculture industry which has grown substantially over the last 10 years [50,51]. The successful cultivation of oysters, scallops and mussels is dependent on the ω-3 fatty acids from microalgal feedstock. The polyunsaturated omega-3 fatty acids EPA and DHA derived from microalgae (e.g. *Isochrysis, Tetraselmis, Chaetoceros, Thalassiosira, Nannochloropsis*) are also known to be essential for

Table 1 Comparison of EPA and DHA fatty acid contents as percentage from total lipids in examples of bacteria, fungi, fish, transgenic plants and microalgae

Organism	% EPA and/or DHA production	Reference
Bacteria		
Shewanella putrefaciens	40.0 EPA	[21]
Alteromonas putrefaciens	24.0 EPA	[22]
Pneumatophorus japonicus	36.3 EPA	[23]
Photobacterium	4.6 EPA	[24]
Fungi		
Thraustochytrium aureum	62.9 EPA + DHA	[1]
Mortierella	20.0 EPA	[25]
Mortierella	13.0 EPA	[26]
Pythium	12.0 EPA	[27]
Pythium irregulare	8.2 EPA	[28]
Fish		
Merluccius productus	34.99 EPA + DHA	[29]
Theragra chalcogramma	41.35 EPA + DHA	[29]
Hypomesus pretiosus	33.61 EPA + DHA	[29]
Sebastes pinniger	29.8 EPA + DHA	[29]
Oncorhynchus gorbusha	27.5 EPA + DHA	[29]
Mallotus villosus	17.8 EPA + DHA	[29]
Sardinops sagax	44.08 EPA + DHA	[29]
Clupea harengus pallasi	17.32 EPA + DHA	[29]
Plant (transgenic)		
Soybean	20.0 EPA	[30]
Brassica carinata	25.0 EPA	[31]
Nicotiana benthamiana	26.0 EPA	[32]
Microalgae		
Nannochloropsis sp.	26.7 EPA + DHA	[33]
Nannochloropsis oceanica	23.4 EPA	[34]
Nannochloropsis salina	~28 EPA	[35]
Pinguiococcus pyrenoidosus	22.03 EPA + DHA	[36]
Thraustochytrium sp.	45.1 EPA + DHA	[37]
Chlorella minutissima	39.9 EPA	[38]
Dunaliella salina	21.4 EPA	[39]
Pavlova viridis	36.0 EPA + DHA	[40]
Pavlova lutheri	27.7 EPA + DHA	[41]
Pavlova lutheri	41.5 EPA + DHA	[42]
Isocrysis galbana	~28.0 EPA + DHA	[43]

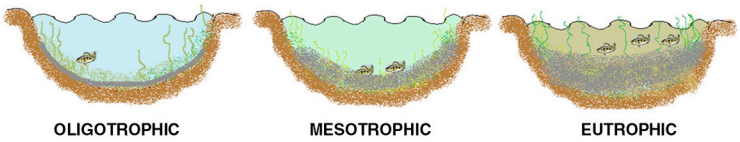

Figure 1 Algal blooms in eutrophic aquatic systems use up nutrients and compete for light. If nutrients become limiting first, microalgae may accumulate large amounts of lipids and/or carbohydrates as a survival strategy. The decay of organic matter by bacteria uses up oxygen causing localized anaerobiosis zones. These zones (here shown as grey areas) are present in all aquatic systems but occur at much deeper levels under mesotrophic or oligotrophic conditions. Photosynthetic microalgae require polar polyunsaturated lipids in particular for membrane where fluidity is critical, while most storage lipid occurs in the form of lipid bodies containing triacylglycerides. These typically vary in their composition and typically contain a mixture of saturated and unsaturated fatty acids for storage.

healthy development of various bivalve larvae [52,53]. Prior research on the scallop *Pecten maximus* has shown a direct relationship between the fatty acid profile of female gonads and the fatty acid composition of the eggs [54]. The increase of EPA and DHA from an algal diet significantly increased the concentration of fatty acids in the digestive gland (78%) of scallops as well as the female (57%) and male gonads (51%). It appears that dietary lipids are stored in the digestive gland and are later transferred to the developing female gonad. These dietary lipids are then incorporated into the eggs and can significantly improve their quality. This in turn improves the hatching rate of eggs and hatching rates have been linked to high contents of EPA and DHA [53]. Aside from bivalve culture, microalgae are also used as food additives to improve the flesh color of salmon [55], as well as inducing a range of other biological activities such as survival and resistance [19].

The selection of suitable microalgae species for aquaculture is very important. Firstly, a candidate species must be adaptable to mass culture with high growth rates and lipid content[34,56]. Furthermore, it must tolerate moderate fluctuations of temperature, light and

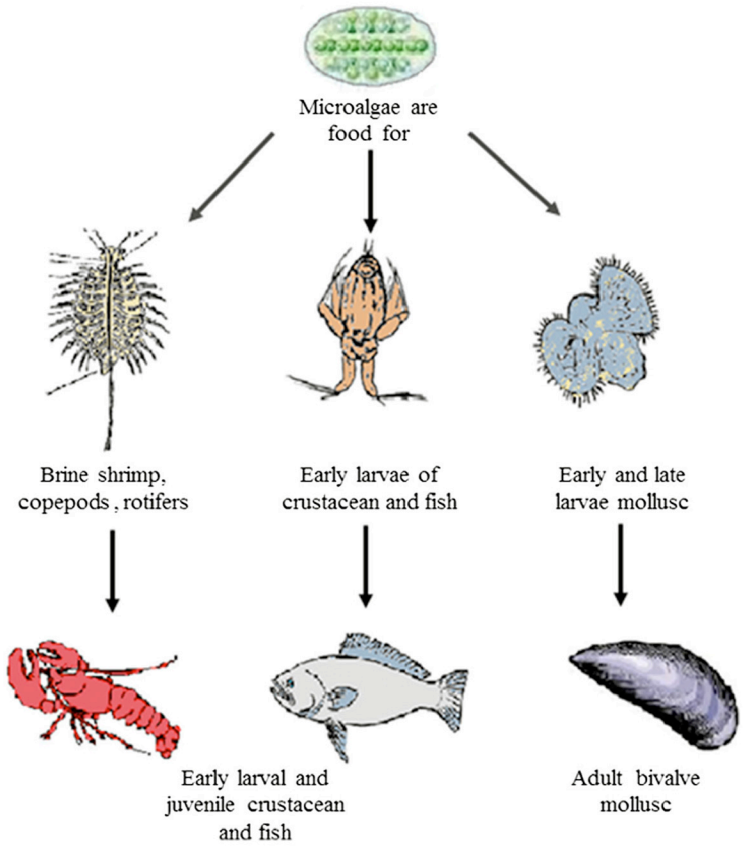

Figure 2 Microalgae are the primary food source of essentially all marine and freshwater food chains.

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nutrients[57,58]. A microalgae species used for aquaculture must also have the appropriate size for ingestion (e.g. from 1 to 15 μm for filter feeders; 10 to 100 μm for grazers) and be readily digestible [56]. Finally, they must possess a suitably high lipid composition with long chain polyunsaturated fatty acids and be free of toxins for target culture species [34,56]. Selection of the suitable microalgal diet is of paramount importance to aquaculture hatchery and nursery success [58]. At present, the most widely cultured species for aquaculture hatcheries and nurseries include *Chaetoceros calcitrans, Isochrysis galbana, Pavlova lutheri, Pseudoisochrysis paradoxa, Tetraselmis suecica* and *Skeletonema costatum.* Other genera include *Spirulina*, *Dunaliella*, *Chlorella*, *Thalassiosira*, *Isochrysis* and *Nannochloropsis* [49].

Health benefits of microalgal omega-3 fatty acids

Omega-3 fatty acids represent an important structural component of human cell membranes, particularly neuronal cells [59]. The consumption of EPA and DHA supplements has been shown to prevent cardiovascular, nervous system and inflammatory conditions [60]. With regards to cardiovascular health, regular consumption of ω-3 fatty acids can help reduce the risk of hypertension, thrombosis, myocardial infarction and cardiac arrhythmias [61]. This occurs because ω-3 fatty acids increase the high-density lipoprotein/low-density lipoprotein (HDL/LDL) ratio and decrease the total cholesterol/HDL ratio [61]. In addition to cardiovascular benefits, omega-3 fatty acids have also demonstrated positive effects on brain function and the nervous system[62]. In pregnant women, the adequate intake of EPA and DHA is crucial for healthy development of the fetal brain [63]. In infants, arachidonic acid (ARA), an omega-6 fatty acid, and DHA are also required for normal growth and functional development [64]. Interestingly, increased consumption of DHA may also diminish the severity of depression [65]. Immuno-modulatory effects have been observed when ω-3 fatty acids were used in the treatment of inflammatory conditions such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, asthma, lupus and cystic fibrosis [66,67]. Children ingesting fish oil more than once a week had a lower probability of suffering from asthma [68]. Increasing the levels of DHA and EPA in patients with rheumatoid arthritis and ulcerative colitis has also been found to reduce pain and improve conditions, although the modes of operation are unclear at this point [69,70].

