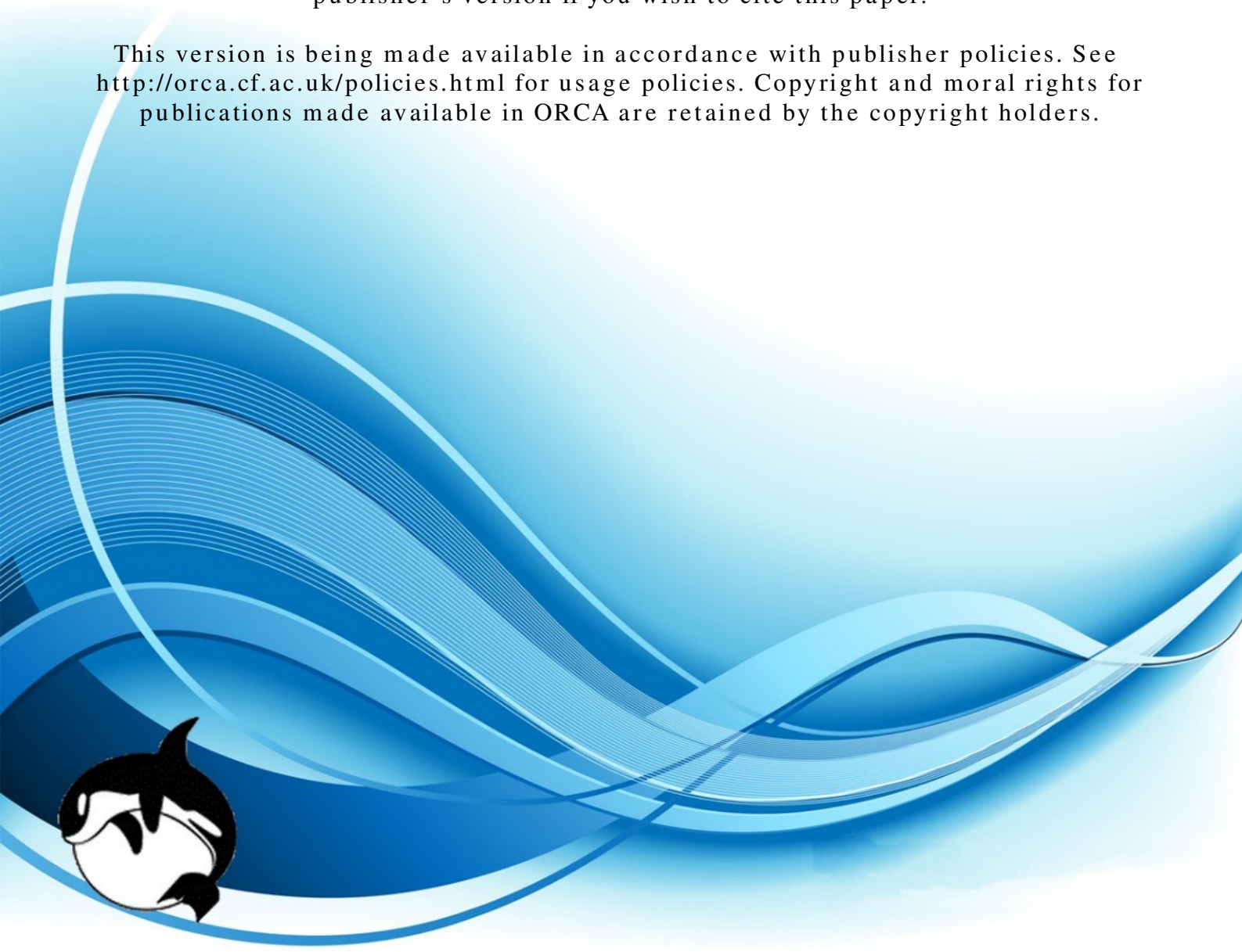

The lipid biochemistry of eukaryotic algae

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Abstract: (<250 words)

Algal lipids have fascinated scientists and entrepreneurs due to the large diversity of fatty acid structures, lipid types and novel lipophyllic compounds they produce. Algae have therefore long been studied as sources of genes for novel fatty acids; and recently due to their superior biomass productivity and high capacity for triacylglycerol (TAG) synthesis, algae are considered a potential feedstock for biofuel. However a major issue in the establishment of a commercially viable "algal TAG-to-biofuel" industry is its high production cost, because most algal species only produce large amount of TAGs after being exposed to stress conditions (e.g. nutrient deficiency, light, temperature, minerals). Therefore recent studies on lipids in algae have mostly focused on the identification of factors involved in TAG metabolism, on the subcellular organization of lipid pathways and on interaction between organelles. This has been accompanied by the development of genetic/genomic and synthetic biological tools not only for the flagship green alga *Chlamydomonas reinhardtii* but also extended to two other algal species, i.e. *Nannochloropsis* and *Phaeodactylum.* Advances in our understanding of enzymes and regulatory proteins of acyl lipid (especially TAG) synthesis and turnover (from *de novo* fatty acid synthesis, to glycerolipid assembly and to their degradation through the β-oxidation pathway) are described here with some focus on carbon and energetic aspects. Inter-organelle communications from peroxisome to chloroplast which have been discovered to be important in acyl lipid metabolism are detailed. We also summarize how changes in environmental factors can impact lipid metabolism and describe present and potential industrial uses of algal lipids.

Key words: Algal lipid metabolism; Acetyl-CoA carboxylase; β-oxidation; Mitochondrial respiration; Reducing equivalents; Triacylglycerols; Environmental effects; Commercial exploitation

Abbreviations: (as a footnote)

α-CT, α-carboxyltransferase; ACCase, acetyl-CoA carboxylase; ACK, acetate kinase; ACAD, acyl-CoA dehydrogenase; ACOX, acyl-CoA oxidase; ACP, acyl carrier protein; ACS, acetyl-CoA synthetase; AOX, alternative oxidase; APX, ascorbate peroxidase; ASC, ascorbate; β-CT, β-carboxyltransferase; BADC, biotin attachment domain-containing protein; BC, biotin carboxylase; BCAA, branched chain amino acid; BCCP, biotin carboxyl carrier protein; CAT, catalase; CEF, cyclic electron flow; CoA, coenzyme A; CTS1, comatose 1; COX, cytochrome oxidase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGDG, digalactosyldiacylglycerol; DHA, docosahexaenoic acid; DH, dehydrogenase; DGTA, 1,2-diacylglyceryl-3-O-2'-(hydroxymethyl)-(*N,N,N*-trimethyl)-β-alanine; DGTS, diacylglyceryl-3-O-4'-(*N,N,N*-trimethyl)-homoserine; DYRK, dual-specificity tyrosine-phosphorylation-regulated kinase; EPA, eicosapentaenoic acid; ER, enoyl-ACP reductase; FA, fatty acid; FAD, fatty acid desaturase; FAT, fatty acyl-ACP thioesterase; FAX1, fatty acid export1; KAS, 3-ketoacyl-ACP synthase; G3P, glycerol-3-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HAD, hydroxyacyl-ACP dehydrase; HL, high light; KAR, ketoacyl-ACP reductase; LACS, long chain acyl-CoA synthetase; LEF, linear electron flow; Lyso-PA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; MCMT, malonyl-CoA:ACP malonyltransferase; Mal, malate; MDA, malondialdehyde; ME, malic enzyme; MFP, multi-functional protein; MGDG, monogalactosyldiacylglycerol; NO, nitric oxide; NRR1, nitrogen response regulator1; OAA, oxaloacetate; PA, phosphatidic acid; PAT, phosphate acetyltransferase; PAP, phosphatidic acid phosphatase; PtdCho, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdGro, phosphatidylglycerol; PGRL1; proton gradient regulation 5 like 1; PL, phospholipid; PLA2, phospholipase A2; PXN, peroxisomal NAD^+ carrier; PPP, pentose phosphate pathway; PUFA, polyunsaturated fatty acid; SAD, stearoyl-ACP desaturase; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol; TAR1, triacylglycerol accumulation regulator1; TCA, tricarboxylic acid; TF, transcription factor; TOR, target of rapamycin; VLCPUFA, very long chain polyunsaturated fatty acid.

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1. Introduction

Algae are a large, diverse and polyphyletic group of photosynthetic organisms. They range from unicellular microalgae (including *Ostreococcus*, the smallest known free-living eukaryote [1]) to the giant kelp, which may reach 45 m in length [2]. Most authors no longer consider prokaryotes, such as cyanobacteria, amongst the algae, but it is from these prokaryotes that algal plastids are derived [3]. While green algae contain primary plastids from endosymbiotic cyanobacteria, diatoms and brown algae contain secondary chloroplasts derived from endosymbiotic red algae [4]. Algae have a range of reproductive strategies and, as implied earlier, can unicellular organisms or possess complex multicellularity [5]. Although there is as yet no accurate tally of the total number of algal species, a recent estimate is that there are 72,500 species worldwide [6].

As befits their diversity, algae can use sunlight for photosynthesis or can exist as mixotrophs or facultative heterotrophs. Some of the latter have lost their ability to photosynthesize and have become obligate heterotrophic parasites, such as *Plasmodium* and *Toxoplasma* [5]. There are also those species of algae that form important symbiotic relationships with other organisms such as in coral reefs [7], lichens [8] and sea sponges. The complexity of algae is manifest in the origins and functions of algal genes [5] as well as in their lipid biochemistry [9].

Algae are prominent in bodies of water (both freshwater and marine) but are also found in unusual environments such as snow and ice or hot springs. In most cases they are at the base of food chains and provide core ecosystem functions such as by supplying half the oxygen we breathe [10]. In high densities, such as algal blooms, algae can outcompete other life forms and cause a health hazard. In other cases, algae can act as indicator organisms to monitor pollution in various situations [11].

Algae have been exploited by humans for hundreds of years and are currently used to produce agar and other alginates, fertilizers, nutritional products and pigments, in addition to their use in bioremediation. With our increasing knowledge of algal genomes and availability of transcriptomes [12], there are more and more opportunities to exploit algae for biotechnological purposes such as for biofuels, nutraceuticals and pharmaceuticals [5]. Opportunities related to lipids are discussed in **section 7**.

For background information on algal lipid biochemistry, please refer to [13-16]. In this review we will concentrate on literature following the review by Guschina and Harwood [9].

2. Lipids in algae

The major lipid classes in algae are the membrane lipids (glycosylglycerides, phosphoglycerides, betaine, ether lipids) and the storage lipids (in the form of triacylglycerol) [17]. Algae also possess small amounts of other lipid classes such as terpenoids, sphingolipids, hydrocarbons, sterols and, of course, pigments that are present in different percentages depending on the class of alga.

There are a number of 'unusual' compounds which have been detected in a limited number of species. No doubt many more will be found. For example, phosphatidylsulphocholine (the sulphonium analogue of phosphatidylcholine) has been identified in diatoms and Euglena [14], halogenated fatty acids (FAs) and their derivatives [18] in various algae and novel hydrocarbons in *Botryococcus braunii* [9].

Table 1 shows the acyl lipid composition of a variety of algae. In keeping with the diverse structure of different algae, the quantitative and qualitative compositions of lipids varies considerably. While the three glycosylglycerides (monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG; sulphoquinovosyldiacyglycerol, SQDG) are major components, their % contributions are distinct. In general there is more MGDG than DGDG, as in higher plants [17] but, in contrast to the latter, SQDG is often a major constituent of algae. Although there has been little effort to examine the subcellular distribution of SQDG, one presumes that in those algae with a high content, it is not just localized to thylakoids (unlike in higher plants). As in land plants, the MGDG of algae tends to contain a higher proportion of polyunsaturated fatty acids (PUFA) than DGDG. Both galactolipids are more unsaturated than SQDG [19]. There is an acylated derivative of SQDG, 2'-O-acyl-sulphoquinovosyldiacylglycerol, which is found in algae such as *Chlamydomonas reinhardtii* [20].

There has been considerable interest in analyzing the molecular species of the glycosylglycerides, especially MGDG. This interest is in relation to the so-called 'prokaryotic' and 'eukaryotic' pathways of acyl lipid synthesis (see [21]) and is discussed in **section 4**. However, it is also relevant to a study in diatoms where MGDG and DGDG molecular species were compared in two centric species (*Skeletonema marinoi, Thalassiosira weissflogii*) with pinnate species (*Phaeodactylum tricornutum, Haslen ostrearia, Navicula perminuta*) [22]. Although monoacyl-glycosylglycerides have been reported in algae [23], their possible formation during extraction cannot be eliminated if careful precautions to inhibit any endogenous lipases are not taken.

Several betaine lipids are important components of algae. DGTS (diacylglyceryl-*O*-(*N,N,N*-trimethy)-homoserine is the most common in nature and is found in green algae (**Table 1**). DGTA (1,2-diacylglyceryl-3-*O*-2'(hydroxymethyl)-(*N,N,N*-trimethyl)-beta-alanine) is typically found in many brown algae. The third betaine lipid is DGCC (diacylglycerylcarboxylhydroxymethylcholine) and was first discovered in the marine genus Haptophyceae, such as in *Pavlova luthera*. The distribution of these betaine lipids in many different species of algae have been reported [24-26]. A recent evaluation of the occurrence and molecular diversity of betaine lipids in marine microalgae has been published [27].

With a few exceptions, the amount of phosphoglycerides in algae is much less than that of the glycosylglycerides (**Table 1**). All the usual phosphoglycerides are found even in minor amounts but phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn) and phosphatidylglycerol (PtdGro) are the main ones. Even in brown algae there is a wide variation in the percentages of different phosphoglycerides [28]. Phosphatidyl-*O*-*N*-(2-hydroxyethyl)glycine (PHEG: preciously called N-CAPE), a ceramidephosphoinositol and an arsenic-containing phospholipid were also detected in a variety of brown algal species [28].

Because of the current interest in algae as sources of particular FAs or in the use of their accumulated triacylglycerol (TAG) for biofuel (**section 7**), there has been much research on evaluating TAG by mass spectrometry [29]. Such research has revealed the evolutionary divergence of the main TAG synthesis pathways in green microalgae [30]. Since TAG is the main lipid accumulated, it may be necessary to rapidly screen many species (or lines) in order to pinpoint those which could be usefully considered by industry. This has led to evaluation of FAs as biomarkers or 'characteristic' components for the quantitation of TAG in algae [31-34].

Remarks about the overall FA composition of algae as well as their location in different lipids have been summarized in [9, 14, 15]. Specific comments in relation to single cell oils are in [35]. A recent important and informative survey by Lang et al [36] has examined the stationary phase compositions of algae within the SAG culture collection. A selection of their analyses are shown in **Table 2**. What is immediately apparent is that the FA compositions of different species vary widely, as noted before [14]. Moreover, even within the same class, there is no very consistent pattern – for example, palmitate concentrations vary widely in Haptophyceae while linoleate concentration varies widely in Conjugatophyceae (**Table 2**). A recent review of diatoms, as the most abundant phytoplankton species, has noted that they tend to have 14:0, 16:0. 16:1 and 20:5 as their main FAs [37]. The presence of 14:0 and a low amount of 18C acids is rather characteristic, as can be seen from the diatom representative, *Phaeodactylum tricornutum* in **Table 2**. For the commercially-important very long chain

PUFAs (VLC-PUFAs), such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, only certain algal classes are productive (see **section 7**). Such acids also tend to be more prevalent in marine or salt-tolerant algae rather than, for example, freshwater green algae (**Table 3**). Moreover, marine algae are also sometimes notable for their high concentrations of arachidonic acid (ARA) as well as EPA (**Table 4**). It should also be noted that different lipid classes will almost invariably have distinct FA compositions as mentioned above and discussed further in [14, 19, 38]. Furthermore, growth conditions, seasonal variations and developmental stages will all play a role in influencing the FA contents of algae and their individual lipid classes [9, 14, 15, 39].

Evaluation of methodology for the extraction of lipids (and FAs)[40-42] and, especially, in their further analysis have continued to be active areas of research. Special attention has been paid to the increasing use of mass spectrometry (MS) [43] which, of course, is sensitive and can provide information about molecular species and confirmation of identities. Nevertheless, some inherent problems with quantification using MS should be considered [44]. The use of MS methods versus the more traditional TLC plus GC techniques have been compared for two microalgae (and Arabidopsis) by [38]. They point out the difficulties of using MS for quantification but suggest a way of reducing the possible bias of MS data by using an external standard.

3. *De novo* FA synthesis in the chloroplast

The pathway and organization of *de novo* FA synthesis in algae is mostly inferred from that of plants wherein the steps and regulatory mechanisms of lipid synthesis have been better characterized [45]. The first *in silico* analysis of the genes encoding proteins of FA synthesis in algae was carried out for the model green alga *Chlamydomonas reinhardtii* in 2005, which allowed a reconstruction of the FA synthetic pathway [46, 47]. Later on, with the advent of affordable genome sequencing and high sensitive RNAseq technologies, many more algal genomes have been sequenced and subjected to *in silico* analyses of metabolic pathways. Up to the time of this writing, ~30 algal genomes have been sequenced, allowing for bioinformatic analyses of algal lipid metabolism and a scaffold for synthetic biological studies. In all known eukaryotic species with a chloroplast (derived either from primary or secondary endosymbiosis), *de novo* FA synthesis is known to occur in the stroma. For example, genome and expressed sequence tags (ESTs) analyses of some algal species from diverse evolutionary origins, including the diatom *Phaeodactylum tricornutum*, the heterokont *Nannochloropsis sp.,* the red alga *Galdieria sulphuraria* and *Cyanidioschyzon merolae* [48-54], revealed that FA

synthesis is likely similar to that of the green lineage namely green algae (Chlorophyta) and higher plants (Embryophyta) [31, 45, 55, 56]. A simplified scheme for *de novo* FA synthesis, highlighting sources for carbon, ATP, and reducing equivalents is outlined in **Figure 1**.

3.1 Reactions and enzymes of FA synthesis

3.1.1 Acetyl-CoA carboxylase (ACCase)

The first committed step for *de novo* FA synthesis is the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA; a two-step reaction catalyzed by the biotin-containing enzyme ACCase. In nature, ACCase occurs in two forms: one is a heteromeric, multisubunit complex containing four different polypeptides including biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP) and α- and β-carboxyltransferase (α- and β-CT); while the other is a homomeric form wherein each of the four aforementioned components are fused in tandem on a single, large polypeptide. The subcellular location of ACCase varies between organisms. Animal ACCase, which is of the homomeric form, is present in both the cytoplasm and mitochondria, while plant and algal ACCase, which is of both homomeric and heteromeric forms, is located in the cytoplasm and plastid [57-59]. The heteromeric form of ACCase, present in all plants except graminaceous monocots, is plastid-localized, while the homomeric form is either cytosolic or plastid-localized, depending on the species [60, 61]. Orthologues to plant ACCase have been identified in all algal species with sequenced genomes [54, 62]. *In silico* sequence analyses of the genome of *C. reinhardtii* identified the occurrence of both types of ACCase [46]. However their subcellular locations have not been verified experimentally. A comprehensive sequence analysis of ACCase across many algal species of diverse evolutionary origin revealed that most algae of primary symbiotic origin (*i.e.* Chlorophyta, Rhodophyta and *Paulinella* spp. with their chloroplast surrounded by two envelope membranes) and contain heteromeric ACCase in their chloroplasts, as evidenced by the presence of heteromeric BCCP in these taxa (**Figure 2**). In contrast, algal and apicoplast-containing species which possess plastids derived from secondary endosymbiosis (*i.e.* Heterokontophyta and Haptophyta), contain only the homomeric ACCase in their chloroplast [63-65]. Thus, in general, the presence of heteromeric or homomeric ACCase is dependent on chloroplast origin. The cytosolic ACCase, which is always of homomeric form, is mostly known for its role in supplying malonyl-CoA for FA elongation or polyketide synthesis.

