Systematic Identification of Microalgal Species for Lipid Production and Genome Based Molecular Characterization of the Oleaginous Microalga *Monoraphidium neglectum*

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Summary

In the light of the depletion of fossil fuel reserves worldwide, the search for sustainable and more environmental friendly alternatives becomes decisive. The generation of liquid fuels as bioethanol or biodiesel from biomass are already considered as economically viable options. However, conflicts of interest arise when the generation of food or animal feedstock competes with biomass production for conversion to biofuels. The use of microalgae could offer a possible solution to several of these problems; however a more wide-spread use is hampered by the requirement of suitable strains for bulk chemical generation. Though a lot of effort has been spent in finding suitable strains in the past years, much of the potential that can be found in the biodiversity of algae remains untouched.

In this work, microalgae that originated from established strain collections but have not been in the focus of biofuel-related research before were investigated on their potential for liquid biofuel production. This approach was complemented by the isolation of new strains from mainly local freshwater and marine water bodies, followed by a detailed characterization in terms of biomass and lipid productivity as well as of their fatty acid profiles. The genus *Monoraphidium* could be identified as highly interesting for these purposes, since many members show robust growth and considerably high lipid productivities. Two strains of interest, *Monoraphidium contortum* and *Monoraphidium neglectum*, were investigated in detail and compared to the model organism *Chlamydomonas reinhardtii*. Both *Monoraphidium* strains showed a pronounced increase of neutral lipids under nitrogen starvation which could be furthermore enhanced in lower culture densities, consequently resulting in better light availability for the cells. The accumulation of lipids was not only superior to that of *C. reinhardtii* but also resulted in a fatty acid profile with a dominance of C18:1 and C16:0 fatty acids, which is promising for liquid biofuel generation. In addition, cells of *M. neglectum* were found to increase their size at defined salt concentrations, thereby offering the possibility for facilitated down-stream processing.

Key element for liquid biofuel production is the content of neutral lipids, namely triacylglycerol (TAG), in the biomass. The elucidation of pathways for TAG synthesis is currently subject to intense research efforts. Therefore the role of two distinct metabolic pathways was investigated in more detail in the model organism *C. reinhardtii*.

To gain further insights into the lipid metabolism of *Monoraphidium* and to allow targeted improvement of these strains, the genome of *M. neglectum* was sequenced. The annotation of pathways of fatty acid synthesis as well as within the glycerolipid metabolism allowed not only detailed insights into the diversity of oleaginous organisms, but also leads to further promising targets to improve this strain.

Within this work, the Selenastraceae could be identified as a highly interesting family with a number of interesting strains for biomass generation that also have a high potential for subsequent conversion to liquid biofuels. The sequencing of *Monoraphidium neglectum* was performed to establish this species as future model organism for the other members of this family and also opens the possibility for future targeted engineering approaches.

Abbreviations

ACC	acetyl-CoA carboxylases
ACP	acyl carrier protein
AFDW	ash free dry weight
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase
ASP	Aquatic Species Program
ATP	adenosine triphosphate
CAD	charged aerosol detector
DAG	diacylglycerol
DGAT	diacylglycerol-acyltransferases type 1
DGTT	diacylglycerol-acyltransferases type 2
DGDG	digalactosyldiacylglycerol
DW	dry weight
Cyt b6f	cytochrome b_6f
DNA	deoxyribonucleic acid
DGTS	diacylglycerol-*N*,*N*,*N*-dimethylhomoserine
EAR	enoyl-ACP reductase
ER	endoplasmic reticulum
FAS	fatty acid synthase
Fd	ferredoxin
FID	flame ionization detection
FNR	ferredoxin-$NADP^+$ oxidoreductase
g	gram
GC	gas chromatography
GPAT	glycerol-3-phosphate O-acyltransferase
HAD	hydroxyacyl-ACP dehydratase
HPLC	high-performance liquid chromatography
KAR	oxoacyl-ACP reductase
KAS I-III	ketoacyl-acyl-carrier-protein synthase I-III
l	liter
MAT	malonyl-CoA:ACP transacylase
MGDG	monogalactosyldiacylglycerol
MLDP	major lipid droplet protein
M	mol
m	meter
mg	milligram
ml	milliliter
µmol	micromole
mM	millimol
MS	mass spectrometry
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
PC	phosphatidylcholine

PDAT	phospholipid:diacylglycerol acyltransferase
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PP	phosphatidate phosphatase
PQ	plastoquinone
PS I	photosystem I
PS II	photosystem II
ROS	reactive oxygen species
s	second
SQDG	sulfoquinovosyldiacylglycerol
UV	ultraviolet
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
TAG	triacylglycerol

1. Introduction

1.1. Sustainability and Bioenergy

An important challenge of modern societies is to create a balance between environmental sustainability, economic growth and security of energy supplies (ESCAP, 2010; IEA, 2012). When the supply with energy is addressed, the world's energy demand is currently primarily met by the utilization of conventional fossil fuels, which are also an important feedstock for plastics and fabrics (Srirangan et al., 2012). The exploitation of these reserves is not only associated with an increase of greenhouse gas emissions but also with a rising demand that resulted in an increase of oil prices, with a direct influence on the economy (Nigam & Singh, 2011). To satisfy the markets hunger for fossil fuels, methods like hydraulic fracturing are increasingly gaining importance, while their environmental and legal implications still need to be understood and assessed by future research (Wiseman, 2009). There is a clear need to overcome the limitations of diminishing fossil oil reserves, which leads to a renewed focus on alternative energy sources, e.g. using biomass for conversion to liquid fuels (Hecht et al., 2009; Sivakumar et al., 2010; Srirangan et al., 2012). Taking into account the large fraction of fuels used for the global energy consumption, it is interesting to note that today renewable alternatives to liquid fuels are far less developed than solutions towards sustainable electricity production (Schenk et al., 2008).

Among liquid fuels, ethanol and biodiesel are probably the most prominent ones (Nigam & Singh, 2011; Sivakumar et al., 2010). Ethanol can be produced by conversion of starch and other carbohydrates deriving from crops as e.g. corn or sugar cane as most prominent feedstock (Merchant et al., 2012; Sivakumar et al., 2010; Srirangan et al., 2012). The use of bioethanol as fuel is fully established in Brazil, with the USA, China and India as further important players in the field (Nigam & Singh, 2011). However, several implications are associated with ethanol production, that include transportation problems, a high water consumption in the case of corn-based ethanol as well as rising prices as for soybean, resulting in misguided incentives for conversion of forests into agricultural land in e.g. South America (Sivakumar et al., 2010). Though switchgrass and giant miscanthus are considered as potential alternatives to produce ethanol from lignocellulosic biomass, the infrastructure as well as down-stream processing is still required to develop (Nigam & Singh, 2011; Sivakumar et al., 2010).

When biodiesel and related liquid biofuels are considered, the compound of interest are oils containing triacylglycerols (TAG), that consist of three fatty acids esterified to a glycerol backbone (Chisti, 2007; Sivakumar et al., 2010). Triacylglycerols can be either used directly or after conversion to fatty acid methyl esters (FAME) as biodiesel, depending on the fatty acid composition (Barnwal & Sharma, 2005; Merchant et al., 2012; Sivakumar et al., 2010). Oils that are characterized by short chain fatty acids with carbon chain lengths between eight and 14 carbons have the potential for direct use as liquid fuel, therewith reducing costs in downstream processing (Sivakumar et al., 2010). TAGs with longer fatty acids with up to 18 carbons can be also used as biofuel, but require prior processing into FAMEs (Sivakumar et al., 2010). Several vegetable oils as derived from canola, palm and soybean contain high contents of C16 and C18 fatty acids and were subject to intense investigations on their fuel properties (Knothe, 2008). For the generation of biodiesel these oils are transesterified to methyl esters, a process catalyzed by acids, alkalis or lipase enzymes, which releases glycerin as side product (Barnwal & Sharma, 2005; Chisti, 2007; Nigam & Singh, 2011). The conversion of the original vegetable oil to biodiesel reduces the viscosity as well as the molecular weight and opens the possibility of blending with conventional fuel in any proportion (Barnwal & Sharma, 2005). A further possibility of conversion of oils into liquid fuel can be found within the hydrogenation of vegetable oils as performed for NExBTL (Aatola et al., 2009; Nigam & Singh, 2011). Instead of fatty esters, hydrocarbons are generated within a process where oxygen is removed from lipids using hydrogen, without the need of additional chemicals (Aatola et al., 2009). Since the term "biodiesel" is reserved for fatty acid methyl esters, hydrotreated oils are therefore known as "renewable diesel fuels" (Aatola et al., 2009). However, the required equipment is rather complex as well as an additional source of hydrogen is needed (Nigam & Singh, 2011). Important points that need consideration when fatty acid derived compounds are intended for biofuel applications include oxidative stability, which is promoted by a high degree of saturation, and cold flow, which generally increases with the degree of unsaturation (Chisti, 2007; Knothe, 2008; Schenk et al., 2008). In addition, it was recently proposed that optimally a biodiesel fuel should consist of only one major component in high concentration, though mixtures might be also acceptable (Knothe, 2008).

Biodiesel is currently produced relying principally on canola, sunflower, soybean and palm oil as feedstock (Nigam & Singh, 2011; Sivakumar et al., 2010; Srirangan et al., 2012). When

compared to ethanol, the use of TAG for liquid biofuel generation has several advantages as it exhibits usually a better energy balance and can be used directly without engine modifications (Merchant et al., 2012; Sivakumar et al., 2010; Srirangan et al., 2012). Biodiesel production from first-generation feedstock is already a commercially viable process as indicated by Srirangan et al., 2012.

1.2. Photosynthesis to capture light energy for biofuel production

Whenever biomass is generated under photoautotrophic conditions, photosynthesis is used to gain energy in combination with the conversion of inorganic carbon into reduced organic compounds as carbohydrates and oils (Chen et al., 2011; Huang et al., 2010; Merchant et al., 2012; Srirangan et al., 2012). In plants and eukaryotic algae these reactions take place in the chloroplast (Nelson & Yocum, 2006). The process of oxygenic photosynthesis relies on light that is captured by light harvesting complex proteins, and their excitation energy is transferred to the reaction centers of the photosystem II (PS II) as summarized previously (Schenk et al., 2008). At the reaction centers, the energy is used to split water for generation of molecular oxygen, releasing the protons into the thylakoid lumen while electrons follow the photosynthetic transport chain of the thylakoid membrane. While they pass plastoquinone (PQ), further protons are released into the thylakoid lumen. The electrons pass cytochrome b_6f (Cyt b6f), plastocyanin and finally reach the photosystem I (PS I) (Schenk et al., 2008). In excited form, PS I is able to reduce ferredoxin (Fd), which converts $NADP^+$ to NADPH via the ferredoxin-$NADP^+$ oxidoreductase (FNR) (Nelson & Yocum, 2006). The proton gradient between thylakoid lumen and the chloroplast stroma is used by an ATP synthase to produce ATP, which is fed together with NADPH into the Calvin cycle besides other biochemical pathways (Schenk et al., 2008). The Calvin cycle mediates the fixation of carbon dioxide, which involves a Rubisco (Ribulose-1,5-bisphosphate carboxylase oxygenase)-catalysed carboxylation of ribulose-1,5-bisphosphate into 3-phosphoglycerates and a subsequent ATP/NADPH-driven reduction, which is followed by the regeneration of glyceraldehyde-3-phosphate to ribulose-1,5-bisphosphate (Raines, 2003; Schenk et al., 2008). The released glyceraldehyde-3-phosphates are then subject to further use within the metabolism of the cell, e.g. synthesis of isoprenoids as well as sucrose and starch after conversion to dihydroxyacetone phosphate (Raines, 2003).

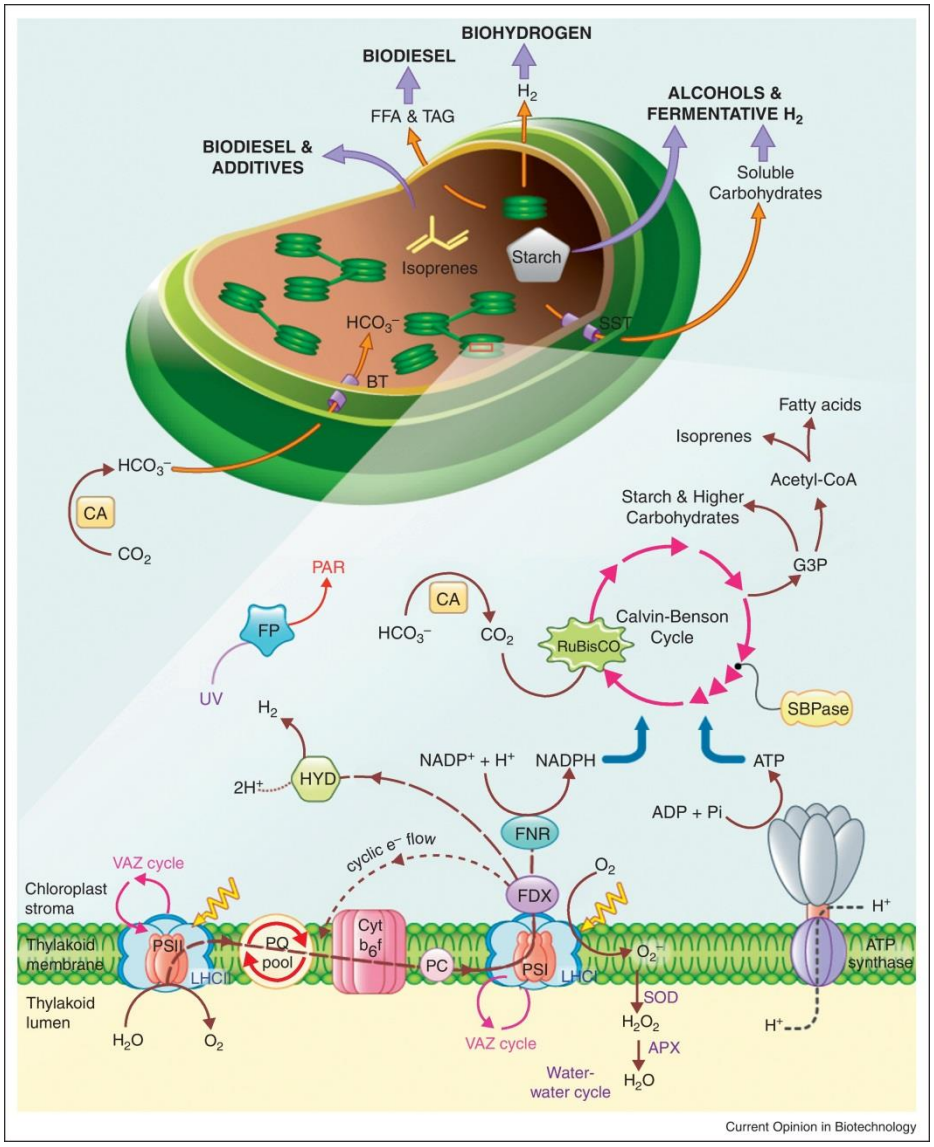

Figure 1 Synthesis of metabolites relevant for fuel production deriving from the capture of light energy by photosynthesis and carbon fixation in green algae. Reprinted from Current Opinion in Biotechnology, Volume 23, Issue 3, Victoria H Work, Sarah D'Adamo, Randor Radakovits, Robert E. Jinkerson, Matthew C. Posewitz. Improving photosynthesis and metabolic networks for the competitive production of phototroph-derived biofuels. 290–297. Copyright (2012), with permission from Elsevier.

In further metabolic steps, these compounds can serve as substrate for the generation of pyruvate, as precursor for fatty acid biosynthesis within the chloroplast (Rawsthorne, 2002). The fatty acids are subsequently converted into glycerolipids by various pathways, serving mainly structural or

storage functions (Merchant et al., 2012; Riekhof & Benning, 2009). Of special interest for the generation of liquid biofuels is the integration of fatty acids into the storage compound TAG, an important source for the production of biodiesel (Huang et al., 2010; Merchant et al., 2012). It should be mentioned that the photosynthetic apparatus as well as the central carbon metabolic network underlies a certain variability and modification if phylogenetically diverse groups are considered, e.g. Chlorophytes and diatoms (Hildebrand et al., 2013).

1.3. Microalgae as sources for liquid biofuels

With the first generation biofuel production heavily relying on the use of agricultural products (Srirangan et al., 2012), several concerns are expressed whether oil crops, animal fat and waste cooking oils are able to replace liquid fossil fuels in larger amounts (Schenk et al., 2008). Furthermore, biofuels are only predicted to be beneficial if environmental sustainability, preservation of biodiversity as well as the debate food versus fuel are properly addressed (Nigam & Singh, 2011). Since competition with agricultural land use for food production purposes is a severely discussed issue, second generation biofuel systems are proposed that are based on the use of lignocellulosic and microalgal biomass (Schenk et al., 2008). There is some overlap in definition, whether microalgae are already considered as second (Schenk et al., 2008) or as third generation accounting rather non-food crops and waste materials to the second generation (Antizar-Ladislao & Turrion-Gomez, 2008; Nigam & Singh, 2011; Srirangan et al., 2012). However, when area and freshwater requirements for oil crops are considered it becomes evident that algal fuel systems can provide a solution addressing several of those concerns e.g. by reducing the competition with agricultural food production (Merchant et al., 2012; Schenk et al., 2008). Currently these factors, land use and availability of freshwater, are considered as the key limiting factors for biofuel production (Srirangan et al., 2012).

Microalgae can be characterized by their unicellularity and the ability to perform photosynthesis thus including photosynthetic eukaryotes as well as cyanobacteria (Li et al., 2008; Mata et al., 2010), a definition which can be furthermore combined with the organisms' ability to fix carbon (Yu et al., 2011). However, these definitions of microalgae and algae in general is rather based on their appearance and functioning, not taking into account that they are a highly diverse and heterogeneous group of organisms with varying phylogenetic origins (Keeling et al., 2005). As

indicated before, the term alga rather describes a life-form than a systematic unit (Hallmann, 2007). Photosynthesis can be found in a wide range of eukaryotic organisms (Parker et al., 2008). The most ancient group of photosynthetic eukaryotes are the members of the Plantae, which acquired their plastids in an event of primary endosymbiosis (Keeling et al., 2005). Primary endosymbiosis occurred when a photosynthetic prokaryotic organism was engulfed by a eukaryote, therewith giving rise to the lineage of green algae, red algae and Glaucophytes (Keeling et al., 2005; McFadden, 2001; Parker et al., 2008). Further groups of algae emerged with the event of secondary symbiosis, when a photosynthetic eukaryote of those ancient lineages was engulfed by another eukaryote and the plastid was integrated in its new cellular environment (McFadden, 2001; Parker et al., 2008). There is evidence for several separate events, resulting in the emergence of groups as the Stramenopiles, Haptophytes, Dinoflagellates, Euglenophytes and Apicomplexa (Bhattacharya et al., 2004; Parker et al., 2008), located at different branches of the three of eukaryotes (Keeling et al., 2005). Two further groups are Cryptophytes and Chlorarachinophytes that are characterized by the presence of a nucleomorph, a remnant of the engulfed primary endosymbiont (Bhattacharya et al., 2004; Parker et al., 2008). Another degree of complexity and diversity is introduced with tertiary endosymbiosis, which is known from some Dinoflagellates, integrating another photosynthetic secondary endosymbiont (Bhattacharya et al., 2004; Parker et al., 2008). All those highly diverse organisms can be considered as algae since they are united through the presence of their plastid, therewith sharing a common link to the photosynthetic prokaryotes (McFadden, 2001).

Since these organisms have such a diverse background, they are also well distinguishable through features in their metabolism, as the preference for specific storage compounds (Table 1). As an example, Bacillariophyceae are known to store carbon as natural oils or chrysolaminarin while green algae rather rely on starch as well as on oil under certain conditions (Sheehan et al., 1998). Not only are the storage compounds rather diverse, they are furthermore found at different cellular locations (Hildebrand et al., 2013). The high diversity is currently investigated in various genome projects and RNA sequencing studies (Sasso et al., 2012), and when the production of biofuels from microalgae is addressed, the before-mentioned storage compounds become decisive. As well as higher plants e.g. canola or soybean, microalgae are able to produce considerable amounts of triacylglycerols (Chisti, 2007; Mata et al., 2010; Sivakumar et al., 2010). The accumulation of TAGs in microalgae usually takes place under environmental stress

conditions e.g. photooxidative stress or nitrogen starvation, resulting in an increase of TAG content to up to 20 – 50% of dry cell weight (Hu et al., 2008).

Table 1 Summary of important storage compounds of various algal groups

Algal group	Important storage compounds according to Sheehan et al., 1998, Work et al, 2012 and Hildebrand et al., 2013
Cyanobacteria	Glycogen
Chlorophytes (green algae)	Starch, lipids
Rodophytes (red algae)	Floridean starch
Phaeophyceae (brown algae)	Laminaran
Bacillariophyceae (diatoms)	Lipids, chrysolaminarin
Chrysophytes (golden-brown algae)	Lipids, chrysolaminarin
Haptophytes	Lipids, chrysolaminarin
Euglenophytes	Paramylon

Many eukaryotic algae have been found to contain very long chain polyunsaturated fatty acids with four or more double bonds as major components (Basova, 2005; Harwood & Guschina, 2009; Lang et al., 2011; Spolaore et al., 2006) which is in contrast to the fatty acid composition of most vegetable oils that show a comparably high degree of saturation (Broun et al., 1999; Chisti, 2007; Knothe, 2008). Therewith the question arises which are the properties that are required for a microalgal strains that should be used for liquid biofuel applications.

1.4. Desired traits of microalgal strains for biofuel purposes

When the economic feasibility is considered to use microalgae for the production of bulk chemicals as crude oil, decisive properties of strains were summarized by Mata et al., 2010, namely abundant growth, the lipid and in particular the TAG content, robustness to fluctuations in the environmental conditions, ease in downstream processing and the potential use for the production of other valuable compounds. The importance of these points was also highlighted by other authors (Chen et al., 2011; Mutanda et al., 2011). Especially the co-production of high value products is considered as important factor, that needs nevertheless careful planning since associated markets are easily saturated (Stephens et al., 2010).

For the production of liquid fuels non- or monounsaturated fatty acids with a carbon chain length of 16 or 18 were reported as preferable sources (Carlsson et al., 2007). Though the degree of unsaturation of fatty acids can be reduced by partial catalytic hydrogenation (Bouriazos et al., 2010), additional efforts in refining could be minimized by choosing strains that readily show suitable lipid profiles. Transesterification was described as a well-established process in the conventional production of biodiesel (Chisti, 2007), though there might be strain-dependent differences in the refinery technologies that are required for catalytic upgrading of the algal material (Tran et al., 2010). Further important factors are the suitability of these strains for the respective cultivation setup, where in principle two main concepts exist: open ponds and closed photobioreactors, whose advantages and disadvantages have been extensively reviewed in the past (Chisti, 2007; Mata et al., 2010; Schenk et al., 2008). In general, the costs of biomass production in photobioreactors is currently estimated to range one order of magnitude higher than those in ponds (Mata et al., 2010), therefore limiting economically viable cultivations to production of high value compounds as astaxanthin and nutraceuticals (Schenk et al., 2008). Open pond systems could provide a low-cost alternative for producing bulk chemicals as for liquid biofuels, but would therefore require competitive strains that are resistant to water loss by evaporation, resulting in increasing salinities, as well as to contaminating organisms (Schenk et al., 2008). To sum up, a strain of interest for biofuel applications should combine a large variety of properties, some of which might be even subject to common trade-offs e.g. high oil content versus high biomass accumulation (Sheehan et al., 1998).

1.5. Previous research and screening programs on microalgae

The suitability of algae for conversion to fuels was intensely investigated within the Aquatic Species Program that was initiated by the U.S. National Renewable Energy Laboratory and lasted from 1978 to 1996 (Mata et al., 2010; Sheehan et al., 1998). The massive screening effort in the frame of this program resulted in a collection of more than 3,000 strains, that was eventually narrowed down to about 300, with the best candidates mainly found among Chlorophytes, diatoms and several Eustigmatophytes, with a comparatively high number of representatives of the genera *Chaetoceros*, *Chlorella*, *Navicula* and *Nitzschia* (Sheehan et al., 1998). As one of the conclusions, diatoms were hypothesized as better choice for biofuel production in terms of highest lipid contents (40 – 60% of dry weight), but they showed disadvantages in the form of

silicate supply and low resistance to temperature fluctuations (Sheehan et al., 1998). Among the other, green algal strains *Monoraphidium minutum* was mentioned by name, assigned as high lipid producer but exhibiting unusual DNA properties (high degree of DNA base modification, 71% GC content) and hard cell walls (Jarvis et al., 1992; Sheehan et al., 1998).

Since the end of the program in 1996 (Sheehan et al., 1998), research efforts to find suitable strains have not ceased, as it is summarized within several comprehensive studies (Chen et al., 2011; Hu et al., 2008; Mata et al., 2010). It has to be noted that important parameters affecting the cellular lipid profile (e.g. medium composition, availability of a reduced carbon source, light intensity and time point of harvesting) were not similar in between many of the published studies, so that large differences of biomass productivities and lipid contents might be reported for one species and even the same strain (Chen et al., 2011). However, some species and strains of interest emerged, showing similar tendencies within the different setups, therefore being recommended for liquid biofuel applications.

Several organisms have been already proposed and investigated in more detail on their suitability for biofuel purposes as *Nannochloropsis* spec. (F&M-M24), which reached lipid productivities of up to 204mg l^{-1} day^{-1} (Rodolfi et al., 2009). A recently isolated strain of *Nannochloropsis* (BR2) was also identified as best performer in terms of fatty acid productivity in another study (Lim et al., 2012). Breuer et al., 2012, recently reported neutral lipid productivities exceeding 300mg l^{-1} day^{-1} for *Scenedesmus obliquus* (UTEX 393), which was identified as best performer of a screening of well-known strains. A further strain of *Scenedesmus*, which was an own isolate, showed also promising lipid productivities in comparison to the other investigated strains by Griffiths et al., 2011. An outstanding lipid productivity was also reported for a strain of *Chlorella vulgaris* (CCALA 256) with 604mg l^{-1} day^{-1} (Přibyl et al., 2012), similarly another strain of this species (IBL-C105) came out as best performer of further screening by Nascimento et al., 2013 with a lipid productivity of 205mg l^{-1} day^{-1}. Best performers of other screening approaches include *Tetraselmis* sp. (Huerlimann et al., 2010) and *Chlorococcum humicola* (Chaichalerm et al., 2012). *Monoraphidium* sp. was selected after a screening of 147 strains for more detailed investigation of its potential for biofuel purposes by (Yu et al., 2012) due to promising oil contents and growth rates. When Dinophytes and Raphidophytes were investigated, *Alexandrium minutum* appeared as most promising, showing lipid productivities of 81 mg l^{-1} day^{-1} in an outdoor bioreactor (Fuentes-Grünewald et al., 2012).

Taking into account the tremendous biodiversity of algae, their actual potential still remains to be explored and promises new insights into their lipid metabolism und principal mechanisms of TAG accumulation (Hu et al., 2008; Liu & Benning, 2013). Bioprospecting is therefore considered of on-going relevance to identify strains with promising properties in terms of biomass generation and lipid accumulation (Merchant et al., 2012; Mutanda et al., 2011). Especially the isolation and selection of strains from local water bodies is currently considered as promising approach to gain strains with competitive growth properties under the respective local climatic conditions (Mutanda et al., 2011; Talukdar et al., 2012).

1.6. Synthesis of oils in microalgae

Intense research was performed in the model organism *C. reinhardtii* (Liu & Benning, 2013; Merchant et al., 2012), providing the understanding of some of the principal mechanisms within fatty acid synthesis and glycerolipid formation. The genes required for the *de novo* synthesis of fatty acids have been identified and annotated for *C. reinhardtii* (Riekhof et al., 2005). In a first step, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylases (ACC) (see Figure 2, reaction 1), followed by the transfer of a malonyl group from a CoA to an acyl carrier protein (ACP) mediated by a malonyl-CoA:ACP transacylase (MAT) (reaction 2). Subsequently the β-ketoacyl-acyl-carrier-protein synthase (KAS III) as part of the heteromultimeric type II fatty acid synthase (FAS) catalyses the condensation reaction between malonyl-ACP and acetyl-CoA forming acetoacetyl-ACP (reaction 3), thereby releasing CO_2 (Hu et al., 2008; Riekhof & Benning, 2009). Acetoacetyl-ACP is then reduced by 3-oxoacyl-ACP reductase (KAR) (reaction 4), followed by dehydration by beta-hydroxyacyl-ACP dehydratase (HAD) (reaction 5). The next step is the reduction by an enoyl-ACP reductase (EAR) (reaction 6), and the acyl-ACP enters subsequently the next condensation reaction with malonyl-CoA catalysed by KAS I (reaction 3) as described in detail previously (Hu et al., 2008; Riekhof & Benning, 2009; Riekhof et al., 2005; Rismani-Yazdi et al., 2011).

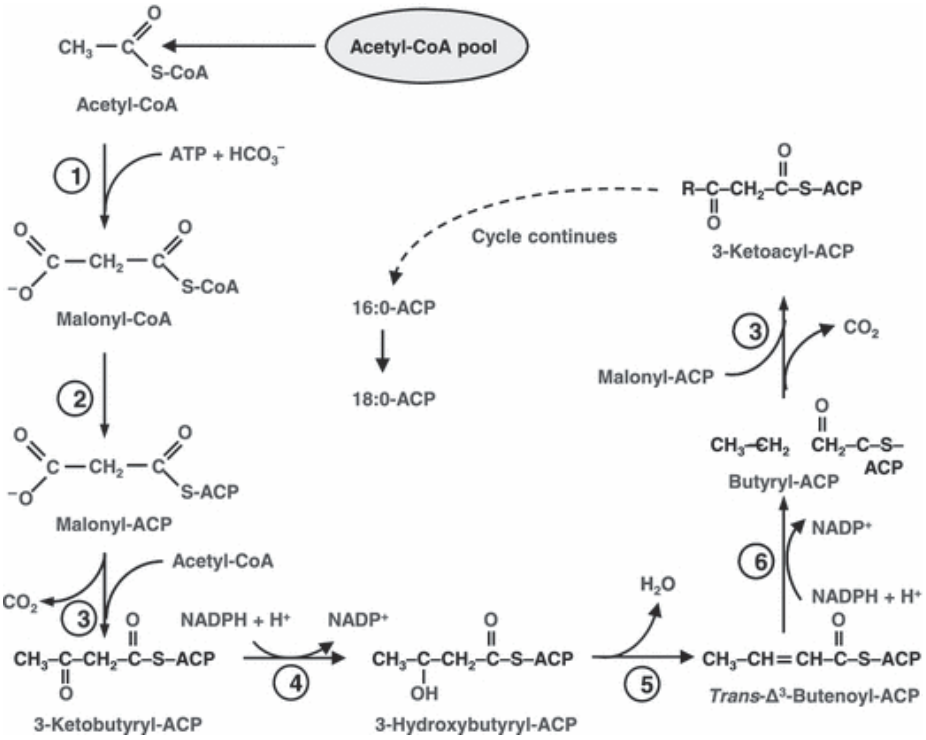

Figure 2 Fatty acid synthesis. Reprinted from Plant Journal, Volume 54, Issue 4, Qiang Hu, Milton Sommerfeld, Eric Jarvis, Maria Ghirardi, Matthew Posewitz, Michael Seibert. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. 621-639. Copyright (2008), with permission from John Wiley and Sons.