There is currently a large demand for microalgae in the nutraceutical and pharmaceutical industry due to their health-promoting effects. Microalgal-derived PUFA, such as ARA and DHA are added as fortifications to infant formulae—an industry that is worth $10 billion per annum alone. To date, microalgal extracts can be found in many face and skin care products, e.g. anti-aging cream, refreshing or regenerative care products, sun cream, emollient and anti-irritant in peelers [19]. Dermochlorella is actually extracted from *Chlorella vulgaris,* which can stimulate collagen synthesis in skin supporting tissue regeneration and wrinkle reduction [71]. Protulines is a protein-rich extract from *Arthrospira* (*Spirulina*), which helps combat early skin aging, exerting a tightening effect and preventing wrinkle formation [72].

Omega-3 fatty acid production in microalgae

Microalgae produce a variety of compounds to help in the adaptation and survival of different environmental conditions. Many marine microalgal strains have oil contents of between 10–50%, (w/w) and produce a high percentage of total lipids (up to 30–70% of dry weight) [1]. The accumulation of fatty acids is closely linked to microalgal growth stages, functioning as an energy stockpile during unfavourable conditions or cell division. Omega-3 is accumulated due to its high energy content, as well as the good flow properties crucial for cellular functions [73,74]. To date, the ω-3 fatty acid content of numerous microalgae strains have been studied. Strains from the genera *Phaeodactylum, Nannochloropsis, Thraustochytrium* and *Schizochytrium* have demonstrated high accumulation of EPA and/or DHA. *Phaeodactylum tricornutum* [38] and *Nannochloropsis* sp. [75] demonstrated an EPA content of up to 39% of total fatty acids, while strains such as *Thraustochytrium* [76] and *Schizochytrium limacinum* [77] contained a DHA percentage of between 30–40% of total fatty acids when grown heterotrophically. High biomass and commercially acceptable EPA and DHA productivities are achieved with microalgae grown in media with optimized carbon and nitrogen concentrations and controlled pH and temperature conditions [78]. High oil production, including DHA from *Schizochytrium* (50% w/w), can be obtained as a result of high growth rate by controlling of nutrients such as glucose, nitrogen, sodium and some other environmental factors, such as oxygen concentrations as well as temperature and pH, achieving high cell densities and DHA productivities [1].

Induction of omega-3 production in autotrophic microalgae

An increase in microalgal lipid content can be induced by a sudden change of growth conditions. The accumulation of starch and/or lipids reserves is considered a survival mechanism in response to growth-limiting stresses [17], such as UV radiation [79], temperature [80] and shock or nutrient deprivation [81,82], as long as light conditions are present that still allow efficient photosynthesis. For example, during nutritional deprivation (e.g. nitrogen) and

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under the provision of light, cellular division of many marine or brackish microalgae is put on hold and cells begin to accumulate lipids [83], leading to a 2–3 fold increase in lipid content. Both total lipid and omega-3 fatty acid production can be adjusted by varying growth conditions. The diatom *Phaeodactylum tricornutum* can be induced to increase its lipid level from 81.2 mg/g of culture dry weight to 168.5 mg/g dry weight [38]. Similarly, *Nannochloropsis* sp. [84] and *Dunaliella* sp. [85] can achieve a total lipid content of up to 47% and 60% of dry ash weight by modifying the light intensity, temperature and salinity levels. Lipid abundance has also been shown to increase due to anaerobic sulphur deprivation [86] or the addition of extra nutrients [87].

Omega-3 fatty acid biosynthesis can be stimulated by a number of environmental stresses, such as low temperature, change of salinity or UV radiation. For example, *Pavlova lutheri* increased its relative EPA content from 20.3 to 30.3 M % when the culture temperature was reduced to 15°C [88]. Similarly, *Phaeodactylum tricornutum* had a higher EPA content when the temperature was shifted from 25°C to 10°C for 12 h [89]. An increase in PUFAs is expected as these fatty acids have good flow properties and would be predominately used in the cell membrane to maintain fluidity during low temperatures. Salinity may also regulate PUFA biosynthesis, although not in a consistent manner. For example, *Crythecodinium cohnii ATCC 30556* increased its total DHA content up to 56.9% of total fatty acids when cultured in 9 g/L NaCl. Other treatments that cause the generation of reactive oxygen species and lipid peroxidation also result in higher PUFA contents. For example, *Phaeodactylum tricornutum* increased its EPA content up to 19.84% when stressed with UV light [90]. Some of the increased PUFAs are used to repair membrane damage but as PUFAs contain many double bonds, these also act as an antioxidant by scavenging free radicals.

Metabolic engineering of microalgae for higher omega-3 contents

Apart for external stresses, metabolic engineering is another promising approach to increase the production of fatty acids in microalgae (for a recent review see Schuhmann et al. [91]). Genes encoding key enzymes involved in the fatty acid biosynthesis have been identified in *Ostreococcus tauri* [92], *Thalassiosira pseudonana* [93-95], *Phaeodactylum tricornutum* [96,97] and in particular the model organism *Chlamydomonas reinhardtii* [98]. At present, the mechanisms involved in the fatty acid biosynthetic pathways in microalgae have not been extensively studied and most information has been gathered from studies on plant metabolism. Briefly, *de novo* fatty acid synthesis occurs in the chloroplast and involves the carboxylation and condensation of acetyl-CoA to malonyl-CoA, with further elongation reactions occurring with malonyl ACP as substrate to create long chain fatty acids. Long chain fatty acids are transferred to glycerol-3-phosphate to form triacylglycerol (TAG) via the metabolic intermediate phosphatidic acid in the endoplasmic reticulum [99]. Synthesis of ω-3 fatty acids occurs via the elongation and desaturation of long chain fatty acids (Figure 3).

Work has been performed to create recombinant sources of ω-3 fatty acids in a variety of systems with some success [101,102]. Canola (*Brassica napus*) seeds have been produced which overexpress the *B. napus* Δ15 desaturase, as well as the Δ6 and Δ12 desaturases from the commercially grown fungus *Mortierella alpina* to synthesize the ω-3 fatty acid stearidonic acid (SDA) [14]. It may be possible in the future to produce ω-3 fatty acids in microalgae in much larger quantities by regulating the expression of similar enzymes. A promising cisgenic approach for microalgae maybe to increase EPA or DHA production by overexpressing at least some of their native elongases and desaturases. It may be necessary to use promoters inducible by external stimuli rather than constitutive promoters that may interfere with normal cell function and growth. Another, yet unexplored option may lie in the inhibition of PUFA degradation. β-oxidation of fatty acids occurs in the peroxisomes but before PUFAs can be metabolized, saturases are required to fill in the double bonds. Mutations in one or several saturases may result in less efficient β-oxidation of PUFA and a higher percentage of these fatty acids. However, at present the mechanism behind the selection and storage of fatty acids for triacylglycerol production remains unclear.

Extraction and purification of omega-3 fatty acids from microalgal biomass

Figure 4 summarizes an integrated system for the large-scale production of microalgal bio-products. A microalgae strain is cultivated to increase cell density using photobioreactors, open ponds, race ways or hybrid systems. Algal cells are separated from culture media by filtration, flocculation or centrifugation, followed by drying to improve extraction [1]. Lipid extraction is then commonly performed using a non-water miscible organic solvent. A typical extraction protocol in small scale is often based on the method of Bligh and Dyer [103], which uses a solvent mixtures made of methanol/chloroform for the cell disruption and lipid extraction. Larger scale extraction is typically carried out with hexane as a solvent. Subsequently, unsaturated fatty acids are separated from the total lipids by fractional (molecular) distillation or winterization, whereby oil temperature is reduced to precipitate the more saturated lipids. Further processing to improve the quality, shelf-life and quantity of

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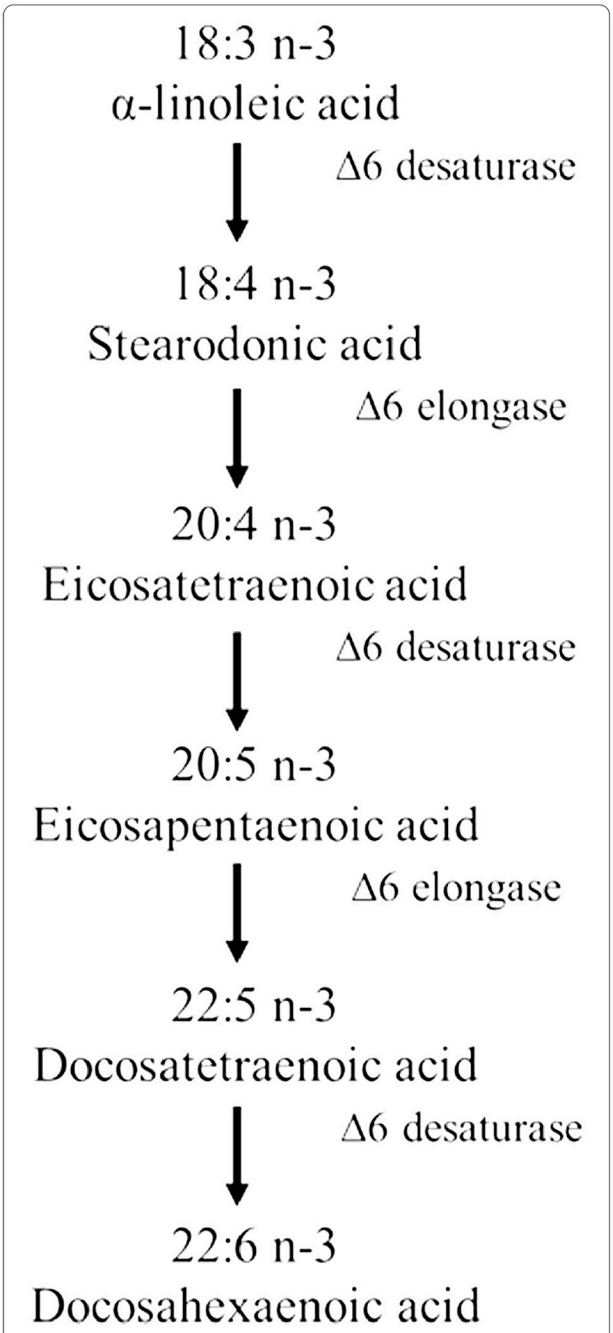

Figure 3 Conventional Δ6-pathway for biosynthesis of EPA and DHA polyunsaturated fatty acids [100].