ACCase plays an important role in the regulation of carbon flux into FA biosynthesis [66], including in algae, wherein a positive correlation between ACCase activity and FA

amount has been observed in *Chlorella vulgaris* [67]. In plants, plastid targeting of a homomeric ACCase to rapeseed plastids produced a 5% increase in seed lipid content [68]. In contrast, overexpression of plant homomeric ACCase in the diatom *Cyclotella cryptica* did not produce a measurable increase in total FAs, despite a 2- to 3-fold increase in ACCase activity [69]. Heterologous overexpression of the homomeric ACCase of the diatom *P. tricornutum* in *Escherichia coli* resulted in a 2-fold increase in neutral lipid production based on Nile red staining, although the identities of these neutral lipids remain unknown due to the absence of diacylglycerol acyltransferase (DGAT) and hence triacylglycerol (TAG) accumulation in most bacteria [70].

The heteromeric form of ACCase is an equally attractive target for metabolic engineering as the homomeric form: overexpression of all four subunits of the heteromeric ACCase in *E. coli* has been shown to result in a >100 fold increase in malonyl-CoA production followed by a six-fold increase in the rate of *de novo* FA synthesis [71]. Although such an experiment has not been performed in plants or algae, individual subunits of heteromeric ACCase have been overexpressed in plants including the subunits β-CT [72], BC [73] and BCCP [74]. In spite of successful overexpression, none of these transformants yielded increased ACCase activity and one (BCCP) produced lower activity due to incomplete biotinylation [74, 75]. Collectively, these results suggest these three subunits are not limiting to ACCase activity *in planta*. This was recently confirmed by absolute quantitation of each of the subunits of the Arabidopsis ACCase during seed development [76]. Surprisingly, the α-CT subunit (which has never been over-expressed in plants) is between 3-10-fold less abundant than its partner β-CT, which is the only plastid-encoded subunit of heteromeric ACCase. Recently, a new plant subunit to the heteromeric ACCase was identified [77]. This subunit, termed BADC (an abbreviation for the tentative annotation as "biotin attachment domain-containing"), resembles the BCCP subunit but is not biotinylated, acting as a negative regulator of ACCase rather than as a carboxyl carrier. The regulation of ACCase by BADC and other factors is discussed in brief in **section 3.8.1** and more comprehensively in a recent review [78].

3.1.2 Malonyl-CoA: ACP malonyltransferase (MCMT)

The malonyl-CoA generated by ACCase enters into the steps dedicated to *de novo* FA synthesis. Malonyl-CoA is first converted to malonyl-acyl carrier protein (ACP) by MCMT. Over-expression of the native gene encoding MCMT in *Nannochloropsis oceanica* resulted in an 31% increase in neutral lipids together with a modified FA composition with eicosapentaenoic acid (20:5, EPA) increased by 8% [79]. This finding is of particular interest because neutral

lipid content is increased together with an increased growth rate and photosynthetic performance.

3.1.3 The FA synthase (FAS) complex

Malonyl-ACP is then ligated to an acetyl-CoA molecule to form a 3-ketoacyl-ACP by ketoacyl-ACP synthase, while releasing a molecule of CO_2. The 4-carbon 3-ketoacyl-ACP is subsequently reduced (by ketoacyl-ACP reductase, KAR), dehydrated (by hydoxyacyl-ACP dehydrase, HD), reduced again (by enoyl-ACP redutase, ER) until finally a 6-carbon-ACP is formed. The enzymes involved (KAS, KAR, HD, ER) collectively form the multi-subunit bacterial type II FA synthase (FAS) complex [80]. In most algal or plant species, the FAS reaction repeats 7 cycles until the formation of a C16-ACP. The C16-ACP has three fates: it can be acylated to glycerol by chloroplast-resident acyltransferases to produce chloroplast lipids; it can also be further elongated to C18-ACP by a KASII; or it can be converted to a C16 free FA by acyl-ACP thioesterase (FAT). C18-ACP is either desaturated by stearoyl-ACP desaturase (SAD) or being converted to free FA by FAT. The saturated and unsaturated C18 fatty acyl ACPs are substrates of FAT and their metabolic products (i.e. non-esterified (free) FAs) are exported out of the chloroplast. Expression of the cyanobacteria KAR in the chloroplast of the red alga *Cyanoidioschyzon merolae* resulted in strains over-accumulating TAG while maintaining cellular growth; transcriptome and metabolome analysis of the overexpressing lines suggest that KAR over-expression and N starvation, although both led to increased TAG accumulation, likely employed different metabolic routes for TAG accumulation [81]. The only algal SAD studied to date is that from *Chlorella zofingiensis* [82], which exhibited a substrate preference for 18:0 similar to the plant enzyme.

In addition to the multi-component type II FAS, some algae also contain a cytosolic type I FAS, which normally is involved in FA elongations (>C18 FA), or may complement type II FAS when the demand for FA synthesis is high. For instance, it was observed that transcription of type I FAS was increased in cells of *Nannochloropsis gaditana* exposed to high light (HL), which is mirrored by a decrease in transcriptions of type II FAS, suggesting a shift in FA synthetic activities from chloroplast to cytoplasm [83].

3.1.4 Acyl-ACP thioesterase (FAT or TE)

During the FAS extension cycles, the acyl chains are covalently bound via a thioester linkage to the prosthetic group of a soluble ACP. Termination of the chain elongation is thus carried out via the action of FAT which hydrolyzes acyl-ACP to form non-esterified FA and ACP. This

step determines quantity and type of FAs that are exported. In 16:3 plants such as *Arabidopsis*, the acyl-flux through FAT has been determined to be ~60% of total FAs made in the chloroplast, and the flux can reach 90% in 18:3 plants [84]. FAT represents a key enzyme in the partitioning of *de novo* synthesized FAs between the prokaryotic and eukaryotic pathways (see **section 4**), and from a biotechnology perspective, FAT is therefore an important target for genetic engineering studies aiming to tailor FA production.

Based on sequence alignments and substrate specificities, FATs have been classified into two major families, FatA and FatB [85, 86]. FatAs from diverse plant/algal species show strict substrate preference towards 18:1-ACP, whereas FatBs primarily hydrolyze saturated acyl-ACPs with 8 to 18 carbons [85, 87, 88]. Several medium-chain specific FatBs have been cloned from *Umbellularia californa* (California bay) and from several species of the genus *Cuphea* known to produce oils rich in medium chain FAs (MCFA, C6-12) in their seeds [89]. Heterologous expression of MCFA-specific FatBs have been shown to produce MCFAs in transgenic oilseed crops [88, 90, 91]. Lately, transgenic expression of some of these specialized plant FatBs have resulted in production of MCFAs in algae including *P. tricornutum* [92], *Dunaliella tertiolecta* [93] and *C. reinhardtii* [94]. Interestingly, heterologous expression of a thioesterase of *Duanliella tertiolecta* in *C. reinhardtii* has resulted in a 50% increase in total FA production [95]. A first report on characterization of algal FAT is the study of a novel thioesterase from *P. tricornutum* where PtTE showed no similarity to characterized plant and bacterial thioesterases [96], but its endogenous overexpression in *P. tricornutum* led to a 72% increase in FA content without altering FA composition [96]. Moreover, recent work in *C. reinhardtii* has identified the important role of protein-protein (ACP-FAT) interaction in chloroplast FA synthesis [97], implying the importance of subcellular context in genetic engineering studies. In summary, current evidence show that FATs play not only a role in determining FA chain length but can also impact FA total amount.

3.1.5 FA export

Currently, no direct evidence is available regarding how the nascent FAs assembled in the stroma pass through the two, three or sometimes four envelope membranes of algal chloroplasts. Genes encoding known protein components of transport pathway in plants, including the fatty acid export 1 (FAX1) [98] and long-chain acyl-CoA synthetase 9 (LACS9) [99], can be identified in algal genomes, but the putative orthologues and their functions in FA export have not been examined in algae. Various similarities and differences in lipid transport between plants and algae are reviewed recently in [31, 100].

3.1.6 FA modifications: elongation and desaturation

Neo-synthesized FAs (C16:0, C18:0; and C18:1) are usually further elongated or desaturated to finally constitute the lipid makeup of a given organism. FA elongations are mostly known to uniquely occur in the endoplasmic reticulum (ER) [101]; while FA desaturations occur both inside the chloroplast and in extra-chloroplast compartments. Except for SAD, mostly known desaturases are membrane-bound [102, 103]. Steps and enzymes required for desaturation of FA in *C. reinhardtii* have mostly been identified and are recently reviewed in [56]. One interesting feature is the occurrence of only one plastidial ω-3 FA desaturase (CrFAD7) in *C. reinhardtii* [104], which often occur in multiple isoforms present in both chloroplast and extra-chloroplast of plant cells [54].

A survey of VLCPUFA synthesis in algae was included in the previous review by [9] and here we provide an update on the pathways involved in the model diatom *Phaeodactylum tricornutum* where high amount of polyunsaturated fatty acids are made (**Figure 3)** {Sayanova, 2017 #2432}. For both the n-3 and n-6 pathways, metabolism begins with Δ6-desaturation in most organisms. However, Δ9-elongation from LA or from LNA provides an alternative route which has been found in *Parietochloris incisa* [105], *Isochrysis galbana* [106], *Pavlova salina* [107], *Emiliania huxleyi* [108] and *Euglena gracilis* [109]. As shown in **Figure 3**, ω3-desaturation can convert n-6 into n-3 PUFAs and such a conversion for ARA into EPA has been shown in *Nannochloropsis* sp. [52], *Monodus subterraneus* [110] and *Porphyridium cruentum* [111]. In some marine eukaryotes of the Thrustochytriaceae, a polyketide synthase (PKS) pathway is used to make VLCPUFA. The pathway is used by *Schizochytrium* but in *Thraustochytrium* a desaturation/elongation pathway is utilised [112]. Due to the nutritional importance of very long chain PUFAs (see **section 7**), elongases and desaturases in algae have been intensively researched, and several excellent current reviews cover this area [49, 54, 113].

3.2 Carbon sources for acetyl-CoA synthesis

Increasing evidence suggests a positive link between the rate of FA synthesis and the amount of carbon precursors in plants and algae [114-118], implying that enhancing the rate of carbon flux into chloroplasts might be a worthwhile approach for genetic engineering attempts to improve FA amount. This finding highlights the importance of understanding the potential sources and their contributions to chloroplast acetyl-CoA production. Various sources and use of acetyl-CoA in plants has been summarized in [119]. In addition to the chloroplast acetyl-

CoA pool, acetyl-CoA is also made by reactions inside the mitochondria and peroxisomes [120]. Activation of the pyruvate dehydrogenase complex (PDC) protein while silencing the pyruvate dehydrogenase kinase (PDK) has boosted acetyl-CoA production therefore neutral lipid content in *Phaeodactylum tricornutum* [121]. Carbons contained in acetyl-CoA can be shuttled to other compartments, but must first be converted into to malate or pyruvate, which are transported across membranes through malate shuttles or by other solute transporters [122, 123]. Thus far, no known acetyl-CoA transporter is reported in any organism. Acetyl-CoA is thus considered not directly imported by chloroplasts [124], but rather generated by chloroplastic enzymes. Four possible routes can lead to acetyl-CoA production, as discussed below. The relative importance of these possible sources varies between species, and between phototrophic or heterotrophic tissues, or trophic style of a given species. Moreover, these carbon sources are by no means exclusive, for instance, through a chemical-genetic screen for oil inducers in *P. tricornutum*, the authors have suggested that sterol metabolism contributes to TAG synthesis probably by providing acetyl-CoA [125].

3.2.1 Chloroplast pyruvate dehydrogenase (PDH)

In plants and algae grown photoautotrophically, acetyl-CoA is mostly produced via the oxidative decarboxylation of pyruvate by the chloroplast pyruvate dehydrogenase complex (PDH)[126]. In addition to acetyl-CoA, this reaction generates CO_2 and NADH [127]. In turn, pyruvate can be made from glycolysis, malate (through malic enzyme, ME), as a side reaction of RuBisCO, or pentose phosphate pathway (PPP) linked to photosynthesis or to sugar oxidation (oxidative PPP = OPPP) (**Figure 1**). The proportion of their contribution to pyruvate formation has not been worked out, but it most likely varies by species and trophic style. The temporal expression of plastid PDH upon N starvation, i.e. during high rate of oil synthesis, is consistent with its role in FA synthesis in several algal species including *C. reinhardtii* [114, 128]. Indeed, silencing of a gene encoding a putative chloroplast E1α subunit of PDH using microRNA in *C. reinhardtii* has resulted in strains producing >40% less total FAs than control strains expressing only an empty vector during photoautotrophic N starvation, but there was little or no impact on lipid accumulation during photoheterotrophic growth (i.e. with the presence of acetate) [129]. In addition, photosynthetic parameters and growth of PDH-E1α silenced strains were also negatively affected, implying the importance of chloroplast PDH not only in FA synthesis but also in general algal physiology and development. Indeed, acetyl-CoA is a key intermediate in a number of different metabolic pathways [130]. Moreover, high CO_2 supply has been observed to increase FA synthesis in several algal species [131-134] (see also

section 6.1.), suggesting the importance of photosynthesis in carbon supply. However, the picture could be different if acetate is present as already observed in the PDH-silenced strains. This is consistent with the recent finding that when acetate is present, *C. reinhardtii* employs principally the CO_2 carbon fixation pathway for starch synthesis whereas acetate is used mainly for FA synthesis [135].

3.2.2 Acetyl-CoA synthetase (ACS)

Acetyl-CoA can also be produced from acetate: a direct conversion through ACS or through a two-step reaction catalyzed by acetate kinase (ACK) and phosphate acetyltransferase (PAT). This feature enables a radio-tracer studies of lipid metabolism in algae via simply feeding cells with radio-labelled C14 acetate. The ACS route is widely present in plants and algae, whereas the ACK/PAT route occur mostly in prokaryotes and some eukaryotic microalgae [136], with both routes requiring ATP. The idea of "acetate-to-acetyl-CoA" for FA synthesis has been discarded in plants due to extensive *in vivo* flux analyses demonstrating the actual source of acetyl-CoA is from PDH [137]. Nevertheless, the ACS route is known to play a major role in heterotrophically or mixotrophically grown algae, because it has been shown recently that *de novo* FA synthesis is boosted by increased acetate supply in *C. reinhardtii* in three independent studies [115, 117, 138], and also in other heterokont species as reviewed in [139]. Furthermore, heterogeneous expression of a bacteria ACS in a marine alga *Schizochytrium sp*. increased its FA proportion by 11.3% [140]. Nevertheless, it remains to be determined what the relative contribution of ACS is versus that of the ACK/PAT pathway for acetyl-CoA formation in those algae where both routes are present.

3.2.3 ATP: citrate lyase (ACL)

Another possible source of acetyl-CoA for *de novo* FAS is the cleavage of citrate by ATP-citrate lyase (ACL). Cytosolic ACL has been shown to play a critical role in determining the oleaginicity of animals and yeasts where *de novo* FAS occur in cytoplasm [141-143], and in plants, cytosolic ACL has been shown to play a critical role in FA elongation [144]. The involvement of ACL in FA synthesis in algae remains to be demonstrated. The ACL enzymatic activities from the glaucocystophyte alga *Cyanophora paradoxa* were found associated to the cytosol; however its involvement in FA synthesis has not been addressed. A single gene (Cre05.g241850) encoding a putative ACL homolog has been identified in the genome of *C. reinhardtii*. The putative protein does not contain any transit peptide based on analyses using Predalgo [145], and is likely cytosolic and therefore it is anticipated to provide a role in filling

the cytosolic acetyl-CoA pool for FA elongation in the cytoplasm, as *C. reinhardtii* does contain 20:1n-9 and 22:1n-9 [146]. Nevertheless, the contribution of plastidial ACL, if it occurs, to *de novo* FAS remains to be examined in algae.

3.3 Sources of reducing equivalents in the chloroplast

As lipids are highly reduced compounds, lipid synthesis requires large quantities of NAD(P)H supplied in a stoichiometric ratio with respect to acetyl-CoA and ATP [45, 59]. Indeed, a positive link between the level of reducing equivalents and FA synthesis has been established in fungi, algae and plants [147, 148]. For instance, recent transcriptomic studies in *P. tricornutum* found that a buildup of precursors such as acetyl-CoA and reducing equivalents may provide a more significant contribution to TAG accumulation than an increase in ACCase activity alone [149, 150]. Neither NADH nor NADPH are permeable to the chloroplast envelope; therefore they have to be produced by chloroplast-located reactions including photosynthesis, ME, PDH, pentose phosphate pathway (PPP) and glycolysis.

3.3.1 Chloroplast PDH

As detailed in **section 3.2.1** considering its contribution as a carbon source, the plastidial PDH also produces one NADH for each molecule of acetyl-CoA produced. The trans 2-enoyl-ACP reductase of *de novo* FAS complex is shown to require one mole of NAD(P)H to ensure *de novo* FAS [151, 152]. This provides a compelling argument for acetyl-CoA coming from the plastid PDH since this is the only known enzyme that produces NADH in plastids.

3.3.2 Photosynthesis

In photosynthetically active cells (algae or plants), photochemical reactions are believed to provide a significant part of reducing equivalents (NADPH) for anabolic reactions inside chloroplast. The finding that cells exposed to HL possessed higher amount of total FAs could be considered an evidence to support this [83, 153-155]. However, upon HL exposure the entire photosynthetic chain is upregulated, increased NADPH production occur together with enhanced CO_2 fixation. Therefore the observed effect on increased lipid amount can be due to a combinatory effect of the increase in both carbon precursors and reducing equivalents.

3.3.3 Glycolysis

Glycolysis is defined as the set of reactions that lead to the generation of pyruvate from glucose. In addition to its obvious role as a carbon source, glycolysis produces two (net) ATP and two

NADH. Glycolysis can occur in both cytoplasm and chloroplast, and parallel pathways operate in both compartments in *A. thaliana* [156]. In *C. reinhardtii* a single pathway operates: the "upper half" of the pathway occurs in the chloroplast, and the 'lower half', i.e. reactions after 3-phosphoglycerate (3-PGA), occurs in the cytoplasm [157]. This compartmentation of glycolysis can have implications on the chloroplast redox state and subcellular energetics. For example, in *C. reinhardtii* glycolysis produces two NADHs inside the chloroplast and two ATPs in the cytoplasm. NADH produced by glycolysis could contribute substantially to FA synthesis especially when starch degradation is high.