After repeated rounds of elongation, the acyl-ACP either serves directly as substrate for generation of glycerolipids within the chloroplast or the fatty acid is cleaved from the acyl carrier protein by thioesterases, releasing the free fatty acid usually as palmitate (C16:0) or stearate (C18:0) (Hu et al., 2008; Riekhof & Benning, 2009; Riekhof et al., 2005; Rismani-Yazdi et al., 2011). The fatty acid can be subjected to several modifications, as elongation or desaturation. Several orthologous genes of the fatty acid desaturase gene complement of *Arabidopsis* could be also identified for *C. reinhardtii* (Riekhof et al., 2005). The presence of further polyunsaturated fatty acids as C18:4 and C18:3 and C16:4 hints towards further desaturases, whereof a Δ5 desaturase has been identified recently (Riekhof & Benning, 2009).

The synthesis of glycerolipids is performed at different cellular sites in *C. reinhardtii*, with monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) exclusively and phosphatidylglycerol (PG) as well as

phosphatidylinositol (PI) partially synthesized by the chloroplast, while diacylglycerol-*N,N,N*-dimethylhomoserine (DGTS) and phosphatidylethanolamine (PE) appear of extraplastidic origin (Giroud et al., 1988). In contrast to higher plants, *C. reinhardtii* lacks the phospholipid phosphatidylcholine (PC), which is possibly functionally replaced by DGTS (Wada et al., 2009), a point which is currently under discussion (Liu & Benning, 2013).

The synthesis of TAG in *C. reinhardtii* is usually hypothesized to be located at the endoplasmic reticulum as in plants (Li-Beisson et al., 2010; Liu & Benning, 2013), but recently evidence arose that TAG can be also assembled in the chloroplast under certain conditions (Fan et al., 2011). Two main pathways for the synthesis of TAG are commonly distinguished, the acyl-CoA dependent pathway, also known as Kennedy pathway, where fatty acids are sequentially transferred to a glycerol-3-phosphate backbone (Coleman & Lee, 2004; Li-Beisson et al., 2010; Riekhof et al., 2005), and an acyl-CoA independent pathway, using phospholipids or diacylglycerol as acyl donors for TAG synthesis (Dahlqvist et al., 2000; Stahl et al., 2004; Yoon et al., 2012) (Figure 3). The acyl-CoA-dependent pathway relies on glycerol-3-phosphate, which is acylated by the action of a glycerol-3-phosphate O-acyltransferase (GPAT), thereby forming lysophosphatidic acid (Figure 3). A further acylation step is mediated by 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) to form phosphatidic acid, which is subsequently dephosphorylated by phosphatidate phosphatase (PP). The resulting diacylglycerol (DAG) is converted by diacylglycerol-acyltransferases (DGAT, DGTT) to TAG (Coleman & Lee, 2004; Hu et al., 2008; Riekhof & Benning, 2009; Rismani-Yazdi et al., 2011). GPAT as well as AGPAT were found to be up-regulated under nitrogen starvation in *Neochloris oleoabundans*, while PP and DGAT did not show pronounced changes in transcript abundance (Rismani-Yazdi et al., 2012). In contrast, an up-regulation of transcript abundance of PP could be shown for *C. reinhardtii* under nitrogen starvation (Miller et al., 2010). In *C. reinhardtii* two different forms of diacylglycerol-acyltransferases corresponding to type-1 (DGAT) and type-2 (DGTT) in *Arabidopsis* were identified (Liu & Benning, 2013). While the presence of DGAT type-2 homologues could be deduced from the published genome sequence of *C. reinhardtii* (Merchant et al., 2007), type-1 DGAT was recently discovered and annotated (Boyle et al., 2012). Both DGAT families are different in their sequence as pronounced by Liu & Benning, 2013.

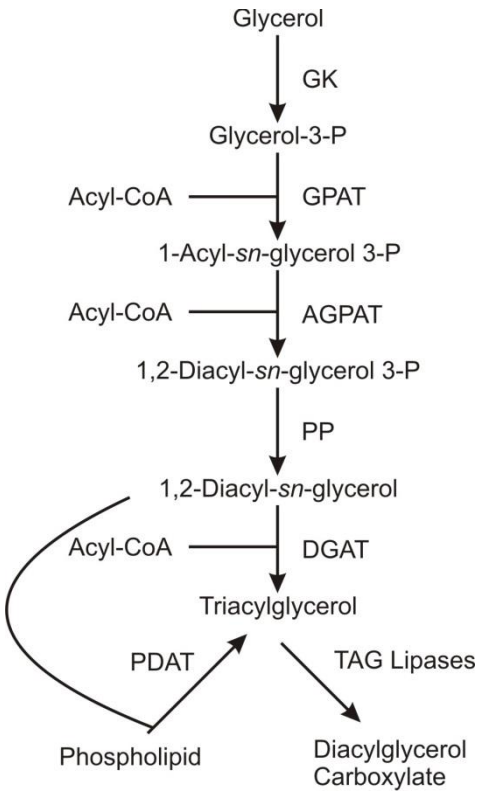

Figure 3 Schematic representation of the Kennedy-pathway leading to TAG synthesis. GK: glycerol kinase, GPAT: glycerol-3-phosphate O-acyltransferase, AGPAT: 1-acylglycerol-3-phosphate O-acyltransferase, PP: phosphatidate phosphatase, DGAT: diacylglycerol-acyltransferase, PDAT: phospholipid:diacylglycerol acyltransferase

It was reported that three DGTTs (DGTT 1-3) were up-regulated under nitrogen starvation (Miller et al., 2010). Recent work showed an increase of transcript abundance in only DGTT1 as well as for the newly discovered type-1 DGAT under nitrogen depleted conditions, while transcript levels of DGTT2 and DGTT3 remained comparatively stable (Boyle et al., 2012). DGTT4 and DGTT5 showed a rather low transcript abundances in both nutrient replete and nitrogen starvation conditions (Boyle et al., 2012). However, when DGTT4 was investigated under nitrogen starvation in photoautotrophic conditions, an increase of transcript abundance could be observed (Msanne et al., 2012). Vice versa, DGAT type 1 showed comparatively low expression, neither exhibiting a strong increase or decrease of transcript abundance (Msanne et al., 2012), therefore indicating differentiating expression patterns following the availability of reduced carbon sources (Boyle et al., 2012).

The acyl-CoA-independent synthesis, mediated by phospholipid:diacylglycerol acyltransferase (PDAT), is hypothesized to also play an important role in TAG generation in *C. reinhardtii*. PDAT unites different functionalities and is capable of handling various substrates, ranging from TAG synthesis via transacylation of DAG with acyl groups of phospholipids or galactolipids to the transacylation of two DAG (Yoon et al., 2012). Its role is discussed in the context of membrane remodelling and TAG homeostasis since it was not only able to hydrolyse phospholipids, galactolipids and cholesteryl esters but also TAG (Yoon et al., 2012). When investigated on the transcriptomic level, the expression pattern appeared less responsive to nitrogen starvation when compared to DGAT and DGTT1 (Boyle et al., 2012). No homologues for PDAT could be identified when the transcriptome of *N. oleoabundans* was investigated (Rismani-Yazdi et al., 2012), while it was present in *Dunaliella tertiolecta* (Rismani-Yazdi et al., 2012). Further roles of the two DGAT forms and PDAT beyond oil accumulation were assumed since all three genes were found with variable expression in a number of different tissues as root, stem and leaves of higher plants (Li et al., 2010a).

Once TAG is formed, it is deposited and stored in oil bodies, also called lipid droplets, whose structure and function have been recently subject to intense research (Huang et al., 2013; Liu & Benning, 2013; Merchant et al., 2012; Moellering & Benning, 2010; Nguyen et al., 2011). In yeast, they are formed at the endoplasmic reticulum (Merchant et al., 2012). In *C. reinhardtii*, the presence of a major lipid droplet protein (MLDP) with a size of 28 kDa was suggested as characteristic structural compound of lipid bodies (Moellering & Benning, 2010; Nguyen et al., 2011), assuming a similar function as of oleosins (Moellering & Benning, 2010). In higher plants, lipid bodies are characterized by the presence of oleosins as structural proteins (Huang et al., 2013; Merchant et al., 2012). The proteome of oil bodies was found rich in proteins in previous studies, including also proteins involved in neutral lipid synthesis as well as in the formation of DGTS (Moellering & Benning, 2010; Nguyen et al., 2011). However, other investigations using immunofluorescence microscopy showed recently the location of MLDP at the ER adjacent to lipid bodies, therewith indicating that MLDP is apparently not directly associated with lipid bodies in *C. reinhardtii* (Huang et al., 2013). This finding corresponds to the results of Wang et al., 2009, where only very low levels of proteins were found associated with lipid bodies and were assigned as apparent contaminants (Wang et al., 2009).

When the synthesis of triacylglycerols is addressed in algae, a further pathway is hypothesized in *C. reinhardtii*. There is evidence that, under certain conditions, TAGs can be produced primarily from chloroplast-derived DAG that is characterized by the occurrence of C16 fatty acids at its *sn*-2 position (Fan et al., 2011). The phenotype of a starchless mutant of *C. reinhardtii* also included the accumulation of neutral lipids in the form of lipid droplets within the chloroplasts (Fan et al., 2011; Goodson et al., 2011), therefore leading to the suggestion that the membranes of the chloroplast might be also sites of neutral lipid synthesis in *C. reinhardtii*. Glycerolipids as lysophosphatidic acid, phosphatidic acid and diacylglycerol are already formed in the chloroplast as precursors for the generation of polar lipids as MGDG, DGDG, SQDG and PG (Riekhof & Benning, 2009). Recently, chloroplast targeting peptides were identified for DGTT4, therefore leading to a model where the overall process of TAG generation was hypothesized to be also located within the chloroplast (Ramanan et al., 2013).

1.7. Function of neutral lipids in photosynthetic organisms

The triacylglycerol pool within the cell serves several functions, with the most prominent one being a storage compound for energy and carbon (Hu et al., 2008; Li-Beisson et al., 2010; Solovchenko, 2012). Usually high biomass productivity and lipid accumulation can be considered as mutually exclusive as stated as one major outcome of the ASP (Sheehan et al., 1998), therefore pronouncing this specific storage function under adverse environmental conditions (Hu et al., 2008; Solovchenko, 2012).

Nitrogen starvation is known as one of the major triggers for TAG accumulation in *C. reinhardtii* (Boyle et al., 2012; Msanne et al., 2012), *Scenedesmus* (Adams et al., 2013; Breuer et al., 2012), *Chlorella* (Adams et al., 2013; Breuer et al., 2012; Griffiths et al., 2011; Herrera-Valencia et al., 2011; Přibyl et al., 2012) as well as in many other members of the green algal lineage (Adams et al., 2013; Breuer et al., 2012; Griffiths et al., 2011; Msanne et al., 2012; Rodolfi et al., 2009; Sheehan et al., 1998; Yamaberi et al., 1998). After the nutrient replete media is depleted of nitrogen sources, cells still sequester carbon dioxide or import further carbon sources, and channel these compounds towards carbohydrate or lipid synthesis instead of protein production (Richardson et al., 1969). Depletion of nitrogen was also shown to be an effective trigger for TAG accumulation in *Phaeodactylum tricornutum* (Breuer et al., 2012; Griffiths et al., 2011),

with silicate deficiency being also an important factor to induce lipid accumulation in diatoms (Sheehan et al., 1998). Among the Eustigmatophytes, strains of the genus *Nannochloropsis* are well-known to increase their neutral lipid as response to nitrogen depletion in the media (Breuer et al., 2012; Pal et al., 2011; Rodolfi et al., 2009; Simionato et al., 2013). It should be however noted that nutrient starvation lead to less promising results in cyanobacteria, where no substantial increase of lipids was observed as shown for *Anacystis nidulans* and *Microcystis aeruginosa* (Piorreck & Pohl, 1984) or *Oscillatoria willei* (Kumar Saha et al., 2003) and *Spirulina platensis* (Griffiths et al., 2011). A further trigger is sulfur starvation, resulting in an increase of neutral lipids in *C. reinhardtii* (Matthew et al., 2009) as well as in strains of *Chlorella* and *Parachlorella* (Mizuno et al., 2013).

Accumulation of TAG could also occur as response to salt stress, as it was shown for *Dunaliella tertiolecta*, where an increase of salt concentrations up to 1M NaCl resulted in a strong accumulation of TAG without any pronounced decrease of growth rate (Takagi et al., 2006). Increasing salt concentrations were also shown to influence lipid contents in freshwater algae as *Chlamydomonas mexicana* and *Scenedesmus obliquus*, where an optimization of salt concentrations at low level to 25mM resulted in improved growth and contents of lipids (Salama et al., 2013). An increase of lipid contents at higher salt concentrations was furthermore observed for *S. obliquus* but however combined with a drastically impaired growth rate (Kaewkannetra et al., 2012). Besides salinity, pH also was discussed on its influence on TAG accumulation (Solovchenko, 2012). Increasing the pH from 8 to 10 resulted in an increase of fatty acid contents in the freshwater organism *Neochloris oleoabundans* albeit also in reduction of growth rate (Santos et al., 2012).

The cellular content of TAG is reported to be increased under high light intensities (Hu et al., 2008; Orcutt & Patterson, 1974; Solovchenko, 2012). The underlying mechanism involves the NADPH-consuming TAG synthesis that relieves the pressure of excess electrons within the electron transport chain that could otherwise result in an over-production of reactive oxygen species (Hu et al., 2008). Therefore a continuing carbon input combined with cell-division inhibiting nutrient limitation are besides light energy important components to produce those high-energy compounds (Přibyl et al., 2012).

A further complex role of TAG homeostasis for cell functioning is suggested since down-regulation of PDAT resulted not only in a reduction of the TAG content but also in a phenotype with impaired growth rate under nutrient replete conditions (Yoon et al., 2012). It was concluded that the TAG pool could be an important source of fatty acids for membrane remodeling under vigorous growth (Yoon et al., 2012). The outstanding role of lipid turnover during vegetative growth is supported by the finding of a further lipase, CrLIP1, that is able to hydrolyze DAG and major polar lipids in *C. reinhardtii* and shows comparatively high transcript abundance under nutrient replete conditions (Li et al., 2012a). Interestingly, CrLIP1 failed to degrade TAG *in vitro*, while its down-regulation resulted in a delayed rate of TAG degradation after nitrogen starved cultures were re-supplied with a nitrogen source (Li et al., 2012a). Furthermore, the incorporation of free fatty acids into glycerolipids by TAG formation could play an additional role for detoxification processes (Listenberger et al., 2003; Petschnigg et al., 2009; Yoon et al., 2012). Therefore, multiple cellular functions appear to be connected to the generation of TAG, being subject to ongoing investigations.

1.8. The model organism *Chlamydomonas reinhardtii*

Among the various algae with sequenced genomes (Merchant et al., 2007; Radakovits et al., 2012; Sasso et al., 2012; Vieler et al., 2012), *Chlamydomonas reinhardtii* is one of the best investigated ones, with a comprehensive molecular toolkit available (Harris, 2009). *C. reinhardtii* is a biflagellate green algae, that can be found in a number of highly diverse habitats as e.g. soils, freshwater ponds and lakes or brackish waters (Harris, 2009). The organism is characterized by the presence of two mating types (plus and minus) combined with a simple cell cycle whose stages can be artificially induced (Harris, 2009).

The various laboratory strains assigned to this species that emerged over the past 60 years of intense research were in addition phylogenetically characterized (Pröschold et al., 2005). Many strains of *C. reinhardtii* lack a cell wall like the strain CC3491 (Casas-Mollano et al., 2008), which is preserved by other strains as CC1690 (Siaut et al., 2011). A notable factor is the loss of the nitrate reductase in many strains, since these strains are not able to metabolize nitrate and have to rely on other nitrogen sources as ammonia or urea (Pröschold et al., 2005). Apart from that, *C. reinhardtii* exhibits a remarkable metabolic flexibility and can be also grown

heterotrophically, while it still retains the elements required for photosynthesis (Merchant et al., 2007).

Nuclear as well as chloroplast transformation protocols have been established for *C. reinhardtii* (Day & Goldschmidt-Clermont, 2011; Walker et al., 2005). Various vectors were created allowing the nuclear overexpression of genes (Fischer & Rochaix, 2001; Neupert et al., 2009). Other vector systems can be used for specific gene down-regulation via artificial microRNAs (miRNAs) that rely on the cell intern RNA silencing machinery of *C. reinhardtii* (Molnar et al., 2009; Schroda, 2006; Zhao et al., 2007). *C. reinhardtii* was found to harbor a complex system of RNA interference, based on miRNAs as well as small interfering RNAs (Zhao et al., 2007), with an extensive duplication of its core polypeptides (Casas-Mollano et al., 2008).

Important aspects of research on *C. reinhardtii* include the study of photosynthesis, flagella as well as more applied aspects as its use for bioremediation purposes or biofuel generation (Merchant et al., 2007). While *C. reinhardtii* is a highly interesting target for hydrogen production (Kruse & Hankamer, 2010; Kruse et al., 2005; Melis & Happe, 2001; Schenk et al., 2008), further applications are emerging to use this organism in the field of liquid, fatty acid-based biofuels (Goodson et al., 2011; Li et al., 2010b).

1.9. Genetic engineering approaches to increase neutral lipids in microalgae

Increasing the cellular TAG contents and lipid productivity is an ongoing effort and therefore several targets have been proposed and were investigated (Merchant et al., 2012; Napier & Graham, 2010; Schenk et al., 2008; Sheehan et al., 1998). Much of this work was carried out in *C. reinhardtii* (Liu & Benning, 2013), but genetic transformation protocols are also available for other groups of algae, as it was e.g. recently established for the oleaginous organism *Nannochloropsis gaditana* (Radakovits et al., 2012). The investigation and sequencing of the genomes of members of the genus *Nannochloropsis* is subject to intense research (Kilian et al., 2011; Radakovits et al., 2012; Rodolfi et al., 2009; Vieler et al., 2012), therefore establishing this group as oleaginous model among Eustigmatophytes (Work et al., 2012).

Addressing the increase of the TAG content within the cell, DGAT was considered one of the main targets, since over-expression was shown to lead to higher oil contents in *Arabidopsis*

thaliana (Jako et al., 2001; Zou et al., 1997) and *Brassica napus* (Weselake et al., 2008; Zou et al., 1997). A particular DGAT1 isoform with a phenylalanine insertion was identified for maize, responsible for a substantial increase of oil contents (Zheng et al., 2008). It was also shown that blocking the fatty acid catabolism resulted in an accumulation of TAG in non-storage tissues (Napier & Graham, 2010; Slocombe et al., 2009), therefore being a further target.

Starchless mutants of *C. reinhardtii* proved to be highly interesting subjects to elucidate the lipid metabolism in green algae (Fan et al., 2011; Goodson et al., 2011; Li et al., 2010b; Li et al., 2010c; Ramanan et al., 2013). In the starchless mutant BAFJ5 an about tenfold increase of TAG compared to the wild type could be observed under highlight conditions and nitrogen starvation, with a concomitant total lipid content of 46% of dry weight (Li et al., 2010c). Moreover, these investigations lead to the hypothesis of an additional pathway for TAG formation that is localized within the chloroplast (Fan et al., 2011; Ramanan et al., 2013). Interestingly, the lipid content increased linearly in starchless mutants of *C. reinhardtii* with acetate as further carbon source in the media (Ramanan et al., 2013). Since lower lipid contents were found under phototrophic conditions, acetate supply was suggested as crucial factor in lipid droplet formation. It should be noted that heterotrophic growth did not result in significant differences in TAG content between wild type and BAFJ5, stressing the role of light availability for this process (Li et al., 2010c). An important function of DGTT4 for plastidic TAG generation was speculated, since DGTT4 was more than fourfold up-regulated under mixotrophic conditions with concomitant about six fold higher lipid contents when compared to phototrophic growth (Ramanan et al., 2013). Additionally, an over-expression of a thioesterase in the diatom *Phaeodactylum tricornutum* resulted in a 72% increase of fatty acids, possibly relieving feedback inhibition mechanisms caused by long-chain fatty acyl-ACPs (Gong et al., 2011). In conclusion, intense investigations were performed to increase the neutral lipid contents, which are an important prerequisite for strains that are intended for liquid biofuel production purposes (Mata et al., 2010).

2. Challenges

A decisive challenge for using microalgae in liquid biofuel production is to increase the economic viability of this process. For this purpose, biorefinery approaches were proposed (Campbell et al., 2011; Chisti, 2007; Mussgnug et al., 2010; Stephens et al., 2010) that allow the conversion of single or a selection of feedstock into either single or multiple products, depending on their categorization (Srirangan et al., 2012). Besides the optimization of each individual step of the biofuel production process, nutrient recycling is also considered an important factor (Figure 4, Mata et al., 2010).

Considering the use of microalgal biomass as feedstock for biorefinery approaches, the co-production of several high value products as beta-carotene in an initial phase would allow a self-subsidizing model (Stephens et al., 2010). However, when high value products are addressed, market size and saturation need to be taken into account (Stephens et al., 2010). After lipid extraction, the residual biomass can be used for biogas production by anaerobic digestion (Chisti, 2007; Mussgnug et al., 2010; Sialve et al., 2009; Yang et al., 2011b) as it has been investigated for oil cakes from e.g. sunflower seeds (Kolesárová et al., 2011). Biogas can be burned for heat and electricity generation and is as well usable as transportation fuel (Srirangan et al., 2012), therewith leading towards a concept that is highly focusing on bioenergy generation. Another biofuel that could be produced in such an approach is hydrogen as indicated previously (Mussgnug et al., 2010; Schenk et al., 2008).

The commercialization of those biofuels is expected to be highly dependent on economic incentives and the possibility of their blending with fossil-derived fuels, and will rely on first generation feedstock until the following generations enter the scope of economic viability (Srirangan et al., 2012). Therefore inexpensive feedstock is considered a major challenge (Srirangan et al., 2012), which was already stated by Sheehan et al., 1998, concerning large scale production systems. The price of algal material varies currently between 15 – 30 € kg^{-1} depending on the cultivation method, therefore being almost tenfold higher than the average price for biomass of other sources as rapeseed ranging between 0.25 and 0.45 € kg^{-1} (Kröger & Müller-Langer, 2012). Other calculations for biomass generation from microalgae indicate ranges between 3 and 13 € kg^{-1} depending on the cultivation method applied (Draaisma et al., 2013).

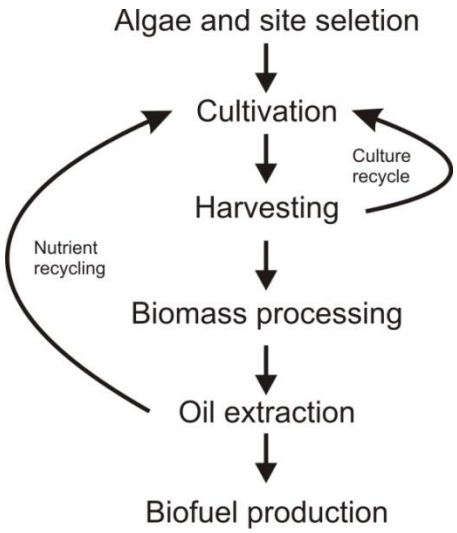

Figure 4 Value chain stages of the process to use microalgae for liquid biofuel generation, redrawn and modified from Mata et al., 2010

One of the identified bottlenecks is the requirement of organisms with high biomass productivities capable to be cultivated in outdoor pond systems (Sheehan et al., 1998). Main challenges for the economic viability include besides, ensuring optimal lipid productivity, the optimization of harvesting and oil extraction processes (Mata et al., 2010). A further important task is to find strains that are suitable for domestication, as it was performed with well-known crop plants in the past (Merchant et al., 2012). As methods of choice to improve strains, genetic engineering and trait selection as well as investigation of higher ploidy lines in algae have been suggested before (Merchant et al., 2012).

3. Aim of this work

Photosynthetic organisms as plants and microalgae are increasingly considered as feedstock for renewable energy production. In the field of renewable energies, the development of solutions for liquid biofuels is of special interest due to their high economic impact. Though microalgae have certain advantages when compared to higher plants, their actual potential is still far from being realized. An important factor is the lack of strains that combine robustness and high biomass productivities with high lipid contents and ease of down-stream processing. While some strains as of *Nannochloropsis* gained increasingly attention over the past years, much of the biodiversity found in algae collections as well as in the environment remains largely untouched.

In this work, collection strains that have not been in the focus of biofuel-related research before as well as recently isolated strains from mainly local water bodies were screened and investigated on robust growth, lipid contents and fatty acid composition. Based on the combination of general robustness and lipid productivity, strains of interest were selected for more detailed investigations. Besides biomass accumulation, high contents of neutral lipids are considered as essential for liquid biofuel generation, albeit usually being the result of exclusive physiological states. Since nitrogen starvation as well as light intensity were reported as important factors for TAG generation within the cells, these conditions were investigated for strains of interest in a two stage setup that includes a growth and a lipid accumulation phase.

The investigation of potential bottlenecks in triacylglycerol synthesis has gained increasing attention in the past years. *C. reinhardtii* can be considered as one of the most important model organism in the field of lipid related research, since its genome sequence and a comprehensive molecular toolbox allow targeted modifications. The final step of TAG synthesis is mediated by DGAT, which was therefore overexpressed to overcome potential limitations, especially under non-stressed conditions. Further pathways that could deprive TAG synthesis of precursors are the generation of polar lipids as DGTS. Since a down-regulation could result in an increase of metabolic flux towards TAG synthesis, the enzyme responsible for DGTS synthesis was down-regulated. These investigations are considered to shed more light into the complex interplay of lipid generation pathways which can be subsequently exploited to create oleaginous strains of microalgae.

When the quality of liquid biofuels is addressed, the fatty acid composition is a decisive factor, demanding mainly saturated and monounsaturated fatty acids. To investigate whether the microalgal strains comply with these requirements, the fatty acid profiles were determined.

A key element for a future economic viability could be found in a biorefinery approach, where the residual biomass is also utilized. Therefore, the performance of the algal biomass in fermentation reactors was investigated to elucidate the strains potential for methane production.

Once an oleaginous strain is found, the next step leads towards its sequencing to elucidate central aspects of its lipid metabolism and to identify targets for genetic manipulations to increase the overall lipid productivity and quality. To provide the basis for this work, the genome of the most promising candidate was sequenced and central parts of its lipid metabolism were reconstructed, allowing a future integration of the results acquired from the intense investigation of the lipid metabolism in *C. reinhardtii* into targeted genetic engineering approaches as well as the establishment of this new promising strain as oleaginous model organism for future domestication.

4. Discussion

This work is dedicated to the finding and investigation of strains of microalgae that could be integrated into liquid biofuel production concepts. Therefore, the approach that characterizes this work relies on the potential found in the biodiversity preserved in strain collections as well as in the environment.

For biofuel applications, candidate strains should not only exhibit high lipid productivities, but also include a resistant and robust growth phenotype, allow targeted genetic modifications and offer ease in down-stream processing. A correspondingly targeted screening can result in effective preselection of strains that comply with several of these requirements. The first part of this discussion focusses on the results of the screening approach and the candidate strains that have been identified in the course of this work.

High overall lipid productivity does not necessarily rely on rapidly growing strains that maintain simultaneously high lipid contents, but can be also reached in a two-stage setup, where an initial rapid growth phase is followed by a period of lipid accumulation, usually under starvation conditions. To ensure that the candidate strains follow this pattern, they were investigated in a comprehensive approach, combining nitrogen starvation with varying culture density that resulted in different light availability.

Besides cultivation parameters, TAG generation can be directly targeted using genetic manipulation. *C. reinhardtii* was chosen as model organism with a comprehensive molecular toolbox. The over-expression and down-regulation approaches on two different targets, DGAT and BTA1 respectively, were performed to elucidate their role on TAG accumulation under varying culturing conditions.

The sequencing of the species of interest, *M. neglectum*, allowed furthermore detailed insights into the potential of its lipid metabolism and can therefore provide a basis for future genetic engineering, thereby integrating the knowledge acquired in *C. reinhardtii*.

The three manuscripts presented in this work are:

[1] La Russa M., Bogen C., Uhmeyer A., Doebbe A., Filippone E., Kruse O., Mussgnug J. H. (2012) Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. Journal of Biotechnology, 162(1), 13-20.

[2] Bogen C., Klassen V., Wichmann J., La Russa M., Doebbe A., Grundmann M., Uronen P., Kruse O., Mussgnug J. H. (2013) Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production. Bioresource Technology, 133(4), 622–626

[3] Bogen C., Al-Dilaimi A., Albersmeier A., Wichmann J., Grundmann M., Rupp O., Blom J., Blifernez-Klassen O., Kalinowski J., Mussgnug J. H., Kruse O. Reconstruction of the lipid metabolism for the microalga *Monoraphidium neglectum* from its genome sequence reveals characteristics suitable for biofuel production. (Submitted)

Further work presented here include the following unpublished results:

[4] Bogen C. Isolation and comprehensive screening of strains on their potential for liquid biofuel generation.

[5] Bogen C. Investigation of the influence of BTA1 down-regulation on neutral lipid content in *C. reinhardtii*.

4.1. Screening of strains on biomass productivities and lipid contents

Whenever a screening for a specific purpose is performed, an important aspect in the early beginning is to choose the most promising starting material. There are different possibilities to gain promising strains for biofuel purposes, either relying on already known strains that have been identified by literature research (Breuer et al., 2012), focussing on strains of a prominent genus or species (Přibyl et al., 2012; Rodolfi et al., 2009) or directly isolating new strains from the environment (Chaichalerm et al., 2012; Lim et al., 2012; Yang et al., 2012) as well as a combination of those strategies (Griffiths et al., 2011).

In this work, strains were considered that originated from algae collections but were not in the focus of biofuel-related research so far [2], and this approach was combined with the

investigation of isolates from local water bodies after a preliminary investigation of growth properties [4]. These organisms were subsequently investigated in the same methodological setup, ensuring their comparability and to overcome limitations of variations in the experimental conditions. Culturing under nutrient replete conditions was chosen to allow maximum biomass productivities and to determine the basic lipid productivities that can be reached without nitrogen starvation.