PUFA oil can include filtration, bleaching, deodorization, polishing and antioxidant addition [1,104] (Table 2).

Efforts have been made to use lipases, hydrolysis and esterification processes to selectively enrich PUFAs. The main application of lipases on PUFAs is the generation of non-natural esters of these products for use as pharmaceutical products or other synthetic bioactive compounds or their precursors [1]. The effectiveness of harvesting and extraction techniques depends on the microalgal strain's physical characteristics (e.g. cell size and cell wall properties) and the use of the end product. In aquaculture, microalgae are used as a fresh product or as dry pellets which preserve the nutritional content of microalgae [57,58,111]. In this case, microalgal biomass is first de-watered either by filtration, dissolved air flotation, flocculation or sedimentation and then dried to form pellets or directly administrated to livestock [111]. When produced for the pharmaceutical industry, further extraction and purification processes are required. Currently, methods such as supercritical fluid extraction, winterization and fractional (molecular) distillation are used for the extraction and purification of PUFA from microalgae [112,113] (Table 2)

Omega-3 fatty acid production: a biorefinery approach

The natural capacity of microalgae to produce multiple products, (e.g. oils, proteins and carbohydrates) has

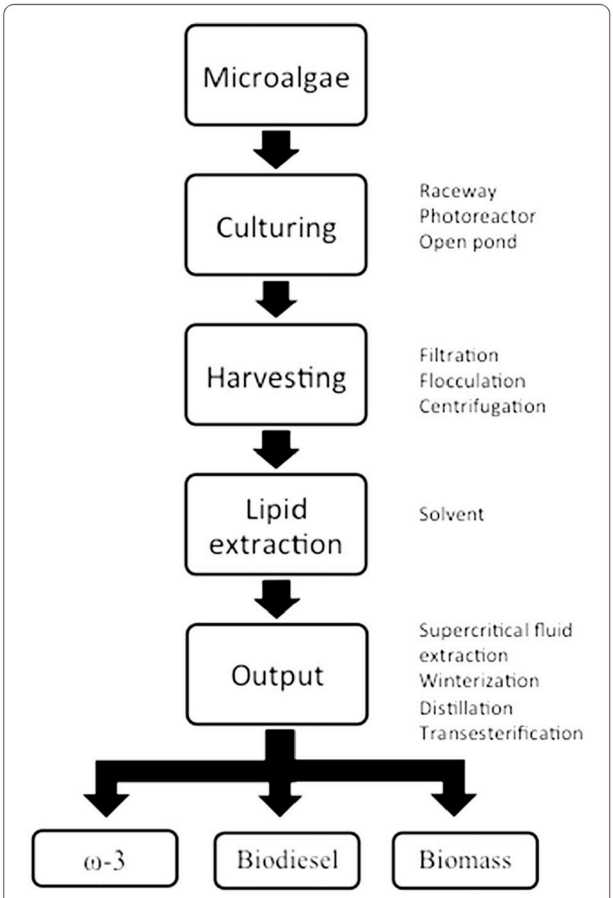

Figure 4 Examples of a bioprocess production chain in a microalgal biorefinery. Apart from omega-3 fatty acids (ω-3), the product portfolio includes biodiesel and protein-rich animal feed from the remaining biomass.

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Table 2 Summary of PUFA enrichment processes

Method	Procedure
Molecular distillation (Fractional distillation)	Purification of fatty acid esters in a vacuum system based on the different boiling points of different fatty acids [105].
Molecular sieves	Separation via membrane permeability and selectivity [106].
PUFA transformations	Esterification of PUFA and free fatty acids to produce esters (ethyl-, glyceryl-, sugar-, other). Inter-esterification to enrich lowly unsaturated fatty acids with PUFA [107].
Super Critical Fluid Extraction	Optimization of lipid solubility and fractionation in supercritical CO_2 [108].
Urea complexation	Solubilization of fatty acids, adding urea and ethanol to saturation point exposing it to heat. Recovery of product by filtration [109].
Winterization	Temperature reduction to render more saturated fats insoluble [110].

encouraged the development of a biorefinery concept for processing. Akin to the petrochemical industry, where crude oil is processed to yield petroleum and a range of other chemicals, microalgae can be processed to produce a range of bioproducts. Different industries are able to use different algal products. For instance, the pharmaceutical and nutraceutical industries use high value bioactive products such as ω-3 fatty acids and carotenoids; the transport industry can use fatty acids from TAG for biodiesel, the chemical industry can use products such as glycerine, while the majority of the biomass can be used by agriculture and aquaculture as animal feed [114,115]. Additional processes that address nutrient recycling and carbon sequestration can be used by anaerobic digestion of wet biomass and pyrolysis for the production of biochar.

Undoubtedly, the biggest interest in microalgal use is for biodiesel production. It potentially represents a more sustainable alternative to fossil fuels as microalgal production facilities do not need to compete for arable land or freshwater. Furthermore, in comparison to land plants, 10–400 times more energy per acre can potentially be produced from microalgae. Although there has been considerable interest and research over the past years into microalgal biofuel production [83], no commercial enterprise has successfully established itself as a supplier of autotrophically derived algal biofuels for any duration. Nevertheless, decreasing fossil fuel reserves and increasing fuel costs continue to drive research targeted towards economically viable production of microalgal biodiesel, with the level of improvement necessary now appearing attainable [15,17]. There is confidence among companies producing microalgae that the production of a high value product, such as omega-3 from microalgae, will further assist in the establishment of the microalgae industry. Several companies have (at least temporarily) shifted their focus from algal biodiesel production, to high value products such as omega-3 and protein-rich biomass as animal feed (e.g. Aurora Algae, MBD, Cellana).

Conclusions

Global fish stocks are declining and cannot provide a sustainable source of omega-3 fatty acids. Heterotrophic microalgae have been used for the production of omega-3 fatty acids, in particular DHA. However, as the primary producers of PUFAs, the use of autotrophic microalgae for large-scale production of omega-3 fatty acids has recently attracted a lot of interest. Autotrophic microalgae do not require an organic carbon source and hence may avoid the problems faced for heterotrophic cultures that can easily get contaminated with other microorganisms. In a biorefinery concept, omega-3 fatty acids can be separated from microalgal lipids which would be widely used for biodiesel production, while biomass can find uses as valuable protein-rich animal feed which could free up arable land for food production. If carried out at a large scale this would address three major areas of importance: human health, transportable energy and food security.

Over the past decade, algae biotechnology has grown steadily into a global industry with increasing numbers of entrepreneurs attempting to utilize its biochemical diversity for a wide array of applications. At present, achieving economically viable production of microalgal lipids is still a major challenge, but strong potential stems from the fact that these microbial cell factories have not been domesticated and are not as well studied compared to agricultural crops [102]. Indeed, of approximately 40,000 algal species, only a few thousand strains are kept in collections, a few hundred are investigated for chemical content and approximately half a dozen are cultivated in industrial quantities. Therefore, continued isolation and screening of microalgae is required, as well as more in depth studies into algal physiology, biochemistry and genetics. Meanwhile the processes for algae cultivation, harvesting and oil extraction need to be further improved in efficiency and costs. As omega-3 fatty acids are one of the most valuable products from microalgae, they are likely to be the "game-changer" towards large-scale economical microalgae cultivation that will catalyze the production of other important algal bioproducts.

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Competing Interest
The authors declare that they have no competing interests.

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Authors' contributions
All authors contributed in data collection from literature and writing of the manuscript including figures and tables. All authors have read and approved the final manuscript.

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Article

Effects of Long Chain Fatty Acid Synthesis and Associated Gene Expression in Microalga *Tetraselmis* sp.

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Abstract: With the depletion of global fish stocks, caused by high demand and effective fishing techniques, alternative sources for long chain omega-3 fatty acids are required for human nutrition and aquaculture feeds. Recent research has focused on land-based cultivation of microalgae, the primary producers of omega-3 fatty acids in the marine food web. The effect of salinity on fatty acids and related gene expression was studied in the model marine microalga, *Tetraselmis* sp. M8. Correlations were found for specific fatty acid biosynthesis and gene expression according to salinity and the growth phase. Low salinity was found to increase the conversion of C18:4 stearidonic acid (SDA) to C20:4 eicosatetraenoic acid (ETA), correlating with increased transcript abundance of the Δ-6-elongase-encoding gene in salinities of 5 and 10 ppt compared to higher salinity levels. The expression of the gene encoding β-ketoacyl-coenzyme was also found to increase at lower salinities during the nutrient deprivation phase (Day 4), but decreased with further nutrient stress. Nutrient deprivation also triggered fatty acids synthesis at all salinities, and C20:5 eicosapentaenoic acid (EPA) increased relative to total fatty acids, with nutrient starvation achieving a maximum of 7% EPA at Day 6 at a salinity of 40 ppt.