3.3.4 Malic enzyme (ME)

Malic enzyme catalyzes the reversible conversion of malate to pyruvate while producing NADPH. The contribution of ME to *de novo* FA synthesis has been demonstrated through an overexpression study where strains over-expressing ME possessed improved FA synthesis in the diatom *P. tricornutum* and also in the green alga *Chlorella pyrenoidosa* [148, 158]. Furthermore, reducing NADPH supply via inhibition of ME activities using sesamol in *Haematococcus pluvialis* and *Nannochloropsis sp* led to reduction in total FAs [159]. Moreover, a high docosahexaneoic acid (DHA) production and total lipid content in the marine alga *Schizochytrium sp* has been found to correlate positively with the cultivation stages when the activities of ME is high [160]. When *Schizochytrium sp* was fed with malate, DHA production is increased by 47% [161]. In addition to supplying the FAS complex with NADPH, the reaction catalyzed by ME also produces pyruvate, which is a substrate for acetyl-CoA synthesis using PDH. Therefore, the contribution of ME to FA synthesis could be two-fold: providing both carbon and reducing equivalents.

3.3.5 Pentose phosphate pathway (PPP)

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the conversion of glucose-6-phosphate to 6-phospho-D-glucono-1,5-lactonate and while generating NADPH for anabolic reactions. G6PDH is a key enzyme of the PPP pathway, and its over-expression led to a 3.7-fold increase in lipid content in *P. tricornutum*, highlighting the critical role of G6PDH in algal lipid accumulation by enhancing NADPH supply [162]. The contribution of OPPP pathway to supply NADPH for FA synthesis has also been observed in the oleaginous diatom *Fistulifera solaris* [163].

3.4 ATP supply for FA synthesis

For every two NAD(P)H molecules supplied to the FAS complex, one molecule of ATP is required (2:1 NAD(P)H:ATP). While sources of acetyl-CoA and NAD(P)H for FA synthesis have been studied intensively [31, 48, 55, 56, 164], of the supply of ATP for the FAS complex is often overlooked. It is generally considered that most of the ATP needed for biosynthetic reactions in algae is provided by photophosphorylation (i.e. photosynthesis) or respiratory oxidative phosphorylation (mitochondrial respiration), but the re-partition of ATP produced between various anabolic reactions has not been investigated, and most current observations in this area remain correlative.

3.4.1 Linear and cyclic electron pathway (LEF and CEF)

When algae are starved for N (i.e. during active TAG synthesis), it has been observed that although the LEF rate and the respiration rate drop, the CEF pathway (as well as the activity of thylakoid membrane-located ATP synthase) increase significantly [165]. The CEF pathway around photosystem I cycles electrons back to the lumen therefore generating a proton motive force to drive ATP synthesis but without O_2 evolution or NADPH production. The CEF pathway has been suggested to supply ATP for lipid production during N starvation in *C. reinhardtii* mutants impaired in the proton gradient regulation 5 like 1 protein (PGRL1), which nonetheless accumulates significantly less neutral lipids under N starvation [166].

3.4.2 Mitochondrial respiration

In the context of lipid metabolism, mention of mitochondrial respiration is reminiscent of the respiration of acetyl-CoA during peroxisomal FA β-oxidation [167-169]. Although less known and often ignored, the interaction between mitochondrial respiration and lipid synthesis has started to be appreciated, mostly due to its role in supplying ATP for FA synthesis in algae [170]. Studies of the ATPase mutant (FUD50) of *C. reinhardtii* have revealed that photosynthetic ATP production is not essential during heterotrophic growth as long as mitochondrial respiration is functioning, implying the occurrence of active ATP transport from mitochondria to chloroplasts [170]. Put another way, defects in ATP synthesis in the chloroplast can be compensated for by ATP derived from mitochondrial respiration. Furthermore, it has been reported that mutants of complex I or complex III lost the capacity to produce lipids like WT *C. reinhardtii* during sulfur deprivation [171, 172]. Interplay between mitochondria metabolism and lipid synthesis could occur at two levels: metabolic (Krebs cycle) as well as energetic (supply of NADH and ATP) (detailed in **section 3.5**). Detailed interactions between

these pathways are yet to be characterized. With the increasing number of respiratory mutants available for *C. reinhardtii* [173, 174], a systematic analysis of the interaction between FA synthesis and mitochondrial respiration should be possible.

3.5 Chloroplast redox poise and FA synthesis

Photosynthesis converts light energy into ATP and NADPH, and the subsequent use of these energies to drive CO_2 assimilation through the Calvin-Benson-Bassham (CBB) cycle. In the absence of exogenous carbon supply, photosynthesis is the only provider of energy and carbon skeletons for all anabolic reactions in the cell. In addition to the CBB cycle, NAD(P)H is a ubiquitous electron carrier also required for starch and FA synthesis. The flux along these pathways is mostly determined by the ratio of NAD(P)H versus NAD(P^+) or ATP levels. Photoautotrophs live in an ever-changing environment where metabolic demand for NAD(P)H and ATP is constantly changing. Multiple strategies have evolved to fine tune photosynthesis to meet downstream metabolic needs because excess production of reducing power may result in an over-reduction of the photosynthetic electron transport chain and consequent photo-oxidative damage [175, 176]. The processes known to poise chloroplast redox balance could have an impact on FA synthesis.

During N starvation, photosynthetic electron transport chain complexes are reduced [177, 178]. Lipid production during N starvation has often been thought to act as an electron sink to accommodate an over-reduced chloroplast [58, 179], but this theory has recently been partly challenged by the observation that heterotrophic cultures accumulate TAGs and starch during N starvation [135]. Nevertheless, this accumulation does occur to a lesser extent – the amount of TAG accumulated during heterotrophic N starvation (in the dark) is four times less than that accumulated in N-starved autotrophic cultures. TAG may thus at least partly serve as an electron sink for N-starved autotrophic algae.

3.5.1 Chloroplast-located alternative electron dissipation pathways

Several chloroplast-located alternative electron pathways (notably the cyclic electron flow, O_2 photoreduction processes, chlororespiration and the water-to-water cycle) are known to play roles in dissipation of photo-reductant [180, 181]. The importance of CEF in metabolism of N-starved cells is further supported by the observation that in N-starved cells of *C. reinhardtii*, LEF fell approximately 15% more than CEF over the first 24 h of N starvation [177]. This is further supported by the observation that the rate of CEF increased while LEF decreased during cells' adaptation to N starvation in *Chlorella sorokiniana* [166]. Perturbation of the above

pathways should have impact on FA synthesis. Thus, for instance, the *pgrl1* mutant defected in the proton gradient regulation 5 like 1 (PGRL1) accumulated 30% less neutral lipids based on BODIPY staining than its corresponding wild type. This finding could be interpreted as indicating that CEF supplies ATP for FA synthesis (discussed in **section 3.4.1**), or could also imply a possible competition for NADPH between CEF and FA synthesis [166]. A systematic analysis of the interaction between various chloroplast electron dissipation pathways and FA synthesis remains to be conducted. With the large number of *Chlamydomonas* mutants available (Chlamydomonas Mutant Library – CLiP library: https://www.chlamylibrary.org/)[182], it should be possible to comprehensively address questions related to the interaction between FA synthesis and various electron dissipation mechanisms.

3.5.2 Electron dissipation through collaboration with mitochondria

Recently, it has also been shown that chloroplast redox poise can be achieved through export of excess reducing equivalents to mitochondria in green algae [183] and diatoms [184]. Structural components involved in the energy trafficking between chloroplasts and mitochondria have not been identified, but malate shuttles and triose phosphate transporters (TPTs) are strong candidates. Once inside mitochondria, reducing equivalents (NADH) are consumed by oxidative respiration operating through two pathways in plants and algae, i.e. cytochrome oxidase (COX) pathway and alternative oxidase (AOX) pathway [171, 174, 185]. The COX pathway couples consumption of NADH to ATP synthesis, while they are uncoupled in the AOX pathway. For a long time, the latter was considered a wasteful process and its physiological significance was uncertain. However, lately it has been observed that expression as well as translation of AOX genes in a number of algal species are upregulated in conditions when photosynthetically-produced reducing equivalents should be attenuated [128, 186-188]. Indeed, inhibition of the AOX pathway using salicylhydroxamic acid (SHAM) under N starvation led to a 23% increase in FA amount in the marine alga *Isochrysis galbana* [186]. These authors suggest that the increase in FA production is likely a result of an increase in chloroplast NADPH (although not measured in above study) due to a defect in its dissipation through the mitochondria AOX pathway. Taken together, these studies suggest that the chloroplast reduction state can be increased, via blocking the mechanisms of NADPH dissipation, therefore boosting FA synthesis.

3.5.3 Energy interactions between peroxisomes and chloroplasts impact FA synthesis

Energetic exchanges between mitochondria and chloroplast have been evidenced, and the impact on FA synthesis has also been evaluated in some cases (discussed in **section 3.5.2**). Until lately, little is known about the energetic interactions between chloroplast and peroxisome, although these two organelles are often seen located in close proximity [189, 190]. We recently showed that, during acclimation of *C. reinhardtii* to N starvation or HL exposure, extensive energetic exchanges occur from peroxisome to chloroplast employing the peroxisomal malate dehydrogenase (MDH2) and redox-based signaling [191]. This interorganelle communication between peroxisome and chloroplast is essential in maintaining chloroplast redox poise, and in its absence, the chloroplast is over-reduced, therefore activating FA and starch syntheses.

3.6 Relationship between FA synthesis and starch accumulation

During a diurnal cycle, most green algae, like plant leaves, accumulate starch during the day and degrade it to provide cells with carbon and energy at night [192]. Lipid synthesis also follows a similar cycle [193, 194]. Upon stress for example N starvation or HL, massive amount of starch and neutral lipids, mostly TAGs, accumulate [31, 55, 154]. Because glyceraldehyde 3-phosphate is a common precursor for both FA and starch synthesis, it has sometimes been suggested that there occurs a competition for carbon precursors between biosynthesis of starch and lipid. Following this idea, the carbon partitioning between starch and TAG upon N starvation has been intensively studied in the past 10 years, mostly based on the starchless *Chlamydomonas* mutant *bafJ5* defected in the small catalytic subunit of ADP-glucose pyrophosphorylase (AGPase) [195]. Some studies reported that there occurs a competition for carbon precursors between starch and lipid synthesis under N starvation [196, 197] yet the competition does not occur during N replete growth [198], while others reported that this competition is minor, if at all, even under N starvation [199, 200].

The contrasting conclusions (above) could be due partly to the way the oil content is expressed (per cell versus per dry biomass), or due to the use of control strains (with or without cell wall), or due to cell culture conditions (light, CO_2 level, cell growth stage). The reported difference could also be due to the fact that interplays between starch and lipids go beyond a mere competition for carbon allocation, and likely also involve a competition for energy (i.e. ATP) and reducing equivalents. Cellular redox context varies depending on genetic as well as environmental factors (such as light quality/quantity, autotrophy versus mixotrophy, CO_2 versus air, growth phases). Increasing evidence suggests the simultaneous occurrence of starch synthesis and turnover in the light [201-203], and starch breakdown eventually generates

reduced carbons, phosphorylating power and reducing equivalent, impacting stromal redox balance and therefore anabolic reactions of starch and FA synthesis. Thus, a much more complex relationship between starch and lipid synthesis likely occurs at both the carbon and energetic level. The energetic aspects of interaction between starch and lipid accumulation have so far mostly been ignored and definitely beg further examination.

It is worth noting that, in addition to the defects at the AGPase locus, the *bafj5* mutant harbors two additional mutations: i.e. a defect in cell wall synthesis and a defect in a gene encoding a respiratory burst oxidase [204]. These additional mutations could impact lipid metabolism, either via competition for carbon precursors (in the case of the cell wall mutations), or via their effect on redox metabolism (in the case of the respiratory burst oxidase). But these hypotheses remain to be tested.

3.7 Relationship between FA synthesis and chrysolaminarin accumulation

Instead of starch, most photosynthetic heterokonts, including *P. tricornutum* and *Halassiosira pseudonana,* accumulate chrysolaminarin, another type of storage polysaccharide [205-207]. Similar to starch, chrysolaminarin accumulates during the day and is mobilized at night, supporting its role as a source of carbohydrates for heterotrophic metabolism in the dark. Two differences are observed between starch and chrysolaminarin: i) starch is stored in chloroplasts, while chrysolaminarin is found in vacuoles [208], and ii) in contrast to massive starch accumulation in N-starved green algal cells, N starvation does not seem to stimulate chrysolaminarin over-accumulation. Silencing the chrysolaminarin synthase gene in *Thalassiosira pseudonana* resulted only in a transient accumulation of TAG [206]. Considering the widespread interest in the use of diatoms for biofuel and biochemical applications, the development of a routine method for quantification of chrysolaminarin [205], and genome editing technologies for diatoms [209, 210], we should expect many more studies on chrysolaminarin metabolism and its relation to lipid synthesis in the near future.

3.8 Regulation of FA synthesis

Due to the central importance of lipids in cell metabolism, physiology, and its interaction with the environment, FA synthesis is subjected to multi-level control.

3.8.1 Regulation at the level of ACCase

ACCase catalyzes the first and committed step in *de novo* FA synthesis and is known to be regulated by a myriad of mechanisms at both the transcriptional and post-transcriptional level.

Plant heteromeric ACCase is activated by light, chloroplast redox status (thioredoxin), and precursor supply, and is inhibited by acyl-ACP; as recently summarized [78]. It remains to be determined which of these regulatory mechanisms are conserved in various algae, and whether algae possess other unique ACCase regulatory mechanisms. We currently know very little about the regulation of homomeric ACCase, which is the exclusive ACCase isoform present in many algal taxa (see **section 3.1.1**). A better understanding of ACCase regulation promises to significantly advance algal lipid biotechnology. Here we will discuss the recent discovery of two new classes of proteins identified as negative regulators of plant ACCase: BADC and PII.

BADC is an abbreviation for the prior (but rather unfortunate) annotation of this protein as a "biotin attachment domain-containing" protein [77]. As mentioned in **section 3.1.1**, BADC is derived from BCCP but lacks the latter's conserved biotinylation motif and biotinyl-Lys residue. As a consequence, rather than acting as a carboxyl carrier, BADC acts as a negative regulator of heteromeric ACCase through its competition with BCCP for binding to the holo-ACCase complex [77]. Additionally, the BADC gene family may be partially responsible for the feedback regulation of ACCase [211]. In Arabidopsis, BADCs are represented by three genes, and all three lack the conserved biotinylation motif and biotinyl-Lys residue. Gene silencing of BADC isoform 1 results in a slight, but significant increase in oil content in seeds of *A. thaliana* [77]. BADC is present in higher plants and in a limited subset of green algae (including *Chlorella* spp., *Volvox* spp. and *Coccomyxa* spp.) but is otherwise absent from eukaryotic algae with a single exception (**Figure 2a**), suggesting that it diverged from BCCP in green algae. The only non-chlorophyte BADC representative is found in the red alga *Galdieria sulphuraria*, the most extremophic red algal species, which has obtained large numbers of archaeal and bacterial genes through horizontal gene transfer [212]. The BADC in *G. sulphuraria* appears likely to have resulted from such a horizontal transfer (although most likely from an ancestral green algae rather than prokaryote).

The second negative regulator of ACCase recently identified is PII (At4g01900), a small homotrimeric protein that acts at the interface of C and N metabolism [213]. PII, acting as a 2-oxoglutarate sensor, inhibits ACCase via binding to the biotin portion of BCCP, and this inhibition can also be relieved by high pyruvate concentrations. The implication is that PII connects carbon and N metabolism by sensing 2-oxoglutarate, pyruvate, and possibly the broader energy and nitrogen (N) status of the chloroplast [214]. Algal PII, which is of bacterial origin, is, like heteromeric ACCase, present in algae with chloroplasts of primary endosymbiotic origin (*i.e.* Chlorophyta, Rhodophyta and *Paulinella spp*.) as well as in higher plants, but is absent from other eukaryotic algal taxa (**Figure 2b**). PII appears, however, to have

been lost from many red algal taxa. As N-starvation has been shown to induce transcription of genes encoding algal ACCase subunits [128, 188, 215], it seems likely that additional mechanisms exist to coordinate N-status and ACCase activity in algae. It is worth noting that PII shares distant orthology with the N-fixation-related nifH from archeae, but plants have not retained a nifH-derived ortholog.

3.8.2 Transcriptional regulation

Although genes encoding the enzymes of lipid metabolism in microalgae can be predicted (based on amino acid sequence identity) to those of plants [31, 56], the regulatory mechanisms of lipid synthesis in algae can not be as easily inferred. Drastic changes in transcript levels of many putative transcription factors (TFs) have been observed in N-deplete versus N-replete Chlamydomonas cells, implying their potential involvement in regulation of lipid metabolism [128, 188, 216]. However, only a couple of them have been experimentally validated to play such a role. A putative zinc-finger protein (Cre14.g624800) has been identified as a regulator of stress-induced lipid synthesis, and overexpression or silencing of the corresponding gene results in altered lipid content [217]. But the regulatory mechanisms and downstream molecular targets of this protein remain to be deciphered. A SQUAMOSA promoter-binding protein domain transcription factor was recently identified in *C. reinhardtii*, and it was named as N response regulator 1 (NRR1) and the insertional knockout mutants accumulated only half amount of the TAG usually found in WT strains upon N starvation, but not under other nutrient stresses (S, P, or Zn) [215]. A correlation between the level of transcription of *NRR1* and that of a major DGAT1 has been observed, but several uncertainties remain. At a molecular mechanistic level, it remains to be determined what the targets of the NRR1 are; and from a biotechnological perspective, it is unknown if overexpression of *NRR1* could impact lipid production.

Another relatively well-studied transcription factor implicated in lipid metabolism is the phosphorus starvation response 1 (PSR1). PSR1 belongs to the MYB-CC (MYB coiled-coil domain) transcription factor family and was originally described as a component of the phosphate starvation pathway [218]. Two recent studies have suggested the role of PSR1 in regulation of lipid metabolism in *C. reinhardtii* [219, 220]. Ngan et al [219] showed that oil content is positively correlated to the expression level of *PSR1*, which were altered by creating knock-out or overexpressor lines. Bajhaiya et al [220] further showed that PSR1 is not only regulating lipid synthesis but also starch synthesis. PSR1 overexpression lines showed increased starch content but reduced neutral lipid content under P starvation, and the phenotype

is persistent regardless of the acetate status. The reason for the contradictory changes in lipid content in *PSR1* overexpressors is not clear, but could be due to the use of different nutrient stress (N versus P). In summary, PSR1 likely plays a role in the regulation of global metabolism, but not specifically limited to lipid synthesis.

In addition to the studies carried out in *C. reinhardtii*, a basic helix-loop-helix (bHLH) TF and a basic leucine zipper (bZIP)-domain containing TF have been identified from *N. salina*, and their overexpression led to an increase in both growth and lipid productivity [221, 222]. Therefore these TFs identified here could serve as genetic engineering targets for improving the production of biofuels and biomaterials in algae. Furthermore, overexpression of known plant TFs, for example the Dof-type (DNA binding with one finger) TF in *C. reinhardtii* [223] and the *Arabidopsis* AtWRI1 in *N. salina* [224], have resulted in transgenic strains with increased lipid production. These studies suggest the conserved nature of some of these regulatory mechanisms between plants and algae.