The broad range of investigations of microalgae on their potential for biofuel applications is based on two main parameters: growth and lipid content (Chen et al., 2011). Biomass accumulation is one of the less problematic parameters to determine, since it is usually reported as dry weight of the total biomass (DW) or as ash free dry weight (AFDW) (Zhu & Lee, 1997). One of the challenges in screening approaches is the determination of lipid contents and the evaluation of their quality, since usually not all lipid classes but specifically triacylglycerols are the compound of interest for liquid biofuel applications (Hu et al., 2008). Traditional approaches apply time-consuming lipid extractions based on methanol and chloroform (Bligh & Dyer, 1959; Folch et al., 1957) or hexane (Davidi et al., 2011), often combined with fatty acid analysis of the total lipids fraction via gas chromatography coupled to flame ionization detection (GC-FID) (Makri et al., 2011; Msanne et al., 2012; Siaut et al., 2011) or to mass spectrometry (GC-MS) (Davidi et al., 2011; Poerschmann et al., 2004; Siaut et al., 2011). More sophisticated methods to determine the amount of lipids in the biomass include Fourier transform infrared micro-spectroscopy (Dean et al., 2010) and neutral lipid determination via high performance liquid chromatography (HPLC) coupled to MS (Fauconnot et al., 2004) or Charged Aerosol Detectors (CAD) (McNichol et al., 2011; Moreau, 2006). However, these investigations are comparably expensive, especially if the analytical equipment is not readily available (Wawrik & Harriman, 2010). Therefore some previous research was dedicated to finding fast and reliable detection methods for lipids, either based on colorimetry in comparatively inexpensive approaches (Wawrik & Harriman, 2010), fluorescent dyes as Nile Red (Chen et al., 2009; Elsey et al., 2007) and BODIPY (Cooper et al., 2010). Due to several advantageous properties as stability and the possibility to distinguish between polar and neutral lipids (Mutanda et al., 2011), much work currently relies on Nile Red (Chen et al., 2009; Lim et al., 2012; Yang et al., 2012). However, due to its shortcomings when inter-species variability is addressed, especially when strains with thick cells walls are considered (Dean et al., 2010; Mutanda et al., 2011), methods of a higher

reliability are required. A wide variety of strains of different phylogenetic origin was investigated in this work [2, 4], therefore harsher and less biased techniques were needed to ensure the comparability between the strains whether they originated from algae collections or were isolated in the course of the screening. It was shown that chloroform-methanol based techniques result in rather complete extraction of lipids when compared to other solvent system as hexane and isopropanol (Lee et al., 1998). Furthermore, the method of Bligh and Dyer (Bligh & Dyer, 1959) had a reduced efficiency in lipid extraction from tissues with increasing lipid contents (Iverson et al., 2001) when compared to the method of Folch (Folch et al., 1957). Therefore, Folch extraction was applied in the course of this work [1-4].

The different cultivation conditions and methods that are used among previous studies (Chen et al., 2011) result in some difficulties within their general comparability, however some common tendencies were observed as well as several differences to previously published work, indicating the presence of several promising candidate strains. As an example, *Dunaliella tertiolecta* has been reported to contain 16.7% oil content of ash free dry weight (Gouveia & Oliveira, 2009), a considerably lower value than the 24.4% of dry matter found here [2, Table 1]. This can be most likely attributed to differences in determining the dry weight as well as a different lipid extraction method used by Gouveia & Oliveira (2009). In contrast, far higher values of 60.6% have been reported by Takagi et al. (2006) from a ten days cultivation of *D. tertiolecta* (ATCC 30929) under phototrophic conditions and a supply of 3% carbon dioxide (Takagi et al., 2006), which could have resulted in unspecific starvation conditions. This finding underlines the high variability of total lipid values reported for the same microalgal species in different studies. The *D. tertiolecta* strain that was investigated here [2] showed comparative contents of lipids as another strain of *Dunaliella* spec. investigated in the same work. Both strains shared a highly similar fatty acid profile, pointing towards their potentially close relationship. Furthermore, the low total lipid yields of *Scenedesmus costatus* (11.9% of dried biomass) [2] correspond well to values reported from *Scenedesmus obliquus* of 12.7% in the stationary phase without supplementary glucose or induction of specific starvation conditions (Mandal & Mallick, 2009). However, other *Scenedesmus* strains were able to accumulate higher amounts of total lipids under phototrophic growth conditions, ranging between 18% and 21% of biomass (Rodolfi et al., 2009). The isolate Br6a II, closely clustering to *Scenedesmus obliquus*, however lays exactly within this

reported range [4], as well as the total lipid content of *S. obliquus* (SAG 276-6) was found on a similar level (17% of dry weight, [3]).

In this initial screening setup, *M. contortum* showed the best overall qualities compared to the other candidate strains, characterized by a good phototrophic biomass productivity (a mean of 239mg dry biomass l^{-1} day^{-1} for the overall cultivation period) and with comparatively high fatty acid abundances [2]. *Navicula salinicola* also showed high productivities, but was not considered for more detailed investigations since its growth performance was rather variable [2]. Other strains of the genus *Monoraphidium* also showed high biomass productivities above 200mg l^{-1} day^{-1}, notably *M. arcuatum*, *M. dybowski*, *M .neglectum* and *M. terrestre* [2]. Therefore this group could be found in the upper range when compared to other investigated strains. Eight further strains (*C. reinhardtii*, *Chlorococcum infusionum*, *Lobochlamys segnis*, *Muriella aurantiaca*, *Nannochloris eucaryotum*, *Navicula salinicola*, *Parachlorella kessleri* and *Pleurastrum insigne*) were also found with comparable biomass productivities, notably *P. kessleri* as best performer with 358mg l^{-1} day^{-1} [2]. This was also the case for the isolate HL1 [4], which shares a high sequence similarity to *P. kessleri* [4]. Recent investigations did not only report high lipid productivities (Li et al., 2012b; Přibyl et al., 2012) but also the scale-up of *P. kessleri* using a thin-layer bioreactor (Li et al., 2012b), therefore underlining the potential of this species and closely related organism when carbon sequestration and lipid production in general is considered.

Similarly *Chlorella vulgaris* was reported with high biomass and lipid productivities (Přibyl et al., 2012). Isolates as Sp2, Spain1 and LS1, which clustered with *C. vulgaris* based on their 23S rDNA sequence, reached also notably high biomass productivities [4], therefore confirming the suitability of these organisms for rapid biomass production. Interestingly, the best performer in terms of biomass production of the screening, Sp2, showed some clear differences in its marker sequence to *C. vulgaris*, therefore indicating a certain phylogenetic distance [4]. These results indicate that besides *Chlorella* and *Parachlorella*, *Monoraphidium* appears as a highly interesting genus for biomass generation especially when carbon sequestration is a further target (Mata et al., 2010; Sankar et al., 2011; Sydney et al., 2010).

4.2. Lipid productivity under photoautotrophic growth conditions

The primary aim of these studies [2, 4] was to identify strains which show high lipid productivity under nutrient replete growth conditions, without unspecific nutrient starvation. The best performers in terms of total lipid productivity, which could be identified in the course of the screening, were *Parachlorella kessleri*, *Navicula salinicola*, *Pleurastrum insigne*, *Monoraphidium contortum* and *Monoraphidium arcuatum* as well as Spain1 and Sp2 with comparable lipid productivities, which were found to range between 67 and 87 mg l^{-1} day^{-1} [Table 2, 3]. Comparable and even lower lipid productivities were reported for a number of photoautotrophically grown strains before (Chen et al., 2011). However, when compared to Griffiths and Harrison, 2009, these values appear low given the lipid productivities of *Amphora*, *Neochloris oleoabundans* and *Ankistrodesmus falcatus*, ranging between 109 and 160mg l^{-1} day^{-1}. As already mentioned by the authors (Griffiths & Harrison, 2009), comparison of lipid productivities obtained from different cultivation conditions, especially from non-optimised conditions, is rather difficult. Nevertheless, the lipid productivity was suggested a helpful parameter to identify key species (Griffiths & Harrison, 2009). In this regard, the best performer strains of *Nannochloropsis* reached 61mg l^{-1} day^{-1} in another broad investigation of various species on their potential for biofuel production purposes (Rodolfi et al., 2009). It should be noted that not the best performer but *Nannochloropsis* sp. with a productivity of 55mg l^{-1} day^{-1} was used for further optimization and more detailed investigation by (Rodolfi et al., 2009).

Under nutrient replete conditions, the lipid productivity of another investigation ranged from 13mg l^{-1} day^{-1} for *Ankistrodesmus falcatus* and *Pavlova* sp. to up to 55mg l^{-1} day^{-1} reached with *Cylindrotheca fusiformis* (Griffiths et al., 2011). The good performance of the diatom *C. fusiformis* (Griffiths et al., 2011) appears to be similar to the comparatively high lipid productivities of *Navicula salinicola* (78mg l^{-1} day^{-1}) in this work [2]. However, with another diatom species, *Phaeodactylum tricornutum*, considerably lower productivities with 30mg l^{-1} day^{-1} were reported (Griffiths et al., 2011), which was also the case for the isolates Br5b and LS2 [4]. These results might therewith be exemplary for the wide range of lipid productivity performance of different strains and species encountered in one specific setup, which in the case of this work was focussed on conditions that could also occur in larger scale cultivations.

Table 2 Mean lipid productivity of the strains originating from algae collections as indicated by Bogen et al., 2013 [2].

	Lipid productivity [mg l^{-1} day^{-1}]
Parachlorella kessleri	81.4
Navicula salinicola	77.9
Pleurastrum insigne	68.1
Monoraphidium contortum	67.9
Monoraphidium arcuatum	67.3
Chlorococcum infusionum	64.4
Monoraphidium dybowski	58.2
Monoraphidium terrestre	57.2
Lobochlamys segnis	52.8
Chlamydomonas reinhardtii	52.3
Nannochloris eucaryotum	50.1
Chloridella simplex	45.0
Muriella aurantiaca	44.2
Monoraphidium neglectum	42.7
Nannochloris spec.	41.3
Ankistrodesmus nannoselene	31.7
Chlorococcum costazygoticum	30.8
Dunaliella spec.	27.8
Coelastrella striolata	27.8
Characium californicum	25.9
Lobochlamys culleus	25.0
Dunaliella tertiolecta	23.8
Scenedesmus costatus	17.5
Chlorella luteo-viridis	15.8
Characium oviforme	14.8
Monoraphidium griffithii	13.6
Chloridella neglecta	13.2
Monoraphidium tortile	10.3
Coccomyxa chodati	3.2
Dunaliella granulata	3.0

Taking into consideration the overall lipid productivity, four different strains of *Monoraphidium* were among the eight best performers obtained from strain collections [2]. Since they showed this phenotype under rather high light intensities, combined with growth under autotrophic conditions

with CO_2 as carbon source, this genus exhibits promising traits for potential industrial applications.

Table 3 Mean lipid productivity of strains isolated in the course of this work [4]

	Lipid productivity [mg l^{-1} day^{-1}]
Spain 1	86.7
Sp2	79.6
Oe11a I	60.5
HL1	59.6
Oe11a III	52.6
LS1	49.9
Oe15a	38.2
Br6a II	34.5
Br7a	30.9
Oe14a I	27.9
Oe2a	27.6
Oe14b II	24.0
AL-1	22.5
LS2	19.6
Oe9b	17.5
Br5b	11.7
Br21	9.6
BS2 I	7.7
BS5 I	3.3
Br10a	2.1

4.3. Fatty acid abundance and composition under phototrophic growth

To evaluate the suitability of a strain for liquid biofuel applications, it was an important first step to quantify the amount of total lipids within the biomass and to calculate the lipid productivity. However, for the production of biodiesel it is equally important to determine the relative abundance of the fatty acids within the total lipid fraction, since compounds like sterols, pigments and hydrocarbons, which are part of the lipid extract, are not suited for the conversion into biodiesel (Pruvost et al., 2009). This can easily lead to an overestimation of the suitability of a strain as biodiesel feedstock as pronounced by McNichol et al., 2011. Therefore it is required to

determine the fatty acid contents of the respective lipid fraction (McNichol et al., 2011) as it was also performed in this work [1-4]. GC-FID is widely used for the determination and quantification of fatty acids from complex lipid samples as indicated before, nevertheless GC-MS was used in this work [1-4] which allows to overcome limitations imposed by GC-FID, which can lead to a misidentification of peaks as fatty acids that result from contaminants, artifacts or other coeluting compounds (Dodds et al., 2005).

Though strains may show similar total lipid contents [2, 4], the relative abundances of fatty acids can vary considerably, e.g. in the case of *Chloridella neglecta* (23% total lipids, relative fatty acid abundance of a value of 111) and *Monoraphidium griffithii* (22% total lipids, relative fatty acid abundance value of 59) [2]. This high variability of fatty acid abundances corresponds well to other recent investigations where distinct differences were found between the total lipid content of algal biomass and their fatty acid content (McNichol et al., 2011). The magnitude of these differences was highly variable between various samples (McNichol et al., 2011), therefore showing the importance to determine the fatty acid abundance. Here, diatom strains as *N. salinicola*, Br5b and LS2 showed the highest overall fatty acid abundances when grown under nutrient replete conditions [2, 4]. Lipids are considered as the main storage compound in diatoms besides chrysolaminarin (Sheehan et al., 1998), which is in accordance with this finding.

In contrast, green algae rather rely on starch for primary storage (Sheehan et al., 1998). Nonetheless, the strains BS2-I, *Coccomyxa chodatii*, *Chloridella neglecta*, *Chloridella simplex* and *Monoraphidium tortile* were also found with comparatively high fatty acid contents (above a threshold of a relative abundance value of 100). With the exception of *C. simplex* [2] these strains showed generally poor biomass productivities [2, 4] and can therefore be considered as a show-case for the trade-off between lipid content and biomass productivity. Since those strains showed rather slow growth, the increased light penetration into the cultures might be a furthermore decisive factor for lipid and fatty acid production. High light intensities can result in neutral lipid accumulation (Solovchenko, 2012), which could be the reason for the comparatively high fatty acid abundances encountered in these strains. This response might be highly species-dependent, since some strains as *Dunaliella granulata* show poor biomass accumulation combined with low fatty acid abundances (a relative abundance value of 49). A further exception was identified with *Chloridella simplex* [2], being possibly a rewarding target for more detailed investigation in the future.

Besides the overall fatty acid abundance the respective fatty acid profile is an important aspect to evaluate the suitability of a species for liquid biofuel applications (Chisti, 2007; Hu et al., 2008; Schenk et al., 2008). Several strains that either belong or cluster to *Chlorella* or *Parachlorella* were identified with high biomass and lipid productivities within this screening [2, 4]. However, with the exception of LS1, comparatively high contents of polyunsaturated fatty acids (especially C18:2, C16:2 and also C16:4 in case of Sp2 and Spain1) could be detected when their fatty acid profiles were investigated [4]. The fatty acid profile of *C. vulgaris* is known for the dominant presence of polyunsaturated C18 (Přibyl et al., 2012). *P. kessleri* also exhibited comparatively high contents of C18:2 (40 - 52%) (Li et al., 2012b). This finding could be partially confirmed in this work, where the investigated *P. kessleri* strain originating from the CCAP as well as the isolate HL1 showed high contents of C18:2 with 34.4% and 41% of the total fatty acids respectively, as well as certain amounts of unsaturated C16 fatty acids [2, 4], that were not reported previously (Li et al., 2012b). While polyunsaturated fatty acids require an additional hydrogenation step during down-stream processing when used e.g. for biodiesel production (Bouriazos et al., 2010), they might be highly valuable subject for other applications, as e.g. for the production of food commodities (Draaisma et al., 2013).

In contrast, the fatty acid profile of several of the *Monoraphidium* strains showed C18:1 dominating the fatty acid profile (above 50% of total fatty acids in *M. dybowski*, *M. neglectum* and *M. tortile*) [2]. Further high abundances of C16:0 [2] reveal fatty acid profiles which appear promising for biodiesel generation, e.g. in terms of oxidative stability as pronounced by Schenk et al., 2008. It could be shown that these strains already possess promising fatty acid profiles when cultured under nutrient replete conditions. Since *Monoraphidium contortum* showed a phenotype that combined robust biomass accumulation, comparatively high fatty acid abundances and a fatty acid profile dominated by the presence of C18:1 and C16:0, it was chosen for more detailed investigation [2]. *M. neglectum*, which appeared with considerably lower fatty acid abundances within this screening but a fatty acid profile with high abundances of C18:1 and C16:0 [2], was also chosen for comparison.

4.4. The impact of nitrogen starvation on lipid accumulation in *Monoraphidium*

The use of nitrogen starvation is a widespread method to induce neutral lipid accumulation in many green algal organisms as e.g. in *Chlamydomonas* (Boyle et al., 2012; Goodson et al., 2011; La Russa et al., 2012; Moellering & Benning, 2010; Msanne et al., 2012; Siaut et al., 2011), *Coccomyxa* (Msanne et al., 2012), *Chlorella* (Adams et al., 2013; Breuer et al., 2012; Laurens et al., 2012; Piorreck & Pohl, 1984; Přibyl et al., 2012), *Neochloris* (Adams et al., 2013; Breuer et al., 2012; Gouveia et al., 2009; Popovich et al., 2012) and *Scenedesmus* (Breuer et al., 2012; Mandal & Mallick, 2009; Piorreck & Pohl, 1984) as well as in groups of other phylogenetic branches as the Eustigmatophytes harbouring the promising genus *Nannochloropsis* (Radakovits et al., 2012; Rodolfi et al., 2009; Simionato et al., 2013). The effects of nitrogen starvation can be tremendous on lipid accumulation as shown for *Scenedesmus obliquus*, where the amount of total lipids was found at 43% of dry weight, compared to 13% from nutrient replete cultivation (Mandal & Mallick, 2009).

When nitrogen starvation was applied in this work, the neutral lipid content as well as the fatty acid abundances increased considerably for all of the investigated strains: *M. contortum* [2], *M. neglectum* and *C. reinhardtii* [3]. A comparative increase of neutral lipids was also observed for *S. obliquus* and *P. kessleri* investigated in the same setup [3]. Interestingly, the increase of neutral lipid contents was similar for both *Monoraphidium* strains: while *M. contortum* increased its neutral lipid content from 3% of dry weight (with a total lipid content of 25% of dry weight) under nutrient replete conditions to 13% under nitrogen starvation [2], *M. neglectum* showed also final neutral lipid contents of 14% of dry weight after nitrogen starvation, starting from about 1% neutral lipids of dry weight reached under nutrient replete conditions [3]. The increase of neutral lipids was even higher, when nitrogen starvation was combined with low culture densities, therewith resulting in an improved light penetration. Here, *M. contortum* reached 20 % neutral lipids [2] and *M. neglectum* a neutral lipid content of 21 % of dry weight [3]. These findings are consistent with other work, where even strains with rather low lipid contents under nutrient replete conditions showed a pronounced increase after nitrogen starvation (Mandal & Mallick, 2009). These gravimetrical determinations were also reflected by the increase of fatty acid abundances within the total lipid fraction as determined via GC-MS for *M. contortum* and *M. neglectum*, with both strains reaching similar abundances of fatty acids under nitrogen starvation in the low density cultures [2, 3]. The reference strain *S. obliquus* showed comparable neutral

lipid yields with 1% of dry weight under nutrient replete and 22% under nitrogen starved conditions [3]. These values are lower than the over 35% TAG of dry weight previously reported (Breuer et al., 2012), though being of identic origin (UTEX 393, SAG 276-6). Since this *S. obliquus* strain was recommended as highly promising for TAG generation (Breuer et al., 2012), the abundantly biomass accumulating *Monoraphidium* strains can be likewise considered as rewarding targets for biofuel generation.

The neutral lipid productivity of *M. neglectum* was calculated with 52 ± 6mg neutral lipids l^{-1} day^{-1} (including three days growth in nutrient replete conditions before transfer to nitrogen starvation conditions for five days) [3]. Similar productivities were reported for *Ankistrodesmus falcatus* (55mg l^{-1} day^{-1}) (Griffiths et al., 2011), though these values are rather difficult to compare due to differences in the growth properties of both investigated strains and the respective experimental setup as indicated before. *Ankistrodesmus braunii*, a further species within the Selenastraceae, was speculated to bear a potential for lipid synthesis that was equal or even superior to *Chlorella spp.*, and was reported to contain lipid contents of up to 73% of DW depending culture age and method of analysis (Williams & McMillan, 1961). This could indicate that a longer period of culturing could also lead towards higher lipid contents in the investigated strains of *Monoraphidium*. Interestingly, the *P. kessleri* strain tested here showed with a total lipid content of 34% of dry weight considerably lower lipid yields in nitrogen depleted conditions [3] than a number of strains investigated previously, ranging from 41% to 51% of dry weight (Přibyl et al., 2012). This could indicate that limiting factors in this experimental setup prevented a higher accumulation of neutral lipids. Since the lipid productivity is highly dependent on the initial biomass concentration, higher productivities of *M. neglectum* appear to be readily attainable in further optimization studies e.g. in regard to higher culture densities (Li et al., 2012b; Přibyl et al., 2012; Rodolfi et al., 2009).

A highly interesting property for biofuel applications is the pronounced increase of C18:1 and C16:0 abundances in both of the investigated *Monoraphidium* strains as response to nitrogen starvation, with both fatty acids dominating the profile (up to 76% of total fatty acids in *M. contortum* and 83% of total fatty acids in *M. neglectum* under optimal lipid accumulation conditions, i.e. comparatively low culture densities) [2, 3]. It should be noted at this point, that the highest lipid content in the biomass does not necessarily correspond to the state of highest lipid productivity of the culture, as it was demonstrated before (Adams et al., 2013).

Considering the accumulation of specific fatty acids, there are further similarities between both *Monoraphidium* strains and *S. obliquus*. *M. contortum* showed a C18:1 content of 55% of total fatty acids under nitrogen starvation [2], while in *M. neglectum* C18:1 contributed to 61% of total fatty acids [3]. Therefore, both strains exhibited a very similar response to nitrogen starvation though a certain phylogenetic difference between *M. neglectum* and *M. contortum* according to their 18S rDNA is probable (Yu et al., 2012). A similar report of a strong increase of C18:1 following nitrogen starvation is available for *Ankistrodesmus falcatus* strain UTEX 242, where oleic acid contents increased from 16% under nutrient replete conditions to 53% of total fatty acids under nitrogen limitation (Griffiths et al., 2011). Interestingly, the content of palmitic acid (C16:0) decreased from 26% to 11% of total fatty acids (Griffiths et al., 2011), contrasting the rather constant 22 - 24% contents of C16:0 of the total fatty acids in *M. neglectum* for both culture densities [3]. A similar pattern was reported for *S. obliquus*, where the C18:1 content increased from 22% in nutrient replete conditions to 50% under nitrogen limitation, while C16:0 decreased from 18% to 14% of total fatty acids under nitrogen starvation (Breuer et al., 2012). Both fatty acids were found dominating the nitrogen starved *S. obliquus* fatty acid profiles of other strains in earlier studies (Mandal & Mallick, 2009; Piorreck & Pohl, 1984). Like *Monoraphidium*, *Scenedesmus* clusters within the Chlorophyceae, where it was shown as member of a neighboring branch that also includes *Neochloris*, *Desmodesmus* and *Bracteacoccus* strains (Krienitz et al., 2001). However, this pronounced increase of C18:1 was also observed in members of other algal groups, e.g. *Coccomyxa* sp., belonging to the Trebouxiophyceae, where 44% were reached as response to nitrogen starvation. Interestingly, the fatty acid content of C18:1 in the TAG fraction was considerably higher, reaching 58% of the fatty acids therein (Msanne et al., 2012).

Interestingly, the fatty acid composition of *C. reinhardtii* showed some pronounced differences [3], although it is also a member of the Chlorophyceae (Harris, 2009). The fatty acid composition of the strain CC1690 which was investigated in this work [3] corresponds in general well to other *Chlamydomonas* strains like CC3491 (La Russa et al., 2012) or CC-3269 (Boyle et al., 2012) or CC125 (Msanne et al., 2012), with notable contents of polyunsaturated fatty acids as C18:2, 18:3 and some minor contents of polyunsaturated C16, as 16:4, detected even in nitrogen starved biomass. The increase of C18:1 appeared less pronounced than in other organisms like

Monoraphidium or *Scenedesmus*, indicating the possibility of a varying priority in the different mechanisms to accumulate fatty acids under nitrogen starvation.

4.5. Light availability as bottleneck for efficient lipid accumulation

A further important factor for the accumulation of lipids is the availability of light. High light intensities were shown to result in neutral lipid accumulation as pronounced before (Hu et al., 2008). It was shown that a higher degree of irradiance was responsible for an increase of saturated and monounsaturated fatty acids in *Nannochloropsis* sp. (Rodolfi et al., 2009). Rodolfi et al. (2009) underlined that those fatty acids were mainly associated with storage lipids. This assessment is supported by the observation made in this work regarding the experiments concerning the BTA1 down-regulation in *C. reinhardtii* [5]. In nutrient replete conditions, *Chlamydomonas* showed comparatively high Nile Red fluorescence values in low culture densities, thereby indicating an increase in neutral lipids within the first hours of cultivation [5]. As pronounced by Hu et al., 2008, the synthesis of triacylglycerols can counterbalance an overproduction of NADPH under photo-oxidative stress, therefore relieving the cell of excess electrons. This is in accordance with the results within this work, since this effect was found to disappear with rising culture densities [5, Figure 2 B therein], with a concomitant decrease of light penetration.

The role of light penetration into the cultures is also decisive for neutral lipid accumulation under nitrogen starvation, which could be also shown in this work [3]. The highest neutral lipid yields were obtained with low density cultures, causal of a better light penetration into the culture vessel. Dense cultures also showed an increase of neutral lipids under nitrogen starvation, but less pronounced. This finding was also reflected in the abundance of fatty acids, which were highest in the low culture densities for the investigated strains [3]. A similar observation was made when higher biomass densities of *Parachlorella kessleri* resulted in a lower accumulation of lipids of nutrient-limited cultures that were grown under identical illumination (Li et al., 2012b). Furthermore, Li et al. (2012) determined that the minimum light intensity required for the production of neutral lipids was about 150µmol m^{-2} s^{-1}.

Likewise, it was observed for *C. reinhardtii* that nitrogen starvation in high density cultures did not result in a pronounced increase of fatty acids within the total lipids, while an overall increase

of fatty acids was only observed at lower culture densities [3]. In high culture densities, *C. reinhardtii* showed an increase of neutral lipids but rather not in its fatty acid content [3]. Only when the light penetration into the culture was increased, a pronounced increase of fatty acids could be observed [3]. PDAT was shown to form triacylglycerol by DAG transacylation, using phospholipids and galactolipids as acyl donors (Yoon et al., 2012). The activity of this enzyme could therewith explain the clear increase of fatty acids in the neutral lipid fraction in the high density cultures, while the fatty acids within the polar fraction showed the tendency to decrease. The role of membrane recycling under nitrogen starvation in *C. reinhardtii* was also discussed recently, since several candidate TAG lipases were found up-regulated under these conditions (Boyle et al., 2012). This lead the authors suggest that lipases could play alternatively a prominent role in releasing fatty acids from membrane lipids for subsequent neutral lipid generation. It was previously suggested that after a two-day period of nitrogen starvation about 30% of TAG could potentially derive from the conversion of membrane lipids as MGDG and PG towards triacylglycerol (Fan et al., 2011), which could explain the phenotype observed for *C. reinhardtii* at high culture densities in this work. For both *Monoraphidium* strains, this picture appeared completely different. Both, *M. neglectum* and *M. contortum* increased their overall fatty acid abundances already about two-fold in the higher density cultures [data not shown, 3]. Compared to *C. reinhardtii* [3], this effect was remarkable and opens the door to comparatively high density culturing with concomitant neutral lipid accumulation under nitrogen starvation.

Nevertheless, very high light intensities can also negatively impact cells, since they can be also responsible for a suppression of triacylglycerol accumulation as stressed by Solovchenko (2012). However, within the investigated range of light intensity in this work, beneficial effects could be confirmed on neutral lipid accumulation for both *Monoraphidium* strains and *C. reinhardtii*. The high contents of C18:1 within the profiles of the investigated strains could be also attributed to the comparatively high light intensities applied in this work. Hu et al., 2008, pronounced that high light intensities favor the production of saturated and monounsaturated fatty acids while increased contents of polyunsaturated fatty acids are usually observed under low light conditions.

Radakovits et al. (2012) reported to reach optimal lipid yields by applying starting culture densities of about 3.6g l^{-1}, a value well above the initial culture density applied for nitrogen starvation here (0.8g l^{-1} in case of *M. neglectum*), so that a further experimental setup optimization is expected to yield substantially higher biomass and lipid productivities, especially

when combined with cultivation in thin layer bioreactors (Přibyl et al., 2012). Since both *Monoraphidium* strains showed a pronounced increase in fatty acids synthesis even in high density cultures, the next step would be to evaluate their response to different nitrate concentrations. It was shown recently that the highest lipid content of cells does not necessarily result in highest lipid productivities (Adams et al., 2013), optimal lipid productivities could be therefore reached by balancing biomass production and lipid accumulation, where the strain should ideally accumulate lipids under mild nitrogen starvation. A further increase of neutral lipid production was reported for *Scenedesmus* when optimized nutrient limitation was combined with a pre-treatment of cultures with glucose (Mandal & Mallick, 2009). The combination of mixotrophic or even heterotrophic growth with subsequent nitrogen starvation under phototrophic conditions could be a further possibility to optimize neutral lipid generation in *M. neglectum*.

These findings show that a detailed investigation of *Monoraphidium*-related strains could be highly rewarding. Since the lipid accumulation patterns appear comparable between those strains as shown for *M. neglectum* and *M. contortum*, taking also into account *Ankistrodesmus falcatus* (Griffiths et al., 2011), as well as the rather distantly related *Scenedesmus* (Breuer et al., 2012; Griffiths et al., 2011; Mandal & Mallick, 2009; Piorreck & Pohl, 1984), the further isolation and screening of those organisms targeting primarily high biomass productivities and resistance to adverse environmental conditions could result into a more widespread application of these strains for biofuel related purposes.