Keywords: nutrients; EPA; fatty acids; omega-3; gene expression

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1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA), eicosatetraenoic acid (ETA) and docosahexaenoic acid (DHA), which are omega-3 fatty acids, and arachidonic acid (ARA), an omega-6 fatty acid, provide significant health benefits, including a reduced risk of hypertension, cardiac arrhythmia, myocardial infarction and thrombosis [1]. LC-PUFAs have also been found to have positive effects on brain function [2] and the healthy development of the foetal brain [3]. LC-PUFAs have primarily been extracted from small fatty marine fish, a limited resource, which hit peak production in the mid-1990s [4]. Concerns about the sustainability of LC-PUFA sources have increased, shifting research towards different sources, such as other marine organisms, transgenic plants and fungi. Interest on the sustainability of the omega-3 sources has moved efforts towards land-based production, including farmed fish, genetically modified plants, regulated krill catches and large-scale production of microalgae [4].

Microalgae are considered a viable and sustainable source of LC-PUFA, including omega-3 fatty acids. They have important advantages for commercial production over transgenic plants or fungi [5], including high areal productivity. They can also be grown on non-potable water and on non-arable land [6,7]. Microalgae have a natural adaptation capacity in diverse and even adverse environmental conditions. Some survival mechanisms include changing their chemical composition, such as modifying cellular fatty acid content to protect themselves from osmotic stress during rapid salinity changes, which may occur in natural environments, such as coastal rock pools [8–14]. The response to environmental stress of an altered salinity can lead to the cessation or slowing of algal growth and biomass accumulation, shifting photosynthetic energy towards the accumulation of chemical energy in the form of fatty acids (FA) or starch [10,15–17]. Marine species, like *Nannochloropsis* sp. [18] and *Dunaliella* sp. [19], can achieve a total lipid content of up to 47% and 60% of dry weight (DW), respectively, by modifying the light intensity, temperature and salinity during cultivation. Similarly, *Phaeodactylum tricornutum* was induced to enhance lipid content from 83.8 mg/g to 108.0 mg/g DW once the salinity of the media had been altered [17]. The response of microalgae to salinity stress is species-specific [20] and probably even strain-specific. Therefore, it is essential to investigate the effect of salinity on algal growth and omega-3 production in microalgal strains with commercial potential.

Research on microalgal metabolic pathways has led to a better understanding of the mechanism for FA synthesis. Genes encoding enzymes involved in particular steps of FA synthesis have been sequenced and studied in diverse microalgal species. The traditional pathway for the synthesis of LC-PUFAs is presented in Figure 1. Most enzymes involved in the final steps of LC-PUFA biosynthesis and derivatization can either use omega-3 or omega-6 FAs as substrates. This pathway has been identified in animals, plants and microorganisms [21].

The synthesis of LC-PUFAs is largely regulated by a series of enzymes that can be classified in two groups: desaturases and elongases. The desaturases are a special group of oxygenases capable of removing hydrogen from a carbon chain, thus catalysing the formation of double bonds. Those enzymes use activated molecular oxygen to remove hydrogens from the carbon chain, creating a carbon/carbon double bond in the FA chain and a molecule of water [22]. The second enzymatic group involved in the synthesis of LC-PUFAs is responsible for increasing the length of the carbon chain and includes elongases [21]. To date, three types of elongases participating in the synthesis of PUFAs

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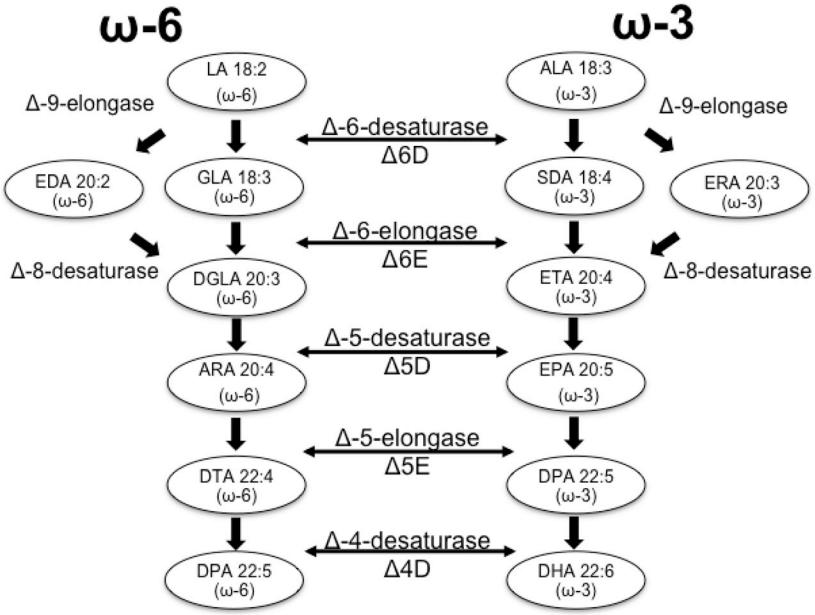
have been characterized: Δ6-elongase, Δ5-elongase and Δ9-elongase; each of these enzymes is substrate-specific. The elongation/desaturation reactions for LC-PUFA synthesis occur in two main pathways (Figure 1): the Δ6-desaturase/elongase and the Δ9-elongase/Δ8-desaturase; both use either linoleic acid (LA) for omega-6 FA or α-linoleic acid (ALA) for omega-3 FAs to make unsaturated fatty acid chains of 20 or more carbons [22].

Figure 1. Biosynthesis of long-chain (LC)-PUFA's via the conventional pathway.



In addition to the previously mentioned enzymes, there is another group of enzymes that can perform the elongation in the FA chain. They are known as microsomal FA elongation complexes. These enzymes mainly participate in the elongation of saturated or monounsaturated FA chains through four consecutive reactions of condensation, reduction, dehydration and a second reduction [23]. The first enzyme of the complex is the β-ketoacyl-coenzyme (BKAS), which catalyses the condensation of the acyl-CoA chain with malonyl-CoA. The additional three enzymes of the complex are 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydratase and enoyl-CoA reductase, which have been studied and characterized in yeast and *Arabidopsis thaliana* [23].

Tetraselmis species are green marine microalgae (Chlorophyta) commonly used in aquaculture, because of their high nutritional value. A number of species have been used as model organisms for physiological and biochemical studies, as well as for survival and adaptation mechanisms to diverse conditions, such as different salinities. Studies on salt tolerance and osmotic regulation have demonstrated that salinity provokes physiological changes, inducing several Na^+-ATPase plasma membrane proteins in *Tetraselmis viridis* at high salinity [24]. Research on membrane pumps regulating the ionic flux in *Tetraselmis viridis* has shown that they are strongly involved in cytosolic homeostasis [25]. Studies on the expression of BKAS have found an increase of gene expression in *Dunaliella salina* as a result of salinity shifts from 0.5 to 3.5 M: this corresponded with an increased proportion of longer chain FAs in cell membranes [26]. Bioinformatics analyses decoding the

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microalgal genome have accelerated the identification of genes participating in the synthesis of molecules involved in microalgal survival mechanisms, such as osmoregulation proteins, as well as FA synthesis [26–29]. The identification of long-chain desaturases has given researches the ability to characterize and study their function in other organisms, such as yeast and plants [30–32]. Understanding FA synthesis in *Tetraselmis* sp. represents an important step towards the production of better nutritional quality microalgal strains for aquaculture in protein, as well as in the FA content and profile, including EPA and ARA. Little is known about the gene expression involved in the FA synthesis of *Tetraselmis* sp. as the salinity of the culture media changes. Therefore, the aim of this study was to profile FAs at various salinity levels in the marine microalga, *Tetraselmis* sp., and evaluate the expression of genes involved in the FA pathway and the osmotic balance for the synthesis of ETA and EPA.

2. Results

This study evaluated the effect of different salinity levels (5 to 50 ppt) on pre-adapted cultures of *Tetraselmis* sp. M8. Cell density, nutrient consumptions, fatty acid profiling and expression differences for genes involved in FA synthesis were profiled over six days after the initial culture inoculation to determine the effect of salinity. This time period includes three growth phases: Days 0–2 (nutrient replete), Days 3–4 (nutrient deplete) and Days 5–6 (nutrient starved). As shown in Figure 2, salinity had a significant effect ($p < 0.05$) on the final day (Day 6) on cell density and growth rates (Table 1). A significant reduction in the final biomass ($p < 0.05$) was observed in high salinity cultures of 50 ppt, as well as in cultures grown at low salinities of 5 and 10 ppt. Cultures grown at 40 and 50 ppt, however, presented the highest growth rates measured during nutrient replete conditions (Table 2).

Figure 2. Optical density (440 nm) for *Tetraselmis* sp. cultivated at different salinities. Data represent mean values ± SDs for three independently grown cultures. Alphabets represent significant differences amongst salinities ($p < 0.05$).