3.8.3 Regulation by kinases and other subcellular processes

Alongside TFs, a given pathway can also be regulated by other mechanisms [219]. For example, alterations of lipid content have also been observed in knock-out mutants of *C. reinhardtii* for two members of the dual-specificity tyrosine-phosphorylation-regulated (DYRK) kinase, i.e. the plant specific DYRKP [225] and the Chlamydomonas triacylglycerol accumulation regulator1 (TAR1) - an orthologue of the yeast Yet another kinase1 (Yak1) subfamily [226]. Lipid production in *N. gaditana* is doubled by knocking out a homolog of fungal Zn(II)2Cys6 encoding a transcriptional regulator of N assimilation pathways [227]. Furthermore, manipulation of the target of rapamycin (TOR) or nitric oxide (NO) signaling pathway is also shown to impact lipid production in algae [228-230]. It is worth noting that molecular targets or the regulatory circuits of above regulatory proteins related to lipid metabolism have not yet been worked out, and biochemical or molecular research in this direction is needed. For additional details on the relation between autophagy and lipid synthesis, readers are referred to [169, 231-233].

4. Glycerolipid synthesis

As noted in **section 2**, eukaryotic algae contain phosphoglycerides, glycosylglycerides and, often, betaine lipids in significant amounts. Because algae carry out oxygen-evolving photosynthesis, their thylakoid membranes contain four lipids also typical of plants and cyanobacteria - MGDG, DGDG, SQDG and PtdGro. When present, betaine lipids are in

extrachloroplast membranes. DGTS is found in many green algae while DGTA and DGCC are found in different algal species such as brown algae (Phaeophyceae). Algae with significant betaine usually have little or no phosphatidylcholine (PtdCho) [19]. Of the phosphoglycerides apart from phosphatidylglycerol (PtdGro), these are in extraplastidial membranes. PtdCho and phosphatidylethanolamine (PtdEtn) are usually the most significant while phosphatidylinositol (PtdIns) and phosphatidylserine (PtdSer) are minor components.

A brief discussion of the origins of algal chloroplasts and the differences between primary plastids (where the two plastid membranes were derived from the cyanobacterial endosymbiont) and complex plastids, which are surrounded by three or four membranes, is given in [19]. Genes involved in glycosylglyceride synthesis often show strong similarity to those of higher plants. These include *MGD1*, *DGD1*, *SQD1* and *SQD2*. Indeed, DGDG synthases in eukaryotic algae have been shown to be similar to the plant type enzymes [20, 234]. However, analysis of chloroplasts is often complicated by the extra membranes in secondary plastids [50].

The Glaucophyta are a small group of rare freshwater algae. Only 13 species are known, none of which is particularly common. Along with red algae, they harvest light through phycobilisomes and also store fixed carbon in the cytosol. There is rather little information about their lipids and, indeed, of their classification---mainly because they have been little studied. They contain the three glycosylglycerides (MGDG, DGDG, SQDG)[235] but there is little consistent information about genes encoding enzymes for MGDG and DGDG formation. It is thought, however, that SQDG synthesis is similar to that in plants and Chlamydomonas [19].

Glycosylglyceride synthesis has been studied most in red and green algae, especially Chlamydomonas. Overall the lipid and fatty acid composition of Chlorophyta resembles that of higher plants. Interestingly, *C. reinhardtii* has some unusual fatty acids -16:4Δ4,7,10,13, pinolenic acid (18:3Δ5,12) and coniferonic acid (18:4Δ5,9,12,15) [234]. An ω13-desaturase is responsible for the Δ5 double bonds introduced into linoleic and α-linolenic acids [236]. A Δ4-desaturase uses 16:3 (Δ7,10,13) bound to MGDG as a substrate to form 16:4 [237] and may be important in controlling overall MGDG synthesis. On the other hand, VLCPUFAs such as arachidonic acid (ARA) or EPA are usually only present in small amounts or absent from green freshwater algae. An exception is *Lobosphaera* (formerly *Parietochloris*) *incisa* which is an oleaginous species that has high amounts of ARA in its membrane lipids and TAG (see **section 7**). In contrast, both ARA and EPA are often present in low proportions in marine green algae.

For example, in *Ulva fenestrata* they are found in significant quantities in DGTS but not in glycosylglycerides [238].

Diatoms are the most abundant phytoplankton species and, consequently, are major producers at the bottom of food chains. They occur ubiquitously in freshwater and marine habitats. Their metabolism, including the production of VLCPUFAs (mainly EPA) through lipid-linked desaturases has been detailed by [37].

One matter which should be borne in mind when describing acyl lipid synthesis in algae is that the biochemistry of the reactions has lagged behind the identification of genes encoding the putative enzymes involved. Thus, in review papers describing metabolism of acyl lipids in diatoms [37] or in *C. reinhardtii* [56] it will be seen that, while genes for most of the enzymes concerned have been identified, functional proof of their activity is much less clear. Nevertheless, for now it can be assumed that the pathways identified in higher plants [239-241] are on the whole followed in eukaryotic algae though, perhaps, in a simpler form with less genetic redundancy [31](**Figure 4**). In an overall survey by comparative genomics and subcellular localisation, it was concluded that the pathways for acyl lipid metabolism in the unicellular red alga *Cyanidioschyzon merolae* were essentially similar to Arabidopsis [242].

Although, as noted above, there has been relatively little biochemistry carried out on the algal enzymes used for acyl lipid formation and the reader is referred to previous work to serve as a background [9]. For example, when studying the distribution of different acyl lipids (and their molecular species as being 'prokaryotic' or 'eukaryotic' in origin: see [243]), Eichenberger's group found that MGDG, DGDG, SQDG and PtdGro in *C. reinhardtii* were clearly of plastidic ('prokaryotic') origin while DGTS and PtdEtn, enriched in 18:3(Δ5,9,12) and 18:4(Δ5,9,12,15) were of 'eukaryotic' origin [244]. These data were followed by radiolabelling experiments to study the time-course of their metabolism which identified, for example, that lipid-linked desaturation on DGTS could give rise to PUFA formation on the sn-2 position while, for MGDG and DGDG, desaturation was at the sn-1 position [245]. Similar radiolabelling experiments in other eukaryotic algae are summarised by [14] and, later, by [9].

4.1 The Kennedy Pathway

Glycerolipids are synthesized using what is commonly known as the Kennedy pathway (**Figure 5**). This is named after Eugene P. Kennedy who discovered and characterised many of the individual reactions for phosphoglyceride formation, although the first two (acylation) reactions were originally reported by Kornberg and Pricer [243]). The penultimate intermediate of the

Kennedy pathway, DAG, is used to form zwitterionic phosphoglycerides (PtdCho, PtdEtn), TAG and the galactosylglycerides MGDG and DGDG (**Figure 5**).

The relative contributions of 'eukaryotic' and 'prokaryotic' pathways of glycerolipid synthesis has been discussed further by [246]. They point to the use of the 'prokaryotic' pathway by *Chlamydomonas* because it lacks thylakoid lipids with 18C acids at the sn-2 position [113, 244, 245]. But this has recently been challenged by the discovery of an ER-located lysophosphatidate acyltransferase (LPAAT) with substrate preference of 16C rather than an 18:0 FA at its sn-2 position in *C. reinhardtii* [247]. In addition, a chloroplast pathway for TAG formation in *C. reinhardtii* was recently reported [248]. In contrast, galactolipids of *Dictyopteris mambranacea* [249] and several other brown algal species [250] are almost completely of the 'eukaryotic' type. On the other hand, green algae such as *Chlorella kessleri* and *Acetabularia mediterranea* and some red and brown algae seem to employ two parallel pathways of lipid formation [246].

Because *Chlamydomonas* lacks PtdCho, which is known to be intimately involved in the 'eukaryotic' pathway in plants, the lack of such synthesis in *C. reinhardtii* has been suggested to be explained by the lack of PtdCho [251]. However, as pointed out by [246], several brown algae that lack PtdCho have thylakoid lipids made by the 'eukaryotic' pathway. This suggests that PtdCho is not essential for the 'eukaryotic' pathway of chloroplast lipid formation.

Three pathways have been suggested for the accumulation of a 'prokaryotic' type of TAG in the cytosol of algae such as *Chlamydomonas* or *Dunaliella bardawil* [246]. The first pathway forms TAG at the chloroplast envelope while, in the second pathway, TAG is assembled in the ER using DAG exported from the plastid. In the third pathway, both DAG and TAG are formed on the ER. For the latter, specific mechanisms are needed to channel 16C fatty acids onto the sn-2 position of DAG [246].

Before describing the Kennedy pathway and its constituent enzymes in detail, it is timely to mention the recent use of metabolomics for the study of lipid synthesis in algae. For example, Juppner et al [193] used GC-MS to study polar metabolites and lipids in synchronous cultures of *C. reinhardtii*. Although this paper was mainly a technical advance, this proof-of-concept study has the potential to be used for further in depth metabolic phenotyping and the identification of biomarkers for various cellular processes, at least in *C. reinhardtii*.

4.2 The Kennedy pathway in detail and phosphoglyceride formation

The Kennedy pathway (**Figure 5**) begins with glycerol 3-phosphate (G3P). This intermediate is produced by reduction of dihydroxyacetone phosphate (DHAP) derived from photosynthesis and/or starch degradation [162]. Under normal growth conditions the supply of G3P can be one controlling factor that may influence total lipid synthesis (and oil accumulation). However, under osmotic stress, hydrolysis of glycerolipids can also take place, leading to an accumulation of glycerol [252]. Moreover, the conversion of G3P back to DHAP under salt stress is also well known [162, 252]. The synthesis and degradation of G3P in *Dunaliella salina* under extracellular salt stress has been well studied (see [253, 254]).

Proteomic [255] and transcriptome analysis [116, 188, 256, 257] have shown that the activity of cytosolic glycerol 3-phosphate dehydrogenase (GPDH) is positively correlated with TAG accumulation in algae. Moreover, overexpression of GPDH in *P. tricornutum* [258] and *C. reinhardtii* [259] increased lipid production. A multiple gene engineering strategy, including overexpression of glycerol kinase, GPDH and acetyl-CoA carboxylase, in *Scenedesmus quadriauela* increased the G3P pool and was paralleled by an increase in oil content [260]. These data suggest that augmentation of G3P levels may be important to enhance lipid accumulation [261].

An interesting recent observation with *C. reinhardtii* is the regulation of a gene GPD2 that encodes a multi-domain enzyme with GPDH and phosphoserine phosphatase activities. This enzyme is, therefore, capable of synthesising either G3P or glycerol, depending on environmental conditions and/or metabolic demands [262].

Candidate genes for the expression of chloroplast-localised glycerol 3-phosphate acyltransferase (GPAT) in *C. reinhardtii* were listed by [251] and by [56]. For diatoms, a gene for GPAT was predicted from the *Phaeodactylum tricornutum* genome [263] and molecular characterisation carried out [264]. The gene was overexpressed in *P. tricornutum* and resulted in a two-fold increase in non-polar lipids (presumably TAG). This altered the overall fatty acid composition in the transgenics with a significant decrease in saturated acids and an increase in PUFA. This was interesting since PUFA such as EPA are thought to be synthesised on the ER [37] even though the GPAT was a chloroplast enzyme [264]. A membrane-bound GPAT was characterised from the marine diatom *Thalassiosira pseudonana* and found to regulate the acyl composition of glycerolipids when expressed in a GPAT-lacking mutant of yeast [265] with increases in 16:0 and decreases in 16:1 and 18:1 in both TAG and phosphoglycerides.

A GPAT-like gene was identified in the freshwater trebouxiophyte *Lobosphaera* (formerly *Parietochloris*) *incisa* which, when over-expressed in *C. reinhardtii*, increased TAG

production [266]. The same alga was examined by Ouyang et al. [267], who considered it localised to chloroplasts. Substitution of Arg to His in the glycerol 3-phosphate binding site increased the enzyme's activity and led to raised phospholipid levels when expressed in yeast.

Two candidate genes for LPAAT were noted in *C. reinhardtii* [56]. The CrLPAAT1 was reported to be located the chloroplast with a preference for 16C at its sn-2 position [268], whereas LPAAT2 has recently been identified as the ER isoform but also with a preference of 16C at its sn-2 position [247]. Both are found to be implicated in TAG synthesis, because plastidial over-expression of *CrLPAAT1* increased oil content [268], and silencing of *CrLPAAT2* reduced oil content during N starvation [247]. The potential regulatory role of AGPAT (acylglycerolphosphate acyltransferase) in *Phaeodactylum tricornutum* for TAG synthesis has been discussed [269]. A genome-wide analysis of LPAAT genes has been carried out in algae [270] and in *Nannochloropsis* two differently-localised enzymes have been found, both of which are needed for TAG formation [271].

Phosphatidic acid, the product of LPAAT activity, is at a branch-point of the Kennedy pathway (**Figure 5**) where it can be converted to CDP-DAG for the formation of anionic phosphoglycerides, PtdIns, PtdGro and DiPtdGro (diphosphatidylglycerol, cardiolipin). Putative genes for phosphatidate cytidylyltransferase, phosphatidylglycerolphosphate (PGP) synthase and phosphatidylinositol synthase were described in *C. reinhardtii* [56, 251] and one for phosphatidylglycerol synthase noted in *P. tricornutum* [37] but, until recently, there has been a dearth of work on the synthesis of such anionic phospholipids in eukaryotic algae except for indirect studies on inoculum size in altering phosphoglyceride profiling [272] and the work on both PGP synthase and PGP phosphatase 1 from *C. reinhardtii* [273, 274]. Two homologues of PGP synthase (CrPGP1 and CrPGP2) were isolated and characterised from *C. reinhardtii* [273]. Their function was demonstrated by complementation of a mutant of *Synechocystis* sp. PCC 6803. In nutrient-starved algae, expression of both homologues was decreased by P-depletion at 4h but restored after 5 days. In contrast, while CrPGP1 was reduced by N-depletion, expression of CrPGP2 was unaffected.

For PGP phosphatase (PGPP), the gene was isolated from *C. reinhardtii* and its function shown using a yeast mutant [274]. While two aspartate residues were essential in yeast PGPP, only the first was needed for function in the algal phosphatase, despite conservation of the putative catalytic motif.

When *C. reinhardtii* was grown in sulphur-deficient medium, synthesis of PtdGro was enhanced and the accumulating phospholipid compensated for the loss of negatively-charged SQDG. Similar activation of PtdGro synthesis was also observed in a SQDG-deficient mutant

under S-replete growth conditions. The data were suggested to indicate a critical role for PtdGro under S-starved conditions in the maintenance of Photosystem I activity [275]. In addition, a role for PtdGro in Photosystem II was suggested in two PtdGro-deficient mutants of *C. reinhardtii* [276]. In these mutants there was a marked reduction of PtdGro and a complete loss of its Δ3-16:1 component. This unique fatty acid is confined to PtdGro in higher plants where there have been studies on its enigmatic function and localisation (see [277, 278]). In algae, it is likely to have similar properties but more research is needed to define these.

Diphosphatidylglycerol (DiPtdGro, cardiolipin) is a characteristic lipid component of the inner mitochondrial membrane, as originally demonstrated in plants (see [279]. Cardiolipin synthase (CLS) was identified in *C. reinhardtii* [280] when it rescued a CLS-mutant of yeast. The sequence for the gene was similar to that from other eukaryotes suggesting that the *C. reinhardtii* CLS catalyses a reaction using CDP-DAG as opposed to the *E.coli* enzyme which uses two molecules of PtdGro [243].

For the formation of the zwitterionic phosphoglycerides, PtdCho and PtdEtn, phosphatidate needs to be dephosphorylated to DAG. Genes for eleven putative plastid phosphatidate phosphatases (PAP) were identified in *C. reinhardtii* [56] and two in *P. tricornutum* [37]. The mRNA level of the CrPAP2 isoform was found to increase in *C. reinhardtii* grown in nitrogen-limiting conditions. RNA interference of the gene reduced total lipid by up to 17% while overexpression caused an increase, indicating that PAP activity can regulate lipid accumulation. The CrPAP2 enzyme showed PAP activity when expressed in *E.coli* [281].

Once DAG has been formed in the Kennedy pathway it can be used for PtdCho and PtdEtn production as well as the synthesis of glycosylglycerides and TAG (**Figure 5**). For the biosynthesis of PtdCho and PtdEtn by the Kennedy pathway, the cytidylyltransferase reaction is considered to show the most flux control, at least in higher plants [282] and animals [243]. The CTP: phosphoethanolamine cytidylyltransferase has been studied in *C. reinhardtii* [283]. It showed a typical signature peptide sequence for the cytidylyltransferase family and was probably localised to mitochondria. It showed cell cycle fluctuations with high activity in the dark. The same group also identified a cDNA encoding CDP-ethanolamine phosphotransferase and expressed it in a yeast mutant deficient in both choline- and ethanolamine phosphotransferase activity and found that it had both activities. This was notable since *C. reinhardtii* only contains PtdEtn and the PtdCho is replaced by DGTS in this alga. Other kinetic properties of the expressed enzyme were measured and parallels with the higher plant enzyme noted [284]. In *Chlamydomonas*, no gene for PtdSer decarboxylase has been found and,

therefore, it can be assumed that PtdEtn is made exclusively by the Kennedy pathway using CDP-Etn [251].

As noted in **section 2**, PtdCho is found in most algae but not in a few species, of which *C. reinhardtii* is one. However, some species of the *Chlamydomonas* genus do contain PtdCho [285]. Accordingly, Sato et al [286] studied the biosynthesis of PtdCho in these algae. In plants PtdCho can be made by the CDP-base pathway as well as by methylation in three steps from PtdEtn [243]. There are also some extra methylation routers (see e.g. [287] Sato et al [286] used radiolabelling studies in conjunction with comparative genomics to elucidate the pathways in three *Chlamydomonas* species together with the red alga *Cyanidioschyzon merolae*. Their results are shown in **Figure 6** and revealed that both *C. sphaeroides* and *C. merolae* form PtdCho from PtdEtn by methyl transfers. In *Chlamydomonas asymmetrica*, PtdCho can be made by the CDP-base pathway or by methylation, as well as the intermediate conversion of PtdEtn to PtdCho. These data revealed an unexpected diversity in the ability of *Chlamydomonas* strains to synthesise PtdCho [286]. Presumably, this will also be reflected in other algae.

Transcriptional analysis (during N stress) of *N. oceanica* indicated that PtdEtn is synthesised be two distinct pathways (PtdSer decarboxylation as well as the Kennedy pathway). In fact, PtdSer was below detection levels in this alga, indicating that it mainly served as a precursor for PtdEtn formation [288]. Such observations make the need for some biochemistry (enzymology) even more urgent.

One subject that should be emphasised when discussing acyl lipid biosynthesis is the fact that individual classes have distinct and usually well preserved fatty acid compositions. This was eluded to in **section 2** but a particular example in *C. reinhardtii* is shown in **Figure 7**. Other algae also show distinct patterns and, of course, these may be quite different to *C. reinhardtii* [289]. So far as the three main phosphoglycerides are concerned, PtdEtn is more unsaturated than PtdGro, which often contains species with the unique trans-3-hexadecenoic acid. For algae containing both PtdCho and PtdEtn, then these classes tend to have a rather similar lipid composition [250]. Presumably this reflects either the formation of both PtdCho and PtdEtn from the same DAG pool or the conversion of PtdEtn to PtdCho by methylation, as discussed above.