4.6. Salt stress and its effect on lipid accumulation

Besides the described role of light intensity, higher salinities are considered a key parameter to allow the exploitation of an organism for biofuel production, therefore using outdoor cultivation systems (Pal et al., 2011). Salt tolerance generally allows an organism to adapt to variations of salinity in culturing conditions, e.g. provoked by evaporation of media or growth in brackish or marine waters (Schenk et al., 2008), therefore less relying on valuable freshwater sources. Both *Monoraphidium* strains showed a certain tolerance to increasing salt concentrations, with *M. neglectum* being more robust than *M. contortum* [2, 3]. *M. neglectum* was not impaired in its growth pattern when 86mM NaCl was applied and reached even slightly higher biomass densities than at lower salt concentrations at the end of cultivation [3]. Compared to other freshwater algae

as *Chlamydomonas mexicana* or *S. obliquus*, which showed growth optima at a salinity of about 25mM (Salama et al., 2013), these values are comparatively high and underline the potential of *M. neglectum* for outdoor cultivation. The final biomass concentrations of *M. neglectum* grown in brackish water conditions with 86mM NaCl amounted up to 6.7g l^{-1} after a culturing period of 15 days [3]. These are much higher biomass concentrations than the 1.12 7g l^{-1} obtained for *A. falcatus* previously (Griffiths et al., 2011), underlining furthermore the robustness of this strain. Interestingly, *M. contortum* showed a considerable increase of neutral lipids when salt concentration increased, from 3% of dry biomass at 17mM to 11% of dry biomass at 86mM and finally 23% of dry biomass after transfer to salt concentrations of 170mM [2]. Takagi et al. (2006) showed previously that an increase of salt concentration from 0.5M to 1M lead to an elevated amount of TAG in cells of the marine strain *Dunaliella tertiolecta*, while maintaining comparative growth and nitrate depletion patterns (Takagi et al., 2006). In the case of *M. contortum*, the increase of neutral lipids was accompanied by a sharp decrease of biomass productivity. This finding indicates a clear trade-off between growth and neutral lipid content in the case of *M. contortum* which is opposite to the described phenotype in *D. tertiolecta* (Takagi et al., 2006). It is therefore likely that the increase of neutral lipids can be rather attributed to the reduction of the growth rate caused by salt stress rather than to a more direct effect of the salt concentration as assumed in the case of *D. tertiolecta* (Takagi et al., 2006). Since the cells were still able to synthesize fatty acids, they retain a certain metabolic viability, though cessation of cell division. Furthermore, the fatty acid profile shifted strongly towards C18:1 and C16:0 fatty acids [2], similarly to the fatty acid pattern observed during lipid accumulation during nitrogen starvation.

Two explanations for the pronounced increase of neutral lipids appear likely. Since triacylglycerols are considered as important storage compound in response to adverse environmental conditions (Hu et al., 2008), the cells could proceed with carbon fixation and conversion of sun energy through photosynthesis while cell division is inhibited by the high salt concentrations (Pal et al., 2011), providing them with a more convenient initial position as soon as environmental conditions improve for the cells (Siaut et al., 2011), e.g. decreasing salinity in water bodies after rain showers. On the other hand, cells show reduced growth rates and are therefore exposed to higher light intensities for a longer period of time. This could result in high photo-oxidative stress caused by generation of reactive oxygen species (ROS), and in cells

coping with these conditions by massive NADPH consumption through fatty acid synthesis as indicated by Hu et al., 2008. At this point further experiments are recommended to investigate the occurrence and the degree of ROS produced under these conditions.

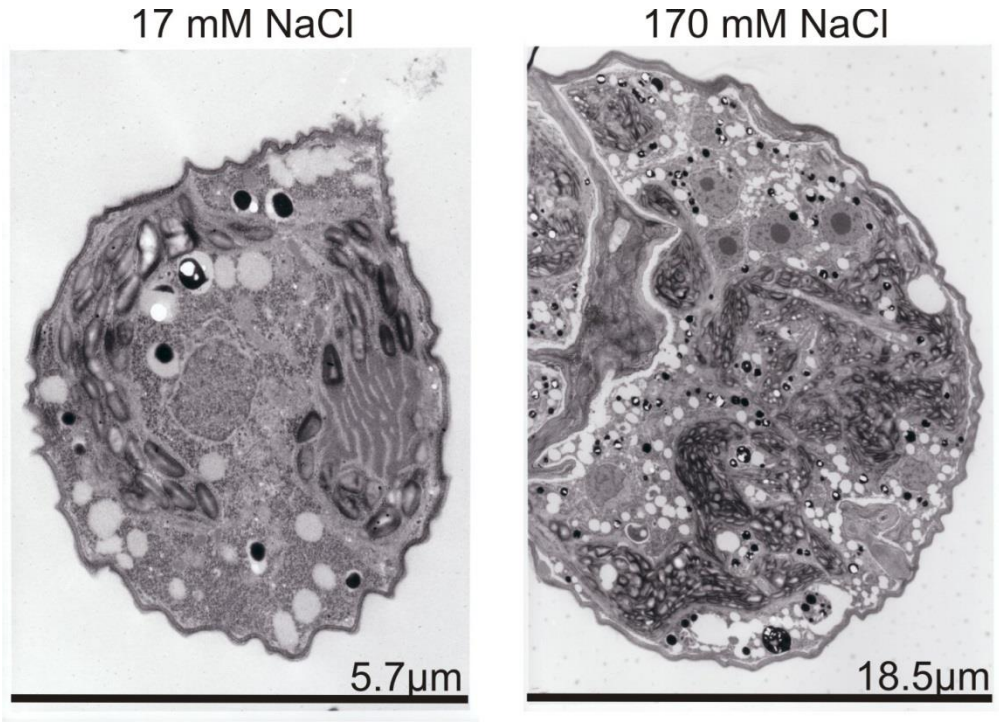

Figure 5 Electron micrographs of *M. neglectum* when grown in two different salt concentrations. Light spots within the cell indicate the presence of lipid droplets. At high salt concentrations, cells formed large complexes with multiple nuclei which were not observed at low salt concentrations (0.1%). Micrographs provided by courtesy of Uwe Kahmann, Bielefeld University.

A similar tendency in the shift in the fatty acid profile was also reported for *Nannochloropsis* sp. and *S. obliquus* (Pal et al., 2011; Salama et al., 2013). *Nannochloropsis* sp. not only increased its total fatty acid content when grown in media with rising salinity, which was even more pronounced when combined with high light intensities (700µmol m^{-2} s^{-1}), but also showed an enrichment of saturated C16 und mono-unsaturated C18 in its fatty acid profile (Pal et al., 2011). An enrichment of C18:1 in the fatty acid profile was also found for *S. obliquus* at salt concentrations of 40mM when compared to inferior concentrations (Salama et al., 2013). When the fatty acid pattern of *M. contortum* under higher salinities is compared to the fatty acid

distribution under nitrogen starvation, the high similarity could lead to the suggestion that similar responses could be triggered for the fatty acid synthesis under both adverse conditions in this species. Though an increase in neutral lipid contents at a sodium chloride concentration of 170mM could be also observed for *M. neglectum* [3], its attribution to a decrease of growth rate appears likely, especially when combined with a better availability of light to the culture as discussed before in the case of *M. contortum*. However, cells of *M. neglectum* showed a distinct response to higher salinities [3, Figure 5], that was not found for *M. contortum*. A distinct phenotype with large cells emerged in cultures with 86mM sodium chloride, which became the dominant form observed in cultures with 170mM sodium chloride after a period of ten days. Electron microscopy revealed the presence of multiple nuclei as well as the presence of lipid droplets and starch granules (Figure 5), indicating the presence of valuable compounds that could be converted to liquid biofuels, whether directly as triacylglycerols (Hu et al., 2008) or after a further conversion step as in the case of starch (Sivakumar et al., 2010).

4.7. *M. neglectum* as potential feedstock for liquid biofuels

M. neglectum could be identified as a highly promising candidate for biofuel applications, showing robust biomass accumulation, supporting various salt concentrations and differences in pH, as well as an efficiently lipid accumulating organism under nitrogen starvation, even in higher culture densities [3]. The next step towards the use of a strain for liquid biofuel application is its optimization, which can be performed on various levels. One example is the optimization of growth conditions or lipid productivities as discussed before, providing an optimal environment for high neutral lipid production. The other possibility is to improve the organism, e.g. by UV mutagenesis or targeted genetic engineering. Since the genome was sequenced in the course of this work, the targeted approach becomes attainable. Therefore, several points require further elucidation, e.g. the underlying mechanisms for this lipid accumulating phenotype and where it can be further improved. Here, the integration of knowledge about the lipid metabolism and neutral lipid accumulating pathways of other organisms is required. The green algae *C. reinhardtii* is one of the most intensely studied model organisms (Harris, 2009; Liu & Benning, 2013). Since the tools for targeted genetic modifications are well established and its genome sequence is publicly available as described before, it offers the possibility to directly interfere into metabolic pathways and to explore specific phenotypes. The availability of a comprehensive

set of transcriptomic, proteomic and metabolomic data allows deep insights and gain of knowledge of its functioning (Harris, 2009; Liu & Benning, 2013). Therefore, intense research has been carried out concerning its lipid metabolism, specifically to elucidate pathways leading towards the formation of triacylglycerols (Liu & Benning, 2013; Merchant et al., 2012).

4.8. Lipid metabolism in model organism *C. reinhardtii* and implications on *M. neglectum*

Two main pathways have been described for triacylglycerol synthesis in eukaryotic organisms, the acyl-CoA dependent pathway, which is also known as Kennedy pathway (Boyle et al., 2012; Coleman & Lee, 2004; Liu & Benning, 2013; Msanne et al., 2012; Yoon et al., 2012), and an acyl-CoA independent route which is mediated by phospholipid:DAG acyltransferases (PDAT) (Boyle et al., 2012; Yoon et al., 2012). Both pathways are present in *C. reinhardtii* and were intensely investigated (Liu & Benning, 2013). Due to recent findings, a further pathway for TAG synthesis is hypothesized which is located in the chloroplast (Liu & Benning, 2013). When metabolic pathways were reconstructed in the genome of *M. neglectum* in the course of this work, the respective enzymes could be also identified.

4.8.1. The role of DGAT for TAG accumulation

Triacylglycerol synthesis within the Kennedy pathway is characterized by a sequential acylation of glycerol-3-phosphate, with the final step being catalyzed by diacylglycerol-acyltransferases (DGATs) (Boyle et al., 2012; Coleman & Lee, 2004; Liu & Benning, 2013). Overexpression of DGAT was already shown to increase neutral lipids content, e.g. in *Arabidopsis thaliana* (Jako et al., 2001). The role of DGTT in neutral lipid accumulation was indicated by previous investigations (Boyle et al., 2012; Miller et al., 2010). Functional redundancy appears as important factor when genes of the lipid metabolism are targeted for over-expression or down-regulation. So, DGAT type-1 showed a similar expression pattern under nitrogen depletion as DGTT1, and both reached a similar degree in transcript abundance after 48 hours of starvation (Boyle et al., 2012). Interestingly the final transcript abundance was for both, DGAT type-1 and DGTT1 at a similar level as DGTT2 and DGTT3, as also stressed by the authors of that study (Boyle et al., 2012). It was furthermore discussed that an increase of triacylglycerols in the

DGTT over-expression strains could have been reversed by lipid degradation [1]. In the light of the recent characterization of PDAT as a multi-functional enzyme, that does not only catalyze TAG synthesis but also functions as TAG hydrolase (Yoon et al., 2012), this reasoning is further supported, especially since PDAT transcript abundance was found up-regulated rapidly after nitrogen starvation was induced (Boyle et al., 2012). Therefore the TAG pool could be controlled by various players. Interestingly the knock-down of PDAT in *C. reinhardtii* resulted in a decrease of TAG contents under nutrient replete conditions but not in a significant change under nitrogen depletion (Yoon et al., 2012), furthermore stressing the potentially prominent role of functional redundancy within PDATs and DGATs for TAG generation.

Table 4 ***List of DGAT type-2 homologues in C. reinhardtii***

Name	NCBI	Name according to Boyle et al., 2012	Augustus transcript ID (Boyle et al., 2012)
CrDGAT2a	XP_001702848.1	DGTT1	Cre12.g557750.t1.3
CrDGAT2b	XP_001691447.1	DGTT2	Cre06.g299050
CrDGAT2c	XP_001694904.1	DGTT3	Cre06.g299050.t1.2
CrDGAT2d	XP_001693189.1	DGTT4	Cre03.g205050.t1.2
CrDGAT2e	XP_001701667.1	DGTT5	Cre02.g079050.t1.3

It is noteworthy, that the carbon source appears to play also a role in DGAT expression, since DGAT type-1 appeared not responsive to nitrogen starvation under photoautotrophic conditions while DGTT3 and DGTT4 showed increased expression levels (Msanne et al., 2012). There is however some uncertainty about the regulation pattern, since DGTT4 was recently reported to be highly up-regulated under mixotrophic growth condition with a pronounced decrease of transcript abundance when investigated under nitrogen starvation in a starchless mutant *of C. reinhardtii* (Ramanan et al., 2013), which appears contrary to results of previous transcriptomic work (Boyle et al., 2012), but could also hint towards strain-specific differences. Since no increased triacylglycerol contents could be observed under neither nutrient replete conditions nor under nitrogen or sulfur starvation, the transcript abundance of DGTT1 to 3 appears not to be the bottleneck for TAG synthesis [1, Table 4]. It should be noted that another study on DGTT, overexpression and knockdown of three DGTT forms (DGTT1, DGTT3 and DGTT4 according to (Liu & Benning, 2013)) lead to an increase of lipids under nutrient replete as well as nitrogen

starvation conditions (Deng et al., 2012). This difference in result could be possibly attributed to varying degrees of transgene expression or the respective methods in lipid analysis as pronounced previously (Liu & Benning, 2013), so that the actual biological function of the various DGTT forms for TAG generation remains to be elucidated.

Interestingly only three DGAT homologues could be found in the genome of *M. neglectum*, thereof one DGAT type-1 homologue and two DGAT type-2 homologues [3]. Higher number of DGAT genes were reported for another oleaginous organism, the Eustigmatophyte *Nannochloropsis gaditana*, where ten DGAT homologues could be found, which is a considerably high number when compared to five in the green algae *C. reinhardtii* or one in the red algae *Cyanidioschyzon merolae* (Radakovits et al., 2012). The role of the high number of gene homologues in *N. gaditana* is however not clear yet, since e.g. only one homologue could be identified for PDAT which was subsequently proposed for overexpression or knockout-studies (Radakovits et al., 2012). It was shown in a transcriptomic study of *Neochloris oleoabundans* that the expression of DGAT as well as phosphatidate phosphatase remained relatively unchanged under nitrogen starvation, while glycerol-3-phosphate O-acyltransferase (GPAT) and 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) were up-regulated, leading the authors to the suggestion that post-transcriptional regulation of DGAT could play a crucial role (Rismani-Yazdi et al., 2012). The interplay between gene expression, functional redundancy and post-transcriptional regulation within the DGAT-PDAT complex are a highly interesting field for understanding and finally optimizing the generation of neutral lipids. A study addressing this issue was recently performed in the oleaginous yeast *Yarrowia lipolytica* (Zhang et al., 2012), showing that the combination of gene disruptions in PDAT, DGAT type 1 and DGAT type 2 resulted in a prominent decrease of oil content.

Taken together, the present results from the investigation of the different DGAT type-1 and the type-2 homologues in *C. reinhardtii*, other factors appear likewise important for the regulation of DGAT functioning as compartmentalization, e.g. in case of DGTT4 which is predicted to be localized in the chloroplast (Ramanan et al., 2013) or the role of metabolic flux as stressed by Boyle et al., 2012.

4.8.2. Down-regulation of BTA1 without impact on neutral lipid accumulation

As an outcome of the up-regulation of the three DGAT transcripts, the down-regulation of enzymes responsible for the synthesis of polar lipids was suggested, to potentially increase the metabolic flux towards TAG formation [1], which therefore could result in increased neutral lipid contents under nutrient replete growth. Considering the role of metabolic flux towards triacylglycerol synthesis, metabolites as phosphatidylcholine (PC) gain importance, since it is known to play a distinct role in fatty acid desaturation in plants (Wallis & Browse, 2010) and was shown to be a substrate to phospholipase activity of PDAT (Yoon et al., 2012). Since PC is replaced in *C. reinhardtii* by DGTS (Giroud et al., 1988; Liu & Benning, 2013), the role of BTA1 appears as interesting target to identify key enzymes involved in TAG synthesis and the lipid metabolism in *C. reinhardtii*. The transcript abundance of BTA1 was reported to be down-regulated as response to nitrogen (Boyle et al., 2012; Miller et al., 2010) as well as sulfur (González-Ballester et al., 2010) starvation and since the protein was initially reported to be associated with lipid droplets in *C. reinhardtii* (Moellering & Benning, 2010), its role for TAG accumulation appeared rather diverse and highly interesting. When down-regulation of BTA1 transcripts were investigated in the course of this work [5], no increase of neutral lipids could be found under nutrient replete conditions, therefore the deprivation of TAG precursors from the Kennedy pathway could have less impact on the basic level of triacylglycerols within the cell. In contrast, PDAT was previously shown to influence TAG contents under nutrient replete conditions, since a knock-down resulted in a significant decrease of the TAG concentration under nutrient replete conditions (Yoon et al., 2012).

Taking into account a putative role of DGTS and its synthesis by BTA1 in the formation of lipid droplets, a distinct phenotype could be expected under nitrogen starvation, when neutral lipids accumulate in the form of lipid bodies. It could be shown in this work that in the investigated range of down-regulation neutral lipid accumulation was not impaired in *C. reinhardtii* [5]. This could be attributed to potential post-transcriptional regulation of this protein, the presence of other enzymes with similar functioning or that still enough transcript was available within the cell. BTA1 showed a rather dominant transcript abundance in various investigations, which is about 4-fold higher than the transcript level of DGTT3 before and about 2-fold higher after 48 hours of nitrogen starvation (Boyle et al., 2012). A direct role of BTA1 in lipid droplet formation

as of phospholipids in plants discussed by Liu et al., 2012, could not be confirmed with the data obtained from BTA1 down-regulation within this work [5].

The presence of two genes in *M. neglectum* that code for BTA1 homologues indicates a potentially important function of this enzyme as well as a certain degree of conservation. As DGTS can be found in a large number of organisms (Dembitsky, 1996), a further important function might consist in overcoming limitation to phosphorous in aquatic environments (Liu & Benning, 2013; Van Mooy et al., 2009). Interestingly, the fatty acid profile of *M. neglectum* shows a higher degree of saturation than *C. reinhardtii* as shown in this work, so expression studies of BTA1 under nutrient replete as well as under nutrient starvation appears to be highly interesting to further elucidate its role, especially since two homologues could be identified within its genome. Since PC is the preferred substrate of PDAT as underlined by Boyle et al., 2012, the interplay between BTA1, PDAT and their substrates should be highly interesting field of further research. However, when the accumulation of neutral lipids needs to be addressed, BTA1 appears apparently with a less pronounced role than previously assumed.

The role of DGTS in *C. reinhardtii* could be possibly found within the fatty acid desaturation process, analogous to PC in plants (Wallis & Browse, 2010). DGTS as well as PC appear to be important substrates for the desaturation of oleic acid in *Parietochloris incisa* as underlined previously (Guschina & Harwood, 2006), therefore corrobating this reasoning. In Eustigmatophytes, DGTS was further discussed as donor of polyunsaturated C20 for the chloroplast (Guschina & Harwood, 2006). As underlined by Liu & Benning, 2012, PC is supposed to hold an important function in acyl exchange mechanisms in plants, so it appears highly interesting whether DGTS is able to substitute this function of PC in *C. reinhardtii* or whether other mechanisms, possibly mediated by PDAT, are involved. For biotechnological applications the possibility to substitute phospholipids by betaine lipids could be exploited by reducing the amount of phosphate within the nutrients supply while still maintaining growth of the organisms.

4.8.3. Further targets in lipid metabolism to increase TAG accumulation

Considering the acyl-Co independent pathway for TAG formation, the overexpression of PDAT was shown to moderately increase the neutral lipid content as discussed previously (Merchant et al., 2012). In the light of recent findings of a rather moderate effect of PDAT on TAG contents (Yoon et al., 2012), other targets might be more promising. There are various other targets that have been proposed to overcome bottlenecks to increase neutral lipid contents, as increasing the activity of acetyl-CoA carboxylase (ACCase) or KAS I (Yu et al., 2011). The overexpression of thioesterases to bypass feedback inhibition mechanisms could be also a worthwhile target, since such an approach resulted in an increase of the triacylglycerol content in the diatom *Phaeodactylum tricornutum* (Gong et al., 2011). Further targets that were proposed to gain higher triacylglycerol contents are the increase of glycerol-3-phosphate levels (Yu et al., 2011). Yu et al., 2011, also speculated that modulating key regulators in lipid metabolism might have more pronounced effects on lipid accumulation. A recent work used a screening where microalgal cultures were supplemented with small organic compounds with subsequent investigation of lipid contents, identifying lipid productivity increasing substances (Franz et al., 2013). This approach appears highly interesting to overcome not only bottlenecks in lipid synthesis pathways, presenting new targets for genetic engineering approaches, but also to elucidate major triggers or check-points in TAG accumulation.

In addition, as highlighted by Liu & Benning, 2012, the mechanisms of triacylglycerol accumulation not only between algae and higher plants but also within the algal realm show certain differences, so that the investigation of the lipid metabolism in its diversity within algae is required for a better understanding of the underlying mechanisms. A highly interesting point considers the storage of neutral lipids within the cells. The formation of cytosolic lipid bodies is well described for *C. reinhardtii* (Fan et al., 2011; Goodson et al., 2011; Moellering & Benning, 2010). Interestingly, starchless mutants also accumulate lipid bodies in the chloroplast (Fan et al., 2011; Goodson et al., 2011) which was not observed in the starch-accumulating wildtype (Goodson et al., 2011). Their exact structure in *C. reinhardtii* is currently under investigation (Huang et al., 2013; Moellering & Benning, 2010), resulting in the latest finding that the major lipid-droplet protein (MLDP) was rather associated with the ER than with lipid droplets (Huang et al., 2013). However, transcripts could be identified that encoded an oleosin-like protein, leading to the hypothesis of the development of oleosins from algae to higher plants. However,

after sequence comparisons with *Chlamydomonas* oleosin (AGG78364.1) and oleosin-like (AGG78363.1), no gene homologues could be identified in the genome of *M. neglectum*.

4.9. *Monoraphidium neglectum* as oleaginous model organism

Within this work, two different strains of the genus *Monoraphidium* were identified that show promising properties for liquid biofuel applications. Both strains, *M. contortum* [2] and *M. neglectum* [3], showed highly similar patterns in neutral lipid contents and fatty acid accumulation under nitrogen starvation. The higher robustness of *M. neglectum* in terms of biomass accumulation, salt tolerance and growth at different pH levels [3] lead to the recommendation of this strain for industrial applications. The large cell phenotype observed under distinct sodium chloride concentrations (Figure 3) indicates furthermore the potential of using *M. neglectum* to study adaptation mechanisms to salt stress. In addition, other *Monoraphidium* strains that were tested within the initial screening setup showed also rather high biomass productivities [2].

In previous work, *Ankistrodesmus falcatus* was presented as a species exhibiting promising lipid productivities (Griffiths & Harrison, 2009). The SAG strain 202-2 which was re-assigned as *A. falcatus* clustered closely to *M. neglectum* (Krienitz et al., 2001), therewith indicating a close relationship. Interestingly, both strains show different morphologies, as different arrangement of autospores and overall cell shape (Krienitz et al., 2001). A further strain of *A. falcatus* was recently isolated and proposed for lipid production (Talukdar et al., 2012). Therefore, the genome sequence of *M. neglectum* could not only become decisive to elucidate the lipid metabolism for this phylogenetic group but also offers the possibility to shed some more light into their general diversity and could offer the possibility of more detailed investigation of TAG accumulation in *A. falcatus*. This could be also the case for studies on the diversity of carbon concentrating and storage mechanisms of the various algal groups as they appear as interesting field of research (Hildebrand et al., 2013), so that comparative studies with the genome *of M. neglectum* can help to explore the underlying mechanisms within the green algal lineage. Combined with RNA sequencing, the draft genome sequence also opens the possibility for detailed comparison to *Neochloris oleoabundans*, another oleaginous organism that was shown to have comparable lipid

productivities (Griffiths & Harrison, 2009) and was recently subject to transcriptomic studies to elucidate its lipid metabolism (Rismani-Yazdi et al., 2012).

4.10. Potential of *M. neglectum* for industrial applications

A distinct feature of *M. neglectum* are the comparatively large cell complexes the organism can reach when cultivated at higher salt concentrations [3], especially when compared to other strains that are characterized by a rather small cell size as 2 - 5µm in the case of *Nannochloropsis* (Rodolfi et al., 2009). Though the cell walls appear also comparatively robust in *M. neglectum* when grown at lower salt concentrations, the larger spheres appear to be more fragile, and could be therefore an easier target for harvesting processes as *Nannochloropsis* exhibiting rather hard cells walls as pronounced by Rodolfi et al., 2009.

Harvesting is an important, cost-critical factor, which needs careful consideration especially if bulk production of compounds for biofuel purposes is targeted (Griffiths & Harrison, 2009), emphasizing the importance of cell sizes that are larger than 20µm to allow low-cost straining and filtration methods. The importance of cheap harvesting methods lead therefore to the consideration, that not the growth system with the maximum yield but with the least effort of harvesting could contribute significantly to the commercialization of microalgal fuel (Griffiths & Harrison, 2009). *M. neglectum* showed an increase of cell size well above this threshold after transfer to brackish water conditions and exhibits a highly promising phenotype, which could be readily exploited for cost-efficient harvesting [Figure 3, 3]. It should be noted that those complex structures furthermore have increased neutral lipid contents already under nutrient replete conditions [3].

The fermentation of the biomass of *M. neglectum* resulted in comparatively high methane yields, which ranged on a similar level as *C. reinhardtii* [2], therefore its inclusion into biorefinery concepts, where the residual biomass is used for biogas generation, can be considered as highly interesting approach for commercial viability.

In conclusion, the combination of robust growth under nutrient replete conditions, high neutral lipid contents under nitrogen starvation and a substantial increase of cell size when transferred to a salt concentration of 170mM NaCl underline the potential of *M. neglectum* as an organism

fitting to the various requirements for promising microalgal strains (Griffiths & Harrison, 2009; Mata et al., 2010). A further important point, when a strain is considered for industrial applications and up-scaling, is its resistance to contamination (Griffiths & Harrison, 2009). Within this work it could be shown that *M. neglectum* is able to tolerate different pH levels, which could be used to apply temporarily pH shifts to reduce contamination in open ponds systems (Mata et al., 2010). Therefore a three-stage process might be recommended, combining a biomass accumulation, lipid production and cell harvesting phase. Ideally, cultures should be grown under nitrogen limitation under outdoor conditions, and sodium chloride could be added after neutral lipid contents increased as response to nitrogen starvation. Increasing salt concentrations could be also easily reached by natural evaporation processes in outdoor pond systems under rather warm, non-humid climatic conditions.

5. Perspective

In this work, *Monoraphidium neglectum* could be characterized as robust strain, able to accumulate a high amount of neutral lipids with a profile that is dominated by favorable fatty acids [3]. Together with its other physiological properties these findings indicate that a bioenergy concept based on this species could be highly rewarding. With the sequenced genome, targeted genetic engineering approaches come into reach to improve the traits desired for large-scale production. As an example, higher biomass productivities could be reached by generating small antenna mutants as shown for *C. reinhardtii* (Mussgnug et al., 2007), since it allows an improved penetration of light into the cultures but also a reduction of photodamage as pronounced previously (Schenk et al., 2008; Work et al., 2012).

Traditional breeding programs with trait selection as mentioned before (Merchant et al., 2012), should be also promising alternatives to improve lipid productivity of strains as *M. neglectum*. Since starchless mutants of *C. reinhardtii* where shown to produce abundantly neutral lipids as storage compound, indicating a direct channeling of photosynthate into triacylglycerols (Liu & Benning, 2013), the generation of starchless *M. neglectum* strains could be a highly rewarding target for biofuel applications. It should be however taken into account that both *Monoraphidium* strains were not easily accessible to the widely used fluorescence dye Nile Red, probably due to their hard cell walls in freshwater conditions. To overcome these limitations, methods using HPLC coupled to CAD as described previously (Moreau, 2006) appear highly promising.

Growth in wastewaters is considered as of high potential to establish microalgae strains for liquid biofuel production (Pittman et al., 2011; Yang et al., 2011a), therefore investigation of the growth properties of *M. neglectum* in recycled water and its improvement by breeding are additionally recommended. As pronounced previously (Merchant et al., 2012), the combination of breeding and molecular engineering can be of great help to create domesticated strains of microalgae that combine those traits that are required for successful large scale cultivations, uniting high lipid contents, strong growth and predation resistance. To reach those goals, *Monoraphidium*

neglectum appears as a highly promising species, showing a phenotype characterized by robust biomass accumulation, strong increase of neutral lipids as response to nitrogen starvation, formation of easy-to-harvest cell complexes under higher salt concentrations, all of which are highly promising awaiting further domestication.

6. References

Aatola, H., Larmi, M., Sarjovaara, T., Mikkonen, S. 2009. Hydrotreated Vegetable Oil (HVO) as a Renewable Diesel Fuel: Trade-off between NOx, Particulate Emission, and Fuel Consumption of a Heavy Duty Engine. *SAE International Journal of Engines*, 1(1), 1251-1262.

Adams, C., Godfrey, V., Wahlen, B., Seefeldt, L., Bugbee, B. 2013. Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous green microalgae. *Bioresource Technology*, 131(0), 188-194.

Antizar-Ladislao, B., Turrion-Gomez, J.L. 2008. Second-generation biofuels and local bioenergy systems. *Biofuels, Bioproducts and Biorefining*, 2(5), 455-469.

Barnwal, B.K., Sharma, M.P. 2005. Prospects of biodiesel production from vegetable oils in India. *Renewable and Sustainable Energy Reviews*, 9(4), 363-378.

Basova, M.M. 2005. Fatty acid composition of lipids in microalgae. *International Journal on Algae*, 7(1), 101.

Bhattacharya, D., Yoon, H.S., Hackett, J.D. 2004. Photosynthetic eukaryotes unite: endosymbiosis connects the dots. *BioEssays*, 26(1), 50-60.

Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Physiology and Pharmacology*, 37(8), 911-7.

Bouriazos, A., Sotiriou, S., Vangelis, C., Papadogianakis, G. 2010. Catalytic conversions in green aqueous media: Part 4. Selective hydrogenation of polyunsaturated methyl esters of vegetable oils for upgrading biodiesel. *Journal of Organometallic Chemistry*, 695(3), 327-337.

Boyle, N.R., Page, M.D., Liu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S.J., Gallaher, S., Johnson, S., Benning, C., Pellegrini, M., Grossman, A., Merchant, S.S. 2012. Three acyltransferases and a nitrogen responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in Chlamydomonas. *Journal of Biological Chemistry*.