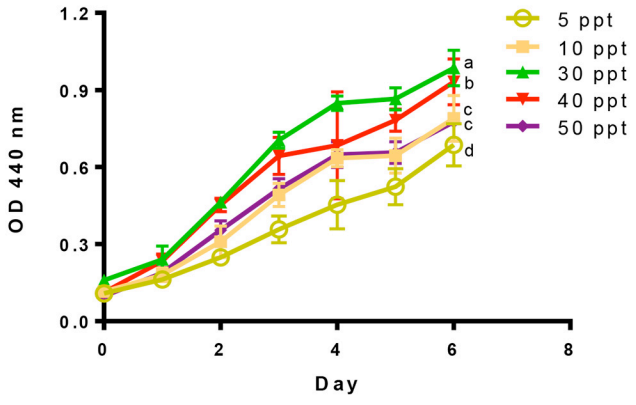

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Table 1. List of genes and primers used for qRT-PCR.

Genes	Primers
Beta-Keto acyl synthase (BKAS)	5′-CAGGCCTTCGAGCATTTCTG-3′
	3′-GCGTCATATCAGGCGACAGC-5′
Delta-5-desaturase (Δ5D)	5′-TGGACGTTGGACATTGTAGGC-3′
	3′-CATTGTCATGCAGATTTGTGTACG-5′
Delta-6-elongase (Δ6E)	5′-CACCTACTACCTGCTTGCTGCC-3′
	3′-CTGGAACATTGTCAGGTAATGCC-5′
Acyl-CoA-synthase (ACSase)	5′-CACGTTGCTGTGCTTAATCTGC-3′
	3′-CGAGTGCAACCCTGAGGATATG-5′
Delta-5-elongase (Δ5E)	5′-TGAGGAAATGGTGCCAGCAG-3′
	3′-ACAAGTTCATCGAGTACCTCGACAC-5′
Glycerol-3-phosphate dehydrogenase (D3PDH)	5′-TCGTACCGCATCCACAAAGG-3′
	3′-GCTAAGGTGAAAGACAACGAGTCC-5′
Glucose-6-phosphate isomerase (G6Pi)	5′-GGGACAGCAGGTTATTGTGGAC-3′
	3′-TGCGCACCTTATCGGAGAAG-5′
Sodium ATPase (PyKPA)	5′-AAGGAAGCTGCGGATATGATTCTC-3′
	3′-TCAAGTTGTCAAAAATCAGACGACC-5′
Phosphate transporter (PHO)	5′-GACTTGGCACCCTTGAAGATAATG-3′
	3′-CTTACGCTCGCTCTTGGTGG-5′
3-ketoacyl-ACP reductase (KAR)	5′-CGGAGGAGATGTTAATGATGCG-3′
	3′-ATCAACCTCACCGGCGTCTT-5′
Delta-8-desaturase (Δ8D)	5′-GTCCGTAAAGGCTCCACTTCG-3′
	3′-GTATTTGACAAGACCACGCAGTTG-5′
Enoyl-ACP reductase (ENR)	5′-CTCCTTGACCTCAGTTGGGACA-3′
	3′-CTCAAACGGGTCCTTAATGGAGT-5′
Phosphatidic acid phosphatase (PP)	5′-TGTGGTCGGAGATCACATACGATA-3′
	3′-CAGTAGAGCGAGAACGACACCAG-5′
Delta-9-desaturase (Δ9D)	5′-GATATGAAAGCGTATGCCGAG-3′
	3′-GTAGCTCTAGCCGCCCCCTT-5′
Diacyl glycerol acyl transferase (DGAT)	5′-ATCAGAGGAACCTGTCCCATCA-3′
	3′-CTGCCATTTTTCACGAGCTAATG-5′
Beta-actin	5′-GCCTCAGAATCCCAAGACCAA-3′
	3′-GGCCTGGATCTGAACGTACATG-5′

Table 2. Specific growth rate (μ) and doubling time (d*t*) of *Tetraselmis* sp. cultures at different salinities.

Salinity (ppt)	Growth Rate (μ)	Doubling Time (d*t*)
5	0.414 ± 0.049	1.689 ± 0.207
10	0.487 ± 0.087	1.457 ± 0.287
30	0.532 ± 0.016	1.303 ± 0.038
40	0.695 ± 0.064 *	1.003 ± 0.088
50	0.644 ± 0.063 *	1.083 ± 0.105

Data represent mean values ± SDs for three independently grown cultures. * Indicates statistically significant differences ($p < 0.05$).

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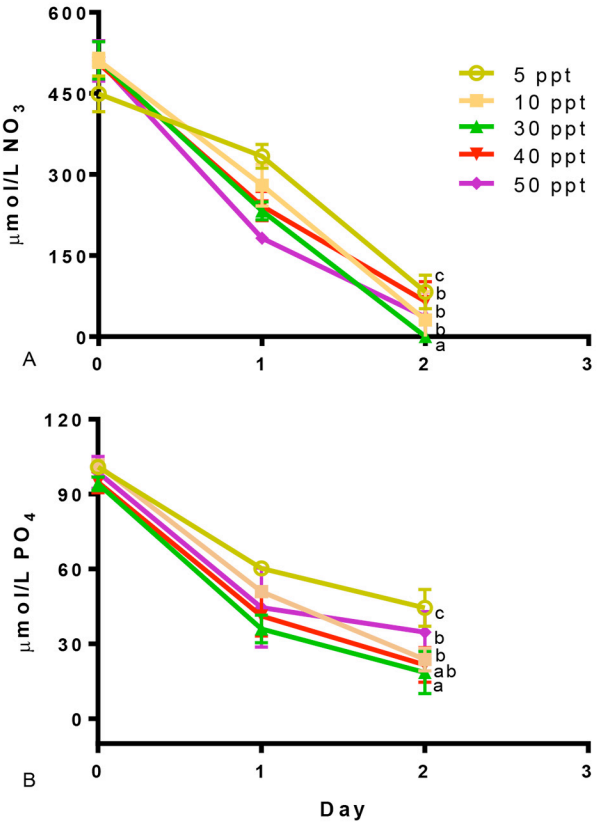
Different salinity levels also had an effect on the nitrogen and phosphate consumption. Figure 3 presents the nutrient drawn down in *Tetraselmis* sp. M8 cultures. Statistically significant differences were found in the uptake of nitrogen and phosphorus ($p < 0.05$). Cultures at 5 ppt were the slowest in nutrient consumption. The fastest use of nutrients was found in culture grown at a salinity of 30 ppt ($p < 0.05$). Although cultures presented differences in nutrient consumption, all reached considerable N depletion within two days.

Figure 3. Nutrient draw down for different salinities in *Tetraselmis* sp. (**A**) Nitrate. (**B**) Phosphate. Data represent mean values ± SDs for three independently grown cultures. Letters represent statistically significant differences amongst salinities ($p < 0.05$).



Furthermore, the expression of fifteen genes involved in FA synthesis was analysed in *Tetraselmis* sp. cultivated at salinities of 5 to 50 ppt and under different nutritional conditions. Four genes, encoding BKAS, Δ5D, Δ6E and ACSace, were differentially expressed according to salinity and nutrient stress; these are presented in Figures 4 and 5. Data of the remaining eleven genes are presented in Supplementary Table S1. The gene, *BKAS*, encodes an enzyme involved in the elongation of long-chain FAs by adding two carbons to the FA chain; its expression was significantly ($p < 0.05$) induced by nutrient deprivation (Figure 4A). On Day 4, the transcript levels were highest in low to medium salinities of 5, 10 and 30 ppt, and on Day 6, the expression was highest in medium to high salinities of 30, 40 and 50 ppt. The enzyme, Δ5D (Figure 1), catalyses the desaturation of C20:3 to C20:4 and of C20:4 to C20:5 in the omega-6 and omega-3 pathways, respectively. Expression of the

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gene encoding Δ5D (Figure 4B) increased with the progression of nutrient stress in all salinities. The upregulation of this gene correlates with EPA levels (Table 3). A consistent increase in percent of EPA was measured in cultures with nutrient depletion through to nutrient starvation. The expression of the ACSase-encoding gene in cultures with different salinities is presented in Figure 4C. Expression increased at all salinities with nutrient depletion (Day 4) and then decreased with nutrient starvation (Day 6).

Figure 4. Expression profiles for three LC-PUFA biosynthesis genes in *Tetraselmis* sp., (**A**) BKAS, (**B**) Δ5D and (**C**) ACSase, under the influence of different salinities (5–50 ppt) and nutrient stress (Day 2, nutrient replete; Day 4, nutrient deplete; Day 6, nutrient starved). Transcript abundances are shown relative to *BETA-ACTIN* (RTA) measured by qRT-PCR from three independently grown cultures. Data represent mean values ± SDs. Letters represent statistically significant differences ($p < 0.05$).