Formation of DAG also allows biosynthesis of glycosylglycerides (**Figure 4**), which are the main membrane constituents of chloroplast thylakoids.

4.3 Biosynthesis of glycosylglycerides

The enzyme synthesising MGDG uses DAG and UDP-galactose substrates and is referred to as MGDG synthase. Only one enzyme is found in Chlorophyta (e.g. *C. reinhardtii*) in contrast to higher plants where there are three MGD genes [46, 50]. Similarly, one DGDG synthase is found in *C. reinhardtii* [46] and many other green algae [19]. Although DGDG synthase also uses a UDP-galactose substrate the galactose link on DGDG is α-anomeric whereas the first galactose (on MGDG) is in the β-anomeric configuration. The DGD in *C. reinhardtii* resembles DGD1 of higher plants [50, 290] but a second isoform is additionally found in *Ostreococcus tauri*, which resembles the plant DGD2 [290]. An interesting short review discusses the pathways for glycosylglyceride synthesis in cyanobacteria and different algae with reference to the endosymbiotic origin of chloroplasts [291]. The authors noted that, even within the red algae, the situation is complicated with *Cyanidioschyzon merolae* differing from, say, *Porphridium purpureum*.

A complication in the formation of MGDG and DGDG in green algae is the variable contribution of the 'prokaryotic' (exclusively in the plastid) and the 'eukaryotic' pathways (where the ER also participates). Green algae can be divided into the first group (e.g. *Chlorella*) where the DAG is derived from PtdCho on the ER and a second, more common, group (e.g. *C. reinhardtii*, *Dunaliella* sp.) where only the 'prokaryotic' pathway is used [19]. In *Dunaliella* two routes exist for the formation of DGDG. The first uses sequential desaturation of 18:1/16:0-DGDG to form 18:2/16:0- and 18:3/16:0-DGDG and the second uses more unsaturated species of MGDG to form DGDG species where further desaturation can lead to 18:3/16:3- or 18:3/16:4-DGDG[19].

The situation in diatoms has been summarised recently by [37], where both 'prokaryotic' and 'eukaryotic' pathways exist. The latter is particularly important for the production of VLCPUFA characteristic of diatoms (**section 2**). For the red alga, *Cyanidioschyzon merolae*, desaturation does not occur in the plastids and PUFA have to be imported, resulting in a coupled pathway for galactolipid synthesis [292].

A thorough review of the evolution of MGDG and DGDG biosynthetic pathways has been published [50]. This compares these pathways based on both molecular and biochemical data and highlights enzyme reactions that have been conserved and those which have diverged. In addition, the *Chlamydomonas* genome encodes an orthologue of the trigalactosyldiacylglycerol (TGD) transport protein, needed for ER to chloroplast lipid trafficking [293]. In a *tgd* mutant, MGDG synthase was strongly stimulated but with TAG accumulation due to the defective lipid trafficking.

The pathway for SQDG synthesis in green algae is similar to that in higher plants. The pathway was first proposed by [294] and the genes involved (SQD1, SQD2) were later isolated by Benning's group [295, 296] to provide independent confirmation (see [297]). SQD1 catalyses a complex overall reaction to generate UDP-sulphoquinovose before the transfer of sulphoquinovose to DAG by SQD2 to generate SQDG. SQD1 sequences are highly conserved in plants and algae. They form three distinct clusters – in green algae, in red algae (and some cyanobacteria e.g. Synochocystis) and in Archaea. The gene was first identified in *C. reinhardtii* by [20]. In addition, two further putative genes for SQDG synthase have been identified (see [56]).

In general, SQDG is thought to play an important role in photosynthesis as discussed by [251]. It is important for the structural integrity and heat-tolerance of Photosystem II [298]. Also of note is the presence in *C. reinhardtii* of 2-O-acyl-SQDG [20], a lipid of unknown function. Various SQDGs or monoacyl derivatives (SQMG) have been suggested to be potential anti-neoplastic agents, which inhibit DNA polymerase [299]. Moreover, while the galactosylglycerides can often serve to supply fatty acids during stress-induced TAG accumulation (see later), SQDG can also provide a major sulphur source for protein synthesis during early phases of sulphur starvation in *C. reinhardtii* [300].

Red algae (Rhodophyta) differ from green algae in often having ARA and EPA as major fatty acids (**Table 5**) [301-303]. Such acids accumulate in the glycosylglycerides as well as other lipid classes. In red algae (e.g. *Porphyridium cruentum*) galactolipids are formed by both the 'prokaryotic' and 'eukaryotic' pathways which give rise to 20C/16C or 20C/20C species, respectively. In the case of *P. cruentum* or *Porphyra yezoensis* the main MGDG species will be 20:5/16:0 and 20:5/20:5 [111, 304]. PtdCho is the source of the 'eukaryotic' moieties [111] while 20C fatty acids can also come from any TAG reserves [305]. In contrast, the DGDG in different Rhodophyta appears to be mainly 20:5/16:0 (i.e. 'prokaryotic') as is the SQDG [304].

MGDG synthases and DGDG synthases of red algae form a clade separated from green algae [306]. Rhodophyta have one MGDG synthase and most of them have one or two plant-like DGDG synthase. DGD2-like isoforms are found in *Chondrus crispus* and many other red algae [50] while the SQDG genes (SQD1, SQD2) are highly related to higher plant orthologues [19].

There is a small group of primitive red algae of the order Cyanidiales, which have a very simple fatty acid composition, lacking the usual 20C fatty acids found in most red algae [292]. The formation of their glycosylglycerides is discussed in [19].

4.4 Betaine lipids

As mentioned in **section 2**, algae often contain betaine lipids as major membrane constituents. DGTS is a significant component of *C. reinhardtii* while DGTA is found in most brown algae as well as some other species. DGTS is formed by reaction of DAG with S-adenosylmethionine and the gene has been identified in *Chlamydomonas reinhardtii*. It codes for a single betaine synthase (BTA1Cr) protein whose function was confirmed by expression in *E. coli* [46]. The reaction contrasts with the two enzymes needed for DGTS biosynthesis in the photosynthetic bacterium, *Rhodobacter spaeroides* [46]. In algae the original pathway proposed involved transfer of a 4C amino acid moiety from methionine (in S-adenosylmethionine) followed by three methylations [307, 308]. DGTS has been proposed as a major source of fatty acids during TAG accumulation following N-starvation in *P. tricornutum* [309]. It also appears to be converted to DGTA in brown algae [310].

For the other betaine lipids, despite being important components of many marine algae, only a little is known of the biosynthetic pathway for DGTA. Early labelling experiments identifying DGTA (then an unidentified lipid) as a rapidly metabolised lipid are summarised in [14]. Experiments using differentially radiolabelled methionine isomers in *Ochromonas danica* suggested that DGTS could be converted to DGTA by decarboxylation and recarboxylation of the polar part (and simultaneous deacylation and reacylation of the glycerol moiety)[310]. The same workers suggested that DGTA could act as a substrate for desaturation. Using a different brown alga, *Ectocarpus fasciculatus*, Eichenberger's group found that, after labelling with [14C]oleate, label rapidly appeared in phosphoglycerides such as PtdGro, PtdEtn and PtdCho but little in DGTA [311]. In contrast, when another brown alga, *Dictyopteris membranacea*, was labelled with 14C-acetate or 14C-oleate [312] label rapidly appeared in DGTA (and PtdGro) and was then transferred to glycosylglycerides during a 6-day chase period. These data confirmed experiments with other brown algae such as *Fucus serratus* [313] or *A. nodosum* [250] in showing that DGTA was actively metabolised and could supply fatty acids to chloroplast glycosylglycerides. They also emphasise that generalisations about metabolism or, indeed, betaine lipid distributions in brown algae cannot be made.

Pavlova lutheri contains not only DGTA and DGCC but also a diacylglycerylglucuronide (DGGA). Radiolabelling experiments in this alga showed that while both DGTA and DGCC were extra-plastidic lipids, only DGCC was important for re-distribution of fatty acids back to plastid components such as MGDG [314].

4.5 Triacylglycerol biosynthesis and accumulation

Recent commercial interest in using oleaginous microalgae for VLCPUFA-enriched oils or for biofuels (see **section 7**) has led to a surge in interest in TAG metabolism. During normal growth, algae usually contain only small amounts of TAG but this is increased remarkably under stress conditions, such as N or P deficiency, elevated temperature or light intensity [315, 316]. *C. reinhardtii* has often been used in studies because this model green alga accumulates TAG rapidly under various stresses [55] such as N-deficiency which also halts cell division and then causes quiescence [317]. Key genes in the process have been identified by forward or reverse genetic approaches [55, 315]. These include forward genetic screening by insertional mutagenesis [48, 179, 318], deep transcriptome analysis by RNA sequencing [177, 188, 204, 319] and proteomics [188, 231, 320]. In the latter case, a major lipid droplet protein (MLDP) was identified in *Chlamydomonas*, which is the equivalent of plant oil crop oleosin [321-323]. In order to evaluate TAG accumulation and, hence, the usefulness of particular algae or growth conditions, efficient analytical methods (especially high-throughput) are needed. Two such procedures are in [29, 289].

For TAG synthesis by the Kennedy pathway, four reaction steps are required (**Figure 5**). In addition it is known from work in higher plants that phospholipid: diacylglycerol acyltransferase (PDAT)[324] can have a prominent role, depending on the plant species [325] [326]. In *Chlamydomonas* there is one PDAT [215, 327] but six diacylglycerol: acyl-CoA acyltransferases (DGATs) of two types, type 1 DGAT and type 2 DGTT (an abbreviation has been adopted for algal researchers). The DGAT genes are called DGAT1 and DGTT1-5 [128, 215, 328]. There is a little confusion in the literature regarding nomenclature of the type 2 DGATs. For example, in *C. reinhardtii* these are referred to as DGTT1-5 or DGAT2 A-E (see [329]) or even as CrDGAT2-1 to CrDGAT2-5 [330]. Nevertheless, regardless of nomenclature, there is a pattern across algal species with, in general, most having a single DGAT1 but multiple DGAT2 genes. Three picoplankton species (*M. pusilla*, *O. taura*, *O. lucimarinus*) did not contain a putative DGAT1 but this could be due to incomplete genome sequences or because their DGAT1 sequences were too divergent to be detected by similarity searches [331]. These authors speculate as to why algae contain multiple copies of DGATs which suggest multiple origins rather than gene duplication events. The same conclusion was reached from a study of DGATs in a wide variety of different organisms from mammals through to fungi and yeasts but including *Chlorella* and *Coccomyxa* sp. [332].

While most attention has been paid to the multiple DGAT2 genes/enzymes, a putative sequence for DGAT1 in *C. reinhardtii* [56] has been reported. For *P. tricornutum*, a DGAT1

was cloned and its activity demonstrated in a yeast mutant [333] where a preference for saturated 16 or 18C fatty acids was displayed.

For the type-2 DGATs in *C. reinhardtii*, CrDGAT2A, B and C were investigated in overexpressing strains but total TAG accumulation was not changed significantly from wild-type under normal growth or after N- or S-depletion [328]. The substrate selectivity of CrDGTT1, 2 and 3 (DGAT2B, E and D, respectively) were assessed. CrDGTT1 preferred PUFAs, CrDGTT2 preferred monounsaturated acyl-CoAs and DGTT3 preferred 16C acyl-CoAs [334]. Knock-down of each of the three genes caused a 20-35% decrease in TAG together with a change in TAG fatty acids. Hung et al. [329] performed heterologous complementation assays for *C. reinhardtii* DGTT1-4 in yeast mutants and showed that DGTT1, 2 and 3 but not 4 complemented the TAG deficiency phenotype. Complementation with DGTT2 was the most effective. In agreement with previous reports, the authors could not detect transcripts for DGTT-5.

In the green alga, *Ostreococcus tauri*, three putative DGAT2 genes were identified. No homologues to DGAT1 or DGAT3 could be detected and two of the three DGAT2 sequences (*OtDGAT2A* and *OtDGAT2B*) gave enzyme activity in TAG-free yeast mutants. OtDGAT2B was shown to have a broad substrate specificity [335]. Gong et al. [336] identified four putative type 2 DGAT genes in *P. tricornutum* (now considered to have five such genes; [37]). The *PtDGAT2B* was expressed in yeast mutants to restore TAG formation. Moreover, up-regulation of *PtDGAT2A* and Pt*DGAT2B* preceded TAG synthesis in the alga. However, *PtDGAT2B* was not regulated by N-starvation [336].

Instead of the Kennedy pathway, using DGAT, TAG can also be produced by phospholipid: diacylglycerol acyltransferase (PDAT) (**Figure 4**). The relative contributions of PDAT versus DGAT in plants is still a matter of debate (see [326]). A gene encoding PDAT was found in *C. reinhardtii* and its activity demonstrated by expression in a TAG-deficient yeast mutant. MicroRNA silencing of PDAT in *C. reinhardtii* altered membrane lipid composition and reduced the growth rate [327]. The *CrPDAT* also had a strong lipase activity and was suggested to be functional during the log phase of growth under normal conditions but not during the large induction of TAG deposition on N-depletion [327]. It is noteworthy that this conclusion contrasts with the increase in PDAT expression following N starvation observed by [215]. In higher plants PDAT is thought to generally use PtdCho, which is not present in *C.reinhardtii* although Boyle et al. [215] reported that the *CrPDAT* complemented TAG-deficient yeast (where it may well have used PtdCho). Moreover, the gene for *CrPDAT* is predicted to be chloroplast-located [337] so the ability of the enzyme to use MGDG (but not

DGDG or SQDG) as acyl donor in this alga is important [327]. It will be interesting to look at the characteristics of other algal 'PDAT' enzymes such as in *P. tricornutum* where a putative gene has been identified [37].

4.6 Regulation of triacylglycerol accumulation

Because of the commercial interest in enhancing TAG accumulation (**section 7**), the regulation of TAG biosynthesis is an active area of research. Recently, the use of transcriptional engineering has been reported [338]. By identifying transcription factors whose expression was enhanced during the TAG accumulation caused by N-deprivation in *N. gaditana*, ZnCys was shown to be a key factor whose expression could be fine-tuned to increase TAG formation with only a minimal reduction in total carbon productivity [227]. These initial experiments are encouraging, although still fall short of commercial requirements [338, 339]. An additional survey about gaps in our knowledge about the biochemistry of TAG accumulation and possible avenues for engineering has been made [340]. Other aspects that impinge on lipid accumulation in green algae are chemical activators [341] and carbon precursor supply [342]. Because TAG biosynthesis is increased by nutrient deprivation, overexpression of important enzymes such as the type 2 DGAT (DGTT4) can be elicited with a P-starvation inducible promoter [343]. Parallel studies have also been made with the commercially-promising *P. tricornutum* (e.g. [149]).

Stress (usually nutrient-deprivation) induction of TAG production is a widely employed method to increase algal oil accumulation. However, it has been noted that our knowledge of the process is largely based on genome predictions which have yet to be experimentally verified [344]. The impact of N-starvation has been examined in nine algal strains (chosen as promising for oil production from 96 candidates) where aspects such as biomass production and the duration of productivity were documented [345]. As an alternative to N-starvation, an RNAi knock-down of nitrate reductase can also enhance lipid biosynthesis in *P. tricornutum* [346]. The authors also noted the changed expression (and binding) of transcription factors, thus heralding their recent use, referred to above [338].

In another oleaginous alga (*Nannochloropsis gaditana*), the availability of detailed genetic information allowed predictions of lipid metabolism to be made [65]. When this alga was grown under N-starvation, the formation of TAG was accompanied by a decrease in galactosylglycerides (and a reorganisation of the photosynthetic apparatus) [347]. Similarly, in *N. oceanica* under N-stress, the amounts of MGDG (as well as DGTS and PtdCho) decreased dramatically and expression analysis accompanying the changes identified a number of genes

that seemed to be involved [288]. In *Chlorella* also, N-stress caused decreases in membrane lipids which accompanied the increase in TAG and this was confirmed by radiolabelling experiments [348]. As an adjunct to increasing TAG accumulation by increased biosynthesis, disrupting lipid catabolism has also been used in the diatom *Thalassiosira pseudonana* [349].

The consistent mobilisation of membrane acyl groups for TAG formation in various algae under N stress begs the question about which enzymes might be responsible. In *N. oceanica*, a phospholipase was upregulated [288] while in *C. reinhardtii* a galactoglyceride lipase is involved [179]. The unusual substrate selectivity of the so-called 'PDAT' in *C. reinhardtii* should also be noted, together with its lipase activity [327]. In *Coccomyxa subellipsoida*, N stress caused extensive chain remodelling of membrane lipids as well as TAG. Over 2/3rds of the chloroplast lipids were lost during TAG accumulation, which was produced by the prokaryotic pathway [350].

Because TAG (especially when accumulated under nutrient stress) is found in lipid droplets, several groups have focussed on these organelles. Most of the work has been with *C. reinhardtii* and the overall composition [351] and a major lipid droplet protein (MLDP) that affects droplet size [322] reported. The MLDP was described by others (see [323, 352]) but, in the studies by Huang et al [353] was found to only interact with the lipid droplet surface intermittently. The whole topic of microalgal lipid droplets, including their composition, formation and function has been reviewed by Goold et al [352].

For further details of the overall subjects of TAG biosynthesis and its elevation under nutrient stress see the reviews [37, 55, 56, 246, 315] as well as the later **sections 6 and 7**.

TAG synthesis under nutrient, temperature or chemical stress is thought to be a protective mechanism to reduce reactive oxidant damage to photosynthetic membranes, as discussed by Du and Benning [315]. It will occur whenever carbon supply outstrips the capacity for starch synthesis [115] and will be observed commonly in other algae (e.g. in the marine alga *Desmodesmus* sp.)[354].

In contrast to *Chamydomonas*, the oleaginous algae *Nannochloropsis* sp. accumulate TAG not only under stress conditions but during normal growth [355]. Under such growth, TAG is around 10% of the dry weight [356]. Nevertheless, following stress (nutrient deprivation), *Nannochloropsi*s will produce substantial amounts of carbohydrates, although TAG is always the major reserve compound [315]. *Nannochloropsis* differs also from *C. reinhardtii* in containing PtdCho as well as DGTS (*C. reinhardtii* only has DGTS; [357] and in the high amounts of VLCPUFA (EPA) in its lipids [356].

The genomes of several *Nannochloropsis* species have been sequenced including *N. oceanica* CCMP1779 [52], *N. oceanica* IMET1 [355] and *N. gaditana* [62]. The first of these has a relatively small genome (28.7 Mb) coding for around 12,000 genes [52]. Some 10 putative genes probably involved in TAG synthesis were identified including those for all the reactions of the Kennedy Pathway (**Figure 5**). There were no less than 13 putative DGAT genes and two for PDAT. In addition, a major lipid droplet protein (LDSP) was identified in *Nannochloropsis* [315]. Such studies provide important background information for the potential industrial exploitation of *Nannochlopsis* (**section 7**).