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H. 2012. The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, 124(0), 217-226.

Broun, P., Gettner, S., Somerville, C. 1999. Genetic engineering of plant lipids. *Annual Review of Nutrition*, 19, 197-216.

Campbell, P.K., Beer, T., Batten, D. 2011. Life cycle assessment of biodiesel production from microalgae in ponds. *Bioresource Technology*, 102(1), 50-56.

Carlsson, A.S., van Beilen, J.B., Möller, R., Clayton, D. 2007. Micro- and macroalgae: utility for industrial applications. Outputs from the EPOBIO project.

Casas-Mollano, J.A., Rohr, J., Kim, E.-J., Balassa, E., van Dijk, K., Cerutti, H. 2008. Diversification of the Core RNA Interference Machinery in *Chlamydomonas reinhardtii* and the Role of DCL1 in Transposon Silencing. *Genetics*, 179(1), 69-81.

Chaichalerm, S., Pokethitiyook, P., Yuan, W., Meetam, M., Sritong, K., Pugkaew, W., Kungvansaichol, K., Kruatrachue, M., Damrongphol, P. 2012. Culture of microalgal strains isolated from natural habitats in Thailand in various enriched media. *Applied Energy*, 89(1), 296-302.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., Chang, J.-S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology*, 102(1), 71-81.

Chen, W., Zhang, C., Song, L., Sommerfeld, M., Hu, Q. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods*, 77(1), 41-47.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnology Advances*, 25(3), 294-306.

Coleman, R.A., Lee, D.P. 2004. Enzymes of triacylglycerol synthesis and their regulation. *Progress in Lipid Research*, 43(2), 134-76.

Cooper, M.S., Hardin, W.R., Petersen, T.W., Cattolico, R.A. 2010. Visualizing "green oil" in live algal cells. *Journal of Bioscience and Bioengineering*, 109(2), 198-201.

Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S. 2000. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proceedings of the National Academy of Sciences*, 97(12), 6487-92.

Davidi, L., Katz, A., Pick, U. 2011. Characterization of major lipid droplet proteins from Dunaliella. *Planta*, 1-15.

Day, A., Goldschmidt-Clermont, M. 2011. The chloroplast transformation toolbox: selectable markers and marker removal. *Plant Biotechnology Journal*, 9(5), 540-553.

Dean, A.P., Sigee, D.C., Estrada, B., Pittman, J.K. 2010. Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae. *Bioresour Technol*, 101(12), 4499-507.

Dembitsky, V.M. 1996. Betaine ether-linked glycerolipids: Chemistry and biology. *Progress in Lipid Research*, 35(1), 1-51.

Deng, X.-D., Gu, B., Li, Y.-J., Hu, X.-W., Guo, J.-C., Fei, X.-W. 2012. The Roles of acyl-CoA: Diacylglycerol Acyltransferase 2 Genes in the Biosynthesis of Triacylglycerols by the Green Algae Chlamydomonas reinhardtii. *Molecular Plant*, 5(4), 945-947.

Dodds, E.D., McCoy, M.R., Rea, L.D., Kennish, J.M. 2005. Proton transfer chemical ionization mass spectrometry of fatty acid methyl esters separated by gas chromatography: quantitative aspects. *European Journal of Lipid Science and Technology*, 107(7-8), 560-564.

Draaisma, R.B., Wijffels, R.H., Slegers, P.M., Brentner, L.B., Roy, A., Barbosa, M.J. 2013. Food commodities from microalgae. *Current Opinion in Biotechnology*, 24(2), 169-177.

Elsey, D., Jameson, D., Raleigh, B., Cooney, M.J. 2007. Fluorescent measurement of microalgal neutral lipids. *Journal of Microbiological Methods*, 68(3), 639-642.

ESCAP, U.N. 2010. Preview Green Growth, Resources and Resilience, Environmental sustainability in Asia and the Pacific, 2010. United Nations.

Fan, J., Andre, C., Xu, C. 2011. A chloroplast pathway for the de novo biosynthesis of triacylglycerol in Chlamydomonas reinhardtii. *FEBS letters*, 585(12), 1985-1991.

Fauconnot, L., Hau, J., Aeschlimann, J.-M., Fay, L.-B., Dionisi, F. 2004. Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry, Vol. 18, John Wiley & Sons, Ltd., pp. 218-224.

Fischer, N., Rochaix, J.D. 2001. The flanking regions of PsaD drive efficient gene expression in the nucleus of the green alga Chlamydomonas reinhardtii. *Molecular Genetics and Genomics*, 265(5), 888-94.

Folch, J., Lees, M., Stanley, G.H.S. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, 226(1), 497-509.

Franz, A.K., Danielewicz, M.A., Wong, D.M., Anderson, L.A., Boothe, J.R. 2013. Phenotypic Screening with Oleaginous Microalgae Reveals Modulators of Lipid Productivity. *ACS Chemical Biology*.

Fuentes-Grünewald, C., Garcés, E., Alacid, E., Rossi, S., Camp, J. 2012. Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors. *Marine Biotechnology*, 1-11.

Giroud, C., Gerber, A., Eichenberger, W. 1988. Lipids of *Chlamydomonas reinhardtii*. Analysis of Molecular Species and Intracellular Site(s) of Biosynthesis. *Plant Cell Physiol.*, 29(4), 587-595.

Gong, Y., Guo, X., Wan, X., Liang, Z., Jiang, M. 2011. Characterization of a novel thioesterase (PtTE) from *Phaeodactylum tricornutum*. *Journal of Basic Microbiology*, 51(6), 666-672.

González-Ballester, D., Casero, D., Cokus, S., Pellegrini, M., Merchant, S.S., Grossman, A.R. 2010. RNA-Seq Analysis of Sulfur-Deprived *Chlamydomonas* Cells Reveals Aspects of Acclimation Critical for Cell Survival. *The Plant Cell Online*, 22(6), 2058-2084.

Goodson, C., Roth, R., Wang, Z.T., Goodenough, U. 2011. Structural Correlates of Cytoplasmic and Chloroplast Lipid Body Synthesis in *Chlamydomonas reinhardtii* and Stimulation of Lipid Body Production with Acetate Boost. *Eukaryotic Cell*, 10(12), 1592-1606.

Gouveia, L., Marques, A.E., da Silva, T.L., Reis, A. 2009. *Neochloris oleabundans* UTEX #1185: a suitable renewable lipid source for biofuel production. *Journal of Industrial Microbiology & Biotechnology*, 36(6), 821-826.

Gouveia, L., Oliveira, A. 2009. Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology and Biotechnology*, 36(2), 269-274.

Griffiths, M., van Hille, R., Harrison, S. 2011. Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions. *Journal of Applied Phycology*, 1-13.

Griffiths, M.J., Harrison, S.T.L. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, 21(5), 493-507.

Guschina, I.A., Harwood, J.L. 2006. Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research*, 45(2), 160-186.

Hallmann, A. 2007. Algal transgenics and biotechnology. *Transgenic Plant Journal*, 1, 81-98.

Harris, E.H. 2009. *The Chlamydomonas Sourcebook*. Elsevier Science.

Harwood, J.L., Guschina, I.A. 2009. The versatility of algae and their lipid metabolism. *Biochimie*, 91(6), 679-684.

Hecht, A., Shaw, D., Bruins, R., Dale, V., Kline, K., Chen, A. 2009. Good policy follows good science: using criteria and indicators for assessing sustainable biofuel production. *Ecotoxicology*, 18(1), 1-4.

Herrera-Valencia, V., Contreras-Pool, P., López-Adrián, S., Peraza-Echeverría, S., Barahona-Pérez, L. 2011. The Green Microalga *Chlorella saccharophila* as a Suitable Source of Oil for Biodiesel Production. *Current Microbiology*, 63(2), 151-157.

Hildebrand, M., Abbriano, R.M., Polle, J.E.W., Traller, J.C., Trentacoste, E.M., Smith, S.R., Davis, A.K. 2013. Metabolic and cellular organization in evolutionarily diverse microalgae as related to biofuels production. *Current Opinion in Chemical Biology*(0).

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*, 54(4), 621-39.

Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G. 2010. Biodiesel production by microalgal biotechnology. *Applied energy*, 87(1), 38-46.

Huang, N.-L., Huang, M.-D., Chen, T.-L.L., Huang, A.H.C. 2013. Oleosin of Subcellular Lipid Droplets Evolved in Green Algae. *Plant Physiology*, 161(4), 1862-1874.

Huerlimann, R., de Nys, R., Heimann, K. 2010. Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. *Biotechnoly and Bioengineering*, 107(2), 245-57.

IEA. 2012. World energy outlook 2012. OECD/IEA.

Iverson, S., Lang, S., Cooper, M. 2001. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids*, 36(11), 1283-1287.

Jako, C., Kumar, A., Wei, Y., Zou, J., Barton, D.L., Giblin, E.M., Covello, P.S., Taylor, D.C. 2001. Seed-Specific Over-Expression of an *Arabidopsis* cDNA Encoding a Diacylglycerol Acyltransferase Enhances Seed Oil Content and Seed Weight. *Plant Physiology*, 126(2), 861-874.

Jarvis, E.E., Dunahay, T.G., Brown, L.M. 1992. DNA Nucleoside Composition and Methylation in Several Species of Microalgae. *Journal of Phycology*, 28(3), 356-362.

Kaewkannetra, P., Enmak, P., Chiu, T. 2012. The effect of CO2 and salinity on the cultivation of *Scenedesmus obliquus* for biodiesel production. *Biotechnology and Bioprocess Engineering*, 17(3), 591-597.

Keeling, P.J., Burger, G., Durnford, D.G., Lang, B.F., Lee, R.W., Pearlman, R.E., Roger, A.J., Gray, M.W. 2005. The tree of eukaryotes. *Trends in Ecology & Evolution*, 20(12), 670-676.

Kilian, O., Benemann, C.S.E., Niyogi, K.K., Vick, B. 2011. High-efficiency homologous recombination in the oil-producing alga Nannochloropsis sp. *Proceedings of the National Academy of Sciences*.

Knothe, G. 2008. "Designer" Biodiesel: Optimizing Fatty Ester Composition to Improve Fuel Properties. *Energy & Fuels*, 22(2), 1358-1364.

Kolesárová, N., Hutňan, M., Bodík, I., Špalková, V. 2011. Utilization of Biodiesel By-Products for Biogas Production. *Journal of Biomedicine and Biotechnology*, 2011 15 pages.

Krienitz, L., Ustinova, I., Friedl, T., Huss, V.A.R. 2001. Traditional generic concepts versus 18S rRNA gene phylogeny in the green algal family Selenastraceae (Chlorophyceae, Chlorophyta) *Journal of Phycology*, 37(5), 852-865.

Kröger, M., Müller-Langer, F. 2012. Review on possible algal-biofuel production processes. *Biofuels*, 3(3), 333-349.

Kruse, O., Hankamer, B. 2010. Microalgal hydrogen production. *Current Opinion in Biotechnology*, 21(3), 238-243.

Kruse, O., Rupprecht, J., Bader, K.-P., Thomas-Hall, S., Schenk, P.M., Finazzi, G., Hankamer, B. 2005. Improved Photobiological H2 Production in Engineered Green Algal Cells. *Journal of Biological Chemistry*, 280(40), 34170-34177.

Kumar Saha, S., Uma, L., Subramanian, G. 2003. Nitrogen stress induced changes in the marine cyanobacterium *Oscillatoria willei* BDU 130511. *FEMS Microbiology Ecology*, 45(3), 263-272.

La Russa, M., Bogen, C., Uhmeyer, A., Doebbe, A., Filippone, E., Kruse, O., Mussgnug, J.H. 2012. Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, 162(1), 13-20.

Lang, I., Hodac, L., Friedl, T., Feussner, I. 2011. Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. *BMC Plant Biology*, 11(1), 124.

Laurens, L.M.L., Quinn, M., Wychen, S., Templeton, D., Wolfrum, E. 2012. Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. *Analytical and Bioanalytical Chemistry*, 403(1), 167-178.

Lee, S., Yoon, B.-D., Oh, H.-M. 1998. Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnology Techniques*, 12(7), 553-556.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M.X., Arondel, V., Bates, P.D., Baud, S., Bird, D., Debono, A., Durrett, T.P., Franke, R.B., Graham, I.A., Katayama, K., Kelly, A.A., Larson, T., Markham, J.E., Miquel, M., Molina, I., Nishida, I., Rowland, O., Samuels, L., Schmid, K.M., Wada, H., Welti, R., Xu, C., Zallot, R., Ohlrogge, J. 2010. Acyl-lipid metabolism. *Arabidopsis Book*, 8, e0133.

Li, R., Yu, K., Hildebrand, D.F. 2010a. DGAT1, DGAT2 and PDAT expression in seeds and other tissues of epoxy and hydroxy fatty acid accumulating plants. *Lipids*, 45(2), 145-57.

Li, X., Benning, C., Kuo, M.-H. 2012a. Rapid triacylglycerol turnover in *Chlamydomonas reinhardtii* requires a lipase with broad substrate specificity. *Eukaryotic Cell*.

Li, X., Přibyl, P., Bišová, K., Kawano, S., Cepák, V., Zachleder, V., Čížková, M., Brányiková, I., Vítová, M. 2012b. The microalga *Parachlorella kessleri*—A novel highly efficient lipid producer. *Biotechnology and Bioengineering*, 110(1), 97-107.

Li, Y., Han, D., Hu, G., Dauvillee, D., Sommerfeld, M., Ball, S., Hu, Q. 2010b. *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. *Metabolic Engineering*, 12(4), 387-391

Li, Y., Han, D., Hu, G., Sommerfeld, M., Hu, Q. 2010c. Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. *Biotechnology and Bioengineering*, 107(2), 258-268.

Li, Y., Horsman, M., Wu, N., Lan, C.Q., Dubois-Calero, N. 2008. Biofuels from microalgae. *Biotechnology Progress*, 24(4), 815-20.

Lim, D.K.Y., Garg, S., Timmins, M., Zhang, E.S.B., Thomas-Hall, S.R., Schuhmann, H., Li, Y., Schenk, P.M. 2012. Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters. *PLoS ONE*, 7(7), e40751.

Listenberger, L.L., Han, X., Lewis, S.E., Cases, S., Farese, R.V., Ory, D.S., Schaffer, J.E. 2003. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proceedings of the National Academy of Sciences*, 100(6), 3077-3082.

Liu, B., Benning, C. 2013. Lipid metabolism in microalgae distinguishes itself. *Current Opinion in Biotechnology*, 24(2), 300-309.

Makri, A., Bellou, S., Birkou, M., Papatrehas, K., Dolapsakis, N.P., Bokas, D., Papanikolaou, S., Aggelis, G. 2011. Lipid synthesized by micro-algae grown in laboratory- and industrial-scale bioreactors. *Engineering in Life Sciences*, 11(1), 52-58.

Mandal, S., Mallick, N. 2009. Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Applied Microbiology and Biotechnology*, 84(2), 281-291.

Mata, T.M., Martins, A.n.A., Caetano, N.S. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*, 14(1), 217-232.

Matthew, T., Zhou, W., Rupprecht, J., Lim, L., Thomas-Hall, S.R., Doebbe, A., Kruse, O., Hankamer, B., Marx, U.C., Smith, S.M., Schenk, P.M. 2009. The Metabolome of *Chlamydomonas reinhardtii* following Induction of Anaerobic H2 Production by Sulfur Depletion. *Journal of Biological Chemistry*, 284(35), 23415-23425.

McFadden, G.I. 2001. Primary and secondary endosymbiosis and the origin of plastids. *Journal of Phycology*, 37(6), 951-959.

McNichol, J., MacDougall, K., Melanson, J., McGinn, P. 2011. Suitability of Soxhlet Extraction to Quantify Microalgal Fatty Acids as Determined by Comparison with In Situ Transesterification. *Lipids*, 1-13.

Melis, A., Happe, T. 2001. Hydrogen Production. Green Algae as a Source of Energy. *Plant Physiology*, 127(3), 740-748.

Merchant, S.S., Kropat, J., Liu, B., Shaw, J., Warakanont, J. 2012. TAG, You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. *Current Opinion in Biotechnology*, 23(3), 352-363.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Marechal-Drouard, L., Marshall, W.F., Qu, L.H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V.V., Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C.L., Cognat, V., Croft, M.T., Dent, R., Dutcher, S., Fernandez, E., Fukuzawa, H., Gonzalez-Ballester, D., Gonzalez-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P.A., Lemaire, S.D., Lobanov, A.V., Lohr, M., Manuell, A., Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J.V., Moseley, J., Napoli, C., Nedelcu, A.M., Niyogi, K.,

Novoselov, S.V., Paulsen, I.T., Pazour, G., Purton, S., Ral, J.P., Riano-Pachon, D.M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S.L., Allmer, J., Balk, J., Bisova, K., Chen, C.J., Elias, M., Gendler, K., Hauser, C., Lamb, M.R., Ledford, H., Long, J.C., Minagawa, J., Page, M.D., Pan, J., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A.M., Yang, P., Ball, S., Bowler, C., Dieckmann, C.L., Gladyshev, V.N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R.T., Brokstein, P., et al. 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science*, 318(5848), 245-50.

Miller, R., Wu, G., Deshpande, R.R., Vieler, A., Gärtner, K., Li, X., Moellering, E.R., Zäuner, S., Cornish, A.J., Liu, B., Bullard, B., Sears, B.B., Kuo, M.-H., Hegg, E.L., Shachar-Hill, Y., Shiu, S.-H., Benning, C. 2010. Changes in Transcript Abundance in *Chlamydomonas reinhardtii* following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiology*, 154(4), 1737-1752.

Mizuno, Y., Sato, A., Watanabe, K., Hirata, A., Takeshita, T., Ota, S., Sato, N., Zachleder, V., Tsuzuki, M., Kawano, S. 2013. Sequential accumulation of starch and lipid induced by sulfur deficiency in *Chlorella* and *Parachlorella* species. *Bioresource Technology*, 129(0), 150-155.

Moellering, E.R., Benning, C. 2010. RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 9(1), 97-106.

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., Baulcombe, D. 2009. Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant Journal*.

Moreau, R.A. 2006. The analysis of lipids via HPLC with a charged aerosol detector. *Lipids*, 41(7), 727-734.

Msanne, J., Xu, D., Konda, A.R., Casas-Mollano, J.A., Awada, T., Cahoon, E.B., Cerutti, H. 2012. Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and *Coccomyxa sp.* C-169. *Phytochemistry*(0).

Mussgnug, J.H., Klassen, V., Schlüter, A., Kruse, O. 2010. Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *Journal of Biotechnology*.

Mussgnug, J.H., Thomas-Hall, S., Rupprecht, J., Foo, A., Klassen, V., McDowall, A., Schenk, P.M., Kruse, O., Hankamer, B. 2007. Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. *Plant Biotechnology Journal*, 5(6), 802-814.

Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., Bux, F. 2011. Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresource Technology*, 102(1), 57-70.

Napier, J.A., Graham, I.A. 2010. Tailoring plant lipid composition: designer oilseeds come of age. *Current Opinion in Plant Biology*, 13(3), 329-336.

Nascimento, I., Marques, S., Cabanelas, I., Pereira, S., Druzian, J., Souza, C., Vich, D., Carvalho, G., Nascimento, M. 2013. Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria. *BioEnergy Research*, 6(1), 1-13.

Nelson, N., Yocum, C.F. 2006. Structure and function of photosystems I and II. *Annual Review of Plant Biology*, 57, 521-565.

Neupert, J., Karcher, D., Bock, R. 2009. Generation of *Chlamydomonas* strains that efficiently express nuclear transgenes. *The Plant Journal*, 57(6), 1140-1150.

Nguyen, H.M., Baudet, M., Cuiné, S., Adriano, J.-M., Barthe, D., Billon, E., Bruley, C., Beisson, F., Peltier, G., Ferro, M., Li-Beisson, Y. 2011. Proteomic profiling of oil bodies isolated from the unicellular green microalga *Chlamydomonas reinhardtii*: With focus on proteins involved in lipid metabolism. *PROTEOMICS*, 11(21), 4266-4273.

Nigam, P.S., Singh, A. 2011. Production of liquid biofuels from renewable resources. *Progress in Energy and Combustion Science*, 37(1), 52-68.

Orcutt, D.M., Patterson, G.W. 1974. Effect of light intensity upon lipid composition of *Nitzschia closterium* (*Cylindrotheca fusiformis*). *Lipids*, 9(12), 1000-1003.

Pal, D., Khozin-Goldberg, I., Cohen, Z., Boussiba, S. 2011. The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. *Applied Microbiology and Biotechnology*, 90(4), 1429-1441.

Parker, M.S., Mock, T., Armbrust, E.V. 2008. Genomic insights into marine microalgae. *Annual review of genetics*, 42, 619-645.

Petschnigg, J., Wolinski, H., Kolb, D., Zellnig, G., Kurat, C.F., Natter, K., Kohlwein, S.D. 2009. Good Fat, Essential Cellular Requirements for Triacylglycerol Synthesis to Maintain Membrane Homeostasis in Yeast. *Journal of Biological Chemistry*, 284(45), 30981-30993.

Piorreck, M., Pohl, P. 1984. Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase. *Phytochemistry*, 23(2), 217-223.

Pittman, J.K., Dean, A.P., Osundeko, O. 2011. The potential of sustainable algal biofuel production using wastewater resources. *Bioresource Technology*, 102(1), 17-25.

Poerschmann, J., Spijkerman, E., Langer, U. 2004. Fatty acid patterns in *Chlamydomonas* sp as a marker for nutritional regimes and temperature under extremely acidic conditions. *Microbial Ecology*, 48(1), 78-89.

Popovich, C.A., Damiani, C., Constenla, D., Martínez, A.M., Freije, H., Giovanardi, M., Pancaldi, S., Leonardi, P.I. 2012. *Neochloris oleoabundans* grown in enriched natural seawater for biodiesel feedstock: Evaluation of its growth and biochemical composition. *Bioresource Technology*, 114(0), 287-293.

Přibyl, P., Cepák, V., Zachleder, V. 2012. Production of lipids in 10 strains of *Chlorella* and *Parachlorella* , and enhanced lipid productivity in Chlorella vulgaris. *Applied Microbiology and Biotechnology*, 94(2), 549-561.

Pröschold, T., Harris, E.H., Coleman, A.W. 2005. Portrait of a Species *Chlamydomonas reinhardtii*. *Genetics*, 170(4), 1601-1610.

Pruvost, J., Van Vooren, G., Cogne, G., Legrand, J. 2009. Investigation of biomass and lipids production with *Neochloris oleoabundans* in photobioreactor. *Bioresour Technol*, 100(23), 5988-95.

Radakovits, R., Jinkerson, R.E., Fuerstenberg, S.I., Tae, H., Settlage, R.E., Boore, J.L., Posewitz, M.C. 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropis gaditana*. *Nature Communications*, 3, 686.

Raines, C. 2003. The Calvin cycle revisited. *Photosynthesis Research*, 75(1), 1-10.

Ramanan, R., Kim, B.-H., Cho, D.-H., Ko, S.-R., Oh, H.-M., Kim, H.-S. 2013. Lipid droplet synthesis is limited by acetate availability in starchless mutant of *Chlamydomonas reinhardtii*. *FEBS Letters*, 587(4), 370-377.

Rawsthorne, S. 2002. Carbon flux and fatty acid synthesis in plants. *Progress in Lipid Research*, 41(2), 182-196.

Richardson, B., Orcutt, D., Schwertner, H., Martinez, C.L., Wickline, H.E. 1969. Effects of nitrogen limitation on the growth and composition of unicellular algae in continuous culture. *Applied microbiology*, 18(2), 245-250.

Riekhof, W.R., Benning, C. 2009. Glycerolipid biosynthesis. *The Chlamydomonas sourcebook: Organellar and metabolic processes vol*, 2, 41-68.

Riekhof, W.R., Sears, B.B., Benning, C. 2005. Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase BTA1Cr. *Eukaryotic Cell*, 4(2), 242-52.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Bibby, K., Peccia, J. 2011. Transcriptome sequencing and annotation of the microalgae *Dunaliella tertiolecta*: Pathway description and gene discovery for production of next-generation biofuels. *Bmc Genomics*, 12.

Rismani-Yazdi, H., Haznedaroglu, B.Z., Hsin, C., Peccia, J. 2012. Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation. *Biotechnology for Biofuels*, 5.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. 2009. Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor. *Biotechnology and Bioengineering*, 102(1), 100-112.

Salama, E.-S., Kim, H.-C., Abou-Shanab, R.I., Ji, M.-K., Oh, Y.-K., Kim, S.-H., Jeon, B.-H. 2013. Biomass, lipid content, and fatty acid composition of freshwater *Chlamydomonas mexicana* and *Scenedesmus obliquus* grown under salt stress. *Bioprocess and Biosystems Engineering*, 1-7.

Sankar, V., Daniel, D.K., Krastanov, A. 2011. Carbon dioxide fixation by *Chlorella minutissima* batch cultures in a stirred tank bioreactor. *Biotechnoly Biotechnolical Equipment*, 25(3), 2468-2476.

Santos, A.M., Janssen, M., Lamers, P.P., Evers, W.A.C., Wijffels, R.H. 2012. Growth of oil accumulating microalga *Neochloris oleoabundans* under alkaline–saline conditions. *Bioresource Technology*, 104(0), 593-599.

Sasso, S., Pohnert, G., Lohr, M., Mittag, M., Hertweck, C. 2012. Microalgae in the postgenomic era: a blooming reservoir for new natural products. *FEMS Microbiology Reviews*, 36(4), 761-785.

Schenk, P.M.P., Thomas-Hall, S.R., Evan, S., Marx, U., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *BioEnergy Research*.

Schroda, M. 2006. RNA silencing in *Chlamydomonas*: mechanisms and tools. *Curr Genet*, 49(2), 69-84.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P. 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae. U.S. Department of Energy's Office of Fuels Development.

Sialve, B., Bernet, N., Bernard, O. 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. *Biotechnology Advances*, 27(4), 409-416.

Siaut, M., Cuine, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylides, C., Li-Beisson, Y., Peltier, G. 2011. Oil accumulation in the model green alga *Chlamydomonas reinhardtii*: characterization, variability between common laboratory strains and relationship with starch reserves. *BMC Biotechnology*, 11(1), 7.

Simionato, D., Block, M.A., La Rocca, N., Jouhet, J., Marechal, E., Finazzi, G., Morosinotto, T. 2013. Response of *Nannochloropsis gaditana* to nitrogen starvation includes a de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids and a reorganization of the photosynthetic apparatus. *Eukaryotic Cell*.

Sivakumar, G., Vail, D.R., Xu, J., Burner, D.M., Lay, J.O., Ge, X., Weathers, P.J. 2010. Bioethanol and biodiesel: Alternative liquid fuels for future generations. *Engineering in Life Sciences*, 10(1), 8-18.

Slocombe, S.P., Cornah, J., Pinfield-Wells, H., Soady, K., Zhang, Q., Gilday, A., Dyer, J.M., Graham, I.A. 2009. Oil accumulation in leaves directed by modification of fatty acid breakdown and lipid synthesis pathways. *Plant Biotechnology Journal*, 7(7), 694-703.

Solovchenko, A. 2012. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. *Russian Journal of Plant Physiology*, 59(2), 167-176.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., egrave, ne. 2006. Commercial Applications of Microalgae. *The Society for Biotechnology, Japan*, 101(2), 87-96.

Srirangan, K., Akawi, L., Moo-Young, M., Chou, C.P. 2012. Towards sustainable production of clean energy carriers from biomass resources. *Applied Energy*, 100(0), 172-186.

Stahl, U., Carlsson, A.S., Lenman, M., Dahlqvist, A., Huang, B., Banas, W., Banas, A., Stymne, S. 2004. Cloning and Functional Characterization of a Phospholipid:Diacylglycerol Acyltransferase from *Arabidopsis*. *Plant Physiology*, 135(3), 1324-1335.

Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Kruse, O., Posten, C., Borowitzka, M.A., Hankamer, B. 2010. An economic and technical evaluation of microalgal biofuels. *Nat Biotech*, 28(2), 126-128.

Sydney, E.B., Sturm, W., de Carvalho, J.C., Thomaz-Soccol, V., Larroche, C., Pandey, A., Soccol, C.R. 2010. Potential carbon dioxide fixation by industrially important microalgae. *Bioresource Technology*, 101(15), 5892-5896.

Takagi, M., Karseno, Yoshida, T. 2006. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. *Journal of Bioscience and Bioengineering*, 101(3), 223-226.

Talukdar, J., Chandra, K., Chandra, G. 2012. Growth, total lipid content and fatty acid profile of a native strain of the freshwater oleaginous microalgae *Ankistrodesmus falcatus* (Ralf) grown under salt stress condition. *International Research Journal of Biological Sciences*, 1(8), 27-35.

Tran, N.H., Bartlett, J.R., Kannangara, G.S.K., Milev, A.S., Volk, H., Wilson, M.A. 2010. Catalytic upgrading of biorefinery oil from micro-algae. *Fuel*, 89(2), 265-274.

Van Mooy, B.A.S., Fredricks, H.F., Pedler, B.E., Dyhrman, S.T., Karl, D.M., Koblizek, M., Lomas, M.W., Mincer, T.J., Moore, L.R., Moutin, T., Rappe, M.S., Webb, E.A. 2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*, 458(7234), 69-72.

Vieler, A., Wu, G., Tsai, C.-H., Bullard, B., Cornish, A.J., Harvey, C., Reca, I.-B., Thornburg, C., Achawanantakun, R., Buehl, C.J., Campbell, M.S., Cavalier, D., Childs, K.L., Clark, T.J., Deshpande, R., Erickson, E., Armenia Ferguson, A., Handee, W., Kong, Q., Li, X., Liu,

B., Lundback, S., Peng, C., Roston, R.L., Sanjaya, Simpson, J.P., TerBush, A., Warakanont, J., Zäuner, S., Farre, E.M., Hegg, E.L., Jiang, N., Kuo, M.-H., Lu, Y., Niyogi, K.K., Ohlrogge, J., Osteryoung, K.W., Shachar-Hill, Y., Sears, B.B., Sun, Y., Takahashi, H., Yandell, M., Shiu, S.-H., Benning, C. 2012. Genome, Functional Gene Annotation, and Nuclear Transformation of the Heterokont Oleaginous Alga *Nannochloropsis oceanica* CCMP1779. *PLoS Genetics*, 8(11), e1003064.

Wada, H., Murata, N., Moellering, E.R., Miller, R., Benning, C. 2009. Molecular Genetics of Lipid Metabolism in the Model Green Alga *Chlamydomonas reinhardtii*. in: *Lipids in Photosynthesis*, (Ed.) Govindjee, Vol. 30, Springer Netherlands, pp. 139-155.