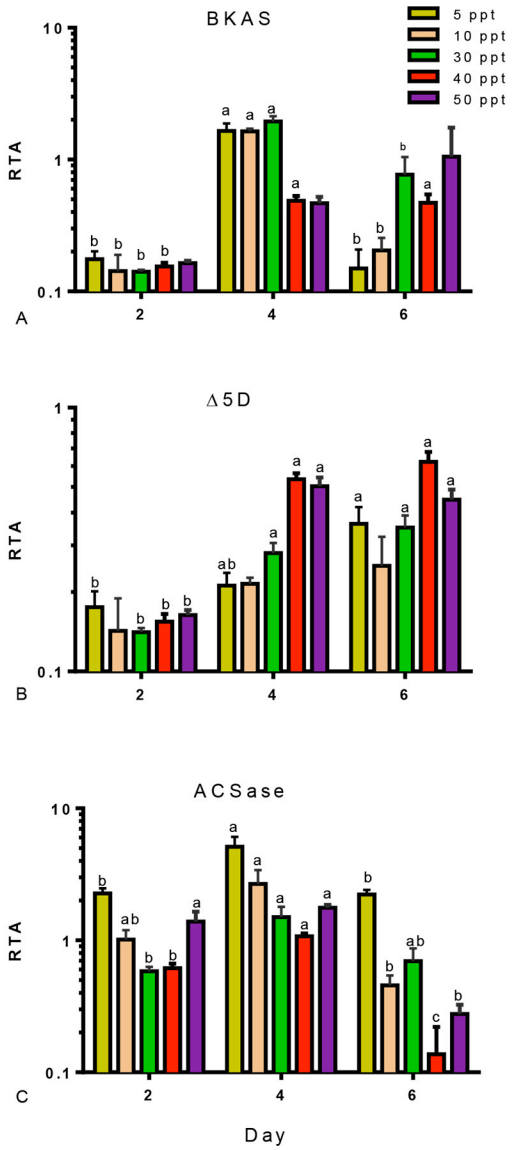

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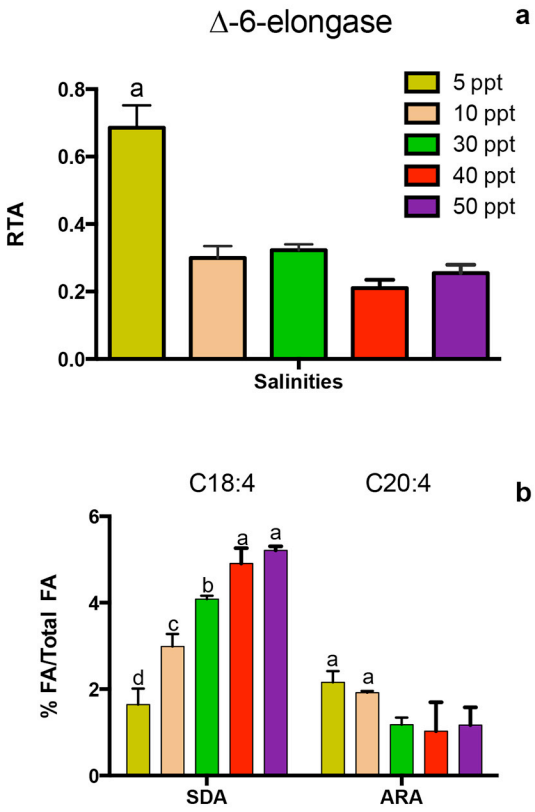
Figure 5. *Tetraselmis* sp. cultivated at different salinities on Day 4 after inoculation. (**A**) Expression profile for Δ-6-elongase-encoding gene. (**B**) Fatty acids C18:4 and C20:4 as a percentage of total FA. Data represent mean values ± SDs from three independently grown cultures. Letters represent statistically significant differences amongst salinities ($p < 0.05$).



The expression of the Δ6E-encoding gene and its relation to FA synthesis on Day 4 is presented in Figure 5. A salinity of 5 ppt led to the highest expression of this gene ($p < 0.05$), which also corresponds to increased efficiency for converting C18:4 to C20:4 (Figure 5B). There was a strong trend of increasing C18:4 and a moderate trend of decreasing C20:4 with increasing salinity. This corresponds to the trend of decreasing expression of the Δ6E-encoding *Tetraselmis* gene with increasing salinity.

Fatty acid profiles for *Tetraselmis* sp. are shown in Table 3. The most abundant FAs were C16:0, C16:4, C18:3 (ALA), accounting for more than 50% of the total FA. The percentage of C16:0 increased in all salinities with a corresponding decrease in C18:3 (ALA) as nutrient stress progressed. There was no significant difference in the percentage of C20:5 (EPA) amongst the different salinities. There was, however, a significant increase ($p < 0.05$) in EPA content with nutrient stress, most notably at a salinity of 40 ppt. C20:4 (ETA) showed statistically higher accumulation at low salinities (5 and 10 ppt) and with nutrient starvation (Day 6) for all salinities.

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Table 3. Fatty acid profile (%TFA) of *Tetraselmis* sp. cultivated at different salinities (5 ppt, 10 ppt, 30 ppt, 40 ppt and 50 ppt), under nutrient stress (Day 2, nutrient replete; Day 4, nutrient deplete; Day 6, nutrient starved).

Fatty Acids	Salinity								
	5 ppt			10 ppt			30 ppt		
	2	4	6	2	4	6	2	4	6
C12:0	0.19 ± 0.07	0.23 ± 0.39	0.26 ±0.40	0.11 ± 0.05	0.11 ± 0.09	0.08 ± 0.09	0.15 ± 0.10	-	0.01 ± 0.01
C14:0	0.38 ± 0.02	0.15 ± 0.27	0.32 ± 0.30	0.30 ± 0.01	0.23 ± 0.01	0.19 ± 0.09	0.23 ± 0.17	0.14 ± 0.04	0.17 ± 0.05
C14:1	1.22 ± 0.01	0.76 ± 0.10	0.72 ± 0.09	1.45 ± 0.05	1.11 ± 0.10	0.77± 0.16	1.67 ± 0.08	1.27 ±0.23	1.01 ± 0.16
C16:0	19.38 ± 0.01	21.22 ± 1.95	22.79 ± 1.36	18.14 ± 0.10	21.81 ± 0.58	22.73 ± 0.52	19.37 ± 0.90	22.15 ± 1.24	23.29 ± 1.00
C16:1	1.56 ± 0.11	1.31 ± 0.13	2.20 ± 1.29	3.63 ± 0.27	1.90 ± 0.07	1.627 ± 0.22	4.71 ± 0.76	2.36 ± 0.30	1.96 ± 0.32
C16:3	6.51 ± 0.55	6.80 ± 0.77	5.89 ± 0.76	5.69 ± 0.18	5.58 ± 0.12	4.73 ± 0.33	4.33 ± 0.37	4.25 ± 0.09	3.44 ± 0.18
C16:4	16.54 ± 0.53	17.37 ± 4.48	13.80 ± 3.49	17.82 ± 0.01	15.02 ± 0.50	16.42 ± 1.61	19.01 ± 1.51	17.51 ± 1.36	17.16 ± 1.57
C18:0	-	-	-	-	-	-	-	-	-
C18:1	10.29 ± 0.38	11.94 ± 3.63	16.25 ± 2.80	7.54 ± 0.12	11.87 ± 0.78	13.30 ± 0.65	7.15 ± 0.79	10.49 ± 0.38	12.11 ± 0.32
C18:2	13.86 ± 1.40	15.15 ±0.88	14.39 ± 0.62	13.07 ± 0.27	15.55 ± 0.18	14.34 ± 0.19	11.37 ± 0.43	13.43 ± 0.46	12.81 ± 0.57
C18:3 (GLA)	0.61 ± 0.03	0.39 ± 0.10	0.79 ± 0.09	0.51 ± 0.01	0.631±0.040	0.81 ± 0.08	0.30 ± 0.18	0.47 ± 0.05	0.74 ± 0.06
C18:3 (ALA)	17.39 ± 1.31	14.35 ± 1.37	11.25 ± 0.79	18.76 ± 0.12	14.219±0.572	12.44 ± 0.37	18.77 ± 0.10	15.15 ± 0.52	13.99 ± 0.40
C18:4	2.26 ± 0.31	1.64 ± 0.37	1.41 ± 0.21	4.04 ± 0.18	2.99 ± 0.29	2.58 ± 0.21	4.84 ± 0.17	4.08 ± 0.08	3.62 ± 0.05
C20:0	-	-	-	0.46 ± 0.65	-	-	-	-	-
C20:1	1.67 ± 0.18	1.12 ± 0.21	1.38 ± 0.27	1.30 ± 0.03	1.21 ± 0.03	1.12 ±0.12	2.02 ± 0.26	1.64 ± 0.12	1.50 ± 0.05
C20:2	0.19 ± 0.26	0.33 ± 0.07	0.27 ± 0.24	0.18 ± 0.25	0.24 ± 0.03	0.31 ± 0.05	0.15 ± 0.14	0.29 ± 0.03	0.21 ± 0.18
C20:3	0.14 ± 0.09	-	-	0.10 ± 0.02	-	-	0.06 ± 0.10	-	-
C20:4	2.12 ± 0.16	2.15 ± 0.26	2.75 ± 0.24	1.63 ± 0.02	1.92 ± 0.04	2.20 ± 0.06	0.94 ± 0.30	1.18 ± 0.16	1.65 ± 0.12
C20:5	5.04 ± 0.11	5.06 ± 0.36	5.49 ± 0.32	4.91 ± 0.04	5.61 ± 0.10	6.13 ± 0.09	4.02 ± 0.43	5.01 ± 0.69	6.10 ± 0.49
SFA	19.96 ± 0.06	21.60 ± 2.59	23.37 ± 2.03	19.01 ± 0.79	22.15 ± 0.48	23.00 ± 0.53	19.76 ± 0.70	22.29 ± 1.26	23.46 ± 1.01
MUFA	14.74 ± 0.31	15.12 ± 3.98	20.55 ± 3.62	13.93 ± 0.23	16.10 ± 0.66	16.82 ± 0.96	15.56 ± 0.44	15.77 ± 0.61	16.58 ± 0.71
PUFA	64.66 ± 0.32	63.25 ± 6.52	56.04 ± 5.09	66.71 ± 0.07	61.76 ± 1.10	59.97 ± 1.48	63.68 ± 0.24	61.38 ± 2.00	59.74 ± 1.53
ω-3	26.81 ± 1.35	23.21 ± 1.66	20.89 ± 0.79	29.34 ± 0.25	24.73 ± 0.87	23.35 ± 0.65	28.57 ± 0.79	25.43 ± 1.14	25.37 ± 0.73