5. Glycerolipid breakdown and β-oxidation of FAs

Cells require the ability to degrade storage TAGs and cell membranes when needed, or to digest extraneously supplied FAs as food source. In laboratories, this condition can be easily mimicked by manipulating the N content in the culture medium. As shown in **Figure 8**, TAGs are made upon N starvation and then degraded rapidly when N is back-introduced. This process is accompanied by a degradation and then re-synthesis of membrane lipids [199]. Based on this observation, several forward genetic screens have been performed aiming to isolate mutants defective in lipid catabolism [179, 194, 317, 318]. Accompanying this process, many genes of lipid hydrolysis exhibit drastic alterations in their expression [128, 188]. The process of lipid breakdown is collectively called lipolysis, which requires highly specialized enzymes called lipases. Most lipases act at the interface of hydrophobic and hydrophilic phases and are membrane proteins, making them very difficult to study; therefore despite intensive research on algal lipid metabolism and several forward genetic screens carried out in the past 10 years [179, 215, 226, 317, 318, 327], only six algal lipases have been identified and studied in more or less detail; yet the major TAG lipase remains to be identified in the most studied algal model *C. reinhardtii*.

5.1 Known algal lipases

The known lipases from algae include two orthologues of the Arabidopsis major TAG lipase (Sugar dependent 1, SDP1) [358-360], a DAG lipase (CrLIP1) from C. *reinhardtii* [361], a *sn*-2 MGDG lipase from *C. reinhardtii* (PGD1)[179], an orthologue of the Arabidopsis CGI58 found in the diatom *Thalassiosira pseudonana* [349], a putative patatin-like phospholipase domain-containing protein 3 (PNPLA3) from *P. tricornutum* [32], and a seipin orthologue from *P. tricornutum* [362]. Moreover the Chlamydomonas PDAT was observed to also possess lipase activity *in vitro* [327]. Expressional manipulation of the above-mentioned genes has often

resulted in strains with modified TAG amount. The lipolytic processes and enzymes involved have recently been reviewed in [169] for microalgae. In this section, we therefore chose to focus on the oxidation of FAs, the major products of lipolysis.

Following their release from a membrane lipid or TAG, non-esterified FAs are first activated to their CoA esters by members of the long chain acyl-CoA synthetase (LACS) family. LACS proteins belong to a multi-protein family, and are ubiquitously present in numerous algal lineages. The resultant activated FA in the form of acyl-CoA is then ready for oxidative attack at the C-3 or β-carbon position, giving rise to the name β-oxidation. An acetyl-CoA (C_2) is cleaved off of the acyl-CoA (C_n) with each round of the β-oxidation spiral, and the remaining acyl-CoA (C_{n-2}) re-enters the spiral to repeat this process until acyl-CoA is completely converted to acetyl-CoA.

5.2 The β-oxidation of FAs

All living organisms have developed the capacity to breakdown FAs to produce acetyl-CoAs, which are further metabolized either for energy production when coupled to mitochondrial electron transport chain, or for synthesis of sugars when coupled to glyoxylate and gluconeogenesis pathways [168, 363]. With the exception of cyanobacteria [364], β-oxidation of FAs is universally present and has been intensively studied in mammals [365], oleaginous yeast [366, 367] and in germinating oilseeds [168] and senesencing leaves [368].

5.2.1 Subcellular location of β-oxidation and phylogenetics of acyl-CoA dehydrogenases/oxidases

FA β-oxidation begins with the enzymes acyl-CoA dehydrogenase (ACAD) or acyl-CoA oxidase (ACOX), which catalyze the dehydrogenation of acyl-CoA to trans-2-enoyl-CoA either via the reduction of O_2 to generate peroxide (ACOX) or via the reduction of FAD to $FADH_2$ (ACAD). It was previously believed that ACOX is exclusively peroxisomal, while ACAD is mitochondrial, but peroxisomal ACAD has since been reported from humans and from the fungus *Ustilago mays* [369]. The β-oxidation of FAs occurs mostly in peroxisomes in yeast and plant cells, in contrast to mammalian cells wherein it occurs principally in mitochondria, with a small peroxisomal contribution [365, 370, 371]. FA degradation has been studied in various algal species, although by no means comprehensively, and the location of FA β-oxidation in algae varies extensively, occurring either in the peroxisome, the mitochondrion or both [372-376]. A better understanding of ACOX/ACAD evolution and localization is essential for the successful engineering (and control) of algal lipid catabolic pathways.

Based on a comprehensive phylogenetic analysis (**Figure 9**), ACOXs and ACADs appear to have diverged and diversified during early prokaryote evolution, as previously reported [377]. Eukaryotes then inherited over twenty distinct ACOXs/ACADs, either via endosymbiosis or via lateral gene transfer, and these inherited prokaryotic genes in turn gave rise to eukaryotic subfamilies. Additional archaeal sequences made available since previous analyses of ACAD evolution [377] now suggest that the major eukaryotic ACAD lineages are of both archaeal and bacterial ancestry rather than solely arising from the latter, while ACOX appears to be exclusively of bacterial origin (**Figure 9**).

As a result of the distinct prokaryotic ancestry of the different eukaryotic ACOX/ACAD subfamilies, inter-subfamily inferences regarding enzyme localization are fraught with peril. Furthermore, the presence of contrasting mitochondrial and peroxisomal members within the same ACAD subfamily indicates that even intra-subfamily localization is not always conserved (e.g. group D-II in **Figure 9**). Nonetheless, some common trends are apparent: all ACOXs thus far characterized are peroxisomal and peroxisomal localization (and catalase activity) may be essential for efficient activity of these peroxide-producing enzymes. By contrast, ACADs are either mitochondrial or peroxisomal.

The conservation of eukaryotic members of the various ACOX/ACAD subfamilies varies extensively by taxonomic group [377], particularly in the case of eukaryotic algae, and may be in part responsible for establishing the organellar location of β-oxidation. Green, heterokont and haptophyte algae possess a variety of ACADs and ACOXs, while red algae (with two exceptions, perhaps due to sequence contamination or lateral gene transfer) possess members of only a single ACOX subfamily (O-III), making them potentially interesting candidates for the engineering of enhanced lipid accumulation through tightened control of β-oxidation. Similarly, Arabidopsis does not contain any ACADs, likely explaining the exclusive peroxisomal localization of β-oxidation in higher plants. However, the lower plants *Physcomitrella patens* and *Marchantia polymorpha* do retain members of the D-X ACAD subfamily, which is also present in green algae (in addition to subfamilies D-II, D-VII, and DXI); it is unknown whether these enzymes are of peroxisomal or mitochondrial localization. By far the widest array of ACADs are present in the heterokont algae, which possess members of up to 10 separate ACAD subfamilies, one of which (D-I) is unique to heterokont and cryptomonad algae. Much work remains to be done in the study of ACOXs/ACADs, with the localization and enzymatic properties of many algae-containing eukaryotic subfamilies remaining completely unexplored.

Below we discuss current literature on the known steps of peroxisomal FA β-oxidation in Chlamydomonas, so far the best studied model alga for lipid catabolism [169, 194].

5.2.2 Core reactions of the peroxisomal β-oxidation

The core peroxisomal pathway requires the acyl-CoA oxidase (ACOX), multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (KAT) to catalyze the sequential oxidation, hydration and dehydrogenation, and thiolytic cleavage of the acyl-CoA molecule (**Figure 10**). Although genes encoding putative orthologues to known proteins of FA β-oxidation can be identified in algal species [56, 215, 378], only the ACOX2 catalyzing the first step in the FA β-oxidation spiral has been characterized experimentally at both genetic and biochemical levels. ACOX2, which is closely related to Arabidopsis ACOX2 (**Figure 9**) exhibited high activity toward a broad range of acyl-chains, and showed highest activity toward C16 and C18 acyl-CoAs [194]. The *acox2* (or *acx2*) mutants lost >50% of the wild-type capacity in remobilization of TAGs upon N resupply following a period of N starvation; and moreover, the *acox2* mutants accumulated 30% more TAG during photoheterotrophic N starvation and with a modified TAG composition [194]. The occurrence of five ACOX isozymes is not surprising [169, 379], since FA β-oxidation is a chain-shortening reaction, and different isozymes should be required to shorten acyl-CoA of various chain lengths. It is worth noting here that the above core enzymatic activities of FA β-oxidation are not sufficient for the oxidation of unsaturated FAs whose degradation normally requires the participation of additional enzymatic reactions. Two alternative pathways are known in plants [45] (Aralip: http://aralip.plantbiology.msu.edu/pathways/pathways), of which only the *Arabidopsis* enoyl-CoA isomerase (ECI) has been studied in detail [380], and none of which have been characterized in algae.

5.2.3 Metabolism of hydrogen peroxide (H_2O_2)

In addition to acetyl-CoAs, peroxisomal FA β-oxidation produces hydrogen peroxide and reducing equivalents in a molar ratio of approximately 1:1:1. In higher plants, the highly oxidative H_2O_2 is usually decomposed to water by peroxisome-resident catalase; which is a major protein in plant peroxisomes and is at the origin for the formation of a crystalloid core apparent under transmission electron microscope [381]. The crystalloid core is however often absent in peroxisomes of *C. reinhardtii* [190]. Based on subcellular fractionation studies, Kato et al [382] showed that catalase activities in *C. reinarhdtii* could be associated to mitochondria fractions. Nevertheless, homology searches using known plant catalase have identified at least

two genes encoding putative catalases in the genome of *C. reinhardtii* [379], but their subcellular localization and biological function remain to be determined. Indeed, it has long been argued that if *C. reinhardtti* contains any H_2O_2-producing activities [383, 384]. We have lately shown that the primitive peroxisomes or microbodies in *C. reinhardtti* do indeed contain reactions that generate H_2O_2, at the first step of FA β-oxidation using ACOX2. It is observed that *in vitro*, the recombinant Chlamydomonas ACOX2 catalyzes the conversion of acyl-CoA to trans-2-enoyl-CoA while producing H_2O_2. This study was the first to demonstrate that *C. reinhardtii* uses a peroxisomal pathway for FA degradation, and that H_2O_2 producing activities had already evolved in green microalgae.

From an evolutionary perspective, it is not clear if there is any advantage of housing FA β-oxidation in peroxisomes instead of mitochondria. The mitochondrial pathway employs an acyl-CoA dehydrogenase at its first step, and this reaction is directly coupled to the mitochondrial respiratory pathway for ATP production, therefore energy is conserved; whereas in the peroxisomal pathway, energy is transferred to O_2 with the production of H_2O_2 and subsequently H_2O; therefore energy is lost. This loss of energy could potentially be advantageous under some conditions, allowing FA degradation to occur without affecting cellular energy/redox status. This also raises the question on what are the possible physiological roles or significance of peroxisome-derived H_2O_2. A study in *Arabidopsis* has shown that in mutant plants defective in the ascorbate peroxidase (APX)/monodehydroascorbate reductase (MDAR) electron transfer system, the escaped H_2O_2 inhibited the activities of the major TAG lipase SDP1 and this slowed TAG hydrolysis [385]. This study implies therefore a possible role of H_2O_2 in coordinating lipolysis to β-oxidation of FAs. H_2O_2 is the most stable form of reactive oxygen species (ROS), and is known to play dual roles in cellular physiology – in excess it can cause oxidative damage, but in sub-lethal levels, chloroplast-derived H_2O_2 is known to play a signaling role [176]. Through characterization of two mutants defected in the peroxisomal malate dehydrogenase 2 (MDH2), we have recently provided evidence that peroxisome-derived H_2O_2 likely plays a role in transmitting the redox state of the peroxisome to the chloroplast, thereby impacting photosynthesis, *de novo* FA synthesis and starch metabolism (discussed in detail in **section 3.5.3**) [191]. However the mechanisms by which peroxisome-derived H_2O_2 passes through the peroxisomal membranes remains largely unknown (i.e. is it by free diffusion or an aquaporin mediated process?).

5.2.4 NADH re-oxidation in peroxisomes

Similar to most oxidative reactions, β-oxidation of FAs also produces NADH through the reaction catalyzed by the 3-hydroxylacyl-CoA dehydrogenase (MFP-DH). *De novo* NAD^+ synthesis occurs in the cytoplasm [386, 387], yet it is required by reactions present in almost every subcellular compartment. Its transport and homeostasis therefore can play a key role in regulation of metabolic pathways [122, 388]. Newly-synthesized NAD^+ is imported from the cytoplasm into the peroxisome by the peroxisomal NAD^+ carrier (PXN) [389, 390]. However, studies in yeasts and plants show that re-oxidation of peroxisomal NADH must occur inside the organelle because the peroxisomal membrane is not permeable to NAD^+ [391]. Lately it has been shown that the peroxisomal malate dehydrogenase 2 (MDH2) in *C. reinhardtii* plays a major role in NADH re-oxidation because the *mdh2* mutants defected in MDH2 protein are impaired by 80% in their capacity to reutilize TAGs following N resupply [191]. MDHs are ubiquitous enzymes, and each subcellular compartment usually contains at least one isoform [122, 379, 388]. Alongside the peroxisomal MDH2, Chlamydomonas genome encodes one $NADP^+$-dependent chloroplast MDH5 [392], and three other NAD^+-dependent MDHs (MDH1, MDH3 and MDH4) [379].

In addition to MDH2, two other mechanisms are known to play a role in NADH re-oxidation in plant peroxisomes. These additional pathways of NADH re-oxidation employ either a peroxisomal hydroxypyruvate reductase (HPR) [393], or the APX/MDAR electron transfer system [385]. Genes encoding putative orthologues to these proteins can be identified in algal genomes, but their function remains unknown. The serious impairment in oil reutilization observed in the *mdh2* mutants suggest that the other mechanisms, if they occur, are not expected to play major roles in *C. reinhardtii*, at least not under the conditions tested (i.e. N resupply following a period of N starvation [191]).

5.3 Additional roles for the β-oxidation spiral

Mostly through phenotypic analyses of *Arabidopsis* mutants defected in various steps of FA β-oxidation, it has become obvious that the β-oxidation spiral does not only play a role in FA breakdown, but also plays a role i) in the production of lipid-based signaling molecules such as jasmonic acid, ii) in the conversion of indole butyric acid to the phytohormone indole acetic acid, and iii) in the later steps of BCAA degradation, therefore impacting plant physiology and development [189, 394-396]. Thus unlike many other metabolic pathways, peroxisomal β-oxidation is multi-functional, and is sometimes called “a pathway with multiple functions” [366]. Jasmonic acid has been identified in *Euglena gracil*is, *Chlorella* and *Spirulina* [397] and

also a variety of marine algae [398] but nothing is known about the enzymes or subcellular locations for their biosynthesis in microalgae.

6. Environmental effects

Early studies of the effects of the environment on lipid metabolism in algae were summarised by Pohl and Zurheide [15]. In our previous review on algal lipids we also discussed effects of nutrition (especially N, P and S limitation), other growth conditions (temperature, light, pH) and some pollutants (e.g. heavy metals) [9]. It is noteworthy that many of these chemical or non-chemical stresses can induce TAG accumulation, which may also be accompanied by alterations in FA and lipid composition [315]. Induction of TAG biosynthesis in algae is also relevant to industrial uses of algal oils (**section 7**).

6.1 Nutrients

Algal species vary in their nutritional requirements although the basic macro-nutrients for all species are carbon, N and phosphorus (P). Some marine microalgae (e.g. many diatoms) need silicon [37]. For a common freshwater green alga like Chlorella, growth will decline once N and P concentrations are below 31.5 and 10.5 mg/l, respectively [399]. For *Chlorella vulgaris*, nitrogen deficiency below 0.5 mg/l gives an optimal induction of lipid production [400]. Indeed, N is the usual stress used to induce TAG production [316]. Under these conditions, green microalgae or diatoms will accumulate TAG at 20-50% dry weight [316]. Similarly, phosphorus deficiency will have major effects on lipid metabolism and, hence, algal oil content [48, 401, 402].

Naturally, because of the interest in using algae for industrial purposes (especially post-induction of oil accumulation), most attention has focussed on species which offer commercial possibilities. Nevertheless, examination of a variety of microalgae showed that most of them increased TAG production when grown in N-deficient conditions. There were, however, significant differences in biomass production, %TAG accumulated and the duration of productivity [345, 403]. For promising feedstocks such as *P. tricornutum*, *Nannochloropsis* spp. and *Chlorella pyrenoidosa*, a variety of laboratories have examined their productivity under N-starvation [288, 347, 401, 404-406]. As mentioned in **section 4**, accumulation of TAG is allowed by *de novo* synthesis accompanied by a decrease in plastid galactolipids and consequent re-organisation of the photosynthetic apparatus in *Nannochloropsis gaditana* [347]. A detailed examination of glycerolipid classes and their molecular species was made in *P. tricornutum*. Most lipids were relatively unaffected although large decreases in MGDG and

PtdGro were noted. For the accumulating TAG there was an enrichment of 16:1 which correlated with its synthesis whereas 20:5 seemed to be transferred from MGDG [401]. In *Nannochloropsis oceanica*, the increase in TAG was accompanied not only by a decrease in MGDG but also of the extra-chloroplastic lipids DGTS and PtdCho [288]. Changes in FA profiles were also reported for *Phaeodactylum tricornutum* [407] and for other algae (*Pavlova viridis*, *Tetraselmis subcordiformis*)[408]. During the increased TAG accumulation on N-starvation in *Chlorella pyrenoidosa*, expression levels of genes for acetyl-CoA carboxylase and DGAT were increased [404]. The latter was also shown to be raised in *C. reinhardtii* along with other acyltransferases and a nitrogen responsive regulator [215]. Overall, it can be concluded that TAG formation is by a combination of *de novo* synthesis as well as transfer of FAs from membrane lipids.

Only limited information is available regarding the mechanisms controlling TAG accumulation during N-limitation. Early on Boyle et al [215] showed that enhanced expression of a nitrogen response regulator accompanied N-starvation induction of TAG production. Furthermore, RNAi knock-down of nitrate reductase can enhance lipid biosynthesis in *Phaeodactylum tricornutum* [346]. Recently, ROC40, a transcription factor involved in circadian rhythm, was found to increase markedly on N-starvation. Further information from mutant analysis supported a role for ROC40 in N-starvation induction of TAG synthesis [340]. Please see **section 3.8** for more regulatory factors involved in novo FA synthesis.

The effect of different N levels in the growth media for *N. oceanica* has been examined with a view to finding the best concentrations for lipid production [406]. The strain studied, DUT01 produced an unusually high amount of 16:2. In a separate study with three different microalgae, the effect of different N concentrations between zero and 1.76 mmol/l were examined. All three algae showed highest lipid accumulation with 0.22 mmol N/l and *Nannochloropsis oceanica* and *Pavlova viridis* showed promise for biodiesel production because of the changes in their FA patterns [408].