Walker, T.L., Collet, C., Purton, S. 2005. Algal transgenics in the genomic era. *Journal of Phycology*, 41(6), 1077-1093.

Wallis, J.G., Browse, J. 2010. Lipid biochemists salute the genome. *Plant Journal*, 61(6), 1092-106.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S., Goodenough, U. 2009. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 8(12), 1856-1868.

Wawrik, B., Harriman, B.H. 2010. Rapid, colorimetric quantification of lipid from algal cultures. *Journal of Microbiological Methods*, 80(3), 262-6.

Weselake, R.J., Shah, S., Tang, M., Quant, P.A., Snyder, C.L., Furukawa-Stoffer, T.L., Zhu, W., Taylor, D.C., Zou, J., Kumar, A., Hall, L., Laroche, A., Rakow, G., Raney, P., Moloney, M.M., Harwood, J.L. 2008. Metabolic control analysis is helpful for informed genetic manipulation of oilseed rape (Brassica napus) to increase seed oil content. *Journal of Experimental Botany*, 59(13), 3543-3549.

Williams, V.R., McMillan, R. 1961. Lipids of *Ankistrodesmus braunii*. *Science*, 133(3451), 459-460.

Wiseman, H. 2009. Untested Waters: The Rise of hydraulic fracturing in oil and gas production and the need to revisit regulation. *Fordham Environmental Law Review*, 20, 115.

Work, V.H., D'Adamo, S., Radakovits, R., Jinkerson, R.E., Posewitz, M.C. 2012. Improving photosynthesis and metabolic networks for the competitive production of phototroph-derived biofuels. *Current Opinion in Biotechnology*, 23(3), 290-297.

Yamaberi, K., Takagi, M., Yoshida, T. 1998. Nitrogen depletion for intracellular triglyceride accumulation to enhance liquefaction yield of marine microalgal cells into a fuel oil. in: *Journal of Marine Biotechnology*, Vol. 6, Springer New York, pp. 44-48.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y. 2011a. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. *Bioresource Technology*, 102(1), 159-165.

Yang, X., Liu, P.H., Hao, Z.D., Shi, J., Zhang, S. 2012. Characterization and Identification of Freshwater Microalgal Strains toward Biofuel Production. *Bioresources*, 7(1), 686-695.

Yang, Z., Guo, R., Xu, X., Fan, X., Luo, S. 2011b. Hydrogen and methane production from lipid-extracted microalgal biomass residues. *International Journal of Hydrogen Energy*, 36(5), 3465-3470.

Yoon, K., Han, D., Li, Y., Sommerfeld, M., Hu, Q. 2012. Phospholipid:Diacylglycerol Acyltransferase Is a Multifunctional Enzyme Involved in Membrane Lipid Turnover and Degradation While Synthesizing Triacylglycerol in the Unicellular Green Microalga *Chlamydomonas reinhardtii*. *The Plant Cell Online*.

Yu, W.-L., Ansari, W., Schoepp, N., Hannon, M., Mayfield, S., Burkart, M. 2011. Modifications of the metabolic pathways of lipid and triacylglycerol production in microalgae. *Microbial Cell Factories*, 10(1), 91.

Yu, X., Zhao, P., He, C., Li, J., Tang, X., Zhou, J., Huang, Z. 2012. Isolation of a novel strain of *Monoraphidium* sp. and characterization of its potential application as biodiesel feedstock. *Bioresource Technology*, 121(0), 256-262.

Zhang, H., Damude, H.G., Yadav, N.S. 2012. Three diacylglycerol acyltransferases contribute to oil biosynthesis and normal growth in *Yarrowia lipolytica*. *Yeast*, 29(1), 25-38.

Zhao, T., Li, G., Mi, S., Li, S., Hannon, G.J., Wang, X.J., Qi, Y. 2007. A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. *Genes Dev*, 21(10), 1190-203.

Zheng, P., Allen, W.B., Roesler, K., Williams, M.E., Zhang, S., Li, J., Glassman, K., Ranch, J., Nubel, D., Solawetz, W., Bhattramakki, D., Llaca, V., Deschamps, S., Zhong, G.-Y., Tarczynski, M.C., Shen, B. 2008. A phenylalanine in DGAT is a key determinant of oil content and composition in maize. *Nat Genet*, 40(3), 367-372.

Zhu, C., Lee, Y. 1997. Determination of biomass dry weight of marine microalgae. *Journal of Applied Phycology*, 9(2), 189-194.

Zou, J., Katavic, V., Giblin, E.M., Barton, D.L., MacKenzie, S.L., Keller, W.A., Hu, X., Taylor, D.C. 1997. Modification of seed oil content and acyl composition in the brassicaceae by expression of a yeast sn-2 acyltransferase gene. *The Plant Cell Online*, 9(6), 909-923.

7. Publications

[1] Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*

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[2] Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production

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[3] Reconstruction of the lipid metabolism for the microalga *Monoraphidium neglectum* from its genome sequence reveals characteristics suitable for biofuel production

Christian Bogen, Arwa Al-Dilaimi, Andreas Albersmeier, Julian Wichmann, Michael Grundmann, Oliver Rupp, Kyle J. Lauersen, Olga Blifernez-Klassen, Jörn Kalinowski, Alexander Goesmann, Jan H. Mussgnug, Olaf Kruse

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[1] Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*

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Contributions:

- The research was designed by M. La Russa, J. H. Mussgnug, E. Filippone and O. Kruse
- M. La Russa, C. Bogen, A. Uhmeyer and A. Doebbe performed the research, with the main part of this work carried out by the first author, M. La Russa.
  C. Bogen assisted in the establishment of lipid analysis and contributed to lipid extractions, chromatographies and fatty acid profiling which resulted in the finding that type-2 DGAT overexpression did not lead to an increased neutral lipid content in the overexpression cell lines. This lead to the suggestion that the amount of DGAT transcript is apparently not the rate limiting step for TAG synthesis and to the down-regulation of BTA1 which is responsible for the synthesis of DGTS, thereby depriving the Kennedy pathway of the direct TAG-precursor DAG.
- Data analysis and interpretation: M. La Russa, C. Bogen, E. Filippone, O. Kruse and J. H.Mussgnug
- Manuscript writing: M. La Russa, C. Bogen, E. Filippone, O. Kruse and J. H. Mussgnug

[2] Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production

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Contributions:

- The research was designed by C. Bogen, V. Klassen, M. La Russa, J. H. Mussgnug, and O. Kruse
- C. Bogen, V. Klassen, J. Wichmann, M. Grundmann, M. La Russa and A. Doebbe performed the research, with the main part of this work carried out by the first author, C. Bogen.
  C. Bogen performed up-scaling, growth analysis, lipid analysis and FAME profiling regarding the screening, lipid analysis and FAME profiling of *M. contortum* under nutrient replete conditions and nitrogen starvation, setup and biogas measurements of the fermentation trials.
  V. Klassen performed setup and biogas measurements as well as determination of the biogas composition of the fermentation trials. J. Wichmann investigated growth of *M. contortum* under nitrogen starvation and performed lipid analysis. M. Grundmann performed investigations of salt tolerance. M. La Russa and A. Doebbe assisted in GC-MS handling.
- Data analysis and interpretation: C. Bogen, V. Klassen (fermentation trials), M. La Russa, O. Kruse and J. H. Mussgnug
- Manuscript writing: C. Bogen, V. Klassen, M. La Russa, P. Uronen, O. Kruse and J. H. Mussgnug

[3] Reconstruction of the lipid metabolism for the microalga *Monoraphidium neglectum* from its genome sequence reveals characteristics suitable for biofuel production

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Submitted to Genome Biology, 2013.

Contributions:

- The research was designed by C. Bogen, A. Al-Dilaimi, J. H.Mussgnug, J. Kalinowski and O. Kruse
- C. Bogen, A. Al-Dilaimi, J. Wichmann, M. Grundmann performed the research, with the main part of this work carried out by the first author, C. Bogen.
- C. Bogen performed growth analysis, lipid analysis and FAME profiling under nutrient replete conditions and nitrogen starvation, was involved in genome sequencing, performed phylogenetic analysis, and *in silico* reconstruction of lipid metabolism, prepared therefore figures and tables and drafted the manuscript.
- Genome sequencing was performed by A. Albersmeier, genome assembly and annotation by A. Al-Dilaimi. The GenDBE project was created and maintained by O. Rupp. Manual gene assignments performed by A. Al-Dilaimi. Comparison of genomes was carried out by J. Blom, O. Rupp and A. Al-Dilaimi. A. Al-Dilaimi prepared figures and tables related to genome assembly and metabolic reconstruction
- J. Wichmann investigated growth of M. contortum under nitrogen starvation and performed lipid analysis. M. Grundmann performed investigations of salt tolerance, O. Blifernez-Klassen of pH tolerance.
- Data analysis and interpretation: C. Bogen, A. Al-Dilaimi, J. H. Mussgnug, O. Kruse
- Manuscript writing: C. Bogen, A. Al-Dilaimi, J. Kalinowski, J. H. Mussgnug, O. Kruse

8. Unpublished results

[4] Isolation and comprehensive screening of strains on their potential for liquid biofuel generation

Christian Bogen

[5] Investigation of the influence of BTA1 down-regulation on the neutral lipid content in *C. reinhardtii*

Christian Bogen

[4] Isolation and comprehensive screening of strains on their potential for liquid biofuel generation

C. Bogen

Summary

Due to the high diversity encountered in the lipid metabolism and fatty acid composition of microalgae, the isolation, identification and characterization of newly isolated strains is considered a worthwhile effort to gain lineages with high potential for liquid biofuel applications, especially when already adapted to local environmental conditions. In the course of this work, new strains of microalgae were isolated from various freshwater as well as marine environments, and characterized on their growth properties and lipid profiles. Several strains were found with a remarkably good performance in terms of biomass accumulation, clustering closely to *Chlorella* and *Parachlorella* based on plastid 23S rDNA analysis. Two diatom strains could be isolated, whose 23S rDNA showed a very high degree of similarity to the plastid of the dinoflagellate *Durinskia baltica*, therefore suggesting a close phylogenetic relationship of the isolate to the tertiary endosymbiont that has been engulfed by the ancestor of the dinoflagellate.

Introduction

Previous screenings of microalgal species

Since the end of the Aquatic Species Program of the U.S. National Renewable Energy Laboratory in 1996 that comprised an intensive screening of a high diversity of microalgal species on their potential as sources for renewable fuels (Mata et al., 2010; Sheehan et al., 1998), many more strains have been investigated regarding lipid content and composition for various purposes, e.g. for use as live food in aquaculture or as a source for food supplements (Carlsson et al., 2007; Sasso et al., 2012; Shamsudin, 1992). In recent years, interest resurged in using microalgal oils for further conversion into liquid biofuels (Chisti, 2007; Hu et al., 2008; Mata et al., 2010;

Schenk et al., 2008; Tran et al., 2010). As a result, lipid contents and fatty acid profiles of several common and well-known algae species based on strains originating from existing algae collections were under further investigation (Bogen et al., 2013; Breuer et al., 2012; Gouveia & Oliveira, 2009; Griffiths et al., 2011; Huerlimann et al., 2010; Li et al., 2008; McNichol et al., 2011; Přibyl et al., 2012; Rodolfi et al., 2009). Nonetheless, much of the potential of microalgae still remains unexplored due to the high heterogeneity of algal fatty acids and lipids that can be encountered among individual algal species even within the same division (Harwood & Guschina, 2009; Lang et al., 2011).

Usually lipids are accumulated under specific stress conditions, when growth is generally decreased or arrested (Hu et al., 2008; Solovchenko, 2012). This tendency is considered to result into a pronounced trade-off between biomass and neutral lipid production (Basova, 2005; Gong & Jiang, 2011; Hu et al., 2008; Huerlimann et al., 2010; Sheehan et al., 1998), therefore reducing the overall lipid productivity.

Lipid productivity is considered as an essential trait and major economic bottleneck to the further commercial utilization of microalgal oils (Griffiths & Harrison, 2009). Important characteristics for liquid biofuel production in the form of e.g. biodiesel comprise the presence of high neutral lipid contents and a fatty acid profile with a comparatively high degree of saturation (Knothe, 2008; Mata et al., 2010; Napier & Graham, 2010). In addition, it has been recommended by Griffiths et al. (2009) to select strains that show fast growth and high productivity under local climatic conditions.

Several species of interest have been identified so far and were intensively studied, e.g. *Botryococcus braunii*, where strains of race B contain high contents of terpenoid hydrocarbons that can be converted to various fuel fractions using conventional catalysts (Tran et al., 2010). However, several critical issues like slow growth rates have constrained its further use for biofuels until today (Chen et al., 2011; Mata et al., 2010; Tran et al., 2010).

Some strains of the genus *Nannochloropsis* showed good performances in terms of lipid productivity; however, critical points as small cell size and hard cell walls currently limit economic downstream processing (Rodolfi et al., 2009).

Thus, further investigation of other algae species is required to identify oleaginous strains with properties that would render them highly promising candidates for future biofuel production (Hu et al., 2008; Tran et al., 2010).

Current publications about recently isolated strains report not only relatively high neutral lipid productivities as for *Pseudochlorococcum* sp. (Li et al., 2011) but also a promising fatty acid composition as shown for *Navicula* spec. (Matsumoto et al., 2010), that makes them possible candidates as biodiesel feedstock and also indicate the potential of yet uninvestigated isolates to serve as a suitable source for diesel production.

In this work, 20 microalgal strains were isolated from predominantly local water bodies and investigated on their potential for biofuel applications. They were cultivated under elevated light conditions and up-scaled to 1.5l batch cultures. Growth parameters were taken and biomass was harvested before the cultures reached the stationary phase to avoid unspecific starvation conditions. Total and neutral lipid contents were determined gravimetrically as well as their fatty acid compositions, which was investigated via GC-MS to identify promising new strains for biofuel applications.

Materials and Methods

Traditional isolation techniques were used for the isolation of new strains (Andersen, 2005; Barsanti, 2006). In detail, samples originating from freshwater or marine water bodies were transferred into defined liquid minimal media as described before (Bogen et al., 2013), without supplementary vitamins or energy rich carbon sources (Fig.1). The resulting enrichment cultures were subsequently sprayed on agar plates containing the respective medium and were inoculated under continuous light (about 50-100µmol m^{-2} s^{-1}).

After a period of approximately three weeks, single colonies were picked and streaked out on further agar plates. Material from these plates was then transferred into liquid media and after several days controlled for homogeneity of cells. In a reiterative step, cells were sprayed on plates and transferred as previously described to reduce the number of contaminating non-target organisms as well as to obtain cultures that potentially derive from individual cells.

Duplicates were identified by visual observation using 1,000x magnified microscopic images and discarded to avoid redundancy.

Culture conditions and growth analysis

An additional criterion for strain selection was successful up-scaling to 1.5l batch cultures under elevated light intensities to obtain strains with a high potential for robust and competitive growth in outdoor cultivation setups (Figure 1).

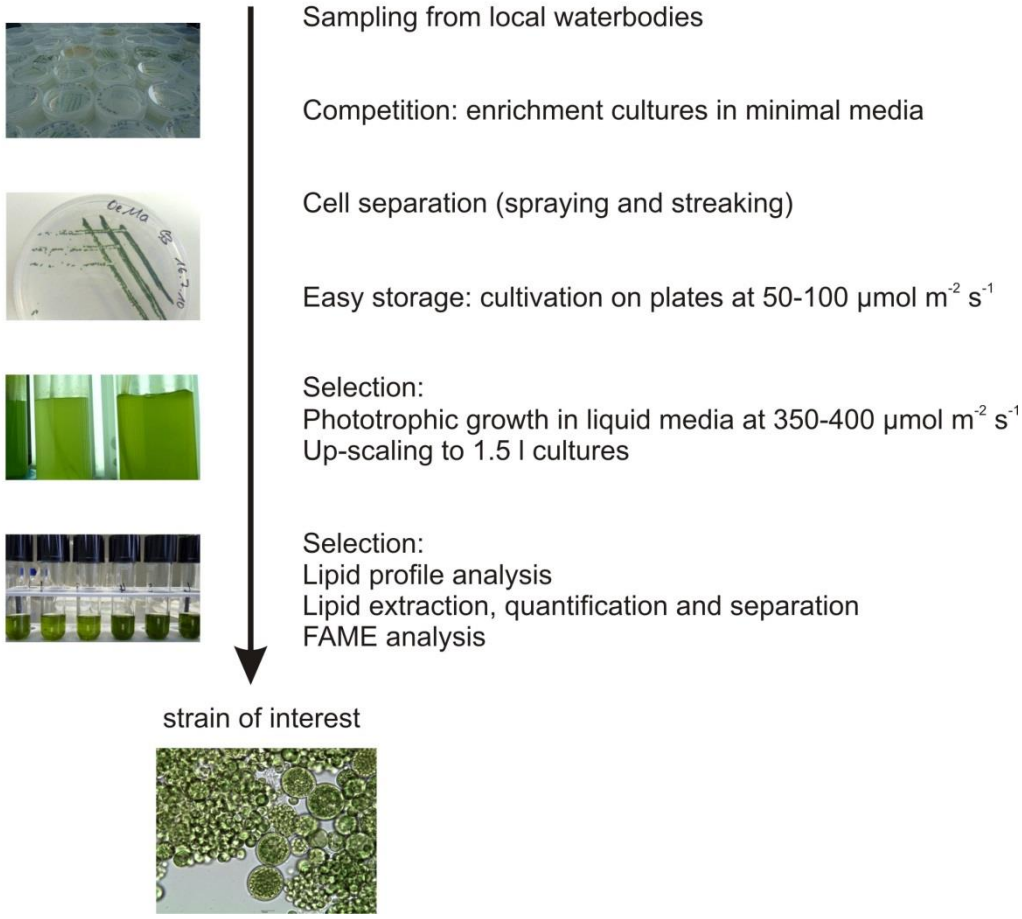

Figure 1 Overview of the screening strategy to identify strains with dominant biomass production and suitable lipid profiles under photoautotrophic conditions

Therefore strains were transferred from agar plates to 20ml of liquid media. After 1-2 weeks, the cultures were transferred into 200ml fresh media that was flushed air enriched with 3% CO_2. For

the following experiments, cells were harvested by centrifugation. The supernatant was discarded, the wet biomass weight was determined and 0.5g were transferred to 1.5l batches of minimal media, flushed with 3% CO_2 enriched air at 350-400µmol m^{-2} s^{-1} at room temperature (25-28°C). Growth was monitored by determination of optical density at 750nm (OD_{750}), cell number (Beckman Coulter Z2, Krefeld, Germany) and dry biomass (cell pellets were washed with de-ionized water, pelleted at 6,000 x g and dried at 105°C overnight).

Determination of total lipids and fatty acid abundances

The strains were investigated on total lipid contents using a modified Folch extraction protocol (Folch et al., 1957), while the overall fatty acid abundances and fatty acid composition were determined by using a TraceGC gas chromatograph connected to a PolarisQ ion trap mass spectrometer following the protocols as described before (Bogen et al., 2013). The fatty acid composition of commercially available rapeseed oil was determined as control.

Phylogenetic characterization of strains

A tentative identification of strains was performed using the plastid 23S rDNA as described previously (Sherwood et al., 2008; Sherwood & Presting, 2007). DNA was extracted using the Chelex method as it was applied for colony PCR on *Chlamydomonas* (Cao et al., 2009).

DNA extraction, 23S rDNA amplification and sequencing of *Parachlorella kessleri* (CCAP 211/11H) and *Monoraphidium contortum* (SAG 47.80) was performed as control. The phylogenetic tree was constructed based on the Maximum Composite likelihood method using Mega5 (Tamura et al., 2011).

Results

The search for new promising strains remains a continuing and challenging task in research that is dedicated to establish microalgal strains for biofuel applications (Hu et al., 2008). Therefore, microalgae were isolated from local water bodies and screened on robust growth properties to identify promising strains for outdoor cultivations.

Isolation and strain selection

In total, 77 strains were isolated from various habitats, thereof 35 strains from a freshwater location in Bielefeld, 21 strains originating from a freshwater location in Gruenberg/Germany, one strain from a well close to the Bielefeld University (Dornberg), five further strains from the Lippesee/Germany, one strain deriving from an algal bloom of a river in Spain and another strain originating from a cooling water circuit in our lab. In addition, a number of saltwater strains were isolated: seven from the North Sea/Denmark and seven from the Baltic Sea/Germany. A further strain was isolated from an acidic lake enrichment culture (Lausatia/Germany). Visual observation of cultures grown in liquid media was performed to identify potential duplicates and resulted in a high reduction of the number of strains. Of the remaining strains, 20 showed generally robust biomass productivities in smaller scale setups and could be finally up-scaled to 1.5l batch cultures (Table 1).

Tentative identification of strains

A further, more detailed characterization is required to assess the novelty of a strain and to compare its general growth pattern and fatty acid profile to well-known organisms. Universal primers were used to amplify a suitable marker sequence of the plastid 23S rDNA (Sherwood et al., 2008), that was subsequently used to assign the isolates to already known organisms (Figure 2).

Most of the isolated strains clustered within the chlorophytes, while only two were found in a separate branch clustering with the evolutionary distinct diatoms (Figure 2).

The comparison of the sequencing data revealed the presence of five potential duplicates concerning the strains isolated from a pond in the Oetker Park (Oe2a, Oe9b, Oe14a I, Oe14b II, Oe15a). Further two duplicates were found to cluster with *Chlamydomonas geitleri* sequences (Oe11a II, Oe11a III). These strains exhibited a rather non-uniform appearance when investigated microscopically; therefore they were not removed after visual examination. This high degree of redundancy was not observed for the isolation of strains from another water body, a freshwater location in Brunnental/Gruenberg, where several distinct lineages could be isolated from the same enrichment culture.

However, these duplicates allowed investigating their micro-biodiversity in terms of biomass accumulation and fatty acid composition, which appears worthwhile since recent reports point out the high diversity in physiological and genetic patterns that can be encountered in individuals of the same species, even within the same bloom (Loret et al., 2002; Medlin et al., 2000).

Table 1 Strains isolated from the environment that have been successfully up-scaled to 1.5l batch cultures

	Origin	**ID**
1	Acidic Lake, Lusatia, Germany	AL-1
2	Pond, Brunnental, Gruenberg, Germany	Br5b
3	Pond, Brunnental, Gruenberg, Germany	Br6a II
4	Pond, Brunnental, Gruenberg, Germany	Br7a
5	Pond, Brunnental, Gruenberg, Germany	Br10a
6	Pond, Brunnental, Gruenberg, Germany	Br21
7	Baltic Sea, Hohwacht, Kiel, Germany	BS2-I
8	Baltic Sea, Hohwacht, Kiel, Germany	BS5-I
9	Cooling water circuit, Bielefeld, Germany	HL1
10	Lake, Lippesee, Germany	LS1
11	Lake, Lippesee, Germany	LS2
12	Pond, Oetker Park, Bielefeld, Germany	Oe2a
13	Pond, Oetker Park, Bielefeld, Germany	Oe9b
14	Pond, Oetker Park, Bielefeld, Germany	Oe11a II
15	Pond, Oetker Park, Bielefeld, Germany	Oe11a III
16	Pond, Oetker Park, Bielefeld, Germany	Oe14a I
17	Pond, Oetker Park, Bielefeld, Germany	Oe14b II
18	Pond, Oetker Park, Bielefeld, Germany	Oe15a
19	Well, Dornberg, Bielefeld, Germany	Sp2
20	River, Rio Yuso, Spain	Spain1

As a further outcome of the 23S rDNA sequence analysis of the isolates, the diatom strain isolate Br5b showed a 100% nucleotide identity in 329 bases of the plastid 23S rDNA to a dinoflagellate's plastid that has been recently published (Imanian et al., 2010). The other diatom isolate LS2, which originated from a different freshwater location, showed also a high sequence similarity with 99%. Their identity as diatoms was however confirmed by microscopic observations.

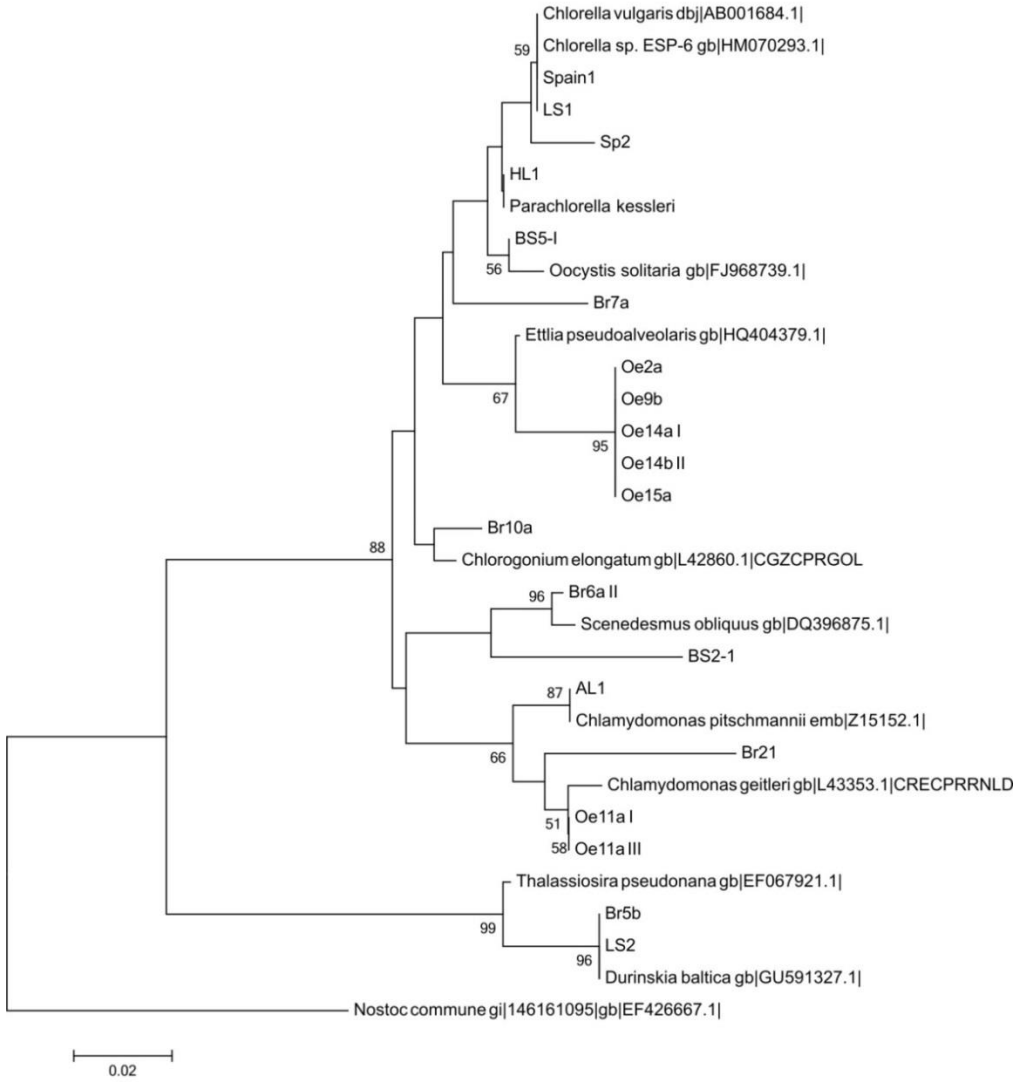

Figure 2 Tentative identification of strains using the evolutionary relationships of taxa. The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky & Nei, 1992). The optimal tree with the sum of branch length of 0.5421 is shown. The percentage of replicate trees in which the associated taxa clustered together above 50% in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree. The analysis involved 32 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 144 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

About half of the strains showed a distinct clustering to known organisms, while the other isolates (namely the five Oe duplicates, BS2-1, Br7a and Br21) appear to be of a certain distance to other well-known organisms according to the investigated stretch of the 23S rDNA sequence.

Biomass productivities and lipid contents

High biomass productivity combined with considerable lipid contents in large-scale cultivation systems are regarded as a crucial property of strains that are considered for biofuel applications (Hu et al., 2008). To identify candidates that could most probably meet those requirements, strains were up-scaled to 1.5l in minimal media and investigated on growth properties and lipid contents.

All investigated strains showed robust growth in minimal media, therefore being able to accumulate biomass without further supplementation with vitamins. Five strains showed mean biomass productivities exceeding 300mg l^{-1} d^{-1} during the cultivation period, including with Sp2 and Spain1 two isolates with productivities even higher than 400mg l^{-1} d^{-1} (Table 2). The lipid content of both strains with 17.6 ± 1.8% and 21.6 ± 0.7% of the dried biomass for Sp2 and Spain respectively was in the medium range.

Highest total lipid contents were determined for Br5b (35.7 ± 1.0% of the dried biomass) and LS2 (30.5 ± 1.8% of the dried biomass), while their biomass accumulation was considerably lower when compared to the other isolated strains (Table 2). LS2 showed with a mean of 64mg l^{-1} d^{-1} higher biomass accumulation than Br5b, that was however 7fold lower than of the best performer Sp2. Total lipid yields were surprisingly low for the Oetker Park isolates, namely for Oe2a, Oe9b, Oe14a I, Oe14b II and Oe15a ranging from 13.5 to 16.3% of dry biomass. Oe11a I and 11a III showed higher total lipid yields (17.4 - 19.4% of dried biomass), also combined with a higher mean biomass productivity.

With the exception of Br6a II and Br7a with a mean increase of biomass of 0.177 and 0.187mg l^{-1} d^{-1} respectively, biomass productivity was comparatively low for the strains isolated from the Brunnental location, a finding also observed for the isolates originating from the Baltic Sea (mean biomass productivities of 55 and 20mg l^{-1} d^{-1}).

Table 2 Mean increase of biomass during the culturing period and total lipid content. Biomass increase was measured by the determination of dry biomass accumulated after the cultivation period, divided by the number of days of cultivation. Total lipid amounts of the dry biomass were determined gravimetrically and the standard deviation determined from three technical replicates.