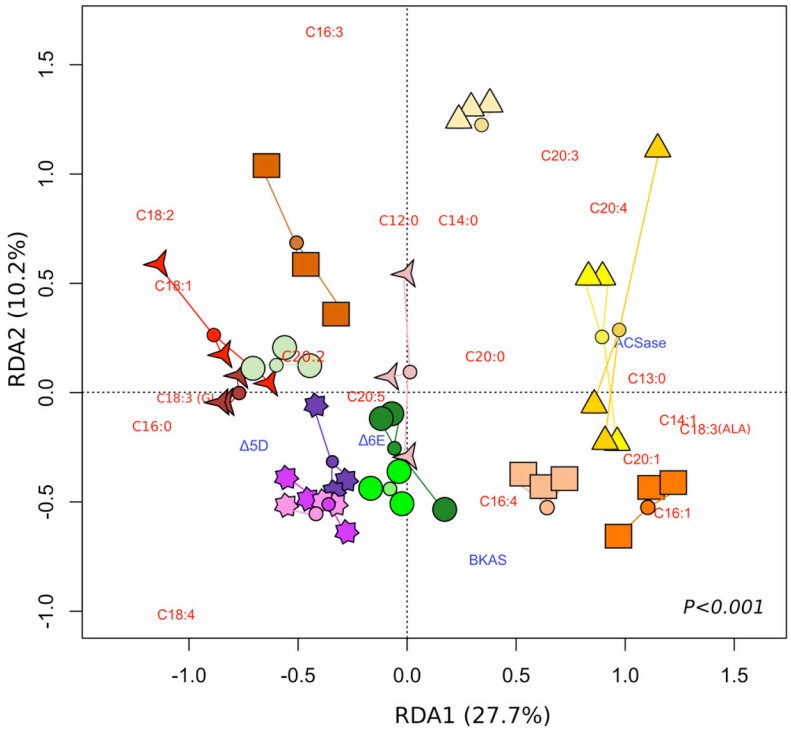
Table 3. *Cont.*

Fatty Acids	Salinity					
	40 ppt			50 ppt		
	2	4	6	2	4	6
C12:0	0.06 ± 0.02	-	0.04 ± 0.07	0.09 ± 0.06	0.22 ± 0.21	0.05 ± 0.06
C14:0	0.19 ± 0.06	0.11 ± 0.1	0.08 ± 0.07	0.24 ± 0.07	0.25 ± 0.22	0.17 ± 0.15
C14:1	1.66 ± 0.06	1.00 ± 0.48	0.65 ± 0.56	1.69 ± 0.05	1.27 ± 0.38	0.98 ± 0.28
C16:0	18.38 ± 0.30	19.44 ± 1.88	20.99 ± 1.24	17.76 ± 1.21	18.70 ± 0.20	20.51 ± 1.83
C16:1	4.26 ± 0.80	2.06 ± 0.80	1.72 ± 0.58	4.64 ± 0.55	2.72 ± 0.60	2.43 ±0.35
C16:3	4.24 ± 0.25	4.70 ± 0.16	3.70 ± 0.12	3.94 ± 0.16	5.38 ± 0.14	4.68 ± 0.64
C16:4	19.37 ± 0.17	22.26 ± 9.65	20.66 ± 4.87	19.47 ± 0.98	18.81 ± 4.11	18.37 ± 5.18
C18:0	-	-	-	-	-	-
C18:1	6.36± 0.18	7.92 ± 3.44	7.12 ± 6.18	6.22 ± 0.36	8.65 ± 1.71	10.50 ± 1.72
C18:2	10.90 ± 0.45	13.20 ± 1.37	13.39 ± 0.34	9.81 ± 0.80	13.15 ± 0.16	13.01 ± 1.10
C18:3 (GLA)	0.32 ± 0.05	0.39 ± 0.34	0.74 ± 0.17	0.35 ± 0.05	0.49 ± 0.33	0.75 ± 0.14
C18:3 (ALA)	20.53 ± 0.30	16.19 ± 0.71	16.00 ± 2.21	20.51 ± 0.16	16.34 ± 0.60	14.69 ± 0.44
C18:4	5.41 ± 0.33	4.90 ± 0.35	4.61 ± 0.84	5.95 ± 0.80	5.21 ± 0.09	4.33 ± 0.54
C20:0	-	-	-	-	-	-
C20:1	2.08 ± 0.26	1.36 ± 0.60	1.12 ± 0.52	2.55 ± 0.36	1.90 ± 0.24	1.59 ± 0.10
C20:2	0.21 ± 0.06	0.15 ± 0.13	0.18 ± 0.09	0.15 ± 0.14	0.33 ± 0.10	0.29 ± 0.02
C20:3	-	-	-	0.05 ± 0.04	0.03 ± 0.04	-
C20:4	1.06 ± 0.04	1.03 ± 0.67	1.73 ± 0.10	1.08 ± 0.07	1.17 ± 0.41	1.46 ± 0.29
C20:5	4.44 ± 0.15	5.12 ± 0.91	7.18 ± 1.46	4.72 ± 0.44	5.29 ± 0.38	5.96 ± 0.30
SFA	16.64 ± 0.24	19.55 ± 1.98	21.11 ± 1.34	18.09 ± 1.12	19.17 ± 0.36	20.74 ± 2.03
MUFA	14.35 ± 0.56	12.34 ± 5.32	10.60 ± 7.83	15.11 ± 0.57	14.54 ± 2.88	15.50 ± 2.38
PUFA	66.49 ± 0.41	67.95 ± 7.44	68.18 ± 9.24	66.02 ± 1.35	66.21 ± 3.18	63.55 ± 4.46
ω-3	31.44 ± 0.22	27.24 ± 0.53	29.51 ± 4.40	32.25 ± 1.13	28.02 ± 0.75	26.44 ± 0.08

Data represent mean values ± SDs for three independently grown cultures. (-) undetected fatty acid. Total amounts of FAs are shown in Supplementary Table S2.

Figure 6 presents the results of a redundancy analysis (RDA), which summarizes in two dimensions the variation of FA production and gene expression that can be attributed to the treatments applied. The primary (RDA1) and secondary (RDA2) axes of the RDA explain 27.7% and 10.2% of this variation, respectively. The gene expression of BKAS, Δ6E, Δ5D and ACSase-encoding genes, as well as FA proportions explained the difference amongst salinity treatments (RDA, Figure 6, $p < 0.001$).

Figure 6. Redundancy analysis (RDA) summarizing the variation in gene expression and fatty acid production of *Tetraselmis* sp. at different salinities. (△) 5 ppt, Day 2; (△) 5 ppt, Day 4; (△) 5 ppt, Day 6; (□) 10 ppt, Day 2; (□) 10 ppt, Day 4; (□) 10 ppt, Day 6; (○) 30 ppt, Day 2; (●) 30 ppt, Day 4; (●) 30 ppt, Day 6; (◁) 40 ppt, Day 2; (◁) 40 ppt, Day 4; (◁) 40 ppt, Day 6; (☆) 50 ppt, Day 2; (☆) 50 ppt, Day 4, (☆) 50 ppt, Day 6. The small coloured circles represent the centroid of the treatment replicates.



Different treatments showed different proportions of certain FAs and transcripts. A clear separation of treatments with a low salinities of 5 ppt (Days 2, 4, 6) and 10 ppt (Days 2, 4) and high salinities of 30 ppt, 40 ppt and 50 ppt was revealed along the primary axis. An induction of the ACSase-encoding gene was observed in salinities of 5 ppt relative to 40 and 50 ppt (Figure 6). The main differences between low and high salinity treatments are particularly attributed to the separation of Fas, such as C18:4 and C20:4 (Table 3), which were present at higher proportions in the 5 ppt and 40–50 ppt treatments, respectively. It can also be noted that these particular FAs are located in contrasting quadrants along both primary and secondary axes of the RDA (RDA1 and RDA2).

On the secondary axes (RDA2), the main differences were observed between salinities of 5 ppt at Day 2 and 10 ppt at Day 4, which was clearly shown by FAs C16:3 and C18:4 (Figure 6). Differences

between low and high salinities were also observed. Salinities of 40 and 50 ppt led to higher proportions of C18:4 relative to 5 ppt, which presented lower percentages of C18:4, but higher C20:4 values.