A second major nutrient needed for algal growth is phosphorus which, of course, is essential for phosphoglyceride biosynthesis. Riekhof et al [20] showed that *C. reinhardtii* had reduced levels of all phosphoglycerides on P-starvation. The 50% reduction in PtdGro, an essential thylakoid (photosynthetic) constituent, was critical but it could be replaced by another anionic membrane lipid, SQDG. In a low-phosphate bleaching mutant of *C. reinhardtii* (*lpb1*) it was shown that normal responses to P-deprivation (and S-deprivation) appeared as usual but that the *lpb1* mutant lacked critical acclimation ability [409]. In the fresh water eustigmatophyte, *Monodus subterraneus*, P-starvation caused increases in DGDG and DGTS

(and TAG) which accompanied the loss of phosphoglycerides. The increase in DGDG (but not MGDG) resembled the response of higher plants to P-deprivation. There were also some changes in the FA contents of individual lipid classes [402]. As noted above, P-starvation triggers lipid (TAG) accumulation in the same way as N-deprivation does [404], but not to the same extent [401]. When considering the time-course of changes in *Phaeodactylum tricornutum*, it was noted that there was a step-wise adaptive response. The authors suggested that phosphoglycerides provided emergency P following their catabolism and that there was some replacement with non-phosphorus lipids, which included SQDG for PtdGro and DGTA for PtdCho [401]. The effect of P-starvation in diatoms is discussed thoroughly by [37].

Other macronutrients, such as carbon dioxide [410] or sulphur can alter lipid metabolism. In the latter case, S-starvation can increase non-polar lipids in *Chlorella ellipsoidea* [411] or *C. reinhardtii* [412]. Sulphur is utilised for the synthesis of proteins and a wide variety of metabolites critical for growth. When *C. reinhardtii* was transferred to S-depleted conditions, some 85% of the SQDG was broken down to yield a major pool of S for protein synthesis [300]. To an extent this utilisation of SQDG is consistent with its role as an important contributor to the global S cycle [413, 414].

Silicon depletion was also noted to induce TAG formation in the diatom *Cyclotella cryptica* [415]. This TAG had a modified FA composition (less unsaturated) compared to cells grown in adequate silicon concentrations. In fact, as noted previously, silicon is a macronutrient for many diatoms [37], including oleaginous species. Thus, silicon depletion will enhance TAG production in those diatoms that need it [416, 417] and, for example, *Thalassiosira pseudonana* will accumulate an average of 24% more lipids than it does under N-starvation [405, 418]. *Phaeodactylum tricornutum*, in contrast, has little, if any, requirement for silicon [418]. However, although silicon does not seem to be required for laboratory or factory cultures of *Phaeodactylum tricornutum,* it seems to be needed for normal expression of miRNAs and growth [419].

Clearly, carbon is a macronutrient, although it will normally be sourced from the atmosphere. Nevertheless, with culture conditions in mind, there has been some attention paid to different concentrations of CO_2 or to various regimes. In *Chlorella kessleri*, CO_2 concentrations have a dramatic effect on lipid metabolism and on the incorporation of [14C]acetate into FAs (and lipids). Part of the changes were due to adjustment of the 'prokaryotic' versus 'eukaryotic' pathways and one result was elevated 18:3 at low CO_2 levels [420]. Likewise, glycerol feeding in batch cultures of *Schizochytrium* sp. [421] or alterations in inorganic carbon regimes supplied to *C. reinhardtii* changed FA patterns [422]. Carbon

metabolism in diatoms, including the impact of environmental factors, has been discussed recently [37, 54]. Furthermore, a general commentary on growth and lipid accumulation by nutrient depletion and supplementation in *C. reinhardtii* has been recently published [423].

Micronutrients, needed in trace amounts (e.g. Co, Cu, Fe, Mg, Mn, Mo, Zn) may have a strong influence on algal growth since they can alter normal enzyme activity [424]. Elements such as Fe and Zn have been shown to influence TAG accumulation in Chlamydomonas [425, 426]. Of course, for many such elements there is a fine line between nutrient deficiency, sufficiency, and toxicity [427]. Iron seems to be a key factor in regulating phytoplankton biomass. When $FeCl_3$ was added to fresh medium at 1.2×10^{-5} mol/l in late exponential phase cultures of *Chlorella vulgaris*, it boosted biomass and % lipid composition whereas lower concentrations led to lower lipid levels [428]. In Fe-starved *C. reinhardtii*, lipid droplets and TAG accumulated. An increased saturation index was noted, suggesting that desaturase activity was compromised. The FA profiles of DGDG and DGTS (but not MGDG) were changed and gene expression of enzymes or proteins involved in TAG accumulation (e.g. the major lipid droplet protein or DGAT) was increased [426]. Effects of Fe-deficiency in *Phaeodactylum tricornutum* were coincident with a partial deficiency of photosynthetic transport and a high sensitivity to light [429].

Like iron, copper is needed for certain enzyme activities [427]. However, toxic effects have been noted in a variety of algae as well as changes to lipid metabolism (see [9, 14]). Copper response regulator1-dependent and –independent responses of *C. reinhardtii* to dark anoxia were shown to be important. Under hypoxic conditions this alga accumulates TAG which, in contrast to during N-depletion, was enriched in unsaturated FAs [430]. When chromium effects were studied in *Euglena gracilis* obtained from a culture or collected from a polluted river, PUFA levels were most affected. Electron microscope examination revealed thylakoid disorganisation in treated cells [431]. Another toxic metal is cadmium, which affects lipid metabolism [9]. As noted for other algae, susceptibility to heavy metal toxicity varies considerably with species (see [14]) and *Phaeodactylum tricornutum* is relatively resistant to Cd probably because it has protective transport and detoxification processes [432].

6.2 Light

Naturally, for photosynthetic organisms, light is a major controlling factor for algal growth [15] [9]. Its duration and intensity influence both biochemical composition and algal mass yield [433]. Of course, algal species vary in their requirements [424]. Increasing light intensity raises the photosynthetic rate and growth until the latter levels off as photosynthesis is balanced by

photorespiration and photoinhibition [434]. Intensities for maximum growth (and lipid production) have been reported for a number of algal species recently [400, 435] as well as the effect of different light intensities in combination with CO_2 levels [436] or salinity and N [437]. These can affect both qualitative and quantitative aspects of lipid metabolism.

In *Pavlova lutheri*, the percentage of EPA and DHA in polar lipids was highest at low light intensities whereas their synthesis was best at intermediate (19 w/m^{-2}) intensities [438]. For the red alga, *Trichocarpus crinitus*, low light conditions favoured an increase in membrane components especially SQDG, PtdGro and PtdCho. However, in contrast to *Pavlova lutheri* there were no differences in total FAs under different light regimes. Nevertheless, there were some changes in individual lipid classes. For example, the percentage of 20:5 in MGDG decreased but in PtdGro it increased. HL increased the proportion of trans-16:1 in PtdGro [439]. Differences in how individual algae respond to light was also emphasised in a study of four freshwater phytoplankton species (including rarely examined Chrysophyceae and Zygnematophyceaea) under different light intensities. While there were significant changes in all algae examined, no generalisations about these alterations could be made [440].

HL intensity tends to increase TAG levels together with decreases in polar lipid classes. This has been observed in the diatom, *Thallassiosira pseudonana* [441], the red alga *Trichocarpus crinitus* [439] and various freshwater species [442]. For *C. reinhardtii* saturating light induces sustained accumulation of TAG in lipid droplets. Interestingly, some of these droplets appear to be located in plastids in contrast to N-deprived growth wherein 60% are of ER-origin [154]. Also in Chlamydomonas, extended (24h) dark periods cause TAG accumulation [430]. Prolonged darkness in *Phaeodactylum tricornutum* has also been studied in detail and information about nuclear transcriptional activity, pigment content and photosynthesis reported during darkness and following re-illumination [443].

Finally, the effect of UV-B irradiation in various algae has been examined. The extent of changes depended on whether the algae were UV sensitive or tolerant. In particular, reduction in VLCPUFAs, such as EPA and DHA, were noted and also that nutrient-deprived cells were more sensitive [444].

6.3 Temperature

Previous studies indicated that temperature can influence not only FA proportions but also the lipid class content in different algae [9, 15]. Optimal algal growth usually occurs at 20-30°C [445] but each species has its optimal value [446]. This has important implications for outdoor

culture systems [424]. Of course, some specialised algae can endure temperatures of 40°C while those growing in hot springs can tolerate temperatures of 80°C.

Naturally, given the diversity of algal species and the temperature range of their habitats, it is unsurprising that lipid analyses have revealed differences. Anesi et al. [447] examined ten dinoflagellates from different freshwater habitats with 4, 13 or 20°C temperatures. They could be grouped depending on the molecular species of their lipid classes. The glycosylglycerides (MGDG, DGDG, SQDG) seemed particularly useful for classification. The range of dinoflagellate tolerance was concluded to be best reflected in thylakoid glycolipids while phylogeny could be better revealed by the distribution of non-thylakoid lipids and their species [447]. A study of a lipid-producing, cold-tolerant yellow green alga (Xanthophyceae) isolated from the Rocky Mountains showed that it produced the highest amount of lipids when grown with HL at 4°C. Under these conditions *Heterococcus* sp. DN1, produced enhanced amounts of PUFA (especially EPA) at the expense of 16:1 [448].

Temperature can be used as a stressor to encourage the production of valuable metabolites [449] and can be used in combination with other parameters to increase lipid yields [450]. Increased growth temperature can be used to elevate lipid contents in several species [315] [9] including Chlamydomonas [320, 451]. The broad effects of culture temperature on growth, lipid composition and FA quality have been studied fairly extensively (see [9]). With the important application of algae in biotechnology (**section 7**), species of commercial interest have formed the main recent focus. Thus, the thrustochytrid, *Aurantiochytrium*, that forms appreciable DHA, was examined in the range 10-30°C and optimised at 10°C [452]. In addition, the combination of N-stress with different growth temperatures has been studied in *C. reinhardtii* in connection with potential biofuel production. In these experiments growth at 32°C seemed optimal for FA content and composition in connection with potential use as biofuel [453]. With increased global warming some relevant studies have been conducted recently with regard to heat stress (in Chlamydomonas) [451, 454] and for climate warming in *Scenedesmus obliquus* [455]. In the latter case, even a relatively moderate increase in ambient temperature (20 to 28°C) resulted in significant changes in endogenous lipids and their metabolism. In particular, the decrease in unsaturation and, consequently, essential PUFAs has implications concerning food quality for higher trophic levels.

6.4 Other factors

Production of lipids in algae can be influenced by such factors as dehydration, salinity, culture age and pH. For more general details on these factors please refer to [9, 315, 424].

Supplementary information for hyper salinity is provided for Chlamydomonas where PtdOH seems to be involved as a second messenger [456], and for various stressors (salinity, N- or P-deprivation, temperature) in combination with elevated carbon dioxide. For *Nannochloropsis,* EPA yields were increased by nitrate, low salinity and low temperature [457]. Lipid accumulation in 50 strains of microalgae during fluctuating brackish and sea water locations has also been examined. From these studies, some promising algae for biodiesel or ω-3 FA production were found [458].

Finally, two special conditions for changes in acyl lipid production have been found for *Chlorella* spp. In *Chlorella sorokiniana*, the impact of inoculum sizes on phospholipid metabolism revealed that PtdGro, PtdEtn and several molecular species of PtdCho may be changed under the experimental conditions. The authors suggested that their data may help in providing potential targets for engineering to improve biofuel production [272]. Additionally, air drying of cells was found to stimulate TAG synthesis in *Chlorella kessleri* by 2.7-fold. The same conditions also stimulated oil accumulation *in C. reinhardtii* but to a much smaller extent [459].

7. Algae for industry

Commercial applications of algae have been reviewed extensively over the years since the article by Guschina and Harwood [9] see [5, 113, 424, 460-467]. Apart from uses in the food or aquaculture industries, algae can be used for pigments, various useful chemicals and, of course potentially for biofuel. While being used for these purposes, algae can also serve environmental applications [462] such as in bioremediation [463]. While much of recent research has concentrated on biotechnological products for wealthy countries [468], but the data are also applicable to developing countries where the use of macroalgae, such as seaweeds is growing significantly [469, 470].

Before discussing uses of algae, some comments should be made about important technical aspects. One of the most expensive stages in the industrial use of algae is extraction of lipids (and other products). This subject is covered in many of the above reviews but is specifically highlighted in others (e.g. [331, 471-473] and, importantly, the use of a forward genetic screen to identify useful oil mutants [318] is notable. Some useful technical aspects include LC-MS technology [474], optimisation of productivity using FTIR analysis [475] and high throughput analysis with MS [476].

Furthermore, there have been important developments in the molecular biology of algae, particularly species which have been earmarked as commercially important. Following

publication of a diatom EST database [477], the *Phaeodactylum* genome [263] and that for *Nannochloropsis gaditana* [62], further comments about diatoms have been up-dated [37]. Additional technical aspects include stable nuclear transformation of *P. tricornutum* [478], gene silencing in the same species [479], RNAi-based gene knockdown in *N. oceanica* [480] and the use of CRISPR technology for genome editing [481].

Of the various uses of eukaryotic algae for industry, two topics have attracted particular attention over the last decade. These are, respectively, algae as sources of VLCPUFAs and for biofuel production.

7.1 Algae as sources of very long chain fatty acids (VLCPUFAs)

Humans and most animals require essential fatty acids of the n-3 and n-6 series [243]. The basic acids of these series are, respectively, α-linolenic (LNA) and linoleic (LA) acids. The usual sources of such are vegetable oils either in the form of spreads or cooking oils but they are also contained in most food products. However, current diets (especially 'Western diets') provide excessive amounts of n-6 PUFA so that a typical ratio of dietary n-6/n-3 PUFA is around 15, whereas the best nutritional advice suggests that a ratio of 3-4 would be more appropriate [482-484]. This is because the main role of the essential fatty acids is to be converted to various 20C or 22C PUFAs which are then oxidised to powerful signalling compounds such eicosanoids, resolvins and protectins. Those signalling molecules derived from n-3 PUFAs are generally anti-inflammatory while those from n-6 PUFAs are generally pro-inflammatory [243, 485]. This is believed to have led to an increase in chronic inflammation and associated diseases (e.g. arthritis, cardio-vascular complaints) in Western societies [486-488].

Although LA and LNA are the main PUFAs in the diet and are thought to be appropriate for normal health requirements [489], they are poorly converted (especially by men) to ARA, EPA and DHA which are the immediate precursors of signalling compounds. Thus, under certain situations there is a 'conditional requirement' for ARA and/or EPA and DHA in the diet [490]. In fact, the perceived need for more n-3 PUFA in diets has led to an increased consumption of fish oils as a convenient source of EPA and DHA. However, given the advised human (and animal) daily requirements [491] and perceived over-fishing in the World, this is not a sustainable situation [492-494].

Following the undeniable demonstration of the importance of VLCPUFA for good health, the commercial market for such products has increased considerably. This has led to a search for algal sources to supplement the obvious limitation to fish oil supplies [35, 495]. Such algal oils have proven useful in infant milk formulations, adult nutraceuticals and in fish feeds.

The first of such oils was 'DHASCO' from *Crypthecodinium cohnii* [496] and, later, those from *Schizochytrium* spp. have proven commercially successful (**Table 5**). Other sources of DHA were discussed in [35, 497], where some of the advantages of algal oils are described. For example, algal oils are usually enriched in a particular VLCPUFA whereas fish oils often have a variable ratio of EPA/DHA (depending on the algae that the fish consumed). Furthermore, the potential problem of toxic compounds in fish oils (or in fish themselves) is obviated by culturing algae [35].

The primary producers of PUFAs are photosynthetic organisms and, in the case of EPA and DHA, are marine algae [460]. In fact, ironically, fish are often as poor as humans in converting LA to ARA or LNA to EPA and DHA [498]. This has led to the increasing use of algae as direct sources of VLCPUFAs in human diets [499] as well as for farmed fish feeds [500-502]. Thus, for example, *Nannochloropsis* spp. and *Phaeodactylum tricornutum* can have an EPA content of up to 40% total fatty acids under autotrophic conditions [495, 503]. Similarly, *Thraustochytrium* and *Schizochytrium limacinum*, when grown under heterotrophic fermentation conditions, can accumulate 30-40%DHA [499]. One comment that should be made here is that EPA and DHA (despite being metabolically interconvertible) seem to have independent effects on humans [504, 505].

Wen and Chen [506] have discussed the production of EPA by microorganisms in some detail. Although considerable interest has been focussed on *Shewanella* spp. (a marine bacterium), factors affecting production in microalgae (e.g. *Nannochloropsis*) including diatoms (e.g. *Phaeodactylum tricornutum*) have been described. These include the use of different systems as well as various environmental factors [506]. Because fish oils contain a mixture of EPA and DHA, the question of a need for EPA *per se* is important (see [507, 508]; and remarks above). Moreover, in terms of nutraceuticals, the subject is complicated by the known ability of dietary DHA to be retro-converted to EPA [509].

The other important VLCPUFA is ARA which, together with DHA, is a prominent component of brain and other nervous tissues [510]. This acid is accumulated in many bryophytes and some marine algae, such as Phaeophyceae [15]. On the other hand, it rarely accumulates to any great extent in most microalgae [36]. However, a freshwater microalga, *Lobosphaera incisa* (formerly *Parietochloris incisa*), accumulates ARA at around 50% of total fatty acids in TAG under N-starvation conditions [511]. To convert oleic acid into ARA requires three desaturases (Δ5, 6 and 8) which have been cloned from *L. incisa* [512] as well as an elongase [513]. In addition, overexpression of a 'GPAT-like' gene from *L. incisa* was shown to increase TAG synthesis [266]. The accumulation of TAG in microalgae, stimulated by

nutrient stress (see **section 6**) is associated not only with increased synthesis but also mobilisation of carbon from membrane lipids, for example those in chloroplasts [128, 188, 401]. This may involve autophagy [514, 515]. Moreover, since N-starvation is a reversible process, the transient production of TAG-enriched lipid bodies gives rise to the transfer of fatty acids back into chloroplast membranes during recovery in *L. incisa* [516].

The commercial production of DHA by *Schizochytrium* has been thoroughly discussed by [517]. These organisms are Thraustochytrids which make up a significant proportion of phytoplankton, although they are often under-reported [518]. The PKS system for making VLCPUFAs (sometimes called PUFA synthetase) first identified by Metz et al. [112] has been studied further with respect to its acyl carrier protein (ACP) domains [519], expression in *E. coli* to make DPAn-6 and DHA [520] and formation of non-esterified fatty acids as end products [521]. Commercial uses of *Schizochytrium* oils have been evaluated for feeds (fish, poultry and cattle – where DHA-enriched milks have been produced), breads, milk drinks, nutritional bars, margarines etc. [517]. A review of how lipid metabolism could be manipulated in *Schizochytrium* and other Chromista, especially *P. tricornutum*, to increase EPA and DHA has been made [139].

A background to the use of algae for producing high-value products, like VLCPUFAs, is given in [522] while for specific aspects of the latter see [523]. A variety of algae from different classes have been or are of interest either as species for basic research or for biotechnological development (**Table 5**).