Species	Mean increase of biomass [g/day]	Total lipids content [%]	S.d.
AL-1	0.117	19.2	0.2
Br5b	0.033	35.7	1.0
Br6a II	0.177	19.5	0.2
Br7a	0.187	16.6	0.1
Br10a	0.015	14.6	0.2
Br21	0.051	18.8	0.3
BS2 I	0.055	13.9	0.4
BS5 I	0.020	16.6	2.5
HL1	0.326	18.2	1.8
LS1	0.219	22.7	0.3
LS2	0.064	30.5	1.8
Oe2a	0.176	15.7	2.7
Oe9b	0.119	14.7	0.4
Oe11a I	0.312	19.4	0.2
Oe11a III	0.302	17.4	0.3
Oe14a I	0.206	13.5	0.2
Oe14b II	0.164	14.6	0.1
Oe15a	0.234	16.3	2.1
Sp2	0.453	17.6	1.8
Spain 1	0.401	21.6	0.7

Fatty acid composition

When the fatty acid compositions of the strains were investigated, distinct differences were found between the strains that were assigned as diatoms and the Chlorophyte strains (Fig.2, Table 3). Both diatom strains (Br5b and LS2) showed the presence of saturated C14 fatty acids as well as comparatively high contents of C16:1 with 49.6% and 43.9% of the total fatty acids (Table 3). Furthermore, about 10% of the total fatty acids could be assigned to C20:5, which were only found to smaller percentages (3.1 – 3.6% of total fatty acids) in the marine isolates BS2-1 and BS5-I. While the Chlorophyte strains exhibited considerable amounts of mono- and dienoic C18 fatty acids, both compounds were below the 1% threshold of total relative abundances in Br5b and LS2. However, when overall abundances of fatty acids were considered, both diatoms

showed highest contents per unit biomass (172 and 144 for Br5b and LS2 respectively, compare to Table 3).

Both marine isolates (BS2-I and BS5-I) showed some amounts of long chained fatty acids (C20:1, C20:3 and C24:0), that did not reach the 1% threshold in any of the other investigated strains. Their overall fatty acid contents were comparatively high, ranging between relative abundance values of 94 and 102 per unit biomass.

Most of the Chlorophyte strains showed high abundances of C16:0 as well as C18:1 and C18:2. Interestingly between 15 and 19% of C16:4 could be detected for a number of strains (AL-1, Br7a, Br10a, Br21, Oe11a I and Oe11a III) as well as some minor amounts of C18:4 (ranging from 5.3 to 7.1% of total fatty acids) in all non-diatom strains isolated from the Brunnental location. The overall fatty acid abundances of the Chlorophytes were typically found to range below 50% of the fatty acid contents encountered in both diatom strains.

Discussion

Screening strategy

Several screenings have been applied before to identify promising strains for biofuel applications, either based on strains that were available from well-known culture collections (Breuer et al., 2012; Griffiths et al., 2011; Rodolfi et al., 2009) or focussing on organisms that have been recently isolated from environmental samples (Chaichalerm et al., 2012; Lim et al., 2012; Yang et al., 2012). While culture collections cultivate generally a wide variety of different organisms as emphasized by Mata et al., 2010, the isolation of new strains from the environment for a specific purpose can already apply targeted selection processes through the use of minimal media and removal of strains with slow growth properties as it was performed in this work.

Table 3 Fatty acid profiles [% of total fatty acids] of the 20 selected isolate strains from nutrient replete conditions. The fatty acid composition was determined by GC-MS and the relative fatty abundances within the total lipid fractions were calculated as specified in the material and methods sections. Values represent the mean of three technical replicates. Relative abundances below 1% were not included.

Isolate	C14:0	C16:4	C16:2	C16:1	C16:0	C18:4	C18:3	C18:2	C18:1	C18:0	C20:5	C20:4	C20:3	C20:1	C22:6	C24:0	Relative abundance
AL-1		14.8	2.8	7.5	17.8		4.2	10.2	42.6								65.91
Br5b	5.7		2.4	49.6	28.8					1.1	9.4	2.5					171.94
Br6a II		15.1	2.9	7.2	13.2	5.3		15.4	40.8								61.25
Br7a		15.1	1.6	6.8	17.8	5.4	5.5	8.6	39.3								61.05
Br10a		9.8	4.7	5.4	16.7	7.1	8.0	13.4	33.0	1.9							57.94
Br21		15.5	1.5	8.1	12.7	6.9		8.5	46.3								60.39
BS2-I			1.9	6.4	18.2			18.4	44.2	1.5	3.6		2.1	1.0		2.7	101.58
BS5-I			1.4	5.8	18.6		0.8	14.2	48.2	2.3	3.1		1.9	1.6		2.2	94.35
HL1			17.8	4.2	15.1			40.5	22.4								72.06
LS1		4.9	6.8	23.3	11.8			18.4	34.8								81.87
LS2	6.4		2.3	43.9	30.7					1.4	11.2	2.8			1.2		143.60
Oe2a			1.9	7.4	23.6			32.0	32.9	2.2							63.30
Oe9b			1.8	11.0	22.8			21.8	40.3	2.3							58.70
Oe11a I		19.3		7.1	15.3			5.3	51.0	2.1							70.60
Oe11a III		18.0	1.9	7.4	14.3			7.7	48.5	2.1							65.63
Oe14a I			2.4	8.1	22.4			29.2	36.3	1.6							52.44
Oe14b II			6.3	9.7	21.0			28.1	33.2	1.8							69.88
Oe15a			2.4	6.5	23.3			31.3	33.8	2.8							67.38
Sp2		7.3	2.0	11.4	15.6			17.8	43.7	2.1							66.14
Spain1		8.8	5.4	15.2	13.8			17.7	39.1					2.5			76.36
Rapeseed oil					5.9			21.9	67.0	2.7							317.33

The screening strategy that was applied here resulted in the selection of 20 strains that are able to grow abundantly under photoautotrophic conditions with elevated light intensities in minimal media, therefore providing the basis to potentially reduce cultivation costs that could arise e.g. from supplementation with high-value substrates as vitamins.

The accumulation of biomass of the isolates showed clear, strain-specific differences with a certain variation even between isolates of the same marker sequence. This heterogeneity in biomass productivity when various strains of the same species is investigated was also shown previously (Chen et al., 2011; Loret et al., 2002), therewith underlining the physiological biodiversity and the potential that can be encountered when new strains are isolated, even when originating from the same water body.

Sufficient supply with carbon plays a crucial role to allow optimal algal growth (Mata et al., 2010). Especially if photoautotrophic biomass generation is used as a sink for CO_2 (Chen et al., 2011; Mata et al., 2010; Schenk et al., 2008), its efficient uptake and conversion to biomass are important parameters for industrial applications. Since all of the strains were supplied with equal amounts of CO_2 in the up-scaled cultivations, the strains Sp2 and Spain1 would be highly interesting targets for carbon sequestration due to their high biomass productivity under these conditions.

It should be noted that the strains of the cultivations in this work not necessarily axenic, similar to other comparative studies before (Huerlimann et al., 2010; Lim et al., 2012).

The cultures showed no considerable increase of contamination during the culturing period in autotrophic conditions as it was controlled by microscopic observation. Nevertheless, it cannot be excluded, that potential symbionts could provide the algal cells with additional essential compounds or growth increasing factors (Lorenz et al., 2005), consequently leading to a decrease of growth when treated with antimicrobial agents to obtain axenic cultures.

Interestingly, solely green algae and diatoms were identified in the course of the screening performed in this work, showing the same tendency as it was reported for the outcome of the Aquatic Species Program (ASP) (Sheehan et al., 1998). When the collection of the ASP was reduced to the most promising candidates, mostly chlorophytes and diatoms came into closer consideration (Hu et al., 2008). Only few other strains from other phylogenetic branches are

currently in the focus of biofuel related research, as for example the Eustigmatophyte *Nannochloropsis* (Pal et al., 2011; Radakovits et al., 2012; Rodolfi et al., 2009; Simionato et al., 2013).

The high number of strains whose 23S rDNA sequence appeared rather distant to other published strains is likely to be reduced as soon as more information on plastid S rDNA is available. In a complementary approach, the nuclear 18S rDNA could be another target to assign those rather distantly related strains to known organisms as it has been performed in some other investigations (Lim et al., 2012; Yang et al., 2012; Yu et al., 2012). This was however not in the scope of this work, which was mainly focused on finding strains for biofuel purposes, but could be however highly interesting for future research on these isolates.

Potential isolation of a close relative of a tertiary endosymbiont

The high degree of nucleotide identity of the two diatom's 23S rDNA with the plastid of the dinoflagellate *Durinskia baltica* (Imanian et al., 2010) points towards a close phylogenetic relationship of their plastids. This finding is unusual since dinoflagellates and diatoms can be found on different phylogenetic branches (Keeling et al., 2005). The diatom origin of the dinoflagellate's plastid in an event of tertiary endosymbiosis has been investigated by Imanian et al., 2010.

The finding of a close relative of the original tertiary endosymbiont could therewith offer the possibility to investigate an apparently relatively recent event of tertiary endosymbiosis in more detail. The fact that even two diatoms have been isolated that apparently share this close relationship, could indicate to the wide distribution of this species in the environment. The genomic and cellular structure of Br5b and LS2 could furthermore possibly help to identify mechanism that could facilitate their integration into the dinoflagellate cell in the frame of tertiary endosymbiotic processes.

Comparison of biomass and lipid production

Usually strains with high basal lipid contents showed the lowest biomass productivities, therewith exemplifying the trade-off between high lipid content of cells and dominant growth as it was stated before (Chen et al., 2011; Rodolfi et al., 2009; Sheehan et al., 1998).

The most abundantly biomass accumulating strains (Sp2, Spain1) clustered closely to *Chlorella*, a genus whose members are well known for their high biomass productivity (Breuer et al., 2012; Laurens et al., 2012; Přibyl et al., 2012). Both isolates showed basic lipid contents and overall fatty acid abundances that were in the medium range when compared to the other strains. The most abundant fatty acids in *Chlorella vulgaris* were found to be C18:3, C18:2, C16:0 and C16:3 under nutrient replete conditions with a pronounced increase of C18:1 under nitrogen starvation (Breuer et al., 2012). This increase of C18:1 in the fatty acid profile was also observed for a *C. vulgaris* strain when grown in a thin-layer photobioreactor, where a total lipid content of 31% was reached (Přibyl et al., 2012). Since lower light intensities were applied by Breuer et al., 2012 and high maximum irradiances between 100 and 960µmol m^{-2} s^{-1} were measured within other studies (Přibyl et al., 2012), the presence of high contents of C18:1 in the fatty acid profiles of Sp2, LS1 and Spain1 could be attributed to the comparatively high and constant irradiance applied in this work. The content of C18:3 was reported to decrease dramatically, even below the detection limit under nutrient depletion (Laurens et al., 2012), so it could be a rather variable factor, responding quickly to the respective culturing conditions in case of *Chlorella* species. It should be noted that pronounced contents of C18:3 could be detected for other strains investigated with the same method in this work as well as previously (Bogen et al., 2013). The content of C18:2 (about 18% of total fatty acids) however corresponds well to other values reported from literature (Laurens et al., 2012; Přibyl et al., 2012).

Another candidate with high biomass productivity was identified with HL1, which clusters with *Parachlorella kessleri*. This species was shown to be able to be scaled up in thin-layer photobioreactors, showing there an outstanding increase of biomass (Li et al., 2012). However, under nutrient replete conditions, the lipid content remained comparatively stable at a low level during the first four days of cultivation (Li et al., 2012). It could be also shown by Li et al, 2012, that the cells required at least light intensities of about 150µmol m^{-2} s^{-1} for neutral lipid production, underlining the importance of light penetration to the cultures to increase the lipid yields. The high irradiance applied here might therefore also contribute to the comparatively high contents of total lipids encountered in HL1 besides potential strain-specific differences. The high contents of C18:2 (41% of total fatty acids) found in this work correspond well to the comparatively stable presence of C18:2 (40-43% of total fatty acids, in most cases) shown by Li et al., 2012, while there were pronounced differences in the contents of C18:0, C18:1 and C16:1.

As well as *C. vulgaris*, *P. kessleri* showed high overall lipid productivities when grown under nutrient depletion (Přibyl et al., 2012), so the application of nitrogen starvation should result in a substantial increase of total lipid contents and overall fatty acid abundances for those promising isolates.

Br6a II clusters with *Scenedesmus obliquus*, which is a well-known organism in biofuel-related research on algae (Breuer et al., 2012; Mandal & Mallick, 2009; Piorreck & Pohl, 1984). Its biomass productivity as it was investigated here was however at an average level. Therefore other isolates of this screening appeared with higher potential, e.g. those strains closely clustering to *Chlorella*.

The finding of polyunsaturated fatty acids with high chain length in case of the two diatom isolates Br5b and LS2 is consistent with the fatty acid profile of the marine diatom *Navicula salinicola*, investigated before (Bogen et al., 2013), showing also notable amounts of C20:5 (14.5% of total fatty acids). Some amounts of C20:5 and C20:4 have been reported previously for diatom strains (Hu et al., 2008). In addition, both isolates show considerably high contents of C16:1. High abundances of C16:1 were also found in *N. salinicola* (39.4% of total fatty acids) (Bogen et al., 2013) and were reported from other diatom strains (Hu et al., 2008). Therefore these fatty acids appear to be characteristic for the investigated diatom species.

When microalgal biomass is converted to liquid biofuels, the fatty acid composition plays a crucial role. Preferable fatty acid profiles for biodiesel should provide C16:1, C18:1 and C14:0 in a ratio of 5:4:1 (Schenk et al., 2008). In this work, C14:0 was only detected to some amounts in the two isolated diatom strains Br5b and LS2. Though both isolates showed considerably high percentages of C16:1 (44 – 50% of total fatty acids), the lack of C18:1 as well as their comparatively poor growth performances are the main obstacles for larger scale applications. In contrast, the isolated strain LS1 also showed high contents of C16:1 (23% of total fatty acids) with a considerable amount of C18:1 and C16:0 (35% and 12% of total fatty acids respectively). Its biomass accumulation was robust (219mg l^{-1} d^{-1}) but not as high as the best performer Sp2 and Spain1, which accumulated almost twice the biomass in the investigated time period.

Recent studies lead to the identification of candidate strains of *S. obliquus* and *Chlorella zofingiensis*, being able to retain high biomass productivities with concomitant prominent neutral lipid accumulation within the first few days of nitrogen starvation (Breuer et al., 2012), which

also appears as an interesting strategy to be applied to robustly growing strains as Sp2 or Spain1. Light penetration was also shown to be a critical factor for neutral lipid accumulation in previous work (Li et al., 2012), therefore optimized culturing and starvation conditions need to be applied to assess the whole potential of those strains of interest.

Summary

A one phase strategy under nutrient replete conditions was applied to identify candidates for liquid biofuel production. Some strains as Sp2 and Spain1 showed a remarkably good performance in terms of biomass accumulation and are therefore highly interesting strains for carbon sequestration. The investigation of total lipid contents and relative abundances of fatty acids revealed strains with comparatively high fatty acid contents, unfortunately combined with rather poor growth properties. The application of nitrogen starvation in a two stage setup is able to increase lipid productivity drastically and is therefore recommended to be applied to the most promising strains of this screening in terms of biomass productivity and lipid profile, which are LS1, Sp2 and Spain1. The finding of two diatoms that share a high similarity of their plastid 23S rDNA with a recently published sequence of a dinoflagellate offer furthermore the possibility for comparative studies of a relatively recent event of tertiary endosymbiosis.

Literature

Andersen, R.A. 2005. Algal Culturing Techniques, Elsevier Academic Press. Burlington.

Barsanti, L. 2006. *Algae : Biochemistry, Physiology, Ecology, and Biotechnology*.

Basova, M.M. 2005. Fatty acid composition of lipids in microalgae. *International Journal on Algae*, 7(1), 101.

Bogen, C., Klassen, V., Wichmann, J., Russa, M.L., Doebbe, A., Grundmann, M., Uronen, P., Kruse, O., Mussgnug, J.H. 2013. Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production. *Bioresource Technology*, 133(0), 622-626.

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H. 2012. The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, 124(0), 217-226.

Cao, M., Fu, Y., Guo, Y., Pan, J. 2009. *Chlamydomonas* (Chlorophyceae) colony PCR. *Protoplasma*, 235(1), 107-110.

Carlsson, A.S., van Beilen, J.B., Möller, R., Clayton, D. 2007. Micro- and macroalgae: utility for industrial applications. Outputs from the EPOBIO project.

Chaichalerm, S., Pokethitiyook, P., Yuan, W., Meetam, M., Sritong, K., Pugkaew, W., Kungvansaichol, K., Kruatrachue, M., Damrongphol, P. 2012. Culture of microalgal strains isolated from natural habitats in Thailand in various enriched media. *Applied Energy*, 89(1), 296-302.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., Chang, J.-S. 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology*, 102(1), 71-81.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnology Advances*, 25(3), 294-306.

Felsenstein, J. 1985. Confidence-Limits on Phylogenies - an Approach Using the Bootstrap. *Evolution*, 39(4), 783-791.

Folch, J., Lees, M., Stanley, G.H.S. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry*, 226(1), 497-509.

Gong, Y., Jiang, M. 2011. Biodiesel production with microalgae as feedstock: from strains to biodiesel. *Biotechnology Letters*, 1-16.

Gouveia, L., Oliveira, A. 2009. Microalgae as a raw material for biofuels production. *Journal of Industrial Microbiology and Biotechnology*, 36(2), 269-274.

Griffiths, M., van Hille, R., Harrison, S. 2011. Lipid productivity, settling potential and fatty acid profile of 11 microalgal species grown under nitrogen replete and limited conditions. *Journal of Applied Phycology*, 1-13.

Griffiths, M.J., Harrison, S.T.L. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, 21(5), 493-507.

Harwood, J.L., Guschina, I.A. 2009. The versatility of algae and their lipid metabolism. *Biochimie*, 91(6), 679-684.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*, 54(4), 621-39.

Huerlimann, R., de Nys, R., Heimann, K. 2010. Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production. *Biotechnoly and Bioengineering*, 107(2), 245-57.

Imanian, B., Pombert, J.F., Keeling, P.J. 2010. The complete plastid genomes of the two 'dinotoms' *Durinskia baltica* and *Kryptoperidinium foliaceum*. *PLoS One*, 5(5), e10711.

Keeling, P.J., Burger, G., Durnford, D.G., Lang, B.F., Lee, R.W., Pearlman, R.E., Roger, A.J., Gray, M.W. 2005. The tree of eukaryotes. *Trends in Ecology & Evolution*, 20(12), 670-6.

Knothe, G. 2008. "Designer" Biodiesel: Optimizing Fatty Ester Composition to Improve Fuel Properties. *Energy & Fuels*, 22(2), 1358-1364.

Lang, I., Hodac, L., Friedl, T., Feussner, I. 2011. Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. *BMC Plant Biology*, 11(1), 124.

Laurens, L.M.L., Quinn, M., Wychen, S., Templeton, D., Wolfrum, E. 2012. Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification. *Analytical and Bioanalytical Chemistry*, 403(1), 167-178.

Li, X., Přibyl, P., Bišová, K., Kawano, S., Cepák, V., Zachleder, V., Čížková, M., Brányiková, I., Vítová, M. 2012. The microalga *Parachlorella kessleri*—A novel highly efficient lipid producer. *Biotechnology and Bioengineering*, 110(1), 97-107.

Li, Y., Han, D., Sommerfeld, M., Hu, Q. 2011. Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under nitrogen-limited conditions. *Bioresour Technol*, 102(1), 123-9.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C. 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology*, 81(4), 629-636.

Lim, D.K.Y., Garg, S., Timmins, M., Zhang, E.S.B., Thomas-Hall, S.R., Schuhmann, H., Li, Y., Schenk, P.M. 2012. Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters. *PLoS ONE*, 7(7), e40751.

Lorenz, M., Friedl, T., Day, J.G. 2005. Perpetual maintenance of actively metabolizing microalgal cultures. in: *Algal Culturing Techniques*, (Ed.) R.A. Andersen, Elsevier Academic Press. New York, pp. 145-156.

Loret, P., Tengs, T., Villareal, T.A., Singler, H., Richardson, B., Mcguire, P., Morton, S., Busman, M., Campbell, L. 2002. No difference found in ribosomal DNA sequences from physiologically diverse clones of *Karenia brevis* (Dinophyceae) from the Gulf of Mexico. *Journal of Plankton Research*, 24(7), 735-739.

Mandal, S., Mallick, N. 2009. Microalga *Scenedesmus obliquus* as a potential source for biodiesel production. *Applied Microbiology and Biotechnology*, 84(2), 281-291.

Mata, T.M., Martins, A.n.A., Caetano, N.S. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*, 14(1), 217-232.

Matsumoto, M., Sugiyama, H., Maeda, Y., Sato, R., Tanaka, T., Matsunaga, T. 2010. Marine Diatom, *Navicula sp.* Strain JPCC DA0580 and Marine Green Alga, *Chlorella sp.* Strain NKG400014 as Potential Sources for Biodiesel Production. *Applied Biochemistry and Biotechnology*, 161(1), 483-490.

McNichol, J., MacDougall, K., Melanson, J., McGinn, P. 2011. Suitability of Soxhlet Extraction to Quantify Microalgal Fatty Acids as Determined by Comparison with In Situ Transesterification. *Lipids*, 1-13.

Medlin, L.K., Lange, M., Nöthig, E.-M. 2000. Genetic diversity in the marine phytoplankton: a review and a consideration of Antarctic phytoplankton. *Antarctic Science*, 12(03), 325-333.

Napier, J.A., Graham, I.A. 2010. Tailoring plant lipid composition: designer oilseeds come of age. *Current Opinion in Plant Biology*, 13(3), 329-336.

Nei, M., Kumar, S. 2000. *Molecular evolution and phylogenetics*. Oxford University Press, USA.

Pal, D., Khozin-Goldberg, I., Cohen, Z., Boussiba, S. 2011. The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. *Applied Microbiology and Biotechnology*, 90(4), 1429-1441.

Piorreck, M., Pohl, P. 1984. Formation of biomass, total protein, chlorophylls, lipids and fatty acids in green and blue-green algae during one growth phase. *Phytochemistry*, 23(2), 217-223.

Přibyl, P., Cepák, V., Zachleder, V. 2012. Production of lipids in 10 strains of *Chlorella* and *Parachlorella* , and enhanced lipid productivity in Chlorella vulgaris. *Applied Microbiology and Biotechnology*, 94(2), 549-561.

Radakovits, R., Jinkerson, R.E., Fuerstenberg, S.I., Tae, H., Settlage, R.E., Boore, J.L., Posewitz, M.C. 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropis gaditana*. *Nature Communications*, 3, 686.

Rodolfi, L., Zittelli, G.C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. 2009. Microalgae for Oil: Strain Selection, Induction of Lipid Synthesis and Outdoor Mass Cultivation in a Low-Cost Photobioreactor. *Biotechnology and Bioengineering*, 102(1), 100-112.

Rzhetsky, A., Nei, M. 1992. A Simple Method for Estimating and Testing Minimum-Evolution Trees. *Molecular Biology and Evolution*, 9(5), 945-967.

Saitou, N., Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4), 406-425.

Sasso, S., Pohnert, G., Lohr, M., Mittag, M., Hertweck, C. 2012. Microalgae in the postgenomic era: a blooming reservoir for new natural products. *FEMS Microbiology Reviews*, 36(4), 761-785.

Schenk, P.M.P., Thomas-Hall, S.R., Evan, S., Marx, U., Mussgnug, J.H., Posten, C., Kruse, O., Hankamer, B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *BioEnergy Research*.

Shamsudin, L. 1992. Lipid and fatty acid composition of microalgae used in Malaysian aquaculture as live food for the early stage of penaeid larvae. *Journal of Applied Phycology*, 4(4), 371-378.

Sheehan, J., Dunahay, T., Benemann, J., Roessler, P. 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae. U.S. Department of Energy's Office of Fuels Development.

Sherwood, A.R., Chan, Y.L., Presting, G.G. 2008. Application of universally amplifying plastid primers to environmental sampling of a stream periphyton community, Vol. 8, Blackwell Publishing Ltd, pp. 1011-1014.

Sherwood, A.R., Presting, G.G. 2007. Universal Primers Amplify a 23S rDNA Plastid Marker in Eukaryotic Algae and Cyanobacteria. *Journal of Phycology*, 43(3), 605-608.

Simionato, D., Block, M.A., La Rocca, N., Jouhet, J., Marechal, E., Finazzi, G., Morosinotto, T. 2013. Response of *Nannochloropsis gaditana* to nitrogen starvation includes a de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids and a reorganization of the photosynthetic apparatus. *Eukaryotic Cell*.

Solovchenko, A. 2012. Physiological role of neutral lipid accumulation in eukaryotic microalgae under stresses. *Russian Journal of Plant Physiology*, 59(2), 167-176.

Tamura, K., Nei, M., Kumar, S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America*, 101(30), 11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*.

Tran, N.H., Bartlett, J.R., Kannangara, G.S.K., Milev, A.S., Volk, H., Wilson, M.A. 2010. Catalytic upgrading of biorefinery oil from micro-algae. *Fuel*, 89(2), 265-274.

Yang, X., Liu, P.H., Hao, Z.D., Shi, J., Zhang, S. 2012. Characterization and Identification of Freshwater Microalgal Strains toward Biofuel Production. *Bioresources*, 7(1), 686-695.

Yu, X., Zhao, P., He, C., Li, J., Tang, X., Zhou, J., Huang, Z. 2012. Isolation of a novel strain of Monoraphidium sp. and characterization of its potential application as biodiesel feedstock. *Bioresource Technology*, 121(0), 256-262.

[5] Investigation of the influence of BTA1 down-regulation on the neutral lipid content in *C. reinhardtii*

C. Bogen

Summary

High contents of neutral lipids are generally considered as important factor to establish strains for liquid biofuel generation. In the past, a number of different targets of the glycerolipid metabolism were investigated on their influence on triacylglycerol (TAG) synthesis. Following an approach to potentially increase the metabolic flux towards TAG generation, BTA1 was down-regulated since it directly relies on the TAG precursor DAG for the synthesis of the non-phosphorous lipid DGTS. The down-regulation using artificial microRNAs resulted in expression levels of about 60% of the transcript level of the wildtype and the empty vector transformant. When the neutral lipid contents were investigated under nutrient replete conditions, no increase could be observed for the down-regulated transformants. This was also the case when nitrogen starvation was applied. Therefore further investigations are required to investigate the role of BTA1 in glycerolipid metabolism in *C. reinhardtii*.

Introduction

The accumulation of neutral lipids, namely triacylglycerols (TAG), in plants and in other photosynthetic organisms has developed to one of the predominant motives in research that is dedicated to the generation of renewable liquid biofuels (Liu & Benning, 2013).

Several pathways and mechanisms were subject to detailed investigations, especially in the plant model organism *Arabidopsis thaliana* (Stahl et al., 2004) and the green algae *Chlamydomonas reinhardtii* (Boyle et al., 2012; Fan et al., 2011; La Russa et al., 2012; Yoon et al., 2012). This work was expanded to other promising organisms, especially to the Heterokontophyte genus *Nannochloropsis* (Radakovits et al., 2012) as well as to diatoms, resulting into the finding that the

lipid metabolism between evolutionary diverse organism shows some pronounced differences (Hockin et al., 2012; Liu & Benning, 2013). It was confirmed that the lipid metabolism of the green algae *C. reinhardtii* exhibits a certain similarity to higher plants, with some notable exceptions (Liu & Benning, 2013). For instance, *C. reinhardtii* is characterized by the absence of phosphatidylcholine (PC), which is potentially replaced by the betaine lipid diacylglycerol-*N,N,N*-trimethylhomoserine (DGTS) (Wada et al., 2009), as well as a distinct pathway of TAG synthesis relying on glycerolipid precursors derived from the chloroplast that has been recently postulated (Fan et al., 2011).

In the past years, several green algal strains gained increasing attention as production host for liquid biofuels, especially due to their high biomass productivity (Bogen et al., 2013; Breuer et al., 2012; Li et al., 2012). Consequently results that were obtained from the investigation of *C. reinhardtii* are expected to promote targeted genetic engineering in other green algae to create strains with superior lipid qualities.

For the elucidation of pathways involved in neutral lipid accumulation in *C. reinhardtii*, over-expression strategies of diacylglycerol acyltransferases (DGAT) enzymes (Deng et al., 2012; La Russa et al., 2012) were applied as well as knock-down of specific enzymes as the phospholipid:diacylglycerol acyltransferase (PDAT) (Yoon et al., 2012) or the characterization of insertional mutants in the case of PDAT and a nitrogen response regulator (NRR) (Boyle et al., 2012). Most of this work resulted in either no increase (La Russa et al., 2012) or even a decrease of TAGs under nutrient replete conditions as shown in the case of PDAT down-regulation, leading to an alteration of polar lipid composition instead (Yoon et al., 2012).

Therefore, it has been suggested that down-regulation of polar lipids depriving the Kennedy pathway of TAG precursors, could result into an increase of metabolic flux towards TAG synthesis. DGTS was proposed as such a candidate (La Russa et al., 2012) since it is directly synthesized from diacylglycerol (Riekhof et al., 2005).

Betaine lipids are distinct in their chemical structure, since a glycerolipid containing two fatty acids is linked to the permethylated derivative of hydroxyamino acid by an ether bond at the *sn*-3 position (Dembitsky, 1996). These lipids are widespread in nature (Dembitsky, 1996; Kato et al., 1996; Künzler & Eichenberger, 1997), but have not been detected in flowering plants so far (Künzler & Eichenberger, 1997). They are however prominent in algae, and have been found in

several chlorophytes, including *C. reinhardtii* (Dembitsky, 1996). The total lipid fraction in *C. reinhardtii* was reported to consist to 18% of DGTS (Mendiola-Morgenthaler et al., 1985), therefore contributing to a prominent part to the polar lipid fraction. Due to its fatty acid composition, a cytoplasmic origin of DGTS could be corroborated (Giroud et al., 1988). Since the contents of DGTS and PC in various investigated organisms appears to be in an inverse relationship, DGTS is expected to functionally replace this phospholipid (Klug & Benning, 2001; Liu & Benning, 2013; Moore et al., 2001). PC is a characteristic phospholipid of extraplastidic membranes in higher plants, with a distinct role in the desaturation of C18:1 fatty acids that were synthesized in the chloroplast and have been subsequently transferred into the endoplasmic reticulum (Wallis & Browse, 2010).

In *C. reinhardtii* it was shown before, that BTA1 is able synthesize DGTS (Riekhof et al., 2005). BTA1 was about 2.6 fold down-regulated in *C. reinhardtii* wild-type under sulfur starvation (González-Ballester et al., 2010), a finding confirmed by microarray data, where BTA1 showed a decrease in abundance under sulfur starvation (Nguyen et al., 2011). This physiological state of the cells coincided with an accumulation of neutral lipids (Matthew et al., 2009), therefore the down-regulation of BTA1 became an interesting target for more detailed investigation. It was expected that the TAG content could increase under nutrient replete growth conditions by an increased flux towards the acyl-CoA dependent diacylglycerol:acyltransferase mediated TAG formation as a result of BTA1 down-regulation.

BTA1 was furthermore reported to be associated with lipid bodies in *C. reinhardtii*, being part of an abundant lipid body protein fraction (Moellering & Benning, 2010), which appears contradictory the decrease of transcript abundance under nitrogen and sulfur starvation (González-Ballester et al., 2010; Miller et al., 2010). Consequently, other studies could not confirm that this protein was connected to lipid bodies (Huang et al., 2013; Wang et al., 2009).