3. Discussion

Tetraselmis sp. was used as a laboratory model strain to study the effect of salinity on growth, FA accumulation and the expression of genes involved in the FA synthesis. Optimal growth conditions are species-specific depending on cellular adaptation mechanisms to environmental stress. This study found that *Tetraselmis* sp. M8 displayed the best growth rate at 40 ppt, but the highest final biomass at 30 ppt. Other studies found that *Tetraselmis suecica* presented a maximum cell density of 6.4×10^6 cell/mL at a salinity of 25 ppt [33]. Diverse microalgal species have been found to have optimal growth when cultured at different salinities. For example, *Chaetoceros wighamii* [10] and *Gracilaria corticata* [34] presented their highest growth rate at 25 and 35 ppt, respectively. *Nannochloropsis* sp. showed a high growth rate at 13 ppt when cultured at low light irradiance (170 μmol photon/m^2 s); however, when *Nannochloropsis* sp. was cultivated under high light irradiance (700 μmol photon/m^2 s), its best growth occurred at 27 ppt [18]. Halotolerant microalgal species *D. salina* demonstrated the highest cell concentration at 1.0 M NaCl (58 ppt) [19]. Although there are several studies related to salt tolerance in microalgal species, the salt tolerance mechanism in several species of commercial interest, such as *Tetraselmis* sp., requires further study. Depending on the strain and its salinity tolerance, FA synthesis can be induced or inhibited. *D. salina* was found to increase its FA content from 60% to 67% when salinity was elevated from 0.5 to 1 M NaCl (58 ppt) [19]. In the present study, *Tetraselmis* sp. M8 was observed to have the highest omega-3 FA proportion in relation to total FAs at Day 2 (nutrient replete) at a salinity of 50 ppt, followed by 40 and 30 ppt. Omega-3 Fas, such as EPA, have been associated with high growth due to their importance in cellular functions. Studies on *Pinguiococcus pyrenoidosus* demonstrated that maximum EPA and DHA production occurred at salinities of 30 ppt [35]. *Schizochytrium limacinum* was found to have high growth rates at salinities between 18 and 27 ppt, while its highest DHA content was found at a salinity of 9 ppt after five days of cultivation [36]. Although the highest EPA production is more commonly associated with nutrient replete conditions optimal for cellular growth, not all species of microalgae have higher ratios of this FA during logarithmic growth. The present study, for example, showed that the proportion of EPA in *Tetraselmis* sp. increased during the progression of nutrient stress. However, total omega-3 FAs decreased with nutrient stress, primarily due to the reduction of ALA.

Gene expression for FA synthesis has previously been studied in several microalgal species, demonstrating that up- and/or down-regulation of genes occurs as a result of the changes of external conditions, like salinity [37,38]. Growth is promoted when cultures are under nutrient replete conditions, enhancing gene expression by using large amounts of anabolic structural components. However, once nutrients are depleted, autophagic processes can be activated to provide intracellular nitrogen for limited *de novo* synthesis, allowing cells to change and adapt [37]. Gene expression for the LC-PUFA synthesis pathway was generally upregulated by nutrient deprivation (Figure 4). Differential gene expression for Δ5D, involved in the desaturation of FA chains for the synthesis of

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ARA and EPA, was higher at high salinity once nutrients were depleted from the media (Figure 4B). On the other hand, lower salinity levels induced higher expression of the Δ6E-encoding gene, involved in the elongation of C18:4 into C20:4 and C18:3 into C20:3 in the omega-3 and omega-6 FA pathways, respectively (Figure 1). Although enzymes have been reported to have a dual activity in each FA pathway; the little or undetectable C20:3 in *Tetraselmis* sp. FA profiles indicates that the omega-3 FA pathway is more likely to be used than the omega-6 pathway.

A salinity shock experiment in *D. salina* found that the proportion of 18, 20 and 22 carbon FAs and desaturation were higher at high salinity (3.5 M; 203 ppt) compared to normal salinity (0.5 M; 29 ppt), which had a higher proportion of saturated 16 carbon FAs. The BKAS-encoding gene was also highly induced with the high salinity treatment. It was therefore suggested that the BKAS elongation reactions provided a sufficient substrate for long-chain desaturases to work. Therefore, salinity can activate FA modification by the elongation and desaturation of FA chains to contribute to the osmoregulation of the salt tolerance of microalgae [26]. In *Tetraselmis* sp., we found an increase in BKAS and ACSase transcript abundance with nutrient depletion, but there was no significant difference in gene expression between salinities and no differences in the proportion of FA carbon chain length or the level of desaturation. The differences we found in the current experiment were probably due to the pre-adaptation to salinity, rather than osmotic shock.

4. Methods

4.1. Algae Culture and Cultivation Conditions

Tetraselmis sp. (strain M8) was isolated from the south-east coast of Queensland, Australia (26°39′39″ S 153°6′18″ E), and stored in the culture collection of the Algae Biotechnology Laboratory at The University of Queensland [39]. Prior to the experiment, the algal strain was pre-adapted in f/2 silicate-free medium [40] that was phosphate enriched (100 μM), with the salinities to be tested using artificial sea water (Acuasonic Ocean-Nature sea salt). The culture in the logarithmic phase was used as inoculum; inoculation concentration varied slightly depending on the starter culture optical densities. Approximately 20 mL of each pre-adapted algal stock culture were transferred to 180 mL of enriched f/2 medium in a 250-mL Erlenmeyer conical flask with artificial salty water adjusted to 5, 10, 30, 40 and 50 parts per thousand (ppt) using three independently-grown cultures. Salinity was determined using a Reed TDS salinity conductivity meter (Toronto, ON, Canada). Cultures were incubated at 25 °C under a 16/8 h light/dark cycle (90 μmol/m^2/s fluorescent lights) regime with constant bubbling. Optical density (OD) 440 nm measurements were performed daily to monitor the growth rate. Nitrogen and phosphorus contents were determined from Day 0 until nutrient depletion. Samples for FA profiling and RNA extraction were collected on Day 2 (nutrient replete), Day 4 (nutrient deplete) and Day 6 (nutrient starved).

4.2. Culture Media Nutrient Analysis

Total nitrate was measured using the commercial colorimetric API Aquarium Pharmaceutical Nitrate NO_3^- test kit; colour intensity was measured using a spectrophotometer at a wavelength of 545 nm. A standard curve was generated and used to determine nitrate concentrations in medium

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samples (algae were previously removed by centrifugation); 0–300 μM was found to have a linear colorimetric relationship to the NO_3^- concentration. Total phosphate was determined using the colorimetric API Aquarium Pharmaceutical Phosphate PO_4^{3-} test kit; colour intensity was measured using a spectrophotometer at a wavelength of 690 nm. A standard curve was generated; 0–60 μM was found to have a linear colorimetric relationship to the PO_4^{3-} concentration.

4.3. Fatty Acid Analysis

Fatty acids were quantified by gas chromatography-mass spectrometry (GC/MS) by Metabolomics Australia at the University of Western Australia, as described previously [41], with the exception that 5 mg of culture was used instead of 2 mL of culture. Hydrolysis and methyl-esterification was performed, as described previously [41].

4.4. Total RNA Extraction and cDNA Synthesis

Total RNA from microalgal biomass was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) using centrifugal pellets obtained from 15 mL of culture. RNA concentrations were measured with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was used for cDNA synthesis using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) for quantitative reverse transcriptase PCR following the manufacturer's instructions.

4.5. Real-Time Quantitative Reverse Transcriptase PCR

Primers used for real-time quantitative reverse transcriptase PCR (qRT-PCR) were designed using Primer Express software (Applied Biosystems, Foster city, CA, USA), based on a recently generated draft transcriptome of *Tetraselmis* sp. by the Algae Biotechnology Laboratory at The University of Queensland, Brisbane, Australia (transcriptome data will be published elsewhere) [42]. Primers were designed in conserved regions to cover the majority of gene family members. Each reaction was performed in a final volume of 10 μL and contained 1 μL of the cDNA (1:4 diluted), 1 μL of each primer (1 μM) and 5 μL SYBR Green using the 7900 HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). *Tetraselmis* sp. M8 transcript levels were normalized to the expression of *β-ACTIN*. Thermal cycling conditions consisted of 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C prior to 2 min at 25 °C.

4.6. Statistical Analyses

The analysis of variance (ANOVA) was performed using the Statistical Package for the Social Sciences (SPSS, IMB, New York, NY, USA). All significant differences ($p < 0.05$) amongst values obtained for different salinities and harvesting times were determined using the Tukey HSD test. Redundancy analysis (RDA) was performed using the package, vegan, implemented in R 3.0.2 [43].

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5. Conclusion

Changes in salinity primarily altered biomass productivity, with 30 and 40 ppt having the highest growth rate and final productivity. Salinity had no significant effect on the percentage of EPA or total FA production (Supplementary Table S2). Under nutrient depletion, most of the genes analysed from the FA synthesis pathway were strongly upregulated, but the gene expression typically decreased once cultures were fully starved (Supplementary Table S1). A correlation between the upregulation of the Δ6E-encoding gene and the conversion of C18:4 to C20:4 was found, indicating that the omega-3 pathway is more likely to be used for the synthesis of LC-PUFAs in *Tetraselmis* sp. Additionally, an increase of EPA (C20:5) and ETA (C20:4) proportions with the progression of nutrient stress was found, especially algal cultures grown at 40 ppt of salinity. However, the trigger for the synthesis of EPA still remains unclear. Hence, further studies are required to determine the responsible factors for the upregulation of the omega-3 pathway synthesizing EPA in *Tetraselmis* sp. under diverse environmental conditions.

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Author Contributions

Conceived and designed the experiments: TCAV, STH, Performed the experiments: TCAV, STH Designed primers: DKYL, Analyzed the data: TCAV, STH. Wrote the paper: TCAV, STH, PMS.

Conflicts of Interest

The authors declare no conflict of interest.

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