Several freshwater or marine species of the genus *Nannochloropsis* (e.g. *N.gaditana, N. oculata*) contain high concentrations of EPA in their chloroplast glycerolipids [524] [347] which can be transferred to TAG. Another eustigmatophyte, *Trachydiscus minatus*, also contains a high % of EPA in storage lipids and may be a potential industrial source [525, 526]. Likewise, diatoms (in particular *Phaeodactylum tricornutum*) have attracted attention not only as a source of EPA but also DHA [527, 528]. *P. tricornutum* has been genetically modified to enhance productivity of n-3 VLCPUFAs [529] and its biotechnological economics assessed [530]. Additionally, haptophytes like *Isochrysis galbana* or *Pavlova lutheri*, are important potential industrial species. Marine macroalgae often contain high amounts of LCPUFAs [9, 14, 15] and their use in nutrition has been considered [469].

As mentioned previously, culture conditions are critical in ensuring good growth and productivity. Some relevant studies are [531-533] [534]. Moreover, some aspects of genetic modification of algae to enhance productivity are discussed by Khozin-Goldberg et al [527]. In particular, information about TAG accumulation in *Chlamydomonas* has been applied to the

oleaginous microalgae *Nannochloropsis* sp. which is often considered one of the best species for industrial utility [356] especially for the production of EPA [535]. Knowledge of its genome and RNA-sequencing of samples from N-replete and -deprived growth, have revealed that many of the genes involved in the Kennedy pathway, as well as PDAT1 and PDAT2 are up-regulated on N-deprivation [315].

As a result of such studies, several commercial companies have exploited algae for the production of EPA and/or DHA. These include the use of *Nannochloropsis* [536] or *Odontella aurita* [537, 538] for EPA production. For DHA, *Crypthecodinium cohnii* (Dynophyta) and *Schizochytrium* (Thruastochytriaceae) have been used for several years in the infant formula market [536] (**Table 6**). Such algal oils have been shown to be efficacious, non-toxic and of high nutritional value [527].

As mentioned previously, the first commercial SCO (single cell oil) containing DHA was from *C. cohnii*, a dinoflagellate. There are over 2000 identified dinoflagellates, of which only about half are photosynthetic. The production of DHA in such species and, especially, in *C. cohnii* is well discussed in [539]. This has included the use of substrates other than glucose to boost or extend production [540]. For the latter, it was demonstrated that higher yields of DHA (compared to glucose) could be obtained using acetic acid or ethanol. Glycerol is also a potential substrate [541] although that produced in surplus from biofuel manufacture is not of food grade [540]. In addition, there have been studies of lipid productivity (in *Chlorella* and *N. salina*) using a lab-scale open pond simulating reactor [542].

General considerations used for the commercial processing of algal oils, particularly as applied to VLCPUFA products, are described by Ratledge et al [543]. Not only are algal VLCPUFAs important in the human nutritional food industry, they are also increasingly used in feed to modify meat, milk or egg characteristics, in pet formulations [544] and in aquaculture [545].

7.2 Biofuels

A second major area for the applied use of algae is in their potential as sources of biofuels [424]. As fossil fuels are diminishing, sustainable replacement sources are required. Moreover such sources would not elevate atmospheric carbon dioxide and, hence, contribute to climate change.

General reviews on the production and uses of algal oils (TAG) for biofuel production (and other purposes) are given by [316, 546-551] in addition to those given in the introduction to **section 7**.

Microalgal-based fuels are eco-friendly and non-toxic and, of course, are formed by fixing atmospheric carbon dioxide [552]. Microalgae grow rapidly and have been estimated to have the potential of transforming 9-10% of solar energy into biomass with a theoretical yield of around 77g/biomass/m2/day (equivalent to 280 tonne/hectare/year) [553, 554]. It is also very important that the growth of algae does not use agricultural land and, therefore, does not compete with food/feed production, unlike most plant crops. Moreover, algae can often use saline or waste water or can even be employed to simultaneously remove pollutants [555-557], including phycoremediation of domestic wastewater [463]. In addition, the great diversity of microalgae provides opportunities for selection of species and strains that can produce oils which can yield biofuels with specific properties. Furthermore, there are also possibilities of using genetic manipulation to enhance productivity and/or modified oil properties [558].

From the above discussion, it can be concluded that algae appear to be one (some would argue the only [559]) source of renewable biodiesel capable of meeting future demands. However, the present high cost makes the use of algae for high-value oils rather than biodiesel currently more attractive and economic [35] (see later discussion). For more information about the use of microalgae to make biodiesel, see [559, 560] [561-563].

Many algae can produce substantial quantities of TAG (up to 80% of total lipids which can be up to 70% dry weight)[558]. However, this usually occurs in response to nutrient deprivation (especially N)[5]. Nevertheless, the green alga, *Botryococcus braunii*, produces >60% of its lipid as hydrocarbons. The bulk of these are accumulated outside cells [558], making recovery somewhat easier. There are different types of *B. braunii*; the A-race, B-race and L-race strains [564].

Clearly, a key consideration in choosing appropriate algae for biofuel production will be the nature of the oil produced (fatty acid composition), as well as the productivity of the strains selected. Such considerations are discussed in [565-567].

As mentioned above, TAG is the lipid normally accumulated in algae and aspects of its biosynthesis are discussed in **section 4.5**. The accumulation of TAG following nutrient stress (see **section 6**) is a major hurdle to be overcome, because growth ceases leading to poor overall productivity [568, 569]. In efforts to address this problem, research has concentrated on enzymes important for TAG synthesis, such as DGAT [331] and seeing which of its isoforms were upregulated on N starvation [128]. Follow- up experiments to use a strong light-responsive promoter of the DGAT genes, however, failed to increase TAG levels, pointing to tight control of lipid synthesis (in *C. reinhardtii*) [5]. In comparison, expression in *Phaeodactylum tricornutum* under control of the light-responsive FCPC promoter increased neutral lipid levels

[570]. Somewhat unexpectedly, it also changed the fatty acid composition of membrane as well as storage (TAG) lipids [5]. Other methods to attempt to overcome the nutritional stress problem are discussed in [5].

Further aspects of the biosynthesis of hydrocarbons {Baba, 2013 #2927} and of TAGs are given in [349, 571-574]. This includes the use of multiple carbon fixation pathways [149], reduction of competing catabolism [349] and the recent use of a transcriptional regulator [227, 339]. Please refer to **sections 4.6 and 6** for additional discussion of the regulation of TAG biosynthesis and accumulation. A recent review has focussed on the use of small molecules (through the process of 'chemical genetics') to improve algal lipid production [574].

Once TAG has been harvested from algae, it has to be converted into fatty acid methyl esters (FAMEs) for biodiesel. Four methods (base-catalysed transesterification, acid-catalysed transesterification, non-catalytic conversion, lipase-catalysed techniques) can be used [558]. The properties of the biodiesel produced is largely dependent on the fatty acid composition of the original TAG [575] and this is very important for the final standard of the finished product [576]. Thus, strain selection [577] and growth conditions (e.g. temperature, light, salinity) [558] are vital considerations.

In terms of technical aspects, the use of Fourier transform infrared spectroscopy to monitor lipid production under various combinations of temperature and cell densities has been reported [475] while the use of various photobioreactors [578] and lipid synthesis in an open pond simulating reactor have been published [579]. The influence of the growth medium has been studied with regard to the use of oil crop biomass residues [580] or palm oil mill effluent [581]. Finally, new developments in biodiesel conversion technology have been reviewed [582].

As mentioned earlier, the main problem with algal-derived biodiesel currently is its cost versus petroleum-based products. A comprehensive evaluation of the economic (and environmental) impacts of microbial biodiesel has been made, including net energy balance, cost of goods sold etc. [583]. Their evaluation is based on current crops but highlights the necessity to mitigate against greenhouse gas emission. Chisti [584] argued strongly that microalgae are better than crops (as used for bioethanol) in terms of their smaller impact on the environment and their efficiency in producing biodiesel. His arguments were disputed by [585] but then further countered by Chisti [586]. With our present technology, algae seem to offer a realistic solution to replacing petroleum and, even more so, as a source of high-value products. For calculation of the theoretical maximum algal oil production (at different global sites) see [587].

Further general aspects of biodiesel production are covered by Ratledge and Cohen [35] and economic analysis by Davis et al. [588] while the future of algal biofuels has been discussed [548]. All the subjects covered in section 7.2 are included in the comprehensive review by [589]. Although this is focussed on a particular programme, the review encompasses the same general area as this current article.

Research on the commercial production of biofuels from algae has been carried out for three decades but, to date, the high cost of production (over an order of magnitude) [9, 551] compared to petroleum supplies, means that economic viability is not yet possible. Reducing costs remains the most important target and, until that has been done significantly, then high-value products (such as VLCPUFA) will remain much more attractive [558].

7.3 Other useful products

There are a number of other valuable chemicals produced by algae which have a commercial niche.

Carotenoids

Carotenoids have utility in the food, cosmetic and pharmaceutical industries [590]. Different algae accumulate various pigments of which the most important commercially are astaxanthin, beta-carotene, phycobiliproteins, phycocyanin and phycoerythrin [186]. Beta-carotene is a useful food supplement and is produced by *Dunaliella salina* at over 10% of its dry mass [591]. Astaxanthin is commercially valuable and it is produced by *Haematococcus pluvialis* at 4-5% dry mass [592]. Phycobiliprotein pigments are fluorescent agents [161] while phycocyanin and other pigments from red algae are used in both the food and cosmetic industries [593]. See [424, 594] for further discussion.

Sterols

Phytosterols are used in the pharmaceutical industry and as nutraceuticals [424]. *Pavlova* and *Thalassiosira* genera are rich in sterols [595-597]. These microalgae have been found to produce up to nearly 3% dry mass as sterols [598]. Some 40 different sterols have been reported in over 100 species of diatoms. Major sterols in Glaucocystophyta are sitosterol, campesterol and stigmasterol, dinoflagellates produce mostly 4α-methyl sterols while 24-propylidene-cholesterol is mainly accumulated in *Pelagophyceae* [424].

Proteins and enzymes

Microalgae produce 2-8 tonnes/hectare/year of proteins [599] and a number of algae, such as *Chlorella*, produce marketable material [424]. Recently, there has been an increasing interest in many algal enzymes for the genetic manipulation of plants, particularly in order to produce VLCPUFAs [600]. A considerable number of genes for such enzymes had already been isolated when we last reviewed the area [9]. Nevertheless, there seems to be constant improvements in the conversion rates of ALA to EPA and DHA by employing newly characterised desaturases and elongases. For example, front-end delta4 and delta6 desaturases from the green alga *Ostreococcus* RCC809 gave 15% desaturation of 22:5 and 54% desaturation of ALA respectively. A Δ6 elongase from the cold-water diatom *Fragilariopsis cylindrus* gave 38% elongation of gamma-18:3. These genes allowed an expansion of activities available for the potential commercial production of EPA and DHA [600].

Any of the enzymes mentioned in **sections 3, 4 and 5** could, potentially, be utilised for commercial purposes. Early work in this area included enzymes useful for the conversion of EPA into DHA [601] and front-end desaturases to produce unusual fatty acids (pinolenic and coniferonic acids) [236]. Another example would be the use of three front-end desaturases from *P. salina* for DHA biosynthesis which could be expressed in higher plants [602]. More information on useful algal lipid enzymes and their utilisation will be found in [589].

8. Conclusions

It should be clear from the preceding text and accompanying references, that significant advances have taken place in the dozen years since the last time we reviewed eukaryotic algae [9]. These advances have taken place in all aspects but with an increasing use of molecular biology to facilitate progress. Much of the research has been driven by the heightened interest in using algae for industrial purposes such as for nutraceuticals or biofuel.

Over the last decade, *C. reinhardtii* has become established as a model organism although, of course, given the diversity of algae this green microalga is not always a good substitute for specific organisms or situations. *Nannochloropsis* spp. and *Phaeodactylum tricornutum* have also been well studied because of their identification as algae of interest for commercialization.

We look forward to future advances in our knowledge which undoubtedly will take place. Perhaps these may eventually include the utilization of algae for biofuels---an area which is urgently needed within the background of climate change. This is just one area where the fascinating and diverse biochemistry of algae can have a global impact.

9. Acknowledgements

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Legend for figures and tables:

Figure 1. *De novo* fatty acid synthesis – carbon and energy sources.

Abbreviations: ACP, acyl-CoA binding protein; ACCase, acetyl-CoA carboxylase; MCMT, malonyl-CoA: ACP malonyltransferase; Dof-type TF, DNA binding with one finger type transcription factor; bHLH, a basic helix-loop-helix; bZIP, a basic leucine zipper-domain containing TF; ER, enoyl-ACP reductase; KAS, keto-acyl-CoA synthase; KAR, ketoacyl-ACP reductase; HAD, hydroxyacyl-ACP dehydrase; ME, malic enzyme; FatA/B, fatty acid thioesterase A/B; PDH, pyruvate dehydrogenase complex; PSR1, Pi Starvation Response 1; SAD, Stearoyl-ACP Desaturase; FAX1, fatty acid export 1; TF, transcription factor.

Figure 2. Maximum likelihood phylogenetic tree of ACCase negative regulators.

(A). BADC/BCCP tree.

(B). PII tree.

Known Arabidopsis sequences were used as PSI-BLAST queries to comprehensively identify eukaryotic algal orthologs from the NCBI non-redundant database, or, for algal species/genes not present on NCBI, from the JGI algal genome database. Representative non-algal sequences of interest were also identified and included. For red algal BCCPs/BADCs, only a single representative sequence per family was retained for the final phylogenetic tree to avoid overcrowding, except in the case of the Cyanidiaceae. Amino acid sequences were aligned with the MAAFT (v. 7.308) plugin in Geneious (v. 11.1.4) using the E-LNS-I option. Target peptides and non-homologous or erroneous sequence regions were manually trimmed, alignments were further refined with MAAFT, and sites with 50% or more gaps were removed from the alignments. Trees were constructed with the RAxML 8.2.11 plugin using the Gamma JTT protein model and 100 rapid bootstrap replicates and were plotted with FigTree 1.4.3 followed by manual formatting in Adobe Illustrator. Bootstrap support is indicated by the darkness of branch lines while clades are colored by taxonomic group.

Figure 3. Fatty acid desaturations in diatoms.

The biosynthesis of LC-PUFAs in diatoms. Schematic representation of Δ6- and Δ8-pathways for LC-PUFAs biosynthesis. Diagram were taken from Sayanova et al [54] (with permission).

Figure 4. Glycerolipid synthesis.

This schema is made partly based on that of Kim et al ([247]. It is worth noting here that PDAT has been shown to use *in vitro* phosphalipids and galactolipids as acyl donors [327], however the situation *in vivo* is not clear.

Abbreviations: CoA, Coenzyme A; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGDG, digalactosyl diacylglycerol; DGTS, diacylglyceryl-3-O-4'-(*N,N,N*-trimethyl)-homoserine; FAX1, fatty acid export 1; G3P, glycerol-3-phosphate; GPAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; MGDG, monogalactosyl diacylglycerol; PDAT, phosphalipid:diacylglycerol acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; TGD, trigalactosyldiacylglycerol; TAG, triacylglycerol.

Figure 5. A simplified Kennedy pathway for lipid biosynthesis.

This schema is made based on that of [243] (with permission).

Abbreviations: G3P, glycerol-3-phosphate; GPAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; DAG, diacylglycerol; PDAT, phosphalipid:diacylglycerol acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase; CoA, Coenzyme A; TAG, triacylglycerol.

Figure 6. Pathways of synthesis of phosphatidylcholine (PtdCho) in various plants and algae. Diagram were taken from Sato et al 2016 [286] (with permission).

Figure 7. Fatty acid and glycerolipid molecular species distribution in *Chlamydomonas.* Data are taken from Li-Beisson et al 2015 [56] (with permission).

Figure 8. Changes in lipid content in response to changes in nitrogen status in the media.

(A). Changes in cellular TAG levels as quantified by Flow cytometry after cells being stained with Nile red.

(B). Changes in polar membrane lipid content.

Abbreviations: A.U.: artificial unit; MM, minimal media; N, nitrogen; TAG, triacylglycerol; TAP, tri-acetate-phosphate media; PL, polar lipids. Data are modified based on [199].

Figure 9. Maximum likelihood phylogenetic tree of eukaryotic algal acyl-CoA oxidases/dehydrogenases.

Phylogenetic analysis was performed as in **Figure 2**, except that PSI-BLAST queries were supplemented with known human and *Ustilago mays* acyl-CoA oxidases/dehydrogenases (ACOXs/ACADs) and sites with 10% of more gaps were removed from the alignment. Dotted lines surround distinct eukaryotic ACOX/ACAD subgroups, with subgroups being designated by a letter indicating their provenance (D = ACAD, O = ACOX, and G = Glutaryl-CoA dehydrogenase) followed by a dash and a roman numeral (e.g. D-XI). The thicker dashed line in the middle of the figure indicates the division between ACOXs and ACADs based on enzyme activities of known members. Note that ACOXs are paraphyletic, with group OIX, which contains *Arabidopsis* acyl-CoA oxidase 4, having likely arisen via horizontal gene transfer of an early eukaryotic glutaryl-CoA dehydrogenase into proteobacteria, followed by another horizontal transfer of the resultant proteobacterial glutaryl-CoA dehydrogenase back into the eukaryotic lineage and then followed by functional mutation into an acyl-CoA oxidase as described more comprehensively in [377].

Figure 10. Lipolysis and β-oxidation of fatty acids in microalgae – carbon and energetic aspects.

Abbreviations: TAG, triacylglycerol; LCS, long chain acyl-CoA synthetase; CTS1, comatose 1; ACOX, acyl-CoA oxidase; CoA, coenzyme A; MFP, multi-functional protein; DH, dehydrogenase; ASC, ascorbate; MDA, malondialdehyde; APX, ascorbate peroxidase; CAT, catalase; Mal, malate; PXN, peroxisomal NAD carrier translocator; OAA, oxaloacetate.

Table 1. The acyl lipid compositions of some algae.

Data taken from Harwood and Jones [14], where original references will be found.

Table 2. Fatty acid composition of selected algae from the SAG culture collection.

The major fatty acids are shown as the % composition recalculated from data in Lang et al 2011 [36] where other compounds (eg phytol) are sometimes listed. 16:1 (9z), 16:2 (9z, 12z), 16:3 (7z, 10z, 13z), 18:1 (9z), *18:1 (11z), 18:2 (9z, 12z), 18:3 (9z, 12z, 15z), 18:4 (6z, 9z, 12z, 15z), 20:4 is ARA, 20:5 is EPA and 22:6 is DHA.

Table 3. Total fatty acid compositions of some algae.

Data taken from Harwood and Jones [14] where original sources are listed. Although the original papers did not always fully define the double bond configuration and position, it can be assumed that these were probably as indicated in **Table 2**, with 16:4 being (6z, 9z, 12z, 15z) and 18:1 being oleic acid, 18:1 (9z). tr = trace (<0.5%).

Table 4. Total fatty acid composition of some marine algae.

Taken from Harwood and Jones [14]. See **Tables 2 and 3** for information about the unsaturated fatty acids. tr = Trace (<0.5%).

Table 5. Very long chain PUFA produced by different algae of industrial interest. For further details see [527].

Table 6. Comparison of the fatty acid composition of *Crypthecodinium cohnii* and *Schizochytrium sp.* and commercial oils produced from them.

Taken from information in [35].

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