Therefore, the down-regulation of BTA1 should not result in a phenotype that is compromised in lipid body formation. This was subsequently tested by applying nitrogen deprivation to the down-regulated transformants.

Material and Methods

Strain and culture conditions

C. reinhardtii wild type CC3491 cw15 mt- (*Chlamydomonas* Resource Center, University of Minnesota, USA) was investigated on BTA1 expression and for the down-regulation approach. The strain was chosen to ensure comparability with previously published work (La Russa et al., 2012; Toepel et al., 2011).

Cultures were grown as liquid cultures in Tris–acetate–phosphate (TAP) medium (Harris, 2009). Transformants were screened in 20ml batch cultivations in Erlenmeyer flasks. Wild type, empty vector transformants and transformants carrying the construct targeting the BTA1 transcript were inoculated to an optical density of 0.05 measured at 750nm (OD_{750}, determined via a Genesys20, Thermo Spectronic, USA) from cultures in the logarithmic growth phase. OD_{750} was chosen since this parameter ensures a similar light penetration of cultures. Cultivations were performed mixotrophically at light intensities of about 150µmol m^{-2} s^{-1} at room temperature on a rotary shaker at 110rpm. Growth was controlled by cell counting (Beckman Coulter Z2, Krefeld, Germany) and OD_{750} as indicated before.

To compare the physiology of the identified down-regulated transformants with wild-type and empty vector control strains, growth was investigated in 200ml batch cultures under nutrient replete conditions.

To induce nitrogen starvation, 50ml of culture were harvested by centrifugation (2,500 x g, 5 minutes), washed twice with medium deprived of a nitrogen source and resuspended in the same amount of nitrogen deficient medium. Cultivation was performed at the same light intensities as indicated above. Following the same procedure, a control was transferred to nutrient replete conditions. For RNA extractions, samples were harvested via centrifugation (2,500 x g, 2 minutes) and frozen in liquid nitrogen.

Downregulation of BTA1

The design of the amiRNA construct, which targets the transcript of BTA1, was carried out as indicated by (Molnar et al., 2009). Oligonucleotides were designed based on the coding sequence

for BTA1 under the accession number AY656806.1 via Web Micro RNA designer 3 (http://wmd3.weigelworld.org) and examined for support by expressed sequence tags via Blast search versus the Kazusa database (http://est.kazusa.or.jp/en/plant/chlamy/EST/index.html, (Asamizu et al., 2004)). After EST support could be confirmed, the phosphorylated (5') and HPLC purified oligonucleotides (amiFor_mm 5' ctagtTTGACCCTTCTGCGCTGTATA-tctcgctgatcggcaccatgggggtggtggtgatcagcgctaTATATGGCGCAGAAGGGTCAAg 3' and amiRev_mm 5'ctagcTTGACCCTTCTGCGCC-ATATAtagcgctgatcaccaccaccccatggtgccgatcag-cgagaTATACAGCGCAGAAGGGTCAAa 3', ordered from Sigma-Aldrich) were annealed and cloned into the *Spe*I restriction site according to Molnar et al., 2009, to generate the construct pChlamiRNA3:BTA. The vector was then amplified in *E. coli* and purified. The insertion site with the target sequence was sequenced to confirm its integrity before the vector was used for the transformation of *C. reinhardtii* CC3491 via the glass beads method as described by (Kindle, 1990). Cultures were plated on TAP agar plates containing paromomycin (10 g l^{-1}) and screened for colonies. Positive transformants were picked and cultivated on paromomcyin containing TAP agar plates for storage in low light intensities (40 µmol m^{-2} s^{-1}). In parallel, transformants were generated carrying the empty vector (eV) as a control.

Expression analysis via RTqPCR

RNA extraction was performed according to (Chomczynski & Sacchi, 1987). Primers (forward GCTCGTACCTGTCCCAAGAC, reverse AGATCGATCGCCAGAAGAAA, with a product size of 117bp) were designed using Primer3 (http://frodo.wi.mit.edu/, (Rozen & Skaletsky, 2000)).

Quantitative real-time polymerase chain reactions (qPCR) were prepared using the SensiMix SYBR One-step Kit (Bioline, Luckenwalde, Germany) in a total reaction volume of 25µl with the following composition: RNA sample 8 µl, 2x Sensimix buffer 12.5µl, primer forward 10µM 1µl, primer reverse 10µM 1µl, RNAse inhibitor 0.5µl and water 2µl. RT qPCR was subsequently performed on a DNA Engine Opticon (MJ Research, Miami, US) and analyzed via the Opticon Monitor Analysis Software (version 1.06) of the same supplier as the instrument.

Expression levels of BTA1 were calculated relative to Cyclophilin (Stürzenbaum & Kille, 2001) (CYN19-3 fw GCCAACCCCTTGGTCTACTT, CYN19-3 rev GAAGTTCTCCGCAGTCTTGG as designed by Lutz Wobbe) using the equation according to (Livak & Schmittgen, 2001):

$$\text{Amount of target} = 2^{-\Delta\Delta CT}$$

$$-\Delta\Delta C_T = (C_T\text{BTA1} - C_T\text{Cyclophilin})$$

Neutral lipid measurements

Neutral lipids were determined using Nile Red as fluorescence dye as described previously (La Russa et al., 2012). Therefore cultures were diluted to cell concentrations between 1-3 × 10^5 cells ml^{-1}. 4µl of Nile-Red (250 µg ml^{-1}) were added to 2ml of cell suspension in 20% DMSO and vortexed for 30 seconds. Fluorescence was measured from 200µl of suspension in a flat transparent Greiner 96 well plate with an excitation wavelength of 530nm and emission wavelength of 580nm using an Infinite Reader M200 (Tecan, Männedorf, Switzerland).

Statistical analysis

Student's T-test was performed for pairwise comparisons to investigate the transcript down-regulation (n=18 for each investigated strain) using EXCEL (Microsoft). P-values were considered significant when < 0.001.

Results

Expression of BTA1 in transformants

BTA1 transcripts were found consistently down-regulated in two (BTA1-1 and BTA1-9) of 48 transformants to about 60 % of the expression level in the wildtype and empty vector transformant (eV) under nutrient replete (BTA1-1 with 63% ± 1 and BTA1-9 with 58 ± 6% of WT, as shown Fig. 1, grey bars) and nitrogen starvation conditions (BTA1-1 with 66% ± 5 and BTA1-9 with 62% ± 6 of WT, Fig.1, white bars). No further transformants were found via a screening applying qPCR that were consistently down-regulated among biological replicates.

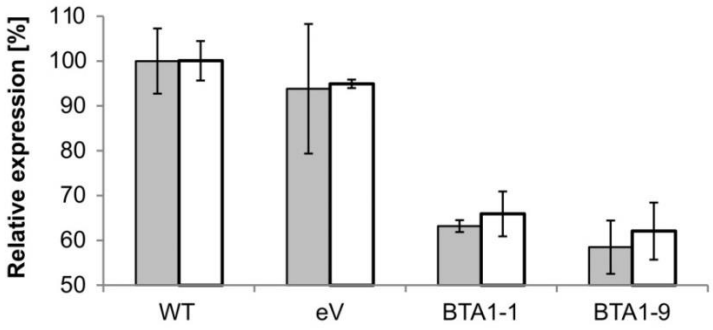

Figure 1 BTA1 down-regulation after 24 hours cultivation in nutrient replete conditions (grey bars) and after 16 hours of nitrogen depletion (white bars). Error bars indicate standard deviation of at least three technical replicates.

The degree of down-regulation was found to fluctuate in a certain range (Figure 1); however was found to be highly significant. When the degree of down-regulation compared to WT was investigated in a pairwise Student's t-test, the two transformants showed highly significant differences ($p>0.001$) in the expression pattern when compared to wild type and eV.

Growth and neutral lipid contents in down-regulated BTA1 transformants

When growth was investigated under nutrient replete conditions, BTA1-1 showed a reduced growth rate in the beginning but eventually reaches the same culture densities as the wild-type and the control eV (Figure 2A). In contrast, BTA1-9 showed a comparable growth pattern to WT and eV, while maintaining the same reduced transcript abundances as BTA1-1 (Figure 1). When the content of neutral lipids was investigated, a pronounced increase of Nile Red fluorescence could be observed within the first 10 hours of growth following the inoculation of denser pre-cultures. However, with rising cell number the Nile Red fluorescence per cell decreased, indicating a reduction of neutral lipids within the cells. The decrease was found less pronounced in the transformant BTA1-1 within the first 56 hours but then reached comparative levels as BTA1-9 and the controls.

Neutral lipids are usually accumulated under nitrogen starvation (Miller et al., 2010). Therefore, growth pattern and neutral lipid content of the two transformants were investigated under these conditions.

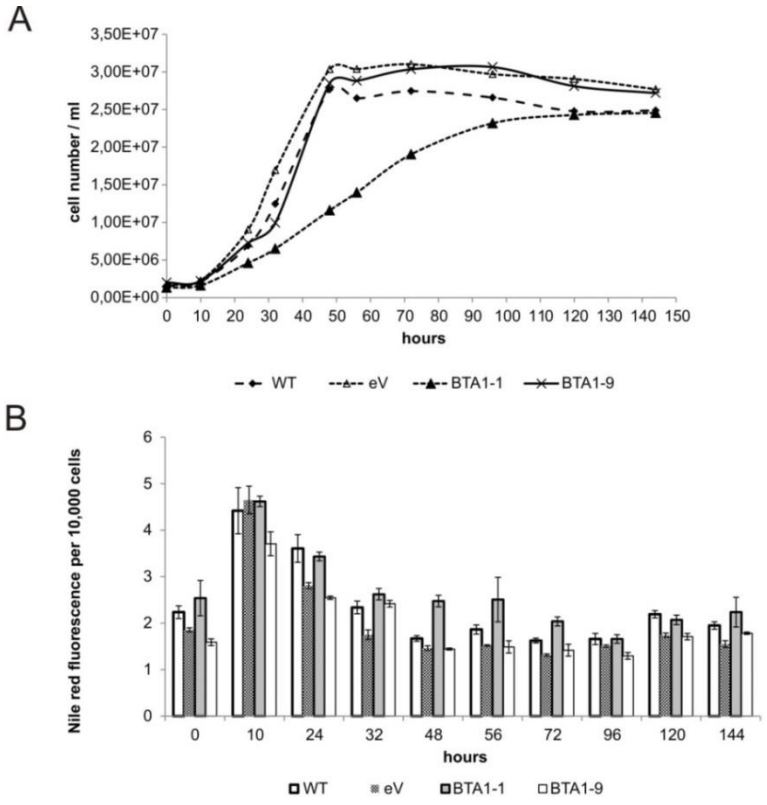

Figure 2 (A) Growth of transformants down-regulated in BTA1 expression (BTA1-1 and BTA1-9) compared to wildtype (WT) and a transformant carrying the basic amiRNA construct without BTA1 targeting sequence (eV) under nutrient replete conditions. The cell numbers represent means of two replicates. (B) Neutral lipid content of the investigated strains at the respective time points of culturing. Error bars indicate standard deviation of at least four technical replicates.

Growth was arrested for down-regulated transformants and controls within 24 hours after starvation was applied, with the number of cells remaining on a similar level (Figure 3A). Furthermore, the content of neutral lipids increased in the cells (Figure 3B), with the highest neutral lipid contents reached in the control carrying the empty vector after 120 and 144 hours of nitrogen deprivation. Wildtype and transformants remained on a similar level of fluorescence. BTA1-9 showed a more pronounced increase of neutral lipids after 120 hours of nitrogen starvation, however not being confirmed by BTA1-1.

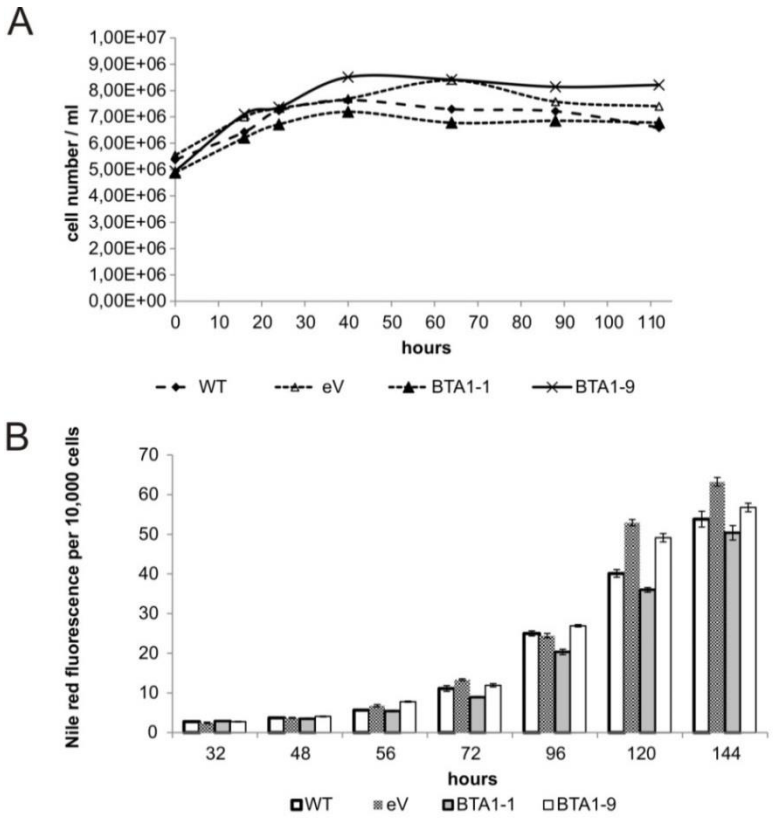

Figure 3 (A) Growth of transformants down-regulated in BTA1 expression (BTA1-1 and BTA1-9) compared to wildtype (WT) and a transformant carrying the basic amiRNA construct without BTA1 targeting sequence (eV) under nitrogen deprivation. The cell numbers represent means of two replicates. (B) Neutral lipid content of the investigated strains at the respective timepoints of culturing. Error bars indicate standard deviation of at least four technical replicates.

These general tendencies could be confirmed by further replicates in view of growth, Nile Red fluorescence and degree of down-regulation.

Discussion

Since comparable levels of neutral lipids were found during the logarithmic growth phase, no persistent change in the metabolic flux towards TAG synthesis via Kennedy pathway could be observed by down-regulating the transcript abundance of BTA1 in the investigated range. Unfortunately, no anti-body was available to confirm the down-regulation via immunoblotting on the protein level.

The TAG pool has been suspected to play not only a role for the storage of carbon and energy, but to represent also an important and flexible system for membrane remodeling with enzymes as PDAT assigned to crucial functions, especially in nutrient replete growth conditions (Yoon et al., 2012). Therefore, even if a down-regulation of BTA1 resulted in an increase of the metabolic flux towards TAG synthesis, multi-functional enzymes as PDAT could act as counterplayer, channeling triacylglycerols immediately to the polar lipid fraction. This flexibility is mirrored by the finding of another enzyme that shuffles acyl-chains between DAG and polar lipids, therewith decreasing the degree of saturation within the neutral lipid fraction (Napier & Graham, 2010). Since it was recently suggested that neutral lipid precursors can be also synthesized in the chloroplast under nitrogen starvation (Fan et al., 2011), DAG from the chloroplast could also potentially bypass main routes of the Kennedy pathway to generate TAG.

The comparatively high number of transcripts found for BTA1 in *Chlamydomonas* under nutrient replete conditions (González-Ballester et al., 2010; Miller et al., 2010), especially when considered with post-transcriptional regulation mechanisms, could result into a sufficiently high translation, leading to a mitigation of the down-regulation effect that was achieved using amiRNAs.

The transformants were furthermore investigated under lipid induction conditions to assess the influence of down-regulated BTA1 transcript abundance on cells suffering nitrogen starvation. The accumulation of neutral lipids under nitrogen starvation was not found to differ consistently between wild-type and down-regulated transformants, leading to the suggestion that the reduction of BTA1 transcript abundance does not result in a phenotype compromised in its lipid accumulation pattern under these conditions.

Whether BTA1 was associated with lipid bodies (Moellering & Benning, 2010) or not (Huang et al., 2013), DGTS was suggested to hold structural roles as it was identified as prominent component of the polar lipid monolayer (Wang et al., 2009). Its function could be possibly replaced by phospholipids in the investigated range of down-regulation; therewith increasing the cells need for phosphorous.

In *Arabidopsis thaliana*, 18:1 fatty acids derived from the chloroplast are incorporated into PC for further desaturation (Wallis & Browse, 2010). Therefore, a down-regulation of BTA1 could result in an alteration of the fatty acid profile of the TAG fraction. This could be used to alter the

fatty acid composition towards a higher degree of saturation, which is in general more favorable for liquid biofuel applications due to higher oxidative stability and cetane number of the fuel (Hu et al., 2008).

However, it remains unclear whether DGTS in *C. reinhardtii* possesses similar function to PC in higher plants as *Arabidopsis*. The ether bond between the diacylglyceryl moiety and the betaine headgroup exhibits a higher stability than the ester bond of phospholipids, therefore a frequent conversion of DGTS into DAG and subsequent TAG appears rather doubtful as pronounced by Liu and Benning (2012).

Here, PDAT could provide a more elegant solution. PDAT could be responsible for the shuffling of fatty acids from DGTS to DAG after they underwent desaturation while connected to DGTS. It has been already shown that PC is a suitable target for its lipase activity (Yoon et al., 2012), so taken the structural similarity with DGTS, it could also lay within the functional range of PDAT.

Since BTA1 down-regulation did apparently not increase metabolic flux or lead to a persistent increase of TAG levels under the conditions tested, other targets might be more promising to increase overall TAG abundance. A different approach to yield higher TAG contents was suggested by blocking the TAG catabolism, which proved to be rather complicated since beta-oxidation plays an important role in the metabolism and unfavorable phenotypes have been reported from various approaches (Radakovits et al., 2010). Further targets could be the over-expression of thioesterases, which were already shown to increase neutral lipid levels in *Phaeodactylum* (Gong et al., 2011), therewith bypassing potential feedback inhibition in fatty acid generation.

Conclusions

The transcript abundance of BTA1, an enzyme responsible for generation of the polar lipid DGTS, was significantly down-regulated to about 60% of the expression found in wildtype. However, down-regulation did not result in a phenotype compromised in growth rate, leading to the suggestion that either the remaining transcript abundance is sufficient to maintain its overall cellular functioning or it could be readily replaced by phospholipids. No sustained increase in neutral lipid accumulation was observed in the down-regulated transformants, so the transcript

abundance of BTA1 appears to be without a major influence on TAG levels. However, further investigation of BTA1 down-regulated mutants are recommended to elucidate the potential role of DGTS in the generation of polyunsaturated fatty acids that are abundantly found in *C. reinhardtii* (La Russa et al., 2012), since a higher content of saturated and monounsaturated fatty acids could promote the organisms suitability for biorefinery concepts.

Literature

Asamizu, E., Nakamura, Y., Miura, K., Fukuzawa, H., Fujiwara, S., Hirono, M., Iwamoto, K., Matsuda, Y., Minagawa, J., Shimogawara, K., Takahashi, Y., Tabata, S. 2004. Establishment of publicly available cDNA material and information resource of *Chlamydomonas reinhardtii* (Chlorophyta) to facilitate gene function analysis. *Phycologia*, 43(6), 722-726.

Bogen, C., Klassen, V., Wichmann, J., Russa, M.L., Doebbe, A., Grundmann, M., Uronen, P., Kruse, O., Mussgnug, J.H. 2013. Identification of *Monoraphidium contortum* as a promising species for liquid biofuel production. *Bioresource Technology*, 133(0), 622-626.

Boyle, N.R., Page, M.D., Liu, B., Blaby, I.K., Casero, D., Kropat, J., Cokus, S., Hong-Hermesdorf, A., Shaw, J., Karpowicz, S.J., Gallaher, S., Johnson, S., Benning, C., Pellegrini, M., Grossman, A., Merchant, S.S. 2012. Three acyltransferases and a nitrogen responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*. *Journal of Biological Chemistry*.

Breuer, G., Lamers, P.P., Martens, D.E., Draaisma, R.B., Wijffels, R.H. 2012. The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains. *Bioresource Technology*, 124(0), 217-226.

Chomczynski, P., Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, 162(1), 156-159.

Dembitsky, V.M. 1996. Betaine ether-linked glycerolipids: Chemistry and biology. *Progress in Lipid Research*, 35(1), 1-51.

Deng, X.-D., Gu, B., Li, Y.-J., Hu, X.-W., Guo, J.-C., Fei, X.-W. 2012. The Roles of acyl-CoA: Diacylglycerol Acyltransferase 2 Genes in the Biosynthesis of Triacylglycerols by the Green Algae *Chlamydomonas reinhardtii*. *Molecular Plant*, 5(4), 945-947.

Fan, J., Andre, C., Xu, C. 2011. A chloroplast pathway for the de novo biosynthesis of triacylglycerol in *Chlamydomonas reinhardtii*. *FEBS letters*, 585(12), 1985-1991.

Giroud, C., Gerber, A., Eichenberger, W. 1988. Lipids of *Chlamydomonas reinhardtii*. Analysis of Molecular Species and Intracellular Site(s) of Biosynthesis. *Plant Cell Physiol.*, 29(4), 587-595.

Gong, Y., Guo, X., Wan, X., Liang, Z., Jiang, M. 2011. Characterization of a novel thioesterase (PtTE) from *Phaeodactylum tricornutum*. *Journal of Basic Microbiology*, 51(6), 666-672.

González-Ballester, D., Casero, D., Cokus, S., Pellegrini, M., Merchant, S.S., Grossman, A.R. 2010. RNA-Seq Analysis of Sulfur-Deprived *Chlamydomonas* Cells Reveals Aspects of Acclimation Critical for Cell Survival. *The Plant Cell Online*, 22(6), 2058-2084.

Harris, E.H. 2009. *The Chlamydomonas Sourcebook*. Elsevier Science.

Hockin, N.L., Mock, T., Mulholland, F., Kopriva, S., Malin, G. 2012. The Response of Diatom Central Carbon Metabolism to Nitrogen Starvation Is Different from That of Green Algae and Higher Plants. *Plant Physiology*, 158(1), 299-312.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal*, 54(4), 621-39.

Huang, N.-L., Huang, M.-D., Chen, T.-L.L., Huang, A.H.C. 2013. Oleosin of Subcellular Lipid Droplets Evolved in Green Algae. *Plant Physiology*, 161(4), 1862-1874.

Kato, M., Sakai, M., Adachi, K., Ikemoto, H., Sano, H. 1996. Distribution of betaine lipids in marine algae. *Phytochemistry*, 42(5), 1341-1345.

Kindle, K.L. 1990. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences*, 87(3), 1228-1232.

Klug, R.M., Benning, C. 2001. Two enzymes of diacylglyceryl-O-4′-(N,N,N,-trimethyl)homoserine biosynthesis are encoded by btaA and btaB in the purple bacterium *Rhodobacter sphaeroides*. *Proceedings of the National Academy of Sciences*, 98(10), 5910-5915.

Künzler, K., Eichenberger, W. 1997. Betaine lipids and zwitterionic phospholipids in plants and fungi. *Phytochemistry*, 46(5), 883-892.

La Russa, M., Bogen, C., Uhmeyer, A., Doebbe, A., Filippone, E., Kruse, O., Mussgnug, J.H. 2012. Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. *Journal of Biotechnology*, 162(1), 13-20.

Li, X., Přibyl, P., Bišová, K., Kawano, S., Cepák, V., Zachleder, V., Čížková, M., Brányiková, I., Vítová, M. 2012. The microalga *Parachlorella kessleri*—A novel highly efficient lipid producer. *Biotechnology and Bioengineering*, 110(1), 97-107.

Liu, B., Benning, C. 2013. Lipid metabolism in microalgae distinguishes itself. *Current Opinion in Biotechnology*, 24(2), 300-309.

Livak, K.J., Schmittgen, T.D. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, 25(4), 402-408.

Matthew, T., Zhou, W., Rupprecht, J., Lim, L., Thomas-Hall, S.R., Doebbe, A., Kruse, O., Hankamer, B., Marx, U.C., Smith, S.M., Schenk, P.M. 2009. The Metabolome of *Chlamydomonas reinhardtii* following Induction of Anaerobic H2 Production by Sulfur Depletion. *Journal of Biological Chemistry*, 284(35), 23415-23425.

Mendiola-Morgenthaler, L., Eichenberger, W., Boschetti, A. 1985. Isolation of chloroplast envelopes from *Chlamydomonas*. Lipid and polypeptide composition. *Plant Science*, 41(2), 97-104.

Miller, R., Wu, G., Deshpande, R.R., Vieler, A., Gärtner, K., Li, X., Moellering, E.R., Zäuner, S., Cornish, A.J., Liu, B., Bullard, B., Sears, B.B., Kuo, M.-H., Hegg, E.L., Shachar-Hill, Y., Shiu, S.-H., Benning, C. 2010. Changes in Transcript Abundance in *Chlamydomonas reinhardtii* following Nitrogen Deprivation Predict Diversion of Metabolism. *Plant Physiology*, 154(4), 1737-1752.

Moellering, E.R., Benning, C. 2010. RNA Interference Silencing of a Major Lipid Droplet Protein Affects Lipid Droplet Size in *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 9(1), 97-106.

Molnar, A., Bassett, A., Thuenemann, E., Schwach, F., Karkare, S., Ossowski, S., Weigel, D., Baulcombe, D. 2009. Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. *Plant Journal*.

Moore, T.S., Du, Z., Chen, Z. 2001. Membrane Lipid Biosynthesis in *Chlamydomonas reinhardtii*. In Vitro Biosynthesis of Diacylglyceryltrimethylhomoserine. *Plant Physiology*, 125(1), 423-429.

Napier, J.A., Graham, I.A. 2010. Tailoring plant lipid composition: designer oilseeds come of age. *Current Opinion in Plant Biology*, 13(3), 329-336.

Nguyen, A.V., Toepel, J., Burgess, S., Uhmeyer, A., Blifernez, O., Doebbe, A., Hankamer, B., Nixon, P., Wobbe, L., Kruse, O. 2011. Time-Course Global Expression Profiles of *Chlamydomonas reinhardtii* during Photo-Biological H_2 Production. *PLoS ONE*, 6(12), e29364.

Radakovits, R., Jinkerson, R.E., Darzins, A., Posewitz, M.C. 2010. Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell*, 9(4), 486-501.

Radakovits, R., Jinkerson, R.E., Fuerstenberg, S.I., Tae, H., Settlage, R.E., Boore, J.L., Posewitz, M.C. 2012. Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropis gaditana*. *Nature Communications*, 3, 686.

Riekhof, W.R., Sears, B.B., Benning, C. 2005. Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase BTA1Cr. *Eukaryotic Cell*, 4(2), 242-52.

Rozen, S., Skaletsky, H. 2000. Primer3 on the WWW for General Users and for Biologist Programmers. in: *Bioinformatics Methods and Protocols*, (Eds.) S. Misener, S. Krawetz, Vol. 132, Humana Press, pp. 365-386.

Stahl, U., Carlsson, A.S., Lenman, M., Dahlqvist, A., Huang, B., Banas, W., Banas, A., Stymne, S. 2004. Cloning and Functional Characterization of a Phospholipid:Diacylglycerol Acyltransferase from *Arabidopsis*. *Plant Physiology*, 135(3), 1324-1335.

Stürzenbaum, S.R., Kille, P. 2001. Control genes in quantitative molecular biological techniques: the variability of invariance. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 130(3), 281-289.

Toepel, J., Albaum, S.P., Arvidsson, S., Goesmann, A., la Russa, M., Rogge, K., Kruse, O. 2011. Construction and evaluation of a whole genome microarray of *Chlamydomonas reinhardtii*. *Bmc Genomics*, 12.

Wada, H., Murata, N., Moellering, E.R., Miller, R., Benning, C. 2009. Molecular Genetics of Lipid Metabolism in the Model Green Alga *Chlamydomonas reinhardtii*. in: *Lipids in Photosynthesis*, (Ed.) Govindjee, Vol. 30, Springer Netherlands, pp. 139-155.

Wallis, J.G., Browse, J. 2010. Lipid biochemists salute the genome. *Plant Journal*, 61(6), 1092-106.

Wang, Z.T., Ullrich, N., Joo, S., Waffenschmidt, S., Goodenough, U. 2009. Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 8(12), 1856-1868.

Yoon, K., Han, D., Li, Y., Sommerfeld, M., Hu, Q. 2012. Phospholipid:Diacylglycerol Acyltransferase Is a Multifunctional Enzyme Involved in Membrane Lipid Turnover and Degradation While Synthesizing Triacylglycerol in the Unicellular Green Microalga *Chlamydomonas reinhardtii*. *The Plant Cell Online*.

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Studium

10/2007 - 11/2009	Studium der Biologie an der Technischen Universität München
	12.11.2009: Master of Science (M.Sc.), Gesamtnote: 1,2 (mit Auszeichnung bestanden)
	Thema der Masterarbeit: Anaerobic Oxidation of Methane in Agriculturally Used Grassland Soils
10/2004 - 06/2007	Studium der Biologie an der Philipps-Universität Marburg
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La Russa, M., **Bogen, C.**, Uhmeyer, A., Doebbe, A., Filippone, E., Kruse, O., Mussgnug, J.H., 2012. Functional analysis of three type-2 DGAT homologue genes for triacylglycerol production in the green microalga *Chlamydomonas reinhardtii*. Journal of Biotechnology, 162(1), 13-20

Bannert, A., **Bogen, C.**, Esperschütz, J., Koubová, A., Buegger, F., Fischer, D., Radl, V., Fuß, R., Chroňáková, A., Elhottová, D., Šimek, M., and M. Schloter. 2012. Anaerobic oxidation of methane in grassland soils used for cattle husbandry. Biogeosciences Discussions, Volume 9, pp.4919-4945

Bogen C., Al-Dilaimi A., Albersmeier A., Wichmann J., Grundmann M., Rupp O., Blom J., Blifernez-Klassen O., Kalinowski J., Mussgnug J.H., Kruse O. Reconstruction of the lipid metabolism for the microalga *Monoraphidium neglectum* from its genome sequence reveals characteristics suitable for biofuel production. Submitted

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst angefertigt habe und nur die angegebenen Quellen und Hilfsmittel verwendet habe. Alle aus der Literatur ganz oder annähernd entnommenen Stellen habe ich als solche kenntlich gemacht.

Weiterhin erkläre ich, dass die vorliegende Dissertation weder vollständig noch teilweise einer anderen Fakultät mit dem Ziel vorgelegt worden ist, einen akademischen Titel zu erwerben. Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Universität Bielefeld.

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